JCB

Structural insights into G domain dimerization and pathogenic mutations of OPA1

Caiting Yu, Jinghua Zhao, Liming Yan, Yuanbo Qi, Xiangyang Guo, Zhiyong Lou, Junjie Hu, and Zihe Rao

Corresponding Author(s): Junjie Hu, Chinese Academy of Sciences

Review Timeline:

Submission Date:	2019-07-15
Editorial Decision:	2019-09-02
Revision Received:	2020-02-18
Editorial Decision:	2020-03-29
Revision Received:	2020-04-08

Monitoring Editor: Richard Youle

Scientific Editor: Marie Anne O'Donnell

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.)

DOI: https://doi.org/10.1083/jcb.201907098

August 28, 2019

Re: JCB manuscript #201907098

Dr. Junjie Hu
Chinese Academy of Sciences
15 Datun Rd. Chaoyang District
Beijing, Beijing 100101
China

Dear Dr. Hu,

Thank you for submitting your manuscript entitled "Structural insights into G domain dimerization and pathogenic mutations of OPA1". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that both reviewers clearly note the question of how the activity of dynamin-related enzymes and their role in fission is affected by their conformation is a very interesting question and that there are significant differences reported here between OPA1 and the recently described Mgm1p structure. However, the comments overall suggest that this study is too preliminary to support its main claims and far more work than would be reasonable for a standard revision period is necessary to substantially address their comments and most, if not all, of the points with new experiments.

Although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

Given interest in the topic, I would be open to resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Richard Youle, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This is a well-written manuscript for Dr. Ju's lab. The paper highlights new structural information about the OPA1 protein, which is the most frequently mutated protein associated with inherited blindness. Building on other work with GTPase domain constructs of dynamin proteins for structural studies, the authors do something similar to isolate a soluble portion of the protein. In this way, they are able to highlight novel attributes within OPA1 compared to other dynamins.

This work follows on the heels of the recent Mgm1p structure. The authors do a nice job of highlighting the similarities and differences that they observed in their structure. Given the region of the protein that they have in their construct, the authors largely focus on GTPase functional assays. This is appropriate, but the biochemical data is not convincing. More rigor should be used to analyze the GTPase activity and define the specific activity of the OPA1 GTPase domain in a way that can be compared with other dynamins, including Mgm1p. Moreover, the authors highlighted work on the s-OPA1 full-length protein, but it is unclear why they could not observe lipid tubulation. Does this reflect issues with their s-OPA1 construct? Any data on lipid templates would greatly strengthen the papers findings. I particularly like the mapping of the patient mutations onto the structure, but it is hard to correlate the impact of their GG structure in the larger context of membrane interactions. I do not know what the dimer interface in their GG crystal structure represents since other interactions are clearly governing dimer formation in the absence of nucleotide. And if the GG dimer only forms during lipid interactions, which would be similar to dynamin, does the assembly correlate with the larger jump in activity when lipid interactions occur. It would be ideal to see the impact of the mutations on lipid-induced assembly.

Overall, I feel that the scope of the manuscript is appropriate for JCB, but I do think that additional experiments and a focused effort to clarify the enzymology would help the manuscript. I offer the following comments to highlight short-comings that I would like to see addressed:

1. The introduction is very brief. Usually, I prefer brevity, but this seems a little incomplete. It is worth expounding more on dynamin-family protein structures, especially for fusion dynamins, to compare with the findings presented here for OPA1.

2. Lines 86-90 - The authors mention that the inclusion of an additional N-terminal helix (residues 217-262) did not impact protein solubility or alter OPA1 GTPase activity, and that "These results suggest that the OPA1-HB1/BSE domain can be a four-helix bundle (4HB)". But this is unfounded without showing this structurally or using a technique that would highlight the formation of an additional helix. They may be correct, but additional data is needed to validate this claim.

3. Lines 93-96 - The authors state, "Similar organization has been reported for dynamin-1 (with a Na+ ion instead) and MFN1 (Fig. 2C,D), but not many other DLPs." To which other DLPs are they comparing and what is observed with Drp1 or Mx or ATL1? Is there any significance to these differences? It is not clear that an important distinction exists.

4. Lines 113-115 - I think it is interesting that GMPPNP is less stable in the nucleotide binding pocket. This is consistent with what other investigators have seen for several DLPs. But GMPPCP generally binds well to these proteins, so I would hope that the authors would test association with this nucleotide analog for completeness.

5. The GTPase assays were a little confusing to me because the authors present µM Pi per min. Initially, the rates seemed higher than I would have expected based on previous GTPase measurements for OPA1 and Mgm1p. But the authors are using 10 µM protein for these assays. I would prefer that they present the data as a kobs measurement by dividing by the concentration of their protein. This would be more consistent with previous results, and for the Mgm1 MGD, the value would actually be higher (~1 min-1) than reported for the full-length Mgm1p (0.5 min-1), but this may be explained by the higher concentration of protein used in the current assays. Ideally, it would worth knowing whether a specific activity could be presented for these constructs (i.e. same rate independent of concentration) since they are not assembling through stalk interactions that generally stimulates the GTPase activity in other DLPs. Given the focus of this paper on the GTP interaction with OPA1, additional studies should be performed to better define how the activity of the GTPase domain is impacted by these interactions.

6. Lines 149-151 - The authors state, "These results suggest that GTP hydrolysis by OPA1 is precisely regulated via additional mechanisms that are not seen with other DLPs." I am not sure that I understand how this can be claimed. I know that Chappie et al examined the impact of cation interactions on GTPase activity, but I don't know other examples with DLPs. And can we say that these mild differences in GTPase activity in response to cation interactions is significant when compared to the stimulated activity that would be observed on a membrane? Perhaps, if the authors provide more of an introduction on the importance of cation interactions in GTPase function, this could provide clarity. But as this is presented, this appears to be a less significant finding.

