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Structural insights into oligomerization and mitochondrial remodeling of dynamin 1-like protein

Chris Fröhlich, Stefan Grabiger, David Schwefel, Katja Faelber, Eva Rosenbaum, Jason Mears, Oliver Rocks, Oliver Daumke

Corresponding author: Oliver Daumke, Max-Delbrueck-Centrum for Molecular Medicine

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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 29 August 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. After some delay due to difficulties with the availability of referees during the current summer holiday period, three referees have now evaluated it, and their comments are shown below.

As you will see, while the referees all consider the study as timely and interesting and support its publication here in principle, they raise serious concerns with respect to the conclusiveness of part of the dataset. In particular, referee 2 and 3 point to major shortcomings within the part of the study that deals with the cooperation of the B-insert and the GPRP motif in DNM1L assembly (figure 3), and referee 3 also raises concerns about a possible role of interface 4 in forming an alternative expanded helical assembly (figure 4). Given that all three referees support publication in principle, we should be able to consider a revised version of the manuscript that addresses the referees' concerns in an adequate manner and to their satisfaction. In particular, it will be important to address the referees' concerns with respect to figures 3 and 4. One possibility would be to refocus the paper to the two alternative interaction surfaces, to strengthen figure 4 and to delete figure 3. Another possibility would be to strengthen the experimental support for all aspects of the study, including figure 3, along the lines suggested by the referees. Please do not hesitate to contact us at any time in case you would like to consult on any aspect of the revision further.

I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment 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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance, and we may be able to grant an extension.

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

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is another outstanding contribution by Daumke and colleagues as they reveal structural insights into dynamin-family GTPase members. The data presented are of high quality and while the authors are unable to build an unambiguous model of assembled Dnm1p, the data clearly show interesting differences with dynamin. This flexibility of assembly strategies will be increasingly interesting as dynamin-related fusion proteins, like atlastin are further studied.

One small point, nicely shown biochemically, but not discussed is the very different nucleotide dependence of membrane association of Dnm1p vs dynamin. Both GTPgS and GDP appear to enhance Dnm1p membrane association, whereas GDP and GTP hydrolysis destabilizes dynamin-liposome interactions. This might be important for processivity needed to 'squeeze' large diameter substrates. The mechanism of Dnm1p-mediated fission, given the still very large diameter tubes (60 nm) remains a mystery

Minor comments. The figures depicting analytical gel filtration experiments need to be better explained. What are the light blue and pink lines? Why do the peaks extend above the lines marking 'dimers', tetramer's etc. I understood the experiment, but not how the results are depicted.

The very low assembly-stimulated GTPase activity may suggest that only a few G domains in an assembly are able to form catalytic dimers. Perhaps the rungs are not in perfect register as depicted. The catalytic machinery seems to be otherwise conserved with dynamin.

Referee #2 (Remarks to the Author):

Frˆhlich et al describe a new 3.5≈ resolution crystal structure of a deletion mutant of human dynamin-like protein-1 (DLP1 or DNM1L) lacking the 'B-insert' region. The structural features appear quite similar to those seen in previous structures of dynamin and MxA. However, the four molecules in the asymmetric unit fell into two classes with respect to the relative orientations of the G domain/BSE unit and the stalk - as a result of changes in a proposed hinge region. Moreover, two modes of DNM1L dimerization are seen. The stalk-mediated DNM1L dimerization seen in these crystals closely resembles that seen for dynamin and MxA - and analyses of a mutated form (K642E) supports the role of the interface shown in Figure 1E in stabilizing DNM1L tetramers and larger oligomers, deformation of liposomes, and mitochondrial fission. Later in the paper, the authors discuss the other (interface 4) dimer, which is a potentially important point of this study. This arrangement is different from what has been seen for MxA and dynamin, and may be functionally relevant. Indeed, the cellular studies of the E426A and R430D mutants suggest so. The authors generate structural models for how the two crystallographically observed interfaces can be used to build structures that resemble the Dnm1 helical arrays described by Hinshaw's group -

explaining the characteristic larger diameters of helical arrays formed by Dnm. These models will be useful for future studies of these interesting proteins.
Although these aspects of the paper are valuable, the authors go on a diversion between the two modes of dimerization that seems less so. They set out to investigate allosteric communication between the B-insert and the GPRP motif in a series of studies that are really very problematic. As listed among the specific points below, some of the biochemical analyses for this, shown in Figure 3, are interpreted in a rather cavalier way. The detail of the assays used simply does not allow the data to support assertions about assembly and lipid binding etc that are made in the text.
In summary, the structural studies and analysis presented here seem suitable for the EMBO Journal, as do the functional studies employed to validate the crystallographically observed interface (although see note 1 below). However, the middle of the paper contains an un-needed diversion into a series of incomplete and over interpreted studies that would be best removed (unless a great deal of addition work is included to extend Figure 3).
Specific points are outlined below:

1. It is slightly disappointing that the efforts to validate 'interface 2' in Figure 2 only involve studies with one mutation. Understanding that several mutations resulted in aggregated protein, it would be useful to have at least some data for another mutant or two if possible. Were Y628F, D638S, or M482A mutations not considered. It would also be useful as a control to describe data for a mutation in this region that would not be expected to disrupt interface 2.

2. An M682D mutation is mentioned on page 9. Presumably the authors mean M482D - mutated the methionine labeled in Figure 1E?

3. On page 12, the authors argue that the B variant of DNM1L elutes from gel filtration columns in a dimer-tetramer equilibrium - but the data presented do not support this. Its elution in Figure 3B seems very similar to that of K642-mutated DNM1L. The B mutation appears to affect assembly. To argue otherwise requires more detailed analysis - with equilibrium approaches such as analytical ultracentrifugation. Loss of self-association as a result of B-insert deletion could easily explain the ability of this deletion to 'rescue' aggregation of the 4A mutation - without implying communication between these two features. Thus, the argument for functional cross talk between GPRP motif and B-insert is quite unconvincing. If the 4A mutation causes a structural change that promotes aggregation, the B deletion could mitigate this by reducing assembly in a structurally unrelated way. This aspect of the manuscript should be substantially further developed with more quantitative analyses - or deleted.

4. Given the stated solubility problems with the B mutant, it is really very difficult to interpret the results in Figure 3C,D - and indeed 3B. The aggregates seen in Figure 3C (top left) will not be seen in gel filtration, for example. Moreover, the mutual 'rescue' of 4A and B solubility here is a phenomenon that cannot reasonably be ascribed to specific communication between the GPRP motif and B-insert without more investigation. Following protein solubility alone to investigate "functional cross talk" cannot yield an unequivocal answer.

5. The statement on page 13 suggesting 'a function of the B-insert in liposome interaction' is also problematic. There is clearly a problem with interpreting this very crude assay in terms of liposome binding. The proteins all self-associate and aggregate, and liposomes may affect that process. Appearance in the pellet can reflect aggregation/assembly or liposome binding or (since aggregated proteins will bind with higher avidity) both - and all are interdependent. More thorough analysis is required for statements about the role of the B-insert in lipid binding to be made. Moreover, the biggest fold difference (+/- PS) is seen for 4A+ B, from which one could even argue that a protein lacking B-insert actually binds PS most strongly.

6. The lack of GTPase stimulation of the B variant in Figure 3E is as likely to reflect the assembly defect shown in Figure 3B as anything else. It really does not support a lack of liposome binding. Also, given the data in Figure 3C, why is basal not higher for B? There is something wrong with this protein - or it aggregates in a way that does not promote (measurable) GTPase activation.

