JCB

Long-lived mitochondrial cristae proteins in mouse heart and brain

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Review Timeline:

Submission Date:	2020-05-26
Editorial Decision:	2020-07-08
Revision Received:	2021-05-06
Editorial Decision:	2021-05-31
Revision Received:	2021-06-03
Editorial Decision:	2021-06-03
Revision Received:	2021-06-14

Monitoring Editor: Jodi Nunnari

Scientific Editor: Tim Spencer

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. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202005193

July 7, 2020

Re: JCB manuscript #202005193

Dr. Jeffrey Savas
Northwestern University
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303 East Chicago Avenue, Ward 12-102
Chicago, IL 60611

Dear Dr. Savas,

Thank you for submitting your manuscript entitled "Long-lived cristae proteins as pillars of mitochondrial architecture in post-mitotic cells". The manuscript has been evaluated by expert reviewers, whose reports are appended below. We sincerely apologize for the delay in sending this decision to you. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

We and the reviewers found the premise of the study and its results interesting, however, as you will read, the reviewers shared numerous conceptual and technical concerns about data and approaches. These valid concerns are significant and potentially undermine the main conclusions of the manuscript. Considerable new experimental work would be needed to strengthen the analyses and validate the core findings, which would be required for publication. We think this work is more substantial than can be addressed in a typical revision period, and therefore, precludes further consideration of your manuscript at JCB. To expedite the publication, it would seem best to pursue publication at another journal. If you were interested in fully addressing the reviewers' concerns however, given interest in the topic, we would be willing to discuss resubmission to JCB of a significantly revised and extended manuscript through our appeal process. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

SILAC based proteomics in mice and significant extension of the labelling/feeding time with isotopes has allowed identifying very long-lived proteins (LLPs), in particular in chromatin and the nuclear pore complex. Here, the authors have analyzed the mitochondrial proteome using this approach and identify LLPs enriched in cristae membranes of post-mitotic tissues of mice. Combining LC-MS/MS based proteomics with BN-PAGE and crosslinking approaches, evidence is provided that LLPs are enriched in higher order assemblies of respiratory complexes and that some complexes, such as MICOS and the ATP synthase, are shown to be very stable and characterized by low exchange rates between newly synthesized and old subunits. The authors propose that longevity of cristae shaping proteins ensures long-term stabilization of mitochondrial cristae.
This manuscript convincingly identifies LLPs in mitochondria and demonstrates a striking stability and low subunit exchange rate of some protein complexes in the IMM. While these findings are compelling, the novelty of the findings is somehow limited by the lack of a time-resolved analysis, which precludes the determination of half-lives in vivo. Extremely long half-lives of mitochondrial proteins have already been described by Fornasiero et al, 2018, including proteins identified here. The observation that LLPs are enriched in large complexes represents therefore the most novel finding. Concerning this point and the interpretation of data, however, I have some concerns that the authors should consider:

1. It remains unclear why the authors focus their interpretation on long-lived cristae shaping proteins such as MICOS and ATP synthase and propose a long-term stabilization of cristae. First, other membrane shaping proteins such as OPA1 are short-lived and recent high-resolution life cell imaging data suggest dynamic behavior of cristae. Second, the identified LLPs (Table 1) also include many respiratory chain subunits in the IMM (which can be expected in post-mitotic tissues).
2. BN-PAGE combined with proteomics suggests that LLPs are enriched in higher order assemblies of respiratory chain complexes such as respirasomes. There is some debate about the relative distribution of monomeric/dimeric complexes and respirasomes in vivo. Do the presented result imply that the ratio between different assemblies remains constant over time?
3. The BN-PAGE experiments were not analyzed in depth on the protein level, but the stability of individual subunit would be very informative.
4. Why are only some subunits of multi-subunit assemblies identified as LLPs? Does this suggest that sub-structures rather than the assembled complexes are extra-ordinarily stable? The authors should discuss their findings in light of reports in the literature demonstrating different turnover rates for the N-module of complex I.
5. The authors argue based on BN-PAGE in gel assays that respiratory complexes harboring LLPs are fully active. However, it is questionable if the used assay for complex activity is sensitive and accurate (error bars) enough to detect functional differences considering that old proteins correspond to approx. 10-12% in the cortex or other tissues.
6. How does protein abundance affect the identification of LLPs? As discussed by Fornasiero et al. (2018), LLPs appears to be often abundant proteins.
7. In contrast to the statement in the text, it seems there is not just a single heavy-light protein crosslink between two mitochondrial proteins: Atp5c1 and Ndufa2. There would be further explanation required why the authors think that the crosslinking approach supports the idea that `proteins reside in the same cristae are co-
preserved with little to no subunit exchange for months in both heart and brain´ (line 296).
8. The authors should discuss their findings in light of available turnover rates for mitochondrial proteins and improve the description of their experiments in figure legends and method section.
- The total number of proteins detected should be provided in absolute numbers for each

experiment. Also provide the coverage of mitochondrial proteins as well as complex subunits (for example judged based on MitoCarta).
- In Figure 2EF, each dot represents an FA value for an individual protein. The reviewer tried to find the respective protein values in the supplementary table 3. There are 4 biological replicates and 10 bands but the reviewer was not able to reproduce the bar plots.
- Figure 1B - Are these experiments based on one biological replicate? Please clarify.
- The GO analysis is missing in the method section. It is necessary to provide information about the background that was used to find enriched proteins.
- Since 14/15N Spectra on MS1 level are not as easy as illustrated in Figure 1A (workflow), please show at least for one LLP the MS1 spectra and indicate light and heavy peaks.

Reviewer #2 (Comments to the Authors (Required)):

In their manuscript "Long-lived cristae proteins as pillars of mitochondrial architecture in post-mitotic cells" Bomba-Warczak and Savas demonstrate that a subset of the mitochondrial proteome persists for months in long-lived post-mitotic cells. These long-lived proteins localize to cristae membranes. Specifically, they show that:
1. Tissue differences in the longevity of the mitochondrial proteome
2. Enrichment of long-lived proteins in the mitochondrial cristae membrane
3. Long-lived functional respiratory supercomplexes in brain
4. Limited exchange of subunits throughout the lifetime of individual cristae
Overall the manuscript is well written and clear. This manuscript advances the field of mitochondrial physiology by tracking the turnover of the mitochondrial proteome in various tissues. This leads to the surprising result that in post-mitotic tissues respiratory complexes, especially the ATP synthase complex are long lived and can remain intact for over four months.

Main point 1: Tissue differences in the longevity of the mitochondrial proteome.
The data are strongly supportive that mitochondrial proteins are capable of lasting for over four months in post mitotic tissues. However, the fractional abundance of these long-lasting proteins is 10%-15% indicating the majority of proteins are turned over. The manuscript would be strengthened by an estimate of the turnover half-life of the different complexes identified, however, given that for the mice in the main experiment only a single time point is taken (4 months) this may not be possible. However, unless I am mistaken the chase experiment described in Figure S2, provides a very rough estimate of ~50 days.

Main point 2: Enrichment of long-lived proteins in the mitochondrial cristae membrane.
The data are supportive of this claim. However, in some places the way the manuscript is written appears to state the findings too absolutely. For example, lines 182-184 states "We found that in the cortex nearly half of the identified cristae proteins (45.1 {plus minus} 4.2%), and over a third of IBM proteins (34.9 {plus minus} 5.9%), persist for at least 4 months". This appears somewhat misleading as, unless I am mistaken the fractional abundance for the N14 labelled peptides is 3.8% in the heart and 11.7% in the cortex as stated in the text (line 143). This indicated that although there is persistence of some proteins for at least 4 months, the majority of the proteins identified have in fact turned over. The above sentence on lines 182-184 should be rewritten, "We found that in the cortex nearly half of the identified cristae proteins (45.1 {plus minus} 4.2%), and over a third of IBM proteins (34.9 {plus minus} 5.9%), can persist for at least 4 months". This is an important distinction that the authors should be careful to make throughout the manuscript.

Main point 3: Long-lived functional respiratory supercomplexes
It is unclear whether the data fully support this claim. Given the Fractional Abundance of old (i.e. 14N proteins) in the cortex supercomplexes is the statistical power of the activity data in Figure S3 sufficient to conclude function of the old proteins? For example, SC1 has a fractional abundance of around 6-15% depending on the constituent complex, if every old SC had zero activity you would expect a drop in total activity of 6-15%, would this level of activity drop show up as statistically significant from your data?

Main point 4: Limited exchange of subunits throughout the lifetime of individual cristae.
The data are supportive of this claim but not entirely clear as presented.

Page 9, line 225-226, the authors state that, "in cortical respirasomes (SC1), Complexes I and III were significantly enriched in old proteins, as compared to the di- and monomeric CI and CIII (Figure 3E)." However, although Figure 3E shows significant enrichment for old CI proteins between SC1 and free CI, it does not show significant enrichment for old CIII proteins between SC1 and dimeric CIII2, only between SC1 and SC3 for CIII proteins.
For the crosslinking data it is unclear whether intralinks, which are most likely self-links within a single molecule, are counted when calculating "homo-isotopic" ratios.

Although it is good to see the crosslinks between subunits of the ATPase, it is surprising not to see more crosslinks between subunits of other know complexes (i.e. between subunits of complex I, complex III2 or complex IV), while at the same time observing crosslinks between complexes that have been established not the tightly interact, such as complex I and complex II (Ndufa10-Sdhb) or complex V and complex II (Atp5a1-Sdha) or complex I and complex V (Ndufa2-Atp5c1). The authors should elaborate on the identified crosslinks and why they think they are real given what we know about the mitochondrial complexes and the cristae architecture.

Minor edits
• The Y-axis label in Figure 1D, E and F, as well as, supplementary figure 1G of has the dividing line in the superscript
• Line 156, the sentence starting with "Hence, represent..." lacks an subject.
• Line 157, "The IMM encloses mitochondrial matrix..." should be "The IMM encloses the mitochondrial matrix..."

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, Bomba-Warczak and Savas use metabolic pulse-chase labeling followed by mass spectrometry analysis to identify long-lived proteins in mouse and rat tissues. Using a very similar approach, Savas and others have previously shown that nuclear pores are long-lived complexes with slow but finite turnover of their individual subcomplexes1,2. The current study now demonstrates that in addition to nuclear pore complex proteins, a subset of the mitochondrial proteome is also long-lived in tissues harboring post-mitotic cells. Other studies have previously identified long-lived mitochondrial proteins that can persist for several days3,4. However, this study is nonetheless interesting because it identifies numerous mitochondrial proteins that persist for months. The results suggest that long-lived mitochondrial proteins are primarily found in high-ordered complexes residing in cristae and the authors hypothesized that the exceptional longevity of some mitochondrial proteins help stabilize cristae architecture.

A major concern with this study is that spectra from long-lived mitochondrial proteins seem far less abundant in the 15N-labeled pulse chase experiment carried out in rat than in the 14N-labeled pulse chase experiment executed in mice. For example, in the rat, the protein UQCRC1 contained only seven 15N spectra (old) out of 494 total spectra while the same protein in mice contained 81 14N spectra (old) out of 143 total spectra. That difference is substantial. Is it species dependent or does the MS analysis favor the detection of 14N over 15N peptides? It seems important to perform a more thorough comparison of the rat and mice samples.

If the authors can explain this important issue, then the evidence related to the extreme longevity of some mitochondrial proteins might be more persuasive. However, the idea that high-ordered complexes experience little to no subunit exchange throughout their lifespan is not adequately substantiated. The results are agglomerated at the high-order levels and are not readily interpretable at the subunit and/or subcomplex levels. This is obviously a major concern since a study has just recently shown that the N-module of mitochondrial complex I is turned over at a higher rate than the rest of the complex5. While this information might be buried within the datasets it has not been successfully analyzed and presented. On a related note, in their interpretation of Figure 2 E and F, the authors omitted to highlight that complexes which assemble into supercomplexes (SC) have different FA values. For example, the mean FA of CIII is almost twice the FA of CI and CIV complexes in SC1 and SC2. This suggests that complexes might be turned over at different rate within a SC. This observation goes against the authors' claim that "the proteins assembled into SCs are preferentially retained together for at least 4 months in cortical and heart tissues" (lines 231-232). This considerably changes the interpretation of the results.

Moreover, only a fraction of the entire ETC subunits have been detected and/or quantified in table 1. Studying the longevity of large protein complexes requires a better coverage of their numerous subunits. Analyzing enriched mitochondrial fractions, peptide fractionation, and data-independent acquisition are options that might be considered. The MS analysis of BN-PAGE gel bands provided better coverage but the longevity of individual proteins was not properly summarized and represented.

In addition, the following comments require attention.

1- Previous studies using similar approaches, including studies from Dr. Savas, did not report (or reported very few) long-lived mitochondrial proteins1,2. In fact, none of the 37 long-lived proteins previously identified in metabolically labeled rat brains after a 6 months chase by Savas et al. in 2013 were localized to mitochondria2. However, using a very similar methodology the current study suggests that long-lived proteins in {greater than or equal to} 4 months old mouse brain are specifically enriched for mitochondrial proteins. The authors need to specify what changes were made, procedural, analytical, or otherwise, to allow the identification of such a high number of previously uncharacterized long-lived proteins in mitochondria. Interestingly, previous studies used a 15N- and not a 14N-labeled pulse chase which revives the concern raised earlier.

2- There is a high correlation between the abundance of total (14N+15N) and 14N spectra. This could indicate that the approach is biased towards more abundant proteins. If that were the case, long-lived mitochondrial proteins would be necessarily more likely to be detected in highly energetic tissues with higher mitochondrial content, such as brain and heart. As the authors noted, supplemental figure 1A&B suggests that "the inter-tissue differences in the longevity of mitochondrial proteins were not due to disproportional identification of mitochondrial proteins" (lines 121-122). However, it would be important to complement this analysis with a comparison of the identified long-lived proteins (mitochondrial and not) across different tissues. Are proteins identified

as long-lived disproportionally represented across different tissues?

3- On a similar note, it is worth noting that abundant cytoskeleton proteins not typically associated with long half-life (e.g. tubulin, actin) were identified has long-lived in this study, especially in the cortex. Also, spectra matched to keratin components originated almost exclusively from 14N peptides in the cortex. The authors should comment on the exceptional longevity of cytoskeleton proteins, particularly in the cortex.

4- If I am not mistaking, the sums of 14N and 15N spectra found in the cortex as presented in supplemental table 1 are respectively 14018 and 33649 while the values shown in figure 1 are 14209 and 31196. This is a slight anomaly but it seems important to identify/explain all inconsistencies.

5- The shaded portions of Figure 2C&D should also be visualized as cluster heatmaps that include all subunits within each protein complexes. That would be more informative than plotting the average of 14N spectra over all proteins identified in a complex. Cluster heatmaps might reveal subunits/subcomplexes that have different longevity profiles than the rest of the complex and would help visualize the number of subunits that are represented within a complex.

6- It is not clear how the numbers for table 1 were calculated. For example, I randomly picked protein NDUFA10 and tried to calculate the percentage of 14N spectra in the cortex based on the following data found in Suppl table 2:

Biological replicate 15N 14N
1 2 7
2 1 4
3 3 7
4 1 1
5 5 0

That should amount to an average 14N+15N of: 6.2 {plus minus} 3.27 i.e. (9+5+10+2+5)/5 and a mean percentage of 14N spectra of: 55.56 {plus minus} 33.23 %. However, the numbers reported were 5.2 {plus minus} 4 and 69.4 {plus minus} 12. That is puzzling and requires some explanation.

7- Figure 2E&F requires additional technical information. It is extremely difficult to perform high resolution protein profiling in native gel accurately and reproducibly, especially for supercomplexes (SCs) which demonstrate limited spatial resolution. Without thorough technical details and a schematic illustrating the location of the 12 bands along the gradient, it is impossible to determine whether the SCs, especially SC1 and SC2, were successfully resolved. Also, bar graphs are not sufficient to visualize such complex datasets. It would be beneficial to also use heatmaps that display the different subunits of a complex across the gel gradient5,6.