7. The dimerization results in Figure 3 are somewhat alarming. You are getting dimerization with the full-length protein that does not further oligomerize in the presence of GTP. So what does the GG interactions with the MGD represent? Is this only occurring in the full-length protein when larger polymers form on lipid templates? If so, then the R445A mutant should be examined in the context of lipid tabulation (EM) and stimulated activity.

8. The authors state that "...under the same conditions used for s-Mgm1, s-OPA1 had no detectable interactions with liposomes." I am confused because OPA1 has been shown to tubulate liposomes with a particular composition, especially when cardiolipin is present. So why did the authors not see tubulation with their s-OPA1? Additional information is needed.

Reviewer #2 (Comments to the Authors (Required)):

Major revisions

Yu et al. describe a 2.4-Å resolution crystal structure of the Minimal GTPase domain (MGD) of human OPA1. This study is timely and extremely interesting in the light of the recent paper of Faelber et al., 2019 describing the structure and the assembly of Mgm1 (the yeast OPA1 homolog) on positively and negatively curved membranes, providing models for the OPA1/Mgm1 remodeling activity of the inner mitochondrial membrane. The exact structure of the OPA1 catalytic machinery and how GTP hydrolysis (both basal and assembly-stimulated) is coupled to the structural

rearrangements needed to mediate membrane fusion are yet open questions.

Here Yu et al. wish to address the properties of nucleotide binding and GTP hydrolysis by OPA1 coupled to dimer formation. While the proposed structure of MGD-OPA1 is similar to those previously published for dynamins and DLPs, the authors report novel nucleotide-MGD and dimer interactions, underling many differences between OPA1 and Mgm1. At the end, the authors try also to explain the effect of some OPA1 mutations on optic atrophy using a cell-based approach. Although the topic is very interesting, some biochemical results are over interpreted or incomplete, and data are insufficient to support the conclusions by the authors. Furthermore, the cell-based experiments on ADOA OPA1 mutants, one the main aspects of the manuscript, are not adequately performed and thus cannot be used to draw any solid conclusion.

Specific points

In the first part of the manuscript, authors describe the overall structure of the crystallized MGD-OPA1, comparing it with some other crystallized DL proteins.

1. A main concern is the absence of the residues 217-263 in the crystal structure of MGD-OPA1. The authors show that the inclusion of these sequence produces more soluble recombinant proteins, with the same GTPase activity. Moreover, since these domain is located downstream the S1 cleavage site, all the l- and s-OPA1 isoforms possess it. Why did the authors prefer to crystallize the MGD-OPA1 and perform the experiments without including this region? A final paper must show the crystal of the MGD-OPA1 with the above mentioned aa sequence.

2. Related to this, authors state that residues 217-263 do not change OPA1 GTPase activity. Authors must report in Figure S1C also the GTPase activity of s-OPA1 and 217-s-OPA1.

3. Did the authors measure the GTPase activity of MGD-OPA1 after removing the His-tag at the C-terminus? Is there a difference in the catalytic activity of the protein?

4. The suggested four-helix bundle domain in OPA1 is really an interesting point. Nevertheless, the authors should elaborate more on this concept taking into consideration at least two points:
• This region seems to be fundamental for the protein, since all the eight human OPA1 isoforms (i.e., the products of the alternative splicing of the OPA1 gene), as well as both l- and s-Opa1 forms (i.e., the products of the Opa1 protein maturation), contain it. Notably, 4 OPA1 isofoms contain the additional alternatively spliced exon 5b with the S2 cleavage site, located between the exon 5 S1 cleavage site and the predicted helix domain (Ishihara et al., 2006; Song et al., 2007). Interestingly, exon 5b contains an additional hydrophobic region with a predicted coiled-coil domain. Since the authors predict the existence of a fourth HB domain, they shall take into consideration the presence of this additional sequence (exon 5b). For example, does this extra region change MGD-OPA1 conformation? What could be the structural role of this alternative region in the long and the short OPA1 forms? Considering a potentially longer 4HB domain, what degree/kind of similarity exists now between the MGD-OPA1 and the MFN-1 model?
• A potential alpha-helix (residues 210-254) has been predicted (COILS program of Lupas) and annotated in UniProt as a coiled-coil domain, along with the C-terminal sequence of OPA1 (residues 895-260), suggesting an interaction between the two. Could the author comment on this? Does it play a role in the s-OPA1 structure rearrangement?

5. Please explain the sentence: "The inclusion of these residues renders soluble and well-behaved OPA1-MGD and s-OPA1 proteins". What does well behaved means? please show experiments to

support this conclusion.

In a following part of the manuscript, authors describe in detail the catalytic core of OPA1 dimers, that resembles that of dynamin-1, and the interactions crucial for GTPase activity and dimer formation.

6. From the ITC experiments in Figure 2E, one would say that the MGD-OPA1 shows lower, rather than similar, affinity with GTPγS compared to GDP. Moreover Mgm1 and OPA1 share comparable affinities with GTPγS, as reported in Faelber et al., 2019 (Kd =9 uM). Please correct.

7. Given the aforementioned similarity between OPA1 and Mgm1, how do the authors comment on the higher GTPase activity of MGD-Mgm1 reported in Figure 2F?

8. In 2010 Chappie revealed the catalytic mechanism of GTP hydrolysis in dynamins. One of the requirements for efficient GTP hydrolysis is the correct position of the water molecule required for the nucleophilic attack of the gamma-phosphate. Authors here do not mention the catalytic water. Why? It must be taken into consideration when they describe domain arrangement and residue interactions.

