Mitofusin 2 but not mitofusin 1 mediates Bcl-XL-induced mitochondrial aggregation

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this work, the authors tried to link the apoptosis-related machinery with proteins involved in mitochondrial fusion, through live-cell FRET, a state-of-the-art technique. In a similar was as the authors have done previously on other proteins using the same technique, the interaction between Bcl-XL and mitofusins (MFNs) in live cells are carefully analyzed, and the stoichiometry of the interaction is calculated.
Although the oligomerization of MFNs are known before, this information is of satisfactory novelty and not able to be gleaned by other means. Overall, this work represents an suitable application and exhibits great value of a high-end biophysical technology in the field of cell biology. On the other hand, some biological issues need to be clarified to show how interaction between MFNs and Bcl-XL impact apoptosis.

Comments for the author

Suggestions:
1. The authors are suggested to perform a co-IP or equivalent assay using YFP/CFP alone as a negative control avoid potential false positive results.

2. The authors claimed that the MFN2(K109A) or MFN1(K88T) was still able to bind Bcl-XL. The mutated lysine here belongs to the conserved G1 motif (or the P-loop) of all GTPases, which coordinate the phosphate groups of the bound guanine nucleotide.
The authors can describe the role of this residue to show the rationale of choosing it in the context.

3. Further to this point, mutation of this residue normally won't affect the overall folding of the protein, but binding of the nucleotide (hence GTP hydrolysis). Therefore, the authors should explain how this mutation affect the interaction between MFNs and Bcl-XL. i.e. is this residue directly involved in the interface, or the interaction is dependent on the loading of GTP/GDP to MFNs? It might be necessary to include these two mutants also in the co-IP/pull-down assay and that is why I feel that point 1 is necessary. If possible, the G domain of MFN1/MFN2 alone should also be tested. If the authors decides to make a pull-down assay, the supply with different guanine nucleotide should be considered.

4. It seems that MFN1 and MFN2 binds to Bcl-XL in more or less the same way (e.g. with 1:1 stoichiometry as the authors claimed), while the author also found that only MFN2 but not MFN1 mediates Bcl-XL induced mitochondrial aggregation. What is the reason for that? The authors should at least make a discussion on it.

5. Some grammatical issues. For example, page 11, line 11, "mitochondrial aggregate cells" is a mistake. Also, this sentence is way too long, better chop it into two parts. Please carefully proofread the manuscript to improve its readability.

Reviewer 2

Advance summary and potential significance to field

The properties and dynamics of mitochondrial membranes are modulated by a variety of specific factors that mediate and/or catalyze the permeabilization of the outer membrane during apoptosis (notably proteins of the Bcl-2 family of proteins) and the fusion and division of mitochondria (notably dynamin-related proteins/Drps like Mfn1 and Mfn2, OPA1, and Drp1). Several reports have revealed links between both processes (permeabilization and dynamics), involvement of Bcl-2 family proteins in fusion/fission dynamics as well as modulation of apoptotic membrane

permeabilization by Drps. The field is challenging with numerous open questions and controversial findings.
The authors of this manuscript have expressed/transfected several outer membrane proteins tagged with CFP or YFP. They have used fluorescence microscopy and/or FRET to investigate protein localization study interactions between CFP and YFP tagged proteins and characterize the consequences of protein expression.
Authors show that overexpressed Mfn1 and Mfn2 localize to mitochondria, provoke mitochondrial aggregation and interact with each other to form homo- and hetero-oligomers. These Mfn-properties are well-established facts. Thorough quantitative analysis of FRET led authors to conclude on 1:1 stoichiometry of Mfn-Mfn interactions.
Authors show that overexpressed Bcl-xL, but not Mfn1 or Mfn2, protect against staurosporine induced apoptosis. The anti-apoptotic activity of Bcl-xL is well established and the results with Mfn1 and Mfn2 apparently contradict findings reported by Sugioka et al. (2004); authors cite this study, but do not discuss this apparent contradiction.
Authors show that overexpressed Bcl-xL can cluster mitochondria and interact with mitofusins, as reported by Delivani et al. 2006. Quantitative analysis of FRET led authors to conclude on 1:1 stoichiometry of Mfn-Bcl-xL interactions.
The FRET experiments are well performed and analyzed and contain appropriate controls.
However, the manuscript does not provide data that improve our understanding on the roles of Mfn1, Mfn2 or Bcl-xL in fusion and/or permeabilization of mitochondrial outer membranes. In addition, the manuscript has several points that would merit revision and/or correction. Some are listed below.

Comments for the author

Introduction:
I.1 reviews cited to describe the role(s) of fusion/fission dynamics or mitofusins in ATP-production (Brookes et al 2004), mtDNA maintenance (Wallace 2005), apoptosis (Wang and Youle 2009) do not describe the roles of fusion/fission dynamics or mitofusins in these processes.
I.2 Lee et al 2016 does not describe any role of fission in caspase activation and cell death (but the involvement of Dyn-2 in mitochondrial fission)
I.3 Cao et al., 2017 describes the 3D structure of MFN1-fragments, not the role/involvement of Mfns in OMM fusion.
I.4 authors assume or affirm that mitochondrial fusion and aggregation are similar (or even identical)
events at least in the context of Bcl-xL expression and/or mitochondrial apoptosis, as described in publications by the Martin group (Delivani 2006, Sheridan 2008). It is not clear that this applies, as inactive Mfn molecules also induce aggregation (see R.1).
I.5 It is surprising that, in the context of this paper, authors do not cite work revealing the capacity of recombinant Bcl-XL to modulate Mfn-dependent fusion (Hoppins et al 2011, PMID 21255726) or the role of Bax and Bak to modulate mitochondrial morphogenesis via mitofusins (Karbowski et al 2006, PMID 17035996)
Materials and Methods M.1 Authors indicate that plasmids were purchased from Addgene. This is not the case, authors need to acknowledge plasmid as indicated in Addgene's web page (https://help.addgene.org/hc/en-us/articles/205432559-How-do-I-cite-a-plasmid-that-I-received-from-Addgene-in-future-publications-):
'The plasmids you have received were created by your colleagues. Please acknowledge the Principal Investigator, cite the article listed on the plasmid datasheet, and include the Addgene plasmid number in future publications.'
M.2 The nature and origin of the YFP-ActA (a control plasmid?) is not indicated.
Results R.1 It is noteworthy that the aggregation induced by overexpression of Mfn1, Mfn2 or Bcl-xL, described as fusion-profile in publications of the Martin group (see I.4), is not observed at lower expression levels achieved by viral transduction (e.g. Chen et al 2003, cited by the authors), but occurs upon high level expression (transfection) of fusion-competent wild-type Mfn and fusion-incompetent mutant Mfn (Eura et al. 2003, cited by the authors).
R.2 FRET efficacy depending on distance and orientation between fluorophores, authors should indicate the position of the FP-tag within the sequence and/or the recently published 3D structures (references cited by authors: Cao et al., 2017; Qi et al., 2016).

R.3 'GTPase domain mutants'. Authors should indicate the nature as well as the predicted and/or known consequences of the mutations studied (the expression 'one traditional mutant' used in the discussion is not precise).
R.4 Given earlier reports on the capacity of Bax or Bcl-xL to stimulate Mfn-mediated fusion (Hoppins et al 2011) and on the capacity of Bax to modulate Mfn2 (but not Mfn1) properties and functions (Karbowski et al 2006), authors may consider using their FRET approach to investigate the specificity of these observations and to compare the behavior of Bcl-xL with that of other anti-apoptotic (Bcl-2 or Mcl-1) or pro-apoptotic proteins (Bax or Bak).
Discussion :
D.1 Authors state : 'Similar to the increased Drp1 GTPase activity by Bcl-XL, Bcl-XL may increase the GTPase activity of MFN2 to induce MFN2-dependent mitochondrial aggregation.' Authors need to cite and discuss relevant literature in the field reporting that Bcl-xL activates Mfn2-mediated mitochondrial fusion (Hoppins et al 2011, PMID 21255726) and increases mitochondrial fission, fusion and biomass in neurons (Berman et al 2009, PMID 19255249).

First revision

Author response to reviewers' comments

Point-by-Point response to the Reviewers' comments
***Note**: the main revisions of our manuscript are marked in red and our responses are in italic and red.*

Dear Jennifer Lippincott-Schwartz,
We would like to thank you very much for your hard works and diligent processing on our manuscript. We are very appreciating these positive and critical comments. According to these comments, we have made some modifications to our manuscript, and supply the revised version to you. Bellow you will find our point-by-point responses to the reviewer's comments.

Best regards, Tongsheng Chen
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Reviewer 1 Advance Summary and Potential Significance to Field:
In this work, the authors tried to link the apoptosis-related machinery with proteins involved in mitochondrial fusion, through live-cell FRET, a state-of-the-art technique. In a similar was as the authors have done previously on other proteins using the same technique, the interaction between Bcl-XL and mitofusins (MFNs) in live cells are carefully analyzed, and the stoichiometry of the interaction is calculated. Although the oligomerization of MFNs are known before, this information is of satisfactory novelty and not able to be gleaned by other means. Overall, this work represents an suitable application and exhibits great value of a high-end biophysical technology in the field of cell biology. On the other hand, some biological issues need to be clarified to show how interaction between MFNs and Bcl-XL impact apoptosis.
Response: Thank you very much for your positive and critical comments. As you say, biological issues need to be clarified to show how the interaction between MFNs and Bcl-XL impacts apoptosis. In Results section, our data showed that Bcl-XL complexed with MFN2/MFN1 maintained its full anti-apoptotic ability to STS. In other words, interaction between MFNs and Bcl-XL has no significant effect on apoptosis at least for STS-induced apoptosis. We also mentioned this question in the fourth paragraph of Discussion section. We proposed two regulation modes for explaining the full anti-apoptotic ability of MFN2-/MFN1-Bcl-XL complex: one mode is that Bcl-XL, MFN2/MFN1 and pro-apoptotic proteins can form heterotrimers; another mode is that association between Bcl-XL and MFN2/MFN1 is unstable, probably may be in kiss and run. According to your comments, we have revised the fourth paragraph of Discussion section as follow,

"A following question is whether Bcl-XL, mitofusins and pro-apoptotic proteins can form heterotrimers. Bcl-XL, an antiapoptotic Bcl-2 family protein, inhibits cells apoptosis mainly through inhibiting the activation of pro-apoptotic proteins (Willis et al., 2005; Hockings et al., 2018; Ku et al., 2011). The BH1, BH2 and BH3 domains of Bcl-XL are important for heterodimerization with other pro-apoptotic proteins (Yin et al., 1994; Cheng et al., 1996; Muchmore et al., 1996; Sattler et al., 1997). Our data showed that neither MFN2 nor MFN1 inhibited STS-induced cells apoptosis, but the Bcl-XL complexed with MFN2/MFN1 maintained its full anti-apoptotic ability to STS. In other words, interaction between MFN2/MFN1 and Bcl-XL has no significant effect on apoptosis at least for the apoptosis induced by STS. Two regulation modes can be used for explaining the full anti-apoptotic ability of MFN2-/MFN1-Bcl-XL complex: one mode is that Bcl-XL, MFN2/MFN1 and pro-apoptotic proteins can form heterotrimers; another mode is that association between Bcl- XL and MFN2/MFN1 is unstable, probably may be in kiss and run. Although previous report showed that the chimera between Bcl-XL and Bax (Bcl-XL/Bax H5) induced substantial mitochondrial fragmentation in healthy cells via binding to mitofusins (Cleland et al., 2011), it's not clear which domain of Bcl-XL plays a key role in binding with mitofusins. It may be worthwhile to explore which regulation mode is dominant."
On the other hand, we also have studied relative questions and modified our manuscript carefully.

References in this part:

Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., & Huang, D. C. (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Gene. Dev. 19, 1294–1305.
Hockings, C., Alsop, A. E., Fennell, S. C., Lee, E. F., Fairlie, W. D., Dewson, G., & Kluck, R. M. (2018) Mcl-1 and Bcl-xL sequestration of Bak confers differential resistance to BH3-only proteins. Cell Death Differ. 25, 721–734.
Ku, B. Liang, C., Jung, J. U. & Oh, B. H. (2011) Evidence that inhibition of BAX activation by BCL-2 involves its tight and preferential interaction with the BH3 domain of BAX. Cell Res. 21, 627–641.
Yin, X. M., Oltval, Z. M. & Korsmeyer, S. J. (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 369, 321–323.
Cheng, E. H. Y., Levine, B. & Boise, L. H. (1996) Thompson CB and Hardwick JM. Bax independent inhibition of apoptosis by Bcl-X(L). Nature 379, 554–556.
Muchmore S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S. L. et. al. (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature 381, 335–341.
Sattler, M., Liang, H., Nettesheim, D., Meadows, R. P., Harlan, J. E., Eberstadt, M., Yoon, H. S., Shuker, S. B., Chang, B. S., Minn, A. J. et. al. (1997) Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science 275, 983–986.
Cleland, M. M., Norris, K. L., Karbowski, M., Wang, C., Suen, D. F., Jiao, S., George, N. M., Luo, X., Li, Z., & Youle, R. J. (2011) Bcl-2 family interaction with the mitochondrial morphogenesis machinery. Cell Death Differ. 18, 235–247.

