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MiD49 and MiD51, novel components of the mitochondrial fission machinery

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(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 23 December 2010

Thank you for the submission of your research manuscript to our editorial offices. It has been sent to three referees, and so far we have received reports from two of them. As both referees agree on the potential interest of the findings, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. We will forward you the third report as soon as we have received it and we expect you to not only address the concerns of the first two referees, but also of the third reviewer once we have received them.

One concern raised by both referees 1 and 2 is that no interaction between MiD proteins and Drp1 was detected and both referees feel that additional experiments towards this end would strengthen the study. In addition, the two referees also bring up other, minor issues that I would like you to address when preparing a revised version. Please note that the main referee concerns (including the ones from referee 3 once we have received them) must be addressed and that acceptance of the manuscript will depend on a positive outcome of a second round of review. 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.

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this limit. When submitting a revised version, may I therefore kindly ask you to shorten the text so that the length of the revised manuscript does not exceed 28,000 characters (including spaces and references) and, including figures, the paper should ultimately fit onto six or seven, pages of the journal. Shortening is facilitated by combining the results and discussion section, which helps to avoid unnecessary redundancy. Should you find the length constraints to be a problem, you may consider including any peripheral data (but not methods in their entirety) in the form of Supplementary information.

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I look forward to seeing a revised form of your manuscript when it is ready and will get back to you once we have received the final report on your study.

Yours sincerely

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REFEREE REPORTS

Referee #1 (Remarks to the Author):

This important study describes two novel, homologous proteins, MiD49 and MiD51, which are tethered by their N-termini to the outer mitochondrial membrane in mammalian cells. These proteins form rings around mitochondrial tubules, promote mitochondrial association with the actin cytoskeleton and play some sort of redundant role in recruiting the fission GTPase Drp1 to mitochondria. Though the mechanism by which these proteins act is not yet clear, the discovery is important as it provides the first concrete demonstration that mitochondrial-actin interactions are important for mitochondrial dynamics in mammals and adds two new players to a growing number of proteins that modulate mitochondrial membrane behavior in mammalian cells. A few minor comments outlined below should be addressed:

- Since it is not clear that either MiD protein has a direct influence on some Drp1 activity (GTP hydrolysis, self-assembly etc.), it is premature to call these proteins effectors in the title.

-Using mutations that block Drp1 GTP binding or phosphorylation, the authors showed that Drp1 is still recruited to mitochondria, indicating that its recruitment is not dependent upon Drp1 phosphorylation or GTPase activity. Using a similar mutagenesis approach, they also concluded that assembly deficient middle domain mutant proteins were not recruited to the outer mitochondrial membrane. In the latter case, the Drp1 tetramer/dimer might be on mitochondria but not assembled. The unassembled Drp1 would not be visible in the light microscope assay and would be less stably associated with the membrane. As a consequence, it may also be difficult to detect on membrane in the mitochondrial sedimentation assay. Thus, rather than saying the middle domain mutant protein is not recruited to mitochondria, it would be more accurate to say that this protein could not be "detected", on mitochondria. Similar studies with a middle domain mutant form of the yeast Dnm1 protein established that the dimer still forms, is recruited to the membrane, but does not assemble

(Bhar et al. 2006).

-The authors were unable to detect MiD proteins in complex with Drp1 using a co-IP approach. Have they tried to perform the co-IP after first using a reversible crosslinker on the intact cells? Have they also tried a yeast two hybrid assay with the MiD cytoplasmic domain(s) and Drp1 as query proteins?

-What is the nature of the marker protein B17.2L? No information is provided about this protein in methods or legends.

-Page 15, paragraph 2, first sentence: The authors state that over expression of MiDs induce fusion but this was not directly shown in paper. No direct evidence for an effect of over expression on the fusion machinery was presented. In fact, the data shown is more consistent with MiD over expression interfering with Drp1-mediated fission. It would be more accurate to state that MiD over expression tips the balance toward fusion.

