EMBO reports

Manuscript EMBOR-2015-40137

Structural basis of intramitochondrial phosphatidic acid transport mediated by Ups1-Mdm35 complex

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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.)

Editor: Barbara Pauly

1st Editorial Decision 09 March 2015

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. All reviewers agree on the potential interest of the findings and recommend publication of the findings in EMBO reports once their (rather minor) concerns have been addressed. In particular referee 1 feels that some additional clarifications on the effects of the mutations introduced into Ups1 should be included and reviewer 2 also makes a comment along those lines.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will 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, but given the rather minor nature of the issues that need to be addressed, I do not anticipate any problems meeting this deadline.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

In this important study, Yu et al. have revealed the crystal structure of the Ups1-Mdm35 complex that transports PA between the two mitochondrial membranes. From the structure, it can be deduced that Ups1 creates a binding pocket for PA while Mdm35 binds Ups1 from the other side. These findings support a model in which Ups1 in mitochondria binds to PA, while Mdm35 maintains the folding and stability of the intrinsically unstable Ups1. From the structural information, the authors identified amino acids in Ups1 that are critical for its interactions with PA and Mdm35. These intriguing data provide novel insight into the molecular mechanisms of lipid transport into the mitochondria. Lipid transport into the mitochondria is critical for the biosynthesis of mitochondrial lipids, and therefore this study would be of great interest to many readers. The manuscript is well written and technically sound.

My concerns relates to the mutational analyses that were carried out in cells. The authors introduced many substitutions in Ups1 near the PA-binding pocket and the interface with Mdm35, and assessed the effects in ups1-null cells. Some of the mutants had defects in mitochondrial morphology and cell growth. It is unclear whether these changes affected the lipid binding, PA transfer activity, or the stability of Ups1.

In Fig. 2, mutations in the PA-binding pocket blocked liposomes containing PA and PC. It is critical to know whether the defects, and therefore the lipid-binding pocket, are specific to PA because Ups1-Mdm35 binds to different phospholipids in addition to PA. Does this pocket also bind to other lipids or does Ups1 have multiple lipid binding mechanisms?

In Fig. 4, mutations in the Mdm35 binding region may decrease Ups1 levels. This needs to be addressed by testing the expression level of Ups1 in the mitochondria or cells of the mutants, using Western blotting.

In Fig. 5, the authors mutated positively charged amino acids near the opening of the PA binding pocket. The resultant mutants did not rescue growth or mitochondrial morphology in ups1-null cells. The authors suggest that these residues are important for interactions with phosphate groups of membrane lipids and therefore important for membrane recruitment of Ups1-Mdm35 prior to PA transfer. This model is certainly plausible, but it is equally possible that these residues maintain the overall structure of the protein or that they facilitate interactions with PA. The authors' conclusion is not well supported, unless associations with PA and other lipids are tested.

Referee #2:

Mitochondria-specific phospholipid cardiolipin (CL) plays important roles in the regulation of mitochondrial dynamics, respiratory function, innate immunity, and apoptosis. It is synthesized in the mitochondrial inner membrane (MIM) from phophatidic acid (PA) that is supplied from the ER via mitochondrial outer membrane (MOM). In yeast, mitochondrial inter membrane space (IMS) protein Ups1 chaperoned by Mdm35 is involved in intramitochondrial PA trafficking. Here, the authors solved crystal structures of the Ups1-Mdm35 and Ups1-Mdm35-PA complexes at 2.0 Å resolution. The analyses revealed a long tunnel-like pocket composed of β-sheets and α-helices to accommodate PA, an α2 helix-containing loop to cover the pocket, and a PA-exit structure surrounded by basic amino acid residues. The experiment also revealed the importance of the interface structure between Ups1 and Mdm35, where Mdm35 wraps around Ups1 as a clamp to form a stable Ups1-Mdm35 complex. The functional importance of these structures were further

confirmed for the domain mutants by measuring repression of Δups1 growth phenotype, lipid binding activity, liposome-dependent lipid transfer activity, and mitochondrial morphology chanages as the markers.

The experiments are carefully performed with appropriate controls. These experiments will provided important insights into the mechanism of intra-mitochondrial phospholipids trafficking at the molecular level. I have only minor comments as follows.

