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Regulation of mitochondrial phospholipids by Ups1/PRELI-like proteins depends on proteolysis and Mdm35

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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 19 May 2010

Thank you for submitting your manuscript to the EMBO Journal. I have now head back from two of the three referees that I asked to review your paper. I am still waiting for the third report, but given the present majority recommendation I can take a prelimenary decision now.

As you can see below, both referees find your analysis intersting, insightful and suitable for publication in the EMBO Journal. However, they also raise a few specific points that should be resolved. In particular, some additional analysis to further look at the role of Mdm35 in the import of Ups1/Ups2 is needed. Given the support and comments provided by the referees, I would like to ask you to start making the requested changes and additions to the manuscript that would render the paper suitable for publication in the view of these two reviewers. I will forward you the comments of the third referee to you as soon as we receive them. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for submitting your intersting manuscript to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Potting et al.: Regulation of mitochondrial phospholipids...

Ups1 and Ups2 are two structurally related proteins of the mitochondrial intermembrane space that play critical roles in the synthesis and/or distribution of cardiolipin and phosphatidylethanolamine. In the present study, the authors identify Mdm35 as an interaction partner of both Ups1 and Ups2. Binding to Mdm35 is critical for mitochondrial import of Ups1 and Ups2 as well as to prevent their degradation by mitochondrial proteases. This study identifies an exciting network present in the intermembrane space of mitochondria that determines the lipid composition of the inner membrane. The authors identified Mdm35 as a central component that physically or functionally interacts with Ups1/Ups2, with the quality control system of mitochondria and with assembly factors of respiratory chain complexes. This is certainly an exciting new aspect of cellular biogenesis and lipid homeostasis that is of high interest for cell biology and molecular medicine. This study is of very high technical quality and the experiments are well controlled. The combination of yeast genetics and biochemistry allows interesting mechanistic insights into the biogenesis of Ups1 and Ups2. Although the specific function of Mdm35 in the translocation of Ups1 and Ups2 into mitochondria remains somewhat unclear, this study presents a thorough analysis of the relevance of the proteolytic system of the intermembrane space for Ups biogenesis. This connection was not described before and makes this study highly interesting.

Specific points:

1. Mdm35 appears to have at least two functions: first, it prevents imported Ups1 and Ups2 from degradation, and second, it facilitates the import of both proteins. While the first aspect is well characterized in this study, the second is only superficially analyzed. Some additional experiments should be performed using the import assay with isolated mitochondria. These should address the following points:
1a: Is Mdm35 really limiting for Ups1/Ups2 import? Does overexpression of Mdm35 increase the amount of imported Ups1 or Ups2?
1b: Fig. 5D, Fig. 6C: Why is there no kinetics of the import reaction and the protein is well imported even after 0 minutes? This may be explained if the import of the protein is not stopped on ice.
1c: At which stage of the import reaction do Ups1 and Ups2 bind to Mdm35? This could be addressed by a co-purification or by a crosslinking approach.

2. The title uses the expression "Regulation" and in the abstract the authors claim that "Mdm35...controls the accumulation" of Ups1 and Ups2. However, there is no direct evidence that the levels of Ups1 and Ups2 are regulated in the sense that they would be balanced in response to specific conditions. The authors therefore should rephrase these sentences or present evidence for a role of Mdm35 as a regulator rather than as a biogenesis factor or complex partner. In the discussion they suggest that Mdm35 might be redox-regulated. This may be the case but as far as I am aware of there is no evidence published thus far.

Minor points:
3. Page 4, last line: "Ups1 is required to maintain normal CL levels, while Ups2 controls PE levels." This sentence is misleading. Both components appear to have counteracting activities that influence (directly or indirectly) both CL and PE. The sentence therefore should be rephrased.

5. Page 6, bottom: "We did not observe binding of Mdm35 to untagged Ups1 and Ups2". This sentence might suggest that the tag is critical for Mdm35 binding. The sentence should be rephrased. It could read something like "When extracts with untagged Ups1 or Ups2 were applied, Mdm35 was not found in the bound fractions"

6. Page 16 upper part: The authors observed a negative effect of Ups2 overexpression on mdm35 null mutants. From this they claim that Ups2 can function independently of Mdm35. Alternatively, it could be that Mdm35 has a backup component that is titrated out under these conditions. For example, if other Cx9C proteins (like Emi1) could replace Mdm35 to a certain degree, overexpression of one Ups protein might lead to adverse effects. The authors should therefore

downgrade the statement of an Mdm35-independent activity of Ups2.

