JCB

Shr3 is a selective co-translational folding chaperone necessary for amino acid permease biogenesis

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October 4, 2022

Re: JCB manuscript #202208060

Prof. Per O Ljungdahl
Stockholm University
Molecular Biosciences, The Wenner-Gren Institute
Svante Arrhenius väg 22B
Stockholm SE-10691
Sweden

Dear Prof. Ljungdahl,

Thank you for submitting your manuscript entitled "In vivo analysis of ER membrane-localized chaperone substrate interactions." Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that Reviewer #1 is supportive and has a few minor requests for text changes. However, Reviewers #2&3 feel that more insight into mechanism is necessary for this study to be suitable for JCB and ask for additional data that supports the model that Shr3 binds to its substrates co-translationally as well as biochemical corroboration of Alphafold predictions. We agree that the claim of co-translational binding must be substantiated by new data. While there are clearly caveats that should be mentioned regarding Alphafold modeling, we believe that this should stay in the paper. Reviewer #2 also asks about the rigor of the scanning mutagenesis screen, this may require a more detailed explanation of the screen and justification of the selected mutations but we do not feel that this requires additional mutagenesis studies.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. If you intend to submit a revision we ask that you first send us a revision plan with a point-by-point response explaining how you will address each comment.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. The typical timeframe for revisions is three to four months. While most universities and institutes have reopened labs and allowed researchers to begin working at nearly pre-pandemic levels, we at JCB realize that the lingering effects of the COVID-19 pandemic may still be impacting some aspects of your work, including the acquisition of equipment and reagents. Therefore, if you anticipate any difficulties in meeting this aforementioned revision time limit, please contact us and we can work with you to find 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.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count is < 40,000, 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.

Figures: Your manuscript may have up to 10 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. Your manuscript may have up to 5 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.

Please note that JCB now requires authors to submit Source Data used to generate figures containing gels and Western blots

with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be made available to reviewers during evaluation of revised manuscripts and, if your paper is eventually published in JCB, the files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

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

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

Sincerely,

Elizabeth Miller, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

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

In this manuscript the authors study the role of the Saccharomyces cerevisiae Shr3 protein, an ER membrane 'tetraspan' chaperone, in the co-translational folding and subsequent biogenesis of amino acid transporters/permeases (AAP). In eukaryotes, newly made transporters and other plasma membrane (PM) proteins are directly sorted from ribosomes to the membrane of the ER via a co-translational translocation process. After ER translocation, properly folded PM proteins are sorted into nascent ER-exit sites (ERes) and enter into COPII secretory vesicles, which constitute to the first step in their trafficking to the PM via the trans-Golgi network/endosome route, or other unconventional Golgi-independent pathways. In addition to interaction with COPII core components, packaging of specific transmembrane cargoes into COPII vesicles often requires additional ER-embedded chaperones or adaptors. Shr3 is such a protein, acting selectively for proper partitioning in COPIIs and ER-exit of AAP.

Previous findings of Ljungdhal group have suggested that Shr3 engages with nascent N-terminal membrane segments of AAP to promote folding of the protein during its translation. The present work shows how this might be achieved. In particular, the authors show that Shr3 facilitates the folding of AAP by selectively and transiently engaging as a structural scaffold, initially interacting with N-terminal transmembrane domains of AAP as they emerge from the translocon, but progressively weakens its interaction and becoming less 'needed', as translated C-terminal transmembrane domains drive and stabilize the proper folding of the holo-transporter. To show this, the Ljungdhal group used extensive systematic mutational analysis of Shr3 combined with very elegant split-ubiquitin assays probing chaperone-cargo interactions in vivo. The final model proposed is very nice and fully convincing by the results obtained.

I have been reviewing manuscripts for more than 30 year and it is extremely rare to have very little to criticize, other than simply saying that this is an excellent piece of work, written and presented in a magnificent way. I have been following the story of Shr3 by the group of Per Ljungdhal since its begging, 30 years ago, and I regretted that there we did not have any 'news' in the last 15 years (since 2007). I am very happy to see the present exquisite manuscript, which advances our knowledge on how Shr3 function to selectively and transiently interact with AAP in order to assist their co-translational folding, seemingly coupled to COPII packaging, ER-exit and biogenesis to the PM.