-Page 19: The authors indicate that mutations in the Drp1 middle domain are proposed to cause defects in higher order assembly of Drp1 at mitochondria and impair mitochondrial fission by preventing Drp1 recruitment and/or retention (Chang et al, 2010). Again, the authors should note the earlier Bhar et al. 2006 study, which showed that although a middle domain mutation prevented higher order assembly, it did not prevent mitochondrial localization of Dnm1 dimers to mitochondria.

-Citation corrections:

Bottom of page 3 sentence reading "...or Fzo1p in yeast
(Hales and Fuller, 1997; Santel et al, 2003)." The yeast citations are not provided. Should cite:

Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p.
Hermann GJ, Thatcher JW, Mills JP, Hales KG, Fuller MT, Nunnari J, Shaw JM.
J Cell Biol. 1998 Oct 19;143(2):359-73.

Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae.
Rapaport D, Brunner M, Neupert W, Westermann B.
J Biol Chem. 1998 Aug 7;273(32):20150-5.

-Page 4, sentence "Two fission mediators conserved between yeast and
mammals have been identified - the mainly cytosolic GTPase Dynamin Related Protein 1 (Drp1; Dnm1p in yeast) and the mitochondrial outer membrane protein Fis1 (Frank et al, 2001; Otsuga et al, 1998; Smirnova et al, 1998)." The Frank et al. ref did not describe Drp1 but showed the link between fission and apoptosis. It should be removed from this citation. In addition to Otsuga 1998 and Smirnova 1998, the following citations showed that Dnm1 was the mitochondrial fission GTPase in yeast and worms and that Fis1 is required for fission in yeast:

Drp1/Dnm1 refs-Bleazard W, McCaffery JM, King EJ, Bale S, Mozdy A, Tieu Q, Nunnari J, Shaw JM., Nat Cell Biol. 1999 Sep;1(5):298-304.

Sesaki H, Jensen RE., J Cell Biol. 1999 Nov 15;147(4):699-706.

Labrousse AM, Zappaterra MD, Rube DA, van der Bliek AM (1999) C.
elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane.
Mol Cell 4: 815-826 (already in the manuscript reference list).

Fis1 ref-Mozdy, A. D., McCaffery, J. M., and Shaw, J. M. (2000) J Cell Biol 151(2), 367-380

-Bottom of page 4 sentence "In yeast, Dnm1p is recruited to mitochondria
by Fis1p and the effector proteins Mdv1p and Caf4p to drive mitochondrial fission
(Griffin et al, 2005; Zhang and Chan, 2007)." The following references should be added for the initial discovery of Mdv1 and Fis1:

Tieu, Q., and Nunnari, J. (2000) J Cell Biol 151(2), 353-366

Fekkes, P., Shepard, K. A., and Yaffe, M. P. (2000) J Cell Biol 151(2), 333-340

Cerveny, K. L., McCaffery, J. M., and Jensen, R. E. (2001) Mol Biol Cell 12(2), 309-321
Mozdy, A. D., McCaffery, J. M., and Shaw, J. M. (2000) J Cell Biol 151(2), 367-380

Referee #2 (Remarks to the Author):