Reviewer 1 Comments for the Author: Suggestions:
1. The authors are suggested to perform a co-IP or equivalent assay using YFP/CFP alone as a negative control avoid potential false positive results.
Response: We agree with your option that negative control should be prepared to avoid potential false positive results. Our CFP and YFP plasmids have no other tags, and they have the same primary GFP antibody, which make co-IP assay between CFP and YFP, CFP and YFP-MFNs, or CFP-Bcl-XL and YFP impossible. However, we have MFN1 plasmid labeled with Myc tag (MFN1- Myc). Thus, we performed Co-IP assay between CFP and MFN1 by using the cells co-expressing CFP+ MFN1-Myc as follow,

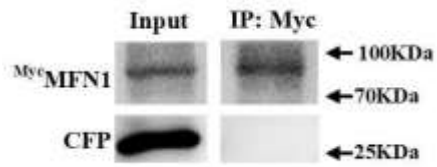

Fig. R1. Co-immunoprecipitation assay between CFP and MFN1. Lysates prepared from Hela cells co-transfected with CFP and MFN1-Myc for 24 h subjected to immunoprecipitation with anti-Myc antibody, followed by anti-Myc and anti-CFP immunoblotting.

We also added Pearson correlation analysis to quantify the distributions of CFP and YFP- MFN2/MFN1 as follow,

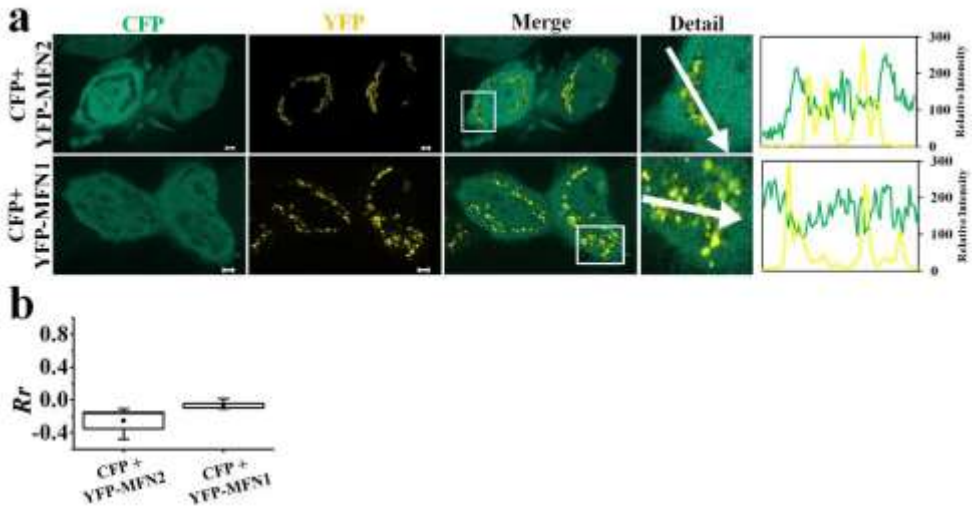

*Fig. R2. Colocalization analysis between CFP and MFN2/MFN1. **a** Fluorescence images of representative living Hela cells co-expressing CFP and YFP-MFN2/YFP-MFN1. Detail panels show information of the white boxes in the merge images, and intensity line profiles show the fluorescence intensities of CFP and YFP- along the selected white arrow lines in the merge panels. Scale bar: 10 μm. **b** Statistical R_r values for the colocalization between CFP and YFP- in cells co-expressing CFP and YFP-MFN2/YFP-MFN1. n ≥ 20 cells each.*

We revised the first paragraph of 3.2 section as follow,

"Quantitative FRET analysis was performed on our Zeiss Apotome.2 imaging system to quantify the homo-oligomerization of MFN2 and MFN1 in living cells co-expressing CFP-MFN2 and YFP-MFN2 (CFP-MFN2 + YFP-MFN2), and CFP-MFN1 + YFP-MFN1. To avoid potential false positive results, the cells co-expressing CFP + YFP, CFP-MFN2 + YFP-ActA (mitochondria- specific Act-A tail-anchor sequence, ActA is a control protein that does not bind to MFN2/MFN1) (Aranovich et al., 2012; Zhu et al., 1996), and CFP-MFN1 + YFP-ActA were used as negative samples. We also evaluated colocalization between CFP and YFP-MFN2/-MFN1 (Fig. S2 A and B), and performed Co-IP assay between CFP and MFN1 by using cells co-expressing CFP+ MFN1- Myc (Myc is a tag labeled with C-terminal of MFN1) (Fig. S2C). Pearson correlation analysis (R_r) showed that CFP cannot colocalized to MFN2/MFN1 (Fig. S2B), and Co-IP assay further confirmed CFP cannot bind to MFN1. Figure 2A showed the fluorescence images (DD, DA, AA) of cells co- expressing CFP + YFP (negative control), CFP-MFN2 + YFP-ActA (negative control), CFP-MFN1 + YFP-ActA (negative control), CFP-MFN2 + YFP-MFN2, and CFP-MFN1 + YFP-MFN1, respectively, and the corresponding pixel-to-pixel pseudo-color donor-centric FRET efficiency (E_D) and acceptor-centric FRET efficiency (E_A) as well as the concentration ratio (R_C) of total acceptor- to-donor images. Pixel-to-pixel E_D, E_A and R_C values from 30 cells were binned with 0.01 bin size of R_C, and the corresponding E_D-R_C and E_A-1/R_C plots were shown in Fig. 2B. The E_D or E_A values of cells co-expressing CFP + YFP, and CFP-MFN2/CFP-MFN1 + YFP-ActA were all lower than 0.05 in 0–4 of R_C or 1/R_C, while E_D or E_A values increased with R_C or 1/R_C, tended to be about 0.4 for the cells co-expressing CFP-MFN2 + YFP-MFN2 and tended to be about 0.2 for the cells co- expressing CFP-MFN1 + YFP-MFN1 when R_C or 1/R_C was larger than 2. E_D or E_A values of the cells co-expressing CFP-MFN2 + YFP-MFN2, or CFP-MFN1 + YFP-MFN1 were significantly higher than that of the cells co-expressing CFP + YFP, or CFP-MFN2/CFP-MFN1 + YFP-ActA in 0–4 of R_C, demonstrating that both MFN2 and MFN1 can form homotypic oligomers.

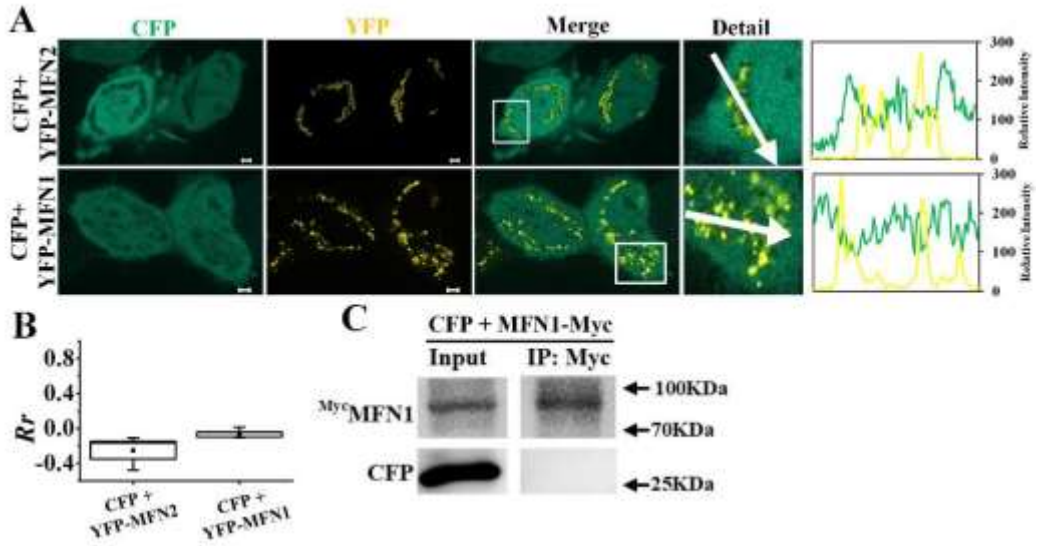

Fig. S2. CFP cannot bind to MFN2/MFN1. A Fluorescence images of representative living Hela cells co-expressing CFP and YFP-MFN2/YFP-MFN1. Detail panels show information of the white boxes in the merge images, and intensity line profiles show the fluorescence intensities of CFP and YFP- along the selected white arrow lines in the merge panels. Scale bar: 10 ¤m. B Statistical Rr values for the colocalization between CFP and YFP- in cells co-expressing CFP and YFP- MFN2/YFP-MFN1. n ≥ 20 cells each. C Co-immunoprecipitation assay between CFP and MFN1. Lysates prepared from Hela cells co-transfected with CFP and MFN1-Myc for 24 h subjected to immunoprecipitation with anti-Myc antibody, followed by anti-Myc and anti-CFP immunoblotting."

References in this part:

Aranovich A, Liu Q, Collins T, Geng F, Dixit S, Leber B, Andrews DW (2012) Differences in the mechanisms of proapoptotic BH3 proteins binding to Bcl-XL and Bcl-2 quantified in Live MCF- 7 cells. Mol Cell 45: 754-763.

Zhu W, Cowie A, Wasfy GW, Penn LZ, Leber B, Andrews DW (1996). Bcl-2 mutants with restricted subcellular location reveal spatially distinct pathways for apoptosis in different cell types. EMBO J 15: 4130-4141.

2. The authors claimed that the MFN2(K109A) or MFN1(K88T) was still able to bind Bcl-XL. The mutated lysine here belongs to the conserved G1 motif (or the P-loop) of all GTPases, which coordinate the phosphate groups of the bound guanine nucleotide. The authors can describe the role of this residue to show the rationale of choosing it in the context.

Response: In fact, our data showed that the MFN2(K109A) or MFN1(K88T) was unable to bind with Bcl-XL. We are sorry for our unclear and rough description about mutant of MFN2/MFN1. According to your comment, we have added the description for mutants in our manuscript and revised the second paragraph of 3.2 section as follow,

"To inspect hetero-oligomerization of MFN2 and MFN1, cells were co-transfected with CFP- MFN1 + YFP-MFN2. Fluorescence images showed that MFN2 co-localized with MFN1 well (Fig. 2C), which was further confirmed by the high *Rr* value up to 0.76 between MFN2 and MFN1 (Fig. 2D). Quantitative FRET two-hybrid assay was used to further quantify the hetero-oligomerization of MFN2 and MFN1. The fluorescence images (DD and AA) of representative cells co-expressing CFP-MFN1 + YFP-MFN2, and the corresponding pixel-to-pixel pseudo-color E_D and E_A as well as R_C images were shown in Fig. 2E. The E_D, E_A and R_C values from 60 cells (with bin size of 0.01 for R_C) were shown in E_D-R_C and E_A-1/R_C plots (Fig. 2F). E_D or E_A rapidly increased with R_C or 1/R_C, tended to be a stable value when R_C was larger than 2. E_{Amax} and E_{Dmax} can be estimated by finding best binding curve (Fig. 2F, white line) during fitting procedure according to equation 5 and 6. Therefore, the stoichiometry ratio (v, n_D/n_A) of MFN1 to MFN2 according to equation 7 by obtaining ratio of E_{Amax} to E_{Dmax} was 1.06 (Fig. 2F). Statistical v values from three independent experiments were 1.15±0.1, implying that MFN1 binds to MFN2 mainly with 1:1 stoichiometry. Live-cell FRET analysis also showed that GTPase domain mutants of MFN2 (MFN2(K109A), alanine replacement of lysine at residue 109 (K109A)) and MFN1 (MFN1(K88T), threonine replacement of lysine at residue 88 (K88T)) still have the ability of

forming homo-/hetero-oligomers (Fig. S3).”

