Neuralized-like protein 4 (NEURL4) mediates ADP-ribosylation of mitochondrial proteins

Maria Cardamone, Yuan Gao, Julian Kwan, Vanessa Hayashi, Megan Sheeran, Junxiang Xu, Justin English, Joseph Orofino, Andrew Emili, and Valentina Perissi

Corresponding Author(s): Valentina Perissi, Boston University School of Medicine and Maria Cardamone, Boston University School of Medicine

Review Timeline:

| Event                  | Date     |
|------------------------|----------|
| Submission Date        | 2021-01-05|
| Editorial Decision     | 2021-02-22|
| Revision Received      | 2021-09-07|
| Editorial Decision     | 2021-10-06|
| Revision Received      | 2021-10-22|

Monitoring Editor: Johan Auwerx

Scientific Editor: Lucia Morgado-Palacin

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.202101021
February 22, 2021

Re: JCB manuscript #202101021

Dr. Valentina Perissi
Boston University School of Medicine
Biochemistry
72 E Concord St
Silvio Conte Building, K-616
Boston, MA 02118

Dear Dr. Perissi,

Thank you for submitting your manuscript entitled "ADP-ribosylation of mitochondrial proteins is mediated by Neuralized-like protein 4 (NEURL4)". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you will see, the reviewers were overall enthusiastic about the paper, but they have each raised a number of concerns that will need to be addressed before the paper would be deemed appropriate for publication in JCB. We hope that you will be able to address each of these concerns in full, including substantial new data to support the main conclusions of the study.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for a Report is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Reports may have up to 5 main text figures. To avoid delays in production, figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu.

Sincerely,

Johan Auwerx
Monitoring Editor
Reviewer #1 (Comments to the Authors (Required)):

Cardemone et al present an interesting investigation consisting of both localisation and functional assessment of NEURL4. The work is both interesting and novel. The results suggest NEURL4 is at least partly located to the mitochondria with the remainder in the cytoplasm and that the KO of NEURL4 leads to an increase in shorter mtDNA amplicons and reduction in apparent PARylation activity, which they are also able to rescue by addition of NEURL4. Perhaps most interestingly they show this to be relevant for fertility and difficulties in generating a NEURL4 mouse model. Finally the authors preform Mass spec and RNA seq investigations as to the impact of NEURL4 KO. Whilst the manuscript is interesting, there are a couple of aspects of the methodological descriptions that require further details to ensure the interpretation of the data is accurate and edits to the figure presentation would substantially improve the manuscript. I have listed below major and minor comments, that I feel will improve the manuscript and its utility to the field.

Major:

For cell staining experiments, the methods do not mention no primary controls, were no primary controls included to exclude non-specific binding of secondary antibodies?

Similarly, Immuno-Gold is particularly affected by non-specific affinity of the gold particles to mitochondria, as such did the authors perform no-primary controls and correct for any non-specific labelling? The addition of a sentence about this in the methods would be beneficial.

From the provided images in figure 1B and description of the methods it is unclear how the authors analysed the fluorescence images to determine mitochondrial localisation? Can the authors please add this to the methods/results section?

Figure 1C it would be helpful for the reader if the authors could include the mitochondrial mass marker and loading control for N4-KO.

Figure 3D is described as showing a significant increase in the frequency of deletions in KO cell lines. There are already deletions present in WT and n4-KO 1 shows a reduction long amlicons and increase in shorter amplicons. Can the authors please adjust the way they explain this in the results and also address in the discussion the presence of mtDNA deletions in the WT and the very low amplification in N4-KO2?

Will the authors deposit the full Mass Spec dataset and include a reference to this in the manuscript?

Minor:

Information for number of repeats is provided in the statistical analysis section, it would be beneficial to also add this to the figure legends or respective methods sections for clarity.

Microscopy (EM, fluorescent and brightfield) images throughout the figures require the addition of scale bars.

For data transparency, it would be useful if bar charts could be replaced by plots on which the datapoints can be seen throughout.

The mtDNA copy number assay measures ND1 (mtDNA) relative to TFAM a nuclear encoded gene. In the figure legend and results this is described as no significant difference in mitochondrial content, but it would be more accurate to say no difference in mtDNA copy number since mtDNA copy number is what is directly being measured here. It should also be acknowledged that since ND1 is sometimes deleted and the authors investigated presence of deletions that this may be a limiting factor for this assay.