8- Still regarding Figure 2E&F, it is unclear what the dots represent. According to the legend, "each dot represents an FA value for an individual protein in a relevant complex" (line 540). However, there are more dots than the number of proteins within each complex. For example, CIII contains 11 proteins but I can discern far more than 11 dots in the "Complex III" panels. Are those dots the FA values of all replicates? If that is the case, then the dots should rather illustrate the average of all replicates. Also, the statistical analysis should be performed on average FA values otherwise it erroneously inflates the sample size.

9- While I appreciate the effort that went in the DSSO crosslinking experiment, I am not sure that such a small number of crosslinked peptides adequately demonstrate that mitochondrial cristae proteins have limited exchange or mixing. To reach such a conclusion would require a much larger number of crosslinked peptides covering several proteins spanning multiple modules/subcomplexes. It is also important to note that the approach identified several protein-protein interactions that have not been validated and might therefore represent artefacts.

Minor points:

- The visualization of the GO terms enrichment analysis is too reductive and cannot be interpreted properly without the supplemental tables. All enriched terms are collapsed down to only 3 categories (mitochondria, nucleus, others). It would be more informative to visualize which terms were enriched in each category.

- "Interestingly, the 14N-content in mitochondrial proteins in olfactory bulb extracts, a brain region known for adult neurogenesis and thus a lower density of old cells, was significantly lower compared to cerebellum, midbrain, and striatum (Altman, 1969; Carleton et al., 2003) (Figure S1G, Table S1)". If that was the case, shouldn't the 'lower density of old cells' in the olfactory bulb also affect the proportion of nuclear/chromatin long-lived proteins?

- Fig 2F: FA axis for CI, CIII, and CIV graphs should be on the same scale.

- The smoothing of the extracted chromatograms seems excessive.

References:

1. Savas, J. N., Toyama, B. H., Xu, T., Yates, J. R. & Hetzer, M. W. Extremely Long-Lived Nuclear Pore Proteins in the Rat Brain. Science 335, 942-942 (2012).
2. Toyama, B. H. et al. Identification of Long-Lived Proteins Reveals Exceptional Stability of Essential Cellular Structures. Cell 154, 971-982 (2013).
3. Fornasiero, E. F. et al. Precisely measured protein lifetimes in the mouse brain reveal differences across tissues and subcellular fractions. Nat. Commun. 9, 4230 (2018).
4. Heo, S. et al. Identification of long-lived synaptic proteins by proteomic analysis of synaptosome protein turnover. Proc. Natl. Acad. Sci. 115, E3827-E3836 (2018).
5. Szczepanowska, K. et al. A salvage pathway maintains highly functional respiratory complex I. Nat. Commun. 11, 1643 (2020).
6. Guerrero-Castillo, S. et al. The Assembly Pathway of Mitochondrial Respiratory Chain Complex I. Cell Metab. 25, 128-139 (2017).

1st Revision - Authors' Response to Reviewers: May 6, 2021

Reviewer #1 (Comments to the Authors (Required)):

SILAC based proteomics in mice and significant extension of the labelling/feeding time with isotopes has allowed identifying very long-lived proteins (LLPs), in particular in chromatin and the nuclear pore complex. Here, the authors have analyzed the mitochondrial proteome using this approach and identify LLPs enriched in cristae membranes of post-mitotic tissues of mice. Combining LC-MS/MS based proteomics with BN-PAGE and crosslinking approaches, evidence is provided that LLPs are enriched in higher order assemblies of respiratory complexes and that some complexes, such as MICOS and the ATP synthase, are shown to be very stable and characterized by low exchange rates between newly synthesized and old subunits. The authors propose that longevity of cristae shaping proteins ensures long-term stabilization of mitochondrial cristae. This manuscript convincingly identifies LLPs in mitochondria and demonstrates a striking stability and low subunit exchange rate of some protein complexes in the IMM. While these findings are compelling, the novelty of the findings is somehow limited by the lack of a time-resolved analysis, which precludes the determination of half-lives in vivo. Extremely long half-lives of mitochondrial proteins have already been described by Fornasiero et al, 2018, including proteins identified here. The observation that LLPs are enriched in large complexes represents therefore the most novel finding.

We thank Reviewer #1 for the positive feedback and careful review of our manuscript. Regarding the questions of novelty - we agree that our conclusions are broadly in line with findings presented by Fornasiero et al. 2018, however, there are important distinctions between the studies which are addressed below.

We extracted the mitochondrial proteins identified as LLPs by Fornasiero et al. and compared them directly to our findings (Fig. 1, just below).

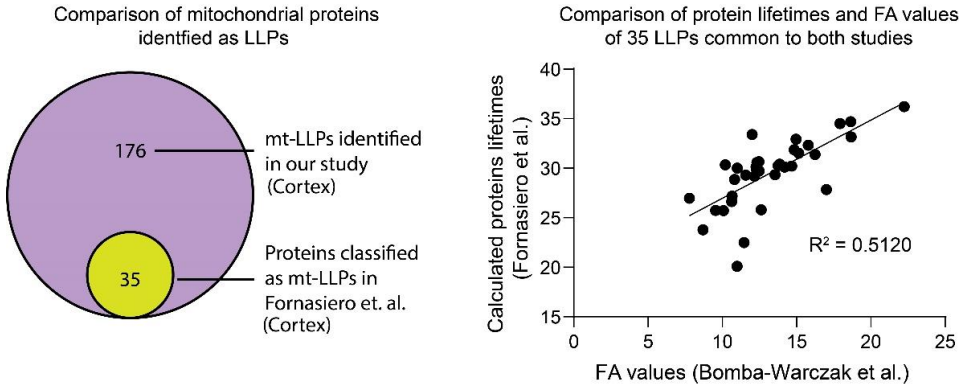


Figure 1. Comparison of mt-LLPs identified in our study versus Fornasiero et al.

Fornasiero et al. 2018 classified a total of 35 mitochondrial proteins as LLPs, i.e. proteins in the 95th to the 98th percentile of the proteome in terms of stability. We confirmed all 35 of the mt-LLPs they reported and identified an additional 176 mt-LLPs in mouse cortical extracts and 200 mt-LLPs in heart (Fig. 1, left). The protein lifetimes reported in Fornasiero et al. 2018 and the FA values reported here loosely correlate (Fig.1, right). In the revised manuscript, we included this data in Table S1.

Secondly, to further highlight the differences between the two studies, we created a table highlighting our main points, as well as advantages and limitations of each method:

	Our study	Fornasiero et al.
Study goal	Identify and characterize the rare pool of mitochondrial proteins with exceptionally long lifespans.	Create a comprehensive catalogue of protein half-lives with computational modeling.
Stable isotope and strategy	Direct identification of ^{15}N and ^{14}N proteins; well suited for monitoring old proteins. Cannot be reliably used for half-life determination. ^{15}N gradually incorporates into the polypeptide backbone and side chains of	$^{13}C_6$-lysine and mathematical modeling of lysine pools; well suited for protein half-life estimation. $^{13}C_6$-lysine incorporates into newly synthesized proteins. Proteins with lysine amino acid(s) will be either light or heavy.

	newly synthesized proteins. Only fully light and heavy proteins (all nitrogen atoms either ^{14}N or ^{15}N) can be identified with LC-MS/MS based proteomics. Cannot confidently identify chimeric proteins. Protein longevity is reported as FA value i.e. fractional abundance of protein pool which persists across pulse or chase periods.	Chimeric proteins, where both L-lysine and $^{13}C_6$-lysine are incorporated into the polypeptide chain, can be identified with LC-MS/MS and measured. Multistep mathematical modeling of lysine pools was used in order to fit experimental data and derive protein's half-life Not well-suited for determination of lifespans of proteins with ''extreme' rates of turnover (i.e. exceptionally short or long-lived proteins)
Labeling efficiency and period	~95% in the brain over 120 days of pulse-labeling. An experimental design well suited for measuring long-lived proteins. After this extended pulse period the majority of the proteome will be turned-over. In effect, by using this method two predominant protein pools are present: newly synthesized (^{15}N) pool, and old (^{14}N) protein pool, which encompasses proteins that persisted for at least 120 days. The ^{14}N (i.e. old) pool of proteins are of interest.	Low level of label incorporation. Two relevant experiments (1) estimated protein half-lives obtained from a short (< 21 days) low level pulse labeling experiments with mathematical modeling, and (2) measured protein lifetimes from a pulse 30 and 60 day more robust labeling periods. ** This is an important difference since 71.8 % of the mitochondrial protein lifetimes increased between the 30 and 60 day pulse-labeling, suggesting that labeling duration can influence apparent protein (Table S16 in Fornasiero et al.).*
Tissue extraction and input	Cortical and Heart tissues. Crude membrane fractions, membrane extracts separated by BN-PAGE, and immuno-captured mitochondria.	Cortical tissue. - Homogenates. - Crude membrane fractions.

We admit that in the first submission, we failed to properly emphasize these points. In the revised manuscript, we have addressed this issue as follows.

1. Provided an improved description our study's goal (in Introduction and Discussion).
2. Extended and improved the coverage of mitochondrial proteome by immuno-capturing method of mitochondria isolation with LC-MS/MS analysis (new data in Figure 2, 3 and 4).
3. Included a comparison of our findings and those published by Fornasiero et al (Table S2, last column).
4. Included an extended pulse-chase experiment where we pulse-chased mice for 0, 2, 4, and 6 months (new data in Figure S2, Table S3).

Concerning this point and the interpretation of data, however, I have some concerns that the authors should consider:

1. It remains unclear why the authors focus their interpretation on long-lived cristae shaping proteins such as MICOS and ATP synthase and propose a long-term stabilization of cristae. First, other membrane shaping proteins such as OPA1 are short-lived and recent high-resolution life cell imaging data suggest dynamic behavior of cristae. Second, the identified LLPs (Table 1) also include many respiratory chain subunits in the IMM (which can be expected in post-mitotic tissues).

The Reviewer raises several important points that we have addressed with modifying the figures and text, and expanding our data analysis.

First, the Reviewer is critical of our focus on "long-lived cristae shaping proteins". We apologize for this misunderstanding. We did not intend to suggest that only cristae-shaping proteins are LLPs, and after reviewing our initial submission, we admit we were not clear in our message. In fact, the identification of many additional respiratory chain subunits, as pointed out by the reviewer, along with many other IMM associated proteins (as shown in new Figures 2 and 3) suggest that not only cristae-shaping proteins that are LLPs, but rather that, most mt-LLP are associated with inner mitochondrial membranes and cristae. We have corrected the text throughout to clarify this point.

Furthermore, in the revised manuscript we expanded our analysis and provided new unbiased bioinformatics of the mt-LLPs, all of which support our conclusion that long-lived proteins are enriched at the IMM and cristae:

(1) New GO analysis in Figure 1, which illustrates terms associated with IMM compartments as significantly enriched in brain and heart.
(2) MitoCarta3.0-based sub-compartment analysis of mt-LLPs which shows enrichment of long-lived proteins in IMM (Figure 2).
(3) MitoCarta3.0 MitoPathway analysis which shows that pathways associated with IMM and cristae selectively harbor mt-LLPs (Figure 3).

Second, we propose that the fact a protein is long-lived does not mean to suggest that it cannot participate in "dynamic behaviors". For example, MICOS complexes in cortical mitochondria might be composed in 10% of old proteins (> 4 months old), and still mediate cristae membrane cycling (if that is its function). On balance, in both our and Fornasiero's results indicate that OPA1 is a relatively long-lived protein. Fornasiero et al. reported that the half-live of Opa1 in the cortex is 17.06 days, which is higher than an average protein in cortex (i.e. 10.70 days).

2. BN-PAGE combined with proteomics suggests that LLPs are enriched in higher order assemblies of respiratory chain complexes such as respirasomes. There is some debate about the relative distribution of monomeric/dimeric complexes and respirasomes in vivo. Do the presented result imply that the ratio between different assemblies remains constant over time?

We thank the reviewer for the on-point comment. Our experiments nor results aim to address questions of whether the abundance of assemblies remain constant during development or aging. In our preliminary studies, we have done some work on the abundance of ATP-synthase complexes in mouse brains at different ages (Figure 2). Based on these results, we are inclined to say that the ratios of the complexes do change with age, at least in the brain.

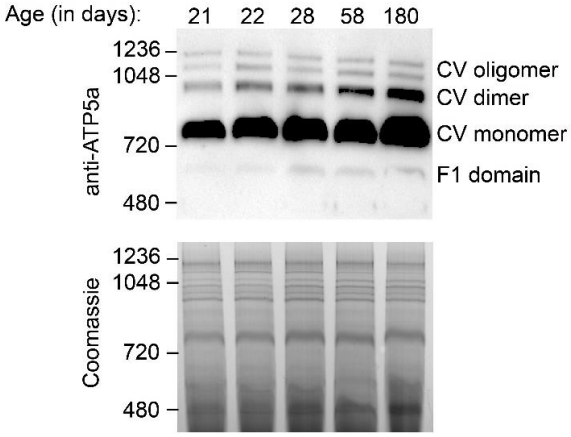


Figure 2. Abundance of ATP-synthase complexes in mouse brains at different ages.

However, by following this and other suggestions from the Reviewers, we have extended the BN-PAGE analysis and come to the conclusion that mt-LLPs are evenly distributed throughout the higher order assemblies of respiratory chain complexes. This new data is presented in Figure S3.

3. The BN-PAGE experiments were not analyzed in depth on the protein level, but the stability of individual subunit would be very informative.

We thank the reviewer for this comment and we apologize for the lack of clarity in our data presentation.

In the initial submission, the protein level analysis of BN-PAGE experiments and the stability of individual OXPHOS subunits were included in Supplemental Table 2. In retrospect, we agree that it would have been more informative and transparent to include this in-depth analysis in the main text and figures in lieu of the excel spreadsheet.

Therefore, in the revised manuscript we have included an extended analysis of individual subunits identified in our BN-PAGE data in heat maps. This new data is now included in Figure 3S.

4. Why are only some subunits of multi-subunit assemblies identified as LLPs? Does this suggest that sub-structures rather than the assembled complexes are extra-ordinarily stable?

We thank the reviewer for this comment.

In the revised manuscript, we extended our data analysis to immuno-captured mitochondria, which increased the coverage of mitochondrial proteome by 3-fold (Figure S2A-B). Thus, we were able to provide a more complete coverage of mitochondrial proteome and yes - we can now state that entire complexes (not just sub-complexes), are extra-ordinarily stable (data now presented in Figure 3).

The authors should discuss their findings in light of reports in the literature demonstrating different turnover rates for the N-module of complex I.

We agree and thank the Reviewer for this constructive comment. In the revised manuscript, we include new data analysis in which demonstrates differences in FA values for several complex I modules (Figure 3). Additionally, we added heat-maps representing individual subunits of OXPHOS complex proteins based on BN-PAGE, which also confirms that FA values are lower for N-module than for the rest of the complex (Figure S3).

5. The authors argue based on BN-PAGE in gel assays that respiratory complexes harboring LLPs are fully active. However, it is questionable if the used assay for complex activity is sensitive and accurate (error bars) enough to detect functional differences considering that old proteins correspond to approx. 10-12% in the cortex or other tissues.

We agree with the reviewer's point and appreciate the validity of this concern. Our thinking was that if 10% of the complex is dramatically impaired, the hope was that we would be able to confidently detect a difference. However, that was not the case and due to this ambiguity, we have removed this inconclusive data from the manuscript.

6. How does protein abundance affect the identification of LLPs? As discussed by Fornasiero et al. (2018), LLPs appears to be often abundant proteins.

We thank the reviewer for raising this important question.

Yes, as with all proteomic-based analyses, protein abundance does have a bearing on the identification and quantitation of individual proteins. Reliable identification of low-abundance proteins remains a limitation in the field and this holds true for our analysis as well. Indeed, in general, we identify more ^{14}N spectral counts from abundant proteins.