7. It is not clear that Figure 4D suggests any reduction in liposome binding by R430D, and interpreting any effect for E426A is complicated by the reduced aggregation in the absence of liposomes.

8. The English needs some attention in places - for both clarity and correctness.

Referee #3 (Remarks to the Author):

Crystal structures of the classical Dynamin GTPase and several Dynamin-Related Proteins (DRPs) including MxA and Atlastin have been published in the last few years. These structures, together with the cryoEM structures of classical dynamin and the yeast mitochondrial dynamin DNM1, have helped refine the domain structure and assembly features of this fundamental family of membrane remodeling proteins. What remains to be learned is how nucleotide-dependent G domain dimerization and/or GTP hydrolysis are coupled to movements within the helix (or disassembly of the helix) that actually catalyze membrane scission. Additional structures of DRP family members are also needed to understand how these proteins mediate membrane scission on such different scales (neck of a clathrin coated pit versus a much larger mitochondrial tubule, peroxisome tubule, or chloroplast).

In this study, Frohlich et al aim to address the second of these open questions, how do the mitochondrial Dynamin proteins accommodate the much larger mitochondrial tubule? They report the first crystal structure of a mammalian mitochondrial dynamin DNM1L (the mammalian ortholog of yeast DNM1). While the structure is similar to those published previously for MxA and dynamin in most respects, the authors report a novel crystalline contact they call interface-4 between the stalk regions. The title of the paper and much of manuscript focuses on testing the hypothesis that the helical lattice formed by DNM1L depends on interface-4 and that this novel interface allows an expanded helical assembly to form around mitochondrial tubules. The experiments and interpretation of this alternative lattice are the weakest part of the manuscript and lead to significant problems that the authors should address.

As experimental support for this alternative assembly model, the authors show that two mutations in interface-4 (R430D and E426A) do not alter the dimer-tetramer distribution of the proteins but the authors claim these mutants can no longer tubulate liposomes in vitro. The TEM images in Figure 4F are too small to see properly and the legend does not report a scale bar, yet it looks like the R430D mutant does tubulate liposomes while the E426A mutant induces extensive liposome aggregation. Both mutants sediment by ultracentrifugation in the presence of GTPγS and appear to bind liposomes. From the data presented it appears that the R430D mutant does not behave differently from the wild type protein. If the E426A mutant really does form dimers and tetramers, assemble in the presence of nucleotide, and bind liposomes, then perhaps they have found a mutant that compromises helical lattice formation. What do the assemblies or partial assemblies of WT versus the E426A mutant look like by TEM? As with other Dynamin GTPases, it should be straightforward to examine the lipid-free assemblies induced by nucleotides to see directly how these assemblies may differ.

Second, the authors suggest that there are interactions between the B-insert/loop and GPRP motif in LS2 of DNM1L. Evidence for this interaction is based solely on the aggregation/solubility of proteins lacking the B-insert and/or containing mutations in GPRP. These data, while again intriguing, are insufficient to conclude that these two regions interact . In addition, on page 10 the authors state, "The deduced position of the B-insert is in direct vicinity of the highly conserved GPRP motif.....". Deduced from what? This "deduction" seems more like a speculation since the B-insert is unstructured and has the potential to adopt a variety of conformations at the base of the stalk. This assumption is worrisome as it forms the basis of the model that a B-insert interaction with the GPRP motif is functionally important. Direct experiments are required that provide a physical demonstration of the proposed interaction (cross-linking, FRET, genetic suppression).

Finally, the docking exercise of the crystal structures with the cryoEM map is an intriguing exercise but raises uncertainties. Even though the Mears et al density map was generated with full-length yeast Dnm1, there does not appear to be any density at all between the protein coat and the membrane surface that could correspond with insert (or any other interaction between the membrane and the protein, is this "action at a distance"?). The authors speculate that insert B is positioned to interact with the membrane in their model lattice, but without any density in this region this speculation is unsupported. Most importantly, at the resolution of this cryoEM map there is no

reason to favor model1 over model2-or some other model-that would be equally compatible with the density.

Additional Comments:
Sedimentation assays are not definitive, especially when assessing liposome binding since these proteins will assemble or aggregate and pellet regardless of the presence of liposomes. Interpreting these results for mutants that may already have compromised stability is problematic. The authors should consider doing liposome flotation or reverse sedimentation assays as a complementary and more rigorous liposome binding assay.

What is the significance of the observation that stimulated GTP hydrolysis was only observed in low (0.5 mM) but not high (>2 mM) MgCl2? Is the same true if the assembly stimulated GTPase assays are performed in the absence of liposomes?

Changes in mitochondrial morphology caused by mutant proteins should be quantified rather than described qualitatively (bottom of page 8: "Furthermore, transfection of this construct led to reduced length of mitochondrial tubes which appeared slightly more fragmented than in control cells.")

In general, the authors have not convinced the reader that the aggregation of the 4A and B mutants is not simply due to reduced solubility. What do the 4A aggregates look like by negative staining TEM? Are they assembling into rings or polymers like DNM1L?

1st Revision - authors' response 29 November 2012

We would like to thank all three referees for their encouraging comments and constructive criticism. Based on their suggestions, we carried out additional experiments, corrected one major result (see comments to referee 2 and 3) and adjusted our conclusions, as outlined below. We are confident that these changes satisfy the concerns of referee 2 and 3 and hope that the manuscript can now be accepted for publication in the EMBO journal.

Referee #1 (Remarks to the Author):

This is another outstanding contribution by Daumke and colleagues as they reveal structural insights into dynamin-family GTPase members. The data presented are of high quality and while the authors are unable to build an unambiguous model of assembled Dnm1p, the data clearly show interesting differences with dynamin. This flexibility of assembly strategies will be increasingly interesting as dynamin-related fusion proteins, like atlastin are further studied.
Thank you very much.

One small point, nicely shown biochemically, but not discussed is the very different nucleotide dependence of membrane association of Dnm1p vs dynamin. Both GTPgS and GDP appear to enhance Dnm1p membrane association, whereas GDP and GTP hydrolysis destabilizes dynamin-liposome interactions. This might be important for processivity needed to 'squeeze' large diameter substrates. The mechanism of Dnm1p-mediated fission, given the still very large diameter tubes (60 nm) remains a mystery.

Sorry, this is a misunderstanding. Only the oligomerization, not the liposome binding assays were carried out in the presence of GTP-γ-S. We could not do the co-sedimentation assays with GTP-γ-S since most DNM1L was already in the pellet without liposomes. We therefore do not know whether GTP-γ-S promotes or prevents liposome binding of DNM1L.
Liposome binding assays were carried out in the presence of GDP where the sedimentation difference in the absence and presence of liposomes was maximal and DNM1L appeared most stable. This is now mentioned in the manuscript.

To allow better comparison of oligomerization and liposome binding assays, we now present both experiments in one graph.

Minor comments. The figures depicting analytical gel filtration experiments need to be better explained. What are the light blue and pink lines? Why do the peaks extend above the lines marking 'dimers', tetramer's etc. I understood the experiment, but not how the results are depicted.

Based on the comments of referee 2 and 3, we moved all analytical gelfiltration experiments to Supp. Fig. 2 and, instead, show sedimentation velocity runs in the main manuscript. In the legend of Supp. Fig. 2, we explain the gelfiltration / RALS experiments in more detail.