Can the authors please include the ladder on the PCR gels.

Figure 4 lacks x axis labels for all plots.
The omics presented in figure 4 present some interesting pathways associated with NEURL4 KO but the discussion does not touch on the relative interests of any of these results and further work that is needed.

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

The manuscript ADP-ribosylation of mitochondrial proteins is mediated by Neuralized-like protein 4 (NEURL4) is an interesting study of Neurl4 a protein previously recognized to play a role in centrosome biology, but also identified from prior investigations as a putative ADPribosyltransferase. The current study solidifies this likely activity, although in most cases the findings are by inference, i.e. ADPribosylation requires Neurl4, but is never DIRECTLY attributed to Neurl4 activity per se. Nevertheless, the study is important, as this protein is barely studied, with only 18 papers pulled up by pubmed search, and almost no direct biochemistry associated with this protein. Thus, the current study, which shows it localizes to mitochondrial matrix, and is required for mitochondrial ADPribosylation is a true leap forward in understanding the biology and biochemistry of this protein. Some preliminary studies showing that Neurl4 is required for proper mitochondrial function (Seahorse), and some indications of defects in sperm function are certainly of interest, but still somewhat preliminary. Taken is sum, a compelling story, which opens up new biochemistry and biology.

Specific concerns:

1. A clearer pileup bioinformatics comparison of Neurl4 should be provided in Figure 1 with comparison to other known ADPribosyltransferases. The authors use a text discussion and a descriptive domain summary. This is probably insufficient.
2. It would be helpful to clarify the nature of the ADPribosylation being observed for Neurl4 activity. Is it all PAR or mono-ADPribosylation or both? This would better clarify the nature of the activity being shown.

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

In this manuscript, Perissi et al described and characterized NEURL4 as an important ADP-ribosyl transferase (ART) in mitochondria and as a key regulator of mitochondrial ADP-ribosylation/DNA repair. They generated two NEURL4-KO cell lines and used them to show the important role of NEURL4 in mitochondrial PARylation. Overall, this manuscript succeeds in the main goal of showing NEURL4 as an important factor for mitochondrial ADP-ribosylation, which is required for mitochondrial stability. Identification of the major mitochondrial ART is one of very important issue in the field, and this manuscript provides a new insight into this aspect. However, this manuscript lacks critical data to support that NEURL4 is an actual ART, such as the in vitro PARylation using purified recombinant NEURL4 (full-length or catalytic domain) and the NEURL4-KO complementation experiments using catalytically inactive mutant (mutations in H-Y-E, in particular E). In addition, authors need to confirm the PARylation of some of identified ADP-ribosylated proteins by recombinant NEURL4 or using N4-KO cells. This reviewer would like to suggest accepting this manuscript after major revision. Here are the specific points.

Major points;
1. As far as I understand, no one has clearly shown that the isolated recombinant full-length or C-terminal part of NEURL4 has the ART activity. Similarly, data in this manuscript also used NEURL4 that was obtained by immunoprecipitation from cell extracts, which may include other ARTs. Authors should use the full-length and/or the C-terminal domain of NEURL4 (i.e., from E. coli) to show their in vitro PARylation activity. In addition, authors should show these recombinant NEURL4 proteins are not inhibited by PARP1 inhibitor, but by BGP-15.
2. Figure 2B and D.
   - In Figure 2B, N4-KO cells complemented with N4-FL show very weak N4-FL expression, compared to WT. In contrast, these N4-FL-complemented cells show very potent PARylation in Fig 2D.
   - This also raises possibility that NEURL4 might be a regulatory protein, such as activator of other ARTs or inhibitor of ubiquitin ligase, rather than a direct ART enzyme.
3. Figure 2D.
   Authors should compare +Ct and catalytically inactive Ct (in H-Y-E, all or in particular E).
4. Figure 2E.
   - Compared to Figure 2D, the overall PARylation signal is too weak and there is no PARylation even in WT. In contrast, in Fig
2C, 2D, and 3E, WT shows very robust PARylation. Authors should explain this discrepancy.