7. Page 17. In addition to Osman et al., the authors should cite the publication of Zeng, Neupert and Tzagoloff, MBC, 2007.

Referee #2 (Remarks to the Author):

In this manuscript, Potting and colleagues study aspects of phospholipid metabolism in mitochondria by addressing the regulation of Ups1 and Ups2 levels - mitochondrial intermembrane space proteins that regulate the accumulation of cardiolipin and PE respectively. The authors show that both proteins separately associate with Mdm35 in the intermembrane space and this protein is involved in their accumulation/stability. The authors identify that Ups2 is turned over by the protease Yme1 while Ups1 is a substrate for Yme1 as well as another protease Atp23 (they are therefore newly identified physiological substrates for these proteases). The biochemical work is nicely complemented with yeast genetic approaches.
The understanding of lipid accumulation in mitochondria is a topical area with changes implicated in general mitochondrial function and disease. The study is therefore important - especially in the context that the machinery for this regulation is evolutionarily conserved. The manuscript is generally well written with appropriate methodologies and the overall conclusions are well supported by the results (although some interpretation of the role of mdm38 should be clarified - see below). I suggest that the authors address the following points:

1. The pull downs using Ups1 or Ups2 in Fig. 1 indicate that only ~1% of total mdm35 is actually associated with each of protein. Thus there is a large excess of mdm35 in the mitochondrial intermembrane space most likely doing something else. It is therefore not clear how there can be a competition between Ups1/Mdm35 and Ups2/Mdm35 complexes. Is it instead possible that the Mdm35 pulled down is in fact bound to the precursor Ups species and is involved in biogenesis (i.e. folding)? The loss of mdm35 might cause the folding kinetics to be slowed in one of the Ups proteins over the other. While I understand that experiments to address this are difficult given that the Ups1 and Up2 proteins are present in such low amounts of mitochondria and subject to rapid turnover, I think the authors should nevertheless discuss this possibility in the text.

2. Fig 1A: marker should read 6.5 kDa, not 6,5 kDa

3. Fig 3B: Are whole cells used in the western blot? If so, the panel should be replaced with mitochondria to illustrate that overexpressed Ups2 in fact accumulates in the intermembrane space in the absence of mdm35.

4. Panel 4B: The stability of Ups1 in yme1 deletion and mutant cells should also be mentioned in the text.

5. Fig. 5D and 6C: the import into the different mitochondrial preparations shows no strong kinetics - especially in Δmdm35 mitochondria. Also, the "0 min" time point has a lot of precursor bound - how is this possible given that no import has occurred? The authors should ensure that the protein has in fact been imported into mitochondria by using protease treatment and also show that the protein is prone to degradation in the assay (e.g. solubilize mitochondria after import and then perform a protease treatment). The intactness of mitochondria should also be confirmed - perhaps by importing a different intermembrane space protein as a control.

6. Fig. 6C the import of Ups1 in the double mutant is much lower than in WT or Δmdm35 mitochondria. The authors have not explained this in the text - please clarify.

7. Page 17: the authors state that the substrate proteins "are apparently unfolded in the intermembrane space". It is not clear what they mean by this. Please clarify.

Additional Correspondence 26 May 2010

I have now received the last report on your manuscript, which I have provided below. As you can see, this referee in agreement with the other two referees is also supportive of publication here pending some revisions. I would therefore like to ask you to submit a revised manuscript that also addresses the concerns raised below. When you submit your revised manuscript, please also include a detailed point-by-point response.