9. Conclusions are erratic on several circumstances. For example, authors first predict that "GTP hydrolysis by OPA1-MGD is tightly linked to dimer formation" but then they say: "OPA1 is insensitive to G-G dimerization in efficient GTP hydrolysis". How can these conclusions be combined?

10. Authors report that only GDP-BeF3- and not GDP, GDP-AlF2-, GTPγS or apo protein, could generate stable dimers of OPA1-MGD in a K+-containing buffer. It is disappointing that they only use K+ buffers to verify their hypothesis. Authors shall include AUC experiments in Na+-containing buffer, not only for GDP-BeF3-, but also for the other ligands.

11. By mutating specific residues in the nucleotide pocket, the authors show reduced or elevated affinity for GTPγS or GDP. Could the authors explain better these results? What is the cause of the differential affinity for the nucleotides?

12. The authors try then to correlate the reduced nucleotide binding affinity with a decrease in the GTPase activity. The results in Figure 2E-F and S3 do not indicate a strong correlation. For example, T503A has more affinity for GDP and GTPγS, while R316A drastically decreases interactions with GTPγS, but not GDP. Surprisingly, both have increase and comparable GTPase activity in K+ buffer. M322A drastically affects GTPγS binding, but it increases GTP hydrolysis; M321A has a mild impact on GTPγS binding, but it decreases GTPase activity. These data are overinterpreted and authors must revise this section completely. As a starting point, perhaps they can consider that mutational studies indicated that the T65 residue is crucial for dynamin catalysis, but its mutation to alanine do not affect nucleotide binding (Marks et al., 2001). The authors should better explain these results in the light of OPA1-MGD structure-function relationship and interaction with the component of core catalytic domain.

13. Authors cannot draw any conclusions on the role of Switch 1 in cooperating with K+ only from these mutational data. The OPA1 specific "additional mechanisms" that regulate GTP hydrolysis are not clear.

14. Authors state that MGD-OPA1 GTPase activity is not dependent on G-G domain dimerization.

To support this model, authors should include GTPase activity also of the other dimer-deficient mutant reported in Figure 3B. It would also be useful, as a control, to report data for a mutation in this region that would not be expected to disrupt dimer formation.

15. The graphics in Figure 3B and C are confusing. The authors use two different techniques to measure nucleotide-dependent dimerization of WT OPA1-MGD only in the presence GDP-BeF3. They shall confirm the data of AUC obtained for the other nucleotides also by MALS coupled with gel filtration. Vice versa, they should add AUC experiments to confirm the absence of dimer formation with the mutants of Figure 3B.

16. In Figure 3D-F, authors want to study the nucleotide-(in)dependent dimerization in the full s-OPA1 protein. These are incomplete and over interpreted experiments that should be removed. Again, control experiments are missing: (i) dimerization should be measured with both MALS coupled with gel filtration and AUC; (ii) dimerization of s-OPA1 should be measured also the other nucleotides, as in Figure 3C, since we do not know if it has a different nucleotide preference for dimerization (both in K+ and Na+-containing buffers); (iii) at least other two mutant of OPA1-MGD dimerization must be tested. To draw solid conclusions the authors should also have the complete structure of s-OPA1 in their hands. Moreover, structure and assembly of Mgm1/OPA1 has been extensively discussed in Faelber et al., 2019, where they demonstrate that Mgm1 stalk mediated the assembly of bent tetramers.

17. Figure 3E. Why do the authors use a 250 mM NaCl buffer, instead of 150 mM KCl (as in the previous AUC experiments) to measure s-OPA1 WT and R445A nucleotide-independent dimerization? Moreover, Figure 3E AUC and Figure 3D MALS coupled with gel filtration report mass of the dimer results that are discordant: 130 kDa in AUC, and >150 kDa in MALS experiments. How do the author explain this?

18. Why did authors test of the ADOA OPA1 mutant only the dimerization ability? They should also test the nucleotide-binding capacity and the GTPase activity of the mutant.

19. The authors claim that they can "deduce the roles of mutations in the remaining parts of s-OPA1 based on sequence comparisons and the structural information from s-Mgm1. s-Mgm1 folds into a dynamin-1-like conformation". Faelber et al., 2019 extensively describe Mgm1 conformation. Moreover, what does ensure the correctness of their predictions since they do not have the crystal structure of OPA1 and repeatedly state that discrepancies between OPA1 and Mgm1 structure are expected? (for example: "key residues for nucleotide-independent assembly of Mgm1 are not conserved in OPA1, and at the same time, key residues for nucleotide-dependent assembly of OPA1 are rarely seen in Mgm1").

The cell-based experiments reported in Figure 4 and Figure S5 are not conclusive. This part of the manuscript should be substantially further developed or deleted.
I. Authors shall study Opa1f/f MEFs, where Opa1 can be acutely deleted by Cre mediated recombination, instead of using Opa1-/- MEFs where chronic Opa1 deletion reduces mtDNA copy number and translation, complicating any interpretation of the results.
II. Along this line, authors fail to rescue mitochondrial elongation by re-expressing OPA1 WT in Opa1-/- MEFs. Thus, they can not conclude anything about the mutant. Moreover, they should also be added the images of the empty vector negative control. They shall use a mitochondrial targeted fluorescent protein, instead of cytochrome c, to stain the mitochondrial network. How did the authors classify the mitochondria as "fragmented", "filamentous" or "intermediate"? Instead, quantify and show length of individual mitochondria.