- Were these performed in the presence or absence of genotoxic or any other stresses to induce PARylation? Is there any evidence that the ART activity of NEURL4 is DNA- or damage-dependent?

5. Fig. 4. Authors should show the PARylation of some of identified ADP-ribosylated proteins by recombinant NEURL4, or the change of their PARylation level in WT and N4-KO cells.

Minor points:
1. Some of the western blots are quite poor, for example Fig 2F and 3F.
Dear Drs. Auwerx and Palacin,

We were extremely happy to see that all three Reviewers were enthusiastic about our findings and we are grateful for their constructive feedbacks. We are also very grateful for your flexibility with the timing of resubmission. We are particularly grateful as this additional time has allowed us to submit a revised manuscript that address all the concerns raised by the Reviewers and, in particular, includes critical new data in support of a direct role for NEURL4 in ADP-ribosylation. As highlighted by two Reviewers, this was necessary to conclude that NEURL4 mediates mitochondrial ADP-ribosylation as opposed to being required to support the activity of another enzyme. We are glad they pushed us to push us to perform the necessary experiments and we hope you’ll agree with us that the revised manuscript has much improved.

Please find below a point-by-point response to the Reviewers’ comments.

We look forward to hearing back from you and hope you will find the revised manuscript suitable for publication.

Best regards,
Valentina Perissi and colleagues

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

Cardamaone et al present an interesting investigation consisting of both localization and functional assessment of NEURL4. The work is both interesting and novel. The results suggest NEURL4 is at least partly located to the mitochondria with the remainder in the cytoplasm and that the KO of NEURL4 leads to an increase in shorter mtDNA amplicons and reduction in apparent PARylation activity, which they are also able to rescue by addition of NEURL4. Perhaps most interestingly they show this to be relevant for fertility and difficulties in generating a NEURL4 mouse model. Finally the authors perform Mass spec and RNA seq investigations as to the impact of NEURL4 KO. Whilst the manuscript is interesting, there are a couple of aspects of the methodological descriptions that require further details to ensure the interpretation of the data is accurate and edits to the figure presentation would substantially improve the manuscript. I have listed below major and minor comments, that I feel will improve the manuscript and its utility to the field.

We are very grateful to this Reviewer for providing fair and constructive criticisms to the manuscript. We have implemented all the suggested changes as detailed below and we thank this reviewer for contributing to a stronger and improved publication.

Major:

For cell staining experiments, the methods do not mention no primary controls, were no primary controls included to exclude non-specific binding of secondary antibodies? Similarly, Immuno-Gold is particularly affected by non-specific affinity of the gold particles to mitochondria, as such did the authors perform no-primary controls and correct for any non-specific labelling? The addition of a sentence about this in the methods would be beneficial.

We thank this Reviewer for pointing out that the Methods were lacking in explaining the controls included in our experimental approaches for cell staining and gold labeling. We have now updated the
Methods to include the description of the controls performed. In each case, images were acquired adjusting for the background of the no-primary control.

From the provided images in figure 1B and description of the methods it is unclear how the authors analyzed the fluorescence images to determine mitochondrial localization? Can the authors please add this to the methods/results section?

Again, we apologize that the Methods sections was not detailed enough. We have now included a sentence in the Methods and the Results to explain that the mitochondrial localization was determined based on colocalization of the staining for NEURL4 (represented in red) and that of classic mitochondrial markers, such as ATP5B or mtHSP70 (represented in green). While the use of color merging (visualized via the presence of yellow color) is not sufficient to define the precise spatial correlation of specific molecules, it can be effectively used for labeling specific organelles or defined subcellular structures.

Figure 1C it would be helpful for the reader if the authors could include the mitochondrial mass marker and loading control for N4-KO.

We thanks the Reviewer for this suggestion. We have added the MW marker to this Figure and added a blot for mitochondrial Hsp70 as loading control for the siRNA transfected sample.

Figure 3D is described as showing a significant increase in the frequency of deletions in KO cell lines. There are already deletions present in WT and n4-KO 1 shows a reduction long amplicons and increase in shorter amplicons. Can the authors please adjust the way they explain this in the results and also address in the discussion the presence of mtDNA deletions in the WT and the very low amplification in N4-KO2?

