EMBO reports

Manuscript EMBO-2015-41273

Mcp3 is a novel mitochondrial outer membrane protein that follows a unique IMP-dependent biogenesis pathway

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Review timeline:

Submission date:	31 August 2015
Editorial Decision:	09 October 2015
Revision received:	19 February 2016
Editorial Decision:	17 March 2016
Revision received:	07 April 2016
Editorial Decision:	13 April 2016
Revision received:	19 April 2016
Accepted:	26 April 2016

Editors: Achim Breiling/Martina Rembold

Transaction Report:

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

1st Editorial Decision 09 October 2015

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

As you will see, all three referees acknowledge that the findings are potentially interesting, in particular the novel import pathway of Mcp3. However, all three referees also point out several concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

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

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. For a normal article there are no length limitations, but the results and discussion section must be separate and the entire materials and

methods included in the main manuscript file. Please use the EMBO reports reference style with non-superscripted numbers.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in all figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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

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

REFEREE REPORTS

Referee #1:

In eukaryotic cells most membrane lipids are synthesized in the ER and then distribute to other cellular membranes either by secretory vesicles or by yet ill-characterized membrane-contacting complexes. Such membrane contacts exist for example between the ER and the mitochondrial outer membrane. In the present study, the authors identified the yeast gene MCP3 as a (weak) multi-copy suppressor of mdm10, a gene coding for a component of the ERMES complex. The function of Mcp3 is directly assessed in this study. The authors present evidence that mitochondria of mcp3 mutants are of normal morphology and lipid composition whereas Mcp3-overexpressors contain an increased fraction of mitochondria with non-wild-type morphology. In the second, much more interesting part of this study, the authors studied the biogenesis of Mcp3. Mcp3 is an outer membrane protein that apparently employs an N-terminal matrix targeting signal for import. The authors present convincing evidence, that the Mcp3 precursor is imported via the TOM and TIM23 complex into the inner membrane where it is cleaved by the IMP protease. Despite its maturation in the inner membrane, the protein appears to accumulate in the outer membrane. Based on these technically sound and compelling observations, the authors come forward with a model which proposes that the protein is exported during its biogenesis from an inner membrane localization to the outer membrane. However, this hypothesis is very speculative as there is no evidence for any export of the C-terminal region of the protein presented. The mature part of the protein might rather stay at the level of the outer membrane and only the N-terminal region is further imported into the mitochondria. This is a very interesting study of high technical quality. Especially the processing of the Mcp3 precursor is very nicely demonstrated here.

Specific points:
1. The authors conclude from their experiments that the MIM complex inserts the Mcp3 precursor from the inside into the MOM. However, there is no evidence for this claim. The Mcp3 C-term might be inserted from the cytosolic side or laterally from the TOM channel into the MOM even if the N-term is imported to the level of the inner membrane. The authors should perform kinetic experiments in which they follow the protease-accessibility of imported protein over time. If their model is correct there should exist a protease-resistant sorting intermediate that can be chased out in the presence but not the absence of Mim1 components.

2. In Fig. 8, the authors show that Mim1 is essential for IMP-dependent processing of Mcp3. However, according to their model, IMP-dependent processing should occur prior to the contact of

Mim1 with the Mcp3 sorting intermediate.

3. The authors should make Mcp3 mutants in which the IMP processing site is changed so that a non-processed form accumulates. Does this species span both membranes of the mitochondria?

4. The yme1 deletion mutant shown in fig. 7D accumulates strongly increased levels of HA-Mcp3. This indicates that the i-AAA protease can degrade the outer membrane protein Mcp3. Does the deletion of YME1 restore the levels of HA-Mcp3 in the mim1 deletion mutant? This would indicate that Yme1 plays a role in the superveillance of the Mcp3 biogenesis removing stalled intermediates.

5. In the yeast genome the MCP3 gene in direct proximity of the MDM10 gene. It is very unlikely that this is by incidence. Is it possible that overexpression of the MCP3 locus resores some misbalance induced by the deletion of the neighboring gene? The mild suppression might be caused indirectly, for example via effects on the gene in between the MCP3 and MDM10 locus which codes for the Spo7 phosphatase regulator. Spo7 strongly influences the growth of yeast cells and the mild effects might just be the consequence of indirect effects on the Spo7 levels in the strains. I do not want the authors to show any further control experiments in this direction but they should consider this aspect just to be sure that they are not on the wrong track when studying the Mcp3 function.

