THE EMBO JOURNAL

Evolutionary adaptation of the folding pathway for secretability

Dries Smets, Alexandra Tsirigotaki, Jochem Smit, Srinath Krishnamurthy, Athina Portaliou, Anastassia Vorobieva, Wim Vranken, Spyridoula Karamanou, and Anastassios Economou
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Transfer from Review Commons:	3rd Apr 22
Editorial Decision:	27th May 22
Revision Received:	7th Jun 22
Editorial Decision:	5th Jul 22
Revision Received:	14th Jul 22
Accepted:	2nd Aug 22

Editor: William Teale

Transaction Report:

This manuscript was transferred to The EMBO Journal following peer review at Review Commons.

1st Editorial Decision **27th May 2022**

Dear Tassos,

Thank you again for submitting your manuscript entitled "Evolutionary adaptation of the folding pathway for secretability" (EMBOJ-2022-111344)for consideration by the EMBO Journal.

We received the submission, along with referee reports and your point by point response to them via Review Commons. Although the referees comments were much appreciated, as you know I decided to send the manuscript to two further referees; the reports from these referees are included at the bottom of this email. Both referees had access to your point by point responses as well as the original manuscript.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of referee #2 below and following your revision plan as outlined upon submission. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

William

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

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

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

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

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

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

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

7) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

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

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

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

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

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

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

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

Further instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines
(https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

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

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (25th Aug 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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

Referee #1:

As the previous reviewers pointed out, the presented work contributes to our understanding of how secretion of proteins through the Sec translocon is facilitated in bacteria by a delayed folding process, that is in addition influenced by the presence of the signal peptide.
The authors responded well to the reviewers comments and clarified, specified, and discussed points that remained open in the first submission. I feel that the paper provides highly relevant insight into this general problem and will find a broad readership.

Referee #2:

The manuscript by Smets et al describes the characterisation of the delayed folding mechanism between two different prolyl isomerase proteins, and the influence of signal peptides in the altered folding between PpiA and PpiB. The authors used pulsed HDX experiments to compare the folding of PpiA and PpiB. The most intriguing part of this study was the generation of a chimera where the signal peptide and part of PpiA were grafted onto PpiB, which changed the rates of folding.

While this is an extremely interesting study I have some concerns in the way data is presented, and the choice for how D2O experiments were performed. There are some major technical issues that I need the authors to more clearly describe or need to address. I have some suggestions how it could be made clearer to increase the accessibility to a broad audience.

Major concerns

1. The supplemental data underlying this work is very difficult to access and understand. Tables are split over multiple pages, and it is hard to get access to the underlying raw HDX data. Could the authors please include all of the underlying HDX data as an excel file.
2. My main concern with all of the local HDX data is that it appears from the details provided in the methods and raw data that this information was carried out in singlicate. In particular Fig 3 where the folding times are based on these measurements This seems to be problematic for the determination of folding time. From looking at the raw data in Fig S4, there appear to be very strange HDX rates that cannot be clearly explained. For example the 2.5 min refolding time has much lower rates across the board than the 1 min refolding time. Is this a replicate issue? Without some additional replicates, at least showing the repeatability of a fraction of the data it does not seem ready for publication in EMBO.
3. Showing some of the raw centroid traces of the HDX exchange for the most important folding regions would also help to increase the confidence in the measurement
4. The authors should include data processing tables for HDX as suggested by the community guidelines in Masson et al Nature Methods 2019.

Revisions

Manuscript: EMBOJ-2022-111344
Corresponding author(s): Anastassios Economou, Spyridoula Karamanou

1. General Statement

We would like to thank both referees for their positive remarks and wish to clarify below some aspects of the local HDX-MS analysis that were unclear before.

2. Description of revisions

Authors:

1. The $t_{50\%}$ lines shown in local HDX-MS analysis (Figures 3 and 5), have been changed to black and grey to avoid confusion with the colours of the foldons.
2. We have noticed that for secretion of (pro)PpiX$_{PhoA}$, the values of pmol protein secreted per protein expressed were too high after processing. The calculations have been corrected now and calculated as amount of active PhoA per 10^8 cells (see Fig. 4F, 6B and Table S9B). Corrections only affected the absolute values of pmol secreted/pmol protein expressed while the comparisons between proteins remains the same.
3. Together with adapting the format to the house style of EMBO J we have rearranged the Materials and Methods section and edited figure legends to improve clarity. In the Materials and Methods, the steps for each of the three MS-based methods (local HDX-MS of protein dynamics, global HDX of refolding and local HDX of refolding) have been bundled together. See all changes highlighted in the file submitted as a dataset: “MS with Highlighted changes”.