Looking forward to seeing the revised manuscript.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee # 3

This is an interesting manuscript that investigates the assembly and function of Ups1 and Ups2 in lipid metabolism in mitochondria. The assembly of lipids is a growing area of development in mitochondrial biology and this manuscript adds additional information. Ups1 and Ups2 are two candidates that are required for assembly of cardiolipin and phosphatidyl ethanolamine, but the specific steps in lipid assembly are not clear because Ups1 and Ups2 do not appear to be direct enzymes in the assembly pathway. Instead, these proteins have unknown functions in the lipid assembly/stability in the mitochondrion. This manuscript adds another layer of regulation by showing that the proteases Yme1 and Atp23 are important for stability of Ups1 and Ups2. It is also interesting that Ups1 and Ups2 do not assemble in a complex but have separate functions.
While this manuscript does not provide a specific mechanism about how Ups1 and Ups2 alter lipid levels in mitochondria, it does sort slices of the growing network of proteins that function in lipid dynamics in the mitochondrion. Thus the manuscript is of interest to a general audience. The model is somewhat speculative, because the arrows suggest that Ups1 and Ups2 regulate CL and PE levels but these proteins are in the IMS and the lipids reside in the membranes. Do the authors propose that CL and PE trafficking are affected by Ups1 and Ups2? CL is also thought to be synthesized on the inner mitochondrial membrane. The model suggests that Yme1/Atp23 degrade Ups1/Ups2 if not assembled with Mdm35, but the import results seem contradictory, because Ups1 and Ups2 are not imported. As outlined in point 2 below, whether Yme1/Atp23 play a direct role in import or a later role in degradation should be sorted out in more detail.
The following points should be addressed to strengthen the conclusions of the manuscript.
1. In Fig. 6A and B, the panels should be explained in more detail in the text. The difference in Ups1 protein levels in Fig 6A (lanes 1 and 2) appear similar compared to the differences in Ups1 protein levels in Fig 6B. Also, in Fig 6B, Mdm35 is not detected in WT mitochondria but is overexpressed from a CEN plasmid.
2. The model suggests that Ups1 and Mdm35 assemble in a complex, so a point mutant in Yme1 that prevents turnover, should result in assembly of the Mdm35/Ups1 complex. This should be tested in the import analysis, because the model suggests that Ups1 should be imported in the absence of Yme1 Do Yme1 and Mdm35 function as import components/scaffolds or do they degrade Ups1 and Ups2 after import via a classical pathway? The model suggests Yme1 and Atp23 at a later step in degradation, but the import assays suggest a function in import and assembly. In the import assays, does a general chelator or a point mutant that inactivates yme1 or mdm35 result in accumulation of Ups1/Ups2? If the point

mutants stabilize the imported precursor, it may suggest a scaffold/import function for Yme1/Mdm35. When Ups1 and Ups2 are imported, are they assembled into complexes? This could be investigated by blue-native gels and one might expect them to assemble when imported into a point mutant that inactivates Yme1 proteolytic activity.
3. Significance is used throughout the paper, but statistics have not been used.
4. In the lipid panels, a lipid is marked with an asterisk. What is this lipid? Its abundance seems to vary also.
5. In the analysis of interactions between Ups1/Mdm35 and Ups2/Mdm35, the extent of interaction should be generally quantitated. Does all of the Ups1 assemble with Mdm35 and Ups2 with Mdm35, for example. Also, in the experiments in which Ups2 is overexpressed (Fig. 3), what is the extent of overexpression? Because Ups2 is difficult to detect in WT mitochondria (Fig 6B), it would help interpretation of results if the increase in protein levels could be included in the manuscript.
6. In Fig. 2D, differences in the CL exist, but have not been discussed. The differences in PE and CL levels should be quantitated. The level of CL seems to be lower in delta ups1 mitochondria and the level of the unknown lipid seems to be lower in delta mdm35 delta ups2 mitochondria.
7. On page 8, the authors comment that the membrane potential ceases. Under these conditions, a mitochondrion is normally nonfunctional and a cell is expected to die. Do the authors suggest that the delta mdm35 delta phb1 cells die, because the membrane potential is decreased to 30% of WT.
8. In the osmotic swelling experiments, altering the lipid content can affect osmotic shock. Controls should be included to verify that osmotic shock was not altered in mutant mitochondria.