Referee #2:

The ER-mitochondria encounter structure in yeast (ERMES; Mdm10/34/12/Mmm1 complex) is involved in the regulation of crucial cellular functions such as mitochondrial fission, mitophagy and lipid exchange, although the underlying molecular mechanisms are less clearly defined. Here the authors have identified a mitochondrial outer membrane (MOM) protein Mcp3 (Mdm10-complementing protein 3) as a high copy suppressor of mdm10△ cells and revealed its novel biosynthetic pathway. It is synthesized as a precursor with the N-terminal mitochondrial targeting sequence (MTS) and two trans-membrane domains (TMDs) in the middle and C-terminal parts of the molecule. It is imported into the mitochondrial inner membrane (MIM) sequentially through Tom70, Tom40 channel and Tim23 complex in the membrane potential-dependent manner, and then retro-integrated into the mitochondrial outer membrane (MOM) by the MIM insertase to establish authentic Mcp3 topology.

This reviewer agree that this pathway is novel, although several control experiments will be required to draw this conclusion since the import response of pre-Mcp3 as analyzed in vivo and in vitro with various mutant cells are weak.

(1) Fig. 5D, F, Fig. 6C, E, Fig. 7B, D, and Fig. 8B: Profiles of long SDS-PAGE gels containing positions of Mcp3 precursor should be provided. They also require size markers.

(2) Fig. 6D & E (tom40-25), Fig. 7A, B, C (tim23ts, +CCCP), Fig. 8A & B (mim1△, mim2△): Intra-mitochondrial localization of the import-intermediates of HA-Mcp30 should be analyzed for either in vitro or in vivo system.

(3) The import behavior of Mcp3-HA should be noted. How about it's mitochondrial targeting and MTS processing?

(4) It would be informative to know the requirement of TMD1 and TMD2 in the rescue of the growth defects of mdm10△ cells.

(5) Fig. 6A: can protease-protection assay detect the import intermediates?
Fig. 6B & C: Import of Su9-DHFR should be analyzed as a
negative control.
Fig. 6D & E: PINK1 or Ugo1 should be analyzed as a negative
control.

(6) Fig. 7A & B: Import of Ugo1 should be analyzed as a negative control.
Fig. 7C: Intra-mitochondrial localization of Mcp3 precursor
in CCCP-treated cells should be analyzed.

(7) Fig. 8A & B: Mcp3 precursor was not processed in these cells.
Why?
Import of pSu9-DHFR should be analyzed in these cells as a
negative control.

Referee #3:

In this manuscript, the authors concentrate on the protein called Mcp3. They identify this protein as a suppressor of ERMES function. The Mcp3 protein is processed but located in the mitochondrial outer membrane (MOM). In principle, the way how the ER communicates with mitochondria, and how other systems can substitute for various functions assigned to the ERMES complex, are of high importance. Equally important is to fully understand how proteins are directed to their final destinations in organelles, including mitochondria.

The current manuscript presents two independent stories, one focused on functional aspects of Mcp3 and another one focused on a unique way of the Mcp3 biogenesis. However, both stories seem to be incomplete with respect to both, function and the sorting pathway. The key to assign the function of Mcp3 is to better describe what happens with mitochodria/ER and the ERMES complex in the absence of Mcp3 and upon Mcp3 overproduction. The manuscript contains limited amount of data on this topic (Fig. 4), in addition to well-proven suppression of the ERMES absence.

The biogenesis pathway of Mcp3 is interesting, because of two aspects, the involvement of the IMP protease complex, and the involvement of the integrase MIM that would take Mcp3 from the IMS site to integrate into the MOM. Also in this case the model presented here is not fully supported by the data leaving the impression that some important aspects have not been addressed. The authors do not comment and do not shown the appearance of the precursor form of Mcp3. Is the precursor form visible in the mitochondrial of the cells lacking Imp1 or Imp2, in the tom mutants or tim23 mutants? What is happening in the mitochondria lacking the MIM complex? These mitochondria should accumulate the processed form in the IMS. Is the mature (and/or precursor) form degraded by the mitochondrial protease, i.e. Yme1 (see Fig. 7D)? Does Mcp3 interacts (transiently) with any of these machines? Probing for some direct interactions is important to exclude indirect effects caused by broad dysfunctions related to translocating and processing machineries defects.

Finally, the pathway of Mcp3 should be placed in a broader context. The case of Pink1, and the similarities and differences of the mitochondrial import of Pink1 should be discussed and compared to Mcp3.

1st Revision - authors' response 19 February 2016

Thank you very much for your message from 09 October 2015 with the comments of the Reviewers referring to our manuscript EMBOR-2015-41273V1, “Mcp3 is a novel mitochondrial outer membrane protein that follows a unique biogenesis pathway” by M. Sinzel et al.

We would like to thank you for handling our manuscript and the Reviewers for their thoughtful comments that helped us to improve the quality of our work.