COMMENTS
(1) It should be clearly stated whether the Ups1-Mdm35 used here is completely PA-free or contains several amounts of 32:1 PA and 34:1 PA.
(2) Fig. 1D: cross-sected position should be indicated in Fig1B or somewhere.
(3) Fig. 2, Fig. 3: the authors should confirm for several mutants the effect of these mutations on the complex formation between Ups1 and Mdm35.
(4) Mitochondrial morphology changes: the authors should briefly discuss why depletion of Ups1 induced mitochondrial fragmentation paralleled with growth defects of the cells.
(5)The authors should mention how newly synthesized Ups1 and Mdm35 translocate across the MOM and assemble in the IMS.
(6)They should also discuss how this complex (with only one opening for the PA passage) mediates transfer of PA from MOM to MIM.

Referee #3:

In this manuscript Yu and co-authors provide structural and functional information about the Ups1-Mdm35 complex and its role in mitochondrial import of phosphatidic acid (PA), which is used in the synthesis of cardiolipin. The problems being addressed are fundamental to understanding the mechanisms of intermembrane transport of phospholipids, and mitochondrial biogenesis.

The authors isolate a Ups1-Mdm35 complex after expression in bacteria. Previously, genetic and biochemical evidence have implicated both Ups1 and Mdm35 in phosphatidic acid import into mitochondria. The authors report that co-expression of Ups1 and Mdm35 are necessary to obtain a soluble protein complex.

The resolution of the structure of the complex reveals that Mdm35 and Ups1 are stably associated. Preparations of the complex also contained lipid ligands that could be recovered and identified as PA by mass spectrometry. Crystals were soaked with various molecular species of PA, and structures harboring di-palmitoyl (di 16:0)PA were ultimately solved.

Three alpha helices (H1, H2, and H3) from the Mdm35 protein are involved as major contributors to the interaction with Ups1. The H1 and H2 helices harbor CX9C motifs found in other mitochondrial proteins with chaperone like function. The principal interactions of Mdm35 are with the external surface of a large beta-sheet domain within Ups1. The PA is located in a tunnel with its sn-1 and sn-2 fatty acids splayed and interacting with different regions of Ups1. The tunnel is capped by a short alpha helix (alpha-2) of Ups1. Multiple hydrophobic contacts define interactions between the sn-1 fatty acid of PA and Ups1. Mutagenesis of these hydrophobic amino acids demonstrates that many of them phenocopy ups1-delta mutants with respect to cell growth and mitochondrial tubulation. Additional mutagenesis studies of the alpha-2 cap of Ups1 also provide evidence that disruption of this domain also phenocopies ups1-delta mutants. Further mutagenesis of the Ups1 amino acids that interface with H1, H2 and H3 of Mdm35, identify residues that also phenocopy ups1-delta mutations for mitochondrial tubulation defects.

Five basic residues characterize the region of Ups1 proximal to the phosphate moiety of di16:0-PA ligand. Mutagenesis of all of these amino acids to negatively charged amino acids, phenocopies the ups1-delta mutation for mitochondrial tubulation defects, but the effects on cell growth are less penetrant.

Collectively, the findings reported in this paper constitute an important advance in the understanding of PA transport within the mitochondria and have important ramifications for understanding other

phospholipid transport phenomena. Several aspects of this paper require further attention by the authors.

Specific Comments

1. The English usage throughout the manuscript requires significant attention, with the major problems being subject-verb agreement and use of definite and indefinite articles.
2. Page 7 para 2 line 5 change to: ...pocket has a bowl like shape...
3. Page 7 para 2 line 7: ...4A in diameter at the joint of the 2 regions...It is unclear what 2 regions are being referred to.
4. Page 7 para 2 line 8: ...comparing with...much improved.. The meaning is unclear and this section needs rewriting.
5. Fig. 4D: The growth phenotypes of the strains in this Figure are unconvincing. Why are the basic phenotypes (WT vs vector alone) observed in Figs. 2D and 3D not as robust in Figs 4D and 5D. The data for defects in growth are weak in many cases, but the data for defects in mitochondrial tubulation appear much more reliable.