The amount of work described is huge and the take-home-message original and very interesting. The design of experiments

described and in particular the mutational analysis presented are excellent. The technically highly demanding split-Ub system employed worked marvelously and led to very rigorous in vivo evidence for the progressive transient interactions that occur between Shr3 and AAP during the co-translational folding of the latter. The main and supplementary figures are all of high quality and self-explanatory.

I only suggest a change in the title, which as it is does not really reflect the original findings of this work. Maybe a title like "The ER-localized Shr3 protein is a selective co-translational folding chaperone necessary for the biogenesis of AAP"?

Some very minor points are the following:

Pg. 78: add coma after (Kuehn et al., 1996)

Pg. 91-93. Spell General Amino Acid Permease in pg.91 and not in in pg.93

Pg. 113. Spell 'Unfolded Protein response' before UPR.

Pg. 134. Please explain the reason why TMS are mutated to Leu and loops to Ala?

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

This manuscript is the next installment in a series of papers from the author's group that explores how the yeast Shr3 membrane chaperone facilitates folding of amino acid permeases (AAP). The authors report several observations. First, Shr3 is quite tolerant to mutations in its membrane and lumenal regions, suggesting that its ability to bind substrates is based more on its overall structural properties than sequence-specific interactions. Second, Shr3 function could be disrupted by several combinations of polar-to-hydrophobic mutations in transmembrane segments or a lumenal loop, consistent with a role for those residues in shielding hydrophilic transmembrane segments that are likely present in AAP intermediates. Third, deletion analysis of Shr3 lumenal loops revealed both loss- and gain-of-function phenotypes, and implicate the L3 loop in conferring some level of substrate selectivity through an unknown mechanism. Fourth, using a well-characterized split-ubiquitin assay, the authors demonstrate that Shr3 interacts with truncated AAP substrates in a progressive manner-with increased binding detected with increasing length, but reduced binding observed to full-length substrates. This leads the authors to a model in which Shr3 chaperones AAP intermediates as their transmembrane segments are sequentially inserted into the ER membrane.

This is a carefully executed study and the data are of generally high quality. The topic is important, as our understanding of multipass membrane protein biogenesis remains at an early stage. Shr3 is an intriguing membrane protein, and the results presented here add to a growing body of literature on intramembrane chaperones. However, several issues reduce my enthusiasm for the paper in its current form:

1. One concern is that the authors "systematic scanning mutagenesis" approach is not particularly comprehensive. It is a bit difficult to know exactly what mutants were made, but a 44-member library is quite small. This biases the structure-function interpretation-did the current screen miss other functionally important regions of Shr3 because they were not included in the library? Perhaps because of this, the mechanistic insight gleaned from the author's mutational analysis is limited. The manuscript would have been greatly strengthened by leveraging the power of yeast genetics to screen much larger libraries.

2. The Alphafold modeling of Shr3 mutants is of questionable value, and should be removed from the results and discussion.

3. The implication throughout the manuscript is that Shr3 interacts with folding intermediates cotranslationally, although this is never formally demonstrated. The truncated substrates used in the split-Ub assays are tagged at their C-terminus, so the assay is reporting on post-translational interactions. While it is not necessarily unreasonable to use this as a proxy for Shr3 binding to true substrate intermediates, the paper would be strengthened if the authors first demonstrate that Shr3 is indeed binding cotranslationally to its substrates.

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

In this paper Myronidi and colleagues investigate the chaperone function of the amino acid permease (AAP)-interacting protein Shr3 in S. cerevisiae. The Ljungdahl lab has previously shown that the N-terminal domain of Shr3, containing 4 transmembrane domains (TMDs), is necessary and sufficient for its chaperone function. Here, the authors identify specific regions required for Shr3 chaperone function and substrate interaction using saturation mutagenesis of the Shr3 N-domain, AAP function-related readouts, and split-ubiquitin assays. The authors identify 3 regions in the Shr3 N-domain that are essential for chaperoning by Shr3. Using truncation constructs of a number of Shr3 substrates they show that their interaction with Shr3 begins when 2-4 TMDs have been synthesized and ceases once the protein is complete. The authors conclude that Shr3 supports the folding of

AAPs by acting as a scaffold.