The very low assembly-stimulated GTPase activity may suggest that only a few G domains in an assembly are able to form catalytic dimers. Perhaps the rungs are not in perfect register as depicted. The catalytic machinery seems to be otherwise conserved with dynamin.

This is an interesting idea which is followed up in the discussion.

Referee #2 (Remarks to the Author):

Fröhlich et al describe a new 3.5Å resolution crystal structure of a deletion mutant of human dynamin-like protein-1 (DLP1 or DNM1L) lacking the 'B-insert' region. The structural features appear quite similar to those seen in previous structures of dynamin and MxA. However, the four molecules in the asymmetric unit fell into two classes with respect to the relative orientations of the G domain/BSE unit and the stalk - as a result of changes in a proposed hinge region. Moreover, two modes of DNM1L dimerization are seen. The stalk-mediated DNM1L dimerization seen in these crystals closely resembles that seen for dynamin and MxA - and analyses of a mutated form (K642E) supports the role of the interface shown in Figure 1E in stabilizing DNM1L tetramers and larger oligomers, deformation of liposomes, and mitochondrial fission. Later in the paper, the authors discuss the other (interface 4) dimer, which is a potentially important point of this study. This arrangement is different from what has been seen for MxA and dynamin, and may be functionally relevant. Indeed, the cellular studies of the E426A and R430D mutants suggest so. The authors generate structural models for how the two crystallographically observed interfaces can be used to build structures that resemble the Dnm1 helical arrays described by Hinshaw's group - explaining the characteristic larger diameters of helical arrays formed by Dnm. These models will be useful for future studies of these interesting proteins.

Although these aspects of the paper are valuable, the authors go on a diversion between the two modes of dimerization that seems less so. They set out to investigate allosteric communication between the B-insert and the GPRP motif in a series of studies that are really very problematic. As listed among the specific points below, some of the biochemical analyses for this, shown in Figure 3, are interpreted in a rather cavalier way. The detail of the assays used simply does not allow the data to support assertions about assembly and lipid binding etc that are made in the text.

In summary, the structural studies and analysis presented here seem suitable for the EMBO Journal, as do the functional studies employed to validate the crystallographically observed interface (although see note 1 below). However, the middle of the paper contains an un-needed diversion into a series of incomplete and over interpreted studies that would be best removed (unless a great deal of addition work is included to extend Figure 3).

Specific points are outlined below:

1. It is slightly disappointing that the efforts to validate 'interface 2' in Figure 2 only involve studies with one mutation. Understanding that several mutations resulted in aggregated protein, it would be useful to have at least some data for another mutant or two if possible. Were Y628F, D638S, or M482A mutations not considered. It would also be useful as a control to describe data for a mutation in this region that would not be expected to disrupt interface 2.

In response to this concern, we additionally tested the E490A and E490R mutants for assembly deficits. Whereas the E490A mutation did not disrupt the interface, the E490R mutation interfered with dimerization leading also to a monomeric variant. Thus, charge reversals in interface-2 can lead

to the dissociation of DNM1L dimers. We also confirmed these assembly deficits by sedimentation velocity experiments (Fig. 2A). Furthermore, we now included RALS data for three more interface-2 mutants (Supp. Fig. 2).

2. An M682D mutation is mentioned on page 9. Presumably the authors mean M482D - mutated the methionine labeled in Figure 1E?

Thanks a lot, this was corrected (indeed, M482D was meant). We also added the RALS data of all mutants in Supp. Fig. 2 and updated Fig. 1F to show all residues which were mutated in this study.

3. On page 12, the authors argue that the ΔB variant of DNM1L elutes from gel filtration columns in a dimer-tetramer equilibrium - but the data presented do not support this. Its elution in Figure 3B seems very similar to that of K642-mutated DNM1L. The ΔB mutation appears to affect assembly. To argue otherwise requires more detailed analysis - with equilibrium approaches such as analytical ultracentrifugation.

We attempted to analyze DNM1L assembly via sedimentation equilibrium experiments. This did not result in reproducible data, most likely because of the fast exchange dynamics of DNM1L oligomeric species. We could infer these fast exchange dynamics for DNM1L in sedimentation velocity experiments. For most of the mutants, we obtained clear data for their assembly with this method which are now included in the manuscript (Fig. 1B, 2A, Supp. Fig. 8). Data for the ΔB mutant are still somewhat ambiguous. Whereas this mutant can clearly form assemblies which are larger than a dimer, it indeed appears to have some deficits in forming higher order assemblies. We mention this now in the manuscript.

Loss of self-association as a result of B-insert deletion could easily explain the ability of this deletion to 'rescue' aggregation of the 4A mutation - without implying communication between these two features. Thus, the argument for functional cross talk between GPRP motif and B-insert is quite unconvincing. If the 4A mutation causes a structural change that promotes aggregation, the ΔB deletion could mitigate this by reducing assembly in a structurally unrelated way. This aspect of the manuscript should be substantially further developed with more quantitative analyses - or deleted.
During the course of the revisions, we noted that we cannot reproduce our data for the 4A mutant. Thus, a newly prepared 4A mutant eluted from gelfiltration not as an aggregate but as a clear dimer and did not show a higher basal GTPase activity. Re-sequencing of all constructs confirmed their correct identity. Preparation of all other mutants described in the main paper did not yield conflicting results. A second preparation of the 4A mutant also resulted in a dimeric species. We do not know the reasons why the first preparation of the 4A mutant showed this massively aggregated phenotype, but we are sure now that the 4A mutation can indeed efficiently interfere with higher-order assembly, as in dynamin and MxA. This also changes our conclusions on the interplay between interface-3 and the B-insert. We very much apologize for this confusion.
We now include the characterization of the assembly of the 4A and ΔB mutant (sedimentation velocity runs and analytical gelfiltration) in the initial biochemical characterization of the crystallized construct. Furthermore, we show the functional data for the 4A and ΔB mutant in Fig. 3, but refrain to postulate any functional cross-talk between the B-insert and the GPRP motif.

4. Given the stated solubility problems with the ΔB mutant, it is really very difficult to interpret the results in Figure 3C,D - and indeed 3B. The aggregates seen in Figure 3C (top left) will not be seen in gel filtration, for example. Moreover, the mutual 'rescue' of 4A and ΔB solubility here is a phenomenon that cannot reasonably be ascribed to specific communication between the GPRP motif and B-insert without more investigation. Following protein solubility alone to investigate "functional cross talk" cannot yield an unequivocal answer.

See above. We do not claim any longer a cross talk between the B-insert and stalk. We are also more careful in interpreting the oligomerization and liposome binding results of Fig. 3. Still, we believe that the functional characterization of the 4A and ΔB mutants is important for this manuscript. The cell biology data clearly show the functional importance of these two elements for mitochondrial remodelling, and the biochemical characterization of these mutants, despite some limitations, helps to understand their biochemical deficits.

5. The statement on page 13 suggesting 'a function of the B-insert in liposome interaction' is also problematic. There is clearly a problem with interpreting this very crude assay in terms of liposome binding. The proteins all self-associate and aggregate, and liposomes may affect that process. Appearance in the pellet can reflect aggregation/assembly or liposome binding or (since aggregated proteins will bind with higher avidity) both - and all are interdependent. More thorough analysis is required for statements about the role of the B-insert in lipid binding to be made. Moreover, the biggest fold difference (+/- PS) is seen for 4A+ΔB, from which one could even argue that a protein lacking B-insert actually binds PS most strongly.