1st Revision - authors' response 21 April 2015

Point by point response to the reviewers' comments

Referee #1:

In this important study, Yu et al. have revealed the crystal structure of the Ups1-Mdm35 complex that transports PA between the two mitochondrial membranes. From the structure, it can be deduced that Ups1 creates a binding pocket for PA while Mdm35 binds Ups1 from the other side. These findings support a model in which Ups1 in mitochondria binds to PA, while Mdm35 maintains the folding and stability of the intrinsically unstable Ups1. From the structural information, the authors identified amino acids in Ups1 that are critical for its interactions with PA and Mdm35. ***These intriguing data provide novel insight into the molecular mechanisms of lipid transport into the mitochondria. Lipid transport into the mitochondria is critical for the biosynthesis of mitochondrial lipids, and therefore this study would be of great interest to many readers. The manuscript is well written and technically sound.***

1. *My concerns relates to the mutational analyses that were carried out in cells. The authors introduced many substitutions in Ups1 near the PA-binding pocket and the interface with Mdm35, and assessed the effects in ups1-null cells. Some of the mutants had defects in mitochondrial morphology and cell growth. It is unclear whether these changes affected the lipid binding, PA transfer activity, or the stability of Ups1.*

……& In Fig. 4, mutations in the Mdm35 binding region may decrease Ups1 levels. This needs to be addressed by testing the expression level of Ups1 in the mitochondria or cells of the mutants, using Western blotting.

RE: In the original manuscript, to test the functions and effects of the PA binding site and the Mdm35 binding region of Ups1, we generated mutations of residues in these regions and tested their effects on mitochondrial morphology and cell growth. In addition, we purified some mutant proteins containing PA-binding pocket mutations and carried out ITC experiments to test their effects on PA binding, and the results shown in **Fig. 2C** (i.e., I78D and V106E) demonstrate that mutations of these residues indeed impair the PA binding, which may in turn affect the PA transfer activity.

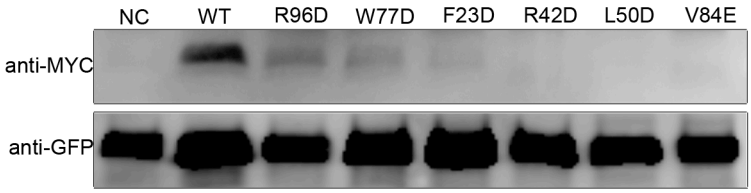
According to the literature, the biological function of Mdm35 is identified to bind with Ups1/2 to ensure their import into the mitochondria and loss of Mdm35 leads to the instability and degradation of Ups1/2 by i-AAA protease Yme1 and metallopeptidase Atp23 (**ref 1**). In the original manuscript, we tested the stability of the Ups1-Mdm35 complex containing mutations of residues in the Mdm35 binding region using anti-His antibody, and the results (shown in **Fig. 4C**) show that these mutations indeed affect the complex stability to various extents *in vitro*. Following this reviewer's suggestion, we further detected the protein level of wild-type and mutant Ups1 in the mitochondria via western

blot using anti-Myc antibody (Ups1 contains a C-terminal Myc tag). The results (attached below) suggest that these mutations also lead to decrease of the Ups1 level *in vivo*. There are two explanations for the decreased protein level *in vivo*: one is that Ups1 is intrinsically unstable and could be degraded by IMS protease once lost the protection of Mdm35 (**refs 1-3**); and the other is that the mutations may decrease the expression level as suggested by the reviewer. The observed decreased level of Ups1 in vivo might be a combined effect of the two factors. Therefore, we have revised the concluding sentence of the section as follows: "These results together **suggest** that the residues at the interaction interface play critical roles in stabilization of the Ups1-Mdm35 complex, and their mutations **may** impair the formation and proper function of the complex." (**Page 12**)

1. Potting C, Wilmes C, Engmann T, Osman C, Langer T (2010) Regulation of mitochondrial phospholipids by Ups1/PRELI-like proteins depends on proteolysis and Mdm35. *EMBO J* **29:** 2888-2898

2. Herrmann JM (2010) Ups delivery to the intermembrane space of mitochondria: a novel affinity-driven protein import pathway. *EMBO J* **29:** 2859-2860