We also revised the fifth paragraph of Discussion section as follow,
“Interestingly, the GTPase domain mutants of MFN2 (MFN2(K109A)) and MFN1 (MFN1(K88T)) still have the ability of forming oligomers, but lose the ability for binding with Bcl- XL. The two mutated lysine here belong to the conserved G2 or G1 (P-loop) motif of GTPases, which coordinate the phosphate groups of the bound guanine nucleotide (Yan et al., 2018), and the two mutants will decrease activity of GTPases (Yan et al., 2018; Ishihara et al., 2004; Detmer et al., 2007). In fact, we performed quantitative FRET assay for cells co-expressing CFP-labeled MFN2(K109A) and YFP-labeled MFN2(K109A), CFP-labeled MFN1(K88T) and YFP-labeled MFN1(K88T), or CFP-MFN1(K88T) and YFP-MFN2(K109A), respectively, and our results showed that MFN2(K109A) and MFN1(K88T) can form homo-/hetero-oligomers (Fig. S3), which is contrast to the previous report that MFN1(K88T)-FLAG does not coprecipitate with MFN1(K88T)-HA (Ishihara et al., 2004). However, FRET assay demonstrated no binding between MFN2(K109A) and Bcl-XL (Fig. S4C), and between MFN1(K88T) and Bcl-XL (Fig. S4C), which is contrast to our results of Co-IP assay (Fig. S4D) between CFP-Bcl-XL and MFN2(K109A)-Myc/ MFN1(K88T)-Myc. One reason is that different environments between living and apoptotic cells lead to different binding capacities between Bcl-XL and MFN2(K109A)/ MFN1(K88T) for explaining the controversial results. It is possible for that MFN2(K109A)/ MFN1(K88T) combined with Bcl-XL in t kiss and run in living cells, and our FRET assay based on averages of large amounts of data will weaken their combination but Co-IP assay may not detect this difference. These results confirm a notion that the GTPase domains of MFN2 and MFN1 play key roles in binding with Bcl- XL. Mutation of this residue normally won’t affect the overall folding of the protein, but binding of the nucleotide (Yan et al., 2018). Is this residue directly involved in the interface or this interaction dependent on the loading of GTP/GDP to MFNs? It may be worthwhile to explore. FRAP analysis and direct observation of fission/fusion all showed that Bcl-XL overexpression significantly increased the rate of mitochondrial fusion (Berman et al., 2009; Delivani et al., 2006). Addition of purified recombinant full-length monomeric soluble Bcl-xL also stimulated mitochondrial fusion in vitro in a dose-dependent manner (Hoppins et al 2011). In vitro GTPase assay performed with purified recombinant full-length Bcl-XL showed that Bcl-XL increased Drp1 GTPase activity (Li et al., 2008), indicating Bcl-XL may induce mitochondrial fragment by activating Drp1. Similar to the increased Drp1 GTPase activity by Bcl-XL, Bcl-XL may increase the GTPase activity of MFN2 to induce MFN2-dependent mitochondrial aggregation.”

References in this part:
Berman, S. B., Chen, Y. B., Qi, B., Mccaffery, J. M., Rucker, E. B., & Goebbels, S., Nave, K. A., Arnold,. K. A. Jonas, E. A., Pineda, F. J., & Hardwick, J. M., et al. (2009) Bcl-xl increases mitochondrial fission, fusion, and biomass in neurons. J. Cell Biol. 184, 707-719
Delivani, P., Adrain, C., Taylor, R. C., Duriez, P. J. & Martin, S. J. (2006) Role for CED-9 and Egl-1 as regulators of mitochondrial fission and fusion dynamics. Mol. Cell 21, 761-773.
Detmer, S. A., & Chan, D. C. (2007). Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. J. Cell Biol. 176, 405-414.
Hoppins, S., Edlich, F., Cleland, M. M., Banerjee, S., McCaffery, J. M., Youle, R. J., & Nunnari, J. (2011). The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes. Molecular cell, 41(2), 150-160.
Ishihara, N., Eura, Y. & Mihara, K. (2004) Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J. Cell Sci. 117, 6535-6546.
Li, H., Chen, Y., Jones, A. F., Sanger, R. H., Collis, L. P., Flannery, R., McNay, E. C., Yu, T., Schwarzenbacher, R., Bossy, B. et. al. (2008) Bcl-xL induces Drp1-dependent synapse formation in cultured hippocampal neurons. Proc. Natl. Acad. Sci. USA 105, 2169-2174.
*Yan, L., Qi, Y., Huang, X., Yu, C., Lan, L., Guo, X., Rao, Z., Hu, J., & Lou, Z. (2018) Structural basis for GTP hydrolysis and conformational change of MFN1 in mediating membrane fusion. Nat. Struct. Mol. Biol. **25**, 233-243.*

3. Further to this point, mutation of this residue normally won’t affect the overall folding of the protein, but binding of the nucleotide (hence GTP hydrolysis). Therefore, the authors should explain how this mutation affect the interaction between MFNs and Bcl-XL. i.e. is this residue directly involved in the interface, or the interaction is dependent on the loading of GTP/GDP to MFNs? It might be necessary to include these two mutants also in the co-IP/pull-down assay, and that is why I feel that point 1 is necessary. If possible, the G domain of MFN1/MFN2 alone should also be tested. If the authors decides to make a pull-down assay, the supply with different guanine nucleotide should

be considered.

Response: We are sorry for our rough description about relation between mutant of MFN2/MFN1 and Bcl-XL. We mentioned GTPase domain mutants of MFN2 (MFN2(K109A)) and MFN1 (MFN1(K88T)) and performed quantitative FRET assay between MFN2(K109A)/ MFN1(K88T) and Bcl-XL. FRET assay demonstrated MFN1 and MFN2 binds to Bcl-XL, but no binding between MFN2(K109A)/ MFN1(K88T) and Bcl-XL (Fig. S3B). However, we did not explain how this mutation affects the interaction between MFNs and Bcl-XL. As you say, mutant residue normally won't affect the overall folding of the protein, there are two reasons for explaining the relation between mutant of MFN2/MFN1 and Bcl-XL: one reason is that this residue directly involved in the interface, another reason is that this interaction is dependent on the loading of GTP/GDP to MFNs. It may be worthwhile to explore which reason is dominant and can be an independent topic to be explored in the further.

According to your comments that we should perform two mutants in the co-IP/pull-down assay, we performed Co-IP assay between Bcl-XL and MFN1/MFN2K109A/MFN1K88T as follow,

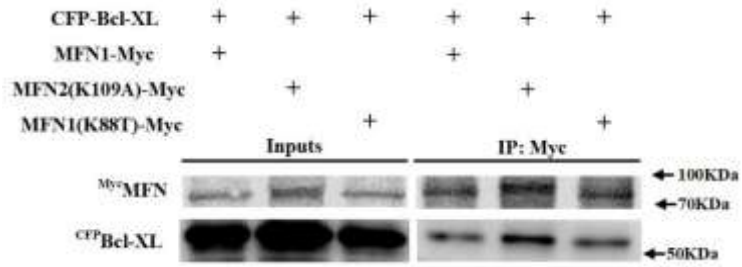

Fig. R3 Co-immunoprecipitation assay between Bcl-XL and MFN1/MFN2(K109A)/MFN1(K88T). Lysates prepared from Hela cells co-transfected with CFP-Bcl-XL and MFN1-Myc/MFN2(K109A)-Myc/MFN1(K88T)-Myc for 24 h subjected to immunoprecipitation with anti-Myc antibody, followed by anti-Myc and anti-CFP immunoblotting.

In contrast to our FRET assay, Co-IP assay showed that Bcl-XL co-immunoprecipitated with not only MFN1 but also MFN2(K109A) and MFN1(K88T). One reason of the controversial results is that different environments between live and apoptotic cells lead to different binding capacities between Bcl-XL and MFN2(K109A)/ MFN1(K88T). It is possible for that MFN2(K109A)/ MFN1(K88T) combined with Bcl-XL in kiss and run in living cells, and our FRET assay based on averages of large amounts of data will weaken their combination, whereas Co-IP assay may not detect this difference. These results confirmed the key roles of the GTPase domains of MFN2 and MFN1 in binding with Bcl-XL in living cells. Though study of GTPase domains of MFNs is not center of our article, their key roles need further study.

We also revised the fifth paragraph of Discussion section as follow,

"Interestingly, the GTPase domain mutants of MFN2 (MFN2(K109A)) and MFN1 (MFN1(K88T)) still have the ability of forming oligomers, but lose the ability for binding with Bcl- XL. The two mutated lysine here belong to the conserved G2 or G1 (P-loop) motif of GTPases, which coordinate the phosphate groups of the bound guanine nucleotide (Yan et al., 2018), and the two mutants will decrease activity of GTPases (Yan et al., 2018; Ishihara et al., 2004; Detmer et al., 2007). In fact, we performed quantitative FRET assay for cells co-expressing CFP-labeled MFN2(K109A) and YFP-labeled MFN2(K109A), CFP-labeled MFN1(K88T) and YFP-labeled MFN1(K88T), or CFP-MFN1(K88T) and YFP-MFN2(K109A), respectively, and our results showed that MFN2(K109A) and MFN1(K88T) can form homo-/hetero-oligomers (Fig. S3), which is contrast to the previous report that MFN1(K88T)-FLAG does not coprecipitate with MFN1(K88T)-HA (Ishihara et al., 2004). However, FRET assay demonstrated no binding between MFN2(K109A) and Bcl-XL (Fig. S4C), and between MFN1(K88T) and Bcl-XL (Fig. S4C), which is contrast to our results of Co-IP assay (Fig. S4D) between CFP-Bcl-XL and MFN2(K109A)-Myc/ MFN1(K88T)-Myc. One reason is that different environments between living and apoptotic cells lead to different binding capacities between Bcl-XL and MFN2(K109A)/ MFN1(K88T) for explaining the controversial results. It is possible for that MFN2(K109A)/ MFN1(K88T) combined with Bcl-XL in kiss and run in living cells, and our FRET assay based on averages of large amounts of data will weaken their combination but Co-IP assay may not detect this difference. These results confirm a notion that the GTPase domains of MFN2 and MFN1 play key roles in binding with Bcl- XL. Mutation of this residue normally won't affect the overall folding of the protein, but binding of the nucleotide (Yan et al., 2018). Is this residue directly involved in the interface or this interaction dependent on the loading of GTP/GDP to MFNs? It may be worthwhile to explore. FRAP analysis and

direct observation of fission/fusion all showed that Bcl-XL overexpression significantly increased the rate of mitochondrial fusion (Berman et al., 2009; Delivani et al., 2006). Addition of purified recombinant full-length monomeric soluble Bcl-xL also stimulated mitochondrial fusion in vitro in a dose-dependent manner (Hoppins et al 2011). In vitro GTPase assay performed with purified recombinant full-length Bcl-XL showed that Bcl-XL increased Drp1 GTPase activity (Li et al., 2008), indicating Bcl-XL may induce mitochondrial fragment by activating Drp1. Similar to the increased Drp1 GTPase activity by Bcl-XL, Bcl-XL may increase the GTPase activity of MFN2 to induce MFN2-dependent mitochondrial aggregation.