Referee #1:

As the previous reviewers pointed out, the presented work contributes to our understanding of how secretion of proteins through the Sec translocon is facilitated in bacteria by a delayed folding process, that is in addition influenced by the presence of the signal peptide. The authors responded well to the reviewers comments and clarified, specified, and discussed points that remained open in the first submission. I feel that the paper provides highly relevant insight into this general problem and will find a broad readership.

Authors response:

We thank the reviewer.

Referee #2:

The manuscript by Smets et al describes the characterisation of the delayed folding mechanism between two different prolyl isomerase proteins, and the influence of signal peptides in the altered folding between PpiA and PpiB. The authors used pulsed HDX experiments to compare the folding of PpiA and PpiB. The most intriguing part of this study was the generation of a chimera where the signal peptide and part of PpiA were grafted onto PpiB, which changed the rates of folding.

Authors response:

We thank the reviewer.

While this is an extremely interesting study I have some concerns in the way data is presented, and the choice for how D2O experiments were performed. There are some major technical issues that I need the authors to more clearly describe or need to address. I have some suggestions how it could be made clearer to increase the accessibility to a broad audience.

Major concerns

1. The supplemental data underlying this work is very difficult to access and understand. Tables are split over multiple pages, and it is hard to get access to the underlying raw HDX data. Could the authors please include all of the underlying HDX data as an excel file.

Authors' response:

In typical HDX-MS studies, a single .xls file is provided with data for a single protein and comparisons can be readily carried out. Here, we compare two proteins (that generate different peptides upon proteolysis and hence cannot be cross-compared), +/- their signal peptide derivatives and at different temperature conditions. To bypass this problem, we resorted to using the 'relative fractional units per residue' function provided by the most updated version of PyHDX (version 0.4.1.) and then plot the results from each protein on the aligned protein sequences. For this reason, all of the HDX-MS data are presented in multiple sheets of the same xls file and post DynamX analysis is presented in separate Tables (see below for details). Previous versions of PyHDX had been used only for the determination of ΔG per residue (Smit et al., 2021, Anal Chem; Krishnamurthy et al., 2021, Structure; Krishnamurthy et al., 2022, Cell reports).

Action taken:

To provide an overview of the complete local HDX-MS analysis pipeline that was carried out in this study and make it easier for the reader to see how the datasets interconnect, we added a schematic pipeline panel (Fig. EV3B) with references to Tables containing data from the various steps. Specific Expanded View Tables appear in the order of this scheme's steps; i.e. DynamX data are in Table EV4, folded fractions per residue in Table EV5 and degree of unfoldedness per residue in Table EV6. Tables contain multiple sheets named in alphabetical order and indicating protein/folding condition. The summary of the contents for each Table is presented in the corresponding README .txt files. Moreover, we have edited the Figure legends to describe accurately which part of the HDX-MS data analysis has been used to generate each figure panel.

2. My main concern with all of the local HDX data is that it appears from the details provided in the methods and raw data that this information was carried out in singlicate. In particular Fig 3 where the folding times are based on these measurements This seems to be problematic for the determination of folding time.

Authors' response:

a. The local HDX-MS data for folding consist of 3 independent biological replicates.

b. These data have been processed using the "relative fractional units per residue" function of PyHDX (version 0.4.1.; see response to point 1 above). The reviewer is right that we had not added these standard errors before to avoid complicating the graphs.

Actions taken:

We have cleared this issue up by adding:

a. a summary of HDX conditions and quality in Table EV4A.

b. a pipeline scheme in Fig. EV3B, to describe the analysis steps and interconnect the relevant data from different steps presented in different Tables.