The enclosed manuscript was revised to address all points raised by the three Reviewers. The revised version includes the results of many novel experiments and control assays that we performed in the last months to address all the Reviewers´ comments. Accordingly, we modified the text of our manuscript to discuss these new results and to address the issues raised by the Reviewers. Furthermore we added another author that contributed experimental data. Descriptions of newly employed methods were also added to the revised Material and Methods section.

We hope that this revised version is suitable for publication in EMBO reports.

Referee #1

We thank the Reviewer for his/her positive opinion and addressed all his/her comments as explained below.

Specific points

1. The authors conclude from their experiments that the MIM complex inserts the Mcp3 pecursor from the inside into the MOM. However, there is no evidence for this claim. The Mcp3 C-term might be inserted from the cytosolic side or laterally from the TOM channel into the MOM even if the N-term is imported to the level of the inner membrane. The authors should perform kinetic experiments in which they follow the protease-accessibility of imported protein over time. If their model is correct there should exist a protease-resistant sorting intermediate that can be chased out in the presence but not the absence of Mim1components.

We fully agree with the Reviewer, that an insertion of Mcp3 precursor by the MIM complex is only one of several potential ways by which the protein can obtain its correct topology. In the revised version we added an *in vitro* experiment that supports a MIM involvement. In this new figure we demonstrate that recombinant Mim1 has the capacity to interact with the radiolabelled precursor of Mcp3 (revised Fig. 9C).

However, despite many *in vitro* experiments employing a variety of conditions (altering temperature, import time, chase conditions) we were not able to observe a processed soluble import intermediate in the intermembrane space that can be chased further.

Whereas most of fully integrated Mcp3 as well as all of the precursor molecules in solution were digested by PK, we always observed a fraction of processed Mcp3 that was resistant to PK (See revised Fig. 8G, 8H, EV3, asterisk). Yet this fraction could not be chased in wild-type mitochondria and no increase of this species in mitochondria isolated from cells lacking MIM complex components could be observed.

One explanation might be that the overall biogenesis pathway is tightly coupled and therefore no isolated intermediate can be obtained. Of note, there are several MOM proteins that are inserted into the membrane after passage through the intermembrane space (OM45, β-barrel proteins). To our knowledge, also for those proteins there is no evidence for soluble, accumulating IMS intermediates. Nevertheless, we included in the revised version an experiment where we can trap a PK-protected full-length intermediate of the D70G mutant that is not cleaved by Imp1 (Full length intermediate D70G, revised Fig 8G see also Reviewer 2, point 5). This observation suggests that under normal conditions the N-terminus reaches the IMP peptidase before the C-terminal transmembrane domain is inserted into the outer membrane. Thus, the option that the C-terminal is inserted directly from the cytosol can be most probably excluded.

Taken together, despite the new supporting results, we cannot exclude a MIM-independent insertion pathway. Therefore in the revised version we toned down our suggestions regarding the potential involvement of the MIM complex and changed original Figure 9 to revised Figure 10, in which the role of MIM complex is only hinted.

2. In Fig. 8, the authors show that Mim1 is essential for IMP-dependent processing of Mcp3. However, according to their model, IMP-dependent processing should occur prior to the contact of Mim1 with the Mcp3 sorting intermediate.

We agree with the Reviewer that if Mim1 would have been involved only in the final membrane insertion step, we should not expect to observe a difference in the processed form of Mcp3 in *mim1/2*Δ strains. The autoradiography used in the original Figure 8A gives the wrong impression that there is hardly any processed form in these mutant strains. However, we always observed a processed form of Mcp3 in all our experiments with mitochondria lacking Mim1 and/or Mim2. Yet the amount of the processed form is strongly reduced (to about 60% of the WT level, see quantification). We thank the Reviewer for this remark and changed in the revised Fig. 9A the autoradiography to an experiment that better reflects the common outcome and the quantification below.

We assume that the lower levels of processed intermediate in the deletion strains in caused a yet unidentified protease that might degrade the non-inserted intermediate molecules and/or by the defects in the TOM machinery.

3. The authors should make Mcp3 mutants in which the IMP processing site is changed so that a non-processed form accumulates. Does this species span both membranes of the mitochondria?

In original Fig. 7F of the manuscript we used the D70G variant of Mcp3, which cannot be processed by IMP peptidase. In a new experiment we now additionally added external PK to analyse the fate of non-processed Mcp3 as suggested by the Reviewer. To present the new data, we omitted the left panel in revised Fig. 8F (numbering changes due to the novel revised Figure 7) and added a novel Figure 8G in the revised manuscript that shows the same experiment as the right part of original Fig 7F, yet including also the PK treatment. The results demonstrate that a non-processed intermediate of the D70G variant is protected against externally-added protease. Since the predicted C-terminal transmembrane domain is followed by only 4 amino acids, the observation that it is not cleaved cannot answer the question whether it spans both membranes. The protease protection might result from the C-terminus being either in the IMS or anchored into the MOM whereas the four amino acids are not accessible by the protease.