This paper deals with the timely and interesting question of how multispanning transmembrane proteins acquire their final structure in the ER membrane with the help of a chaperone. The work is technically thorough, the paper is well written, and the figures are excellent. The data, however, only partially support the authors' conclusions.

Major criticisms:

1. The authors claim repeatedly that Shr3 acts as a 'scaffold' for folding of AAPs. Other than a general interaction between Shr3 and its substrates, however, the authors do not explain the 'scaffold' function of Shr3 nor do they address the actual mechanism by which Shr3 works. Do multiple TMDs of the substrates sequentially interact with the same domains of Shr3? In which case Shr3 would just guide the TMDs from the Sec61 lateral gate into the bilayer, somewhat similar to YidC. Or do multiple TMDs of the substrate really interact at the same time with different parts of Shr3 which orients them towards each other before releasing the whole folded protein into the bilayer (only the latter would be a scaffolding function).

2. Based on the alpha-fold structures and the fact that only multiple amino acid replacements have effects, the authors claim that it is the Shr3 N-domain structure that is affected in the mutants that no longer interact with the substrate AAPs. It would be good to have some biochemical evidence supporting this notion.

Minor points:

- YidC should be mentioned in the introduction in the sentence about MS-chaperones
- Figures 6 and 7 could be combined
- In Figure 8 the label 'native AAP' should be under the folded protein (purple), not to the side

JCB manuscript #202208060 April 15, 2023

Point-by-Point Response to Reviewers' Comments

Reviewer #1:
I only suggest a change in the title, which as it is does not really reflect the original findings of this work. Maybe a title like "The ER-localized Shr3 protein is a selective co-translational folding chaperone necessary for the biogenesis of AAP"?

We greatly appreciated the reviewer's very positive general comments regarding the high-quality and impact of our work. Although, the suggested title is accurate, we are reluctant to use it as is for several reasons. First, Shr3 is already known to be a selective chaperone required for AAP biogenesis. Second, we are reticent to include co-translational folding in the title, although this is consistent with our findings, formal proof of this is lacking. Third, the suggested title does not adequately reflect one of the key aspects of our work, i.e., that we assessed events occurring in the proper context of living cells. Finally, the suggested title has too many characters to comply with JCB's state requirement of 100 characters (with spaces). We have adjusted the title, condensed title and Summary to incorporate aspects of the reviewer's suggestion.

Original title: *"In vivo analysis of ER membrane-localized chaperone substrate interactions"* (74 characters w/ spaces)

Modified title*: "In vivo analysis of ER membrane chaperone interactions that facilitate amino acid permease folding"* (98 characters w/ spaces)

Condensed title: "*Shr3 acts as a scaffold that guides AAP folding"* (47 characters w/ spaces)

Modified summary: *The yeast ER membrane chaperone Shr3 specifically guides the co-translational folding of amino acid permeases (AAP) comprised of 12 membrane segments (MS). Structural rather than sequence-specific interactions enable Shr3 to transiently engage with N-terminal MS, acting as a scaffold that facilitates the folding of C-terminal MS as translation proceeds to completion.*

Some very minor points are the following:

Pg. 78: add coma after (Kuehn et al., 1996) DONE

Pg. 91-93. Spell General Amino Acid Permease in pg.91 and not in in pg.93 DONE

Pg. 113. Spell 'Unfolded Protein response' before UPR. DONE

Pg. 134. Please explain the reason why TMS are mutated to Leu and loops to Ala?
We have modified the text as follows:

To maintain compatibility with the hydrophobic nature of membranes, the intramembrane residues were mutated to leucine; the length of consecutive substitution mutations varied, ranging from 2 to 13 residues. To minimize negative folding artefacts, the extramembrane residues within ER lumenal loops L1 and L3 and cytoplasmic oriented NT and loop L2 where mutated to alanine; the length of consecutive alanine replacements ranged from 2 to 3.