However, and more importantly: **not every high-abundance protein is, by default, a long-lived protein, and not every low-abundance protein is short-lived** (Figure 3, left)**.**

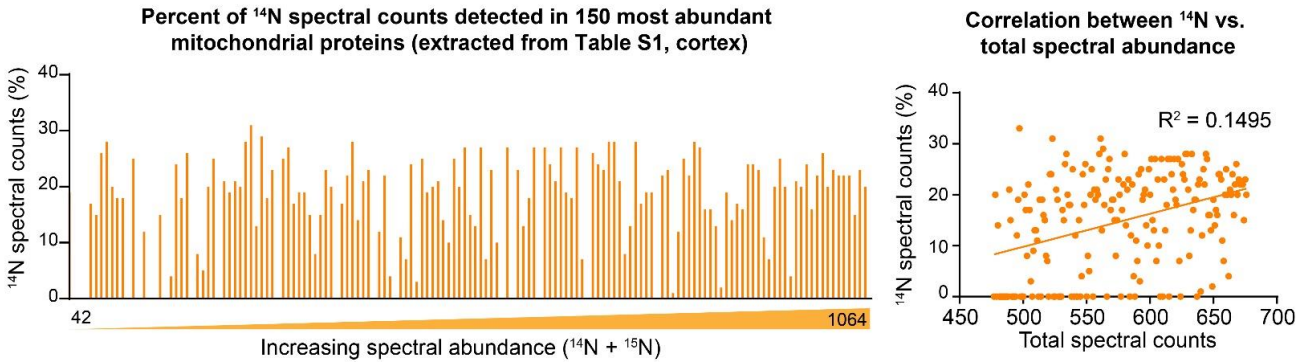


Figure 3. Comparison of protein abundance based on spectral counts.

Here we show the percent of ^{14}N-spectral counts (i.e. ^{14}N spectra # / (^{14}N+^{15}N spectra #)*100) for the top 150 most abundant mitochondrial proteins, rank ordered according to number of total identified spectral counts ranging from 42 to 1064. As illustrated, for many of the highly abundant proteins, percent of ^{14}N-spectra counts are low and for some even zero, indicating that in spite of being abundant, these proteins are not LLPs. Similarly, the correlation between ^{14}N-

spectral counts and total spectral abundance for the top 150 most abundant mitochondrial proteins is low, with an R^2=0.1495 (Figure 3, right).

7. In contrast to the statement in the text, it seems there is not just a single heavy-light protein crosslink between two mitochondrial proteins: Atp5c1 and Ndufa2. There would be further explanation required why the authors think that the crosslinking approach supports the idea that `proteins reside in the same cristae are co-preserved with little to no subunit exchange for months in both heart and brain´ (line 296).

We thank the Reviewer for this fair comment. The limited coverage of mitochondrial interactome was a concern raised by all three Reviewers. We are happy to report that we have made substantial improvement in our cross-linking methods and data analysis, which increased the number of identified cross-links by 10-fold in the heart tissue and allowed us for an in-depth analysis of interactions between old and new proteins. This advancement was primarily driven by crosslinking immuno-captured mitochondria rather than crude membrane fractions.

Here we provide a side-by-side comparison of old and new data, highlighting the dramatic improvement in the number of cross-links identified (Figure 4).

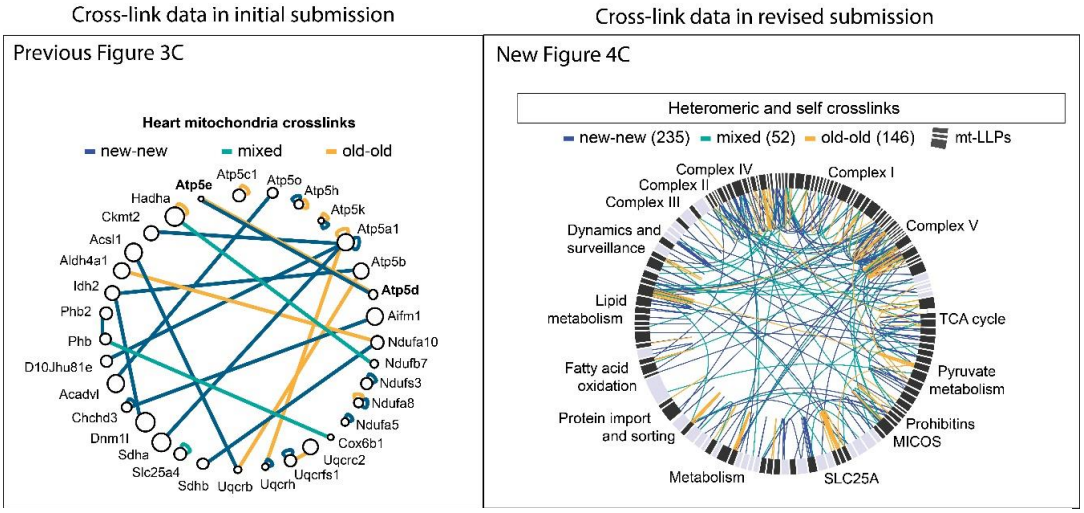


Figure 4. Comparison of crosslinking results from previous and current submissions.

In the revised manuscript the expanded cross-link data is presented in Figures 4, 5, S4, and S5.

To recap the new data, with a nearly 10-fold increase in the number of identified cross-links in the dynamically labeled heart extracts, we were able to clearly delineate the following:

1. Vast majority (i.e. 94.3%, or 866/919) of the identified cross-links in heart mitochondria were “homo-isotopic”, meaning between two old or two new peptides, suggesting limited mixing of old and new proteins.
2. Mixed “hetero-isotopic” cross-links made up only 5.8% (53/919) of the entire cross-link population. Out of those, only 12 cross-links were identified between or within OXPHOS complexes, indicating that these complexes are stable and have restricted subunit exchange.
3. The majority of the identified mixed cross-links were between proteins within an OXPHOS complex and other mitochondrial proteins (e.g. Atp5a1 and Hadha), and in a few other rare cases between two non-OXPHOS proteins (e.g. Mpc2 and Idh3a).

Based on these results we hypothesize that many mt-LLPs are co-preserved together. The reasoning behind this hypothesis is that if mt-LLPs were randomly scattered throughout mitochondria and mitochondrial networks, we would expect the majority of cross-links to be of mixed nature, i.e. between the old LLP and other new proteins. However, our

cross-linking analysis we found the opposite - nearly all cross-links are formed between either two old or two new proteins.

8. The authors should discuss their findings in light of available turnover rates for mitochondrial proteins and improve the description of their experiments in figure legends and method section.
- The total number of proteins detected should be provided in absolute numbers for each experiment. Also provide the coverage of mitochondrial proteins as well as complex subunits (for example judged based on MitoCarta).

We now clearly state the number of proteins identified in each figure panel, as well as coverage based on MitoCarta3.0.

- In Figure 2EF, each dot represents an FA value for an individual protein. The reviewer tried to find the respective protein values in the supplementary table 3. There are 4 biological replicates and 10 bands but the reviewer was not able to reproduce the bar plots.

We thank the reviewer for this valid comment and apologize for lack of clarity. In the initial submission, the FA values listed in Supplementary Table 3 corresponded to "^{15}N-remaining" values (Toyama et al., 2013), whereas in the figure we plotted "^{14}N-remaining" values. ^{14}N-remaining is calculated as follows [100] – [^{15}N-remaining"].

In the revised manuscript we have corrected this error and presented the data in a consistent manner throughout the manuscript.

- Figure 1B - Are these experiments based on one biological replicate? Please clarify.

Yes, in the initial submission data presented in Figure 1B were based on one biological replicate. In the revised manuscript we extended this analysis to 3 biological replicates (i.e. mice) per tissue. New data is included in Figure 1C-E.

- The GO analysis is missing in the method section. It is necessary to provide information about the background that was used to find enriched proteins.

Thank you for this comment and we apologize for the omission in the methods section. In the revised manuscript, we included the following text:

"Gene Ontology Analysis

GO analysis was performed using the Pantherdb (Mi et al., 2019). The "query" is defined as proteins identified as long-lived in the analyzed tissue (based on ^{14}N-peptide identification), and the reference is defined as all proteins identified in the same tissue analyzed (^{14}N and ^{15}N-peptide identification)."

- Since ^{14}/^{15}N Spectra on MS^{1} level are not as easy as illustrated in Figure 1A (workflow), please show at least for one LLP the MS1 spectra and indicate light and heavy peaks.

We have now included representative raw annotated MS^{1} spectra for ^{14}N and ^{15}N peptides mapping to three proteins – histone H4, Atp5a, and Lrpprc – from the cortex, heart, and spleen. The reasoning behind the choice of these proteins is that Histone H4 is an LLP in all three tissues, Atp5a is an mt-LLP in cortex and heart, but not spleen, and Lrpprc is not long-lived in any of the tissues. This new data is included in Figure S1A.

Reviewer #2 (Comments to the Authors (Required)):

In their manuscript "Long-lived cristae proteins as pillars of mitochondrial architecture in post-mitotic cells" Bomba-Warczak and Savas demonstrate that a subset of the mitochondrial proteome persists for months in long-lived post-mitotic cells. These long-lived proteins localize to cristae membranes. Specifically, they show that:
1. Tissue differences in the longevity of the mitochondrial proteome
2. Enrichment of long-lived proteins in the mitochondrial cristae membrane
3. Long-lived functional respiratory supercomplexes in brain
4. Limited exchange of subunits throughout the lifetime of individual cristae
Overall the manuscript is well written and clear. This manuscript advances the field of mitochondrial physiology by

tracking the turnover of the mitochondrial proteome in various tissues. This leads to the surprising result that in post-mitotic tissues respiratory complexes, especially the ATP synthase complex are long lived and can remain intact for over four months.

We thank Reviewer 2 for the assessment and supportive feedback.

Main point 1: Tissue differences in the longevity of the mitochondrial proteome.
The data are strongly supportive that mitochondrial proteins are capable of lasting for over four months in post mitotic tissues. However, the fractional abundance of these long-lasting proteins is 10%-15% indicating the majority of proteins are turned over. The manuscript would be strengthened by an estimate of the turnover half-life of the different complexes identified, however, given that for the mice in the main experiment only a single time point is taken (4 months) this may not be possible. However, unless I am mistaken the chase experiment described in Figure S2, provides a very rough estimate of ~50 days.

The Reviewer raises several important points which we would like to address.

First, we acknowledge the request for additional protein turnover measurements, since in our first submission most of our data was from a single time point. In the revised manuscript we provide results from additional pulse-chase experiments in mice across a broader chase period and provide protein lifetime measurements after 0 ,2, and 6-months of chase (Figure S2 and Table S3).

Our ^{15}N-metabolic labeling approach is specifically designed for studying proteins with exceptionally long-lifespans and cannot be reliably used for calculating protein half-lives. Precise half-life measurements require extensive mathematical modeling since there are several different protein pools *in vivo*, along with the ability to measure partially labelled peptides. In our experiments we can only identify fully light or heavy peptides. Please note that, comprehensive measurements of protein half-lives have been reported by the Rizzoli group in 2018 (Fornasiero et al., 2018). In the revised manuscript, we have appended their findings on mt-LLPs to our own data, which can be found in Table S1, last column.

Lastly, to make our point more complete, we including a table comparing our method with that which was used by Rizzoli group. We are hoping that this side-by-side view will help in clarifying our stance.

	Our study	Fornasiero et al.
Study goal	Identify and characterize the rare pool of mitochondrial proteins with exceptionally long lifespans.	Create a comprehensive catalogue of protein half-lives with computational modeling.
Stable isotope and strategy	Direct identification of ^{15}N and ^{14}N proteins; well suited for monitoring old proteins. Cannot be reliably used for half-life determination. ^{15}N gradually incorporates into the polypeptide backbone and side chains of newly synthesized proteins. Only fully light and heavy proteins (all nitrogen atoms either ^{14}N or ^{15}N) can be identified with LC-MS/MS based proteomics. Cannot confidently identify chimeric proteins. Protein longevity is reported as FA value i.e. fractional abundance of protein pool which persists across pulse or chase periods.	$^{13}C_6$-lysine and mathematical modeling of lysine pools; well suited for protein half-life estimation. $^{13}C_6$-lysine incorporates into newly synthesized proteins. Proteins with lysine amino acid(s) will be either light or heavy. Chimeric proteins, where both L-lysine and $^{13}C_6$-lysine are incorporated into the polypeptide chain, can be identified with LC-MS/MS and measured. Multistep mathematical modeling of lysine pools was used in order to fit experimental data and derive protein's half-life Not well-suited for determination of lifespans of proteins with ''extreme' rates of turnover (i.e. exceptionally short or long-lived proteins).

Labeling efficiency and period	~95% in the brain over 120 days of pulse-labeling. An experimental design well suited for measuring long-lived proteins. After this extended pulse period the majority of the proteome will be turned-over. In effect, by using this method two predominant protein pools are present: newly synthesized (^{15}N) pool, and old (^{14}N) protein pool, which encompasses proteins that persisted for at least 120 days. The ^{14}N (i.e. old) pool of proteins are of interest.	Incomplete label incorporation. Two relevant experiments (1) estimated protein half-lives obtained from a short (< 21 days) low level pulse labeling experiments with mathematical modeling, and (2) measured protein lifetimes from a pulse 30 and 60 day experiments. ** This is an important difference since 71.8 % of the mitochondrial protein lifetimes increased between the 30 and 60 day pulse-labeling, suggesting that labeling duration can influence apparent protein lifetimes (Table S16 in Fornasiero et al.).*
Tissue extraction and input	Cortical and Heart tissues. Crude membrane fractions, membrane extracts separated by BN-PAGE, and immuno-captured mitochondria.	Cortical tissue. - Homogenates. - Crude membrane fractions.

Main point 2: Enrichment of long-lived proteins in the mitochondrial cristae membrane.
The data are supportive of this claim. However, in some places the way the manuscript is written appears to state the findings too absolutely. For example, lines 182-184 states "We found that in the cortex nearly half of the identified cristae proteins (45.1± 4.2%), and over a third of IBM proteins (34.9 ± 5.9%), persist for at least 4 months". This appears somewhat misleading as, unless I am mistaken the fractional abundance for the N14 labelled peptides is 3.8% in the heart and 11.7% in the cortex as stated in the text (line 143). This indicated that although there is persistence of some proteins for at least 4 months, the majority of the proteins identified have in fact turned over. The above sentence on lines 182-184 should be rewritten, "We found that in the cortex nearly half of the identified cristae proteins (45.1 ± 4.2%), and over a third of IBM proteins (34.9 ± 5.9%), can persist for at least 4 months". This is an important distinction that the authors should be careful to make throughout the manuscript.

This is point is well taken. In the revised manuscript we have taken great care to ensure the description of our results are not overstated. We apologize, in hindsight it's also clear that we did not present or describe our data on protein longevity in an understandable manner. In the revised manuscript, we have completely reworked Figures 1-3 to avoid this pitfall.

First, to simplify the narrative and to avoid confusion between spectra, peptide, and protein-based measures, we removed FA values from Figure 1. In this way - Figures 1 and 2 are now based solely on the identification of long-lived proteins (based solely on ^{14}N-peptide identification with MS/MS, no quantification or FA value measurements) and a description of their associated GO terms across tissues. Subsequently, FA values are reserved exclusively for Figure 3 and S3. To further improve the clarity of the message, in the revised manuscript we removed all of the data analysis based on ^{14}N spectra counts (previously shown in Figure 2) and replaced all relevant analysis with either protein identifications or FA values.

Main point 3: Long-lived functional respiratory supercomplexes
It is unclear whether the data fully support this claim. Given the Fractional Abundance of old (i.e. ^{14}N proteins) in the cortex supercomplexes is the statistical power of the activity data in Figure S3 sufficient to conclude function of the old proteins? For example, SC_1 has a fractional abundance of around 6-15% depending on the constituent complex, if every old SC had zero activity you would expect a drop in total activity of 6-15%, would this level of activity drop show up as statistically significant from your data?

We agree with the Reviewer and acknowledge the validity of this concern. Our thinking was that if 10% of the complex is dramatically impaired (i.e. the mt-ELLPs) we may be able to detect a difference in this "bulk" fractional assay. However, we do not observe that phenomena and agree the results are inconclusive, thus, we have removed this data of unclear importance from the revised manuscript.

Main point 4: Limited exchange of subunits throughout the lifetime of individual cristae.
The data are supportive of this claim but not entirely clear as presented.

We thank the reviewer for this comment. In the revised manuscript, we expanded the datasets and re-worked our figures in hope of providing a clearer understanding of mt-LLPs. New data are presented in updated Figures 4, 5, S4, and S5.