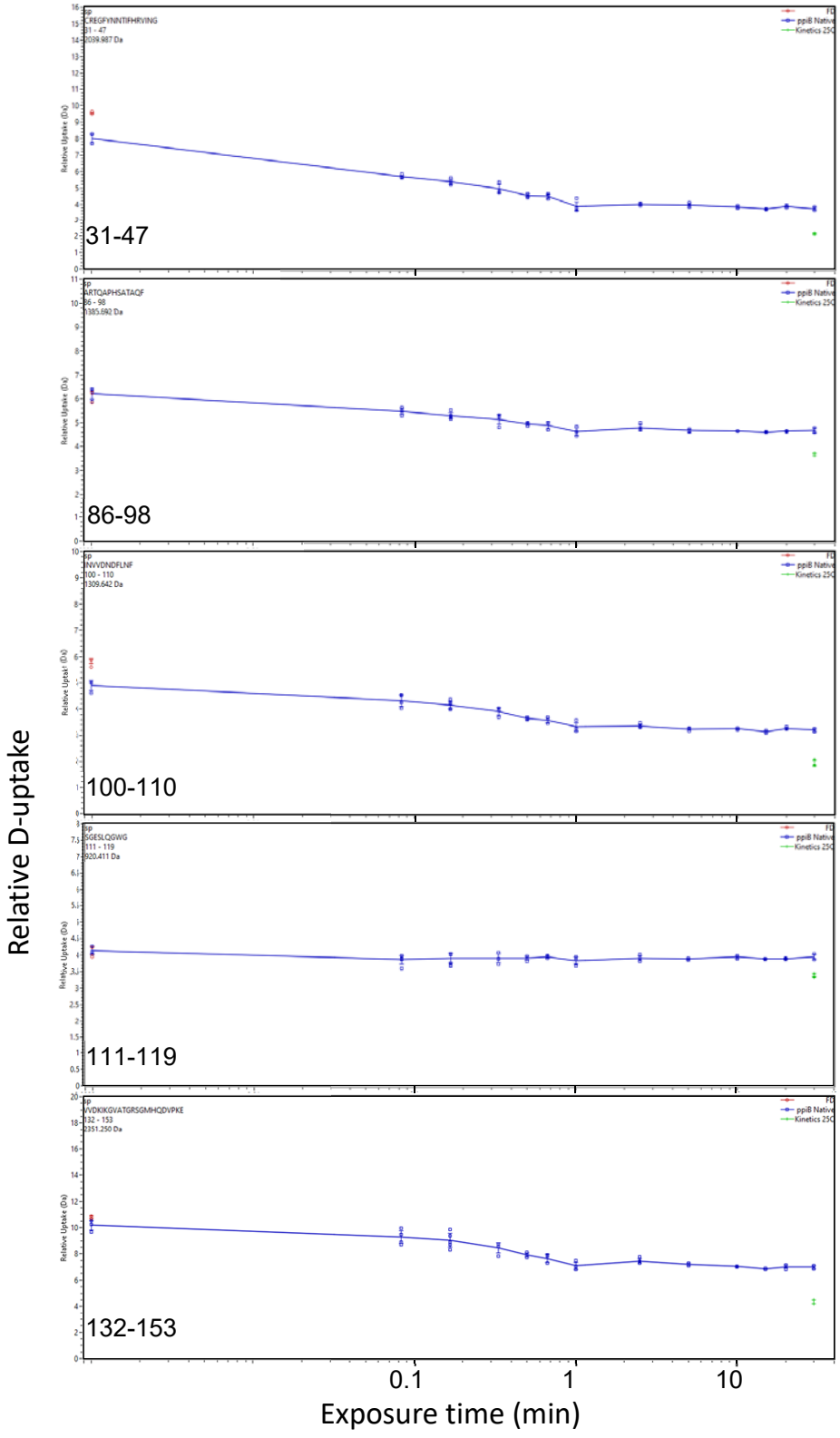
c. the local HDX data with standard deviation values in Table EV4D-I.

d. the folded fractions per residue data with standard deviation values in Table EV5 [following analysis of local HDX data (Table EV4) by PyHDX]. Table EV5 now also includes graphs in which the standard deviations in the PyHDX analysis have been plotted.

e. All other standard deviations that may be requested by the reader can be found in their corresponding Table.

From looking at the raw data in Fig S4, there appear to be very strange HDX rates that cannot be clearly explained. For example the 2.5 min refolding time has much lower rates across the board than the 1 min refolding time. Is this a replicate issue? Without some additional replicates, at least showing the repeatability of a fraction of the data it does not seem ready for publication in EMBO.

Authors' response:
There is a minor issue for folding timepoint 1 min in the current Table EV5B (folding of PpiB at 25°C) which shows slightly deviating folded fractions compared to those of the flanking timepoints.