This Reviewer is correct in observing that there is a basal level of deletions present in the WT cells, which we do not find surprising considering that Hela are cancer cells. For comparison, please see Figure 3I, in which the amplification of WT sperm mtDNA shows a prevalence of the expected full size mtDNA amplicon of about 16kB. Despite the presence of basal amplification of amplicons of variable sizes, a reduction in long amplicons in favor of shorter amplicons is the expected result when the amplification of the circular mtDNA from divergent primers is impaired by an increase in deletions along the template. Depending on the position and the frequency of deletions in specific positions, it is possible to observe an increase in the abundance of specific short amplicons (with a stronger signal), or a more diffuse increase in small amplicons of different sizes (hence the ‘lower’ signal observed in N4-KO2)

Will the authors deposit the full Mass Spec dataset and include a reference to this in the manuscript?

We are happy to provide the full Mass Spec datasets in appendix to the manuscript. Please see the Supplementary Material for a Table including the complete dataset (Supplementary Table 1)

Minor:

Information for number of repeats is provided in the statistical analysis section, it would be beneficial to also add this to the figure legends or respective methods sections for clarity.

Thanks for the suggestions, we have now included this information in the figure legends.
Microscopy (EM, fluorescent and brightfield) images throughout the figures require the addition of scale bars.

Thanks for the suggestion, we have now added a scale bar to all imaging figures.

For data transparency, it would be useful if bar charts could be replaced by plots on which the datapoints can be seen throughout.

Thanks for the suggestion, we have changed the bar chart whenever possible to include single datapoints (See Figure 3C and 3G). In the case of chromatin immunoprecipitation, we clarified in the methods and legends that the data is presented as technical triplicates of a single experiment, which is representative of at least 3 separate biological triplicates. We’ll be happy to provide access to the raw data for transparency if that can be helpful.

The mtDNA copy number assay measures ND1 (mtDNA) relative to TFAM a nuclear encoded gene. In the figure legend and results this is described as no significant difference in mitochondrial content, but it would be more accurate to say no difference in mtDNA copy number since mtDNA copy number is what is directly being measured here. It should also be acknowledged that since ND1 is sometimes deleted and the authors investigated presence of deletions that this may be a limiting factor for this assay.

This is an excellent point, we have changed wording in the text as suggested and described the results as no difference in mtDNA copy number rather than mitochondrial content. Our understanding of the literature is that ND1 is in an area of the mtDNA that is more protected from deletions, but we agree that any deletion of this area would affect the results of this assay. Nonetheless, our conclusions regarding DNA replication would not differ as the assay would fail to detect an increase, not a decrease, in mtDNA copy number.

Can the authors please include the ladder on the PCR gels.

Figure 4 lacks x axis labels for all plots.

Thanks for catching these mistakes, we have fixed both in the revised figures.

The omics presented in figure 4 present some interesting pathways associated with NEURL4 KO but the discussion does not touch on the relative interests of any of these results and further work that is needed.

We fully agree that there is some interesting work to be done! We have now discussed these results in more details in the Discussion.

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

The manuscript ADP-ribosylation of mitochondrial proteins is mediated by Neuralized-like protein 4 (NEURL4) is an interesting study of Neurl4 a protein previously recognized to play a role in centrosome biology, but also identified from prior investigations as a putative ADPribosyltransferase. The current study solidifies this likely activity, although in most cases the findings are by inference, i.e.
ADPribosylation requires Neurl4, but is never DIRECTLY attributed to Neurl4 activity per se. Nevertheless, the study is important, as this protein is barely studied, with only 18 papers pulled up by pubmed search, and almost no direct biochemistry associated with this protein. Thus, the current study, which shows it localizes to mitochondrial matrix, and is required for mitochondrial ADPribosylation is a true leap forward in understanding the biology and biochemistry of this protein. Some preliminary studies showing that Neurl4 is required for proper mitochondrial function (Seahorse), and some indications of defects in sperm function are certainly of interest, but still somewhat preliminary. Taken is sum, a compelling story, which opens up new biochemistry and biology.

We thank this Reviewer for his positive evaluation of our studies and for raising the critical point of whether NEURL4 carries independent ADP-ribosylation activity. To address this point we have expressed the catalytic domain of NEURL4 in E.Coli and performed in vitro ribosylation assays with the recombinant protein alone. The results, included in the new Figure 2F, confirm that NEURL4 Ct domain indeed mediates ADP-ribosylation in absence of any other ART. Importantly, mutation of key residues in the catalytic domain (as suggested by Reviewer #3) fully abrogates the enzymatic activity, further confirming its direct role in these reactions.