Page 9, line 225-226, the authors state that, "in cortical respirasomes (SC1), Complexes I and III were significantly enriched in old proteins, as compared to the di- and monomeric CI and CIII (Figure 3E)." However, although Figure 3E shows significant enrichment for old CI proteins between SC1 and free CI, it does not show significant enrichment for old CIII proteins between SC1 and dimeric CIII2, only between SC1 and SC3 for CIII proteins.

We apologize for the ambiguity and appreciate this on-point criticism. Per requests from all three reviewers, we performed more in-depth BN-PAGE analysis of OXPHOS complexes in cortical mitochondria by increasing the number of gel slices analyzed with LC-MS/MS from 12 to 40. By doing so, we increased the resolution of higher-order complexes and, we must admit, we found that the differences in LLP enrichment in higher-order complexes became negligible. Therefore, in the revised manuscript we stepped away from the conclusion that LLPs are enriched in higher-order complexes. We have modified the figures and text to accommodate these findings and new data is now presented in Figure S3.

For the crosslinking data it is unclear whether intralinks, which are most likely self-links within a single molecule, are counted when calculating "homo-isotopic" ratios.

The Reviewer raises an important point. Yes - in the first submission, intralinks were included in our calculation of “homo-isotopic” ratios. However, in the revised manuscript, we have corrected this issue by clearly delineating what type of a cross-link is considered at each step of data analysis. We divided the cross-links to three categories: interlinks (between two different proteins), self: non-overlapping peptides, and self: overlapping peptides (shown in Fig. 4C). In the revised manuscript, the reported crosslinks are further systematically delineated based on if they are formed between two ^{14}N peptides (i.e. “old-old”), between two ^{15}N peptides (i.e. “new-new”), or a between a ^{14}N and ^{15}N peptide (“mixed”) (Fig. 4F).

All three cross-link types are included in the combined isotopic ratio distribution shown in Fig. 4D, and these data are further delineated into separate types in Fig. 4F. We note that this data set has been extensively improved upon (as detailed in the next point, below), and we increased the number of identified cross-links in heart mitochondria by 10-fold.

In the revised manuscript, the new data is presented in Figures 4, 5, S4 and S5.

Although it is good to see the crosslinks between subunits of the ATPase, it is surprising not to see more crosslinks between subunits of other know complexes (i.e. between subunits of complex I, complex III2 or complex IV), while at the same time observing crosslinks between complexes that have been established not the tightly interact, such as complex I and complex II (Ndufa10-Sdhb) or complex V and complex II (Atp5a1-Sdha) or complex I and complex V (Ndufa2-Atp5c1). The authors should elaborate on the identified crosslinks and why they think they are real given what we know about the mitochondrial complexes and the cristae architecture.

This is an important concern, which was raised by all three Reviewers. We are happy to report that we have developed a new workflow, which allowed us to identify 10-fold more cross-links in heart mitochondria. The biggest limitation in our previous workflow was the heterogenous membrane extracts used for crosslinking that were only slightly enriched with mitochondria. We were able to improve upon this by cross-linking immuno-captured mitochondria using Miltenyi technology, as published by Fecher et al. (2019).

Below, we provide a side-by-side comparison of the previously included and revised figure panel, highlighting the dramatic improvement in the number of cross-links identified in the heart. In the revised manuscript, this new data is presented in Figures 4, 5, S4 and S5.

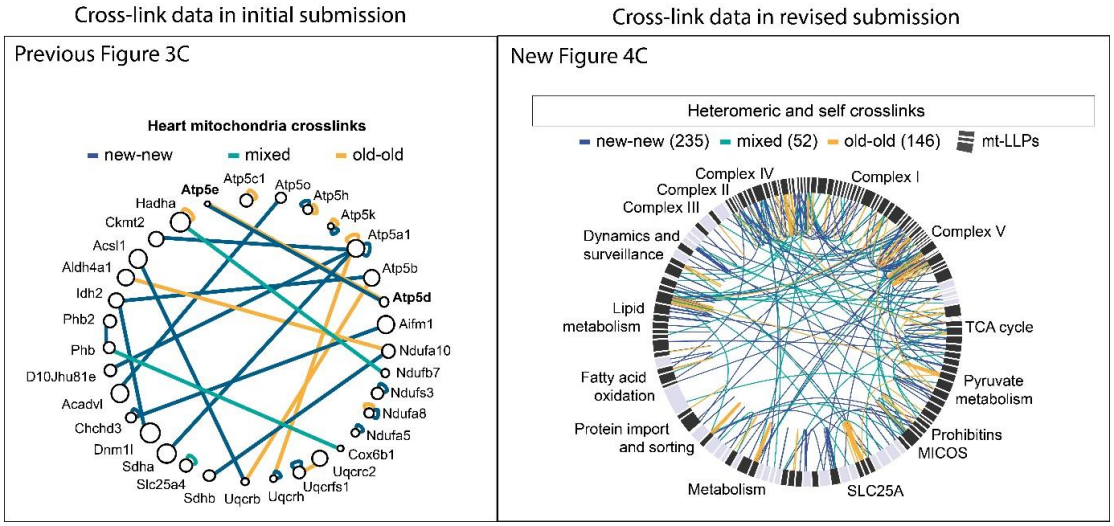


Figure 1. Comparison of identified cross-linked peptides from heart extracts reported in the previous and current submissions.

Minor edits

- The Y-axis label in Figure 1D, E and F, as well as, supplementary figure 1G of has the dividing line in the superscript
- Line 156, the sentence starting with "Hence, represent..." lacks an subject.
- Line 157, "The IMM encloses mitochondrial matrix..." should be "The IMM encloses the mitochondrial matrix..."

We thank the Reviewer for these suggested edits - we have now made these changes in the revised manuscript.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, Bomba-Warczak and Savas use metabolic pulse-chase labeling followed by mass spectrometry analysis to identify long-lived proteins in mouse and rat tissues. Using a very similar approach, Savas and others have previously shown that nuclear pores are long-lived complexes with slow but finite turnover of their individual subcomplexes [1,2]. The current study now demonstrates that in addition to nuclear pore complex proteins, a subset of the mitochondrial proteome is also long-lived in tissues harboring post-mitotic cells. Other studies have previously identified long-lived mitochondrial proteins that can persist for several days [3,4]. However, this study is nonetheless interesting because it identifies numerous mitochondrial proteins that persist for months. The results suggest that long-lived mitochondrial proteins are primarily found in high-ordered complexes residing in cristae and the authors hypothesized that the exceptional longevity of some mitochondrial proteins help stabilize cristae architecture.

We thank the Reviewer for the positive comments and their appreciation of our research. We must add that the comments and suggestions raised by Reviewer #3 played a key role in guiding our efforts to revise our manuscript and led to major improvements of our analysis, data presentation, and overall conclusions.

A major concern with this study is that spectra from long-lived mitochondrial proteins seem far less abundant in the ^{15}N-labeled pulse chase experiment carried out in rat than in the ^{14}N-labeled pulse chase experiment executed in mice. For example, in the rat, the protein UQCRC1 contained only seven ^{15}N spectra (old) out of 494 total spectra while the same protein in mice contained 81 ^{14}N spectra (old) out of 143 total spectra. That difference is substantial. Is it species dependent or does the MS analysis favor the detection of ^{14}N over ^{15}N peptides? It seems important to perform a more thorough comparison of the rat and mice samples. If the authors can explain this important issue, then the evidence related to the extreme longevity of some mitochondrial proteins might be more persuasive.

We acknowledge the Reviewer's point regarding the differences in the ratio of ^{14}N / ^{15}N spectral counts for some proteins reported in the rat versus mouse analyses. Please let us explain the apparent discrepancy between these two datasets.

First, to address the point of the MS analysis favoring detection ^{14}N over ^{15}N peptides. We would like to remind the reviewer that MS^1 spectra of ^{14}N and ^{15}N peptide are captured simultaneously in the same scans. However, peak selection for MS^2 is a stochastic process influenced by several factors including peak intensity, co-eluting peptides from other proteins, and instrument method settings such dynamic exclusion that are required for identification of low-abundance proteins. Taken altogether, ^{14}N and ^{15}N peptides are subjected to the same constraints and limitations during acquisition of the MS data.

However, in terms of quantitation, over the past 12 years working with ^{15}N-metabolic labeling of animals, we have observed a slight discordance in the measured abundance of ^{14}N versus ^{15}N-peptides. In fact, we recently reported this phenomenon in our analysis of Alzheimer's Disease mouse models and directly compared the long-lived proteins from ^{15}N-dynamically labelled mice with ^{15}N-pulse chased mice. In this way we confirmed the results with a "mirror control" Hark et al. (2021). In summary the same pool of proteins was identified in both paradigms, however the percentage of old protein remaining was greater in the ^{14}N proteins from the dynamic pulse experiment. To ensure rigor, in the revised manuscript we took the same strategy and provide measures of mitochondrial long-lived proteins from mice in both dynamic- pulse and pulse-chase analyses (Figure 2, S2, Table S2, and S3). Overall the identified mt-LLPs are nearly identical, however again the abundance of the old proteins was higher in the dynamic ^{15}N-pulse paradigm.

In addition to the points discussed above, the discrepancy between previously presented rat and mouse data can be further attributed to the intrinsic differences in the experimental designs.

1. Length of pulse/chase: 4 months for mice vs. 6 months for rat.
2. Reference protein database for mouse is more comprehensive and accurate than for rat (Hark et al., 2021; Savas et al., 2012).
3. The samples analyzed are not equivalent:
    a. Rat data was acquired from the whole brain homogenates, while mouse was from isolated cortex.
    b. Rat brain samples were measured as total homogenates, whereas for mouse samples were analyzed as crude membrane fractions, which are enriched with mitochondria.

Lastly, we note that, while we stand by the rat data presented in the initial submission, but in the interest of clarity and consistency, we decided to replace this data with new and more comprehensive analysis in mice.

However, the idea that high-ordered complexes experience little to no subunit exchange throughout their lifespan is not adequately substantiated. The results are agglomerated at the high-order levels and are not readily interpretable at the subunit and/or subcomplex levels. This is obviously a major concern since a study has just recently shown that the N-module of mitochondrial complex I is turned over at a higher rate than the rest of the complex 5. While this information might be buried within the datasets it has not been successfully analyzed and presented.

We apologize for the inadequate presentation of our data in the first submission. In the revised manuscript we present our data in a systematic and uniform format and used several new data visualization strategies to better convey our findings. For example, we included detailed heatmaps (Figure S3) and high-resolution protein models (Figures 3 and 5) illustrating the longevity of proteins at the subunit and sub-complex levels. The raw data is also included in Table S2.

Importantly, per requests from all three reviewers, we performed a more robust BN-PAGE based analysis of OXPHOS complexes and sub-complexes in cortical mitochondria by increasing the number of gel slices analyzed by LC-MS/MS from 12 to 40. By doing so, we increased the resolution of higher-order complexes and, admittedly, found that the differences in LLP enrichment in higher-order complexes became negligible. Therefore, in the revised manuscript we stepped down from the conclusion that LLPs are enriched in higher-order complexes. We have modified the figures and text to accommodate these findings and new data is now presented in Figure S3.

Additionally, as suggested by the Reviewer, we parsed the ^{14}N fractional abundance from each subunit of complex I and indeed we observed that the N- and Q- modules of Complex I have significantly less old protein and are turned-over at a shorter time-frame than rest of the complex. This data is presented in Figure 3D. While our results cannot be directly compared to those published by Szczepanowska et al. 2020, our data is congruent with the overall result.

On a related note, in their interpretation of Figure 2 E and F, the authors omitted to highlight that complexes, which assemble into supercomplexes (SC) have different FA values. For example, the mean FA of CIII is almost twice the FA of CI and CIV complexes in SC1 and SC2. This suggests that complexes might be turned over at different rate within a SC. This observation goes against the authors' claim that "the proteins assembled into SCs are preferentially retained together for at least 4 months in cortical and heart tissues" (lines 231-232). This considerably changes the interpretation of the results.

We acknowledge this helpful critique. In the revised manuscript we now include a plot illustrating the differences between the average mt-LLP FA of the ETC complexes (Figure 3C).

In revised manuscript, the interpretation that mt-LLPs are enriched in higher-order complexes has been removed.

Moreover, only a fraction of the entire ETC subunits have been detected and/or quantified in table 1. Studying the longevity of large protein complexes requires a better coverage of their numerous subunits. Analyzing enriched mitochondrial fractions, peptide fractionation, and data-independent acquisition are options that might be considered. The MS analysis of BN-PAGE gel bands provided better coverage but the longevity of individual proteins was not properly summarized and represented.

The Reviewer raises a fair point that we have gone to great lengths in order to address with new experiments. To increase coverage of the mitochondrial proteome, we repeated many of our experiment using Mitenyi biotech micro-bead based affinity capture of Tom20-positive mitochondria with LC-MS/MS analysis (Fecher et al., 2019). Using this method, we were able to increase the coverage of mitochondrial proteome 3-fold (Figure S2).

In the revised manuscript we also provide much improve data presentation which allows the reader to assess the depth of our protein analysis (Figure 3A). Moreover, new heatmaps illustrating our BN-PAGE data serve a second confirmation of the coverage of mitochondrial OXPHOS protein subunits and their FA values (Figure S3). Please note that during the revision process, Table 1 was removed and replaced with Figure 3 and Supplementary Table 2.

In addition, the following comments require attention.

1- Previous studies using similar approaches, including studies from Dr. Savas, did not report (or reported very few) long-lived mitochondrial proteins[1,2]. In fact, none of the 37 long-lived proteins previously identified in metabolically labeled rat brains after a 6 months chase by Savas et al. in 2013 were localized to mitochondria [2]. However, using a very similar methodology the current study suggests that long-lived proteins in ≥ 4 months old mouse brain are specifically enriched for mitochondrial proteins. The authors need to specify what changes were made, procedural, analytical, or otherwise, to allow the identification of such a high number of previously uncharacterized long-lived proteins in mitochondria. Interestingly, previous studies used a ^{15}N- and not a ^{14}N-labeled pulse chase which revives the concern raised earlier.

The Reviewer raises an important question regarding the relationship between our new results and those we have previously published. We understand the concern and we have added an explanation of how our method changed, as well as the reasoning behind the change, in the main text.

First, its true that the methodology used in the current study is similar to those we have used before, but there are several important differences:

1. Our previous publication Toyama et al. (2013), serves as the premise for studying long-lived mitochondrial proteins. In that initial publication, we identified a handful of spectra mapping to mitochondrial proteins (the only data on mitochondrial proteins can be found in Fig. 1B of the 2013 publication by Toyama and Savas). While this result provided key support that for the existence of mi-LLPs, however the identified ^{15}N-labelled peptides did not meet our preset criteria of $\geq$ 5% FA values, which is what we used to define ELLPs required to meet our threshold for inclusion in Table 1.
2. Since after 6-months of chase the FA values of mt-LLPs proved to be too low to allow for in-depth analysis of this phenomena in brain homogenates, we turned to shorter labelling periods in order to gain a better insight into mt-LLPs.
    a. This was confirmed with new experiments presented in Figure S2, where we show that after 6 months of chase, we can only quantify six (6) mt-LLPs and their FA values falls below 5% as published in Toyama et al. (2013).
3. In the initial submission we performed the analysis on crude membrane fractions and BN-PAGE separated material. In the revised manuscript, we performed additional analysis on Mitenyi biotech micro-bead based capture of Tom20-positive mitochondria (Fecher et al., 2019). This method significantly increased the coverage of the mitochondrial proteome and allowed for a more thorough identification and analysis of mt-LLPs.
4. In order for a protein to be classified as long-lived in the current study, we used the criteria of ^{14}N-peptide identification (Savas et al., 2012), rather than the 5% FA value cut off (Toyama et al., 2013).

Concerning the question of ^{14}N vs ^{15}N peptide identification, in the revised manuscript we include a direct comparison between ^{15}N- dynamic labeling method vs ^{15}N-pulse-chase method of animal labeling. This data is shown in Figure S2. While the number of old peptides mapping to mitochondrial proteins in ^{5}N-pulse-chase method is lower than that of ^{15}N-dynamic method, we find a consistent set of mitochondrial proteins to be long-lived.