3. Tamura Y, Iijima M, Sesaki H (2010) Mdm35p imports Ups proteins into the mitochondrial intermembrane space by functional complex formation. *EMBO J* **29:** 2875-2887



2. *In Fig. 2, mutations in the PA-binding pocket blocked liposomes containing PA and PC. It is critical to know whether the defects, and therefore the lipid-binding pocket, are specific to PA because Ups1-Mdm35 binds to different phospholipids in addition to PA. Does this pocket also bind to other lipids or does Ups1 have multiple lipid binding mechanisms?*

RE: The structure of the Ups1-Mdm35-PA complex shows that PA binds in the pocket via its two hydrophobic acyl chains, while the head group points to the small opening of the PA binding site. These observations suggest that other lipids may also bind to the pocket in a similar mode (via acyl chains). This notion is supported by a modeling study showing that CL can indeed bind to the lipid binding pocket of Ups1-Mdm35 with two of its four acyl chains. The modeling results have been discussed in the text (**Pages 13-14**).

3. *In Fig. 5, the authors mutated positively charged amino acids near the opening of the PA binding pocket. The resultant mutants did not rescue growth or mitochondrial morphology in ups1-null cells. The authors suggest that these residues are important for interactions with phosphate groups of lipids and therefore important for membrane recruitment of Ups1-Mdm35 prior to PA transfer. This model is certainly plausible, but it is equally possible that these residues maintain the overall structure of the protein or that they facilitate interactions with PA. The authors' conclusion is not well supported, unless associations with PA and other lipids are tested.*

RE: We agree with the reviewer on this point and have revised the sentence in the manuscript as follows: "These data suggest that the hydrophilic patch plays an important role in the function of Ups1-Mdm35. It is possible that the positive charges of this patch might facilitate the Ups1-Mdm35 complex to interact with the phosphate group of PA or other lipids in the membrane." (**Page 13**)

Referee #2:

Mitochondria-specific phospholipid cardiolipin (CL) plays important roles in the regulation of mitochondrial dynamics, respiratory function, innate immunity, and apoptosis. It is synthesized in the mitochondrial inner membrane (MIM) from phophatidic acid (PA) that is supplied from the ER via mitochondrial outer membrane (MOM). In yeast, mitochondrial inter membrane space (IMS) protein Ups1 chaperoned by Mdm35 is involved in intramitochondrial PA trafficking. Here, the authors solved crystal structures of the Ups1-Mdm35 and Ups1-Mdm35-PA complexes at 2.0 Å resolution. The analyses revealed a long tunnel-like pocket composed of β-sheets and α-helices to accommodate PA, an α2 helix-containing loop to cover the pocket, and a PA-exit structure surrounded by basic amino acid residues. The experiment also revealed the importance of the interface structure between Ups1 and Mdm35, where Mdm35 wraps around Ups1 as a clamp to form a stable Ups1-Mdm35 complex. The functional importance of these structures were further confirmed for the domain mutants by measuring repression of Δups1 growth phenotype, lipid binding activity, liposome-dependent lipid transfer activity, and mitochondrial morphology changes as the markers.

The experiments are carefully performed with appropriate controls. These experiments will provide important insights into the mechanism of intra-mitochondrial phospholipids trafficking at the molecular level. I have only minor comments as follows.

COMMENTS

1. *It should be clearly stated whether the Ups1-Mdm35 used here is completely PA-free or contains several amounts of 32:1 PA and 34:1 PA.*

RE: We expressed and purified the Ups1-Mdm35 complex using *E. coli* expression system. The mass spectrometry data of the protein sample indicate that there is trace amount of PA bound with the protein, with 32:1 (16:0-16:1) PA and 34:1 (16:0-18:1) PA as the highest contents (**Figure EV3**), which were apparently co-purified with the protein from the expression system. We added PA of various lengths including di 16:0, di 18:1 and 16:0-18:1 PAs in the co-crystallization experiments but only obtained co-crystals of the Ups1-Mdm35-PA (di 16:0) complex. Following this suggestion, we have revised the text to convey the above information more clearly (**Page 7**).

2. *Fig. 1D: cross-sected position should be indicated in Fig1B or somewhere.*

RE: We have taken this suggestion and indicated the cross-sected position in **Fig. 1C**.