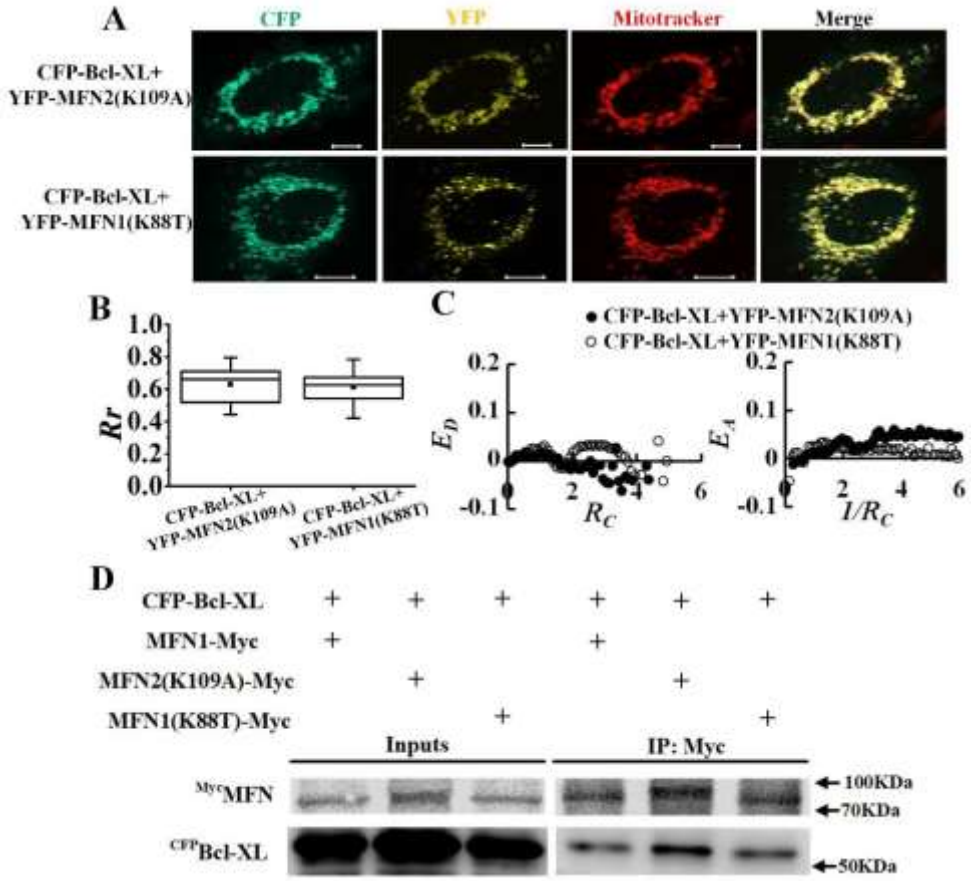

Figure S4. Interaction between Bcl-XL and GTPase domain mutants of both MFN2 and MFN1 (MFN2(K109A) and MFN1(K88T)). A Fluorescence images of representative living Hela cells co-expressing CFP-Bcl-XL + YFP-MFN2(K109A), CFP-Bcl-XL + YFP-MFN1(K88T), and the corresponding merge images. B Statistical Rr values for the colocalization between Bcl-XL and MFN2(K109A), and between Bcl-XL and MFN1(K88T). n ¤ 20 cells each. C ED-RC and EA-1/RC plots for cells co-expressing the indicated plasmids. n ¤ 60 cells each. D Co-immunoprecipitation assay between Bcl-XL and MFN1/MFN2(K109A)/MFN1(K88T). Lysates prepared from Hela cells co-transfected with CFP-Bcl-XL and MFN1-Myc/MFN2(K109A)-Myc/MFN1(K88T)-Myc for 24 h subjected to immunoprecipitation with anti-Myc antibody, followed by anti-Myc and anti-CFP immunoblotting."

References in this part:

Berman, S. B., Chen, Y. B., Qi, B., Mccaffery, J. M., Rucker, E. B., & Goebbels, S., Nave, K. A., Arnold,. K. A. Jonas, E. A., Pineda, F. J., & Hardwick, J. M., et al. (2009) Bcl-xl increases mitochondrial fission, fusion, and biomass in neurons. J. Cell Biol. 184, 707-719

Delivani, P., Adrain, C., Taylor, R. C., Duriez, P. J. & Martin, S. J. (2006) Role for CED-9 and Egl-1 as regulators of mitochondrial fission and fusion dynamics. Mol. Cell 21, 761-773.

Detmer, S. A., & Chan, D. C. (2007). Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. J. Cell Biol. 176, 405-414.

Hoppins, S., Edlich, F., Cleland, M. M., Banerjee, S., McCaffery, J. M., Youle, R. J., & Nunnari, J. (2011). The soluble form of Bax regulates mitochondrial fusion via MFN2 homotypic complexes. Molecular cell, 41(2), 150-160.

Ishihara, N., Eura, Y. & Mihara, K. (2004) Mitofusin 1 and 2 play distinct roles in mitochondrial

fusion reactions via GTPase activity. J. Cell Sci. 117, 6535-6546.
Li, H., Chen, Y., Jones, A. F., Sanger, R. H., Collis, L. P., Flannery, R., McNay, E. C., Yu, T., Schwarzenbacher, R., Bossy, B. et. al. (2008) Bcl-xL induces Drp1-dependent synapse formation in cultured hippocampal neurons. Proc. Natl. Acad. Sci. USA 105, 2169-2174.
*Yan, L., Qi, Y., Huang, X., Yu, C., Lan, L., Guo, X., Rao, Z., Hu, J., & Lou, Z. (2018) Structural basis for GTP hydrolysis and conformational change of MFN1 in mediating membrane fusion. Nat. Struct. Mol. Biol. **25**, 233-243.*

4. It seems that MFN1 and MFN2 binds to Bcl-XL in more or less the same way (e.g. with 1:1 stoichiometry as the authors claimed), while the author also found that only MFN2 but not MFN1 mediates Bcl-XL induced mitochondrial aggregation. What is the reason for that? The authors should at least make a discussion on it.

Response: We are sorry for our unclear description. In fact, in the second paragraph, we discuss why only MFN2 but not MFN1 mediates Bcl-XL induced mitochondrial aggregation. Different roles of MFN2 and MFN1 in regulating mitochondrial morphology may result in more key roles of MFN2 than MFN1 in Bcl-XL induced mitochondrial aggregation. We revised the second paragraph of Discussion section as follow,

"More key roles of MFN2 than MFN1 in Bcl-XL induced mitochondrial aggregation may be due to the different roles MFN2 and MFN1 in regulating mitochondrial morphology. Live-cell FRET two-hybrid assays showed that both MFN2 and MFN1 bind to Bcl-XL for forming heterotypic oligomers, which is consistent with previous co-IP assays (Cleland et al., 2011) in Hela cells. However, in HEK293T cells, previous Co-IP assays showed that Bcl-XL selectively coprecipitated MFN2 but not MFN1 (Delivani et al., 2006), implying different functions between MFN2 and MFN1. Indeed, previous report showed that knocked down MFN2 induced more cells with thick fragmented mitochondria, but knocked down MFN1 induced more cells with short tubular mitochondria (Eura et al., 2003), consistent with our results (Fig. 4C and 4E). MFN2 mutant embryos have a severe disruption of the placental trophoblast giant cell layer, but MFN1-deficient giant cells are normal (Chen et al., 2003). Katsuyoshi's group also showed that MFN1 and MFN2 exhibited distinct activity in a GTP-dependent mitochondrial tethering reaction and purified recombinant MFN1 exhibited about eightfold higher GTPase activity than MFN2 (Ishihara et al., 2004). Recent structural studies of truncated MFN1 and MFN2 (Cao et al., 2017; Li et al., 2019) revealed that unlike MFN1, MFN2 forms sustained dimers even after GTP hydrolysis to maintain its high membrane tethering efficiency. Our data that the values of E_D/E_A for cells co-expressing CFP-MFN2 and YFP-MFN2 were higher than that of the cells co-expressing CFP-MFN1 and YFP- MFN1 (Fig. 2A) support the view that membrane tethering efficiency of MFN2 is higher than that of MFN1. Accordingly, we propose that MFN1 play a direct role in maintaining mitochondrial elongation, while MFN2 play a direct role in maintaining mitochondrial tethering. Mitochondrial aggregation induced by Bcl-XL is mainly by regulating MFN2-mediated mitochondrial tethering. Our data verify the notion that MFN2 and MFN1 play different roles in regulating mitochondrial morphology."

References in this part:

Cao, Y. L., Meng, S., Chen, Y., Feng, J. X., Gu, D. D., Yu, B., Li, Y. J., Yang, J. Y., Liao, S., Chan, D. C. & Gao, S. (2017) MFN1 structures reveal nucleotide-triggered dimerization critical for mitochondrial fusion. Nature 542, 372-376.
Chen, H., Detmer, S. A., Ewald, A. J., Griffin, E. E., Fraser, S. E., & Chan, D. C. (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J. Cell Biol. 160, 189-200.
Cleland, M. M., Norris, K. L., Karbowski, M., Wang, C., Suen, D. F., Jiao, S., George, N. M., Luo, X., Li, Z., & Youle, R. J. (2011) Bcl-2 family interaction with the mitochondrial morphogenesis machinery. Cell Death Differ. 18, 235-247.
Delivani, P., Adrain, C., Taylor, R. C., Duriez, P. J. & Martin, S. J. (2006) Role for CED-9 and Egl-1 as regulators of mitochondrial fission and fusion dynamics. Mol. Cell 21, 761-773.
Eura, Y., Ishihara, N., Yokota, S. & Mihara, K. (2003) Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. J. Biochem. 134, 333-344.
Ishihara, N., Eura, Y. & Mihara, K. (2004) Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J. Cell Sci. 117, 6535-6546.
Li, Y. J., Cao, Y. L., Feng, J. X., Qi, Y., Meng, S., Yang, J. F., Zhong, Y. T., Kang, S., Chen, X., Lan, L. et. al. (2019) Structural insights of human mitofusin-2 into mitochondrial fusion and CMT2A

onset. Nat. Commun. 10, 1-14.

5. Some grammatical issues. For example, page 11, line 11, "mitochondrial aggregate cells" is a mistake. Also, this sentence is way too long, better chop it into two parts. Please carefully proofread the manuscript to improve its readability."
*Response: We are very sorry for this unintentional mistake and this sentence "*Interestingly, results from three independent experiments showed that mitochondrial aggregate cells occupied approximately 45% in the cells expressing Bcl-XL and transfected with siCtrl, and 44% in the cells expressing Bcl-XL and transfected with siMFN1 (Fig. 4F).*" has been revised as*
"Results from three independent experiments showed that cells with aggregated mitochondria occupied approximately 45% in the cells expressing Bcl-XL and transfected with siCtrl.

Interestingly, cells with aggregated mitochondria still occupied approximately 44% in the cells expressing Bcl-XL and transfected with siMFN1 (Fig. 4F)."
We also carefully proofread the manuscript to improve its readability.

Reviewer 2 Advance Summary and Potential Significance to Field:
The properties and dynamics of mitochondrial membranes are modulated by a variety of specific factors that mediate and/or catalyze the permeabilization of the outer membrane during apoptosis (notably proteins of the Bcl-2 family of proteins) and the fusion and division of mitochondria (notably dynamin-related proteins/Drps like Mfn1 and Mfn2, OPA1, and Drp1). Several reports have revealed links between both processes (permeabilization and dynamics), involvement of Bcl-2 family proteins in fusion/fission dynamics as well as modulation of apoptotic membrane permeabilization by Drps. The field is challenging, with numerous open questions and controversial findings.
The authors of this manuscript have expressed/transfected several outer membrane proteins tagged with CFP or YFP. They have used fluorescence microscopy and/or FRET to investigate protein localization, study interactions between CFP and YFP tagged proteins and characterize the consequences of protein expression.
Authors show that overexpressed Mfn1 and Mfn2 localize to mitochondria, provoke mitochondrial aggregation and interact with each other to form homo- and hetero-oligomers. These Mfn-properties are well-established facts. Thorough quantitative analysis of FRET led authors to conclude on 1:1 stoichiometry of Mfn-Mfn interactions.
Authors show that overexpressed Bcl-xL, but not Mfn1 or Mfn2, protect against staurosporine induced apoptosis. The anti-apoptotic activity of Bcl-xL is well established and the results with Mfn1 and Mfn2 apparently contradict findings reported by Sugioka et al. (2004); authors cite this study, but do not discuss this apparent contradiction.
Response: Our results that overexpresion of Bcl-xL but not Mfn1 or Mfn2 protects against staurosporine (STS)-induced apoptosis seems to have contradict the findings that Fzo1 inhibits etoposide/anti-Fas antibody-induced apoptosis (Sugioka et al., 2004). Etoposide is in the topoisomerase inhibitor family of medication and it is believed to work by damaging DNA (Loike and Horwitz, 1976; Kalwinsky et al., 1983). Fzo1 may delay apoptosis by inhibiting apoptotic mitochondrial fission to compensate DNA damage induced by etoposide. Mitochondrial fusion may be sufficient to delay Fas-induced apoptosis, but not enough to delay STS-induced apoptosis. It is also possible that mitochondrial fusion more or less delay apoptosis, but at least in our experiment, anti-apoptotic function of MFN2/MFN1 is not comparable to that of Bcl-XL.
We have added one paragraph after the third paragraph of Discussion section to discuss this question as follow,
"Our results that overexpresion of Bcl-XL but not MFN1 or MFN2 protects against STS- induced apoptosis seems to have contradict the findings that Fzo1 inhibits etoposide/anti-Fas antibody-induced apoptosis (Sugioka et al., 2004). Etoposide is in the topoisomerase inhibitor family of medication and it is believed to work by damaging DNA (Loike and Horwitz, 1976; Kalwinsky et al., 1983). Fzo1 may delay apoptosis by inhibiting apoptotic mitochondrial fission to compensate DNA damage induced by etoposide. Mitochondrial fusion may be sufficient to delay Fas-induced apoptosis, but not enough to delay STS-induced apoptosis. It is also possible that mitochondrial fusion more or less delay apoptosis, but at least in our experiment, anti-apoptotic function of MFN2/MFN1 is not comparable to that of Bcl-XL."