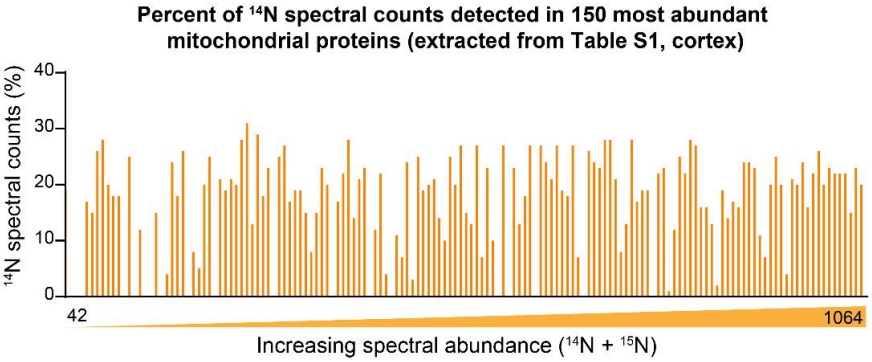
We also refer the Reviewer to (1) our response above, and (2) to the extensive literature on ^{14}N/^{15}N peptide MS analysis (Hark et al., 2021; Park et al., 2008; Savas et al., 2016; Savas et al., 2012; Toyama et al., 2013).

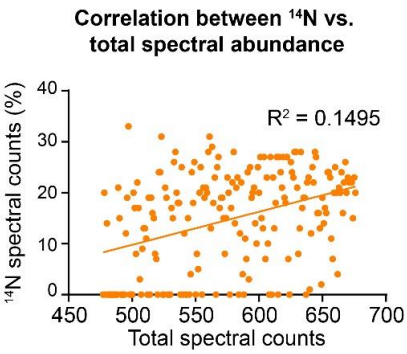
2- There is a high correlation between the abundance of total (^{14}N+^{15}N) and ^{14}N spectra. This could indicate that the approach is biased towards more abundant proteins. If that were the case, long-lived mitochondrial proteins would be necessarily more likely to be detected in highly energetic tissues with higher mitochondrial content, such as brain and heart. As the authors noted, supplemental figure 1A&B suggests that "the inter-tissue differences in the longevity of mitochondrial proteins were not due to disproportional identification of mitochondrial proteins" (lines 121-122). However, it would be important to complement this analysis with a comparison of the identified long-lived proteins (mitochondrial and not) across different tissues. Are proteins identified as long-lived disproportionally represented across different tissues?

This is a valid point of concern.

First, we would like to address the question of a bias in LLP identification towards more abundant proteins. Yes, as with any proteomic-based analysis, protein abundance does have a bearing on the overall identification and quantitation of individual proteins, and identification of low-abundant proteins has been, and continues to be, and important limitation in the field of proteomics. This holds true for our analysis as well. Indeed, we identify more ^{14}N spectral counts for more abundant proteins.

However, and more importantly: **not every high-abundant protein is, by default, a long-lived protein, and not every low-abundant protein is short-lived.** To illustrate this, we present the following data analysis:





Here we show the percent of ^{14}N-spectral counts (i.e. ^{14}N spectra # / (^{14}N+^{15}N spectra #)*100)) for the top 150 most abundant mitochondrial proteins in cortex, rank ordered according to total number of identified spectral counts ranging from 42 to 1064. As illustrated, for many of the highly abundant proteins, percent of ^{14}N-spectra counts is zero, indicating that in spite of being abundant, these proteins are not LLPs. Similarly, the correlation between ^{14}N-spectral counts and total spectral counts for the top 150 most abundant mitochondrial proteins is low, with an R^2=0.1495.

Secondly, in the revised manuscript we present two additional pieces of evidence that address the concern that a (dis)proportional number of mitochondrial proteins are identified in different tissues. In revised Figures 1D and S1B we show that overall number of protein identifications does not correlate with our ability to identify LLPs across different tissue types. Moreover, in revised Figures 2 and 3 we include a control tissue, spleen, for which we have not identified any mitochondrial proteins as mt-LLPs. This data is also presented in Table S1, where spectral counts can be compared. Here, for convenience, we include a snapshot of modified table to illustrate our point:

Here we show spectral counts identified in spleen, cortex and heart, sorted from most to least abundant in spleen (the tissue which does not harbor mt-LLPs). Top 48 proteins are shown. As illustrated, protein abundance does not correlate with identification of ^{14}N-spectral counts as (1) none of the highly abundant proteins in spleen are identified in ^{14}N-search, and (2) abundance of proteins within cortex and heart also does not correlate with ^{14}N-identification, even though these tissues are shown to harbor mt-LLPs.

	Spleen	Cortex	Heart	Spleen	Cortex	Heart	Spleen	Cortex	Heart
Gene	total spectral # (avg)			^{14}N spectral # (avg)			% ^{14}N spectra		
Atp5b	2292	930.7	5668	0.0	282.70	494.0	0.0%	30.4%	8.7%
Atp5a1	1758	1064.0	5603	0.0	269.00	462.0	0.0%	25.3%	8.2%
Hspd1	1230	431.7	747	0.0	119.70	60.0	0.0%	27.7%	8.0%
Slc25a5	1068	385.7	1518	0.0	93.70	162.0	0.0%	24.3%	10.7%
Uqcrc1	1019	551.3	2520	0.0	156.70	231.0	0.0%	28.4%	9.2%
Mdh2	974	310.7	822	0.0	100.30	89.0	0.0%	32.3%	10.8%
Hadha	761	179.3	3076	0.0	26.00	288.0	0.0%	14.5%	9.4%
Aldh2	707	133.3	247	0.0	0.00	0.0	0.0%	0.0%	0.0%
Sdha	699	273.3	1487	0.0	11.00	84.0	0.0%	4.0%	5.6%
Slc25a4	628	659.7	3090	0.0	120.30	350.0	0.0%	18.2%	11.3%
Aco2	595	471.0	1511	0.0	134.00	144.0	0.0%	28.5%	9.5%
Uqcrc2	570	330.7	1263	0.0	116.30	155.0	0.0%	35.2%	12.3%
Phb	488	146.7	295	0.0	56.70	34.0	0.0%	38.7%	11.5%
Glud1	405	237.7	138	0.0	57.70	8.0	0.0%	24.3%	5.8%
Cox5a	388	166.3	653	0.0	48.00	79.0	0.0%	28.9%	12.1%
Phb2	368	135.7	250	0.0	47.30	42.0	0.0%	34.9%	16.8%
Ndufs1	359	311.0	1636	0.0	59.00	125.0	0.0%	19.0%	7.6%
Hspa9	357	184.3	484	0.0	3.70	0.0	0.0%	2.0%	0.0%
Slc25a12	346	408.0	507	0.0	125.00	95.0	0.0%	30.6%	18.7%
Slc25a3	333	191.3	764	0.0	44.30	38.0	0.0%	23.2%	5.0%
Vdac2	324	90.7	749	0.0	27.00	55.0	0.0%	29.8%	7.3%
Got2	322	251.0	539	0.0	83.00	79.0	0.0%	33.1%	14.7%
Tomm22	321	158.3	164	0.0	2.30	2.0	0.0%	1.5%	1.2%
Acsf2	302	29.7	68	0.0	0.00	0.0	0.0%	0.0%	0.0%
Immt	301	262.3	942	0.0	67.30	142.0	0.0%	25.7%	15.1%
Hadhb	285	96.3	2557	0.0	0.00	63.0	0.0%	0.0%	2.5%
Gpd2	282	293.3	154	0.0	72.70	0.0	0.0%	24.8%	0.0%
Ogdh	278	229.3	638	0.0	16.30	0.0	0.0%	7.1%	0.0%
Atp5c1	274	323.0	471	0.0	90.70	63.0	0.0%	28.1%	13.4%
Etfa	265	87.7	474	0.0	16.00	58.0	0.0%	18.2%	12.2%
Vdac1	263	287.7	2745	0.0	77.30	131.0	0.0%	26.9%	4.8%
Ndufv1	253	215.7	866	0.0	42.30	48.0	0.0%	19.6%	5.5%
Dld	252	227.0	566	0.0	67.00	47.0	0.0%	29.5%	8.3%
Acaa2	249	75.7	1293	0.0	5.30	119.0	0.0%	7.0%	9.2%
Letm1	236	174.7	170	0.0	34.30	11.0	0.0%	19.6%	6.5%
Pdhb	235	136.3	419	0.0	42.00	42.0	0.0%	30.8%	10.0%
Cyc1	234	139.3	452	0.0	55.00	70.0	0.0%	39.5%	15.5%
Acat1	224	140.0	520	0.0	55.70	41.0	0.0%	39.8%	7.9%
Uqcrfs1	224	162.0	1554	0.0	53.70	85.0	0.0%	33.1%	5.5%
Lrpprc	213	152.3	250	0.0	0.00	0.0	0.0%	0.0%	0.0%
Atp5o	208	132.3	495	0.0	50.00	81.0	0.0%	37.8%	16.4%
Cps1	207	0.0	22	0.0	0.00	0.0	0.0%	#DIV/0!	0.0%
Dlst	206	104.7	230	0.0	38.30	29.0	0.0%	36.6%	12.6%
Mthfd1l	204	40.3	22	0.0	9.30	0.0	0.0%	23.1%	0.0%
Slc25a11	203	158.3	508	0.0	48.30	50.0	0.0%	30.5%	9.8%
Ndufs2	196	214.3	948	0.0	44.30	36.0	0.0%	20.7%	3.8%
Tufm	193	119.7	257	0.0	31.00	33.0	0.0%	25.9%	12.8%
Prdx3	187	61.7	215	0.0	22.70	18.0	0.0%	36.8%	8.4%

3- On a similar note, it is worth noting that abundant cytoskeleton proteins not typically associated with long half-life (e.g. tubulin, actin) were identified has long-lived in this study, especially in the cortex.

Yes, we do identify some cytoskeletal proteins as long-lived in our study.

However, we would like to point out that we took a discovery-based approach and performed a GO analysis and did not find terms associated with the cytoskeleton proteins to be significantly enriched in cortex or any other brain region. We did find that LLPs are associated with GO terms related to cytoskeleton proteins in pancreas, spleen, and lung extracts. In the revised manuscript, these findings are presented in Figure 1E.

We also note that according to the previous report by Fornasiero et al. (2018), the half-live of tubulins (alpha and beta) is between 17 and 34 days, which in fact meets their defined criteria of LLPs. Therefore, broadly speaking, our findings are in agreement with previously published data indicating that some of the cytoskeletal proteins are long-lived.

Also, spectra matched to keratin components originated almost exclusively from ^{14}N peptides in the cortex. The authors should comment on the exceptional longevity of cytoskeleton proteins, particularly in the cortex.

We apologize for this oversight in our initial submission. Please note that the keratin is very likely coming from the human sources and is considered as a contaminant. Similar to trypsin, in the revised manuscript, it is uniformly omitted from our datasets since it is considered an artifact.

4- If I am not mistaking, the sums of ^{14}N and ^{15}N spectra found in the cortex as presented in supplemental table 1 are respectively 14018 and 33649 while the values shown in figure 1 are 14209 and 31196. This is a slight anomaly but it seems important to identify/explain all inconsistencies.

We thank the reviewer for their keen attention to detail. This was an honest error on our end. In the revised manuscript we expanded this data set to include more biological replicates per tissue, new data is included in Figure 1 and Table S1.

We note that, per request from reviewers, we have moved away from spectra-based analysis and re-focused our paper on (1) ^{14}N/^{15}N peptide identification and (2) FA values. Therefore, this type of analysis has been removed from the revised manuscript.

5- The shaded portions of Figure 2C&D should also be visualized as cluster heatmaps that include all subunits within each protein complexes. That would be more informative than plotting the average of ^{14}N spectra over all proteins identified in a complex. Cluster heatmaps might reveal subunits/subcomplexes that have different longevity profiles than the rest of the complex and would help visualize the number of subunits that are represented within a complex.

This is an insightful comment and we are very grateful for the suggestion to use heatmaps. In the revised manuscript we replaced graphs shown previously in Figure 2C&D with heatmaps. New data is shown in Figure S3.

6- It is not clear how the numbers for table 1 were calculated. For example, I randomly picked protein NDUFA10 and tried to calculate the percentage of ^{14}N spectra in the cortex based on the following data found in Suppl table 2:

Biological replicate 15N 14N
1 2 7
2 1 4
3 3 7
4 1 1
5 5 0

That should amount to an average ^{14}N+^{15}N of: 6.2 ± 3.27 i.e. (9+5+10+2+5)/5 and a mean percentage of ^{14}N spectra of:

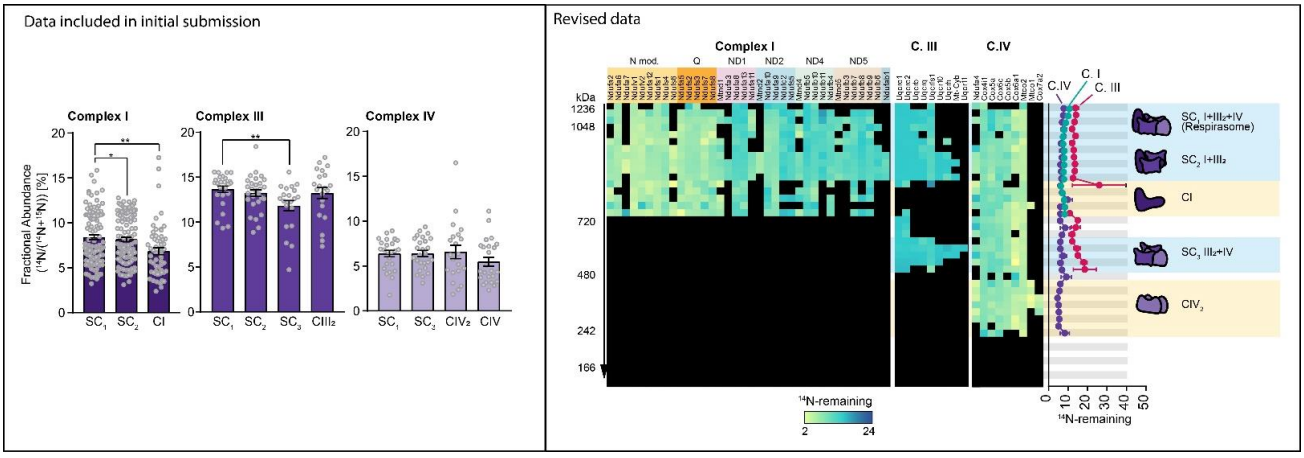
55.56 ± 33.23 %. However, the numbers reported were 5.2 ±4 and 69.4 ± 12. That is puzzling and requires some explanation.

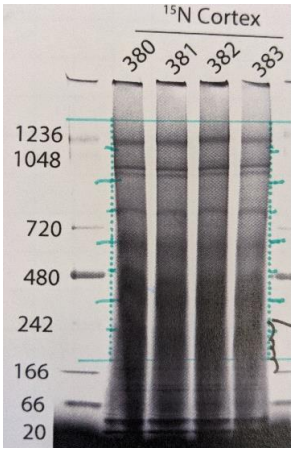
We thank the reviewer for the comment and sincerely apologize for this error. The value calculated by the reviewer is correct, and an error occurred on our end where, by mistake, the values for biological replicate #5 values were accidentally dropped.

After careful consideration, we decided to remove Table 1 from the revised manuscript. In lieu of the table, we have generated color coded, modeled and PDB-based, protein illustrations representing our data, shown in Figure 3. Summary data is included in the new Supplemental Table S2.

7- Figure 2E&F requires additional technical information. It is extremely difficult to perform high resolution protein profiling in native gel accurately and reproducibly, especially for supercomplexes (SCs) which demonstrate limited spatial resolution. Without thorough technical details and a schematic illustrating the location of the 12 bands along the gradient, it is impossible to determine whether the SCs, especially SC1 and SC2, were successfully resolved. Also, bar graphs are not sufficient to visualize such complex datasets. It would be beneficial to also use heatmaps that display the different subunits of a complex across the gel gradient [5,6].

