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Mitochondria-type GPAT is required for mitochondrial fusion

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Editor: Anne Nielsen

1st Editorial Decision 07 December 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. As you will see from the reports, all referees express great interest in the findings reported in your manuscript; however, they also raise a number of concerns that you will need to address in full before submitting a revised version of the manuscript. I would especially like to emphasize the request by referee #2 for further experimental evidence on the mitochondrial targets for LPA. While we understand that delineating the downstream pathway in full may be outside the scope of the current study, we would need you to provide additional mechanistic insight as outlined by the referee.

Given the referees' positive recommendations, we offer you the opportunity to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

REFEREE REPORTS

Referee #1

This is a comprehensive study of GPAT mutants and their effects on mitochondrial fusion in C. elegans with an additional foray into mammalian cell biology. One of the GPATs goes to mitochondria while others go to the ER. The combination of mutations in ER and mitochondrial GPATs is lethal. A deletion in only one of them, the GPAT encoded by acl-6, which is also the one that goes to mitochondria, causes sterility and it causes mitochondrial fragmentation. These phenotypes are suppressed by injecting LPA, by mutations in LPAAT and by RNAi for Drp1. The main conclusion then is that the LPA generated by acl-6 on mitochondria is necessary for mitochondrial fusion.

In general, the data look very solid. This story is interesting because it provides a new twist to one that was begun a while ago with mammalian cells. It was shown there that mitoPLD promotes fusion by converting cardiolipin to PA, while another downstream-acting enzyme, lipin-1, promotes fission by converting PA to DAG. Curiously, in the current study LPA was identified as the fusion-promoting agent, while further conversion to PA by LPAAT inhibited fusion. The authors provide a balanced discussion of their data and that provided by previous studies, but there is a little bit of an unresolved difference here with the role of mitoPLD in fusion. I actually think that this makes the story more interesting. Perhaps there are multiple ways in which lipids act on fusion.

One small point: the idea that fzo-1 RNAi is weaker than the fzo-1 deletion and can therefore be suppressed by the acl-1 mutation is poorly explained. It is also open for other interpretations. The fzo-1 deletion could, for example, lead to a compensatory response that can no longer be influenced by LPA levels. These compensatory responses are not that uncommon in worm deletion strains.

Another small point: it is unclear how membrane destabilization by LPA would promote fusion. The authors should elaborate on that and maybe also point to the possibility that there are intracellular receptors for LPA signaling.

Referee #2

Mitochondria-type GPAT is required for mitochondrial fusion

In this manuscript, Ohba et al. describe a likely new molecular component of the mitochondrial fusion machinery. They identify the mitochondrial variant of enzyme glycerol-3-phosphate acyltransferase (Mt-GPAT) in C. elegans and demonstrate the importance of the enzyme's catalytic product lysophosphatidic acid (LPA) in the fusion of mitochondrial networks.
The work expands the field of lipid biology with regards to mitochondrial morphology regulation. Previous work has demonstrated important roles for cardiolipin and phosphatidylethanolomine in yeast and the activity of mitochondrial phospholipase D in converting cardiolipin to phosphatidic acid in mammalian cells.
Since this study broadens our understanding of the role of lipid subspecies in mitochondrial morphology regulation, it could be of high general interest. However, some of the data need to be strengthened and experiments adding more mechanistic understanding of how GPAT (or GPAT products) regulate mitochondrial fusion machinery need to be added before the manuscript can be recommended for publication.

Specific concerns:

1. Figure 3 requires quantification of the abnormal mitochondria present in addition to the presentation of selected representative images.
2. MitoTracker Red is used extensively throughout the submission to demonstrate mitochondrial morphology. MitoTracker Red relies on active mitochondria thus it would be more correct to state

