EMBO reports

Dynamics of SLC25A51 Reveal Preference for Oxidized NAD+ and Substrate Led Transport

Shivansh Goyal, Akhilesh Paspureddi, Mu-Jie Lu, Hsin-Ru Chan, Scott Lyons, Crystal Wilson, Marc Niere, Mathias Ziegler, and Xiaolu Cambronne
DOI: 10.15252/embr.202256596

Corresponding author(s): Xiaolu Cambronne (lulu@austin.utexas.edu)

Review Timeline:

Submission Date:	1st Dec 22
Editorial Decision:	25th Jan 23
Revision Received:	12th Jun 23
Editorial Decision:	19th Jul 23
Revision Received:	23rd Jul 23
Accepted:	27th Jul 23

Editor: Martina Rembold

Transaction Report:

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

Dear Dr. Cambronne

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

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also raise a number of technical concerns and suggest experiments to strengthen and validate your conclusions. I think that all suggestions are valid and addressing them will substantially strengthen your study. Regarding HAP1 knock-out cell lines, it will be good to at least repeat key experiments using this cell line.

I realize that the revision will involve a lot of additional work and am happy to discuss the revision and its timeline further via e-mail or a video call, if you wish.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (Arpil 25th). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

Your manuscript contains currently 4 figures and will therefore be published as a short report. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice.

*****IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

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

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our

Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

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

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

9) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

10) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

12) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

While structural characterizations for SLC25A family carriers are available to illustrate the overall protein fold and to support the alternative access transport mechanism, the molecular detail how different family members transport metabolites represents an important question. Here, the authors applied molecular dynamic simulation and mutagenesis studies to SLC25A51, the recently discovered mitochondrial NAD carrier to address this problem. The previously developed NAD fluorescent sensor provides a nice cellular system to screen for functional mutants predicted by computational analysis. The study provided several interesting insights on NAD transport, and would benefit by digging deep into some of these mechanisms and by validating using another orthologous assay. For instance:

(1) The significance of cardiolipin binding. R82, R174 and R270 residues are predicted to bind cardiolipin and the triple mutants failed to transport NAD. Direct interaction with cardiolipin through these residues needs to be demonstrated experimentally.
(2) The salt bridges. Different from the ADP/ATP carrier, the study found that SLC25A51 exhibits a weak matrix salt bridge and almost dispensable cytoplasmic gate -- this result is interesting especially if relevant to its unidirectional NAD import. For instance, while single mutants (E139Q or K236Q) are non-functional, how about double mutant swapping residues (E139K, K236E), introducing another salt bridge (Q142E, Q52K), or changing the single salt bridge to Q52-Q142 position (E139Q, Q142E,Q52K)? Will a larger NAD gradient compensate for the impaired salt bridge and permit NAD transport? Another transport assay might be helpful too.

(3) The ligand specificity. The authors referred to the contact sites proposed for the ADP/ATP carrier -- this comparison requires a similar binding model for NAD and adenine nucleotide, two structurally different nucleotides. It would be helpful to perform docking for ADP/ATP with its carrier, and present a superimposed figure showing SLC25A51 and ADP/ATP carrier to demonstrate that the orientation of bound ligands and contact sites are similar. If this is case, one might be able to explore ligand specificity for NAD, over NAD(P), NMN, and other dinucleotides.

(4) For experimentally validated residues important for NAD transport, are they conserved in SLC25A51 paralogs and orthologs, in comparison with other nucleotide carriers? For the most interesting mutants, it might be helpful to perform an orthologous transport assay to confirm.

Minor points:

Figure 1, the specific mutant needs to be named in the figure.

A cartoon diagram summarizing all the mutants studied and their putative functions might improve communication.

The relative expression level of the FLAG-tagged recombinant protein in comparison to the endogenous carrier needs to be shown.

Referee #2:

The article reports the molecular characterization of SLC25A51, a mitochondrial NAD+ transporter in mammals. The major finding is that SLC25A51 requires cardiolipin and that the matrix gate of SLC25A51 is comprised of a single salt bridge interaction between E139 and K236 and that this interaction is required for SLC25A51 activity. This is supported by a combination of modeling, simulations and biochemical assays. The study also found that the glutamine braces and arginine cap

residues contribute to regulating SLC25A51 activity but they are not required for its activity. The overall impression of the manuscript is that it presents a detailed and thorough analysis of the molecular mechanisms of SLC25A51. The study's strengths include the use of multiple methods to confirm the findings, and the use of simulations, which allowed the researchers to identify the matrix gate, and the use of the mitochondrial NAD+ sensor assay to test the requirement of the matrix gate for SLC25A51 activity. The study's limitations include that the research is based on computational models. In particular, They found that mutation of all three cardiolipin binding sites resulted in loss of SLC25A51 activity, indicating that cardiolipin is likely required for SLC25A51 activity. The strong association between an SLC25A family member and cardiolipins has been shown before for the ADP/ATP transporter.

Overall, the claims in this manuscript are novel and convincing. The authors use a combination of different techniques to gain insights into the mechanism of SLC25A51. The use of different techniques helps to strengthen the conclusions made in the manuscript. The authors also provide clear and detailed explanations for their results and conclusions. The authors provide a comprehensive literature review on the topic, which helps to put their results and conclusions in the context of earlier literature. They also cite previous studies that have been conducted on SLC25A51 and other mitochondrial NAD+ transporters. This manuscript and its finding will be of interest to mitochondrial biology.

The main short coming of this manuscript is that the authors use only one type of biochemical assay to validate their computational findings. The biochemical is not well described in the manuscript. In order to strengthen the claims being made, the authors needs to validate with another biochemical method as described above. However, I do believe that the experimental data of sufficient quality to justify the conclusions.