In the relative uptake DynamX plots, the 1 min timepoint appears as a minor outlier without, however, really falling off the folding kinetics shaped by the rest of the data points (see examples from 5 different peptides across the protein including replicates; corresponding DynamX data in Table EV4E).
We would propose to leave these datasets intact unless advised otherwise.

3. Showing some of the raw centroid traces of the HDX exchange for the most important folding regions would also help to increase the confidence in the measurement
Actions taken:
We have added some extra peptide spectra examples in Appendix Fig. S4 that display raw centroid traces of two peptides from two initial foldons of PpiA and proPpiA. For more examples on D-uptake changes, Tables EV4 and EV5 that contain information for each timepoint can be visited or, we can provide the spectra upon request as noted in the Data Availability section.

4. The authors should include data processing tables for HDX as suggested by the community guidelines in Masson et al Nature Methods 2019.
Actions taken:
Table EV4A now contains a summary of the HDX conditions/quality and on separate sheets (B-I) the D-uptake per peptide for each protein condition tested, including the standard deviation values, as indicated in the guidelines of Masson et al Nature Methods 2019. Separate tables now contain the PyHDX “per residue – analysis” on the folded fractions (Table EV5) and degree of unfoldedness (Table EV6) as shown in the schematic pipeline (Figure EV3B).

1st Revision - Editorial Decision **5th Jul 2022**

Dear Tassos,

We have now received re-review reports from the final referee, and you have addressed all of his/her concerns satisfactorily. Before we can move to publication, however, there are a number of small editorial points that I would like you to consider.

Please:
- Reduce the number of key words to five
- Update the Conflict of Interest statement to Disclosure and Competing Interests Statement according to the instructions given in our guidelines for authors.
- Change the reference format according to the instructions given in our guidelines for authors.
- Update the figure callouts so that Fig 3B is called out before 3C. In addition, Fig 4F, EV1G, EV3G, EV5D callouts (as well as Appendix Fig. SSB_F, S3A_C callouts) are missing, and EV4E is only called out in the figure legends.
- Change the name of tables EV2, EV4, EV5, EV6, EV7, EV8 and EV9 to Dataset EV#. The legends should be either added into the excel files or ZIPed along with each table, and removed from the manuscript file.
- Add Appendix 1 file with a table of contents, which is currently missing
- Merge Fig 1 into one file.
- Also re-check the image in Figure EV5A and see if it also relates to figure 2B last image bottom right.
- In figure EV2D, error bars are shown for n=2 in Figure EV2D. See Word file for additional comments.
- Format the Structured Methods by removing instructions from the template.
- remove the Appendix Figure legends from the manuscript and insert in the Appendix Figures pdf with a Table of Contents.

CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. You are encouraged to use the free text boxes beneath each contributing author's name to add specific details on the author's contribution. Please follow the instructions in our guide to authors.

All Appendix Tables (see our guidelines) need to be contained in a single Merged PDF along with the figures and a Table of contents (Appendix files are not typeset by our publisher so all formatting has to be done by the Authors). If the files cannot be converted into a pdf without loss of data then the Tables will need to be changed to Datasets.
Alternatively your Appendix tables could be turned into Datasets (Dataset EV1 etc.)
To do this, please remove the table legends from the manuscript and insert the corresponding legend into the correct Table/Dataset xlsx, using a in a separate worksheet.

We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. Source Data can also include Excel tables to accompany your graphs. We anticipate that their inclusion will make your work more discoverable and useable to scientists in the future.

Please therefore upload your main figure source data: a single pdf file per main figure (not zipped together).

We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.
We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

Best wishes,

William

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

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also figure legend guidelines: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines
(https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

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

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

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (3rd Oct 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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

Referee #2:

The authors have answered my questions, I am okay with this proceeding to publication

Revisions

Manuscript: EMBOJ-2022-111344
Corresponding author(s): Anastassios Economou, Spyridoula Karamanou

1. General Statement

2. Description of revisions

- Reduce the number of key words to five
Author's response/Action taken: Done.

- Update the Conflict of Interest statement to Disclosure and Competing Interests Statement according to the instructions given in our guidelines for authors.
Author's response/Action taken: Done.