that mutants with smaller mitochondria as determined by MitoTracker Red staining have smaller active mitochondria, and the authors should use a mitochondrial label that does not require actively respiring mitochondria to characterize morphology. For example, the authors are encouraged to include some analyses using worms expressing fluorescent protein targeted to the mitochondria (e.g. mito-GFP). Combination of MitoTracker Red with mito-GFP staining would also reveal whether GPAT-mutation linked mitochondrial dysfunctions result or not in the accumulation of low membrane potential organelles (compare to Mfn1 or 2 KO mouse embryonic fibroblasts).
3. Figure 5 demonstrates the ability of injected LPA to rescue the observed mitochondrial defect in C. elegans germ cells. A number of species of other lipids are tested as well, but noticeably absent is cardiolipin, a critical lipid for mitochondrial inner membrane structure and enzyme activity. It seems that this would be a good control to include, as LPA and CL are linked by the cardiolipin remodeling enzyme lysocardiolipin acyltransferase 1 which also converts LPA into phosphatidic acid in mammals (C. elegans ortholog is acl-8).
4. Although the requirement for Mt-GPAT in the mitochondrial morphology regulation is well demonstrated, any mechanistic insights of how Mt-GPAT (and/or its products) affects molecular components of mitochondrial fusion are essential to move this paper into the quality and resolution normally required for the publication in the EMBO Journal. For example, GTPase activity of fzo-1, oligomeric status of known components of the fusion machinery, or alternatively proteolysis of Opa1 could be determined. Any experiments adding specific links between Mt-GPAT and mediators of mitochondrial fusion would significantly strengthen this promising study.

Referee #3

In their manuscript, Ohba et al show that the mitochondrial glycerol-3-phosphate acyltransferase (GPAT) is required for mitochondrial fusion. The authors use C. elegans as a model organism and start by identifying the microsomal and mitochondrial GPAT homologs in C. elegans. Two microsomal (acl-4 and acl-5) and one mitochondrial (acl-6) GPAT were found. Their function was studied by generating mutants. Biochemical analysis of the mutant worms showed that the different GPATs can at least partly compensate for each others deficiency. The acl-6 mutant produced a clear phenotype. In addition to embryonic lethality, adult worms were often sterile, which was caused by impaired oogenesis. The authors continue and find that the acl-6 mutants have abnormal mitochondrial morphology, more specifically fragmented mitochondria, indicating that the fusion or fission of mitochondria is affected. Other experiments show that the effect of acl-6 is cell autonomous and caused by depletion of mitochondrial lysophosphatidic acid. In addition, the effect of the acl-6 mutation can be counteracted by knocking down the pro-fission protein Drp-1. Importantly, GPAT1 knockdown in HeLa cells produced a similar defect in mitochondrial fusion, indicating that GPAT funtion in mammals is also required for mitochondrial fusion.
This is a technically sound and well written paper. The experiments are well done. I have only few points that the authors could address:
- It is unclear why the authors started to study mitochondrial morphology. Previous studies did not suggest effects on the mitochondrial fusion/fission.
- The experiment in HeLa cells. Did the authors try to rescue the fusion defect by adding lysophosphatidic acid to the cells. This would be a nice addition to show that the mechanism of action is fully conserved between C. elegans and mammals.
- drp-1 RNAi rescues the fragmentation of mitochondria. Does it also rescue the oogenesis phenotype?
- Do the results from this study shed new light on the findings in the GPAT1 KO mice? Please discuss.

1st Revision - authors' response 14 February 2013

Referee #1

This is a comprehensive study of GPAT mutants and their effects on mitochondrial fusion in C. elegans with an additional foray into mammalian cell biology. One of the GPATs goes to mitochondria while others go to the ER. The combination of mutations in ER and mitochondrial GPATs is lethal. A deletion in only one of them, the GPAT encoded by acl-6, which is also the one that goes to mitochondria, causes sterility and it causes mitochondrial fragmentation. These

phenotypes are suppressed by injecting LPA, by mutations in LPAAT and by RNAi for Drp1. The main conclusion then is that the LPA generated by acl-6 on mitochondria is necessary for mitochondrial fusion.

In general, the data look very solid. This story is interesting because it provides a new twist to one that was begun a while ago with mammalian cells. It was shown there that mitoPLD promotes fusion by converting cardiolipin to PA, while another downstream-acting enzyme, lipin-1, promotes fission by converting PA to DAG. Curiously, in the current study LPA was identified as the fusion-promoting agent, while further conversion to PA by LPAAT inhibited fusion. The authors provide a balanced discussion of their data and that provided by previous studies, but there is a little bit of an unresolved difference here with the role of mitoPLD in fusion. I actually think that this makes the story more interesting. Perhaps there are multiple ways in which lipids act on fusion.