Referee #3:

SLC25A51 is a mitochondrial carrier recently shown to mediate NAD+ transport into mitochondria, a very important function for mitochondrial and cellular metabolism. In this manuscript, entitled "Dynamics of SLC25A51 reveal preference for oxidised NAD+ and substrate led transport", the authors explore the molecular mechanism of NAD+ binding and translocation via molecular dynamic simulations, mutagenesis and assessments of NAD+ fluctuations in mitochondria using a genetically-encoded fluorescent sensor for free NAD+.

Understanding the molecular mechanism of NAD+ transport is of fundamental biological importance; hence, the topic is significant and important for the field. The manuscript is generally well-written and easy to follow, although more information should be added in the Methods section, as described below. The major issue with this work is that the authors attempt to cover all aspects of substrate binding and translocation with a functional assay that cannot define the details of the molecular mechanism. Despite great effort to study mutations at positions flagged by the in silico analyses, the functional data do not provide strong evidence for the conclusions made. This is critical, especially because the two SLC25A51 models used here appear to differ in important positions (as stated in the Results, page 5). Regarding the functional assay, first, the mutants have been expressed in the background of endogenously expressed SLC25A51 in HeLa cells. Second, this assay is not a direct assessment of substrate transport by SLC25A51 and substrate kinetics are not provided. Moreover, the authors propose substrate binding residues without performing any type of substrate binding studies. Additional assays would be necessary to establish the validity of the hypotheses driven by the MD simulations and for this manuscript to be reconsidered.

Major comments

1. It would be appropriate to study the SLC25A51 mutations in a null background. A HAP1 knock-out cell line for SLC25A51 has been previously reported and is viable. I can't see why the authors would not apply their biosensor assay in this cell line, especially if it is already available.
2. It is not mentioned whether or how the authors have normalised their flow cytometry data against the expression levels of the individual mutants. Although most mutants appear to express well, there are differences in expression levels that could account, at least in part, for the observed changes in the functional data. The authors should either explain how they normalised the data, if they have done so, or perform normalisation, making sure the signal on their blots is not saturated, as currently seen is some figures (see Figure 1 and 2).
3. The evaluation of the expression levels has been made based on total lysates rather than isolated mitochondria. How can the authors exclude the possibility that some mutants have biogenesis defects and are not properly localised in the mitochondrial membrane? This could be addressed either by imaging experiments or by performing subcellular fractionation and testing the expression levels in isolated mitochondria by WB. Also, a mitochondrial marker should be used as control for Western Blots.
4. The technical limitations of the sensor should be discussed. What is the response time and the sensitivity of the sensor? Depending on the nature of the substitution introduced in certain residues, could there be modest but significant changes that have been missed with this approach?
5. It will be useful if the authors can provide an alignment of SLC25A51 across species. Are the functionally important residues conserved? For example, one would expect the substrate binding site residues to be conserved. Are the functionally important residues conserved in NAD transporters from yeast and Arabidopsis?
6. For the models used, it is stated that "...several residues in the central pore that were hydrophobically buried in one model but

exposed to the hydrophilic pore in the other". Which model was finally supported by the mutagenesis analysis?
7. Regarding the NAD+ binding site, to support substrate co-ordination by a given residue, substrate binding studies are necessary. With the current approach one cannot conclude whether a residue plays a role in the binding step versus any other step in the transport cycle. If there is no binding assay available, the authors could at least show kinetic analyses of substrate transport in isolated mitochondria.
8. It is claimed that the cytoplasmic gate is not necessary for function based on the finding that mutating the two residues of the putative ionic gate K198/E291 to glutamines (double mutant) had no effect on activity. However, there is a possibility to have hydrogen bond formation by mutating both residues to glutamine. Is the result the same when the pair is mutated to alanine?

Minor comments

1. It is stated that all original data are included but I can only see processed data. There are no raw data for the functional assays and biological repeats or figures of the full-size Western blots.
2. The authors should give important details on the experimental design for NAD+ measurements for this particular study, beyond referring to a previous publication. For example, what were the timelines for transfection of the mutants and the NAD+ measurements?
3. It will be good to rationalise the choice of the specific substitutions introduced in each position (also see major comment 8).

Dear Referees, Dr. Rembold, and Editorial Staff,

Thank you for the generally supportive and thoughtful reviews. We appreciate the acknowledgement that this study addresses an “important question” and that “the topic is significant and important for the field”. We appreciate the assessment that that we have striven to provide “a detailed and thorough analyses of the molecular mechanisms of SLC25A51”; that “the claims in this manuscript are novel and convincing” and that “the study’s strengths include the use of multiple methods.”

The suggestions to provide additional supporting evidence for our central claims were excellent, and we have worked to address all comments. We submit for your consideration an improved manuscript with multiple additional experiments and figures, as well as point-by-point responses.

Our major revisions are summarized below, and we have included a full list of the new figures. Text edits are highlighted in blue font in the accompanying manuscript for easy tracking of revisions.

Major Revisions:

1. We repeated key sensor experiments in knockout cell lines to test the effects of expressing SLC25A51 variants without the presence of endogenous SLC25A51 activity and with initially lowered mitochondrial NAD^+ concentrations.
2. We incorporated new uptake data as an orthogonal test of SLC25A51 activity throughout all figures. Additionally, this assay allowed a direct comparison of NAD^+ or NADH as potential ligands.
3. We tested how cardiolipin bound to wildtype and mutated SLC25A51.
4. We confirmed that expression of SLC25A51 variants colocalized with mitochondrial inner membrane enzyme Cox IV. We also included analyses of Western Blots that were normalized using mitochondrial protein HSP60 instead of cytoplasmic actin protein.
5. We tested new mutations that mis-aligned the c-state salt-bridge from the ligand binding pore to assess the importance of positioning the positively charged nicotinamide ring in NAD^+ so that it may directly interact with the negatively charged residue in the salt bridge.