We attempted to demonstrate liposome binding of DNM1L by floatation assays. However, in the presence of DNM1L, PS liposomes did not float any more, which might be caused by DNM1L-induced liposome remodeling (see Supp. Fig. 4). Although we agree on the limitations of the co-sedimentation assays, we also confirm by EM the formation of highly ordered DNM1L oligomers at the surface of tubulated PS liposomes, which was not observed for any other tested mutant. In the current manuscript, however, we are more careful in interpreting the liposome-binding results.

6. The lack of GTPase stimulation of the ΔB variant in Figure 3E is as likely to reflect the assembly defect shown in Figure 3B as anything else. It really does not support a lack of liposome binding. Also, given the data in Figure 3C, why is basal not higher for ΔB? There is something wrong with this protein - or it aggregates in a way that does not promote (measurable) GTPase activation.

In our GTPase assays, we cannot distinguish between a lack of liposome binding or missing oligomerization. Thus, these data are now more carefully interpreted in the manuscript.

7. It is not clear that Figure 4D suggests any reduction in liposome binding by R430D, and interpreting any effect for E426A is complicated by the reduced aggregation in the absence of liposomes.

We agree, R430D behaves similar to DNM1L in these assays (see current manuscript). The E426A mutant indeed shows reduced oligomerization but otherwise behaves similar in these assays.

8. The English needs some attention in places - for both clarity and correctness.

We revised the manuscript at several positions and hope that this point is now adequately addressed.

Referee #3 (Remarks to the Author):

Crystal structures of the classical Dynamin GTPase and several Dynamin-Related Proteins (DRPs) including MxA and Atlastin have been published in the last few years. These structures, together with the cryoEM structures of classical dynamin and the yeast mitochondrial dynamin DNM1, have helped refine the domain structure and assembly features of this fundamental family of membrane remodeling proteins. What remains to be learned is how nucleotide-dependent G domain dimerization and/or GTP hydrolysis are coupled to movements within the helix (or disassembly of the helix) that actually catalyze membrane scission. Additional structures of DRP family members are also needed to understand how these proteins mediate membrane scission on such different scales (neck of a clathrin coated pit versus a much larger mitochondrial tubule, peroxisome tubule, or chloroplast).

In this study, Frohlich et al aim to address the second of these open questions, how do the mitochondrial Dynamin proteins accommodate the much larger mitochondrial tubule? They report the first crystal structure of a mammalian mitochondrial dynamin DNM1L (the mammalian ortholog of yeast DNM1). While the structure is similar to those published previously for MxA and dynamin in most respects, the authors report a novel crystalline contact they call interface-4 between the stalk regions. The title of the paper and much of manuscript focuses on testing the hypothesis that the helical lattice formed by DNM1L depends on interface-4 and that this novel interface allows an expanded helical assembly to form around mitochondrial tubules. The experiments and interpretation of this alternative lattice are the weakest part of the manuscript and lead to significant problems that the authors should address.

Based on this criticism and the new data for interface-3, we changed the title of our manuscript. The focus is now on the characterization of DNM1L assembly.

As experimental support for this alternative assembly model, the authors show that two mutations in interface-4 (R430D and E426A) do not alter the dimer-tetramer distribution of the proteins but the authors claim these mutants can no longer tubulate liposomes in vitro. The TEM images in Figure 4F are too small to see properly and the legend does not report a scale bar, yet it looks like the R430D mutant does tubulate liposomes while the E426A mutant induces extensive liposome aggregation.

In these EM assays, some PS liposomes tubulated even in the absence of DNM1L. However, these tubules never had a protein coat which is completely different for DNM1L. This is now mentioned in the manuscript. Furthermore, upon addition of DNM1L, the majority of liposomes are tubulated, whereas addition of liposomes to interface-4 mutants never showed tubulation above background (see representative images in Supp. Fig. 9).

Both mutants sediment by ultracentrifugation in the presence of GTPγS and appear to bind liposomes. From the data presented it appears that the R430D mutant does not behave differently from the wild type protein. If the E426A mutant really does form dimers and tetramers, assemble in the presence of nucleotide, and bind liposomes, then perhaps they have found a mutant that compromises helical lattice formation. What do the assemblies or partial assemblies of WT versus the E426A mutant look like by TEM? As with other Dynamin GTPases, it should be straightforward to examine the lipid-free assemblies induced by nucleotides to see directly how these assemblies may differ.

We attempted to carry out the proposed experiments already before submitting the manuscript and repeated them during the revisions. However, we were never able to observe oligomers of DNM1L in the presence of GTP-γ-S by negative stain EM. Possibly, the oligomers are still too dynamic, or our staining protocol using uranyl-acetate interferes with the formation of these protein oligomers in solution.

Second, the authors suggest that there are interactions between the B-insert/loop and GPRP motif in LS2 of DNM1L. Evidence for this interaction is based solely on the aggregation/solubility of proteins lacking the B-insert and/or containing mutations in GPRP. These data, while again intriguing, are insufficient to conclude that these two regions interact . In addition, on page 10 the authors state, "The deduced position of the *B-insert is in direct vicinity of the highly conserved GPRP motif.....". Deduced from what? This "deduction" seems more like a speculation since the B-insert is unstructured and has the potential to adopt a variety of conformations at the base of the stalk. This assumption is worrisome as it forms the basis of the model that a B-insert interaction with the GPRP motif is functionally important. Direct experiments are required that provide a physical demonstration of the proposed interaction (cross-linking, FRET, genetic suppression).*

As mentioned already to referee 2, we could not reproduce the data for the 4A mutant. As demonstrated in two independent new preparations, the 4A mutant was dimeric and not able to further oligomerize. These data are in agreement with data from MxA and dynamin. We thus refrain from inferring any functional cross-talk between the B-insert and 4A mutant in the new manuscript. However, we are still convinced that the biochemical and functional characterization of the 4A and ΔB mutant are important for this manuscript.

Finally, the docking exercise of the crystal structures with the cryoEM map is an intriguing exercise but raises uncertainties. Even though the Mears et al density map was generated with full-length yeast Dnm1, there does not appear to be any density at all between the protein coat and the membrane surface that could correspond with insert (or any other interaction between the membrane and the protein, is this "action at a distance"?). The authors speculate that insert B is positioned to interact with the membrane in their model lattice, but without any density in this region this speculation is unsupported.

The B-insert is predicted to be unstructured, and unstructured regions do not result in defined electron density in EM reconstructions since they average out during the reconstructions. The

absence of electron density around the lipid tubule is therefore indeed an indication that DNM1L interacts via an unstructured region with the lipid tubule, and the only unstructured region of this size at that position is the B-insert. This is now explained in the main text.

Most importantly, at the resolution of this cryoEM map there is no reason to favor model 1 over model 2-or some other model-that would be equally compatible with the density.

With the new data for interface-3, we now provide evidence that DNM1L uses similar assembly interfaces to form filaments as MxA and dynamin. We therefore exclude the first oligomerization model in which interface-3 is not involved in oligomerization.
In the revised manuscript, we now exactly describe our assumptions that lead us to propose the model. These assumptions include the helical spacing of the EM reconstruction and the presence of interfaces1-4. We strongly believe that such working model will guide further experiments to explore structure and oligomerization of DNM1L, as already mentioned by referee 2.