4. The yme1 deletion mutant shown in fig. 7D accumulates strongly increased levels of HA-Mcp3. This indicates that the i-AAA protease can degrade the outer membrane protein Mcp3. Does the deletion of YME1 restore the levels of HA-Mcp3 in the mim1 deletion mutant? This would indicate that Yme1 plays a role in the superveillance of the Mcp3 biogenesis removing stalled intermediates.

As correctly stated by the Reviewer, higher levels of HA-Mcp3 are observed in the crude mitochondrial fractions of *yme1Δ* cells in comparison to their levels in wild-type cells (original Figure 7D). Following the suggestion of the Reviewer, we tried to construct in the W303 wild-type background double deletion strains *yme1*Δ/*mim1*Δ and *yme1*Δ/*mim2*Δ. In the case of *MIM1* we were not able to retrieve the double deletion strain neither by sequential homologous recombination nor by a mating and tetrad-dissection approach suggesting synthetic lethality of both genes. In contrast, we were able to retrieve *yme1*Δ/*mim2*Δ double deletion strains by tetrad dissection and performed the same experiment as in Figure 7D. Figure R1 herein shows that on the one hand HA-Mcp3 levels are indeed marginally increased in freshly curated *yme1*Δ cells. On the other hand, as we reported in the original version, HA-Mcp3 levels are reduced in *mim2*Δ and there was no accumulation in the *yme1*Δ/*mim2*Δ cells. Hence Yme1 seems to have no special role in surveillance of Mcp3 levels.
We believe that these new results can distract the general reader and are outside the focus of the current manuscript. Therefore we did not change the manuscript in this regard and included Figure R1 only for the Reviewer.

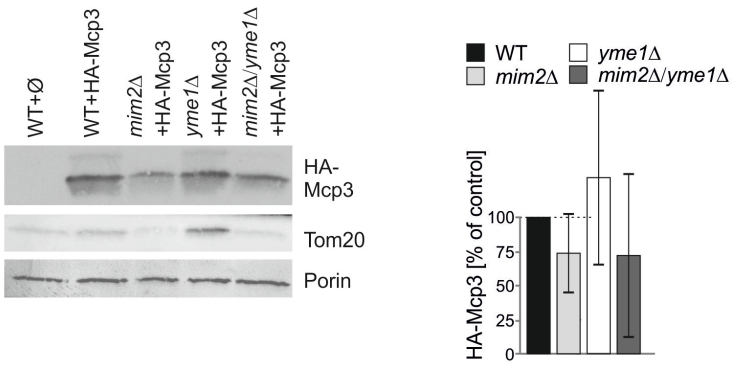

Figure R1. Steady state levels of HA-Mcp3 in cells lacking Mim2 and/or Yme1. Crude mitochondria were obtained from wild-type cells containing an empty plasmid (Ø), and wild-type cells, *mim2*Δ, *yme1*Δ or *mim2*Δ/*yme1*Δ cells containing a plasmid expressing internally HA-tagged Mcp3 (HA-Mcp3). Samples were analysed by SDS-PAGE and immunodecoration with antibodies against the HA-tag, Tom20 as control substrate of MIM complex and Porin as loading control. HA-Mcp3 levels were quantified in relation to Porin and wild-type levels were set to 100%. The bar diagram shows the mean with standard deviation of three independent experiments.

5. In the yeast genome the MCP3 gene is in direct proximity of the MDM10 gene. It is very unlikely that this is by incidence. Is it possible that overexpression of the MCP3 locus restores some misbalance induced by the deletion of the neighboring gene? The mild suppression might be caused indirectly, for example via effects on the gene in between the MCP3 and MDM10 locus which codes for the Spo7 phosphatase regulator. Spo7 strongly influences the growth of yeast cells and the mild effects might just be the consequence of indirect effects on the Spo7 levels in the strains. I do not want the authors to show any further control experiments in this direction but they should consider this aspect just to be sure that they are not on the wrong track when studying the Mcp3 function.

We thank the Reviewer for his/her thoughtful comment and will keep this aspect in mind. Nevertheless Mcp3 over-expression also partially rescues the deletion of the other ERMES genes *MDM12*, *MDM34* and *MMM1* (Fig. 3). These genes are located in other regions of the yeast genome and have to our knowledge no connection to Spo7 levels. In addition, when *MCP3* cDNA was cloned into yeast over-expression vector and transformed into *mdm10*Δ cells, a similar complementation capacity as from the genomic library was observed. Thus, we believe that these findings support a general role of Mcp3 as a real suppressor of non-functional ERMES complex and not solely of Mdm10.