List of New Figures:

Fig. 1C	Representative Western Blot with HSP60 loading control for cardiolipin binding mutant
Fig. 1D	Pulldown assay with Flag-SLC25A51 variants and cardiolipin-coated beads
Fig. 1E	Representative ^{32}P-NAD^{+} uptake comparing wildtype and cardiolipin binding mutant
Fig. 1F	Quantitation of uptake assays
Fig. 2D	Representative Western Blot with HSP60 loading control for matrix gate mutants
Fig. 2E	Quantitation of uptake assays
Fig. 2F	Representative ^{32}P-NAD^{+} uptake comparing wildtype and matrix gate mutants
Fig. 3C	Representative Western Blot with HSP60 loading control for E132 mutants
Fig. 3D	NADH Competition Assay with E132A mutant
Fig. 3F	^{32}P-NAD^{+} uptake data with mutated contact sites in recombinant E. Coli
Fig. 3G	Representative images of ^{32}P-NAD^{+} uptake with contact site mutants
Fig. 3H	Representative Western Blot with HSP60 loading control for contact site mutants
Fig. 4E	Sensor Assay to test new mutants and representative Western Blot with HSP60 loading control
Fig. EV1B	Alignment of SLC25A51 orthologs and paralogs
Fig. EV3A	Representative IF colocalization of transiently expressed variants and Cox IV
Fig. EV3B	Quantitation of mean expression level of variant relative to wildtype SLC25A51 in HeLa cells
Fig. EV3C	Representative fractionation of E. Coli membranes and E132A mutant
Fig. EV3D	Expression of Cardiolipin-binding mutant in E. Coli membranes; TolC loading control
Fig. EV3E	Time-dependent uptake of ^{32}P-NAD^{+} in recombinant E.Coli expressing wildtype (red) or mutant (black) SLC25A51
Fig. EV3F	Western Blots showing loss of endogenous SLC25A51 protein in KO cells and overexpressed ^{Flag}SLC25A51 in wildtype cells
Fig. EV3G	Biosensor Assay with predicted cardiolipin-binding mutants and HSP60 Western Blot control in SLC25A51 KO cells
Fig. EV3H	Biosensor Assay with cytoplasmic gate mutants and HSP60 Western Blot control in SLC25A51 KO cells
Fig. EV3I	Expression of variants in E.Coli membranes; TolC loading control
Fig. EV3J	Biosensor Assay with binding site mutants and HSP60 Western Blot control in SLC25A51 KO cells
Fig. EV4B	Representative Western Blot with HSP60 loading control for predicted cap mutants
Fig. EV4E	Additional cytoplasmic gate mutations and HSP60 Western Blot control
Fig. EV4F	Quantitated ^{32}P-NAD^{+} uptake assays with new cytoplasmic gate mutants
Fig. EVG	Representative ^{32}P-NAD^{+} uptake assay with new cytoplasmic gate mutants
Fig. EVH	Expression of cytoplasmic gate mutant in E. Coli membranes; TolC loading control
Fig. EV5	Cartoon Representation of mutations in this study

Referee #1:

Thank you for the supportive comments and excellent suggestions to improve the work.

(1) Direct interaction with cardiolipin through R82, R174 and R270 residues.

To strengthen our examination of whether cardiolipin regulates SLC25A51 activity, we performed two additional assays. We tested whether cardiolipin interacted with SLC25A51 at the computationally identified sites and whether this impacted uptake activity in vitro. We found that mutation of R82, R174, and R270 (1) impaired SLC25A51 pulldown by cardiolipin-coated beads and (2) impaired the uptake of ^{32}P-NAD$^+$ in a recombinant assay. Together, the revised Fig.1 presents the corroborating data indicating the significance of cardiolipin binding on SLC25A51 activity via sites identified in this study. The simulations are now further supported by in vitro pulldown assays with cardiolipin-coated beads (*Figure 1D*), quantitation of recombinant uptake activity (*Figures 1E and 1F*), biosensor measurements of free mitochondrial NAD$^+$ in intact and respiring cells (*Figure 1G*), and biosensor measurements of free mitochondrial NAD$^+$ in SLC25A51-knockout cells lacking endogenous uptake activity (*Figure EV3C*).

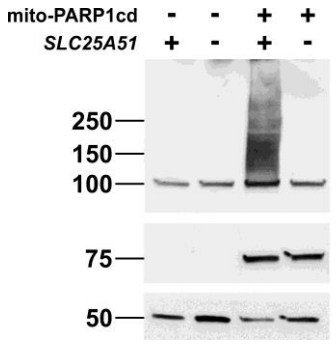
(2) Functions and position of the salt bridges.

We independently generated and expressed these interesting variants suggested by the reviewer: the reversed salt bridge (reversed SB, E139K-K236E), two salt bridges (extra SB, Q52E-Q142K) and a shifted salt bridge position (shifted SB, E139Q-K236Q-Q52E-Q142K) (*Figure 4E*). We tested effects of the mutations on SLC25A51 activity using sensor assays in cells lacking endogenous SLC25A51. All three variants had impaired function compared to wildtype. The reversed and shifted salt-bridges are energetically equivalent to wildtype. However, E139 is now positioned farther away from E132, which we have shown engages the positively charged nicotinamide ring on NAD$^+$ (*Figure 3*). We propose that this misalignment affects efficient ligand channeling and ligand-led gate opening, and thus there is an issue with the mechanics of gate opening. In the case of two matrix salt-bridges relative to the single cytosolic salt-bridge, interestingly there is some residual activity despite major impairment. This indicates that the preserved E139-K236 salt bridge is positioned to be functional. Nevertheless, the extra bond appears to hamper the free energy of gate opening leading to significantly diminished activity.