Additional Comments:
Sedimentation assays are not definitive, especially when assessing liposome binding since these proteins will assemble or aggregate and pellet regardless of the presence of liposomes. Interpreting these results for mutants that may already have compromised stability is problematic. The authors should consider doing liposome flotation or reverse sedimentation assays as a complementary and more rigorous liposome binding assay.

We carried out the suggested liposome floatation experiments. However, addition of DNM1L prevented liposomes floating leading to non-interpretable results (Supplementary Figure 5a). Therefore, we have to rely here on the co-sedimentation assays but are more careful with their interpretation.

What is the significance of the observation that stimulated GTP hydrolysis was only observed in low (0.5 mM) but not high (>2 mM) MgCl2?

We now show by EM that PS liposomes fuse and disintegrate in the presence of higher $MgCl_2$ concentrations (Supp. Fig. 5C).

Is the same true if the assembly stimulated GTPase assays are performed in the absence of liposomes?

Since we do not observe assembly-stimulated GTPase of DNM1L in the absence of liposomes, it is difficult to address this point. However, as shown in Supp. Fig. 5B, higher $MgCl_2$ concentrations appear to increase rather than decrease the basal GTPase rate.

Changes in mitochondrial morphology caused by mutant proteins should be quantified rather than described qualitatively (bottom of page 8: "Furthermore, transfection of this construct led to reduced length of mitochondrial tubes which appeared slightly more fragmented than in control cells.")

We quantified the relative mitochondrial length by our FRAP-based assay and confirm the described effect in Figure 2G.

In general, the authors have not convinced the reader that the aggregation of the 4A and ΔB mutants is not simply due to reduced solubility. What do the 4A aggregates look like by negative staining TEM? Are they assembling into rings or polymers like DNM1L?

Based on our new data on the 4A mutant, we refrain from such interpretation now. As mentioned before, we were not able to visualize the DNM1L oligomers by negative-stain EM.

Additional correspondence (editor) 22 February 2013

Please, accept my sincere apologies for the absolutely exceptional delay with the reevaluation of your manuscript. To briefly explain, I sent it back to the two referees that had raised the most relevant concerns during the initial round of review; as you remember, my colleague Isabel had originally emphasized the importance of satisfactorily addressing their concerns through decisive additional experiments and/or re-focussing of the study.

Referee 3 soon came back with a still very critical assessment, making it clear that the paper is in their opinion still not ready for acceptance and publication at this stage. In order to exclude that this referee may simply be exceedingly critical, I chose to wait for referee 2's additional input, which was unfortunately very much delayed. We only heard back from referee 2 last week, his/her comments generally being more supportive. But given the serious and well-taken reservations of referee 3 we still want to hear referee 2's opinion on the importance of those criticisms for publication of this work, and again we've been promised feedback but haven't received it yet.

I realize that this further delay is very unsatisfactory, but I hope you appreciate that the sole purpose and justification for this process rests on us trying to find a way to proceed further in the face of what appears to be very major concerns from a trusted expert in the field. As you will know, our editorial policies usually don't allow a second round of major revision, especially when key concerns have already been raised in the initial round of review.

While we are still waiting for this additional feedback, I decided that it would be fairest to in the meantime forward you the reports as we have received them so far - you will find the copied at the end of this email. With this I would also like to give you an opportunity to consider the key critical points, and maybe to compile a brief response letter describing your view on these criticisms and how they could possibly be answered/reconciled.

Thank you very much for your outstanding patience with this re-review, and once again we are very sorry for this significant delay.

Referee #2

(Remarks to the Author)
Fröhlich et al. have responded thoughtfully to the comments made in the first round of review. The result is a much clearer and more compelling manuscript that represents an important contribution in understanding DNM1L and its relatives. This very nice paper seems acceptable for publication in the EMBO J. in its current form, although there are a few minor revisions that the authors might like to consider:

1. On page 7, second paragraph, the authors state that the "domain boundaries" in their new structure "deviate from the sequence derived domain boundaries (Figure 1A)" and go no further. Having made this comment, the authors should describe how the boundaries deviate in the main text - referring to the alignment presented in Supplemental Figure 4. Revision to clarify this would be helpful.

2. On page 10, first sentence, the following statement is slightly confusing when looking at Figure 2B: "...DNM1L binding to negatively-charged liposomes composed of phosphatidylserine (PS) was most evident in the presence of GDP (Figure 2B)".
The figure legend or text might be clarified to indicate that only the GDP condition contains PS (so no comparison can currently be made in assessing the data). Perhaps it would be useful to add a PS alone lane here (nucleotide free).

3. On page 13 (top), the reference to Figure 3C,D in discussing enhanced sedimentation of the deltaB mutant seems misplaced. Fig. 3C should be referred to 9-10 lines later.

4. It is not clear from Fig. 4B that the R430D mutant shows reduced liposome binding - this should be corrected on p14 (para2).

Referee #3

(Remarks to the Author)
The authors have addressed many (but not all) criticisms raised in the initial review.
The first crystal structure of a mitochondrial dynamin is noteworthy. The data for interface 2 presented in the paper is solid but is not unexpected given studies of previous dynamin family members. However, the data for interface 3 and the newly proposed interface 4 remain problematic. In addition, the study still relies heavily on the sedimentation assay, which cannot distinguish between aggregation and true assembly and does not directly measure liposome binding. Thus, the ability of many of the mutant proteins to partially or fully oligomerize remains in question. In addition, for some mutant proteins (see numbered comments below), there remain contradictions between the conclusions drawn and the data presented.

Two of the assays considered standard in the dynamin field are missing from this analysis: 1) negative staining TEM of mutant proteins under conditions that produce pelleting in the sedimentation assay (with and without liposomes), and 2) flotation analysis of proteins under conditions that promote pelleting in the presence of liposomes. In the response letter, the authors indicate that they were never able to observe oligomers of DNM1L in the presence of GTPgammaS by negative stain TEM. This is worrisome, as the protein should self-assemble in the absence of lipids under the same or similar conditions used to generate the panels shown in figure 2D. The authors need to get this assay working so that they can use it to visualize the assemblies (or aggregates) formed by the various mutant protein that pellet in the sedimentation assay. The authors also indicate they cannot get wildtype DNM1L to behave in a flotation assay)(Supplemental Figure 5) (despite the fact that this protein clearly deforms liposomes in figure 2D in the presence and absence of nucleotide). If the protein can bind and deform liposomes, it should be possible to monitor this species by flotation. It is possible that the authors are using too much protein and lengthy incubation times, such that the ends of tubules formed by DNM1L assembly are rupturing (if so, the liposomes will no longer float). If they have not done so yet, the authors should try to vary protein:liposome ratios and incubation times to find conditions where the DNM1L protein assembled on lipids will float. They will then be able to use this assay to analyze the mutants that appear to bind liposomes in the sedimentation assay.

Without these additional assays, the effects of the mutations introduced to query interfaces 3 and 4 of the crystal structure are unclear. Some examples of problematic results reported in the paper are outlined below.