Reviewer #2:
This is a carefully executed study and the data are of generally high quality. The topic is important, as our understanding of multipass membrane protein biogenesis remains at an early stage. Shr3 is an

intriguing membrane protein, and the results presented here add to a growing body of literature on intramembrane chaperones. However, several issues reduce my enthusiasm for the paper in its current form:

We appreciate reviewer #2's positive general comments regarding the high-quality and impact of our work.

1. One concern is that the authors "systematic scanning mutagenesis" approach is not particularly comprehensive. It is a bit difficult to know exactly what mutants were made, but a 44-member library is quite small. This biases the structure-function interpretation-did the current screen miss other functionally important regions of Shr3 because they were not included in the library? Perhaps because of this, the mechanistic insight gleaned from the author's mutational analysis is limited. The manuscript would have been greatly strengthened by leveraging the power of yeast genetics to screen much larger libraries.

We have clarified the description regarding our mutagenesis to indicate that it affects all 159 amino acid residues in the N-terminal membrane domain of Shr3 that possesses the chaperone function. This represents an efficient approach to assess all amino acid residues and should not be considered a small set of mutations. The text now reads (starting with line 139:

The biological activity of the 44 mutant proteins, which collectively alter all 159 aa residues comprising the N-terminal membrane domain of Shr3, was initially assessed using growth-based assays on high-amino acid content YPD medium supplemented with metsulfuron-methyl (MM).

2. The Alphafold modeling of Shr3 mutants is of questionable value, and should be removed from the results and discussion.

We disagree with the reviewer and note that the alphafold structures added considerably to forming our thoughts regarding the mechanism of Shr3 chaperone function. We believe that potential readers will find them equally stimulating and perhaps encourage independent experimental approaches. We have gone forward to test the alphafold structures but our results are preliminary and remain out of the scope of this manuscript. We note that the JCB editors indicated the appropriateness of including the alphafold structures.

3. The implication throughout the manuscript is that Shr3 interacts with folding intermediates cotranslationally, although this is never formally demonstrated. The truncated substrates used in the split-Ub assays are tagged at their C-terminus, so the assay is reporting on post-translational interactions. While it is not necessarily unreasonable to use this as a proxy for Shr3 binding to true substrate intermediates, the paper would be strengthened if the authors first demonstrate that Shr3 is indeed binding co-translationally to its substrates.

We are aware that physical proof of Shr3 functioning in a co-translational manner is lacking. To date, we have been frustrated in our attempts to unequivocally demonstrate this. For the reviewer's information, we have tried to exploit the recently published Sdd1 arrest peptide that reportedly works to stall translation of proteins in yeast. We inserted this arrest peptide into Gap1 after MS 10, however, we have not observed efficient arrest. Clearly, there are aspects that can be optimized and we are continuing to pursue this approach.

In parallel, we have successfully pursued co-IP experiments to obtain additional biochemical evidence to support the specificity of Shr3 – AAP interactions. Here we have found that Shr3 selectively co-IPs with C-terminal truncated AAPs and not with similar C-terminal truncated hexose transporters.

In lieu of "hard" data to confirm co-translational interactions, we have adjusted the text to "tone down" statements to be more accurate.

In the abstract, we have replaced "indicate that" with "align with" as follows:

The data align with Shr3 engaging nascent N-terminal chains of AAP, functioning as a scaffold to facilitate folding as translation completes.

Reviewer #3:
This paper deals with the timely and interesting question of how multispanning transmembrane proteins acquire their final structure in the ER membrane with the help of a chaperone. The work is technically thorough, the paper is well written, and the figures are excellent. The data, however, only partially support the authors' conclusions.

We appreciate reviewer #3's positive comments regarding the technical thoroughness and writing.