We especially thank the Reviewer for this constructive comment and we are pleased to say that we have replaced the graphs in question with detailed heat maps, as illustrated below (as well as in response to 'major concerns' above).





By displaying our data as heat-maps we resolved both of the concerns: (1) the identification of different subunits across the gel gradient and (2) the location of each band along the molecular weight gradient.

For the Reviewer, we also decided to include our raw image of BN-PAGE gel from Dr. Bomba-Warczak's lab notebook showing how the gel was processed:

The image shows cortical extracts from four ^{15}N-labeled mice (#380,381,382 and 383). Marked ladder is shown to the left, and green dots/lines illustrate how the gel was cut. Bottom four bands were extended as marked in black on the right bottom side of the gel. To ensure consistency all four replicates were cut and processed at the same time.

8- Still regarding Figure 2E&F, it is unclear what the dots represent. According to the legend, "each dot represents an FA value for an individual protein in a relevant complex" (line 540). However, there are more dots than the number of proteins within each complex. For example, CIII contains 11 proteins but I can discern far more than 11 dots in the "Complex III" panels. Are those dots the FA values of all replicates? If that is the case, then the dots should rather illustrate the average of all replicates. Also, the statistical analysis should be performed on average FA values otherwise it erroneously inflates the sample size.

We thank the reviewer for this comment and apologize for the lack of clarity in the description of the experiment. We performed the FA analysis at the peptide level, which we averaged for each protein within the biological replicate. Therefore, as stated in the legend, each dot indeed represents a protein within the complex, and each biological replicate is shown independently.

In the revised manuscript, we replaced the plots with heat maps, and we performed statistics on average of four biological replicates (Figure S3).

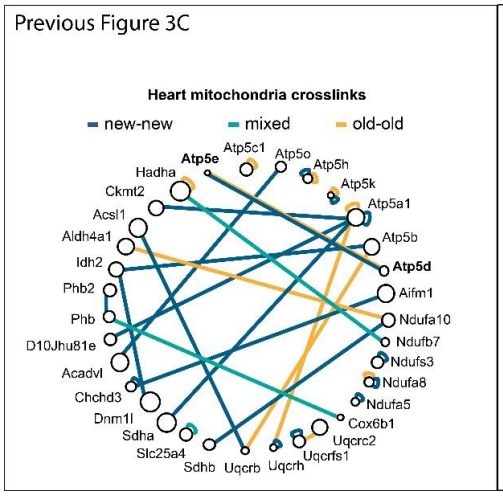
9- While I appreciate the effort that went in the DSSO crosslinking experiment, I am not sure that such a small number of cross-linked peptides adequately demonstrate that mitochondrial cristae proteins have limited exchange or mixing. To reach such a conclusion would require a much larger number of crosslinked peptides covering several proteins spanning multiple modules/sub-complexes. It is also important to note that the approach identified several protein-protein interactions that have not been validated and might therefore represent artefacts.

This is an important concern, which was raised by all three reviewers. We are pleased to say that we have developed a new workflow, which allowed us to identify 10-fold more cross-links in mitochondria isolated from heart tissue. The biggest variable in our ability to identify cross-links turned out to be the abundance and purity of the input material used for cross-linking studies. We were able to improve upon this essential step by immono-capturing mitochondria with Miltenyi technologies Fecher et al. (2019).

Below, we provide a side-by-side comparison of the previous and new crosslinking results, highlighting the dramatic improvement in the number of cross-links identified in the heart. In the revised manuscript, this new data is presented in Figures 4, 5, and S4.

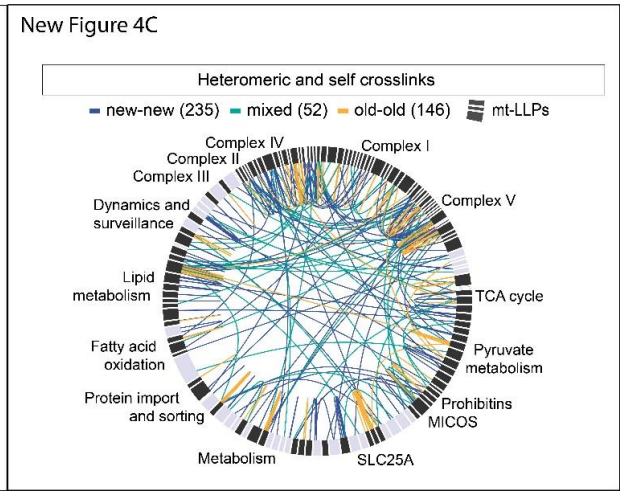
Cross-link data in initial submission

Previous Figure 3C



Cross-link data in revised submission

New Figure 4C



Minor points:

- The visualization of the GO terms enrichment analysis is too reductive and cannot be interpreted properly without the

supplemental tables. All enriched terms are collapsed down to only 3 categories (mitochondria, nucleus, others). It would be more informative to visualize which terms were enriched in each category.

We agree and apologize for not doing this in the initial submission. We included an expanded version of the GO analysis in Figure 1E of the revised manuscript.

- "Interestingly, the ^{14}N-content in mitochondrial proteins in olfactory bulb extracts, a brain region known for adult neurogenesis and thus a lower density of old cells, was significantly lower compared to cerebellum, midbrain, and striatum (Altman, 1969; Carleton et al., 2003) (Figure S1G, Table S1)". If that was the case, shouldn't the 'lower density of old cells' in the olfactory bulb also affect the proportion of nuclear/chromatin long-lived proteins?

Interesting discussion here, but we have removed data on the olfactory bulb due to a low number of replicates.

- Fig 2F: FA axis for CI, CIII, and CIV graphs should be on the same scale.

We thank the reviewer for this fair comment. In the revised manuscript this data has been replaced with heatmaps shown in Figure S3F where we graphed FA values for CI, CIII and CIV subunits together on a single plot (and thus same scale).

- The smoothing of the extracted chromatograms seems excessive.
Thank you for the comment. The chromatograms were smoothened as previously described by Park et al. (2008). This strategy has been used in hundreds of studies that have used the Census quantitative analysis software.

It is well established that chromatogram smoothing is important for peptides measured in only a relatively small number of MS1 scans. It is also well known that this strategy does not robustly change the overall measurement at the protein level.

Nonetheless, in the revised manuscript, the extracted chromatograms were replaced with raw MS1 scans. This new data is shown in Figure S1.

References:

1. Savas, J. N., Toyama, B. H., Xu, T., Yates, J. R. & Hetzer, M. W. Extremely Long-Lived Nuclear Pore Proteins in the Rat Brain. Science 335, 942-942 (2012).
2. Toyama, B. H. et al. Identification of Long-Lived Proteins Reveals Exceptional Stability of Essential Cellular Structures. Cell 154, 971-982 (2013).
3. Fornasiero, E. F. et al. Precisely measured protein lifetimes in the mouse brain reveal differences across tissues and subcellular fractions. Nat. Commun. 9, 4230 (2018).
4. Heo, S. et al. Identification of long-lived synaptic proteins by proteomic analysis of synaptosome protein turnover. Proc. Natl. Acad. Sci. 115, E3827-E3836 (2018).
5. Szczepanowska, K. et al. A salvage pathway maintains highly functional respiratory complex I. Nat. Commun. 11, 1643 (2020).
6. Guerrero-Castillo, S. et al. The Assembly Pathway of Mitochondrial Respiratory Chain Complex I. Cell Metab. 25, 128-139 (2017).

References for the 'Response to Reviewers":

Fecher, C., Trovo, L., Muller, S.A., Snaidero, N., Wettmarshausen, J., Heink, S., Ortiz, O., Wagner, I., Kuhn, R., Hartmann, J.*, et al.* (2019). Cell-type-specific profiling of brain mitochondria reveals functional and molecular diversity. Nat Neurosci *22*, 1731-1742.
Fornasiero, E.F., Mandad, S., Wildhagen, H., Alevra, M., Rammner, B., Keihani, S., Opazo, F., Urban, I., Ischebeck, T., Sakib, M.S.*, et al.* (2018). Precisely measured protein lifetimes in the mouse brain reveal differences across tissues and subcellular fractions. Nat Commun *9*, 4230.
Hark, T.J., Rao, N.R., Castillon, C., Basta, T., Smukowski, S., Bao, H., Upadhyay, A., Bomba-Warczak, E., Nomura, T., O'Toole, E.T.*, et al.* (2021). Pulse-Chase Proteomics of the App Knockin Mouse Models of Alzheimer's Disease Reveals that Synaptic Dysfunction Originates in Presynaptic Terminals. Cell Syst *12*, 141-158 e149.
Mi, H., Muruganujan, A., Ebert, D., Huang, X., and Thomas, P.D. (2019). PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res *47*, D419-D426.

Park, S.K., Venable, J.D., Xu, T., and Yates, J.R., 3rd (2008). A quantitative analysis software tool for mass spectrometry-based proteomics. Nat Methods *5*, 319-322.
Savas, J.N., Park, S.K., and Yates, J.R., 3rd (2016). Proteomic Analysis of Protein Turnover by Metabolic Whole Rodent Pulse-Chase Isotopic Labeling and Shotgun Mass Spectrometry Analysis. Methods Mol Biol *1410*, 293-304.
Savas, J.N., Toyama, B.H., Xu, T., Yates, J.R., 3rd, and Hetzer, M.W. (2012). Extremely long-lived nuclear pore proteins in the rat brain. Science *335*, 942.
Toyama, B.H., Savas, J.N., Park, S.K., Harris, M.S., Ingolia, N.T., Yates, J.R., and Hetzer, M.W. (2013). Identification of long-lived proteins reveals exceptional stability of essential cellular structures. Cell *154*, 971-982.

May 31, 2021

Re: JCB manuscript #202005193R-A

Dr. Jeffrey Savas
Northwestern University
Department of Neurology
Feinberg School of Medicine
303 East Chicago Avenue, Ward 12-102
Chicago, IL 60611

Dear Dr. Savas,

Thank you for submitting your revised manuscript entitled "Long-lived mitochondrial proteins as pillars of cristae architecture". The manuscript has been seen by the original reviewers, whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that, while two reviewers recommend publication, one referee (#3) had remaining technical concerns. In addition, Rev#3 did not feel like the conclusion that "mito LLPs are essential to maintain or shape the architecture of cristae" is sufficiently supported.

Our general policy is that papers are considered through only one revision cycle; however, we agree that final, limited revisions are needed for publication, and given that the suggested changes are relatively minor, we are open to one additional short round of revision. Please address the remaining minor points from all reviewers. These should be text/figure edits, clarifications based on existing data, and possibly additional analyses of the data, but no new experimentation should be needed. While we are supportive of the degree of advance for JCB, we agree with Rev#3 that the title is misleading and recommend revising it.

Please submit the final revision within one month, along with a cover letter that includes a point-by-point response to the remaining reviewer comments.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief, Journal of Cell Biology

Melina Casadio, Ph.D.
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have carefully and comprehensively addressed previous concerns of the reviewers and significantly improved the manuscript. Rather than analyzing crude mitochondrial fractions, they now employed immune-capturing of mitochondria which allowed them to drastically increase the coverage of mitochondrial proteins and significantly extend previous findings by Fornasiero et al. Moreover, they significantly improved the crosslink interactome data and now also include a time-resolved analysis, examining mice at different ages. Moreover, the text has been improved and does not only focus on cristae shaping proteins. The authors have addressed all my comments satisfactorily and I therefore recommend publication of this interesting manuscript.

Reviewer #2 (Comments to the Authors (Required)):

In their manuscript "Long-lived mitochondrial proteins as pillars of cristae architecture" Bomba-Warczak et al. demonstrate that a subset of the mitochondrial proteome persists for months in long-lived post-mitotic cells. These long-lived proteins localize to cristae membranes. Specifically, they show that:

1. Tissue differences in the longevity of the mitochondrial proteome
2. Enrichment of long-lived proteins in the mitochondrial cristae membrane
3. Long-lived respiratory supercomplexes in the heart and brain
4. Limited exchange of subunits throughout the lifetime of individual cristae

Overall, the manuscript is well written and clear. This manuscript advances the field of mitochondrial physiology by tracking the turnover of the mitochondrial proteome in various tissues. This leads to the surprising result that in post-mitotic tissues respiratory complexes, especially the ATP synthase complex are long lived and can remain intact for over four months.

This paper is a resubmission of a previously reviewed manuscript, and the authors clearly address all of the previous points brought up by the reviewers. The paper is its current state is greatly improved with respect to methodology (using immune-purified mitochondrial samples), data analysis (addressing the specific concerns of the previous reviews) and data presentation (the current figures are much cleaner and easier to interpret). Overall, if the authors can address the point below, this manuscript is appropriate for publication in JCB.

Main point 1: Line 235 states that 218 crosslinks were inter-links but in Fig. 4C it appears that there are less than 100 inter-links. Is this discrepancy due to total cross-links vs. unique cross-links? If so, please state this more clearly in the text and figure legend.

Minor edits:
Typo in line 186 should be CII not CIII.

Line 311 "but are rather" should be "but rather."

Reviewer #3 (Comments to the Authors (Required)):

In this revised manuscript, Bomba-Warczak et al. provided additional data and analysis that substantially increase the quality of data presentation and visualization. The authors convincingly identified LLPs in mitochondria of long-lived cells of brain and heart tissues and showed that within mitochondria, LLPs are enriched in the IMM. The addition of novel DSSO-crosslinking experiments further supports the idea that some protein complexes are extremely stable with limited subunit exchange within the IMM. Furthermore, the authors provided additional BN-PAGE analysis that helped clarify the longevity of higher-order OXPHOS assemblies, complexes, and subcomplexes. However, despite these improvements, I still have concerns that the authors should consider.

The idea that mtLLPs are essential to maintain or shape the architecture of cristae is not fully supported. Cristae are known to be dynamics, the abundance of mtLLPs is low (~10% or less based on the authors data), and the authors provide no evidence indicating that mtLLPs are "pillars of cristae architecture". At the very least, the manuscript title should be toned down.

The additional crosslinking experiments are interesting but raise several fundamental questions that are left unanswered. For example, in the control experiment presented in Figure S4A, one would expect a 50:50 H-H/L-L ratio in the heavy-light 50:50 mixture. However, that ratio is closer to ~25:75 which indicates that the analysis is possibly skewed towards the detection of 14N peptides. This also apparent in Figures 4D and S4C displaying the number of crosslinks identified in the heart and cortex, respectively. While the fraction of mtLLPS is ~10% or lower in all subcompartments in both tissues (see Figure S3B) the number of L-L (14N, old-old) crosslinked peptides always outnumbers the number of H-H (15N, new-new) crosslinked peptides. This is particularly noticeable in Figure S4C where the number of old-old crosslinked peptides is 3-4 times higher than the number of new-new crosslinked peptides. This is rather unexpected considering the small fractional abundance of mtLLPs and suggests that a bias was introduced during the analysis.

According to the Methods, 15N-labeled pups for the pulse-chase experiment originated from 15N-labeled female (two generations) and remained on the 15N-milk until weaning. In Figure S2F-G, the proportion of 14N proteins at T = 0 is surprisingly high assuming that the animals were fed a conventional 15N chow diet with 98%+ 15N. The authors recently published that similar metabolic labeling resulted in ~98% labeling efficiency (Hark et al. 2021). What was the 15N-labeling efficiency of these pups in the current study? The data in Figure S2G suggests it is much lower than 98%. What is the origin of the 14N proteins at T = 0?

In Figure S2, it is unclear whether the N15 pulse-chase experiments presented in S2E and S2F correspond to the same experiment or constitute two separate pulse-chase with different experimental designs. Only one pulse-chase experiment was described in the Methods section, so one has to assume that the same experimental design was followed and that Fig S2D-E represents a single timepoint taken from the time-course experiment presented in Fig S2F-G. The schematics should not be duplicated and Fig S2D-G should be presented and interpretated together. Also, the color of the "N15-chase" label should be changed to blue to reflect the type of chow and the "mt-LLPs HEAVY 14N" label should be changed to "mt-LLPs HEAVY 15N".

Figure S2E presents 79 N15-labeled mt-LLPs while Figure S2G only contains 30 (24 + 6). That represents a substantial difference which lacks a proper explanation. Even if Figure S2D-E and S2F-G constitute two separate experiments, the authors should explain why they identified over 2.5 more mt-LLPs in S2D-E vs S2F-G. Also, was the table for S2D-E provided?