III. No experiments on mitochondrial function, or cristae biogenesis, are shown. They must be included

20. The authors conclude that "MGD and the short form of Mgm1 possess nearly identical GTPase activity, whereas the GTPase activity of s-OPA1 is higher than that of OPA1-MGD". Again, (as already asked above) did the try to measure GTPase activity of OPA1-MGD after His-tag removal?

21. Vis-à-vis the presented data, it is impossible to conclude that Mgm1 and OPA1 have a different mechanism of action. There are no data or references supporting that "under the same conditions used for s-Mgm1, s-OPA1 had no detectable interactions with liposomes". As a matter of fact, there are many works that demonstrate the contrary. For example, l-OPA1 and cardiolipin (CL) cooperate in heterotypic mitochondrial IM fusion (Ban et al., 2017).

22. references are missing.

23. English needs revision, for both clarity and grammar.

Minor points

1. Result and discussion, line 66: Please, correct "dyanamin-1" with "dynamin-1".
2. Result and discussion, line 219: Presumably the authors are referring to S298N mutation, as mentioned previously?
3. Figure 1A: the transmembrane domain in the cartoon is labelled as "TM". Please correct "TMD" in the legend as "TM".
4. Figure 1C: the PDB code of Mgm1 crystal structure is missing.
5. Figure 3E: the figure legend might be clarify indicating also the R445A mutant.
6. Figure S1A: the position of the predicted a4 domain is not correct.
7. Figure S1B: What is the second band that appear at lower molecular weight in 217-s-OPA1?
8. The Figure S3 is referred as Figure 3 in the text.
9. In the material and methods the author do not describe the production and purification of Mgm1.

Reviewer #1 (Comments to the Authors (Required)):

This is a well-written manuscript for Dr. Ju's lab. The paper highlights new structural information about the OPA1 protein, which is the most frequently mutated protein associated with inherited blindness. Building on other work with GTPase domain constructs of dynamin proteins for structural studies, the authors do something similar to isolate a soluble portion of the protein. In this way, they are able to highlight novel attributes within OPA1 compared to other dynamins.

This work follows on the heels of the recent Mgm1p structure. The authors do a nice job of highlighting the similarities and differences that they observed in their structure. Given the region of the protein that they have in their construct, the authors largely focus on GTPase functional assays. This is appropriate, but the biochemical data is not convincing. More rigor should be used to analyze the GTPase activity and define the specific activity of the OPA1 GTPase domain in a way that can be compared with other dynamins, including Mgm1p. Moreover, the authors highlighted work on the s-OPA1 full-length protein, but it is unclear why they could not observe lipid tubulation. Does this reflect issues with their s-OPA1 construct? Any data on lipid templates would greatly strengthen the papers findings. I particularly like the mapping of the patient mutations onto the structure, but it is hard to correlate the impact of their GG structure in the larger context of membrane interactions. I do not know what the dimer interface in their GG crystal structure represents since other interactions are clearly governing dimer formation in the absence of nucleotide. And if the GG dimer only forms during lipid interactions, which would be similar to dynamin, does the assembly correlate with the larger jump in activity when lipid interactions occur. It would be ideal to see the impact of the mutations on lipid-induced assembly.

Overall, I feel that the scope of the manuscript is appropriate for JCB, but I do think that additional experiments and a focused effort to clarify the enzymology would help the manuscript. I offer the following comments to highlight short-comings that I would like to see addressed:

1. The introduction is very brief. Usually, I prefer brevity, but this seems a little incomplete. It is worth expounding more on dynamin-family protein structures, especially for fusion dynamins, to compare with the findings presented here for OPA1.

We rewrote the introduction accordingly.

2. Lines 86-90 - The authors mention that the inclusion of an additional N-terminal helix (residues 217-262) did not impact protein solubility or alter OPA1 GTPase activity, and that "These results suggest that the OPA1-HB1/BSE domain can be a four-helix bundle (4HB)". But this is unfounded without showing this structurally or using a technique that would highlight the formation of an additional helix. They may be correct, but additional data is needed to validate this claim.

We synthesized a peptide according to the sequences of 217-262 and tested its helical propensity using CD spectroscopy. Our new results showed that this region is indeed helical (**Fig. 2A**). Furthermore, we showed that this region confers nucleotide-independent dimerization through a coiled-coil type of interaction (**Figs. 2E and 4E,F**), instead of complementing the HB.

3. Lines 93-96 - The authors state, "Similar organization has been reported for dynamin-1 (with a Na+ ion instead) and MFN1 (Fig. 2C,D), but not many other DLPs." To which other DLPs are they comparing and what is observed with Drp1 or Mx or ATL1? Is there any significance to these differences? It is not clear that an important distinction exists.

We changed wording accordingly.

4. Lines 113-115 - I think it is interesting that GMPPNP is less stable in the nucleotide binding pocket. This is consistent with what other investigators have seen for several DLPs. But GMPPCP generally binds well to these proteins, so I would hope that the authors would test

association with this nucleotide analog for completeness.

We tested GMPPCP as suggested and found no interaction (**Fig. S1A**).

5. The GTPase assays were a little confusing to me because the authors present μM Pi per min. Initially, the rates seemed higher than I would have expected based on previous GTPase measurements for OPA1 and Mgm1p. But the authors are using 10 μM protein for these assays. I would prefer that they present the data as a kobs measurement by dividing by the concentration of their protein. This would be more consistent with previous results, and for the Mgm1 MGD, the value would actually be higher (~1 min-1) than reported for the full-length Mgm1p (0.5 min-1), but this may be explained by the higher concentration of protein used in the current assays. Ideally, it would worth knowing whether a specific activity could be presented for these constructs (i.e. same rate independent of concentration) since they are not assembling through stalk interactions that generally stimulates the GTPase activity in other DLPs. Given the focus of this paper on the GTP interaction with OPA1, additional studies should be performed to better define how the activity of the GTPase domain is impacted by these interactions.