Major criticisms:

1. The authors claim repeatedly that Shr3 acts as a 'scaffold' for folding of AAPs. Other than a general interaction between Shr3 and its substrates, however, the authors do not explain the 'scaffold' function of Shr3 nor do they address the actual mechanism by which Shr3 works. Do multiple TMDs of the substrates sequentially interact with the same domains of Shr3? In which case Shr3 would just guide the TMDs from the Sec61 lateral gate into the bilayer, somewhat similar to YidC. Or do multiple TMDs of the substrate really interact at the same time with different parts of Shr3 which orients them towards each other before releasing the whole folded protein into the bilayer (only the latter would be a scaffolding function).

In our previous work, we have shown that Gap1 partitions efficiently into the ER membrane in the absence of Shr3 with each of the MS correctly oriented, therefore Shr3 is not required to simply guide MS into the bilayer. Thus, contrary to what the reviewer suggests, Shr3 does something more. Furthermore, split Gap1 constructs, i.e., co-expressed MS I-V and MS VI-XII, assemble to form functional amino acid-competent Gap1. The assembly is dependent on Shr3 being present; Shr3 interacts with MS I-V, but not MS VI-XII, to hold the N-terminal portion of Gap1 in an assembly-competent form. In the absence of Shr3, similar to full length Gap1, MS I-V aggregates and cannot interact with MS VI-XII. The C-terminal portion of Gap1, MS VI-XII, aggregates unless both MS I-V and Shr3 are present. These previous results strongly suggest that Shr3 interacts with the N-terminal MS of Gap1 as they partition out of the Sec61 gate, and carries out a function that apparently differentiates it from the chaperone functions associated with YidC. In our current manuscript, we developed the split-ubi assay to obtain more detailed information using C-terminal truncations as proxies of translation intermediates. Our data are consistent with Shr3 engaging early, but it does not interact efficiently with Gap1unless multiple N-terminal MS are present. Consistently, Shr3-AAP interactions fade as translation of all MS completes.

2. Based on the alpha-fold structures and the fact that only multiple amino acid replacements have effects, the authors claim that it is the Shr3 N-domain structure that is affected in the mutants that no longer interact with the substrate AAPs. It would be good to have some biochemical evidence supporting this notion.

To be clear, previous work has shown that single amino acid modifications can affect Shr3-AAP interactions. Shr3 was originally identified based on three single point mutations affecting the N-terminal membrane domain of Shr3 (see Ljungdahl et al., 1992 *Cell*). Also, quite long ago, we showed that AAP did not co-purify with the non-functional shr3-23 mutant protein carrying a single amino

acid substitution at thr19 (Gilstring 1999 *Mol. Biol. Cell*). We now include new biochemical data demonstrating the specificity of Shr3 – AAP interactions; Shr3 selectively co-IPs with C-terminal truncated AAPs and not with similar C-terminal truncated hexose transporters.

Minor points:

- YidC should be mentioned in the introduction in the sentence about MS-chaperones. DONE

- Figures 6 and 7 could be combined.
We have considered this, but feel that the data regarding Can1 in Fig. 6 is very important and significantly adds to our understanding, and thus, deserves being separated from the more general results presented in Fig 7. To be clear, Fig. 6 documents that Shr3 specifically and transiently interacts with translation intermediates of an AAP that does not efficiently interact when it is fully folded, i.e., we failed to detect efficient interactions between Shr3 and full length Can1 when all 12 MS are present but clearly detect interactions with truncated forms of Can1.

- In Figure 8 the label 'native AAP' should be under the folded protein (purple), not to the side.
DONE

June 6, 2023

RE: JCB Manuscript #202208060R

Prof. Per O Ljungdahl
Stockholm University
Molecular Biosciences, The Wenner-Gren Institute
Svante Arrhenius väg 22B
Stockholm SE-10691
Sweden

Dear Prof. Ljungdahl:

Thank you for submitting your revised manuscript entitled "In vivo analysis of ER membrane chaperone interactions that facilitate amino acid permease folding." The revised paper was re-assessed by two of the original reviewers. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines as well as to address the remaining reviewer comments (see details below).