In Figure S2G, the left panel indicates that a total of 223 mitochondrial proteins were quantified at T

= 0. However, the panel on the right suggests that only 220 proteins (105 + 85 + 24 + 6) were identified. Where did the 3 missing proteins go?

These sentences from lines 158-160 are misleading: "Consistent with our previously published findings, in nearly all instances measures of protein longevity are reduced in the pulse-chase compared to the dynamic pulse experiments (Hark et al., 2021) (Table S3). Altogether, these results provide independent confirmation of the mtLLPs identified in the dynamic 15N pulse analysis paradigm." These sentences suggest that the same mtLPPs were identified but with lower longevity in the 15N pulse-chase experiment. However, the data provided indicate that the overall number of LLPs identified is substantially reduced in the 15N pulse-chase. And while I agree with the statement presented on lines 152-153 indicating that 97.5% of the mtLLPs identified in the 15N pulse-chase analysis were also identified in the 14N chase analysis (77 mtLLPs out of 79), the opposite is simply not true. Only ~37% of the mtLLPs identified in the 14N chase analysis were also identified in the 15N pulse-chase analysis (77 mtLLPs out of 209) and this value is potentially lower if we consider that only 30 mtLLPS were identified in the 15N pulse-chase analysis presented in Figure S2G. The discordance should be explained unequivocally and an explanation should be provided. Also, the authors should indicate whether the mtLLPs identified in the 15N pulse-chase analysis are also enriched in the IMM as in the 14N chase analysis.

In Figure 2C, the authors "identified a total of 677 mitochondrial proteins in extracts from the cortex, 773 from the heart, and 758 from the spleen". However, the numbers for the cortex and heart do not match those presented in Figure S2A while the numbers for the spleen do match. If the numbers of the Venn diagrams are interpreted as in Figure S2E (as they should) the total number of mitochondrial proteins identified in the cortex should be 888 (677 + 211) and 978 in the heart (773 + 205). However, these values still differ from the values presented in Figure S2A (868 and 962 respectively). This statement on lines 124-125: "Notably, even though the total number of mitochondrial proteins identified in the spleen was higher than in the cortex and heart" seems to contradict the results presented in Figure S2A. These numbers should be reviewed and corrected.

The following sentence on lines 182-183 is misleading: "We then plotted the mt-LLPs, along with their FA values, according to their localization patterns within mitochondria, reinforcing the finding that mt-LLPs are enriched at IMM (Fig. 3B)". While Figure 3B is a great visualization aid, it does not illustrate that mtLLPs are enriched at IMM. This panel does not contain any statistical analysis but only displays a subset of FA values handpicked from Figure 3A by the authors. While Figure 2C 14N spectral count data indicates that the IMM has a larger proportion of proteins that be can be long-lived, Figure S3B suggests that ~10% of 14N proteins remains in each subcompartment, hence suggesting that the fractional abundance of long-lived proteins is similar between different subcompartments.

On lines 183-184: "On a global scale, we found no significant differences in the average FA values between the various MitoPathways". Where is the statistical analysis supporting that statement and assuming the lack of significance, why have some pathways been highlighted and handpicked by the authors in Figure 3A-B?

Figure S3F, heatmap color legend is wrong.

RESPONSE TO THE REVIEWERS

Reviewer #1 (Comments to the Authors (Required)):

The authors have carefully and comprehensively addressed previous concerns of the reviewers and significantly improved the manuscript. Rather than analyzing crude mitochondrial fractions, they now employed immune-capturing of mitochondria which allowed them to drastically increase the coverage of mitochondrial proteins and significantly extend previous findings by Fornasiero et al. Moreover, they significantly improved the crosslink interactome data and now also include a time-resolved analysis, examining mice at different ages. Moreover, the text has been improved and does not only focus on cristae shaping proteins. The authors have addressed all my comments satisfactorily and I therefore recommend publication of this interesting manuscript.

We thank the reviewer for the recommendation to publish.

Reviewer #2 (Comments to the Authors (Required)):

In their manuscript "Long-lived mitochondrial proteins as pillars of cristae architecture" Bomba-Warczak et al. demonstrate that a subset of the mitochondrial proteome persists for months in long-lived post-mitotic cells. These long-lived proteins localize to cristae membranes. Specifically, they show that:

1. Tissue differences in the longevity of the mitochondrial proteome
2. Enrichment of long-lived proteins in the mitochondrial cristae membrane
3. Long-lived respiratory supercomplexes in the heart and brain
4. Limited exchange of subunits throughout the lifetime of individual cristae

Overall, the manuscript is well written and clear. This manuscript advances the field of mitochondrial physiology by tracking the turnover of the mitochondrial proteome in various tissues. This leads to the surprising result that in post-mitotic tissues respiratory complexes, especially the ATP synthase complex are long lived and can remain intact for over four months.

This paper is a resubmission of a previously reviewed manuscript, and the authors clearly address all of the previous points brought up by the reviewers. The paper is its current state is greatly improved with respect to methodology (using immune-purified mitochondrial samples), data analysis (addressing the specific concerns of the previous reviews) and data presentation (the current figures are much cleaner and easier to interpret). Overall, if the authors can address the point below, this manuscript is appropriate for publication in JCB.

We thank the reviewer for the recommendation to publish.

Main point 1: Line 235 states that 218 crosslinks were inter-links but in Fig. 4C it appears that there are less than 100 inter-links. Is this discrepancy due to total cross-links vs. unique cross-links? If so, please state this more clearly in the text and figure legend.

We thank the reviewer for this comment. Indeed the reviewer is correct - we previously reported unique cross-links only. For consistency, we have replaced the graph in Figure 4C to illustrate the total number of inter-crosslinks rather than unique cross-links.

Minor edits:
Typo in line 186 should be CII not CIII.
Thank you, the text has been revised.

Line 311 "but are rather" should be "but rather."
Thank you, the text has been revised.

Reviewer #3 (Comments to the Authors (Required)):

In this revised manuscript, Bomba-Warczak et al. provided additional data and analysis that substantially increase the quality of data presentation and visualization. The authors convincingly identified LLPs in mitochondria of long-lived cells of brain and heart tissues and showed that within mitochondria, LLPs are enriched in the IMM. The addition of novel DSSO-crosslinking experiments further supports the idea that some protein complexes are extremely stable with limited subunit exchange within the IMM. Furthermore, the authors provided additional BN-PAGE analysis that helped clarify the longevity of higher-order OXPHOS assemblies, complexes, and subcomplexes. However, despite these improvements, I still have concerns that the authors should consider.

The idea that mtLLPs are essential to maintain or shape the architecture of cristae is not fully supported. Cristae are known to be dynamics, the abundance of mtLLPs is low (~10% or less based on the authors data), and the authors provide no evidence indicating that mtLLPs are "pillars of cristae architecture". At the very least, the manuscript title should be toned down.

We thank the reviewer for the comment. We have revised the title to tone down this conclusion, which now reads “Long-lived mitochondrial cristae proteins in mouse heart and brain”.

The additional crosslinking experiments are interesting but raise several fundamental questions that are left unanswered. For example, in the control experiment presented in Figure S4A, one would expect a 50:50 H-H/L-L ratio in the heavy-light 50:50 mixture. However, that ratio is closer to ~25:75 which indicates that the analysis is possibly skewed towards the detection of ^{14}N peptides. This also apparent in Figures 4D and S4C displaying the number of crosslinks identified in the heart and cortex, respectively. While the fraction of mtLLPS is ~10% or lower in all subcompartments in both tissues (see Figure S3B) the number of L-L (14N, old-old) crosslinked peptides always outnumbers the number of H-H (15N, new-new) crosslinked peptides. This is particularly noticeable in Figure S4C where the number of old-old crosslinked peptides is 3-4 times higher than the number of new-new crosslinked peptides. This is rather unexpected considering the small fractional abundance of mtLLPs and suggests that a bias was introduced during the analysis.

We thank the reviewer for this concern. We would like to remind Reviewer #3 that this additional substantial control experiment was not requested in the previous critique. Rather, we provided this important experiment to further document our system and convince you of our results. The purpose of this experiment was to confirm the specificity of the identified heavy and light cross-linked peptides, rather than validate Nitrogen-15 based quantitative proteomics. Numerous peer-reviewed studies documenting the value of using Nitrogen-15 for quantitative proteomics have been previously published (1-16).

We have investigated this point and found that in this control experiment, the dissimilar number of identified heavy versus light cross linked peptides directly correlates with the total number of identified light or heavy peptides. Which is consistent with simply under loading the heavy mitochondria during mixing.

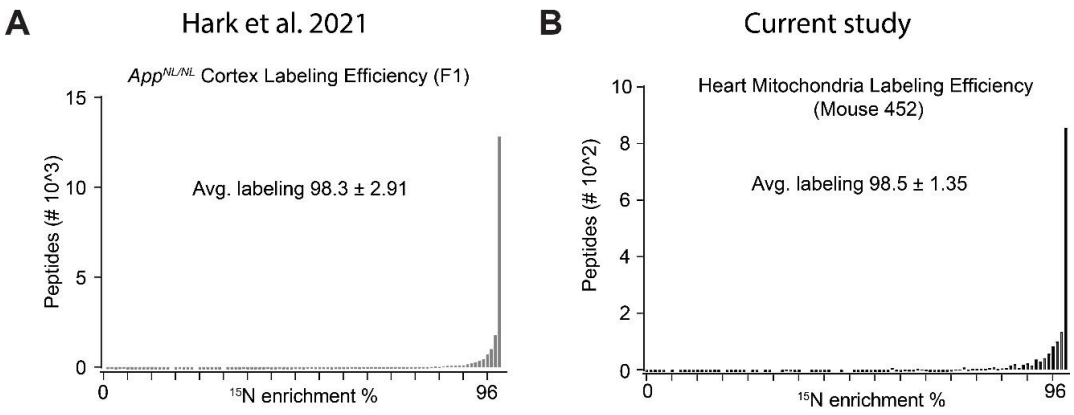
We would like to remind the Reviewer that our previous publications on ELLPs relied on tracking the Nitrogen-15 labeled proteins rather than Nitrogen-14. Furthermore, in our recent publication using metabolic Nitrogen-15 based labeling of AD mouse models we found nearly an identical set of ELLPs in

both paradigms (i.e. dynamic pulse versus pulse-chase). Finally, we now include a pulse-chase experiment with consistent results (Figure S2).

According to the Methods, 15N-labeled pups for the pulse-chase experiment originated from 15N-labeled female (two generations) and remained on the 15N-milk until weaning. In Figure S2F-G, the proportion of 14N proteins at T = 0 is surprisingly high assuming that the animals were fed a conventional 15N chow diet with 98%+ 15N. The authors recently published that similar metabolic labeling resulted in ~98% labeling efficiency (Hark et al. 2021). What was the 15N-labeling efficiency of these pups in the current study? The data in Figure S2G suggests it is much lower than 98%. What is the origin of the 14N proteins at T = 0?

Figure S2F-G shows select mitochondrial proteins that persisted at multiple time points examined, not all proteins are identified at this time point, and thus cannot be used to assess labeling efficiency.

The two generational method of metabolic ^{15}N-labeling of mammals has been consistently shown to generate tissues which are ~98% labeled with ^{15}N. This is also the case in this study, and we provide the figure below, which shows representative plots of labeling efficiency from Hark et al. 2021 (A) and current study (B). Again, consistently with labeling efficiency is above 98%.



In the revised manuscript, we added two graphs illustrating labelling efficiency in **Figure S2H**.

In the main text, we also added the following sentences:

> First, we confirmed efficient labeling efficiency of the proteome. In the pups born to ^{15}N-labelled dam, at the time of weaning (t = 0) we achieve an average labelling efficiency of ≥ 98.3% (**Fig. S2H**).

In Figure S2, it is unclear whether the N15 pulse-chase experiments presented in S2E and S2F correspond to the same experiment or constitute two separate pulse-chase with different experimental designs. Only one pulse-chase experiment was described in the Methods section, so one has to assume that the same experimental design was followed and that Fig S2D-E represents a single timepoint taken from the time-course experiment presented in Fig S2F-G. The schematics should not be duplicated and Fig S2D-G should be presented and interpretated together.

Yes - that is correct, the same data sets was used in these two panels. This was done in order to illustrate two separate points (1) to demonstrate the consistency between the two paradigms at one time point (in this case 4 months) and (2) to track the longevity of the same proteins across multiple time points (0, 2, 4, and 6 months).

The schematics are intentionally re-drawn twice to clearly show what is being compared in each panel and we believe that removing them, or combining them, would lead to unnecessary confusion and will make the conclusions less clear.

To clarify this, in the revised manuscript, we included the following text in the Figure S2 (G) legend:

> We note that the 4-month ^{14}N pulse chase time-point shown here represents same data set as in panels (D-E).

Also, the color of the "N15-chase" label should be changed to blue to reflect the type of chow and the "mt-LLPs HEAVY 14N" label should be changed to "mt-LLPs HEAVY 15N".

Thank you, these corrections have been made.

Figure S2E presents 79 N15-labeled mt-LLPs while Figure S2G only contains 30 (24 + 6). That represents a substantial difference which lacks a proper explanation. Even if Figure S2D-E and S2F-G constitute two separate experiments, the authors should explain why they identified over 2.5 more mt-LLPs in S2D-E vs S2F-G.

We apologize for the lack of clarity. Since the goal of Figure S2D-E was to illustrate protein longevity ***across multiple time points*** we only plotted proteins that were (1) *identified at t = 0, AND* (2) *were then identified across at least 2 consecutive time-points*, i.e. t =0 AND t = 2 months, or t = 0 AND t = 2 months AND t = 4 months.

Therefore, while we identified 79 proteins at the 4-month time-point, only 30 of these proteins were also identified at t = 0 and t =2 months, thus only those proteins were plotted in Figure S2D-E.

Also, was the table for S2D-E provided?

It was not. In the revised manuscript, this data is now shown in column R in Supplemental Table 2.

In Figure S2G, the left panel indicates that a total of 223 mitochondrial proteins were quantified at T = 0. However, the panel on the right suggests that only 220 proteins (105 + 85 + 24 + 6) were identified. Where did the 3 missing proteins go?

Thank you for this comment, this is a typo. N = 105 should have been N = 108 and we corrected this error in the revised manuscript.

These sentences from lines 158-160 are misleading: "Consistent with our previously published findings, in nearly all instances measures of protein longevity are reduced in the pulse-chase compared to the dynamic pulse experiments (Hark et al., 2021) (Table S3). Altogether, these results provide independent confirmation of the mtLLPs identified in the dynamic 15N pulse analysis paradigm." These sentences suggest that the same mtLPPs were identified but with lower longevity in the 15N pulse-chase experiment. However, the data provided indicate that the overall number of LLPs identified is substantially reduced in the 15N pulse-chase. And while I agree with the statement presented on lines 152-153 indicating that 97.5% of the mtLLPs identified in the 15N pulse-chase analysis were also identified in the 14N chase analysis (77 mtLLPs out of 79), the opposite is simply not true. Only ~37% of the mtLLPs identified in the 14N chase analysis were also identified in the 15N pulse-chase analysis (77 mtLLPs out of 209) and this value is potentially lower if we consider that only 30 mtLLPS were identified in the 15N pulse-chase analysis presented in Figure S2G. The discordance should be explained unequivocally and an explanation should be provided. Also, the authors should indicate whether the mtLLPs identified in the 15N pulse-chase analysis are also enriched in the IMM as in the 14N chase analysis.

This is a fair point that we are happy to address.

First, in the revised manuscript we added the following sentence to more fairly describe the result:

> However, in the label-swap experiment, in general, we identified fewer mt-LLPs, which is consistent with our previously published findings (17) (**Table S3**).

We also refer the Reviewer to the previous publications using ^{15}N-based proteomic, as listed in the response above.

Secondly, we have completed the requested GO analysis on the mt-LLPs identified in the ^{14}N-chase analysis (Fig. S2F, Table S1). The results are consistent with our findings in ^{15}N-dynamic pulse.