References in this part:
Kalwinsky, D. K., Look, A. T., Ducore, J., & Fridland, A. (1983) Effects of the epipodophyllotoxin VP-16-213 on cell cycle traverse, DNA synthesis, and DNA strand size in cultures of human leukemic lymphoblasts. Cancer Res. 43, 1592-1597.
Loike, J. D., & Horwitz SB. (1976) Effect of VP-16-213 on the intracellular degradation of DNA in HeLa cells. Biochemistry. 15, 5443-5448.
Sugioka, R., Shimizu, S. & Tsujimoto, Y. (2004) Fzo1, a protein involved in mitochondrial fusion, inhibits apoptosis. J. Biol. Chem. 279, 52726-52734.

Authors show that overexpressed Bcl-xL can cluster mitochondria and interact with mitofusins, as reported by Delivani et al. 2006. Quantitative analysis of FRET led authors to conclude on 1:1 stoichiometry of Mfn-Bcl-xL interactions.
The FRET experiments are well performed and analyzed and contain appropriate controls. However, the manuscript does not provide data that improve our understanding on the roles of Mfn1, Mfn2 or Bcl-xL in fusion and/or permeabilization of mitochondrial outer membranes. In addition, the manuscript has several points that would merit revision and/or correction. Some are listed below.
Response: Thank you very much for your positive and critical comments. In this report, we firstly performed live-cell FRET assay to explore the regulation between mitofusins and Bcl-XL. our data showed MFN2/MFN1 associates with Bcl-XL to form complexes with 1:1 stoichiometry, and MFN2 but not MFN1 participates in Bcl-XL-mediated mitochondrial aggregation. Although our results did not break out of roles of MFN2/MFN1 in fusion and Bcl-XL in anti-apoptosis, our data verify the notion that MFN2 and MFN1 play different roles in regulating mitochondrial morphology. We propose that MFN1 play a direct role in maintaining mitochondrial elongation, while MFN2 play a direct role in maintaining mitochondrial tethering. Mitochondrial aggregation induced by Bcl-XL is mainly by regulating MFN2-mediated mitochondrial tethering. Our data also showed MFN2/MFN1 cannot protect cells against staurosporine induced apoptosis and neither MFN2 nor MFN1 alters the anti-apoptotic ability of Bcl-XL, which indicates that mitochondrial fusion and apoptosis are also two independent events in cells. These findings encourage us to continue exploring the relationship between mitochondrial dynamics and apoptosis. We also studied these relative questions and carefully modified our manuscript.

Reviewer 2 Comments for the Author: Introduction:
I.1 reviews cited to describe the role(s) of fusion/fission dynamics or mitofusins in ATP-production (Brookes et al 2004), mtDNA maintenance (Wallace 2005), apoptosis (Wang and Youle 2009) do not describe the roles of fusion/fission dynamics or mitofusins in these processes.
Response: According to your comments, we revised the first paragraph of Introduction section as follow,
"Mitochondria undergoes continuous cycles between fission and fusion to maintain proper morphology and physiological functions, including ATP production (Brookes et al., 2004), mitochondrial DNA (mtDNA) maintenance (Wallace, 2005) and regulation of apoptosis (Jagasia et al., 2005; Wang and Youle, 2009). Dynamic mitochondrial behavior is believed to ensure appropriate mitochondrial distribution to provide ATP to localized cytosolic regions (Brookes et al., 2004). Frequent fusion and fission may be an efficient means of intermitochondrial DNA complementation through exchange of genomes between fusing mitochondria (Nakada et al., 2001). Mitochondrial fragmentation may participate in apoptosis (Olichon et al., 2002). Mitochondrial fusion defect results in fragmented mitochondria, even participates in the pathogenesis of Charcot– Marie–Tooth disease type 2A (Züchner et al., 2004), whereas reduced mitochondrial fission leads to elongated, hyper-fused mitochondria even delays caspase activation and cell death (Labbé et al., 2014; Karbowski et al., 2002; Sugioka et al., 2004; Breckenridge et al., 2003). Mitochondrial dynamics are regulated by a set of dynamin-related proteins. In mammalian cells, mitochondrial fission is mainly mediated by GTPase dynamin-related protein 1 (Drp1) (Smirnova et al., 2001) which are recruited from cytoplasm to mitochondria by several outer mitochondrial membrane (OMM) proteins (mitochondrial fission factor, Mid51, Mid49, and fission 1) (Gandre-Babbe and van der Bliek, 2008; Palmer et al., 2011; James et al., 2003), assembling into a helical ring-like structure to 'drawstring' constrict mitochondria. Mitochondrial fusion involves OMM fusion and inner mitochondrial membrane (IMM) fusion. Although OMM fusion is highly coordinated with IMM fusion to maintain integrity of organelle under normal conditions, they can also be uncoupled in cells (Labbé et al., 2014; Legros et al., 2002; Meeusen et al., 2004). Mitofusin 1 and 2 (MFN1 and MFN2), two dynamin-related GTPases embedded in OMM, are essential for mitochondrial outer membrane fusion in mammalian cells (Cao et al., 2017), while optic atrophy 1 (OPA1) mediates IMM fusion (Ishihara et al., 2006)."

References in this part:

Breckenridge, D. G., Stojanovic, M., Marcellus, R. C. & Shore, G. C. (2003) Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome c release to the cytosol. J. Cell Biol. 160, 1115-1127.
Brookes, Yoon, Y., Robotham, J. L., Anders, M. W. & Sheu, S. S. (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am. J. Physiol. Cell Physiol. 287, C817-33.
Cao, Y. L., Meng, S., Chen, Y., Feng, J. X., Gu, D. D., Yu, B., Li, Y. J., Yang, J. Y., Liao, S., Chan, D. C. & Gao, S. (2017) MFN1 structures reveal nucleotide-triggered dimerization critical for mitochondrial fusion. Nature 542, 372-376.
Gandre-Babbe, S. & van der Bliek, A. M. (2008) The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. Mol. Biol. Cell 19, 2402- 2412.
Ishihara, N., Fujita, Y., Oka, T. & Mihara, K. (2006) Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO J. 25, 2966-2977.
Jagasia, R., Grote, P., Westermann, B. & Conradt, B. (2005) DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. Nature 433, 754-760.
James, D. I., Parone, P. A., Mattenberger, Y. & Martinou, J. C. (2003) hFis1, a novel component of the mammalian mitochondrial fission machinery. J. Biol. Chem. 278, 36373-36379.
Karbowski, M., Lee, Y. J., Gaume, B., Jeong, S. Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C. L., & Youle, R. J. (2002) Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J. Cell Biol. 159, 931-938.
Labbé, K., Murley, A., & Nunnari, J. (2014) Determinants and functions of mitochondrial behavior. Ann. Rev. Cell Dev. Biol. 30, 357-391.
Legros, F., Lombès, A., Frachon, P. & Rojo, M. (2002) Mitochondrial fusion in human cells is efficient, requires the inner membrane potential, and is mediated by mitofusins. Mol. Biol. Cell 13, 4343-4354.
Meeusen, S., McCaffery, J. M. & Nunnari, J. (2004) Mitochondrial fusion intermediates revealed in vitro. Science 305, 1747-1752.
Nakada, K., Inoue, K., Ono, T., Isobe, K., Ogura, A., Goto, Y. I., Nonaka, I. & Hayashi, J. I. (2001) Inter-mitochondrial complementation: mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. Nat. Med. 7, 934-940.
Olichon, A., Baricault, L., Gas, N., Guillou, E., Valette, A., Belenguer, P. & Lenaers, G. (2002) Loss of OPA1 perturbates the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. J. Biol. Chem. 278, 7743-7746.
Palmer, C. S., Osellame, L. D., Laine, D., Koutsopoulos, O. S., Frazier, A. E., & Ryan, M. T. (2011) MiD49 and MiD51, new components of the mitochondrial fission machinery. EMBO Rep. 12, 565-573.
Smirnova, E., Griparic, L., Shurland, D. L. & Van Der Bliek, A. M. (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol. Biol. Cell 12, 2245-2256.
Sugioka, R., Shimizu, S. & Tsujimoto, Y. (2004) Fzo1, a protein involved in mitochondrial fusion, inhibits apoptosis. J. Biol. Chem. 279, 52726-52734.
Wang, C. & Youle, R. J. (2009) The role of mitochondria in apoptosis. Annu. Rev. Genet. 43, 95-118.
Züchner, S., Mersiyanova, I. V., Muglia, M., Bissar-Tadmouri, N., Rochelle, J., Dadali, E. L., Zappia, M., Nelis, E., Patitucci, A., Senderek, J. et. al. (2004) Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot–Marie–Tooth neuropathy type 2A. Nat. Genet. 36, 449- 451.

I.2 Lee et al 2016 does not describe any role of fission in caspase activation and cell death (but the involvement of Dyn-2 in mitochondrial fission).

Response: We are sorry for our mistake and we have deleted this reference in the first paragraph of Introduction section as follow,

"Mitochondrial fusion defect results in fragmented mitochondria, even participates in the pathogenesis of Charcot-Marie-Tooth disease type 2A (Züchner et al., 2004), whereas reduced mitochondrial fission leads to elongated, hyper-fused mitochondria even delays caspase activation and cell death (Labbé et al., 2014; Karbowski et al., 2002; Sugioka et al., 2004; Breckenridge et al., 2003)."

I.3 Cao et al., 2017 describes the 3D structure of MFN1-fragments, not the role/involvement of Mfns in OMM fusion.

*Response: We are sorry for our mistake and this sentence "*Mitofusin 1 and 2 (MFN1 and MFN2,

mitofusins), two dynamin-related GTPases embedded in OMM, are essential for mitochondrial outer membrane fusion in mammalian cells (Cao et al., 2017), while optic atrophy 1 (OPA1) mediates IMM fusion (Ishihara et al., 2002).*" has been revised as*
"Mitofusin 1 and 2 (MFN1 and MFN2, mitofusins), two dynamin-related GTPases embedded in OMM, are essential for mitochondrial outer membrane fusion in mammalian cells (Santel and Fuller, 2001), while optic atrophy 1 (OPA1) mediates IMM fusion (Ishihara et al., 2002)."

I.4 authors assume or affirm that mitochondrial fusion and aggregation are similar (or even identical) events at least in the context of Bcl-xL expression and/or mitochondrial apoptosis, as described in publications by the Martin group (Delivani 2006, Sheridan 2008). It is not clear that this applies, as inactive Mfn molecules also induce aggregation (see R.1).
*Response: Indeed, we don't distinguish mitochondrial fusion and aggregation, as described in publications by the Martin group (Delivani 2006, Sheridan 2008). Fusion is different from aggregation in mitochondrial morphology. Fused mitochondria should display extensive mitochondrial networks consisting of long tubules and aggregated mitochondria should display mitochondrial clustering in the perinuclear region. Inactive Mfn molecules also induce aggregation but not fusion. We agree with your option that mitochondrial fusion should distinguished from mitochondrial aggregation. This sentence "*Expression of Bcl-XL in Hela cells produced two distinct mitochondrial phenotypes including fused mitochondria (aggregated mitochondria) and fragmented mitochondria: aggregated mitochondria increased but fragmented mitochondria decreased with increasing Bcl-XL (Delivani et al., 2006; Sheridan et al., 2008).*" has been revised as*

"Expression of Bcl-XL in Hela cells produced two distinct mitochondrial phenotypes including aggregated mitochondria (mitochondrial clustering in the perinuclear region) and fragmented mitochondria: aggregated mitochondria increased but fragmented mitochondria decreased with increasing Bcl-XL (Delivani et al., 2006; Sheridan et al., 2008)."