1. The behavior of the B mutant protein differs depending upon the assay used.
For example, in Figure 1B, the analytical ultracentrifugation sedimentation velocity experiment shows a long 'tail' on the plots for B. This is indicative of protein aggregation. Yet the protein appears well behaved by analytical gel filtration in Supplemental figure 2 (though shifted slightly toward monomer relative to DNM1L).
The B protein behavior is very similar to DNM1L in Figure 3B, though it sedimentsl ess well in the presence of liposomes. However, this sedimentation assay does not distinguish between aggregation and self-assembly. The punctate structures marked by white arrowheads in 3D (and the pelleting in 3B) could be due to assembly rather than aggregation. Indeed, the puncta in 3D are very regular in size and look very much like DNM1L assemblies rather than aggregates. In the response to reviewers, the authors admit that the data for B are still ambiguous. Due to these discrepancies, the authors should work out a protocol that allows negative stain TEM of B assembled in the absence of nucleotide or presence of GTPgammaS (Fig. 3B) with and without liposomes. If this mutant protein is able to form ordered or partially ordered assemblies, it should be apparent by negative stain TEM.

2. On page 13, the authors state that the 4A + B mutant showed reduced sedimentation in the presence of nucleotides and liposomes. This statement is not consistent with all of the data presented. In 3B (top), the 4A + B mutant sediments quite well in the presence of GTPgammaS (compared to nucleotide free or GDP). In addition, compared to 4A + B without nucleotide or with

GDP, the 4A + B protein does sediment with GDP/liposomes. These data suggest that this mutant self assembles (or aggregates) without liposomes in the presence of GTPgammaS and may also assemble on liposomes to some extent. This is not consistent with a role for insert B in liposome binding as suggested by the authors. Once again, it would be very helpful to visualize the putative assembly states detected in this assay by negative stain TEM.

3. In contrast to the statements in the text on page 14, pelleting of the E426 mutant is quite good in the presence of liposomes when compared to its pelleting in the absence of nucleotide and presence of GDP. As stated by the authors, the R430D mutant behavior is more similar to wildtype. Despite the fact that neither mutant protein tubulates lipids well in vitro (Figure 4D), pelleting in the sedimentation assay could be interpreted as some sort of assembly. Once again, the putative assembly states need to be visualized by negative stain TEM.

The in vivo data shown for the interface 4 mutants is consistent with an affect on some DNM1L assembly step. However, the results are not strong enough to support the author's conclusion that the mutations provide evidence for the proposed interface 4 interaction. According to the model, the interface 4 interaction should occur after membrane recruitment and spiral assembly. However, neither of the interface 4 mutants are recruited to mitochondria in vivo, even though interfaces 1, 2 and 3 are intact. Since nonphysiological contacts can occur in crystals and this interaction would be entirely novel compared to other dynamin family members, additional evidence in support of this interaction is warranted prior to publication.

If the data (and model) for interface 4 were removed from the manuscript (and the comments outlined above were addressed), the crystal structure of the first mitochondrial dynamin would still be noteworthy and of great interest to researchers in the dynamin and membrane remodeling fields. However, it would fall to the editors to determine whether this, in and of itself, would be suitable for publication in the EMBO Journal.

Additional correspondence (author) 26 February 2013

Our manuscript describes the crystal structure of THE central eukaryotic molecule mediating division of mitochondria. This interesting crystal structure is followed by an extensive structure-function analysis, to include analytical ultracentrifugation analysis, oligomerization assays, liposome binding assays, GTPase assays, membrane deformation studies and also quite demanding cellular studies and FRAP-based assays for quantifying mitochondrial morphology. Finally, the manuscript introduces a new oligomerization model of DNM1L which is based on a previous EM reconstructions of human DNM1L and our new crystal structural data. The manuscript includes 5 figures with 26 subpanels and 8 Supplementary Figures with 21 subpanels. As trusted experts in the field of structural studies on dynamin superfamily proteins (Daumke /et al./, 2007;Gao /et al./, 2010;Gao /et al./, 2011;Faelber /et al./, 2011;Chappie /et al./, 2011;Mears /et al./, 2011), we are very much convinced, as are referees 1 and 2, that this study represents an important contribution to the dynamin and mitochondrial remodeling field which is very well suited for publication in the EMBO Journal.

As much as we appreciate the critical analysis of referee 3, the more we would like to emphasize that this comprehensive functional study of DNM1L is everything else than being a "standard". We fully agree that liposome sedimentation assays, despite their wide use, have certain limitations. However, also the suggested liposome flotation assays can be problematic. We found a single reference using liposome flotation assays for dynamin-related proteins (in this case for an Arabidopsis thaliana fusion protein); the small liposome tubules created by dynamins might just be too fragile to float efficiently, as intact vesicles do. Furthermore, the floating agent (in this case 30% glucose or accudenz) likely interferes with the labile, electrostatic interactions of DNM1L with negatively-charged PS liposomes. Thus, although we would be willing to provide these additional data (and of course, we tested various protein:liposome ratios, incubation times, floating times, and temperatures as suggested), these experiments are just not feasible. To avoid any over-interpretation of the PS liposome data, we have already much toned down our conclusions on these assays. In fact, the paragraphs on the B insert, interface-3 and -4 just describe the data which we obtained in the PS liposome sedimentation assays. It is not true that our interpretations rely much

on these experiments. The functional importance of the B-insert and interface-3 is mostly derived from the cellular data, and for interface-3 on the new AUC data. Our conclusion that the B insert is involved in liposome binding is mostly based on the EM reconstruction of Dnm1, where this was first proposed, and on the position of the B-insert at the same sequence site as the lipid binding PH domain of dynamin and loop L4 of MxA. The whole discussion whether the B-insert mediates PS liposomes binding or not might be without physiological relevance: In vivo, DNM1L binding to mitochondrial membrane mostly depends on recruitment factors, and we would be much willing to include such statement in the manuscript (including some more adjustments to satisfy other comments of referee 3).

As correctly pointed out by referee 3, the analysis of interface-4 was based on contacts in the crystal lattice which are often without physiological relevance. To find out the importance of such contacts, one has to examine them by mutagenesis, exactly as we did. While we have not found major differences in liposome binding, GTPase activity and oligomerization for these mutants (and we fully agree with that with referee 3), we see differences in the potential of interface-4 mutants to assemble a regular oligomeric coat on liposomes and to be recruited to mitochondria. Similar data were obtained for a mutant localizing close to this interface by another group (Strack, Cribbs 2012). As a referee, I would ask for mutagenesis data in interface-4 to complete a proper structure-function analysis, and we are therefore not willing to exclude these data from the manuscript. From the previously reported EM reconstruction, it is clear that DNM1L assembles in a different mode compared to dynamin, and our data on interface-4 could rationalize such assembly. It is obvious and explicitly stated that the low resolution EM reconstruction does not allow an unambiguous fitting of DNM1L molecules in the electron density, and more work needs to be done before the exact oligomerization mode via interface-4 is clarified (for example, the timely order in which the interfaces are formed is entirely unclear). However, our current model is an excellent basis for further experiments exploring the function of interface-4 for DNM1L-mediated membrane remodeling.