One small point: the idea that fzo-1 RNAi is weaker than the fzo-1 deletion and can therefore be suppressed by the acl-1 mutation is poorly explained. It is also open for other interpretations. The fzo-1 deletion could, for example, lead to a compensatory response that can no longer be influenced by LPA levels. These compensatory responses are not that uncommon in worm deletion strains.

We agree with the reviewer's comment. We revised the discussion to raise the possibility that the *fzo-1* deletion leads to an alternative compensatory response that is not influenced by LPA levels (p 15, lines 25-27 in the revised manuscript).

Another small point: it is unclear how membrane destabilization by LPA would promote fusion. The authors should elaborate on that and maybe also point to the possibility that there are intracellular receptors for LPA signaling.

As the reviewer suggested, we added the following to the discussion (p 17, lines 2-7 in the revised manuscript).

LPA is an inverted cone-shaped lipid that causes the membrane to have a positive curvature (Haucke and Di Paolo, 2007). In lipid bilayer fusion, hemifusion structures and fusion pores are characterized as sequences of the intermediate structures (Chernomordik and Kozlov, 2008). Lipids inducing positive curvature facilitate the formation of a fusion pore (Haucke and Di Paolo, 2007; Chernomordik and Kozlov, 2008). Therefore, LPA produced by Mt-GPAT may assist Mfn-mediated mitochondrial outer membrane fusion.

We agree that it is possible that LPA has an intracellular receptor/target molecule, although we do not have any strong evidence to support this idea. In the revised manuscript, we showed that the GTPase activity of the recombinant Mfn1 is slightly but significantly increased by LPA, which suggests that Mfn itself is a target molecule of LPA (p 16, lines 17-26 in the revised manuscript).

Referee #2

Mitochondria-type GPAT is required for mitochondrial fusion

In this manuscript, Ohba et al. describe a likely new molecular component of the mitochondrial fusion machinery. They identify the mitochondrial variant of enzyme glycerol-3-phosphate acyltransferase (Mt-GPAT) in C. elegans and demonstrate the importance of the enzyme's catalytic product lysophosphatidic acid (LPA) in the fusion of mitochondrial networks.
The work expands the field of lipid biology with regards to mitochondrial morphology regulation. Previous work has demonstrated important roles for cardiolipin and phosphatidylethanolomine in yeast and the activity of mitochondrial phospholipase D in converting cardiolipin to phosphatidic acid in mammalian cells.
Since this study broadens our understanding of the role of lipid subspecies in mitochondrial morphology regulation, it could be of high general interest. However, some of the data need to be strengthened and experiments adding more mechanistic understanding of how GPAT (or GPAT products) regulate mitochondrial fusion machinery need to be added before the manuscript can be recommended for publication.

Specific concerns:

1. Figure 3 requires quantification of the abnormal mitochondria present in addition to the presentation of selected representative images.

We quantified the abnormal mitochondria in the gonads and wall muscle cells of wild-type, *acl-6* mutants (Figure 3 of the original manuscript) and *acl-6; acl-1* double mutants (Figure 4 of the original manuscript), and added the data to Figure 4 of the revised manuscript.

2. MitoTracker Red is used extensively throughout the submission to demonstrate mitochondrial morphology. MitoTracker Red relies on active mitochondria thus it would be more correct to state that mutants with smaller mitochondria as determined by MitoTracker Red staining have smaller active mitochondria, and the authors should use a mitochondrial label that does not require actively respiring mitochondria to characterize morphology. For example, the authors are encouraged to include some analyses using worms expressing fluorescent protein targeted to the mitochondria (e.g. mito-GFP). Combination of MitoTracker Red with mito-GFP staining would also reveal whether GPAT-mutation linked mitochondrial dysfunctions result or not in the accumulation of low membrane potential organelles (compare to Mfn1 or 2 KO mouse embryonic fibroblasts).

We examined the mitochondrial morphology in Mt-GPAT mutants by expressing mitoGFP in the wall muscle cells. Mitochondria observed with mitoGFP, like those observed with MitoTracker, were significantly fragmented in Mt-GPAT mutants. The mitoGFP fluorescence mostly matched the MitoTracker stain, indicating that fragmented mitochondria still maintain membrane potential. MitoGFP also detected very small portions of mitochondria that were not detected by MitoTracker.