We showed K_{obs} as suggested and measured the activity of OPA1 with various protein concentrations (**Fig. S1B**). As suggested by the other reviewer (see below), we now provide a more comprehensive analysis of the OPA1 GTPase.

6. Lines 149-151 - The authors state, "These results suggest that GTP hydrolysis by OPA1 is precisely regulated via additional mechanisms that are not seen with other DLPs." I am not sure that I understand how this can be claimed. I know that Chappie et al examined the impact of cation interactions on GTPase activity, but I don't know other examples with DLPs. And can we say that these mild differences in GTPase activity in response to cation interactions is significant when compared to the stimulated activity that would be observed on a membrane? Perhaps, if the authors provide more of an introduction on the importance of cation interactions in GTPase function, this could provide clarity. But as this is presented, this appears to be a less significant finding.

We rewrote this part accordingly. The fold of stimulation by proper cation, in this case K^+ vs. Na^+ (~2-fold higher with K^+), is statistically reproducible and has been compared to human MFN1 (~5-fold higher with K^+). The GTPase activity in K^+ can be further stimulated in the presence of proper lipids (**Fig. 4G**), with the fold of increase equivalent to that by proper cation. We therefore believe the difference is significant. As mentioned in the text, the preference for K^+ is consistent with its cellular concentrations.

7. The dimerization results in Figure 3 are somewhat alarming. You are getting dimerization with the full-length protein that does not further oligomerize in the presence of GTP. So what does the GG interactions with the MGD represent? Is this only occurring in the full-length protein when larger polymers form on lipid templates? If so, then the R445A mutant should be examined in the context of lipid tabulation (EM) and stimulated activity.

We showed that stimulated activity is more prominent with the 217-960 construct (217-s-OPA1) that the 263-960 construct (263-s-OPA1). We performed the stimulated GTPase assay and found that R445A abolishes the stimulation (**Fig. 4G**). We also tried the lipid tubulation assay by EM as suggested, but did not obtain satisfactory results.

8. The authors state that "...under the same conditions used for s-Mgm1, s-OPA1 had no detectable interactions with liposomes." I am confused because OPA1 has been shown to tubulate liposomes with a particular composition, especially when cardiolipin is present. So why did the authors not see tubulation with their s-OPA1? Additional information is needed.

We found that the key discrepancy here is residues 217-263, when including this region, the s-OPA1 can now interact with lipids more efficiently. We also found that like Mgm1 tested in our

hands, the MGD has some lipid association (**Fig. 2F**). Unfortunately, we did not obtain satisfactory results with the tubulation assay, even when 217-s-OPA1 was used.

Reviewer #2 (Comments to the Authors (Required)):

Major revisions

Yu et al. describe a 2.4-Å resolution crystal structure of the Minimal GTPase domain (MGD) of human OPA1. This study is timely and extremely interesting in the light of the recent paper of Faelber et al., 2019 describing the structure and the assembly of Mgm1 (the yeast OPA1 homolog) on positively and negatively curved membranes, providing models for the OPA1/Mgm1 remodeling activity of the inner mitochondrial membrane. The exact structure of the OPA1 catalytic machinery and how GTP hydrolysis (both basal and assembly-stimulated) is coupled to the structural rearrangements needed to mediate membrane fusion are yet open questions.

Here Yu et al. wish to address the properties of nucleotide binding and GTP hydrolysis by OPA1 coupled to dimer formation. While the proposed structure of MGD-OPA1 is similar to those previously published for dynamins and DLPs, the authors report novel nucleotide-MGD and dimer interactions, underling many differences between OPA1 and Mgm1. At the end, the authors try also to explain the effect of some OPA1 mutations on optic atrophy using a cell-based approach. Although the topic is very interesting, some biochemical results are over interpreted or incomplete, and data are insufficient to support the conclusions by the authors. Furthermore, the cell-based experiments on ADOA OPA1 mutants, one the main aspects of the manuscript, are not adequately performed and thus cannot be used to draw any solid conclusion.

Specific points

In the first part of the manuscript, authors describe the overall structure of the crystallized MGD-OPA1, comparing it with some other crystallized DL proteins.

1. A main concern is the absence of the residues 217-263 in the crystal structure of MGD-OPA1. The authors show that the inclusion of these sequence produces more soluble recombinant proteins, with the same GTPase activity. Moreover, since these domain is located downstream the S1 cleavage site, all the l- and s-OPA1 isoforms possess it. Why did the authors prefer to crystallize the MGD-OPA1 and perform the experiments without including this region? A final paper must show the crystal of the MGD-OPA1 with the above mentioned aa sequence.

As mentioned to the other reviewer, we now show that this region confers nucleotide-independent dimerization and facilitate lipid interactions (**Fig. 2**). We have tried extensively to crystalize this version of MGD, but with no success so far. Additional biochemical analysis with residues 217-262 provides important insight into OPA1 activity.

2. Related to this, authors state that residues 217-263 do not change OPA1 GTPase activity. Authors must report in Figure S1C also the GTPase activity of s-OPA1 and 217-s-OPA1.

We performed this experiment as suggested, and found no significant changes in the activity (**Fig. 2D**).