(3) Will a larger NAD gradient compensate for the impaired salt bridge and permit NAD transport?

We do not yet have the ability to test SLC25A51 activity in fully reconstituted proteoliposomes, which would be ideal for establishing a variety of gradients including increasing interior NAD$^+$ concentration. We instead altered the gradient experienced by the salt-bridge mutants by expressing them in HEK293 SLC25A51 knockout (KO) cells with lowered NAD$^+$ concentrations selectively in the mitochondrial matrix. The lowered concentration of mitochondrial NAD$^+$ was determined by diminished Mito-Paraplay activity (*Response Figure A*); Mito-Paraplay is an assay that subcellularly localizes a constitutively active version of the PARP1 catalytic domain (PARP1cd) to the mitochondrial matrix. Thus Mito-Paraplay, as a probe for NAD$^+$-dependent PARylation, was used to assay for local NAD$^+$ concentrations. The K_D of PARP1 for NAD$^+$ is ~40 µM. This indicates that concentrations in the matrix in KO cells were less than 40 µM. We therefore expect that in

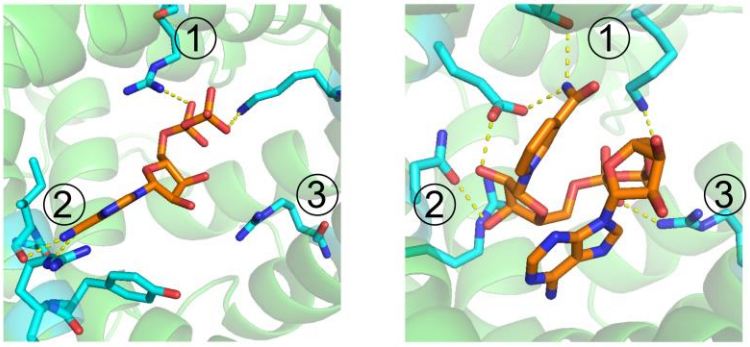
KO cells the gradient experienced by SLC25A51 would be either eliminated or even reversed compared to wildtype cells. In other words, the transporter would experience in wildtype cells ~50-100 µM cytosolic NAD^+ and ~250 µM matrix NAD^+; in a KO cell, it would now experience a shift in gradient of ~50-100 µM cytosolic NAD^+ and < 40 µM matrix NAD^+. Altering the gradient in this manner did not affect the requirement of this salt bridge (*Figure EV3C*), indicating that the salt-bridge mutation was insensitive to relative NAD^+ concentrations and did not convert the transporter into a gradient-dependent channel.



Response Figure A. *The catalytic domain of PARP1 (Mito-PARP1cd) is expressed and localized to the mitochondrial matrix in HEK 293 wildtype (+) or SLC25A51 KO (-) cells, as indicated. NAD^+-dependent PARP1 activity is assessed by detection of poly-ADP-ribosylation of co-expressed PARP1-Flag (PAR).*

(4) Ligand specificity, comparing ADP in its carrier to NAD^+ in SLC25A51.

We adopted the terms “contact site 1, 2, and 3” to align with the vocabulary used to describe the ADP/ATP carrier and didn’t intend to imply that the sites were similar. We have updated the text for clarification (page 11). We docked ADP onto the human SLC25A4 homology model based on the solved structure of the bovine ADP/ATP carrier(*Response Figure B*). We broadly observed that ADP adopted a pose with similarities to its reported binding (Mavridou et al 2022), including the interaction of the adenine ring of ADP with a hydrophobic pocket at contact site 2 in SLC25A4. SLC25A51 lacks hydrophobic residues at its contact site 2 (R182, N183 and S186, with E132 nearby), which may explain how it discriminates against binding of adenine nucleotides. Moreover, in contrast to the clear positioning of the adenine in SLC25A4, the adenine in the NAD^+ molecule did not consistently orient in any dedicated way, and thus it appears that the NR moiety dominates the guiding of NAD^+ in SLC25A51. As for SLC25A51’s selectivity against other related ligands, we hypothesize that the smaller NMN is unable to easily make contacts with all three sites and that this prevents it from being a good substrate. And while there is not obvious steric hindrance for the additional phosphate in NADP+ and NADPH, the phosphate group would increase the overall negative charge of the molecule by -2 and this may not be compatible for transport by the binding site in SLC25A51.



Response Figure B. *Left,* ADP docked in HsSLC25A4 with interacting residues indicated in contact sites 1, 2, and 3. *Right,* NAD^+ docked to SLC25A51 after MD simulations.

(5) Are experimentally validated residues conserved in SLC25A51 paralogs and orthologs, in comparison with other nucleotide carriers?

Thank you for this suggestion. We have included a sequence alignment of SLC25A51 paralogues and orthologues from different species (*Figure EV1B*). Experimentally validated residues, such as E132, are highlighted. We observed conservation among SLC25A51 paralogs and orthologs, but not with Ndt1-type carriers in yeast or in plant. Together, this indicates a distinct mechanism for SLC25A51 homologues.

(6) An orthologous transport assay for confirmation.

All reviewers requested an orthologous transport assay, which is one of the major revisions in this study to support the main claims of our work.

To monitor transport, we followed previously published assays that used bacterial cells (e.g. Haferkamp et al 2002, Ravaud *et al*, 2012; Mifsud *et al*, 2013). We ectopically induced expression of recombinant human SLC25A51 and its variants individually in *E. Coli* cells, then monitored the resulting uptake of ^{32}P-NAD^{+} compared to cells expressing control YFP protein (*Figures 1-3 and EV3-EV4*). Although recombinant bacterial systems do not accurately recapitulate the kinetics of uptake (Haferkamp et al 2002 and *Figure EV3E*), they have been used successfully for determining relative activity (Haferkamp et al 2002, Ravaud *et al*, 2012; Mifsud *et al*, 2013). The bacterial system avoids the need to isolate active mitochondria immediately prior to the assay. Consequently, this method increased the reproducibility of results and facilitated the analyses when comparing activity across numerous variants.