Specific concerns:

1. A clearer pileup bioinformatics comparison of Neurl4 should be provided in Figure 1 with comparison to other known ADPribosyltransferases. The authors use a text discussion and a descriptive domain summary. This is probably insufficient.

We thank this Reviewer for the excellent suggestion. We have modified Figure 1 to include a sequence alignment between the catalytic domain of NEURL4 and PARP1, including the labeling of known motifs and areas of interest. We have also highlighted in the alignment the position of the putative H/E residues that were mutated to generate a catalytically inactive mutant as required to further confirm that NEURL4-dependent ADP-ribosylation is mediated via the predicted catalytic domain.

2. It would be helpful to clarify the nature of the ADPribosylation being observed for Neurl4 activity. Is it all PAR or mono-ADPribosylation or both? This would better clarify the nature of the activity being shown.

This is an excellent question. The identification of NEURL4-mediated ADP-ribosylation using an anti-PAR antibody suggests that NEURL4 can promote poly-ADP ribosylation, which is in agreement with the presence of a conserved E residue in the catalytic domain. At the same time, results of the in vitro ADP ribosylation assay shown in Figure 2F suggest that the recombinant Ct domain can at least mediate PAR formation. While more biochemical and structural studies will be necessary to fully uncover the nature of NEURL4 enzymatic activity, we don’t exclude the possibility that NEURL4 could mediate both MARylation and PARylation. We have now directly addressed this issue in the Discussion and revised the text to avoid confusion in the nomenclature used and to use the most recently approved nomenclature in the field (ADP-ribosyltransferases, an update on function and nomenclature. Luscher et al., 2021).

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

In this manuscript, Perissi et al described and characterized NEURL4 as an important ADP-ribosyl transferase (ART) in mitochondria and as a key regulator of mitochondrial ADP-ribosylation/DNA repair. They generated two NEURL4-KO cell lines and used them to show the important role of NEURL4 in
mitochondrial PARylation. Overall, this manuscript succeeds in the main goal of showing NEURL4 as an important factor for mitochondrial ADP-ribosylation, which is required for mitochondrial stability. Identification of the major mitochondrial ART is one of very important issue in the field, and this manuscript provides a new insight into this aspect. However, this manuscript lacks critical data to support that NEURL4 is an actual ART, such as the in vitro PARylation using purified recombinant NEURL4 (full-length or catalytic domain) and the NEURL4-KO complementation experiments using catalytically inactive mutant (mutations in H-Y-E, in particular E). In addition, authors need to confirm the PARylation of some of identified ADP-ribosylated proteins by recombinant NEURL4 or using N4-KO cells. This reviewer would like to suggest accepting this manuscript after major revision. Here are the specific points.

We are extremely grateful to this Reviewer for the positive evaluation and for the constructive feedback that have helped us strengthen the original manuscript. As discussed in the response to Reviewer #2, we fully acknowledge the importance of showing that recombinant NEURL4 can mediate ADP-ribosylation in vitro in order to prove that NEURL4 is an actual ART – as opposed to a modifier/regulative partner of another enzyme. In response to both Reviewers suggestions, we have now included data confirming NEURL4 enzymatic activity through in vitro AD-ribosylation assays with the C-terminal catalytic domain alone.

Major points;
1. As far as I understand, no one has clearly shown that the isolated recombinant full-length or C-terminal part of NEURL4 has the ART activity. Similarly, data in this manuscript also used NEURL4 that was obtained by immunoprecipitation from cell extracts, which may include other ARTs. Authors should use the full-length and/or the C-terminal domain of NEURL4 (i.e., from E. coli) to show their in vitro PARylation activity. In addition, authors should show these recombinant NEURL4 proteins are not inhibited by PARP1 inhibitor, but by BGP-15.