We included these data in Supplementary Figure S3 and the comments in the revised manuscript (p 8, line 24 to p 9, line 4 in the revised manuscript). We did not examine the gonad with mitoGFP because overexpressed mRNA tends to decay rapidly in the gonad.

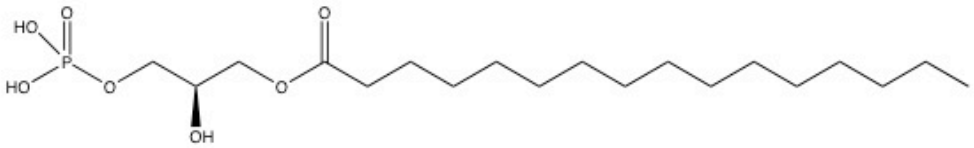
3. Figure 5 demonstrates the ability of injected LPA to rescue the observed mitochondrial defect in C. elegans germ cells. A number of species of other lipids are tested as well, but noticeably absent is cardiolipin, a critical lipid for mitochondrial inner membrane structure and enzyme activity. It seems that this would be a good control to include, as LPA and CL are linked by the cardiolipin remodeling enzyme lysocardiolipin acyltransferase 1 which also converts LPA into phosphatidic acid in mammals (C. elegans ortholog is acl-8).

As the reviewer suggested, we tried to inject cardiolipin (CL). However, we found that unlike LPA, CL could not be suspended homogeneously in the injection buffer, possibly because it is much less soluble in water than LPA (LPA and CL possesses one fatty acyl chain and four acyl chains, respectively).

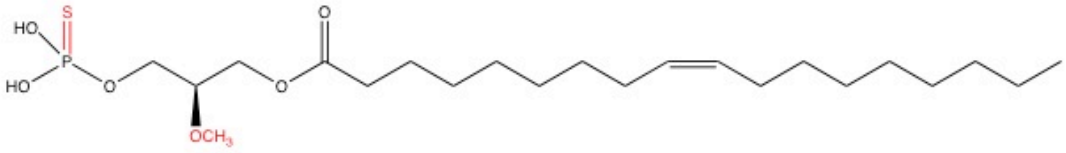
Instead, we examined whether further metabolism of LPA is required for mitochondrial morphology. For this, we injected metabolically stabilized LPA analogues to see whether they can rescue the mitochondrial morphology in Mt-GPAT mutants. OMPT is an acyltransferase- and phosphatase-resistant analogue. XY-26 is a phosphatase-resistant LPA analogue. XY-47 is an alkyl LPA and resistant to phospholipase A (chemical structures of these analogues are shown below). We found that all these LPA analogues also suppressed the mitochondrial defects. Thus, the conversion of mitochondrial LPA to downstream metabolites such as PA or monoaclylglycerol is not critical for the mitochondrial morphology. Although these LPA analogues may have ability to suppress LPAAT activity, which also rescues the mitochondrial defects in Mt-GPAT mutants, we found that these analogues didn't inhibit LPAAT activity. Finally, we also showed that the CL content was not changed by the Mt-GPAT mutation. We included these results in Figure 5 and the comments in the revised manuscript (p 11, lines 9-20 and p18, lines 3-4 in the revised manuscript).

Structures of LPA and LPA analogues

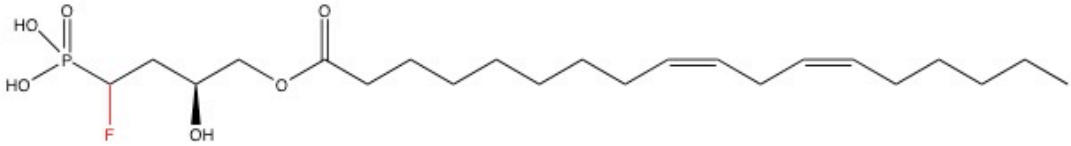
LPA



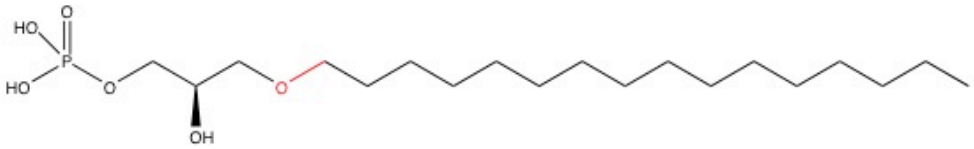
OMPT



XY-26



XY-47



Structures of LPA and LPA analogues are shown.
The structural differences between LPA and LPA analogues are pointed out by red letter and line.