We used this assay to determine effects of mutations on transport of cardiolipin-binding mutants (*Figure 1E,F*), matrix and cytoplasmic gate mutants (*Figures 2E, F and Figure EV4F, G*), and ligand contact site variants (*Figure 3D, F, G*). These represent new data for this revision.

Minor points:

- *Figure 1, the specific mutant needs to be named in the figure.*

  Thank you, we have included labeling to specify the mutation in *Figure 1*.

- *A cartoon diagram summarizing all the mutants studied and their putative functions might improve communication.*

  This is a great idea. We have added a new cartoon figure of all the residues that were tested in this study (*Figure EV5*).

- *The relative expression level of the FLAG-tagged recombinant protein in comparison to the endogenous carrier needs to be shown.*

  To determine the relative expression of the proteins, we used Western Blotting with an antibody targeting amino acids 1-35 of human SLC25A51 to compare endogenous levels in HeLa cells with ectopically overexpressed ^{Flag}SLC25A51 on the same blot. To identify endogenous SLC25A51, we resolved lysate from HEK 293 wildtype and HEK 293 SLC25A51 knockout (KO) cells. The bands corresponding to endogenous SLC25A51 are indicated by red dots in *Figure EV3F*, and ^{Flag}SLC25A51 is indicated by green dots. Transiently transfected plasmid for CMV-^{Flag}SLC25A51 resulted in dramatically higher levels of ectopic SLC25A51 variants compared to its endogenous protein (*Figure EV3F*).

Referee #2:
We thank the reviewer for their overall supportive comments.

(1) Validate with another biochemical method.

This suggestion to validate was helpful for strengthening the major claims of the work and was echoed by the other reviewers. We adopted a second biochemical activity assay to test the uptake of ^{32}P-NAD$^+$ from ectopically expressed variants in a recombinant *e.coli* based system. This assay was adapted from published works. We have described the assay in the main text methods, the revised text is highlighted in blue, as well as in response #6 to reviewer 1. Although recombinant bacterial systems do not accurately recapitulate the kinetics of uptake (Haferkamp et al 2002 and *Figure EV3E*), they have been successfully used for determining relative activity (Haferkamp et al 2002, Ravaud *et al*, 2012; Mifsud *et al*, 2013). The bacterial system bypasses the isolation of active mitochondria immediately prior to the assay. Consequently, this method increased the reproducibility of results and facilitated the analyses when comparing activity across numerous variants.

We have now included new figures depicting the uptake activity from cardiolipin-binding mutants (*Figure 1E,F*), matrix and cytoplasmic gate mutants (*Figures 2E, F and EV4F, G*), and ligand contact site variants (*Figure 3D, F, G*).

Referee #3:

Thank you for the expert review and for highlighting that "*the molecular mechanism of NAD+ transport is of fundamental biological importance; hence, the topic is significant and important for the field."* As well as acknowledging that *"[t]he manuscript is generally well-written and easy to follow…"*

- *…more information should be added in the Methods section, as described below. The major issue with this work is that the authors attempt to cover all aspects of substrate binding and translocation with a functional assay that cannot define the details of the molecular mechanism.*

  We have expanded the Methods to include details about the NAD$^+$ sensor measurements used in this study. We have further strengthened the support for our main conclusions with additional experimental data; a list of multiple new figures is included at the top of this document.

- *"…the two SLC25A51 models used here appear to differ in important positions (as stated in the Results, page 5)." "Additional assays would be necessary to establish the validity of the hypotheses driven by the MD simulations and for this manuscript to be reconsidered."*

  The structural models produced by Alpha Fold and Swiss Model were similar and readily superimposed, but there were some differences in the orientation of specific residues. Our hypotheses were generated around residues that were similarly positioned between the models. We have since tested the contributions of differentially positioned residues with new assays, including: sensor assays in cells without endogenous SLC25A51 (knockout cells); sensor assays in cells with respiring mitochondria by

monitoring the contribution from overexpressed variants; recombinant ^{32}P-NAD$^+$ uptake assays; and competition activity assays with unlabeled NAD$^+$ or NADH.

Major comments

(1) Study of the SLC25A51 mutations in a null background.

We have analyzed the effects of SLC25A51 variants in a sensor-expressing HEK 293 clonal cell line that was genetically knocked out for SLC25A51(KO) using a CRISPR-Cas9 guided approach. We validated that the KO cell line did not express endogenous SLC25A51 protein using Western Blotting (*Figure EV3F*). The activities of individual variants were re-tested in these KO cells (*Figure EV3B*). The variants showed similar relative retention or deficiencies in their activity regardless of whether they were overexpressed in HeLa cells or in HEK 293 KO cells. This confirmed that the resulting mitochondrial NAD$^+$ sensor measurements in wildtype HeLa cells reflected the effects of expressing the particular SLC25A51 variant in general. Accordingly, we have since confirmed that the variants were greatly overexpressed compared to endogenous SLC25A51 levels using Western Blot analyses (*Figure EV3F*).

Loss of SLC25A51 resulted in loss of cell respiration, mitochondrial oxidative reactions, and presumably an altered NAD$^+$ gradient experienced by the transporter (Luongo et al, 2020, Kory et al, 2020, Girardi et al 2020). Thus, because of these potentially confounding factors and that we could readily distinguish SLC25A51 activity when overexpressed, we had originally opted not to evaluate the variants in SLC25A51-deficient cells.

(2) Variability in abundance of SLC25A51 mutants.