Referee #2:

This reviewer agree that this pathway is novel, although several control experiments will be required to draw this conclusion since the import response of pre-Mcp3 as analyzed in vivo and in vitro with various mutant cells are weak.

We thank the Reviewer for the positive evaluation of our work and addressed his/her concerns as described below.

(1) Fig. 5D, F, Fig. 6C, E, Fig. 7B, D, and Fig. 8B: Profiles of long SDS-PAGE gels containing positions of Mcp3 precursor should be provided. They also require size markers.

As the Reviewer suggested, we exchanged the panels of HA-Mcp3 in these figures with wider panels that include also the size range of the precursor and added molecular weight markers. However, the precursor form was not always observed and is often only detectable, if at all, upon

very long exposures (see for example Figure R2 for the Reviewer only, which is a long exposure of Figure 5G).

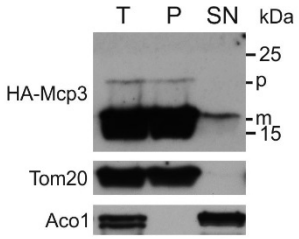

Figure R2. Mcp3 is not extractable by alkaline treatment (Long exposure of Figure 5G).
Mitochondria isolated from *mcp3*Δ cells expressing HA-Mcp3 were either left untreated (T) or subjected to alkaline extraction. The supernatant (SN) and pellet (P) fractions were analysed by SDS-PAGE and immunodecoration with antibodies against the indicated proteins. Tom20 an integral MOM protein; Aco1, a soluble matrix protein. The precursor and mature forms of HA-Mcp3 are indicated with p and m, respectively.

(2) Fig. 6D & E (tom40-25), Fig. 7A, B, C (tim23ts, +CCCP), Fig. 8A & B (mim1?, mim2?): Intra-mitochondrial localization of the import-intermediates of HA-Mcp30 should be analyzed for either in vitro or in vivo system.

We performed import of HA-Mcp3 in the respective mitochondria isolated from wild-type and mutant cells for 15 minutes and treated the mitochondria with proteinase K. The results are included as revised Figure EV3A. For CCCP treatment we added the novel revised Fig. 8H. These experiments with protease treatment show that the full length precursor is completely degraded if it is not processed by IMP. We suggest that precursor that does not reach the mitochondrial inner membrane is "slipping out" again. Furthermore we do observe a protease-protected fraction of the processed form as was discussed in addressing points #1 & 3 of Reviewer 1. However this intermediate could not be chased into fully-inserted species. We speculate that this is a non-productive intermediate that fails to be inserted into the outer membrane.

(3) The import behavior of Mcp3-HA should be noted. How about it's mitochondrial targeting and MTS processing?

We agree with the Reviewer that analysis of the consequences of C-terminally tagging of Mcp3 can provide interesting information on the role of various domains in the structure/function relationship of the protein. In the revised version we included the following new information (i) an experiment showing that C-terminally tagged Mcp3-HA is still targeted to mitochondria and processed (novel panel Fig. 5F and corresponding Results section) and (ii) An experiment demonstrating that the modified protein is still processed by Imp1 (novel Fig. 8E and corresponding Results section). A long exposure was chosen in the latter experiment to detect the unprocessed precursor. Taken together this data suggest that C-terminally HA-tagging leads to non-functional Mcp3 although the protein is correctly localized to mitochondria and processed by Imp1.

(4) It would be informative to know the requirement of TMD1 and TMD2 in the rescue of the growth defects of mdm10Δ cells.

The Reviewer raises an interesting question. To gain insight into the relevance of the two predicted transmembrane domains we constructed Mcp3 variants that lack either TMD1 (a.a. 106-128) or TMD2 (a.a. 172-198). Next, we performed a growth phenotype rescue assay for *mdm10*Δ cells with these constructs. The results of this new experiment demonstrate that both variants are not functional (revised Fig. 5J and corresponding Results section). Since no rescue was observed, we also monitored the levels of the different constructs with a new antibody raised against Mcp3. The results

show that the variant without TMD1 cannot be detected (probably due to its low stability and high turn-over) whereas the one without TMD2 is detected in lower levels than full-length Mcp3 (revised Fig. 5K and corresponding Results section). Taken together, the deletion of either TMD results in a less stable protein in comparison to native Mcp3.

(5) (i) Fig. 6A: can protease-protection assay detect the import intermediates?

As was discussed in addressing points #1 & 3 of Reviewer 1, a protease-protected processed intermediate was detected in many cases but this intermediate could not be chased into fully-inserted species. A protease protected full-length form was detected only with the D70G variant that cannot be processed by Imp1 (see also Reviewer 1, point #3).

(ii) Fig. 6B & C: Import of Su9-DHFR should be analyzed as a negative control.