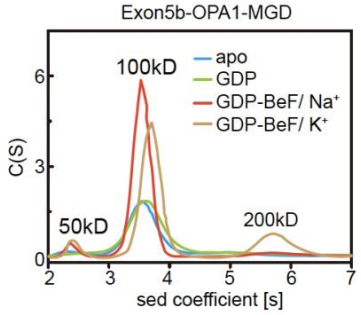
3. Did the authors measure the GTPase activity of MGD-OPA1 after removing the His-tag at the C-terminus? Is there a difference in the catalytic activity of the protein?

We performed this experiment as suggested, and found no significant changes in the activity (**Fig. S1C**).

4. The suggested four-helix bundle domain in OPA1 is really an interesting point. Nevertheless, the authors should elaborate more on this concept taking into consideration at least two points:

• This region seems to be fundamental for the protein, since all the eight human OPA1 isoforms (i.e., the products of the alternative splicing of the OPA1 gene), as well as both l- and s-Opa1 forms (i.e., the products of the Opa1 protein maturation), contain it. Notably, 4 OPA1 isofoms contain the additional alternatively spliced exon 5b with the S2 cleavage site, located between the exon 5 S1 cleavage site and the predicted helix domain (Ishihara et al., 2006; Song et al., 2007). Interestingly, exon 5b contains an additional hydrophobic region with a predicted coiled-coil domain. Since the authors predict the existence of a fourth HB domain, they shall take into consideration the presence of this additional sequence (exon 5b). For example, does this extra region change MGD-OPA1 conformation? What could be the structural role of this alternative region in the long and the short OPA1 forms? Considering a potentially longer 4HB domain, what degree/kind of similarity exists now between the MGD-OPA1 and the MFN-1 model?
• A potential alpha-helix (residues 210-254) has been predicted (COILS program of Lupas) and annotated in UniProt as a coiled-coil domain, along with the C-terminal sequence of OPA1 (residues 895-260), suggesting an interaction between the two. Could the author comment on this? Does it play a role in the s-OPA1 structure rearrangement?

We now show that the 217-262 region forms a helix and confers nucleotide-independent dimerization as the reviewer predicted (**Figs. 2E and 4E,F**). We purified an extended construct including the exon 5b for comparison. The 5b-MGD behaved poorly after purification (tend to precipitate), and the 5b-s-OPA1 did not even express. With the residual 5b-MGD, we performed AUC analysis and found that it forms dimer in the apo state (see below), consistent with what has been observed with 217-MGD. It is difficult to test whether exon 5b offers additional coiled coil interactions, given the aggregation-prone behavior of the protein.



5. Please explain the sentence: "The inclusion of these residues renders soluble and well-behaved OPA1-MGD and s-OPA1 proteins". What does well behaved means? please show experiments to support this conclusion.

We meant that these proteins are purifiable and usable for further biochemical analysis. We changed the wording accordingly.

In a following part of the manuscript, authors describe in detail the catalytic core of OPA1 dimers, that resembles that of dynamin-1, and the interactions crucial for GTPase activity and dimer formation.

6. From the ITC experiments in Figure 2E, one would say that the MGD-OPA1 shows lower, rather than similar, affinity with GTPyS compared to GDP. Moreover Mgm1 and OPA1 share comparable affinities with GTPyS, as reported in Faelber et al., 2019 (Kd =9 uM). Please correct.

We changed the wording accordingly. As discussed in the text, the variation in affinities is likely due to subtle chemical modifications in the analogs.

7. Given the aforementioned similarity between OPA1 and Mgm1, how do the authors comment

on the higher GTPase activity of MGD-Mgm1 reported in Figure 2F?

In terms of GTP binding, OPA1 and *Ct*Mgm1 are equivalent (~9-10 μM). The discrepancy in GTPγS binding seen with *Sc*Mgm1 (81.3 μM) is likely due to its sensitivity to chemical modification of the nucleotide. In contrast, there would be no complication when interpreting the GDP data: OPA1 has a higher affinity for GDP (5.5 μM) when compared to that of *Sc*Mgm1 (34.9 μM), suggesting that it would release GDP and begin a new cycle slower than Mgm1. Collectively, these findings reasonably explain the differences in GTPase activity between OPA1 and Mgm1. We have added this point in the text.

8. In 2010 Chappie revealed the catalytic mechanism of GTP hydrolysis in dynamins. One of the requirements for efficient GTP hydrolysis is the correct position of the water molecule required for the nucleophilic attack of the gamma-phosphate. Authors here do not mention the catalytic water. Why? It must be taken into consideration when they describe domain arrangement and residue interactions.

We did observe the two water molecules at the related positions in our structure. We have added this point in the figure (**Figs. 3D and S1D, left panel**) and text.

9. Conclusions are erratic on several circumstances. For example, authors first predict that "GTP hydrolysis by OPA1-MGD is tightly linked to dimer formation" but then they say: "OPA1 is insensitive to G-G dimerization in efficient GTP hydrolysis". How can these conclusions be combined?

We modified or removed these sentences. We found that basal GTP hydrolysis is generally not affected but membrane-stimulated hydrolysis is sensitive to key residues at the dimer interface.

10. Authors report that only GDP-BeF3- and not GDP, GDP-AlF2-, GTPγS or apo protein, could generate stable dimers of OPA1-MGD in a K+-containing buffer. It is disappointing that they only use K+ buffers to verify their hypothesis. Authors shall include AUC experiments in Na+-containing buffer, not only for GDP-BeF3-, but also for the other ligands.

We performed these experiments as suggested (**Fig. 4C**). As expected, dimers only formed with GDP and BeF_3^- in K^+-containing buffer.

11. By mutating specific residues in the nucleotide pocket, the authors show reduced or elevated affinity for GTPγS or GDP. Could the authors explain better these results? What is the cause of the differential affinity for the nucleotides?