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

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

1) Text limits: Character count for Articles is < 40,000, 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) Figure formatting: Articles may have up to 10 main text figures. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. Please avoid pairing red and green for images and graphs to ensure legibility for color-blind readers. If red and green are paired for images, please ensure that the particular red and green hues used in micrographs are distinctive with any of the colorblind types. If not, please modify colors accordingly or provide separate images of the individual channels.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Please, indicate whether 'n' refers to technical or biological replicates (i.e. number of analyzed cells, samples or animals, number of independent experiments).
*** If independent experiments with multiple biological replicates have been performed, we recommend using distribution-reproducibility SuperPlots (please see Lord et al., JCB 2020) to better display the distribution of the entire dataset, and report statistics (such as means, error bars, and P values) that address the reproducibility of the findings.

Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if 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."

4) Title: While your current title describes what was done in the study we do not feel that it fully conveys the advance and novel findings. We therefore suggest the following title: "ER-localized Shr3 is a selective co-translational folding chaperone necessary for AAP biogenesis".

5) 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." Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

6) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or

gifted to you by other investigators (and provide references where appropriate). Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary. If antibodies are not commercial, please add a reference citation if possible.

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

8) 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.

9) Supplemental materials: Articles may have up to 5 supplemental figures and 10 videos. You currently exceed this limit but, in this case, we will be able to give you extra space if it is needed. Your current Fig. S2 is 4 pages long but for JCB formatting figures cannot exceed a single page. Please consolidate the supplemental data as much as possible and reorganize into single page 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. Please include one brief sentence per item.

10) 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.

11) 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."

12) 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/).

13) 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.

14) JCB requires authors to submit Source Data used to generate figures containing gels and Western blots with all revised manuscripts. This Source Data consists of fully uncropped and unprocessed images for each gel/blot displayed in the main and supplemental figures. Since your paper includes cropped gel and/or blot images, please be sure to provide one Source Data file for each figure that contains gels and/or blots along with your revised manuscript files. File names for Source Data figures should be alphanumeric without any spaces or special characters (i.e., SourceDataF#, where F# refers to the associated main figure number or SourceDataFS# for those associated with Supplementary figures). The lanes of the gels/blots should be labeled as they are in the associated figure, the place where cropping was applied should be marked (with a box), and molecular weight/size standards should be labeled wherever possible. Source Data files will be directly linked to specific figures in the published article.

Source Data Figures should be provided as individual PDF files (one file per figure). Authors should endeavor to retain a minimum resolution of 300 dpi or pixels per inch. Please review our instructions for export from Photoshop, Illustrator, and PowerPoint here: https://rupress.org/jcb/pages/submission-guidelines#revised

15) Journal of Cell Biology now requires a data availability statement for all research article submissions. These statements will be published in the article directly above the Acknowledgments. The statement should address all data underlying the research presented in the manuscript. Please visit the JCB instructions for authors for guidelines and examples of statements at (https://rupress.org/jcb/pages/editorial-policies#data-availability-statement).

B. FINAL FILES:

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

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

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

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Sincerely,

Elizabeth Miller, PhD
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Dan Simon, PhD
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Reviewer #2 (Comments to the Authors (Required)):

1. It would be helpful if the authors included a supplementary figure that graphically summarizes the 44 mutant constructs that were made and tested. Even though these cover 159 residues, the library remains far from "systematic" and it remains puzzling to me why the authors refer to it as such (e.g., "The foundation of our model of Shr3 is based on saturation scanning mutagenesis of the N-terminal membrane domain...").

2. To be clear, my concern is not with the use of Alphafold to model the structure of Shr3, nor with mapping the locations of various mutations to the model. Alphafold is powerful and the authors are wise to leverage it for this work. What remains a concern, however, is the use of Alphafold to model the structural consequences of point mutations, as done in Fig 3E. It is hardly surprising that the authors report: "although the leucine substitutions at positions 17 through 19 (shr3-35) result in a clearly defective and non-functional protein (Fig 3A), no obvious structural alterations were predicted...". This is almost certainly because Alphafold does not do a good job predicting the structural consequences of point mutations. See the Alphafold website FAQ: "AlphaFold has not been validated for predicting the effect of mutations. In particular, AlphaFold is not expected to produce an unfolded protein structure given a sequence containing a destabilising point mutation." See also Pak et al., PLOS One 2023 and others. The mutant modeling shown in Fig 3E and the associated text (~lines 271-284 and ~line 482) is of little value and the manuscript would be strengthened by its removal.