We agree with the Reviewer that the import of Su9-DHFR is a good negative control for these experiments. In the revised Fig. 6B we show that the import of this precursor protein is not affected in the *tom70/71Δ* strain.

(iii) Fig. 6D & E: PINK1 or Ugo1 should be analyzed as a negative control.

As suggested by the Reviewer, we analysed the *in vitro* import of Ugo1-2HA in *tom40-25* mitochondria by BN-PAGE according to published assay (Becker, T. *et al.* 2011). The experiment is added as novel panel and quantification in the revised Fig. 6D. Furthermore we added a panel to revised Fig. 6E that provides immunodecoration of endogenous Ugo1 levels. The results demonstrate that, as expected, Ugo1 is, not affected in this mutant. These new data is described in the revised Results section.

(6) (i) Fig. 7A & B: Import of Ugo1 should be analyzed as a negative control.

As suggested by the Reviewer, we analysed the *in vitro* import of Ugo1-2HA in *tim23ts* mitochondria by BN-PAGE (Becker, T. *et al.* 2011). The experiment is added as novel panel and quantification in the revised Fig. 8A. Also we added a panel to revised Fig. 8B that provides immunodecoration of endogenous Ugo1 levels. Both the in vitro imported amounts and the endogenous Ugo1 levels are unaltered in the *TIM23* mutant.

(ii) Fig. 7C: Intra-mitochondrial localization of Mcp3 precursor in CCCP-treated cells should be analyzed.

We thank the Reviewer for this suggestion. We performed the experiment, added the result as novel revised Fig. 8H and changed accordingly the Results section. The Mcp3 precursor is degraded by PK in the presence of the uncoupler. This finding is in line with the hypothesis that non-imported precursor can be degraded rapidly in the cytosol.

(7) Fig. 8A & B: Mcp3 precursor was not processed in these cells. Why? Import of pSu9-DHFR should be analyzed in these cells as a negative control.

Indeed the original Fig. 8A might give the impression that hardly any processed Mcp3 is present after import. Yet the quantification of import in three independent experiments shows that about 60% of wild-type levels are reached. We thank the Reviewer for this remark and changed in the revised Fig. 9A the autoradiography to an experiment that better reflects the common outcome and the quantification below.

We assume that the lower amount of processed Mcp3 derives from two different outcomes of the absence of Mim1 and/or Mim2: (i) The fast degradation inside mitochondria of non-inserted Mcp3 intermediates, and (ii) The assembly defects in TOM complex in mitochondria lacking MIM complex that cause less precursor to reach the intermembrane space.

We agree with the Reviewer that in theory pSu9-DHFR could be used as a negative control for mitochondria isolated from cell lacking MIM components, if all import machineries were independent, and MIM complex was solely responsible for insertion of proteins in the outer

membrane. Yet loss of either Mim1 or Mim2 leads to secondary effects since TOM complex assembly is strongly reduced (Ishikawa et al., 2004; Waizenegger *et al.*, 2005; Hulett et al., 2008; Dimmer, *et al.*, 2012). Indeed Mim1 was identified in a screen as affecting import of matrix proteins (Mitochondrial IMport, Mnaimneh S, *et al.* 2004). Hence, it is not surprising that the import of pSu9-DHFR was affected in these *MIM* deletion strains. We added revised Appendix Figure S4 that shows the import defect on Su9-DHFR. We describe these new data in the revised Results section.

Referee #3:

The current manuscript presents two independent stories, one focused on functional aspects of Mcp3 and another one focused on a unique way of the Mcp3 biogenesis. However, both stories seem to be incomplete with respect to both, function and the sorting pathway. The key to assign the function of Mcp3 is to better describe what happens with mitochodria/ER and the ERMES complex in the absence of Mcp3 and upon Mcp3 overproduction. The manuscript contains limited amount of data on this topic (Fig. 4), in addition to well-proven suppression of the ERMES absence.

We would like to thank the Reviewer for acknowledging the relevance of our studies. We agree with the Reviewer that currently we cannot describe completely the function of Mcp3. Nevertheless we are convinced that the data included in this work provide a valuable basis to understand the molecular mechanism of Mcp3. Furthermore the novel insights from employing deletion variants or C-terminally tagged version of Mcp3 provide first information about structure-function relationship of Mcp3 (See addressing points #3 & 4 of Reviewer 2)

We are grateful to the Reviewer for the suggestion to test whether loss of Mcp3 or its over-expression has an effect on ERMES complex formation. We performed the suggested experiments by fluorescence microscopy of the ERMES complex. Using RFP-Mmm1, we could detect the puncta structures of the ERMES complex and could not observe any change upon deletion or over-expression of Mcp3. These new experiments are included as revised Figure EV2 and are discussed in the revised Results section.