4. Although the requirement for Mt-GPAT in the mitochondrial morphology regulation is well demonstrated, any mechanistic insights of how Mt-GPAT (and/or its products) affects molecular components of mitochondrial fusion are essential to move this paper into the quality and resolution normally required for the publication in the EMBO Journal. For example, GTPase activity of fzo-1, oligomeric status of known components of the fusion machinery, or alternatively proteolysis of Opa1 could be determined. Any experiments adding specific links between Mt-GPAT and mediators of mitochondrial fusion would significantly strengthen this promising study.

As the reviewer suggested, we performed several experiments.

First, we examined Opa1 proteolysis. As previously reported (Griparic *et al,* 2007), Opa1 can be resolved into several bands on Western blots in HeLa cells, and this band pattern was not altered by Mt-GPAT knockdown. We included these data in Supplementary Figure S8 and comments in the revised manuscript (p 16, lines 7-10 in the revised manuscript).

As presented in the original manuscript, *in vitro* oligomerization of Mfn was stimulated by GTP. However, addition of LPA did not significantly stimulate the oligomerization. In fact, we sometimes observed that LPA or LPA analogue XY47 stimulated (but never inhibited) Mfn oligomerization. However, we do not want to include these data in the manuscript because they were not always reproducible.

We finally examined the effect of LPA on the GTPase activity of Mfn by using a recombinant rat Mfn produced by *E. coli*. The GDP production was increased time-dependently, and the specific

activity was nearly comparable to that reported previously (Ishihara *et al,* 2004). Interestingly, addition of LPA to the reaction mixture slightly but significantly stimulated the Mfn1 GTPase activity. Lysophosphatidylcholine (LPC), which is inactive in rescuing the mitochondrial defect of Mt-GPAT mutants, did not stimulate GTPase activity. Although the stimulation was small in the detergent (TritonX-100)-containing *in vitro* assay, LPA produced on the mitochondrial outer membrane may stimulate GTPase activity of Mfn and enhance mitochondrial fusion.

We included these results in Supplementary Figure S11 and also mentioned the possibility that LPA regulates Mfn GTPase activity (p 16, lines 17-26 in the revised manuscript).

Referee #3

In their manuscript, Ohba et al show that the mitochondrial glycerol-3-phosphate acyltransferase (GPAT) is required for mitochondrial fusion. The authors use C. elegans as a model organism and start by identifying the microsomal and mitochondrial GPAT homologs in C. elegans. Two microsomal (acl-4 and acl-5) and one mitochondrial (acl-6) GPAT were found. Their function was studied by generating mutants. Biochemical analysis of the mutant worms showed that the different GPATs can at least partly compensate for each others deficiency. The acl-6 mutant produced a clear phenotype. In addition to embryonic lethality, adult worms were often sterile, which was caused by impaired oogenesis. The authors continue and find that the acl-6 mutants have abnormal mitochondrial morphology, more specifically fragmented mitochondria, indicating that the fusion or fission of mitochondria is affected. Other experiments show that the effect of acl-6 is cell autonomous and caused by depletion of
mitochondrial lysophosphatidic acid. In addition, the effect of the acl-6 mutation can be counteracted by knocking down the pro-fission protein Drp-1. Importantly, GPAT1 knockdown in HeLa cells produced a similar defect in mitochondrial fusion, indicating that GPAT funtion in mammals is also required for mitochondrial fusion.
This is a technically sound and well written paper. The experiments are well done. I have only few points that the authors could address:

- It is unclear why the authors started to study mitochondrial morphology. Previous studies did not suggest effects on the mitochondrial fusion/fission.