We fully agree with this Reviewer that showing that isolated recombinant NEURL4 can mediate ADP-ribosylation is a critical result. In the past, we had struggled with expressing the full size NEURL4 due to its size and multiple repetition of conserved sequences within the NEUZ domains promoting recombination. We have now followed the suggestion of using the catalytic domain alone and we are happy to be able to include in the manuscript confirmation that the recombinant C-terminal domain of NEURL4 carries ART activity, which is abrogated by mutagenesis of the conserved H and E residues.

As expected, in vitro assays with the recombinant catalytic domain confirmed that NEURL4 is not inhibited by PARP1 inhibitor Olaparib. However, to our surprise, we found that BGP-15 was also unable to inhibit NEURL4 catalytic domain. We have added text in the manuscript to discuss how this result may be due to a few different reasons, including the fact that inhibition may be indirect or occurring through binding to a region distinct to the catalytic domain per se as the mechanism of action of BGP-15 is currently unknown. Future work to identify specific inhibitors for NEURL4 will be essential to have tools to better investigate the biological outcomes of NEURL4 activity in vivo.

2. Figure 2B and D.
- In Figure 2B, N4-KO cells complemented with N4-FL show very weak N4-FL expression, compared to WT. In contrast, these N4-FL-complemented cells show very potent PARylation in Fig 2D.

We apologize for failing to acknowledge in the legend that in fact different amount of plasmid where transfected in the two experiments. We have now modified the figure legend to clearly indicate that a
lower amount of the full length construct was expressed in Fig. 2B (200ng) as compared to the usual amount used in Fig. 2D (2ug). The reason behind this choice is to avoid blot saturation that would have masked the signal of the endogenous protein.

- This also raises possibility that NEURL4 might be a regulatory protein, such as activator of other ARTs or inhibitor of ubiquitin ligase, rather than a direct ART enzyme.

This is an excellent point. We have now included text to address the possibility that NEURL4 may regulate the activity of another enzyme, including other ARTs or ubiquitin ligases. This is certainly a possibility, particularly considering that i) PARP1 was found among proteins with increased PARylation in our proteomics dataset, ii) NEURL4 has been shown to activate the E3 ligase Herct2, and iii) indirect effects through modulation of separate enzymes have been reported as a mechanism of action of other ARTs – such as PARP9, for example. However, the new data with the recombinant catalytic domain indicates that at least some of its effect are due to its independent PAR activity.

3. Figure 2D.
Authors should compare +Ct and catalytically inactive Ct (in H-Y-E, all or in particular E).

Please see Fig 2F for this data and alignment in Figure 1A for description of the mutations (we mutated H and E, while Y does not appear to be conserved.

4. Figure 2E.
- Compared to Figure 2D, the overall PARylation signal is too weak and there is no PARylation even in WT. In contrast, in Fig 2C, 2D, and 3E, WT shows very robust PARylation. Authors should explain this discrepancy.

We agree with this Reviewer that the basal ADP-ribosylation signal in Figure 2E appears lower than in other panels, this is due to reduced exposure to avoid saturation of the lanes with inhibitor treatments.

- Were these performed in the presence or absence of genotoxic or any other stresses to induce PARylation? Is there any evidence that the ART activity of NEURL4 is DNA- or damage-dependent?

We agree with this Reviewer that this is an important point. None of the experiment were performed in presence of genotoxic stress and we have no evidence that indicates that the ART activity of NEURL4 is activated by stress as we observe significant activity under physiological conditions. At the same time, in vitro assays with both immunoprecipitated full length NEURL4 or recombinant catalytic domain alone show increased enzymatic activity in presence of DNA. We have highlighted these conclusions in the text.

5. Fig. 4. Authors should show the PARylation of some of identified ADP-ribosylated proteins by recombinant NEURL4, or the change of their PARylation level in WT and N4-KO cells.

As suggested, we show that the PARylation of LIG3 and CPS1 is significantly diminished in NEURL4 KO cells. Please see Figure 3E and Sup. Fig. 2B

Minor points;
1. Some of the western blots are quite poor, for example Fig 2F and 3F.
October 6, 2021

RE: JCB Manuscript #202101021R

Dr. Valentina Perissi
Boston University School of Medicine
Biochemistry
72 E Concord St
Silvio Conte Building, K-616
Boston, MA 02118

Dear Dr. Perissi,

Thank you for submitting your revised manuscript entitled "ADP-ribosylation of mitochondrial proteins is mediated by Neuralized-like protein 4 (NEURL4)". We are pleased to say that the reviewers are now supportive of publication. Please be sure to address all the remaining concerns raised by reviewers in the final version of the manuscript. Pending these revisions and any revisions necessary to meet our length and other formatting guidelines (see details below), we would be happy to publish the paper in JCB.