The authors do not comment and do not show the appearance of the precursor form of Mcp3.

We are grateful to the Reviewer for the comment. As already suggested by Reviewer 2 (point 1), we now added in several experiments broader panels of the immunodecorations with antibodies against the HA-tag. As shown in revised Fig. 8E (Mcp3-HA) and the Reviewers exclusive Figure R2, the precursor of HA-Mcp3 is usually hardly detectable. We assume that not imported full-length precursor HA-Mcp3 molecules are rapidly degraded in the cytosol and therefore, are barely detectable, if at all.

Is the precursor form visible in the mitochondrial of the cells lacking Imp1 or Imp2, in the tom mutants or tim23 mutants?

As expected, no processed form is observed in cells lacking Imp1 or Imp2. As discussed in the previous point, the full length precursor was never visible in experiments like the one presented in revised Fig. 8D for HA-Mcp3. Only a weak band representing the non-processed form is detected in the case of Mcp3-HA (revised Fig. 8E). Along this line no full-length precursor protein is observed in *tom40-25* and *tim23ts* strains. As discussed above, we believe that non-imported molecules are unstable and therefore are degraded by cellular proteases.

What is happening in the mitochondria lacking the MIM complex? These mitochondria should accumulate the processed form in the IMS.

We agree with the comment of the Reviewer. Yet these mitochondria do not accumulate the expected intermediate. We discussed this point in length in our answers to points #1 and 2 of Reviewer 1 and point #7 of Reviewer 2.

Is the mature (and/or precursor) form degraded by the mitochondrial protease, i.e. Yme1 (see Fig. 7D)?

Indeed the levels of HA-Mcp3 seem to be higher in *yme1*Δ cells in the original Fig. 7D. However, we investigated this point by further experiments and could not observe a statistically significant change in the levels of HA-Mcp3 in a strain lacking Yme1 (see Figure R1 for Reviewers only, and answer to point #4 of Reviewer 1).

Does Mcp3 interacts (transiently) with any of these machines? Probing for some direct interactions is important to exclude indirect effects caused by broad dysfunctions related to translocating and processing machineries defects.

As suggested by the Reviewer we performed several experiments to show direct interactions between import machineries and radiolabelled Mcp3. Since several experiments were performed we include in the revised version a novel Figure 7 which presents the interaction with the TOM machinery. Accordingly original Figure 7 becomes revised Figure 8 etc.: The following additional experiments are included in the revised version and are described and discussed in the revised Results and Discussion sections.

(i) The soluble cytosolic domains of the import receptors Tom70, Tom20 and Tom22 were expressed as recombinant GST-fusion proteins in *E. coli* cells. All three constructs could specifically pull-down radiolabelled Mcp3 (revised Fig. 7A). Of note Tom70, which is required for Mcp3 import, binds better than Tom20 that has no influence on Mcp3 import.

(ii) To substantiate the observed Tom22-Mcp3 interaction we imported radiolabelled Mcp3 into mitochondria isolated from a strain harboring His-tagged Tom22. Affinity-binding with the solubilized organelles (revised Fig. 7B) demonstrates an interaction between Tom22 and Mcp3.

(iii) As a further support for the interaction with Tom22 we imported radiolabelled Mcp3 into the mitochondria described in (ii) and performed an antibody-shift assay analysed by BN-PAGE. The specific shift as observed with the anti-His antibody confirms the interaction with Tom22-His containing TOM core complex (revised Fig. 7C).

(iv) As already described above in the response to Reviewer #1, point 1, we added an experiment in which we can show that recombinant MBP-Mim1 can specifically interact *in vitro* with radiolabelled precursor of Mcp3 (revised Fig. 9C).

Finally, the pathway of Mcp3 should be placed in a broader context. The case of Pink1, and the similarities and differences of the mitochondrial import of Pink1 should be discussed and compared to Mcp3.

We thank the Reviewer for his/her comment. PINK1 is a nice example how understanding the biogenesis pathway of a (mitochondrial) protein is crucial for unraveling its physiological function. In the revised Discussion section we briefly discuss the current model of PINK1 import into mitochondria and the similarities to the newly described pathway of Mcp3.

2nd Editorial Decision 17 March 2016

Thank you for the submission of your revised manuscript to our editorial offices. Your manuscript has now been seen again by our referees, whose comments you will find below. As you will see, all three now support the publication of your manuscript in EMBO reports. However, some further minor revisions are necessary before we can proceed with the formal acceptance of your manuscript.

Referee #3 has a concern/suggestion regarding your model, which should be addressed.

Further, the main text (including references) has currently nearly 68000 characters including spaces. Even if we have no strict limitation for articles, it would be useful if you could shorten your manuscript to below 60000 characters. We also would need a conflict of interest statement before the author contribution list in the main text!