We rewrote this part accordingly. We also acknowledged that a few observations are unexpected. The complications could be caused by the structural plasticity of the catalytic core upon mutagenesis and varied sensitivity to chemical modification of the nucleotide (from GTP to GTPγS).

12. The authors try then to correlate the reduced nucleotide binding affinity with a decrease in the GTPase activity. The results in Figure 2E-F and S3 do not indicate a strong correlation. For example, T503A has more affinity for GDP and GTPγS, while R316A drastically decreases interactions with GTPγS, but not GDP. Surprisingly, both have increase and comparable GTPase activity in K+ buffer. M322A drastically affects GTPγS binding, but it increases GTP hydrolysis; M321A has a mild impact on GTPγS binding, but it decreases GTPase activity. These data are overinterpreted and authors must revise this section completely. As a starting point, perhaps they can consider that mutational studies indicated that the T65 residue is crucial for dynamin catalysis, but its mutation to alanine do not affect nucleotide binding (Marks et al., 2001). The authors should better explain these results in the light of OPA1-MGD structure-function relationship and interaction with the component of core catalytic domain.

We rewrote this part accordingly. In general, a decrease in affinity for GTPγS would cause a correlated decrease in GTPase activity, as GTP binding is a necessary first step. T65 in dynamin is equivalent to T323 in OPA1, which we showed that by mutating this residue to Ala, the GTPase activity is much reduced (**Fig. 3F**). We also performed ITC for this mutant to confirm that nucleotide binding is not significantly affected (**Table S1**).

13. Authors cannot draw any conclusions on the role of Switch 1 in cooperating with K+ only from these mutational data. The OPA1 specific "additional mechanisms" that regulate GTP hydrolysis are not clear.

We performed a more comprehensive analysis, combining analysis for nucleotide interaction (ITC), dimerization (MALS/AUC) and GTP hydrolysis. These results underscore the plasticity of Switch 1 upon mutagenesis and suggest that GTP hydrolysis by OPA1 is precisely regulated by coordinated G motifs, particularly Switch 1, using mechanisms in addition to previously identified one with other DLPs. The OPA1specific mechanisms include: 1) the guanine stabilization by R316 in Switch 1; 2) the Switch 1 bending by R324; 3) and the hydrophobic gating of the nucleotide pocket by M321.

14. Authors state that MGD-OPA1 GTPase activity is not dependent on G-G domain dimerization. To support this model, authors should include GTPase activity also of the other dimer-deficient mutant reported in Figure 3B. It would also be useful, as a control, to report data for a mutation in this region that would not be expected to disrupt dimer formation.

We clarified that we are comparing both basal activity and assembly-stimulated activity. As suggested, we used E320A as a negative control. E320 is in the dimer interface, its mutation cause no changes in nucleotide binding, minor defects in dimerization and no defects on basal or assembly-stimulated GTP hydrolysis.

15. The graphics in Figure 3B and C are confusing. The authors use two different techniques to measure nucleotide-dependent dimerization of WT OPA1-MGD only in the presence GDP-BeF3. They shall confirm the data of AUC obtained for the other nucleotides also by MALS coupled with gel filtration. Vice versa, they should add AUC experiments to confirm the absence of dimer formation with the mutants of Figure 3B.

We improved the completeness of the experiments here (**Fig. 4D** and **S2C**).

16. In Figure 3D-F, authors want to study the nucleotide-(in)dependent dimerization in the full s-OPA1 protein. These are incomplete and over interpreted experiments that should be removed. Again, control experiments are missing: (i) dimerization should be measured with both MALS coupled with gel filtration and AUC; (ii) dimerization of s-OPA1 should be measured also the other nucleotides, as in Figure 3C, since we do not know if it has a different nucleotide preference for dimerization (both in K+ and Na+-containing buffers); (iii) at least other two mutant of OPA1-MGD dimerization must be tested. To draw solid conclusions the authors should also have the complete structure of s-OPA1 in their hands. Moreover, structure and assembly of Mgm1/OPA1 has been extensively discussed in Faelber et al., 2019, where they demonstrate that Mgm1 stalk mediated the assembly of bent tetramers.

We removed this part as suggested, and tested stimulated GTPase activity using various mutants (**Fig. 4G**).

17. Figure 3E. Why do the authors use a 250 mM NaCl buffer, instead of 150 mM KCl (as in the previous AUC experiments) to measure s-OPA1 WT and R445A nucleotide-independent dimerization? Moreover, Figure 3E AUC and Figure 3D MALS coupled with gel filtration report mass of the dimer results that are discordant: 130 kDa in AUC, and >150 kDa in MALS experiments. How do the author explain this?

As mentioned above, we removed this part to avoid confusion. Notably, we compared GTPase activity of wt proteins with 150 vs 250 mM salts and found no significant difference. The mass estimated by these methods are of limited accuracy.

18. Why did authors test of the ADOA OPA1 mutant only the dimerization ability? They should also test the nucleotide-binding capacity and the GTPase activity of the mutant.

We improved the completeness of the experiments here (**Table S1** and **Fig. 5E**).

19. The authors claim that they can "deduce the roles of mutations in the remaining parts of s-OPA1 based on sequence comparisons and the structural information from s-Mgm1. s-Mgm1 folds into a dynamin-1-like conformation". Faelber et al., 2019 extensively describe Mgm1 conformation. Moreover, what does ensure the correctness of their predictions since they do not have the crystal structure of OPA1 and repeatedly state that discrepancies between OPA1 and Mgm1 structure are expected? (for example: "key residues for nucleotide-independent assembly of Mgm1 are not conserved in OPA1, and at the same time, key residues for nucleotide-dependent assembly of OPA1 are rarely seen in Mgm1").