I.5 It is surprising that, in the context of this paper, authors do not cite work revealing the capacity of recombinant Bcl-XL to modulate Mfn-dependent fusion (Hoppins et al 2011, PMID 21255726) or the role of Bax and Bak to modulate mitochondrial morphogenesis via mitofusins (Karbowski et al 2006, PMID 17035996).
Response: We apologize for our no description of capacity of recombinant Bcl-XL to modulate Mfn-dependent fusion, and the role of Bax and Bak to modulate mitochondrial morphogenesis via mitofusins. We have added corresponding content and revised the third paragraph of Introduction section as follow,
"Bcl-2 family proteins, as central players of the mitochondrion-dependent apoptotic program, participate in mitochondrial dynamic regulation (Gross and Katz, 2017). Bcl-2 family proteins is normally divided into multidomain antiapoptotic Bcl-2, Bcl-XL and Mcl-1, multidomain pro- apoptotic Bax and Bak, and pro-apoptotic BH3-only proteins (Chen et al., 2015). Apoptotic stimuli activate Bax and Bak to coalesce on the surface of the OMM, and form large foci which are responsible for mitochondrial outer membrane permeabilization (MOMP) (Chen et al., 2015) and culminate in release of cytochrome c into the cytosol, subsequent caspase activation and apoptosis. In addition to apoptotic role, Bax and Bak colocalize with DRP1 and MFN2 at prospective mitochondrial fission sites during apoptosis (Nechushtan et al., 2001; Valentijn et al., 2003; Karbowski et al., 2002; Youle and Karbowski, 2005). Quantitation of mitochondrial dynamics by photolabeling mitochondrial matrix shows that mitochondrial fusion is blocked during Bax activation phase of apoptosis independently of caspase activation (Karbowski et al., 2004). In 2006, Karbowski et al. demonstrated that Bax or Bak is required for normal fusion of mitochondria into elongated tubules in healthy cells (Karbowski et al., 2006) and Bax seems to induce activation of MFN2. Moreover, another study shows that Bak interacts with MFN1 and MFN2 (Brooks et al., 2007) in healthy Hela cells using co-immunoprecipitation (co-IP) assay and acceptor photobleaching-based fluorescence resonance energy transfer (FRET) measurement, but during apoptosis, Bak dissociates from MFN2 and enhances the association with MFN1 using co-IP assay. Indeed, it was demonstrated in a vitro experiment system of mitochondrial fusion that soluble form of Bax, positively regulates mitochondrial fusion exclusively through homotypic MFN2 trans complexes (Hoppins et al., 2011). Antiapoptotic proteins (Bcl-2, Bcl-XL and Mcl-1) preserve mitochondrial integrity to inhibit cells apoptosis mainly through inhibiting activation of pro- apoptotic proteins, and Bcl-XL have higher ability than Bcl-2 and Mcl-1 in preventing apoptosis owing to its dual inhibition of pro-apoptotic Bax and Bak as well as higher protein stability (Chen et al., 2015; Willis et al., 2005; Hockings et al., 2018). Interestingly, Bcl-XL may tip the balance in favor of fusion or fission depending on Bcl-XL relative expression level (Delivani

et al., 2006; Sheridan et al., 2008). Expression of Bcl-XL in Hela cells produced two distinct mitochondrial phenotypes including fused mitochondria (aggregated mitochondria) and fragmented mitochondria: aggregated mitochondria increased but fragmented mitochondria decreased with increasing Bcl-XL (Delivani et al., 2006; Sheridan et al., 2008). However, Mcl-1 failed to perturb mitochondrial networks in HeLa cells at any of the plasmid concentrations tested (Sheridan et al., 2008). Previous report also showed that add of purified recombinant full-length monomeric soluble Bcl-XL stimulated mitochondrial fusion in vitro in a dose-dependent manner (Hoppins et al., 2011). This finding is consistent with the observation that overexpression of Bcl-xL increases mitochondrial connectivity in cells (Delivani et al., 2006; Berman et al., 2009). In vitro GTPase assay with purified recombinant full-length Bcl-XL and full-length human Drp1 showed that Bcl-XL increased Drp1 GTPase activity (Li et al., 2008). Bcl-XL selectively co-immunoprecipitated MFN2 but not MFN1 or OPA1 in HEK293T cells (Delivani et al., 2006). However, co-IP experiments showed that Bcl- XL bond to both MFN1 and MFN2 in Hela cells (Cleland et al., 2011) and a Bcl-XL-Bax chimera (containing helix 5 (H5) of Bax replacing H5 of Bcl-XL (Bcl-XL/Bax H5)) bond to mitofusins better than to either wild-type Bax or Bcl-XL and this chimera can induce substantial mitochondrial fragmentation in healthy cells (Cleland et al., 2011)."

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Materials and Methods

M.1 Authors indicate that plasmids were purchased from Addgene. This is not the case, authors need to acknowledge plasmid as indicated in Addgene's web page (https://help.addgene.org/hc/en- us/articles/205432559-How-do-I-cite-a-plasmid-that-I-received-from-Addgene-in-future- publications-): ' The plasmids you have received were created by your colleagues. Please acknowledge the Principal Investigator, cite the article listed on the plasmid datasheet, and include the Addgene plasmid number in future publications.'

Response: we have revised the first paragraph of Materials and Methods section as follow, "MFN2-YFP was a gift from RichardYoule (Addgene plasmid #28010; http://n2t.net/addgene:28010; RRID:Addgene_28010) (Karbowski et al., 2002). MFN1-Myc was a gift from David Chan (Addgene plasmid #23212; http://n2t.net/addgene:23212; RRID:Addgene_23212) (Chen et al., 2003). MFN2(K109A)-16xmyc was a gift from David Chan (Addgene plasmid #26051; http://n2t.net/addgene:26051; RRID:Addgene_26051). MFN1(K88T)-10xmyc was a gift from David Chan (Addgene plasmid #26050; http://n2t.net/addgene:26050; RRID:Addgene_26050). ECFP-Bak was a gift from Richard Youle (Addgene plasmid #31501; http://n2t.net/addgene:31501; RRID:Addgene_31501) (Nechushtan et al., 2001). pEYFP-C1-Drp1 was a gift from Richard Youle (Addgene plasmid #45160; http://n2t.net/addgene:45160; RRID:Addgene_45160) (Frank et al., 2001). pcDNA3-CFP was a gift from Doug Golenbock (Addgene plasmid #13030; http://n2t.net/addgene:13030; RRID:Addgene_13030). pcDNA3-YFP was a gift from Doug Golenbock (Addgene plasmid #13033; http://n2t.net/addgene:13033 ; RRID:Addgene_13033). CFP-Bcl-XL and CFP-Bax were kindly supplied by A. P. Gilmore (Valentijn et al., 2003). The YFP-G4-CFP (CFP-YFP dimer, C4Y) was kindly provided by Christian Wahl-Schott (Butz et al., 2016). mCherry-ActA (mitochondria-specific Act-A tail-anchor sequence, ActA is a control protein that does not bind to MFN2/MFN1) were kindly provided by David W. Andrews (Aranovich et al., 2012). For synthesis of CFP-Mcl-1 plasmid as previously described (Wang et al., 2020). To generate a plasmid encoding CFP fused to MFN2/MFN1/MFN2(K109A)/MFN1(K88T) (CFP-MFN2/CFP-MFN1/CFP-MFN2(K109A)/CFP-MFN1(K88T)), the coding region for MFN2/MFN1/MFN2(K109A)/MFN1(K88T) was prepared by PCR from the MFN2/MFN1/MFN2(K109A)/MFN1(K88T) cDNA of MFN2-YFP/MFN1-Myc/MFN2(K109A)-16xMyc/MFN1(K88T)-10xMyc and was ligated into pECFP-C1 vector obtained by double enzyme digestion (XhoI-BamHI) of ECFP-Bak plasmid. YFP-MFN2/YFP- MFN1/YFP-MFN2(K109A)/YFP-MFN1(K88T) was generated by ligating coding region of MFN2/MFN1/MFN2(K109A)/MFN1(K88T) into pEYFP-C1 vector obtained by double enzyme digestion (XhoI-BamHI) of pEYFP-C1-Drp1 plasmid. CFP/YFP was fused to N-terminal of MFN2/MFN1/MFN2(K109A)/MFN1(K88T) to generate CFP-MFNs or YFP-MFNs, similar to described by Brooks et al (Brooks et al. 2007). Schematic to YFP-MFNs and CFP-Bcl-XL were shown in Fig. S1. We previously constructed YFP-ActA using coding region of ActA from mCherry-ActA and YFP- from YFP-Bak (Yang et al., 2019)."

References in this part:

Aranovich, A., Liu, Q., Collins, T., Geng, F., Dixit, S., Leber, B., & Andrews, D. W. (2012) Differences in the mechanisms of proapoptotic BH3 proteins binding to Bcl-XL and Bcl-2 quantified in live MCF-7 cells. Mol. cell 45, 754-763.

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M.2 The nature and origin of the YFP-ActA (a control plasmid?) is not indicated.
Response: We are sorry for our no describing origin of the YFP-ActA. mCherry-ActA (mitochondria-specific Act-A tail-anchor sequence, ActA is a control protein that does not bind to MFN2/MFN1) were kindly provided by David W. Andrews (Aranovich et al., 2012). We previously constructed YFP-ActA using coding region of ActA from mCherry-ActA and YFP- from YFP-Bak (Yang et al., 2019). We have added above content about origin of YFP-ActA in DNA Constructs and siRNAs section as follow,
"MFN2-YFP was a gift from Richard Youle (Addgene plasmid #28010; http://n2t.net/addgene:28010 ; RRID:Addgene_28010) (Karbowski et al., 2002). MFN1-Myc was a gift from David Chan (Addgene plasmid #23212; http://n2t.net/addgene:23212; RRID:Addgene_23212) (Chen et al., 2003). MFN2(K109A)-16xmyc was a gift from David Chan (Addgene plasmid #26051; http://n2t.net/addgene:26051; RRID:Addgene_26051). MFN1(K88T)- 10xmyc was a gift from David Chan (Addgene plasmid #26050; http://n2t.net/addgene:26050; RRID:Addgene_26050). ECFP-Bak was a gift from Richard Youle (Addgene plasmid #31501; http://n2t.net/addgene:31501; RRID:Addgene_31501) (Nechushtan et al., 2001). pEYFP-C1-Drp1 was a gift from Richard Youle (Addgene plasmid #45160; http://n2t.net/addgene:45160; RRID:Addgene_45160) (Frank et al., 2001). pcDNA3-CFP was a gift from Doug Golenbock (Addgene plasmid #13030; http://n2t.net/addgene:13030; RRID:Addgene_13030). pcDNA3-YFP was a gift from Doug Golenbock (Addgene plasmid #13033; http://n2t.net/addgene:13033 ; RRID:Addgene_13033). CFP-Bcl-XL and CFP-Bax was kindly supplied by A. P. Gilmore (Valentijn et al., 2003). The YFP-G4-CFP (CFP-YFP dimer, C4Y) was kindly provided by Christian Wahl-Schott (Butz et al., 2016). mCherry-ActA (mitochondria-specific Act-A tail-anchor sequence, ActA is a control protein that does not bind to MFN2/MFN1) were kindly provided by David W. Andrews (Aranovich et al., 2012). For synthesis of CFP-Mcl-1 plasmid as previously described (Wang et al., 2020). To generate a plasmid encoding CFP fused to MFN2/MFN1/MFN2(K109A)/MFN1(K88T) (CFP-MFN2/CFP-MFN1/CFP-MFN2(K109A)/CFP-MFN1(K88T)), the coding region for MFN2/MFN1/MFN2(K109A)/MFN1(K88T) was prepared by PCR from the MFN2/MFN1/MFN2(K109A)/MFN1(K88T) cDNA of MFN2-YFP/MFN1-Myc/MFN2(K109A)-16xMyc/MFN1(K88T)-10xMyc and was ligated into pECFP-C1 vector obtained by double enzyme digestion (XhoI-BamHI) of ECFP-Bak plasmid. YFP-MFN2/YFP- MFN1/YFP-MFN2(K109A)/YFP-MFN1(K88T) was generated by ligating coding region of MFN2/MFN1/MFN2(K109A)/MFN1(K88T) into pEYFP-C1 vector obtained by double enzyme digestion (XhoI-BamHI) of pEYFP-C1-Drp1 plasmid. CFP/YFP was fused to N-terminal of MFN2/MFN1/MFN2(K109A)/MFN1(K88T) to generate CFP-MFNs or YFP-MFNs, similar to described by Brooks et al (Brooks et al. 2007). Schematic to YFP-MFNs and CFP-Bcl-XL were shown in Fig. S1. We previously constructed YFP-ActA using coding region of ActA from mCherry-ActA and YFP- from YFP-Bak (Yang et al., 2019)."