Could figures 2 and 3 be fused?

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information should also be provided in a paragraph in the methods section.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.
I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors satisfyingly addressed all points that I raised on the original version. This is a very interesting study which addresses a novel and important aspect of mitochondrial protein biogenesis.

Referee #2:

The paper is well revised and answers satisfactorily the points that the reviewers have raised. I believe this is an important report identifying a novel biogenesis pathway of the cytosol-exposed mitochondrial outer membrane protein.

Referee #3:

The authors adequately addressed the criticism. I feel that the manuscript has been greatly improved by strengthening several conclusions concerning the biogenesis steps of Mcp3 in particular.
I have only one minor comment for further consideration. The model implies that Mcp3 is laterally sorted into the inner membrane. This step is not shown and is less likely in my opinion. Placing the arrow in the IMS would probably represent a more likely scenario.

2nd Revision - authors' response 07 April 2016

Thank you very much for your message from 17 March 2016 with the final comments of the Reviewers referring to our manuscript EMBOR-2015-41273V2, “Mcp3 is a novel mitochondrial outer membrane protein that follows a unique biogenesis pathway” by M. Sinzel et al.
We would like to thank you for handling our manuscript and the Reviewers for their final comments.

As you can read below, in the new modified version we addressed all the Editorial comments and the one of Reviewer #3.

1) As Reviewer #3 suggested, we placed the arrow from step 3 to step 4 of our model in the IMS in the revised Figure 9.

2) As suggested by the Editor, we shortened our Manuscript (main text including references excluding figure legends) from 68 000 characters including spaces to now approx. 60 900. Since we added several new experiments during the revision process our manuscript has become an extensive

one. However, we believe that further shortening will result in reduced clarity and readability of our manuscript.

3) Figures 2 and 3 were fused to revised Figure 2 and all other Figures renamed accordingly.

4) We added the information of experiment number n and type of error bars (SD in all cases) to all figure legends.

5) We included a novel synopsis image in jpeg format that is based on the model in Figure 9 (550x400 pixels).

6) Furthermore, we extended the bullet points to short sentences.

7) We added a sentence to declare lack of conflict of interest.

We hope our manuscript is now ready for its formal acceptance.

3rd Editorial Decision 13 April 2016

Thank you for the submission of your revised manuscript to our editorial offices. While going through your manuscript I noted that further minor revisions are necessary before we can proceed with the formal acceptance of your manuscript.

Maybe this was not clear from my last decision letter, but we need statistics for several of the figures of your manuscript. We would require statistical tests for the data shown in Figures 1B, 2C, 3B, 3C, 3E, 5B-5E, 7A, 8A and 8B. Please test those differences shown in these figures you consider or claim to be significant using the appropriate test and indicate the significant differences (e.g. by asterisks) and also the p-values in the figure or the figure legend. Please could you also add a paragraph in the methods section describing this and mention the test(s) used to calculate p-values? I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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

3rd Revision - authors' response 19 April 2016

Thank you very much for your messages from 13 and 15 March 2016 with editorial minor points for revision of our manuscript EMBOR-2015-41273V3, “Mcp3 is a novel mitochondrial outer membrane protein that follows a unique biogenesis pathway” by M. Sinzel et al.

In the modified version we included the following changes:

1) As you recommended, we changed the title to “Mcp3 is a novel mitochondrial outer membrane protein that follows a unique *IMP-dependent* biogenesis pathway”.

2) As you suggested, we performed two-sided student t-tests for the bar diagrams in Figs. 1B, 2C, 3B, 3E, 5C, 5E, 7B and 8B and determined the corresponding *p*-values.

3) In Figs. 3C and D we do not observe any difference in lipid composition of wt or *mcp3*Δ mitochondria, hence there is no statistical significance to be determined.

4) In the evaluation of the mitochondrial morphology rescue in Fig. 1B the p-value equals 0.052, which we included directly in the bar diagram.

5) In Figure 3B and 3E the values are 0.084 and 0.088 respectively. Considering only values lower than 0.05 as significant, we changed the manuscript text in the Results part in a way that we

explicitly mention this. In our opinion providing the data in this way is clearer than including a table with numbers only.

Again we would like to thank you for handling our manuscript and your help during the revision process. We hope our manuscript is now ready for its formal acceptance.

4th Editorial Decision 26 April 2016

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Kai Stefan Dimmer
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2015-41273V2

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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For animal studies, include a statement about randomization even if no randomization was used.	NA
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
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Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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http://www.ebi.ac.uk/ega

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used in this study are "home made" laboratory stocks used in many previous studies and raised in rabbits against yeast proteins. The antibody against HA-tag is commercially available from Roche Diagnostics (cat. number 11 867 423 001).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

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13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
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21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
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