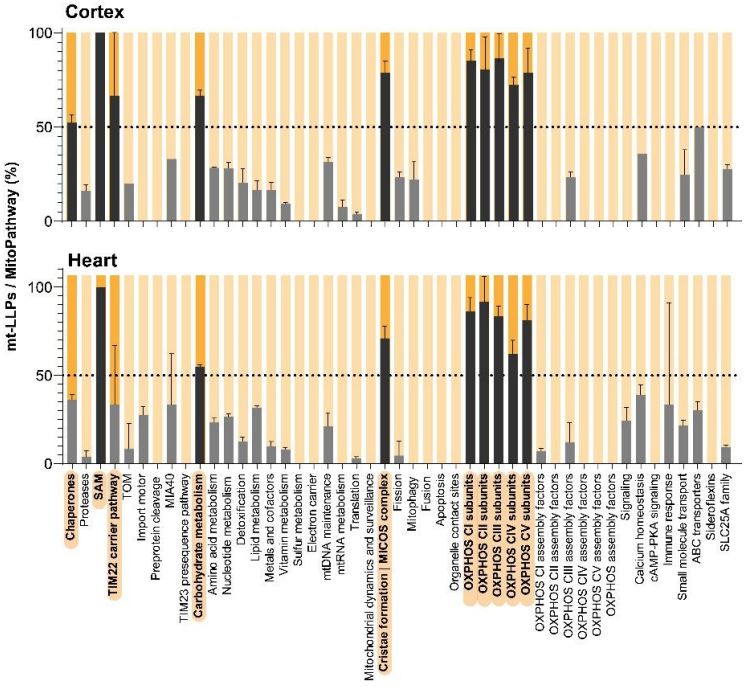
In the main text, we also added the following sentences:

> Moreover, GO analysis of mt-LLPs identified in the label-swap experiment revealed enrichment of same terms as with the 15N-dynamic pulse experimental design, further demonstrating consistency between the two paradigms (**Fig. S2F**).

In Figure 2C, the authors "identified a total of 677 mitochondrial proteins in extracts from the cortex, 773 from the heart, and 758 from the spleen". However, the numbers for the cortex and heart do not match those presented in Figure S2A while the numbers for the spleen do match. If the numbers of the Venn diagrams are interpreted as in Figure S2E (as they should) the total number of mitochondrial proteins identified in the cortex should be 888 (677 + 211) and 978 in the heart (773 + 205). However, these values still differ from the values presented in Figure S2A (868 and 962 respectively). This statement on lines 124-125: "Notably, even though the total number of mitochondrial proteins identified in the spleen was higher than in the cortex and heart" seems to contradict the results presented in Figure S2A. These numbers should be reviewed and corrected.

Thank you, we have corrected these typos in the figure.

The following sentence on lines 182-183 is misleading: "We then plotted the mt-LLPs, along with their FA values, according to their localization patterns within mitochondria, reinforcing the finding that mt-LLPs are enriched at IMM (Fig. 3B)". While Figure 3B is a great visualization aid, it does not illustrate that mtLLPs are enriched at IMM. This panel does not contain any statistical analysis but only displays a subset of FA values handpicked from Figure 3A by the authors. While Figure 2C 14N spectral count data indicates that the IMM has a larger proportion of proteins that be can be long-lived, Figure S3B suggests that ~10% of 14N proteins remains in each subcompartment, hence suggesting that the fractional abundance of long-lived proteins is similar between different subcompartments.



We thank the reviewer for this comment. There are two separate issues, which we would like to address.

1. FA values shown in Figure 3B are not handpicked, but rather are based on the enrichment of mt-LLPs per MitoPathway, as shown on the graphs to the right. We defined enrichment as pathways for which **at least 50% of identified proteins were identified as long-lived.**

These pathways are highlighted within this figure.

Figure 3A was meant to be a comprehensive illustration of the enrichment, while Figure 3B was meant to visually illustrate the localization of the identified mt-LLPs, along with their FA values.

We included Tim23 and Oxa1 with an intention of illustrating that not all mitochondrial proteins in IMM are long-lived, but in retrospect, we believe that this may have led to confusion. Therefore, we have modified Figure 3B to only include MitoPathways enriched in mt-LLPs, as defined above.

Also, the graphs shown above are now included in the revised Figure S3.

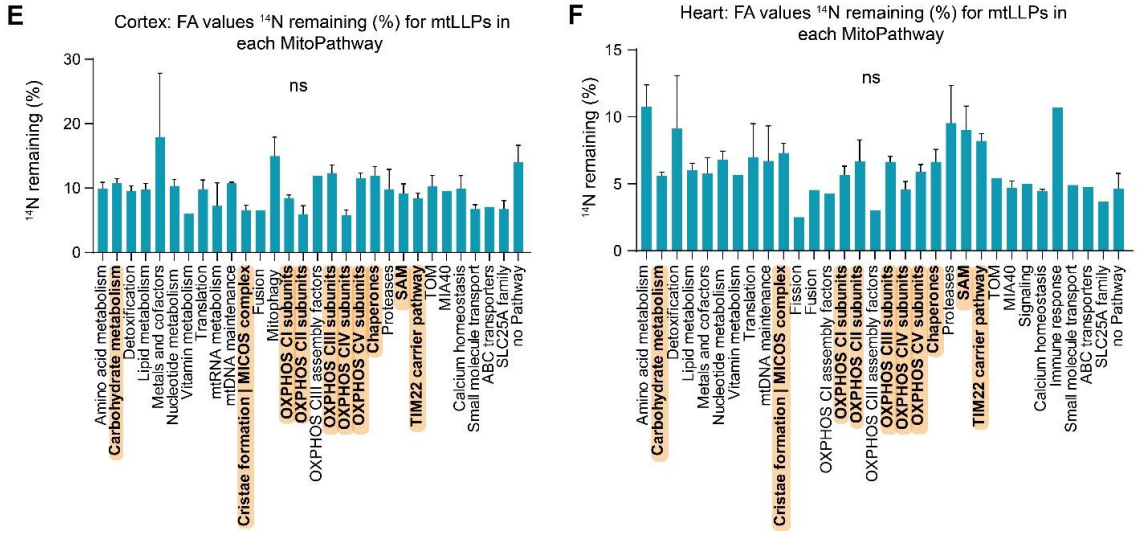
2. Yes, that is the precise conclusion: IMM has a larger proportion of proteins that can be long lived BUT the long-lived proteins, in general, have similar FA values, regardless of their localization. To illustrate this rationale we provide the following example:
    a. mitoribosome is composed of over 80 proteins, and yet only one protein, Mrps36, was identified as an LLP, with an FA value of 12.44 (Supplementary Table 2, Row 49). Hence, we do not classify mitoribosome as enriched in mt-LLPs since only one out of over 80 proteins was identified as long-lived. Moreover, if we consider that all non-long-lived proteins have a presumed FA value of <2 (or close to 0), then the average combined FA value for this complex is about 2.
    b. On the other hand, Complex I is composed of >40 proteins and ALL of the identified Complex I proteins were identified as long-lived. Therefore, the average FA value of the entire complex is ~8, and we consider the complex as enriched in long-lived proteins.

    Therefore, it is not the FA value but rather the mt-LLPs identification that guided the conclusion that IMM is enriched in mt-LLPs. This is clearly shown in Figure 2C.

On lines 183-184: "On a global scale, we found no significant differences in the average FA values between the various MitoPathways". Where is the statistical analysis supporting that statement and assuming the lack of significance, why have some pathways been highlighted and handpicked by the authors in Figure 3A-B?

We reiterate that pathways shown in Figures 3A-B were not handpicked, and we apologize for the lack of clarity in our manuscript. The highlighted MitoPathways represent the complete set of pathways with at least 50% of the proteins being long-lived in cortical extracts. We included Tim23 and Oxa1 with an intention of illustrating that not all mitochondrial proteins in IMM are long-lived, but in retrospect we believe that this may have led to confusion. Therefore, we have modified the figure to only include MitoPathways enriched in mt-LLPs, as defined above.

Here we also provide graphs illustrating the statistical analysis behind our statement that “no significant



differences in the average FA values between the various MitoPathways". The graphs shown below are now included in a revised Figure S3.

Figure S3F, heatmap color legend is wrong.

Thank you, we have corrected this error that occurred during file conversion.

1. Butko MT, Savas JN, Friedman B, Delahunty C, Ebner F, Yates JR, 3rd, Tsien RY. In vivo quantitative proteomics of somatosensory cortical synapses shows which protein levels are modulated by sensory deprivation. Proc Natl Acad Sci U S A. 2013;110(8):E726-35. Epub 2013/02/06. doi: 10.1073/pnas.1300424110. PubMed PMID: 23382246; PMCID: PMC3581967.
2. McClatchy DB, Savas JN, Martinez-Bartolome S, Park SK, Maher P, Powell SB, Yates JR, 3rd. Global quantitative analysis of phosphorylation underlying phencyclidine signaling and sensorimotor gating in the prefrontal cortex. Mol Psychiatry. 2016;21(2):205-15. doi: 10.1038/mp.2015.41. PubMed PMID: 25869802; PMCID: PMC4605830.
3. Parkhurst CN, Yang G, Ninan I, Savas JN, Yates JR, 3rd, Lafaille JJ, Hempstead BL, Littman DR, Gan WB. Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. Cell. 2013;155(7):1596-609. doi: 10.1016/j.cell.2013.11.030. PubMed PMID: 24360280; PMCID: PMC4033691.
4. Savas JN, Park SK, Yates JR, 3rd. Proteomic Analysis of Protein Turnover by Metabolic Whole Rodent Pulse-Chase Isotopic Labeling and Shotgun Mass Spectrometry Analysis. Methods Mol Biol. 2016;1410:293-304. doi: 10.1007/978-1-4939-3524-6_18. PubMed PMID: 26867752.
5. Savas JN, Ribeiro LF, Wierda KD, Wright R, DeNardo-Wilke LA, Rice HC, Chamma I, Wang YZ, Zemla R, Lavallee-Adam M, Vennekens KM, O'Sullivan ML, Antonios JK, Hall EA, Thoumine O, Attie AD, Yates JR, 3rd, Ghosh A, de Wit J. The Sorting Receptor SorCS1 Regulates Trafficking of Neurexin and AMPA Receptors. Neuron. 2015;87(4):764-80. doi: 10.1016/j.neuron.2015.08.007. PubMed PMID: 26291160; PMCID: PMC4692362.
6. Savas JN, Toyama BH, Xu T, Yates JR, 3rd, Hetzer MW. Extremely long-lived nuclear pore proteins in the rat brain. Science. 2012;335(6071):942. doi: 10.1126/science.1217421. PubMed PMID: 22300851; PMCID: PMC3296478.
7. Toyama BH, Savas JN, Park SK, Harris MS, Ingolia NT, Yates JR, 3rd, Hetzer MW. Identification of long-lived proteins reveals exceptional stability of essential cellular structures. Cell. 2013;154(5):971-82. doi: 10.1016/j.cell.2013.07.037. PubMed PMID: 23993091; PMCID: PMC3788602.
8. Wan J, Savas JN, Roth AF, Sanders SS, Singaraja RR, Hayden MR, Yates JR, 3rd, Davis NG. Tracking brain palmitoylation change: predominance of glial change in a mouse model of Huntington's disease. Chem Biol. 2013;20(11):1421-34. doi: 10.1016/j.chembiol.2013.09.018. PubMed PMID: 24211138; PMCID: PMC3880188.
9. Maccarrone G, Chen A, Filiou MD. Using (15)N-Metabolic Labeling for Quantitative Proteomic Analyses. Methods Mol Biol. 2017;1546:235-43. Epub 2016/11/30. doi: 10.1007/978-1-4939-6730-8_20. PubMed PMID: 27896773.
10. Liu P, Xie X, Jin J. Isotopic Nitrogen-15 Labeling of Mice Identified Long-lived Proteins of the Renal Basement Membranes. Sci Rep. 2020;10(1):5317. Epub 2020/03/27. doi: 10.1038/s41598-020-62348-6. PubMed PMID: 32210336; PMCID: PMC7093503.
11. Gouw JW, Tops BB, Mortensen P, Heck AJ, Krijgsveld J. Optimizing identification and quantitation of 15N-labeled proteins in comparative proteomics. Anal Chem. 2008;80(20):7796-803. Epub 2008/09/24. doi: 10.1021/ac801249v. PubMed PMID: 18808151.
12. Frank E, Kessler MS, Filiou MD, Zhang Y, Maccarrone G, Reckow S, Bunck M, Heumann H, Turck CW, Landgraf R, Hambsch B. Stable isotope metabolic labeling with a novel N-enriched bacteria diet for improved proteomic analyses of mouse models for psychopathologies. PLoS One. 2009;4(11):e7821. Epub 2009/11/17. doi: 10.1371/journal.pone.0007821. PubMed PMID: 19915716; PMCID: PMC2773927.
13. Oda Y, Huang K, Cross FR, Cowburn D, Chait BT. Accurate quantitation of protein expression and site-specific phosphorylation. Proc Natl Acad Sci U S A. 1999;96(12):6591-6. Epub 1999/06/09. doi: 10.1073/pnas.96.12.6591. PubMed PMID: 10359756; PMCID: PMC21959.
14. McClatchy DB, Yates JR, 3rd. Stable isotope labeling in mammals (SILAM). Methods Mol Biol. 2014;1156:133-46. Epub 2014/05/06. doi: 10.1007/978-1-4939-0685-7_8. PubMed PMID: 24791985.

15. Wu CC, MacCoss MJ, Howell KE, Matthews DE, Yates JR, 3rd. Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis. Anal Chem. 2004;76(17):4951-9. Epub 2004/09/18. doi: 10.1021/ac049208j. PubMed PMID: 15373428.
16. MacCoss MJ, Wu CC, Matthews DE, Yates JR, 3rd. Measurement of the isotope enrichment of stable isotope-labeled proteins using high-resolution mass spectra of peptides. Anal Chem. 2005;77(23):7646-53. Epub 2005/12/01. doi: 10.1021/ac0508393. PubMed PMID: 16316172.
17. Hark TJ, Rao NR, Castillon C, Basta T, Smukowski S, Bao H, Upadhyay A, Bomba-Warczak E, Nomura T, O'Toole ET, Morgan GP, Ali L, Saito T, Guillermier C, Saido TC, Steinhauser ML, Stowell MHB, Chapman ER, Contractor A, Savas JN. Pulse-Chase Proteomics of the App Knockin Mouse Models of Alzheimer's Disease Reveals that Synaptic Dysfunction Originates in Presynaptic Terminals. Cell Syst. 2021;12(2):141-58 e9. doi: 10.1016/j.cels.2020.11.007. PubMed PMID: 33326751; PMCID: PMC7897324.

June 3, 2021

RE: JCB Manuscript #202005193RR

Dr. Jeffrey Savas
Northwestern University
Department of Neurology
Northwestern University, Feinberg School of Medicine
303 East Chicago Avenue, Ward 12-102
Chicago, IL 60611

Dear Jeff:

Thank you for submitting your revised manuscript entitled "Long-lived mitochondrial cristae proteins in mouse heart and brain". We have now had an opportunity to assess the revised manuscript and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Reports is normally < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. As discussed, we will be able to give you the extra characters in this case.
However, please note that in the JCB Report format, the Results and Discussion sections should be merged into a single "Results and Discussion" module. Please revise the text accordingly. (Given the current orientation of the manuscript, you may be able to simply retitle the section and remove the "Discussion" title but, if you can, reworking the discussion elements into the results would make the paper read a bit better.)

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please add weight markers to the silver stain gel in figure 4B.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test

(for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.).Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."
Also, the materials and methods should be included with the main manuscript text and not in the supplementary materials.

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.
Also, please note that JCB does not allow "supplementary references". Please remove that list and incorporate any non-duplicated references into the main reference list.

8) Supplemental materials: As you know, Reports may normally have up to 3 supplemental figures. However, as discussed, we can give you the extra space in this instance.
However, please also note that tables, like figures, should be provided as individual, editable files. Also, a detailed summary of all supplemental material should appear at the end of the Materials and methods section (that is, in addition to the supplementary figure/table legends - please see page 12 of the attached JCB paper for an example of what we mean).

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements

regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

12) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Jodi Nunnari, Ph.D.
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