References in this part:
Aranovich, A., Liu, Q., Collins, T., Geng, F., Dixit, S., Leber, B., & Andrews, D. W. (2012) Differences in the mechanisms of proapoptotic BH3 proteins binding to Bcl-XL and Bcl-2 quantified in live MCF-7 cells. Mol. cell 45, 754-763.
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Results

R.1 It is noteworthy that the aggregation induced by overexpression of Mfn1, Mfn2 or Bcl-xL, described as fusion-profile in publications of the Martin group (see I.4), is not observed at lower expression levels achieved by viral transduction (e.g. Chen et al 2003, cited by the authors), but occurs upon high level expression (transfection) of fusion-competent wild-type Mfn and fusion-incompetent mutant Mfn (Eura et al. 2003, cited by the authors).

Response：Indeed, mitochondrial aggregation only occurs upon overexpression of Mfn1, Mfn2 or Bcl-xL. Mfn-deficient cells had dramatically fragmented mitochondria, but Mfn-deficient cells infected with Mfn-expressing retrovirus showed predominantly tubular (Chen et al., 2003). High level expression (transfection) of Mfn1, Mfn2, Bcl-xL or incompetent mutant Mfn will induce mitochondrial aggregation (Delivani et al., 2006; Sheridan et al., 2008; Eura et al., 2003). Mitochondrial aggregation may be one step probably one necessary step for mitochondrial fusion. Other steps or factors, such as nucleotide, are also needed to assist mitochondrial aggregation to achieve mitochondrial fusion. We should describe it clearly in the text and we have added these discussions into the third paragraph of Discussion section as follow,

“Our data also indicate that both MFN2 and MFN1 form homo-dimers to regulate mitochondrial aggregation. Analysis of crystal structure in the HR2 domain of MFN1 resolved that HR2 polypeptide folds into a dimeric antiparallel coiled coil (Koshiba et al., 2004). Later, structural and biochemical analysis revealed that the HR2 in the MFN1 may form part of the predicted helix bundle region and membrane tethering likely needs active hydrolysis of GTP to form nucleotide- dependent dimerization of MFN1 (Qi et al., 2016). In 2017, Gao’s group highlighted that dimerization of the GTPase domains of MFN1 was regulated by guanine nucleotide (Cao et al., 2017). Recently, Gao’s group also proposed that MFN2 remained dimerized after GTP hydrolysis, which is contrast to the GTP hydrolysis-dependent dimerization of MFN1 (Li et al., 2019). Here, live-cell FRET assays demonstrate the homo-oligomerization of both MFN2 and MFN1 (Fig. 2A). To further resolve the MFN2/MFN1 homo-oligomers, we used another FRET analysis method (*ED = (1-(1/(1+RC))^(n-1))*EDmax*, where n is the number of homo-oligomers) just as described previously (Adair and Engelman, 1994; Meyer et al., 2006) to evaluate the data in Fig. 2A, and the n value obtained by fitting ED-RC plots was 2.4 for the cells co-expressing CFP-MFN2 and YFP- MFN2, and was 2.0 for the cells co-expressing CFP-MFN1 and YFP-MFN1, indicating that both MFN2 and MFN1 regulate mitochondrial fusion likely through forming dimers. Mitochondrial aggregation only occurs upon overexpression of MFN1, MFN2 or Bcl-XL but not lower expression levels of that. MFN-deficient cells had dramatically fragmented mitochondria, but MFN-deficient cells infected with MFN-expressing retrovirus showed predominantly tubular (Chen et al., 2003). High level expression of MFN1, MFN2, Bcl-xL or incompetent mutant MFN will induce mitochondrial aggregation (Delivani et al., 2006; Sheridan et al., 2008; Eura et al., 2003). Mitochondrial aggregation may be just because of much more MFN2 and MFN1 forming dimers. Mitochondrial aggregation may be just one step, probably one necessary step, for mitochondrial fusion. Other steps or factors, such as nucleotide, are also needed to assist mitochondrial aggregation to achieve mitochondrial fusion. Specific regulation mechanism of mitochondrial fusion or mitochondrial aggregation calls for further study.”

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Sheridan, C., Delivani, P., Cullen, S. P. & Martin, S. J. (2008) Bax-or Bak-induced mitochondrial fission can be uncoupled from cytochrome C release. Mol. Cell, 31, 570-585.

R.2 FRET efficacy depending on distance and orientation between fluorophores, authors should indicate the position of the FP-tag within the sequence and/or the recently published 3D structures (references cited by authors: Cao et al., 2017; Qi et al., 2016).
Response: In this report, CFP/YFP was fused to N-terminal of MFN2/MFN1/MFN2(K109A)/MFN1(K88T) to generate CFP-MFNs or YFP-MFNs, similar to described by Brooks et al (Brooks et al. 2007). According to your comments, we added schematic to indicate position of the FP-tag within the sequence of target proteins as follow,

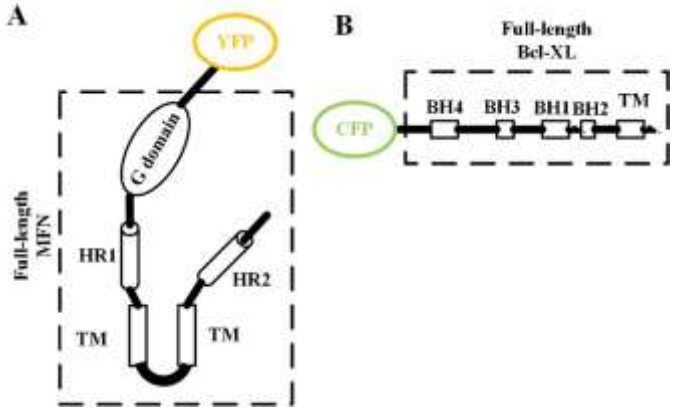

Fig. S1 Schematic to YFP-MFN (A) and CFP-Bcl-XL(B). G domain, GTPase domain; HR1, heptad repeat region 1; HR2, heptad repeat region 2; TM, transmembrane region. BH, conserved Bcl-2 homology domains.
We also revised the responding description in Material and methods as follow,
"MFN2-YFP was a gift from Richard Youle (Addgene plasmid #28010; http://n2t.net/addgene:28010 ; RRID:Addgene_28010) (Karbowski et al., 2002). MFN1-Myc was a gift from David Chan (Addgene plasmid #23212; http://n2t.net/addgene:23212; RRID:Addgene_23212) (Chen et al., 2003). MFN2(K109A)-16xmyc was a gift from David Chan (Addgene plasmid #26051; http://n2t.net/addgene:26051; RRID:Addgene_26051). MFN1(K88T)- 10xmyc was a gift from David Chan (Addgene plasmid #26050; http://n2t.net/addgene:26050; RRID:Addgene_26050). ECFP-Bak was a gift from Richard Youle (Addgene plasmid #31501; http://n2t.net/addgene:31501; RRID:Addgene_31501) (Nechushtan et al., 2001). pEYFP-C1-Drp1 was a gift from Richard Youle (Addgene plasmid #45160; http://n2t.net/addgene:45160; RRID:Addgene_45160) (Frank et al., 2001). pcDNA3-CFP was a gift from Doug Golenbock (Addgene plasmid #13030; http://n2t.net/addgene:13030; RRID:Addgene_13030). pcDNA3-YFP was a gift from Doug Golenbock

(Addgene plasmid #13033; http://n2t.net/addgene:13033 ; RRID:Addgene_13033). CFP-Bcl-XL and CFP-Bax were kindly supplied by A. P. Gilmore (Valentijn et al., 2003). The YFP-G4-CFP (CFP-YFP dimer, C4Y) was kindly provided by Christian Wahl-Schott (Butz et al., 2016). mCherry-ActA (mitochondria-specific Act-A tail-anchor sequence, ActA is a control protein that does not bind to MFN2/MFN1) were kindly provided by David W. Andrews (Aranovich et al., 2012). For synthesis of CFP-Mcl-1 plasmid as previously described (Wang et al., 2020). To generate a plasmid encoding CFP fused to MFN2/MFN1/MFN2(K109A)/MFN1(K88T) (CFP-MFN2/CFP-MFN1/CFP-MFN2(K109A)/CFP-MFN1(K88T)), the coding region for MFN2/MFN1/MFN2(K109A)/MFN1(K88T) was prepared by PCR from the MFN2/MFN1/MFN2(K109A)/MFN1(K88T) cDNA of MFN2-YFP/MFN1-Myc/MFN2(K109A)-16xMyc/MFN1(K88T)-10xMyc and was ligated into pECFP-C1 vector obtained by double enzyme digestion (XhoI-BamHI) of ECFP-Bak plasmid. YFP-MFN2/YFP- MFN1/YFP-MFN2(K109A)/YFP-MFN1(K88T) was generated by ligating coding region of MFN2/MFN1/MFN2(K109A)/MFN1(K88T) into pEYFP-C1 vector obtained by double enzyme digestion (XhoI-BamHI) of pEYFP-C1-Drp1 plasmid. CFP/YFP was fused to N-terminal of MFN2/MFN1/MFN2(K109A)/MFN1(K88T) to generate CFP-MFNs or YFP-MFNs, similar to described by Brooks et al (Brooks et al. 2007). Schematic to YFP-MFNs and CFP-Bcl-XL were shown in Fig. S1. We previously constructed YFP-ActA using coding region of ActA from mCherry- ActA and YFP- from YFP-Bak (Yang et al., 2019)."

References in this part:

Aranovich, A., Liu, Q., Collins, T., Geng, F., Dixit, S., Leber, B., & Andrews, D. W. (2012) Differences in the mechanisms of proapoptotic BH3 proteins binding to Bcl-XL and Bcl-2 quantified in live MCF-7 cells. Mol. cell 45, 754-763.

Brooks, C., Wei, Q., Feng, L., Dong, G., Tao, Y., Mei, L., Xie, Z. J., & Dong, Z. (2007). Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. Proc. Natl. Acad. Sci. 104(28), 11649-11654.

Butz, E. S., Ben-Johny, M., Shen, M., Yang, P. S., Sang, L., Biel, M., Yue, D. T., & Wahl-Schott, C. (2016) Quantifying macromolecular interactions in living cells using FRET two-hybrid assays. Nat. Protoc. 11, 2470-2498.

Chen, H., Detmer, S. A., Ewald, A. J., Griffin, E. E., Fraser, S. E., & Chan, D. C. (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J. Cell Biol. 160, 189-200.

Frank, S., Gaume. B., Bergmann-Leitner, E. S., Leitner, W. W., Robert, E. G., Catez, F., Smith, C. L., & Youle, R. J. (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell. 1, 515-525.

Karbowski, M., Lee, Y. J., Gaume, B., Jeong, S. Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C. L., & Youle, R. J. (2002) Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J. Cell Biol. 159, 931-938.

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Wang, Y., Su, W. H., Mai, Z. H., Yu, S., & Chen, T. S. (2020) Anti-apoptotic capacity of mcl-1Δ127. Biochem. Biophys. Res. Commun. 526, 1042-1048.

Yang, F., Qu, W., Du, M., Mai, Z., Wang, B., Ma, Y., W, X., & Chen, T. (2019) Stoichiometry and regulation network of Bcl-2 family complexes quantified by live-cell FRET assay. Cell. Mol. Life Sci. https://xs.scihub.ltd/https://doi.org/10.1007/s00018-019-03286-z.

R.3 'GTPase domain mutants'. Authors should indicate the nature as well as the predicted and/or known consequences of the mutations studied (the expression 'one traditional mutant' used in the discussion is not precise).

Response: We are sorry for our unclear and rough description about mutant of MFN2/MFN1. According to your comment, we have added the description for mutants in our manuscript and revised the second paragraph of 3.2 section as follow,

"To inspect hetero-oligomerization of MFN2 and MFN1, cells were co-transfected with CFP- MFN1 + YFP-MFN2. Fluorescence images showed that MFN2 co-localized with MFN1 well (Fig. 2C), which was further confirmed by the high *Rr* value up to 0.76 between MFN2 and MFN1 (Fig. 2D). Quantitative FRET two-hybrid assay was used to further quantify the hetero-oligomerization of MFN2 and MFN1. The fluorescence images (DD and AA) of representative cells co-expressing CFP-MFN1 + YFP-MFN2, and the corresponding pixel-to-pixel pseudo-color *ED* and *EA* as well as *RC* images were shown in Fig.