Osellame and co-workers identified in this study two novel mammalian proteins involved in the control of mitochondrial morphology acting on the level of mitochondrial fission. Two homologous proteins, MiD49 and MiD51, were shown to localize to mitochondrial membranes in biochemical and cell-biological assays. The authors used both overexpression and knockdown experiments in several cellular systems to investigate the role of MiD proteins in mitochondrial dynamics. Whereas enforced overexpression of MiD proteins resulted in actin-attached tubular extensions of perinuclear clustered mitochondria, moderate expression of MiD¥s revealed the presence of ring-shaped structures around mitochondrial tubules accompanied by recruitment of the mitochondrial fission factor Drp1 to mitochondria. Enhanced mitochondrial relocation of Drp1 depended on its assembly properties but occurred independent of GTPase activity leading the authors to conclude that MiD proteins might assist Drp1 assembly at the mitochondrial surface. Consistent with these observations, RNAi-mediated downregulation of MiD proteins was associated with reduced levels of Drp1 at mitochondria resulting in fused mitochondrial networks indicative of compromised fission, a finding that was also corroborated by the reversal of CCCP-mediated fragmentation upon MiD downregulation.
The identification of novel Drp1 effector proteins is intriguing and allows to further refine our understanding of the regulation of mitochondrial fission. The manuscript is well-written, experiments are technically sound and support the authors conclusions that MiD proteins are involved in mitochondrial fission presumably by regulating Drp1 assembly. The apparent co-localization of overexpressed MiD proteins with F-actin is indeed intriguing, although, as pointed out by the authors, its physiological relevance needs to be clarified. The only drawback of the study is the lack of evidence for a physical interaction of MiD proteins with Drp1. This is rather unexpected as their accumulation at foci and constriction sites is highly reminiscent of Drp1. Moreover, the observation that over-expressed MiD proteins recruit only assembly-competent Drp1 molecules regardless of their activity points to a rather specific interaction. I am wondering whether the authors used chemical crosslinking and Drp1 variants to detect a direct interaction of MiD proteins with Drp1? Moreover, did the authors assess oligomerization of Drp1 in the presence or absence of MiD proteins?

Additional points:
1. The authors should assess directly whether the assembly status of Drp1 depends on MiD proteins.
2. The formation of ring-shaped MiD structures around mitochondrial tubules suggest higher-order assemblies. Are the authors able to detect complex formation of MiD proteins in sucrose gradient or BN-PAGE experiments?
3. An ER marker protein should be included in Fig. 1D to exclude contamination of the mitochondrial fraction with microsomes.
4. What is the degree of MiD overexpression when distinguished between low and high expression (Fig. 2)?
5. When appropriate, the papers by Otera et al., JCB 2010 and Koirala et al., JCB 2010, which were published during the editorial process of this manuscript, should be cited.

1st Revision - authors' response 01 March 2011

Thank you and the reviewers for your positive comments regarding our manuscript. As requested, we have made substantial changes to the manuscript and added new experiments which strengthen the manuscript. The specific changes requested by you are as follows:

1. The manuscript has been shortened from 64,000 characters to 28,000 characters as requested for an EMBO Reports article. The reviewers may not be aware that the manuscript was originally formatted as an article for EMBO but was then directly transferred to EMBO reports – hence the reason for its length. This has meant that we have had to prioritise the requests of the reviewers. We trust that the reviewers will understand. Nevertheless our findings are the same.
2. We have added data (Fig. 4E,F) now showing a direct interaction between the MiD proteins and Drp1. The data has come from both yeast 2-hybrid work and also co-immunoprecipitation studies.
3. Due to the addition of new figure panels, we have had to remove Fig 4D which showed still images from the supplemental Movie 5 (the movie remains in the submission).
4. The author order has also changed such that the first named author is now Catherine Palmer.

A point-by-point response to the reviewer's concerns are noted below:

Referee #1:

a. Since it is not clear that either MiD protein has a direct influence on some Drp1 activity (GTP hydrolysis, self-assembly etc.), it is premature to call these proteins effectors in the title.

We agree with the reviewer. The title has been changed to "MiD49 and MiD51, novel components of the mitochondrial fission machinery"

b. …. rather than saying the middle domain mutant protein is not recruited to mitochondria, it would be more accurate to say that this protein could not be "detected", on mitochondria.

We have modified this in the text as requested.

c. The authors were unable to detect MiD proteins in complex with Drp1 using a co-IP approach. Have they tried to perform the co-IP after first using a reversible crosslinker on the intact cells? Have they also tried a yeast two hybrid assay with the MiD cytoplasmic domain(s) and Drp1 as query proteins?