1st Revision - authors' response 24 June 2010

Reply to the comments of the reviewers

Reviewer 1:

1. We appreciate the positive evaluation of our work by the reviewer and have performed additional experiments to characterize the role of Mdm35 during import of Ups1 and Ups2. We would like to stress, however, that we favor the hypothesis that deficiencies in the import and stability of Ups1-like proteins in Δmdm*35* mitochondria are the consequence of one molecular function of Mdm35, namely binding to Ups1-like proteins.
We discuss this point now more clearly in the manuscript on p.19.
a) We have compared the kinetics of import of Ups1 and Ups2 in mitochondria containing different levels of Mdm35 (WT, Δmdm*35*, Mdm35 overexpressed). These experiments, which are shown in Fig. 5D, clearly demonstrate that Mdm35 is not rate-limiting for import.
b) We would like to thank the reviewer for this notion that helped us to improve our import assay conditions. As suspected by the reviewer, the import of both Ups1 and Ups2 is not halted on ice. We therefore stopped the import reactions by directly treating samples at the indicated time points with protease. These experiments revealed fast import kinetics and are now shown in Fig. 5D (includes former Fig. 6C).
c) We performed initial crosslinking experiments as suggested by the reviewer but could not detect Mdm35-specific crosslinks at early time points of import. These (negative) results might simply reflect the limited sensitivity of our assay to detect an interaction and is therefore not conclusive. As we can now demonstrate that Mdm35 is not rate-limiting for import (new Fig. 5D) and that Ups1 and Ups2 assemble quantitatively with Mdm35 (new Fig. 6E), we feel that demonstration of early binding of Mdm35 during import would not allow additional conclusions on the function of Mdm35.

2. We completely agree with the notion of the reviewer that it remains to be determined whether Mdm35 regulates Ups1 and Ups2 levels in response to specific physiological demands and therefore use a more careful wording throughout the manuscript. The word “regulation” in the title refers to the role of Ups1 and Ups2 in phospholipid metabolism which, in our opinion, is justified based on the published information on these proteins (Osman et al., 2009a; Tamura et al., 2009).

Minor points:
3. We have rephrased the sentence on pp. 4/5.
4. We have rephrased the sentence following the suggestion of the reviewer.
5. We completely agree that another component might be involved and therefore changed the wording in this paragraph on p. 17.
6. We cite now the important work of Zeng et al., 2007 on p. 17.

Reviewer 2:

1. We thank the reviewer for the thoughtful comments. We agree completely that our previous experiments did not exclude the possibility that Mdm35 might only bind to newly imported Ups1 and Ups2 molecules and might be released from Ups1 and Ups2 at later time points. To examine the association of both proteins with Mdm35, we analyzed complex formation by gelfiltration experiments using Δyme*1* mitochondria (which accumulate higher levels of Ups1 and Ups2). These experiments are shown in the new Fig. 6E. We observe co-elution of Mdm35, Ups1 and Ups2, demonstrating that Mdm35 does not only bind to precursor forms of Ups1 and Ups2. Rather, both proteins assemble quantitatively with Mdm35.

2. We have corrected the labeling of Fig. 1A.

3. Mitochondria purified by sucrose-gradient centrifugation were analyzed in these experiments as now stated more clearly in the legend to Fig. 3B. We also added a sentence in the text on p. 9 to emphasize that overexpressed Ups2 accumulates within mitochondria.

4. We mention now the stability of a deletion of *YME1* on Ups1 level already on p. 10, but discuss this result in more detail on p. 14.

5. As pointed out in reply to point 1B of reviewer 1, we have now optimized our import conditions and followed the suggestion of both reviewers to stop the import reaction by protease treatment of the samples. We also included now the intermembrane space protein Tim9 as an additional import control (Fig. 5D).

6. Growth of Δyme*1*Δmdm*35* cells in a W303 background is severely impaired while both mutations are synthetic lethal in a S288C background (Fig. 5A). This is likely explained by concomitantly decreased levels of both PE and cardiolipin. Accordingly, we explain the severely impaired import of Ups1 in mitochondria as an indirect consequence of an impaired mitochondrial integrity in these cells, as now discussed in more detail on p. 13. In agreement with this hypothesis we observed also a decreased membrane potential across the inner membrane and an impaired import of Su9(1-69)-DHFR into the matrix space as now shown in Fig. S4.

7.We can indeed only speculate that binding to Mdm35 supports folding of Ups1-like proteins. We have therefore rephrased corresponding sentences for clarification.