3. *Fig. 2, Fig. 3: the authors should confirm for several mutants the effect of these mutations on the complex formation between Ups1 and Mdm35.*

RE: In the original manuscript, we used the purified Ups1-Mdm35 complex (Wt or mutants shown in **Fig.2** and **3**) for biochemical analyses. We have taken this advice and added the purification results of these mutant complexes in the revision (**Page 9**). The results are incorporated in **Figure EV5,** which show that these mutations do not affect formation of the Ups1-Mdm35 complex.

4. *Mitochondrial morphology changes: the authors should briefly discuss why depletion of Ups1 induced mitochondrial fragmentation paralleled with growth defects of the cells.*

RE: We have taken this advice in the revision. Depletion of Ups1 induced mitochondrial fragmentation paralleled with growth defects of the cells is caused by the GTPase protein Mgm1p that is regulated by Ups1 (**refs 1-5**). We have revised the text and added the related references in "The lipid-binding pocket" section (**Page 9**).

1. Jones BA, Fangman WL (1992) Mitochondrial DNA maintenance in yeast requires a protein containing a region related to the GTP-binding domain of dynamin. *Genes Dev* **6:** 380–389

2. Sesaki H, Dunn CD, Iijima M, Shepard KA, Yaffe MP, Machamer CE, Jensen RE (2006) Ups1p, a conserved intermembrane space protein, regulates mitochondrial shape and alternative topogenesis of Mgm1p. *J Cell Biol* **173:** 651-658.

3. Sesaki H, Southard SM, Yaffe MP, Jensen RE (2003) Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. *Mol Biol Cell* **14:** 2342-2356.

4. Shepard KA, Yaffe MP (1999) The yeast dynamin-like protein, mgm1p, functions on the mitochondrial outer membrane to mediate mitochondrial inheritance. *J Cell Biol* **144:** 711-720.

5. Wong ED, Wagner JA, Scott SV, Okreglak V, Holewinske TJ, Cassidy-Stone A, Nunnari J (2003) The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion. *J Cell Biol* **160:** 303-311.

5. *The authors should mention how newly synthesized Ups1 and Mdm35 translocate across the MOM and assemble in the IMS. ....They should also discuss how this complex (with only one opening for the PA passage) mediates transfer of PA from MOM to MIM.*

RE: We appreciate these advices. In the revision, we have added a short discussion about the translocation of Ups1 and Mdm35 in the last section. In addition, we have also added a new figure (**Figure EV9**) to illustrate how the Ups1-Mdm35 complex mediates translocation of PA from MOM to MIM (**Pages 15-16**).

Referee #3:

In this manuscript Yu and co-authors provide structural and functional information about the Ups1-Mdm35 complex and its role in mitochondrial import of phosphatidic acid (PA), which is used in the synthesis of cardiolipin. The problems being addressed are fundamental to understanding the mechanisms of intermembrane transport of phospholipids, and mitochondrial biogenesis.

The authors isolate a Ups1-Mdm35 complex after expression in bacteria. Previously, genetic and biochemical evidence have implicated both Ups1 and Mdm35 in phosphatidic acid import into mitochondria. The authors report that co-expression of Ups1 and Mdm35 are necessary to obtain a soluble protein complex.

The resolution of the structure of the complex reveals that Mdm35 and Ups1 are stably associated. Preparations of the complex also contained lipid ligands that could be recovered and identified as PA by mass spectrometry. Crystals were soaked with various molecular species of PA, and structures harboring di-palmitoyl (di 16:0)PA were ultimately solved.

Three alpha helices (H1, H2, and H3) from the Mdm35 protein are involved as major contributors to the interaction with Ups1. The H1 and H2 helices harbor CX9C motifs found in other mitochondrial proteins with chaperone like function. The principal interactions of Mdm35 are with the external surface of a large beta-sheet domain within Ups1. The PA is located in a tunnel with its sn-1 and sn-2 fatty acids splayed and interacting with different regions of Ups1. The tunnel is capped by a short alpha helix (alpha-2) of Ups1. Multiple hydrophobic contacts define interactions between the sn-1 fatty acid of PA and Ups1. Mutagenesis of these hydrophobic amino acids demonstrates that many of them phenocopy ups1-delta mutants with respect to cell growth and mitochondrial tubulation. Additional mutagenesis studies of the alpha-2 cap of Ups1 also provide evidence that disruption of this domain also phenocopies ups1-delta mutants. Further mutagenesis of the Ups1 amino acids that interface with H1, H2 and H3 of Mdm35, identify residues that also phenocopy ups1-delta mutations for mitochondrial tubulation defects.