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

A. MANUSCRIPT FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/site/misc/ifora.xhtml. Submission of a paper that exceeds these limits without prior discussion with the journal office will delay scheduling of your manuscript for publication.

1) Text limits:
Character count for Reports is < 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.

2) Figures limits: Reports may have up to 5 main text figures.

3) Figure formatting:
Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. *** Please, add molecular weight markers to main figures 1E, 2A-E, 3E-F, 3H and supplementary figures 1A, 2B.

Scale bars must be present on all microscopy images, including inset magnifications. *** Also, please avoid pairing red and green for those images in which separate channels or quantification graphs are not shown to ensure legibility for color-blind readers. Please change the color scheme of main figures 1B, 4C.

4) 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. We are aware that, in most of the cases, n is indicated in the figure, but there are some panels (i.e. main figure 5G) in which this information is missing.

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.). As you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience.

The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.
While your title is fine, we would suggest something in the active voice: “Neuralized-like protein 4 (NEURL4) mediates ADP-ribosylation of mitochondrial proteins.”

6) 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.”

7) 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.

8) Microscope image acquisition:

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

9) 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.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Reports may have up to 3 supplemental figures.

Please also note that tables, like figures, should be provided as individual, editable files.

*** A summary of all supplemental material should appear at the end of the Materials and methods section.

11) 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.

12) 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.”

13) *** 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/).

14) 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.

15) Materials and data sharing: All datasets included in the manuscript must be available from the date of online publication, and the source code for all custom computational methods, apart from commercial software programs, must be made available either in a publicly available database or as supplemental materials hosted on the journal website. Numerous resources exist for data storage and sharing (see Data Deposition: https://rupress.org/jcb/pages/data-deposition), and you should choose the most appropriate venue based on your data type and/or community standard. If no appropriate specific database exists, please deposit your data to an appropriate publicly available database.

*** Please, indicate in the methods section the accession numbers corresponding to the RNAseq and proteomics datasets generated in this study.

B. FINAL FILES:
In order to accept and schedule your paper, we need you to upload the following materials to eJP. If you have any questions about the online submission of your final materials, please contact JCB's Supervising Manuscript Coordinator, Lindsey Hollander (lhollander@rockefeller.edu).

1) Electronic version of the text: An editable version of the final text is needed for copyediting (no PDFs).

2) High-resolution figure and video files: Individual high-resolution, editable figure files must be provided for each figure. Acceptable figure file formats are .eps, .ai, .psd, and .tif. JCB cannot accept PowerPoint files. All images must be at least 300 dpi for color, 600 dpi for greyscale and 1,200 dpi for line art. Videos must be supplied as QuickTime files.

3) It is JCB policy that if requested, original data images must be made available to the editors. Please ensure that you have access to all original data images prior to final submission.

4) Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the cover or table of contents. Images should be uploaded as .tif or .eps files and must be at least 300 dpi resolution.

**The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.**

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

You can contact me or the scientific editor listed below at the journal office with any questions, jcellbiol@rockefeller.edu.

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

Sincerely,

Johan Auwerx
Monitoring Editor
Journal of Cell Biology

Lucia Morgado-Palacin, PhD
Scientific Editor
Journal of Cell Biology

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

I am happy that the authors have responded to my points appropriately and that this paper is ready for publication. I did however note a typing error in Mitofillin (should be one L not two).

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

The authors have for the most part satisfied the raised points from the prior review.

Specific comment
A key observation made from new Figure 2F is that ADPribosyltransfer activity appears to be DNA dependent. However, the authors really dont discuss this aspect. This DNA dependence should be further discussed in both the results as well as the discussion. In the further discussion of what biochemical questions remain about the biochemical activity of NEURL4 the DNA-aspect as well as caveats with the nature of the ADP-ribosylation should be emphasized (i.e. poly or mono-ADPribosylation).

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

All points were addressed. In method, purification of recombinant NEURL4-Ct (WT and mutant) is missing.