More than just a ticket canceller: The mitochondrial processing peptidase tailors complex precursor proteins at internal cleavage sites

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Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

RE: Manuscript #E20-08-0524
TITLE: "More than just a ticket canceller: The mitochondrial processing peptidase tailors complex precursor proteins at internal cleavage sites"

Dear Dr. Boos:

I have read your manuscript entitled "More than just a ticket canceller: The mitochondrial processing peptidase tailors complex precursor proteins at internal cleavage sites" and the review documents resulting from its previous submission to eLife that you provided.

This paper could be acceptable for publication in MBoC if the reviewer comments can be addressed. In particular, the key issue is to more firmly establish that MPP is indeed the protease responsible for the internal cleavage of the Arg6-Arg5 precursor. Thus, I think you need to pay particular attention to reviewer 1's point #2 (which is echoed by reviewer 2). I'm also wondering whether temperature shift experiments using mas1 and/or mas2 temperature sensitive cells could be informative here.

With your revised manuscript, please send us a point by point summary of your responses to the referees' comments, and the revisions you make to the paper.

Thank you for submitting this very interesting work to Molecular Biology of the Cell.

best wishes,

Thomas D. Fox

Sincerely,
Thomas Fox
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Boos,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

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Sincerely,

Eric Baker
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Kaiserslautern
September 21, 2020

Revision of our manuscript #E20-08-0524 for *MBoC*

Dear Tom Fox,

Thank you very much for evaluating our manuscript. We were pleased to see that you found our paper interesting and in principle suitable for publication in *MBoC*. We addressed the points that were raised by the reviewers, especially (but not only) those emphasized in your decision letter. You find our point-by-point answers to the reviewers' comments below.

We feel that these additional experiments considerably strengthened our manuscript and firmly established MPP as processing protease for internal cleavage of the Arg5,6 precursor. We included several new experiments in the revised manuscript (novel Figures 1E, 2B, 2C, 6B, S1B and S1F) as well as some clarifications and additional comments suggested by the reviewers. Even though be we did our best to keep the text as concise as possible, we now exceeded the limits of a Brief Report to an extent that makes it difficult to shorten without sacrificing content. In addition, we deem it beneficial to present the findings on MPP being the protease responsible for cleavage (Fig. 1) and the determination of the precise cleavage site (Fig. 2) in two separate figures, thereby increasing the number of display items to six. In our opinion, the new data and the textual additions made during the revision are important and would be misplaced in the supplementary material. Therefore, we would like to request the conversion of our manuscript to the Article format. Please let us know in case that you feel that this is not appropriate. In that instance, we would be grateful for your advice on how to shorten the manuscript to adhere to the Brief Report restrictions.

We hope that our revisions satisfactorily addressed the concerns of the reviewers. We are convinced that our work on internal precursor processing as a novel *bona fide* function of MPP is of broad interest for the cell biology community. Please feel free to contact us with any questions that may have remained open.

All best,

This paper could be acceptable for publication in MBoC if the reviewer comments can be addressed. In particular, the key issue is to more firmly establish that MPP is indeed the protease responsible for the internal cleavage of the Arg6-Arg5 precursor. Thus, I think you need to pay particular attention to reviewer 1's point #2 (which is echoed by reviewer 2). I'm also wondering whether temperature shift experiments using mas1 and/or mas2 temperature sensitive cells could be informative here.

We thank the editor for the positive evaluation of our manuscript. We addressed the points raised by the reviewers, especially those emphasized by the editor, as detailed below. We particularly thank the editor for his valuable suggestion to use a *mas1* temperature sensitive mutant, which we now used for *in vitro* and *in vivo* experiments that are presented in the revised version of the manuscript.

Reviewer #1 (General assessment and major comments (Required)):
1. The authors use purified MPP to show that in vitro synthesized Arg5,6 precursor can be processed to the correct sized products. At that point, the authors "conclude that Arg5,6 is imported into the mitochondrial matrix and processed twice by MPP". This is plausible, but is premature based on the data, which show that MPP is able to process Arg5,6. However, the conclusion that MPP actually does process Arg5,6 in vivo is not documented, and the alternative that something else does this job is not formally excluded. This caveat should be acknowledged unless the authors are able to show necessity of MPP, not just sufficiency.

As detailed in our response to point 2, we now added several new experiments to address the necessity of MPP for internal cleavage, both *in vitro* and *in vivo*. We therefore now have much stronger evidence that MPP is *the* protease responsible for internal processing of the Arg5,6 precursor in the mitochondrial matrix.

2. The experiment showing cleavage with purified MPP (Fig. 1E and S1A) would be strengthened with control experiments using a catalytically inactive mutant of MPP, and a Arg5,6 substrate with a mutated site for cleavage. The first control would rigorously exclude any contaminants, and the second would help verify the site of cleavage.