- Change the reference format according to the instructions given in our guidelines for authors.
Author's response/Action taken: Done.
The EMBO Journal style of Endnote was used (https://endnote.com/style_download/embo-journal/)

- Update the figure callouts so that Fig 3B is called out before 3C. In addition, Fig 4F, EV1G, EV3G, EV5D callouts (as well as Appendix Fig. SSB_F, S3A_C callouts) are missing, and EV4E is only called out in the figure legends.
Author's response/ Actions taken: Done.
-*Fig. 3B* (1st reference on line 319) vs *Fig. 3C* (1st reference later in the same line).
-*Fig. 4F* (lines 416, 418).
- *EV1G* (line 241).
- *EV3G* does not exist; we presume the editor meant EV3E which was not referenced before. *EV3E* is now referenced in line 321.
-*EV5D* (line 454).
-We presume '*Appendix Fig. SSB_F*' refers to Appendix Fig. S2B-F.
Fig.S2A-D (line 323, 326).
Fig.S2E-H (line 331).
- *Appendix Fig. S3A_C*
Fig.S3A (line 452).
Fig.S3B (line 462, 464).
Fig.S3C (line 452).
Fig.S3D (lines 462, 464).
-*Fig. EV4E* (line 417).

- Change the name of tables EV2, EV4, EV5, EV6, EV7, EV8 and EV9 to Dataset EV#. The legends should be either added into the excel files or ZIPed along with each table, and removed from the manuscript file.
Author's response/ Actions taken: Done.
-Tables were renamed to Dataset EV# and relevant references in the manuscript were adjusted.
-Legends were removed from the manuscript and added into the dataset excel files.

- Add Appendix 1 file with a table of contents, which is currently missing
Author's response/Actions taken: Done.
The table of contents (previously on page 2) was moved to page 1. Previous information on page 1 has now been minimized.

- Merge Fig 1 into one file.
Author's response/ Actions taken: Presuming that the editor refers to the Source Data relating to Fig 1**,** we merged the relevant data into one excel file.

- Also re-check the image in Figure EV5A and see if it also relates to figure 2B last image bottom right.
Author's response/ Actions taken:
-EV5A left panel and Fig. 2B bottom right panel are the same data. Duplication facilitates direct comparison of PpiA to proPpiA (EV5A, left to right panel).
-Labels and legends have been readjusted in Fig. EV5A.

- In figure EV2D, error bars are shown for n=2 in Figure EV2D. See Word file for additional comments.
Author's response: We are not clear on this comment. We could not find an additional word file. Please clarify.

- Format the Structured Methods by removing instructions from the template.
Author's response/Actions taken: Done.

- remove the Appendix Figure legends from the manuscript and insert in the Appendix Figures pdf with a Table of Contents.
Author's response/ Actions taken: Done.
-The manuscript contained legends only for Figures and Extended View Figures.
-A table of contents has been added to the first page of the appendix.

CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. You are encouraged to use the free text boxes beneath each contributing author's name to add specific details on the author's contribution. Please follow the instructions in our guide to authors.
Author's response/ Actions taken: Done.

All Appendix Tables (see our guidelines) need to be contained in a single Merged PDF along with the figures and a Table of contents (Appendix files are not typeset by our publisher so all formatting has to be done by the Authors). If the files cannot be converted into a pdf without loss of data then the Tables will need to be changed to Datasets.
Alternatively your Appendix tables could be turned into Datasets (Dataset EV1 etc.)
To do this, please remove the table legends from the manuscript and insert the corresponding legend into the correct Table/Dataset xlsx, using a in a separate worksheet.
Author's response/ Actions taken: Done.
All Extended View Tables were changed to Datasets (see above).

We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures. The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. Source Data can also include Excel tables to accompany your graphs. We anticipate that their inclusion will make your work more discoverable and useable to scientists in the future.
Please therefore upload your main figure source data: a single pdf file per main figure (not zipped together).
Author's response/ Actions taken: Done.
Source data have been submitted as excel or pdf files.

We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.
We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.
Author's response/ Actions taken: Done.
A synopsis (with a summary figure, statement and bullet points), had been uploaded in the last version as well. It is included again.

2nd Revision - Editorial Decision **2nd Aug 2022**

Dear Tassos,

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

Congratulations on a really elegant study!

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
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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
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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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
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  - 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.

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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?	Not Applicable	

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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	Reagents and Tools Table

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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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	
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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.	Yes	Table
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?	Not Applicable	

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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)
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