3. I appreciate the author's attempts to demonstrate that Shr3 is acting co-translationally. In the absence of a decisive experiment showing this, I suggest that the authors include a clear statement that the split-Ub assay reports on post-translational interactions between Shr3 and truncated but fully translated (terminated) AAPs, which the authors are using as a proxy to monitor what are presumed to be co-translational interactions.

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

In the revised version the authors answered to all my previous queries satisfactorily.

JCB manuscript #202208060 June 18, 2023

Point-by-Point Response to Reviewers' Comments

Reviewer #2:

1. It would be helpful if the authors included a supplementary figure that graphically summarizes the 44 mutant constructs that were made and tested. Even though these cover 159 residues, the library remains far from "systematic" and it remains puzzling to me why the authors refer to it as such (e.g., "The foundation of our model of Shr3 is based on saturation scanning mutagenesis of the N-terminal membrane domain...").

Figure 3 represents a thorough summary showing the results from the systematic mutagenesis approach. The library of mutant plasmids cover each of the residues comprising the membrane domain of Shr3 (1 – 163 aa). Table S2, in the accompanying supplementary material, lists a detailed description of each plasmid and the exact residues affected and the nature of the mutations. For clarity, we have now indicated whether alanine (A; extra-membrane regions) or leucine (L; membrane spanning segments) residues replace the endogenous amino acids. Hopefully, this will enable readers to more easily grasp the systematic approach we have used.

2. To be clear, my concern is not with the use of Alphafold to model the structure of Shr3, nor with mapping the locations of various mutations to the model. Alphafold is powerful and the authors are wise to leverage it for this work. What remains a concern, however, is the use of Alphafold to model the structural consequences of point mutations, as done in Fig 3E. It is hardly surprising that the authors report: "although the leucine substitutions at positions 17 through 19 (shr3-35) result in a clearly defective and non-functional protein (Fig 3A), no obvious structural alterations were predicted...". This is almost certainly because Alphafold does not do a good job predicting the structural consequences of point mutations. See the Alphafold website FAQ: "AlphaFold has not been validated for predicting the effect of mutations. In particular, AlphaFold is not expected to produce an unfolded protein structure given a sequence containing a destabilising point mutation." See also Pak et al., PLOS One 2023 and others. The mutant modeling shown in Fig 3E and the associated text (~lines 271-284 and ~line 482) is of little value and the manuscript would be strengthened by its removal.

The reviewer is correct in pointing out that there is growing information demonstrating that AlphaFold poorly predicts structural alterations that likely result from point mutations, and thus is not considered to be a useful tool to account for the phenotypic manifestations arising from mutations. However, it is worth pointing out that our analysis examined larger deletion mutations and we did observe some interesting predicted changes. Also, it is still early going in AI-based structural predictions and our analysis may eventually be helpful for improving algorithms. We also not that we did refer to the Pak et al paper, albeit in its pre-publication BioRxiv form, and are grateful for the reviewer pointing out that this paper has recently been published in PLoS One, we have updated the reference.

3. I appreciate the author's attempts to demonstrate that Shr3 is acting co-translationally. In the absence of a decisive experiment showing this, I suggest that the authors include a clear statement that the split-Ub assay reports on post-translational interactions between Shr3 and truncated but fully translated (terminated) AAPs, which the authors are using as a proxy to monitor what are presumed to be co-translational interactions.

We adjusted line 380 to more clearly indicate that our split-Ub assay assesses interactions between fully translated forms of AAPs and Shr3. Line 380 now reads: "We posited that if fully translated truncations of AAP are indeed proxies of translation intermediates, then truncations of Can1 . . . "

We trust that readers will understand the significance of this.

Reviewer #3:

We thank reviewer 3 for initial comments that enabled us to improve our manuscript. We are appreciative that we could fully address the concerns.