We addressed this point by various approaches. We used a temperature-sensitive *mas1*^{ts} mutant and tested its ability to cleave Arg5,6 both *in vivo* and *in organello*. Both approaches concordantly showed that this mutant has defects in processing of Arg5,6 both at its MTS (as expected for an MPP mutant) and at its internal cleavage site (novel Figure 1E and Suppl. Figure 1B). In addition, we predicted MPP cleavage sites in the iMTS-L region of Arg5,6 with *in silico* sequence analysis (novel Figure 2B) and generated Arg5,6 mutants in which we changed these putative MPP recognition motifs. These variants could be imported into isolated mitochondria *in vitro*. When the RSY motif at amino acid position 523-525 was mutated, no internal processing was observed, while mutation of the RGY motif at amino acid position 549-551 did not affect internal cleavage of the precursor (novel Figure 2C). Hence, the internal cleavage site of Arg5,6 adheres to the classical R-2 motif at position 524, and the cleavage by MPP is presumably followed by an Icp55-mediated processing of the newly formed N-terminus of Arg5.

3. The conclusion that MPP processes Arg5,6 at the correct site in their in vitro experiments is based on size by SDS-PAGE. The resolution is not sufficient to draw this conclusion, which should be adjusted to say that processing occurs at approximately the correct site (unless the authors perform additional analysis to document the precise cleavage site). Mutagenesis of the putative site (point 2 above) would also be helpful in establishing the site more precisely.

As described above, our mutagenesis experiments identified the site of cleavage to occur at position 524, which is in perfect agreement with the fragment sizes observed by SDS-PAGE.

4. The smaller products seen in Fig. 1E would seem to suggest that MPP exhibits a degree of promiscuity in vitro that is not seen in vivo. This should be noted in the text.

We discussed this possibility in the revised version in the context of cleavage site recognition *in vitro* versus *in vivo* (line 236). See also our response to point 6 for further details.

5. The authors observe that Arg6(1-343) cannot replace Arg6(1-502). They conclude that residues 344-502 are needed for enzyme activity, but this could be for many reasons. For example, Arg6(1-343) might not associate with Arg5. It is premature to imply that catalytic activity is impaired without making such measurements. The conclusion should be adjusted.

We adjusted the paragraph on the enzymatic activity as suggested by the reviewer (line 202).

6. It is worth testing whether Arg5(344-862) produced by in vitro translation can be processed by purified MPP. This would help distinguish between some intrinsic problem with access versus a more nuanced issue relating to how import is mediated by the iMTS-L versus a bona fide MTS (e.g., with only the latter recruiting MPP as speculated by the authors).

We thank the reviewer for her/his suggestion – this is a very interesting point. We tested whether purified MPP can process Arg5(344-863) in an *in vitro* reaction. Indeed, Arg5(344-863) can be cleaved internally by MPP in this organelle-free assay (novel Suppl. Figure 1F). An N-terminal presequence was not required for the *in vitro* cleavage (even though it appears as if the efficiency of processing was slightly higher when a Su9 presequence was fused to the construct), in contrast to the results we obtained *in organello* and *in vivo*. This clearly shows that the lack of internal cleavage of the Arg5(344-863) precursor is not an artificial problem of this truncated construct, since the cleavage site is accessible in this minimal system. Instead, internal processing of proteins by MPP obviously is a regulated process inside mitochondria. As pointed out by the reviewer, this finding is best compatible with our model of MPP recruitment by a *bona fide* N-terminal MTS and subsequent "scanning" for internal cleavage sites once loaded onto the precursor. It will be exciting to study the mode of regulation in detail in the future.

Reviewer #2 (General assessment and major comments (Required)):
Arg5, 6, a polyprotein is cleaved to produce two proteins Arg5 and Arg6. The authors report that production of these two proteins is mediated by a mitochondrial protease that is known for its function in N-terminal cleavage. The in vitro analysis is interesting, but the possibility of a contaminating activity cannot be ruled out. This needs to be tested by additional experiments, preferably by more data in intact cells.

We addressed this point by various approaches as specified above in the response to point 2 of reviewer #1. Thereby, we now provide ample data from *in vitro*, *in organello* and *in vivo* experiments that firmly establish MPP as the protease responsible for internal processing of Arg5,6.

RE: Manuscript #E20-08-0524R
TITLE: "More than just a ticket canceller: The mitochondrial processing peptidase tailors complex precursor proteins at internal cleavage sites"

Dear Dr. Boos:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell. As you suggested, the paper will be published as a regular Article.

Sincerely,
Thomas Fox
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Boos:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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