Five basic residues characterize the region of Ups1 proximal to the phosphate moiety of di16:0-PA ligand. Mutagenesis of all of these amino acids to negatively charged amino acids, phenocopies the ups1-delta mutation for mitochondrial tubulation defects, but the effects on cell growth are less penetrant.

Collectively, the findings reported in this paper constitute an important advance in the understanding of PA transport within the mitochondria and have important ramifications for understanding other phospholipid transport phenomena. Several aspects of this paper require further attention by the authors.

Specific comments

1. *The English usage throughout the manuscript requires significant attention, with the major problems being subject-verb agreement and use of definite and indefinite articles.*

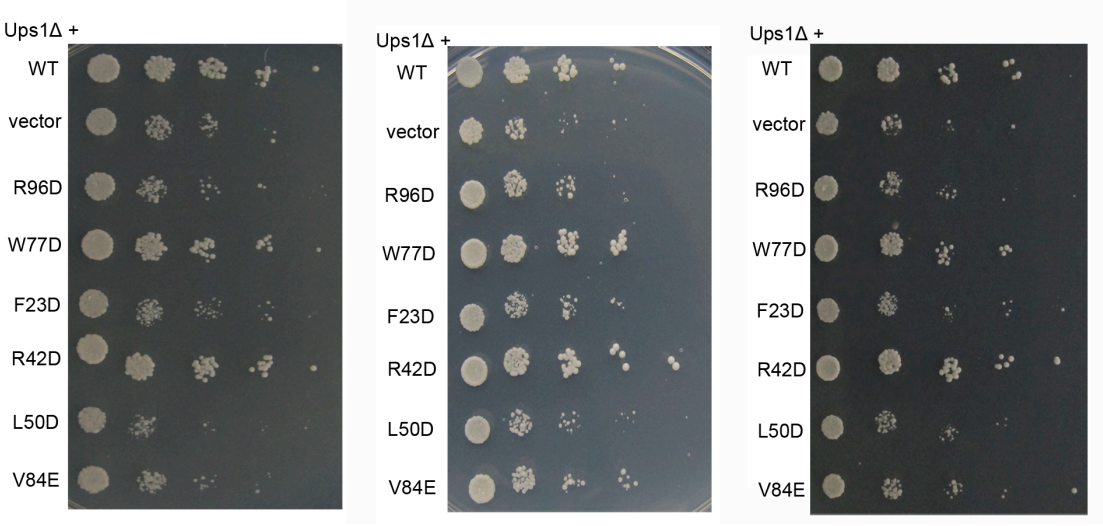
RE: We thank the reviewer for the criticism. In the revision, we have carefully checked the English usage throughout the text and improved the writings.

2. *Page 7 para 2 line 5 change to: ...pocket has a bowl like shape...*
Page 7 para 2 line 7: ...4A in diameter at the joint of the 2 regions...It is unclear what 2 regions are being referred to.
Page 7 para 2 line 8: ...comparing with...much improved.. The meaning is unclear and this section needs rewriting.

RE: We have rewritten this paragraph accordingly (**Page 8**).

3. *Fig. 4D: The growth phenotypes of the strains in this Figure are unconvincing. Why are the basic phenotypes (WT vs vector alone) observed in Figs. 2D and 3D not as robust in Figs 4D and 5D. The data for defects in growth are weak in many cases, but the data for defects in mitochondrial tubulation appear much more reliable.*

RE: The cell growth experiments were repeated several times originally. Following this comment, we have repeated the experiments twice again and all the results are consistent. Here, we have attached the results of three independent experiments as follows, one of which is shown in Figure 4D.