Referee 3 is asking repeatedly for EM-based oligomerization assays in the absence of membranes. We are not convinced that these assays will contribute much to our story here. We know from our AUC and oligomerization assays that both interface-4 mutants can still efficiently assemble and form GTP-dependent oligomers (also the D426A mutant which is just assembling with somewhat reduced efficiency). However, how such artificial oligomers in solution are structurally related to oligomers formed on the membrane surface is completely unclear. Furthermore, it will be impossible to figure out by these EM assays whether DNM1L or the interface-4 mutants form different filaments or not (e.g. single- or double helical filaments). We agree with referee 3 that it is unclear whether the deltaB insert mutant can form regular oligomers or not. However, this point is also not important for our conclusions that deltaB mutant cannot remodel membranes and is functionally inactive (and there is little doubt for that from our data and data from others). Our electron microscope at the MDC might technically not well suited for visualization of small protein oligomers with high contrast (we tried several times now the suggested experiments). To comply with the requests of referee 3, we would be willing to give these experiments another try in the lab of Jason Mears and to come back with the results in a final manuscript within the next three weeks (including a detailed point-by-point response to referee 3; for referee 2, there are no problems). However, we also need to clearly state that we will not be able to answer all questions related to DNM1L-mediated mitochondrial remodeling based on this single manuscript. Please let me know whether this is an acceptable solution.

2nd Decision letter 27 February 2013

Thank you for sending your helpful response letter.

Referee 2 has in the meantime also offered very valuable feedback on the remaining concerns of referee 3, with the bottom line that these criticisms are to some extent justified, but affecting only a relatively minor aspect of this work; and the opinion that extensive further experimental work as requested would not substantially add to the main conclusions of the study within its present scope.

Taking all this into consideration, I feel that we shall be able to publish the manuscript without

further experimental data (although your offer to try repeating EM studies in another lab is appreciated), once the remaining points are textually addressed and answered in a point-by-point letter as suggested.

In the absence of necessary further changes to the data and figures, I would therefore like to invite you to simply send me via email a modified text file as well as a brief response letter. When preparing the modified text file, please also (a) remove any figures from the DOCX file (only legends should remain in there) and (b) make sure to include the PDB accession code at this stage.

Once we will have received and uploaded this modified final version, we should be in the position to proceed with acceptance and production of the paper for The EMBO Journal!

2nd Revision - authors' response 12 March 2013

Referee #2

(Remarks to the Author)
Fröhlich et al. have responded thoughtfully to the comments made in the first round of review. The result is a much clearer and more compelling manuscript that represents an important contribution in understanding DNM1L and its relatives. This very nice paper seems acceptable for publication in the EMBO J. in its current form, although there are a few minor revisions that the authors might like to consider:

Thanks a lot.

1. On page 7, second paragraph, the authors state that the "domain boundaries" in their new structure "deviate from the sequence derived domain boundaries (Figure 1A)" and go no further. Having made this comment, the authors should describe how the boundaries deviate in the main text - referring to the alignment presented in Supplemental Figure 4. Revision to clarify this would be helpful.

We now explain in more detail how the domain boundaries deviate from the predicted sequence-derived domain boundaries.

2. On page 10, first sentence, the following statement is slightly confusing when looking at Figure 2B: "...DNM1L binding to negatively-charged liposomes composed of phosphatidylserine (PS) was most evident in the presence of GDP (Figure 2B)".
The figure legend or text might be clarified to indicate that only the GDP condition contains PS (so no comparison can currently be made in assessing the data). Perhaps it would be useful to add a PS alone lane here (nucleotide free).

We clarify this in the text and added Supplementary Figure 5a to show that DNM1L also binds to liposomes composed of a typical mitochondrial lipid mixture. In this Figure, we also provide liposome-sedimentation assays in the presence of different nucleotides.

3. On page 13 (top), the reference to Figure 3C,D in discussing enhanced sedimentation of the deltaB mutant seems misplaced. Fig. 3C should be referred to 9-10 lines later.

Thanks, this has been corrected.

4. It is not clear from Fig. 4B that the R430D mutant shows reduced liposome binding - this should be corrected on p14 (para2).

We fully agree that the E426A and R430D mutants behave similarly to DNM1L in AUC, sedimentation and AUC assays and stated this now explicitly in the manuscript (see also comments to referee 3).

Referee #3

(Remarks to the Author)
The authors have addressed many (but not all) criticisms raised in the initial review. The first crystal structure of a mitochondrial dynamin is noteworthy. The data for interface 2 presented in the paper is solid but is not unexpected given studies of previous dynamin family members.

We previously demonstrated dimeric assembly of MxA via interface-2 (Gao et al., 2010). We also postulated a similar dimerization mode for dynamin (Faelber et al, 2011), but other dimeric assembly models including domain swapped dimers have been put forward (Chappie et al., 2011). Data of this manuscript now unequivocally demonstrate that dimerization of DNM1L is mediated by the central interface-2.

However, the data for interface 3 and the newly proposed interface 4 remain problematic. In addition, the study still relies heavily on the sedimentation assay, which cannot distinguish between aggregation and true assembly and does not directly measure liposome binding. Thus, the ability of many of the mutant proteins to partially or fully oligomerize remains in question. In addition, for some mutant proteins (see numbered comments below), there remain contradictions between the conclusions drawn and the data presented.

Comments see below.

Two of the assays considered standard in the dynamin field are missing from this analysis: 1) negative staining TEM of mutant proteins under conditions that produce pelleting in the sedimentation assay (with and without liposomes), and 2) flotation analysis of proteins under conditions that promote pelleting in the presence of liposomes. In the response letter, the authors indicate that they were never able to observe oligomers of DNM1L in the presence of GTPgammaS by negative stain TEM. This is worrisome, as the protein should self-assemble in the absence of lipids under the same or similar conditions used to generate the panels shown in figure 2D. The authors need to get this assay working so that they can use it to visualize the assemblies (or aggregates) formed by the various mutant protein that pellet in the sedimentation assay.

We are not convinced that additional EM-based assays will much contribute to our story. We know from our AUC and oligomerization assays that both interface-4 mutants can still efficiently assemble and form GTP-dependent oligomers. However, how such artificial oligomers in solution are structurally related to oligomers formed on the membrane surface is completely unclear. Furthermore, it will be impossible to figure out by these EM assays whether DNM1L or the interface-4 mutants form different filaments or not (e.g. single- or double helical filaments). Finally, we are likely limited by our electron microscope which is not optimized for visualization of small protein oligomers with high contrast - to comply with the requests of referee 3, we attempted several times the suggested experiments but failed to detect these DNM1L assemblies.

The authors also indicate they cannot get wildtype DNM1L to behave in a flotation assay (Supplemental Figure 5), despite the fact that this protein clearly deforms liposomes in figure 2D in the presence and absence of nucleotide. If the protein can bind and deform liposomes, it should be possible to monitor this species by flotation. It is possible that the authors are using too much protein and lengthy incubation times, such that the ends of tubules formed by DNM1L assembly are rupturing (if so, the liposomes will no longer float). If they have
not done so yet, the authors should try to vary protein:liposome ratios and incubation times to find conditions where the DNM1L protein assembled on lipids will float. They will then be able to use this assay to analyze the mutants that appear to bind liposomes in the sedimentation assay.

We agree on the limitations of the liposome co-sedimentation assay. However, also the proposed liposome flotation assay can be problematic. Thus, liposome tubules created by dynamin superfamily proteins might be unable to float efficiently, as intact vesicles do. Furthermore, the floating agents (in this case 30% glucose or accudenz) might well interfere with the labile, likely electrostatic interactions of DNM1L with negatively-charged PS liposomes and might additionally destabilize liposome tubules by the osmotic shock they are creating. As suggested by the referee, we indeed tested various protein:liposome ratios, incubation times, floatation times, and temperatures and were never able to observe a homogenous fraction of floating lipid tubules, despite the presence of a clearly ordered DNM1L coat, as observed by EM. This appears to be a general problem for members of the dynamin superfamily and might be the reason why liposome flotation assays are not commonly used for these proteins.
To avoid any over-interpretation of the PS liposome data, we have further toned down our conclusions on these assays. In fact, the paragraphs on the B insert, interface-3 and -4 merely describe the data which we obtained in the PS liposome sedimentation assays. It is not true that our interpretations rely much on these data. The functional importance of the B-insert and interface-3 is mostly derived from the AUC and/or cellular data.