Reviewer 3:

We thank the reviewer for acknowledging the general interest of our work and his/her thoughtful comments. Indeed, it remains to be determined how Ups1 and Ups2 proteins regulate the accumulation of PE and cardiolipin on a molecular level. This question certainly will foster future studies, but was not the focus of the experiments presented here. We would like to point out that an association of both proteins with mitochondrial membranes has been reported previously by Osman et al. (2009a) and Tamura et al. (2009), as now pointed out in the legend to Fig. 7. A direct role for phospholipid metabolism is therefore likely, a role for phospholipid trafficking at least one possibility that we have discussed in our previous publication (Osman et al., 2009a).
We do not agree with the reviewer, however, that a requirement of Mdm35 for import contradicts necessarily a function after import. As pointed out now more clearly on p. 17, we propose that binding of Mdm35 promotes folding of Ups1-like proteins in the intermembrane space. This may trap newly imported Ups1 and Ups2 within mitochondria and protect them against degradation by Yme1/Atp23.

1. We describe now the experiments shown in Figs. 6A and B in more detail in the text on pp. 13/14 to improve clarity.

2. We have examined the steady state level of Ups1 and Ups2 in mitochondria harboring the proteolytic site variant Yme1^{E541Q} (Fig. 4B). We observe the same increase as found in Δyme*1* mitochondria, demonstrating that the accumulation depends on the proteolytic function of Yme1 *in vivo*. We thus have no evidence for other activities of Yme1, like a scaffold function. Import assays are made difficult by the fact that the membrane potential across the inner membrane is reduced in Δyme*1* or *yme1*^{E541Q} mitochondria and that the function of Δyme*1*Δmdm*35* mitochondria (or *yme*^{E541Q}Δmdm*35*) is severely impaired due to the concomitant decrease of PE and cardiolipin in these mitochondria (see Fig. 5).

3. We carefully revised the manuscript and use significance now only in experiments which have been evaluated statistically.

4. We know from ^{32}P-labeling studies that this lipid does not contain phosphate but still do not know the nature of this lipid (as indicated in the legend). Experiments to identify the nature of this lipid are ongoing. However, we would like to point out that the abundance of this lipid appears to vary in a non-reproducible manner in different experiments and we do not observe any reproducible effect on its abundance when interfering with the regulatory network described here.

5. To assess the extent of the interaction, we used Δyme*1* mitochondria which contain increased levels of Ups1 and Ups2 facilitating their detection by immunoblotting. Our experiments (now shown in new Fig. 6E) revealed that both Ups1 and Ups2 (though accumulating at increased levels) co-elute quantitatively with Mdm35 in fractions corresponding to a molecular mass of ~60 kDa. We therefore conclude that Mdm35 remains bound to Ups1 and Ups2 after import.
The quantitative binding of Ups1 and Ups2 to Mdm35 even when overexpressed (in Δyme*1* mitochondria) suggest that Mdm35 is present in molar excess over Ups1 and Ups2 (consistent with our observation that Mdm35 is not rate-limiting for import). We therefore tried to determine the abundance of endogenous Mdm35 relative to Ups1 and Ups2 and compared the accumulation of variants of these proteins harboring a Myc-tag (as we have done for Ups1 and Ups2; see Fig. 6B). However, we observed effects of the Myc-tag on the protein level of Mdm35 (independent whether added to the N- and C-terminus) precluding a direct comparison of the endogenous protein levels. Our antiserum directed against Ups2 does not allow detection of the endogenous protein in wild type mitochondria which precludes to directly compare protein levels with that in mitochondria in Ups2-overexpressing cells.

6. CL levels are decreased in the absence of Ups1 or Mdm35, but not of Ups2. We have quantified CL and PE levels in these mitochondria by mass spectrometry in our original publication on Ups1 and Ups2/Gep1 (Osman et al., 2009a) and refer now to this study for clarity. The level of the yet to be identified lipid species varies slightly in different preparations but these effects are not reproducible.

7. Depletion of Mdm35, as previously observed for Ups2 (Osman et al., 2009a), cause death of prohibitin-deficient cells. We do not want to conclude from our experiments shown in Fig. 2B that a reduction of DY to ~30% causes cell death. Our experiments, however, indicate a progressive decrease in DY (as pointed out on p. 8) which likely results ultimately in a complete dissipation of DY.

8. We routinely check the efficiency of osmotic disruption of the outer membrane using immunoblotting against marker proteins localized in the intermembrane space (e.g. Yme1) and the matrix space (e.g. Mge1) and did not observe any difference between wild type and Δmdm*35* mitochondria. We have included this control in Fig. 2C to comply with the request of the reviewer.