We rewrote and tuned down the conclusion here. We performed secondary structure prediction for OPA1 and then aligned these elements with those of Mgm1 for comparison. To avoid confusion, we have removed the original Fig. S1.

The cell-based experiments reported in Figure 4 and Figure S5 are not conclusive. This part of the manuscript should be substantially further developed or deleted.
I. Authors shall study Opa1f/f MEFs, where Opa1 can be acutely deleted by Cre mediated recombination, instead of using Opa1-/- MEFs where chronic Opa1 deletion reduces mtDNA copy number and translation, complicating any interpretation of the results.
II. Along this line, authors fail to rescue mitochondrial elongation by re-expressing OPA1 WT in Opa1-/- MEFs. Thus, they can not conclude anything about the mutant. Moreover, they should also be added the images of the empty vector negative control. They shall use a mitochondrial targeted fluorescent protein, instead of cytochrome c, to stain the mitochondrial network. How did the authors classify the mitochondria as "fragmented", "filamentous" or "intermediate"? Instead, quantify and show length of individual mitochondria.
III. No experiments on mitochondrial function, or cristae biogenesis, are shown. They must be included

We improved data quality here using virus-based stable cell lines and add EM analysis for cristae. Our new results show that stably expressed OPA1 offers much improved rescue efficiency. We added images for empty vector, changed markers and provided quantitative parameters for the morphological categories. Finally, we analyzed cristae morphology by EM for the rescue experiments (**Fig. 5F** and **S3**).

20. The authors conclude that "MGD and the short form of Mgm1 possess nearly identical GTPase activity, whereas the GTPase activity of s-OPA1 is higher than that of OPA1-MGD". Again, (as already asked above) did the try to measure GTPase activity of OPA1-MGD after His-tag removal?

We tested this as suggested and saw no difference (**Fig. S1C**).

21. Vis-à-vis the presented data, it is impossible to conclude that Mgm1 and OPA1 have a different mechanism of action. There are no data or references supporting that "under the same conditions used for s-Mgm1, s-OPA1 had no detectable interactions with liposomes". As a matter of fact, there are many works that demonstrate the contrary. For example, l-OPA1 and cardiolipin (CL) cooperate in heterotypic mitochondrial IM fusion (Ban et al., 2017).

We found that the key discrepancy here is residues 217-263, when including this region, the s-OPA1 can now interact with lipids more efficiently. We also found that like Mgm1 tested in our hands, the MGD has detectable lipid association (**Fig. 2F**).

22. references are missing.

We double checked references and corrected them accordingly.

23. English needs revision, for both clarity and grammar.

Done.

Minor points

1. Result and discussion, line 66: Please, correct "dyanamin-1" with "dynamin-1".
2. Result and discussion, line 219: Presumably the authors are referring to S298N mutation, as mentioned previously?
3. Figure 1A: the transmembrane domain in the cartoon is labelled as "TM". Please correct "TMD" in the legend as "TM".
4. Figure 1C: the PDB code of Mgm1 crystal structure is missing.
5. Figure 3E: the figure legend might be clarify indicating also the R445A mutant.
6. Figure S1A: the position of the predicted a4 domain is not correct.
7. Figure S1B: What is the second band that appear at lower molecular weight in 217-s-OPA1?
8. The Figure S3 is referred as Figure 3 in the text.
9. In the material and methods the author do not describe the production and purification of Mgm1.

Done.

March 29, 2020

RE: JCB Manuscript #201907098R-A

Dr. Junjie Hu
Chinese Academy of Sciences
15 Datun Rd. Chaoyang District
Beijing, Beijing 100101
China

Dear Dr. Hu:

Thank you for submitting your revised manuscript entitled "Structural insights into G domain dimerization and pathogenic mutations of OPA1". We would be happy to publish your paper in JCB provided the remaining comments from Reviewer#1 are addressed by revisions to the text where appropriate and pending final revisions necessary to meet our formatting guidelines (see details below).

- Please provide a short eTOC statement
- Provide the main and supplementary texts as separate, editable .doc or .docx files
- Provide main and supplementary figures as separate, editable files according to the instructions for authors on JCB's website *paying particular attention to the guidelines for preparing images and blots at sufficient resolution for screening and production*
- Provide tables as excel files
- Format references for JCB
- Add paragraph after the Materials and Methods section briefly summarizing all "Online Supplementary Materials"
- Add author contributions

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Richard Youle, Ph.D.
Monitoring Editor

Marie Anne O'Donnell, Ph.D.
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This paper clearly lays out the structure of a truncated OPA G domain construct. The structure in the presence of GDP-BeFx highlights the dimer interface that is well conserved with other dynamin family members. Moreover, a robust collection of mutants are used to assess functional interfaces and the specific impact of patient mutations in this region of the protein. The authors attempt to examine OPA1-lipid interactions, and the effects on activity are mild. But the abundance of structural and functional insight into the G domain of OPA1 makes this a meaningful contribution to the field. The cellular studies provide a nice complement, and also highlight the complexity of recovery experiments for mitochondrial dynamics proteins.

1. I still find it puzzling that they are unable to get decorated tubules with the s-OPA1 construct, and nothing was obviously different in their methods section other than that previous studies have

used higher molar ratios of cardiolipin in their liposome preps. Regardless, the data is the data.

2. I do think that there was tremendous effort to incorporate synthetic and patient-defined mutants into this construct. And that really makes the story for me. The detailed information from these differences is very informative.

3. The writing is much better and the paper was easy for me to follow.

4. I did find a small typo on line 80 where dynamin is misspelled, "...dyanamin-1 G domain construct..."