The relative expression of SLC25A51 mutants used in the original sensor measurements was obtained with quantitative Western Blots and fluorescent Imaging using LiCOR technology. Images were not saturated due to the large dynamic range of the method and there were some fluctuations in expression level that we quantified in *Figure EV3B*. We determined the relative mean expression from three independent transfections for each mutant relative to the parallel expression and detection of transfected wildtype SLC25A51 that was resolved on the same blot. By this method, there were several variants that were expressed at lower levels than wildtype and so we further evaluated each with orthogonal approaches.

We evaluated the proposed cardiolipin binding mutant using an in vitro pulldown assay with equivalent levels of protein compared to wildtype. The mutant was less efficient in binding (*Figure 1D*). We also expressed this variant in E.Coli membranes; here the mutant was more abundant than wildtype (*Figure EV3D*). In the E.Coli system, the mutant was significantly deficient compared to wildtype for uptake for ^{32}P-NAD$^+$ (*Figure 1E-F*).

Although the individual mutations E139Q and E139A were destabilized, mutation of the paired K236 residue in the proposed cytoplasmic saltbridge was equivalently expressed to wildtype. Because mutation of K236 impaired SLC25A51's ability to sustain mitochondrial NAD$^+$ levels in cells and was also impaired ^{32}P-NAD$^+$ uptake, we have confidence that the proposed cytoplasmic saltbridge is required (*Figure 2*). We further determined that the double mutant E139Q-K236Q lost its ability to uptake ^{32}P-NAD$^+$, and this mutant expressed robustly in E.Coli membranes compared to wildtype (*Figure EV3I*).

Mutations in ligand-binding sites that destabilized SLC25A51 in HeLa cells were orthogonally evaluated and determined to be deficient in uptake assays in E.Coli (*Figure 3F, G*), where they expressed equivalently or better than wildtype SLC25A51 (*Figure EV3I*).

For putative cap residues, at least one mutant R57L expressed equivalently to wildtype (*Figure EV3B*). Similarly for matrix gate residues, R194A expressed equivalently to wildtype (*Figure EV3B*).

(3) Expression and localization of variants

We have redone all the western blots in this work to now include mitochondrial marker HSP60 as a loading control such that readers can determine the expression of the variants relative to another mitochondrial protein (*list of new Figures at top of this response*). We have also included immunofluorescence assays (*Figure EV3A*) to determine the co-localization of transiently transfected Flag-tagged variants with endogenous inner mitochondrial membrane protein Cox IV. In all cases, the mutants colocalized with Cox IV within the constraints of light microscopy. We confirmed localization of mutants to E.Coli membranes by fractionation and Western Blotting (*Figure EV3*); endogenous TolC protein was blotted as a marker for E.Coli membranes.

We did not observe any gross effects on mitochondrial morphology from ectopic expression of SLC25A51 variants; loss of SLC25A51 expression additionally is not known to have any significant impact on mitochondrial volume per cell or morphology (Luongo et al 2020, Kory et al 2020, Girardi et al 2020).

(4) Response time and the sensitivity of the sensor

The assays using the NAD^+ sensor reported on steady-state free mitochondrial NAD^+ levels 24 - 48 hours post-transfection of the variant. This is well within the sensor's response time; the NAD^+ sensor can respond to fluctuations in NAD^+ levels within seconds and has been used to obtain in-cell turnover rates (Cambronne et al 2016).

We re-performed the sensor experiments in SLC25A51 KO cells to increase the sensitivity of the assay by lowering the lower limit, as well as independently evaluated the mutants using sensitive ^{32}P-NAD^+ uptake assays. In both cases, these complementary assays did not reveal significantly new results and corroborated the original sensor data.

(5) An alignment of SLC25A51 across species.

An alignment was also requested by Review 1. We have included a sequence alignment of SLC25A51 paralogues and orthologues from different species (*Figure EV1B*). Experimentally validated residues, such as E132 and contact sites, are highlighted. We observed conservation among SLC25A51 paralogs and orthologs, but not with Ndt1-type carriers in yeast or in plant (*Figure EV1A*). Together, this indicates a distinct mechanism for SLC25A51 homologues.

(6) Which model was supported by the mutagenesis analysis?

It wasn't clear that there was a correct or incorrect model per se. While we observed different initial positioning of some residues between the models (*Figure EV2D*), over the course of the MD simulations many of the residues became aligned. We have edited the text for clarification (*page 4*). For example, the side chain of E132 was originally positioned slightly differently between the models but after simulation its

positioning became the same; F229 and L225 sidechains originally faced the pore in the Swiss Model but became buried after simulation, as predicted by the Alpha-Fold model.

Side chain positions of L280 and W283 were buried in Alpha-Fold but faced the hydrophilic core in Swiss Model. Introducing W283F did not significantly interfere with function as measured by the NAD^+ sensor and W283L only modestly impaired activity. W283A was reported in Kory et al 2020 and had no significant effect on proliferation rate in galactose. Together the data indicated that W283 was not a critical residue for SLC25A51 activity.

The side chain of K91 retained slight differences in the models after simulation; in Swiss Model K91's side chain was oriented upward of its horizontal plane in the pore and in Alpha-Fold it was in the same plane as its backbone. Mutation of K91 in this work and in Kory et al 2020 indicated that this is a critical residue for function. We observed that in Swiss-Model K91's positioning made it unable to readily engage NAD^+ but in Alpha Fold it engaged the phosphates of NAD^+.

(7) Binding studies to support substrate co-ordination by a given residue

Competition assays with unlabeled NAD^+ and NADH were carried out to compare effects on ^{32}P-NAD^+ uptake by wildtype and E132A variants recombinantly expressed in E. Coli (*Figure 3D*). The data showed that loss of E132 permitted NADH to compete with ^{32}P-NAD^+ uptake at 100 µM and 250 µM concentrations. This indicates that E132 plays a critical role in the active site and helps to differentiate ligand identity.