We include new data that strengthens our work. Yeast 2-hybrid work shows that the soluble domain of MiD49 and Drp1 can self associate under stringent selection (Fig 4E). In addition, we now show using cross-linking and co-immunoprecipitation, that Drp1 can co-precipitate with MiD49-GFP (Fig 4F).

d. What is the nature of the marker protein B17.2L? No information is provided about this protein in methods or legends.

We clarify that B17.2L is a mitochondrial matrix protein that serves as a control. This is detailed in the figure legends. Supplementary materials and methods contain a reference for this antibody.

e. Page 15, paragraph 2, first sentence: The authors state that over expression of MiDs induce fusion but this was not directly shown in paper. No direct evidence for an effect of over expression on the fusion machinery was presented. In fact, the data shown is more consistent with MiD over expression interfering with Drp1-mediated fission. It would be more accurate to state that MiD over expression tips the balance toward fusion.

The reviewer is correct. We have changed this statement accordingly.

f. Page 19: The authors indicate that mutations in the Drp1 middle domain are proposed to cause defects in higher order assembly of Drp1 at mitochondria and impair mitochondrial fission by preventing Drp1 recruitment and/or retention (Chang et al, 2010). Again, the authors should note the earlier Bhar et al. 2006 study, which showed that although a middle domain mutation prevented higher order assembly, it did not prevent mitochondrial localization of Dnm1 dimers to mitochondria.

We thank the reviewer for this comment and have clarified this in the text and included the reference as suggested.

g. Citation corrections:

We thank the reviewer for correcting the citations. To meet the strict character limit, we have reduced the text and the number of references. We have checked that the references used (while minimal) are nevertheless, accurate.

Referee #2:

a. The only drawback of the study is the lack of evidence for a physical interaction of MiD proteins with Drp1…..I am wondering whether the authors used chemical crosslinking and Drp1 variants to detect a direct interaction of MiD proteins with Drp1?

We now include new data that strengthens our work. We now show using chemical cross-linking and co-immunoprecipitation that endogenous Drp1 can be co-precipitated with MiD49-GFP (Fig 4F). In addition, we have included yeast 2-hybrid work that shows that the soluble domain of MiD49 and Drp1 can self associate under stringent selection (Fig 4E).

Additional points:

1. The authors should assess directly whether the assembly status of Drp1 depends on MiD proteins.

We have mentioned in the text details of chemical crosslinking experiments in HeLa cells both over expressing and depleted of MiD49/51, in which Drp1 dimer/trimer formation was evident. Because of size constraints we have not included these results in the figures. Loss or formation of higher oligomeric species cannot be examined using this method due to the size limits of in gel analysis. Furthermore, owing to insolubility of purified hMiD49 and hMiD51, in vitro oligomerization assays and gel filtration analysis were not possible.

2. The formation of ring-shaped MiD structures around mitochondrial tubules suggest higher-order assemblies. Are the authors able to detect complex formation of MiD proteins in sucrose gradient or BN-PAGE experiments?

Our BN-PAGE data does show that the MiD proteins are present in higher ordered assemblies, however as the results are preliminary, and due to space constraints, we would prefer to omit this aspect of the work.

3. An ER marker protein should be included in Fig. 1D to exclude contamination of the mitochondrial fraction with microsomes.

Although we agree with the reviewer that this is a useful control, all of our GFP (and other tagged constructs) show that the MiD proteins are clearly mitochondrial. In addition, MiD49 is also found in the Mitocarta database as a result of proteomic analyses of highly purified mitochondria. In this case, we do not feel it is necessary to include an ER control.

4. What is the degree of MiD overexpression when distinguished between low and high expression (Fig. 2)?

We have included in Supplemental Fig S1 a description and comparison of low and high expression as determined by low and high level of fluorescence.

5. When appropriate, the papers by Otera et al., JCB 2010 and Koirala et al., JCB 2010, which were published during the editorial process of this manuscript, should be cited.

We thank the reviewer for this suggestion. Due to changes in manuscript length we have had to tailor both the manuscript and the references cited. We have included Otera et al., 2010.