We agree with the reviewer that the data for defects in mitochondrial tubulation are more reliable while the data for defects in growth are weaker in some cases. Ups1 forms a stable complex with Mdm35 to transfer lipids across the mitochondrial membranes, which is important for maintaining the membrane lipid composition. Additionally, Ups1 can regulate the sorting of Mgm1p which is required for fusion, inheritance and morphology of yeast mitochondria **(refs 1-5)**. Therefore, the defects in Ups1-Mdm35 can severely affect the mitochondrial tubulation. However, the growth of the yeast cells is much more complicated, which could be affected by the mitochondria and many other reasons. We presented the results of these two experiments to show the effects of these mutations.

1. Jones BA, Fangman WL (1992) Mitochondrial DNA maintenance in yeast requires a protein containing a region related to the GTP-binding domain of dynamin. *Genes Dev* **6:** 380–389

2. Sesaki H, Dunn CD, Iijima M, Shepard KA, Yaffe MP, Machamer CE, Jensen RE (2006) Ups1p, a conserved intermembrane space protein, regulates mitochondrial shape and alternative topogenesis of Mgm1p. *J Cell Biol* **173:** 651-658.

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5. Wong ED, Wagner JA, Scott SV, Okreglak V, Holewinske TJ, Cassidy-Stone A, Nunnari J (2003) The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion. *J Cell Biol* **160:** 303-311.

2nd Editorial Decision 13 May 2015

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

1. Most importantly, referee 1 recommends that the new results for Ups1 stability that are currently only shown in your point-by-point response should be included in Fig. 4.

2. I have taken the liberty of modifying the abstract slightly and paste it below for your information. Please do let me know whether you are okay with these changes.

3. Could you please also indicate for the experiments quantified in figures 3F, 4F and 5D, how often they were repeated independently?

4. I should also point out that we recently changed our reference style to a number-based one. I am sorry for having to ask you to do this, but could you please change the style before submitting your revised manuscript? I am attaching the relevant endnote file to this email.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

REFEREE REPORTS:

Referee #1:

All of my concerns have been addressed. I believe that this interesting manuscript is now ready for publication.

Minor point. The authors present new results for Ups1 stability in the letter but not in the manuscript. These data should be included in Fig. 4.

Referee #2:

Since this MS has been nicely revised, I have no further comments. This is very important paper to be published soon.

Referee #3:

The revised ms has adequately addressed all the criticisms raised in the first review with the exception of the quality of English usage. Although significant improvements have been made, additional editing is still needed.

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edited abstract:

Ups1 forms a complex with Mdm35 and is critical for the transport of phosphatidic acid (PA) from the mitochondrial outer-membrane to the inner-membrane. We report the crystal structure of the Ups1-Mdm35-PA complex and the functional characterization of Ups1-Mdm35 in PA binding and transfer. Ups1 features a barrel-like structure consisting of an anti-parallel β-sheet and three α-helices. Mdm35 adopts a three-helical clamp-like structure to wrap around Ups1 to form a stable complex. The β-sheet and α-helices of Ups1 form a long tunnel-like pocket to accommodate the substrate PA, and a short helix α2 acts as a lid to cover the pocket. The hydrophobic residues lining the pocket and helix α2 are critical for PA binding and transfer. In addition, a hydrophilic patch on the surface of Ups1 near the PA phosphate-binding site also plays an important role in the function of Ups1-Mdm35. Our study reveals the molecular basis of the function of Ups1-Mdm35, and sheds new light on the mechanism of intramitochondrial phospholipid transport by the MSF1/PRELI family proteins.

2nd Revision - authors' response 14 May 2015

We are pleased to submit our revised manuscript (MS# **EMBOR-2015-40137V2**), entitled "*Structural basis of intramitochondrial phosphatidic acid transport mediated by Ups1-Mdm35 complex*", to ***EMBO Reports*** for consideration of publication.

The manuscript was revised and the changes have been highlighted for easy track, which include:

1) Suggested by reviewer-1, we redraw **Figure 4** which includes the results of Ups1 in vivo stability (**Fig 4D**). A description of the results is added on Page-12. We also updated the method of this experiment.
2) The modified abstract is quite good to convey the meaning.
3) The times of the quantification experiments in Figures **3F**, **4F** and **5D** are indicated in the figure legends.
4) The reference style has been updated.

We hope that these modifications satisfy the concerns of the reviewer and editor, and that the manuscript is now suitable for publication.

3rd Editorial Decision 15 May 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