(8) Is the cytoplasmic gate not necessary for function when the pair is mutated to alanine?

We tested the requested double mutant K198A-E291A using the biosensor assay in intact cells (*Figure EV4E*), as well as a recombinant ^{32}P-NAD^+ uptake assay in E. coli (*Figure EV4F, G, H*). Both datasets indicated that K198A-E291A was active at least to the extent of wildtype—and possibly even hyperactive—for the import of NAD^+.

Minor comments

1. Original data

Source data and copies of raw images have been included with this submission and can additionally be found at Texas Data Repository, Cambronne XA Lab Dataverse https://doi.org/10.18738/T8/TIHMRO.

2. Experimental details about NAD^+ sensor measurements

The information has been added to the methods section.

3. Choice of specific substitutions.

For each targeted residue we aimed for 2 to 3 different substitutions and analyzed each variant that resulted in stable protein. Not all substitutions resulted in stable protein and those that were not detectable or expressed significantly less than wildtype were not used for analyses. To test charge of a side-chain, we made substitutions that retained either similar size or geometry of the side chain.

Dear Dr. Cambronne

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and request only minor changes to clarify methodology and data interpretation in some instances and to discuss limitations.

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

- Your manuscript will be published as Scientific Reports. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references). The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice.

- Please remove the Author Contributions from the manuscript file and make sure that the author contributions in our online submission system are correct and up-to-date. The information you specified in the system will be automatically retrieved and typeset into the article. You can enter additional information in the free text box provided, if you wish.

- Figures and their panels should be called out in a numerical and alphabetical order. We note that Fig EV1B is called out after Fig. EV4E and ask to reorganize these figures, if possible.

- All movies need to be ZIPd with their legend (simple README.txt file). The movie legends must be removed from the manuscript file.

- Data availability section: Please only refer to deposited data and please specify the database and the kind of data deposited there (see also comments in attached word file).

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

- On a different note, I would like to alert you that EMBO Press offers a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
https://www.embopress.org/video_synopses
https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

- Finally, EMBO Reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

The authors have presented significant new experimental data that addresses all the major and minor questions raised. This reviewer has no additional comments.

Referee #2:

The authors addressed most of my points.

Referee #3:

The authors have put a lot of effort in addressing the concerns raised and have succeeded in the most part. I am not impressed by the transport assay but I understand the difficulties of setting one up on demand and it seems that the results match the biosensor assay data. I believe this manuscript should now be accepted for publication but it is important that the authors first clarify some issues in the text and explain the transport assay they have introduced.
The following minor issues need to be addressed:
1. Unless I have missed it, it has not been indicated if the data from the biosensor assay, shown throughout the manuscript, have been normalised based on the Western Blot signals. If not done already, the data should be normalised to facilitate interpretation.
2. The cardiolipin binding assay should be explained a bit better. What is the "enriched sample" made of? Is it mitochondria enriched sample from mammalian cells or a protein enriched sample from E. coli expressed protein?
3. The transport assay rationale is not explained and the time course is bit peculiar. What driving force is in place for NAD+ transport in this E.coli assay system? What keeps driving the uptake for hours (Figure EV3, E)? Has the non-specific signal been defined and subtracted? It is understandable that no other transport assay is available at the moment but it is important to discuss the limitations of this assay and explain how it works.

Xiaolu Ang Cambronne, Ph.D.
Department of Molecular Biosciences, College of Natural Sciences

100 E. 24th St., Stop A5000 • Austin, Texas 78712 • 512-232-1928
www.utexas.edu • lulu@austin.utexas.edu

EMBO Reports

July 21st, 2023.

Dear Dr. Rembold,

We appreciate this opportunity and excitedly submit our revised manuscript that has been edited for length, clarification of methodology and data interpretation, and with an additional statement of its limitations.

We have made the following edits:

- Combined the Results and Discussion sections; reduced character count to 26,942 characters including spaces.
- Removed the Author Contributions section from the manuscript file and confirmed the accuracy of the online information
- Ordered the call-out of all figures and panels in the manuscript file.
- Removed Movie legends from the manuscript file and included in Movie ZIP file
- Added requested information to the Data Availability section
- Addressed all comments and tracked changes for all edits this round.
- Included a synopsis with figure for the online version.

We would like to also extend our appreciation to the referees and the editorial team for a productive and timely review of our work.

I can also respond to the most recent comments from Referee #3.

1. "…it has not been indicated if the data from the biosensor assay, shown throughout the manuscript, have been normalised based on the Western Blot signals. If not done already, the data should be normalised to facilitate interpretation."

Normalization of the data is not straightforward because it is unclear what percent change of transporter expression results in changes in mitochondrial NAD^+ steady-state concentrations. Additionally, the fluorescent readout of the sensor is not strictly linear. In lieu, we had worked to identify mutations that did not significantly destabilize SLC25A51 in mammalian cells (Fig. EV3B). We did not include in our study any mutation that destabilized SLC25A51 protein > 50%, and this is why some sites had multiple mutations, eg. K236Q and K236A, R194A (stable) and R194Y (slightly destabilizing).

For mutations where we observed a significant difference in expression in HeLa cells, we tested the same mutation in additional experimental systems such that in at least one paradigm the mutant expressed at equivalent levels to wildtype. We drew conclusions only from

data that was corroborated by multiple assays. I present below a table listing each mutation that significantly differed from wildtype SLC25A51 when expressed in HeLa cells (Fig. EV3B) and the corroborating data in other cell types. We have now clarified this in the 3rd to last sentence of the main text.