The primary goal of our study was to identify the functional difference between ER-GPAT and Mt-GPAT. To this end, we generated both ER-GPAT and Mt-GPAT mutants, and found that only Mt-GPAT mutants are sterile. We then focused on the reproductive systems of Mt-GPAT mutants, and found abnormal oogenesis in the mutants. Because Mt-GPAT is localized in mitochondria, we then examined the mitochondrial morphology in the gonad of Mt-GPAT mutants and found abnormal mitochondrial structures. We then examined the mitochondrial morphology in the muscle cells, and found a significant fragmentation of the mitochondria, which is very similar to the abnormality reported in the *fzo-1* mutants. Then, we examined the relationship between Mt-GPAT/LPA and Mfn (*fzo-1*)/Drp1 (*drp-1*).

- The experiment in HeLa cells. Did the authors try to rescue the fusion defect by adding lysophosphatidic acid to the cells. This would be a nice addition to show that the mechanism of action is fully conserved between C. elegans and mammals.

LPA cannot easily diffuse through the plasma membrane when simply added to the culture medium because it has a negatively charged phosphate group. Therefore, it is necessary to inject LPA directly into the cytosol of mammalian cells. We tried this experiment, but in most cases the cells died after injection. It is not clear whether this is due to the toxicity of injected LPA. *C. elegans* gonad is a syncytium and therefore much larger than HeLa cells, which makes easy to inject lipids into the gonad. So far we could not optimize the experimental conditions such as lipid concentration, injection speed, buffer composition, temperature etc in HeLa cells.

Instead, we tried another set of experiments. In *C. elegans*, the mitochondrial abnormality in Mt-GPAT mutants was rescued by LPAAT depletion. We performed similar experiments using HeLa cells, and found that LPAAT knockdown rescued the mitochondrial fragmentation in Mt-GPAT-depleted cells, suggesting that LPA is required for mitochondrial fusion in mammals as well as in *C. elegans*. We included these data in Supplementary Figure S10 and comments in the revised manuscript (p 13, lines 12-14 in the revised manuscript).

- drp-1 RNAi rescues the fragmentation of mitochondria. Does it also rescue the oogenesis phenotype?

We looked into this and found that *drp-1* RNAi did not rescue significantly the oogenesis defect of Mt-GPAT mutants. The new data are shown in Supplementary Figure S8 of the revised manuscript. A possible explanation is that mitochondrial function is not restored enough to rescue the fertility. Alternatively, LPA produced by Mt-GPAT may have an as yet unknown function in oogenesis. We included these results and the comments in the revised manuscript (p 12, lines 14-18 in the revised manuscript).

- Do the results from this study shed new light on the findings in the GPAT1 KO mice? Please discuss.

Hammond *et al*. reported that mitochondria obtained from the liver of GPAT1 knockout mice are more sensitive to Ca^{2+}-induced mitochondrial permeability transition, a common marker of mitochondrial dysfunction, though mitochondrial morphology appears normal. In mammals, GPAT2, another mitochondrial GPAT, may function to produce LPA, which may account for the apparent normal mitochondrial morphology in GPAT1 knockout mice. We expect that double knockout of GPAT1 and GPAT2 would induce a mitochondrial fragmentation as observed in HeLa cells. We added these comments to the discussion (p 14, lines 18-25 in the revised manuscript).

Acceptance letter 12 March 2013

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below. As you will see they both find that all criticisms initially raised have been addressed in an adequate manner, and I am therefore pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

REFEREE REPORTS

Referee #1

In this revised manuscript, Ohba et al. adequately addressed comments from the first round of review. Changes in the text that were asked for by the reviewers were made, including a more thorough discussion of the differences with earlier mitoPLD and lipin results. The authors also added a few more experiments, as requested, among others testing the effects of LPA on Mfn/Fzo1 activities in vitro, but these effects were weak, so it is unclear this truly reflect mechanism. Despite not getting to the bottom of these mechanistic questions, I do think the paper makes enough of an advance to be of general interest. It should set the stage for future studies in which the mechanisms of Mfn/Fzo1 mediated fusion are finally are finally worked out.

Referee #2

The authors significantly strengthened this work. I believe that in the current form this manuscript is appropriate for publication in the EMBO journal.