Referee #3:

One at the end wonders whether these proteins are receptor for Drp1, or if they have a different mechanism of action. The logical prediction would be that if they were to recruit Drp1 on mitochondria, their overexpression should result in fragmentation, but this apparently is not the case. The data is self contradictory in the sense that they see similar effects when they overexpress or downregulate the proteins. …….. The weakest point of the paper is then: how can increased recruitment of Drp1 to mitochondria lead to increased fusion and clustering?

We thank the reviewer for this insight, and have adjusted the discussion of the results accordingly. In summary, we propose that at higher expression levels, MiD-GFP results in Drp1 still binding to mitochondria but Drp1 is unable to organize into active scission complexes, blocking fission and resulting in unbiased fusion. This is supported by the change in distribution of Drp1 from punctate to uniform on the outer mitochondrial membrane following over expression of the MiD proteins. This is most evident following induction of MiD51 in MEFs transfected with GFP-Drp1.

For instance, A395D & G363D mutant have pro-elongation effect, because they are shown to be recruited less to mitochondria (Chuang-Rung Chang et al 2010). K38A mutant has pro-elongation effect although it is recruited to mitochondria because it cannot hydrolyze GTP and it has also shown to be tubulating membranes (Yoon Y, 2001). So, in order to verify why increased recruitment of Drp1 to mitochondria induces elongation:

- the authors should show that either it is unable to hydrolyze GTP (which is not the case since they are wild type version).

We agree with the reviewer that Drp1 GTPase activity does not seem affected or required for recruitment by MiD over expression, as seen also with the recruitment of the GTPase mutant Drp1^{K38A}.

- Or, that Mid proteins somehow disrupts the oligomers.

Due to the insolubility of purified hMiD49 and hMiD51, in vitro oligomerization assays and gel filtration analyses were not possible. While we were unable to assess loss or formation of higher oligomeric species of Drp1 due to the size limits of in gel analysis, again, we note that GFP-Drp1 distribution in transfected MiD51 in MEFs changes form discrete punctae to uniform distribution at the outer mitochondrial membrane providing evidence that the MiD51 over expression disrupts endogenous Drp1 association with mitochondria.

- Or that Mid proteins compete with Drp1 for binding to mitochondria.

We cannot rule out that MiD49/MiD51 compete with other mitochondrial proteins for binding to mitochondria such as Mff or Fis1 which also influence Drp1 action, and have noted this in our discussion. However, we feel that the newly added yeast 2-hybrid and co-immunoprecipitation following chemical crosslinking data strengthens this manuscript, and supports our hypothesis of a direct interaction between MiD49 and Drp1.

Fig 1B

Some other mitochondrial marker like Tom20 staining would be nice to confirm the tubules as mitochondria. Since they do not know the function of these proteins, using cytochrome c, an apoptotic marker is not so wise.

We have now included an additional image of HeLa cells over expressing MiD49-GFP and probed for endogenous Tom20. Our results confirm the mitochondrial localization of MiD proteins.

Figure 3C

Would be nice to see levels of MiD49 expression by western blot. Actually with low level of expression, mitochondria seems to be fragmented and this could really solve all the previously mentioned concerns about this paper.

As the expression levels are variable in cells, western blot analysis is not instructive. We have instead included in supplementary Fig S1 a description of the difference between low and high fluorescence. We have also included additional explanation and discussion of the hypothesised reason for the mitochondrial phenotypes following knockdown and over expression of the MiD proteins.

Figure 4C &E

Western blot analysis of all the mutants for translocation of Drp would be better. In addition, authors should show the localization of the Drp1 mutants before the induction of MIDs.

We thank the reviewer for this suggestion and have included images of the localization of the Drp1 mutants prior to the induction of MiD51. We have not included western analysis due to the size limits of the manuscript and feel that the images provided are convincing. We note that such an approach was recently published by Otera et al., 2010.

2nd Editorial Decision 18 March 2011

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