Mutation	**Relative expression <u>in HeLa cells</u> (Sensor Experiment)**	**Effect of variant on mito NAD+ levels**	**Distinct Experimental paradigm with equivalent expression to wildtype variant**	**Does 2nd experimental paradigm corroborate HeLa Sensor data?**
R82Q-R174Q-R270Q	~60% of WT	Indicated diminished activity	Expressed robustly in E.Coli membranes (Fig. EV3D, higher than wildtype). Uptake activity was near null (Fig. 1E)	YES
E139Q and E139A	Both ~60% of WT	Indicated diminished activity	Complementary disruption of salt-bridge partner (K236Q and K236A) indicates requirement of this interaction. Double mutation E139Q-K236Q was expressed robustly in E.Coli membranes (Fig. EV3I, higher than wildtype). Uptake activity was near null (Fig. 2E, F)	YES
Q90L	~110% of WT	Indicated comparable activity to wildtype	Expression in HEK 293 cells KO background resulted in similar expression to wildtype (Fig. EV3J). Sensor activity was comparable to wildtype in 293 KO cells.	YES
T94V	~75% of WT	Indicated diminished activity	All attempts to mutate or express in different cell types resulted in partially destabilized protein. We included the data in the figures but did not draw conclusions from this site.	Inconclusive.
R182L and R182Q N183V and N183Q	Ranges between 60-80% of WT	Indicated diminished activity	Expressed robustly in E.Coli membranes (Fig. EV3I, higher than wildtype). Uptake activity was near null (Fig. 3G)	YES
R278L	~80% of WT	Indicated diminished activity	Expressed robustly in E.Coli membranes (Fig. EV3I, higher than	YES

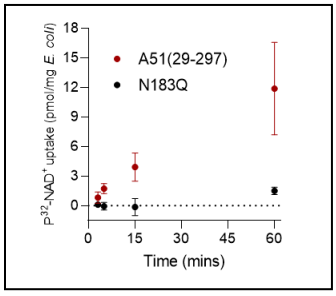
			wildtype). Uptake activity was near null (Fig. 3G)	
R140L and R238L	~50-60% of WT	Indicated diminished activity	Mutation of partner cap residue R57L was not destabilizing and corroborated activity of these mutants	YES
R194Y	~80% of WT	Indicated diminished activity	Mutation R194A did not destabilized and corroborated diminished activity (Fig. EV4E).	YES

2. *"The cardiolipin binding assay should be explained a bit better. What is the "enriched sample" made of? Is it mitochondria enriched sample from mammalian cells or a protein enriched sample from E. coli expressed protein?"*

   From a text search, I believe this reference to "enriched" was from the methods section where we had split the cardiolipin binding assay into two easier-to-read sections (1) Immunoprecipitation of Flag-SLC25A51 variants and (2) Cardiolipin Binding Assay. Enriched was intended to indicate the immunoprecipitated Flag-tagged protein fraction obtained via a Flag bead pulldown done in native conditions. We have edited the sentence for clarity as follows: "Equivalent amounts of ~~enriched~~ *immunoprecipitated* wildtype and mutant ^{Flag}SLC25A51 from HEK293T cells were incubated with 20 µL of cardiolipin-conjugated beads…"

3. *"The transport assay rationale is not explained and the time course is bit peculiar. What driving force is in place for NAD+ transport in this E.coli assay system? What keeps driving the uptake for hours (Figure EV3, E)? Has the non-specific signal been defined and subtracted? It is understandable that no other transport assay is available at the moment but it is important to discuss the limitations of this assay and explain how it works."*

   We had adopted an assay that was published by multiple labs (Haferkamp et al, 2002; Ravaud et al, 2012; Mifsud et al, 2013). Within 0 to 60 min, the observed uptake curve is comparable to that reported in Haferkamp et al 2002 for the uptake of ^{32}P-nucleotides (Insert, zoomed in data from Fig. EV3E from 0 to 60 min). Notably uptake begins earlier than 60 min. At 180 min, we hypothesize that the cellular integrity starts to compromise because we observed influx of ^{32}P in control cells (Fig EV3E, black), thus guiding our experimentally determined timepoint of 60 minutes when there is a clear separation of data points. In our data, any non-specific signal is defined by the negative control, YFP protein with no transport activity. YFP expressing cells were tested in parallel with each experiment and its data used to subtract background.



   In Mifsud et al 2013 the uptake curve for ^{13}C-ADP is continuous. We do not know exactly what regulates the timing or influx. To control for non-specific leakiness of the cells, which is the critical issue, we expressed mutant N183Q in parallel cells (Fig. EV3E, black line). This is why we hypothesize that at 180 minutes the cell membrane integrity begins to compromise, as the baseline drifted upward, indicating ^{32}P influx in N183Q expressing E.Coli cells.

Not having a fully reconstituted proteoliposome assay is a limitation of this study, and we indicate such in the 2nd to last sentence of the main text.

Thank you to all referees and the editorial team for your time and input that improved and strengthened this piece. I hope this final version is acceptable and meets all expectations.

Sincerely,

Lulu Cambronne, Ph.D.
Assistant Professor, Dept of Molecular Biosciences, University of Texas at Austin

Xiaolu Cambronne
The University of Texas at Austin
United States

Dear Dr. Cambronne,

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

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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

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

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

THINGS TO DO NOW:

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

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

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2022-56596V3 and be addressed to emboreports@wiley.com.

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

EMBO Press Author Checklist

Corresponding Author Name: Dr. Xiaolu Ang Cambronne
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2022-56596-T

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

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:

  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;

  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	New materials are described in Materials and Methods. All the materials are available free of cost upon request.

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	The information is available in the Materials and Methods section. TolC antibody is obtained from the Mavridou lab at the University of Texas at Austin and has been previously published (Furniss et al. 2022, Pubmed ID 35025730)

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	The details are provided in the Materials and Methods section
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Cells were obtained from a reputable source and were routinely tested for Mycoplasma contamination.

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	The details are provided in the Materials and Methods section

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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

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

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

Ethics

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

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

Reporting

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

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

Data Availability

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