Without these additional assays, the effects of the mutations introduced to query interfaces 3 and 4 of the crystal structure are unclear. Some examples of problematic results reported in the paper are outlined below.

1. The behavior of the deltaB mutant protein differs depending upon the assay used. For example, in Figure 1B, the analytical ultracentrifugation sedimentation velocity experiment shows a long 'tail' on the plots for deltaB. This is indicative of protein aggregation. Yet the protein appears well behaved by analytical gel filtration in Supplemental figure 2 (though shifted slightly toward monomer relative to DNM1L). The deltaB protein behavior is very similar to DNM1L in Figure 3B, though it sediments less well in the presence of liposomes. However, this sedimentation assay does not distinguish between aggregation and self-assembly. The punctate structures marked by white arrowheads in 3D (and the pelleting in 3B) could be due to assembly rather than aggregation. Indeed, the puncta in 3D are very regular in size and look very much like DNM1L assemblies rather than aggregates.
Indeed, the deltaB protein behaves similar to DNM1L in AUC and assembly assays, but shows reduced sedimentation in the presence of PS liposomes (this is what we wrote). In cells, we cannot distinguish between unspecific aggregates and ordered self-assemblies at an undefined cytoplasmic site and have therefore removed this statement.

In the response to reviewers, the authors admit that the data for deltaB are still ambiguous. Due to these discrepancies, the authors should work out a protocol that allows negative stain TEM of deltaB assembled in the absence of nucleotide or presence of GTPgammaS (Fig. 3B) with and without liposomes. If this mutant protein is able to form ordered or partially ordered assemblies, it should be apparent by negative stain TEM.

As mentioned above, it is unclear whether the deltaB protein can form regular oligomers or not. Our conclusion that the B-insert is important for membrane remodeling and mitochondrial fission is derived from liposome deformation and cellular assays.

2. On page 13, the authors state that the 4A + delta B mutant showed reduced sedimentation in the presence of nucleotides and liposomes. This statement is not consistent with all of the data presented. In 3B (top), the 4A + deltaB mutant sediments quite well in the presence of GTPgammaS (compared to nucleotide free or GDP). In addition, compared to 4A + deltaB without nucleotide or with GDP, the 4A + deltaB protein does sediment with GDP/liposomes. These data suggest that this mutant self assembles (or aggregates) without liposomes in the presence of GTPgammaS and may also assemble on liposomes to some extent.

Both the 4A and the 4A+deltaB proteins can assemble to some extent in the presence of GTP-gamma-S. This is different from the corresponding mutation in dynamin but similar for the

corresponding MxA mutation (Gao et al., 2010). Likely, the 4A mutation in DNM1L is not fully disruptive. This is now mentioned in the manuscript.

This is not consistent with a role for insert B in liposome binding as suggested by the authors.

It is unclear why 4A+deltaB protein still shows some residual co-sedimentation with liposomes (20%). This might be unspecific binding or indeed some sort of aggregation. Our conclusion that the B insert is involved in liposome binding is mostly based on the EM reconstruction of Dnm1 and on the position of the B-insert at the same sequence site as the lipid binding PH domain of dynamin and loop L4 of MxA. The question whether the B-insert mediates direct membrane binding or indirect, via recruitment factors, will be a topic of further investigation.

Once again, it would be very helpful to visualize the putative assembly states detected in this assay by negative stain TEM.

See above.

3. In contrast to the statements in the text on page 14, pelleting of the E426 mutant is quite good in the presence of liposomes when compared to its pelleting in the absence of nucleotide and presence of GDP. As stated by the authors, the R430D mutant behavior is more similar to wildtype. Despite the fact that neither mutant protein tubulates lipids well in vitro (Figure 4D), pelleting in the sedimentation assay could be interpreted as some sort of assembly. Once again, the putative assembly states need to be visualized by negative stain TEM.

We fully agree that the E426A mutant and R430D mutant behave similarly to DNM1L in these assays, it is now even more clearly stated in the manuscript.

The in vivo data shown for the interface 4 mutants is consistent with an affect on some DNM1L assembly step. However, the results are not strong enough to support the author's conclusion that the mutations provide evidence for the proposed interface 4 interaction. According to the model, the interface 4 interaction should occur after membrane recruitment and spiral assembly. However, neither of the interface 4 mutants are recruited to mitochondria in vivo, even though interfaces 1, 2 and 3 are intact.

We show a function of interface-4 for ordered assembly on a membrane and for mitochondrial remodeling. This shows that interface-4 mutants have not only functional deficit in a cellular context (e.g. in the presence of recruitment factors) but have also some assembly deficit on their own. This is mentioned in the Discussion. We do not know the timely order and the steps of assembly events leading to the ordered recruitment of DNM1L to mitochondria. Based on our results, experiments addressing this issue can now be designed.

Since nonphysiological contacts can occur in crystals and this interaction would be entirely novel compared to other dynamin family members, additional evidence in support of this interaction is warranted prior to publication. If the data (and model) for interface 4 were removed from the manuscript (and the comments outlined above were addressed), the crystal structure of the first mitochondrial dynamin would still be noteworthy and of great interest to researchers in the dynamin and membrane remodeling fields.

As correctly pointed out, the analysis of interface-4 was based on contacts in the crystal lattice which are often without physiological relevance. To find out the importance of such contacts, one has to examine them by mutagenesis, as we did. While we have not found major differences in liposome binding, GTPase activity and GTP-gamma-S induced oligomerization for these mutants, we see differences of interface-4 mutants to assemble a regular oligomeric coat on liposomes and to be recruited to mitochondria. Similar cellular data were obtained by another group for the R376E mutant localizing close to this interface (Strack, Cribbs 2012). We strongly believe that these data

will guide future experiments addressing the detailed function of interface-4. It is therefore important to include data for interface-4 in this manuscript.

However, it would fall to the editors to determine whether this, in and of itself, would be suitable for publication in the EMBO Journal.

Our manuscript describes the crystal structure of THE central eukaryotic molecule mediating division of mitochondria. This crystal structure is followed by an extensive structure-function analysis, to include analytical ultracentrifugation analysis, oligomerization assays, liposome binding assays, GTPase assays, membrane deformation studies and also quite demanding cellular studies and FRAP-based assays for quantifying mitochondrial morphology. Finally, the manuscript introduces a new oligomerization model of DNM1L which is based on a previous EM reconstruction of human DNM1L and our new crystal structural data. The manuscript includes 5 figures with 26 subpanels and 9 Supplementary Figures with 22 subpanels. We are much convinced that this paper represents an important contribution for the dynamin and mitochondrial remodelling field, with numerous novel mechanistic insights, which is well suited for publication in the EMBO Journal.

Acceptance letter 13 March 2013

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal. I'd like to congratulate you on a successful publication, and once more apologies for the delays associated with the re-review process. Nevertheless I hope you will consider us again in the future for your most exciting work!