2E. The *ED*, *EA* and *RC* values from 60 cells (with bin size of 0.01 for *RC*) were shown in *ED*-*RC* and *EA*-1/*RC* plots (Fig. 2F). *ED* or *EA* rapidly increased with *RC* or 1/*RC*, tended to be a stable value when *RC* was larger than 2. *EAmax* and *EDmax* can be estimated by finding best binding curve (Fig. 2F, white line) during fitting procedure according to equation 5 and 6. Therefore, the stoichiometry ratio (*v, nD*/*nA*) of MFN1 to MFN2 according to equation 7 by obtaining ratio of *EAmax* to *EDmax* was 1.06 (Fig. 2F). Statistical *v* values from three independent experiments were 1.15±0.1, implying that MFN1 binds to MFN2 mainly with 1:1 stoichiometry. Live-cell FRET analysis also showed that GTPase domain mutants of MFN2 (MFN2(K109A), alanine replacement of lysine at residue 109 (K109A)) and MFN1 (MFN1(K88T), threonine replacement of lysine at residue 88 (K88T)) still have the ability of forming homo-/hetero-oligomers (Fig. S1).”

We also revised the fifth paragraph of Discussion section as follow,

“Interestingly, the GTPase domain mutants of MFN2 (MFN2(K109A)) and MFN1 (MFN1(K88T)) still have the ability of forming oligomers, but lose the ability for binding with Bcl- XL. The two mutated lysines here belong to the conserved G2 or G1 (P-loop) motif of GTPases, which coordinate the phosphate groups of the bound guanine nucleotide (Yan et al., 2018), and the two mutants will decrease activity of GTPases (Yan et al., 2018; Ishihara et al., 2004; Detmer et al., 2007). In fact, we performed quantitative FRET assay for cells co-expressing CFP-labeled MFN2(K109A) and YFP-labeled MFN2(K109A), CFP-labeled MFN1(K88T) and YFP-labeled MFN1(K88T), or CFP-MFN1(K88T) and YFP-MFN2(K109A), respectively, and our results showed that MFN2(K109A) and MFN1(K88T) can form homo-/hetero-oligomers (Fig. S3), which is contrast to the previous report that MFN1(K88T)-FLAG does not coprecipitate with MFN1(K88T)-HA (Ishihara et al., 2004). However, FRET assay demonstrated no binding between MFN2(K109A) and Bcl-XL (Fig. S4C), and between MFN1(K88T) and Bcl-XL (Fig. S4C), which is contrast to our results of Co-IP assay (Fig. S4C) between CFP-Bcl-XL and MFN2(K109A)-Myc/ MFN1(K88T)-Myc. One reason is that different environments between living and apoptotic cells lead to different binding capacities between Bcl-XL and MFN2(K109A)/ MFN1(K88T) for explaining the controversial results. It is possible for that MFN2(K109A)/ MFN1(K88T) combined with Bcl-XL in the form of kiss and run in living cells, and our FRET assay based on averages of large amounts of data will weaken their combination but Co-IP assay may not detect this difference. These results confirm a notion that the GTPase domains of MFN2 and MFN1 play key roles in binding with Bcl-XL. Mutation of this residue normally won’t affect the overall folding of the protein, but binding of the nucleotide (Yan et al., 2018). Is this residue directly involved in the interface or this interaction dependent on the loading of GTP/GDP to MFNs? It may be worthwhile to explore. FRAP analysis and direct observation of fission/fusion all showed that Bcl-XL overexpression significantly increased the rate of mitochondrial fusion (Berman et al., 2009; Delivani et al., 2006). Addition of purified recombinant full-length monomeric soluble Bcl-xL also stimulated mitochondrial fusion in vitro in a dose-dependent manner (Hoppins et al 2011). In vitro GTPase assay performed with purified recombinant full-length Bcl-XL showed that Bcl-XL increased Drp1 GTPase activity (Li et al., 2008), indicating Bcl-XL may induce mitochondrial fragment by activating Drp1. Similar to the increased Drp1 GTPase activity by Bcl-XL, Bcl-XL may increase the GTPase activity of MFN2 to induce MFN2-dependent mitochondrial aggregation.”

References in this part:

Berman, S. B., Chen, Y. B., Qi, B., Mccaffery, J. M., Rucker, E. B., & Goebbels, S., Nave, K. A., Arnold,. K. A. Jonas, E. A., Pineda, F. J., & Hardwick, J. M., et al. (2009) Bcl-xl increases mitochondrial fission, fusion, and biomass in neurons. J. Cell Biol. 184, 707-719

Delivani, P., Adrain, C., Taylor, R. C., Duriez, P. J. & Martin, S. J. (2006) Role for CED-9 and Egl-1 as regulators of mitochondrial fission and fusion dynamics. Mol. Cell 21, 761-773.

Detmer, S. A., & Chan, D. C. (2007). Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. J. Cell Biol. 176, 405-414.

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Ishihara, N., Eura, Y. & Mihara, K. (2004) Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J. Cell Sci. 117, 6535-6546.

Li, H., Chen, Y., Jones, A. F., Sanger, R. H., Collis, L. P., Flannery, R., McNay, E. C., Yu, T., Schwarzenbacher, R., Bossy, B. et. al. (2008) Bcl-xL induces Drp1-dependent synapse formation in cultured hippocampal neurons. Proc. Natl. Acad. Sci. USA 105, 2169-2174.

Yan, L., Qi, Y., Huang, X., Yu, C., Lan, L., Guo, X., Rao, Z., Hu, J., & Lou, Z. (2018) Structural basis for GTP hydrolysis and conformational change of MFN1 in mediating membrane fusion. Nat. Struct.

Mol. Biol. 25, 233-243.

R.4 Given earlier reports on the capacity of Bax or Bcl-xL to stimulate Mfn-mediated fusion (Hoppins et al 2011) and on the capacity of Bax to modulate Mfn2 (but not Mfn1) properties and functions (Karbowski et al 2006), authors may consider using their FRET approach to investigate the specificity of these observations and to compare the behavior of Bcl-xL with that of other anti- apoptotic (Bcl-2 or Mcl-1) or pro-apoptotic proteins (Bax or Bak).
Response: According your comments, we used FRET approach to investigate interaction between MFNs and other anti-apoptotic Mcl-1 or pro-apoptotic proteins Bax and Bak. Our data showed that MFN2/1 binds with clustering Bax but not monomeric Bax. MFN1 rather than MFN2 binds with Bak. MFN1 and MFN2 can also bind with Mcl-1. We also added these data to Results section as follow,

"3.6 Interactions between MFN2/MFN1 and other Bcl-2 family proteins

We also studied the combination between MFN2/MFN1 and pro-apoptotic proteins Bax/ Bak or anti-apoptotic Mcl-1 in the same way. To research co-localization of MFN2 or MFN1 with Bax in healthy (control) or apoptosis cells, cells co-transfected with CFP-Bax and YFP-MFN2 or YFP- MFN1 for 24 h were treated without (control) or with STS for 4h, and then stained by using DilC1(5) for 30 min before fluorescence imaging. Our previous report (Yang et al., 2019) has proved that STS indeed induce Bax translocation from cytosol to mitochondria and subsequent Bax clusters formation. In contrast to uniform distribution of CFP-Bax in control cells (Fig. 6A, upper panel), CFP-Bax distribute punctuate clusters in STS-treated cells (Fig. 6A, upper panel) and partially colocalized with YFP-MFN2. Similarly, CFP-Bax clusters also partially colocalized with YFP- MFN1 (Fig. 6A, lower panel). Pearson correlation analysis showed that R_r values (Fig. 6B) between CFP-Bax and YFP-MFN2, or CFP-Bax and YFP-MFN1 were 0.12 ± 0.06 or 0.10 ± 0.07 in control
cells, but were 0.57±0.13 or 0.50 ± 0.15 in STS-treated cells, suggesting that MFN2 or MFN1 colocalizes with Bax in apoptotic cells rather than healthy cells.
Next, we further analyzed combination between MFN2 or MFN1 and Bax using quantitative FRET analysis. Fig. 4C showed representative fluorescence images of cells co-expressing CFP-Bax and YFP-MFN2, or CFP-Bax and YFP-MFN1 in control cells or STS-treated cells, and the corresponding pixel-to-pixel pseudo-color E_D and E_A as well as R_C images. E_D values in 0-6 of R_C or E_A values in 0-6 of $1/R_C$ between MFN2/1 and Bax in control cells were about 0, much lower than that in STS-treated cells (Fig. 6D), further confirming that MFN2/1 can only bind with clustering Bax but not monomeric Bax. We next assessed stoichiometry of Bax and MFN1 in apoptosis cells by using FRET two-hybrid assay. Results from three independent experiments (62 cells for each experiment) showed that v values (Fig. 6E) were 0.96 ± 0.1 for cells co-expressing CFP-Bax and YFP-MFN2, and 0.9 ± 0.1 for cells co-expressing CFP-Bax and YFP-MFN1, revealing that either MFN2 or MFN1 binds with clustering Bax with 1:1 stoichiometry ratio.

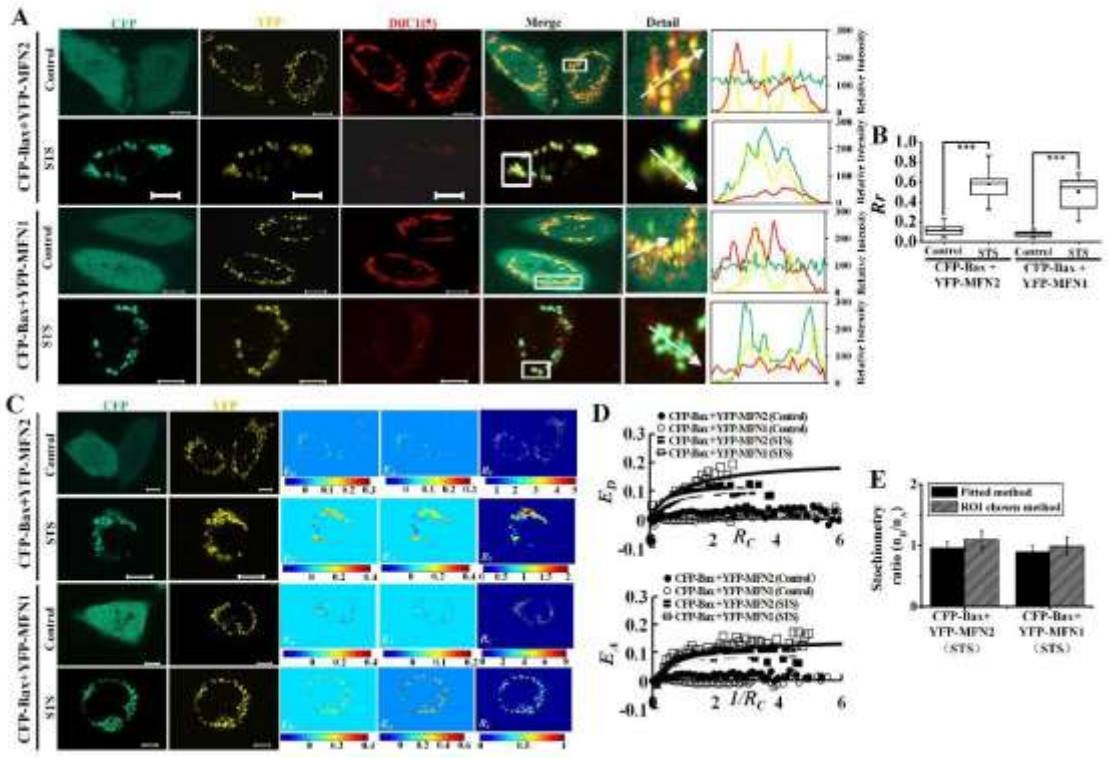

Fig. 6. MFN2/MFN1 binds with clustering Bax but not monomeric Bax. **A** Fluorescence images of representative living or STS-treated Hela cells co-expressing CFP-Bax and YFP-MFN2/MFN1, and stained with DilC1(5). Detail panel show information of white boxes in the merge images, and intensity line profiles show the fluorescence intensities of CFP-, YFP- and DilC1(5) along the selected white arrow line in the merge panels. **B** Statistical *Rr* values for the colocalization between Bax and MFN2/MFN1 in living or STS-treated Hela cells expressing CFP-Bax and YFP- MFN2/MFN1, n =20 cells. **C** Fluorescence images (DD and AA) of representative living or STS- treated cells co-expressing CFP-Bax and YFP-MFN2/MFN1, and corresponding pixel-to-pixel pseudo-color *ED* and *EA* as well as *RC* images. Scale bar: 10 ¤m. **D** *ED*-*RC* and *EA*-1/*RC* plots (0.01 bin size of *RC*) from living or STS-treated cells co-expressing CFP-Bax and YFP-MFN2/MFN1 (n= 62 cells each). **E** Stoichiometry ratio (*v*, *nD*/*nA*) of Bax to MFN2/MFN1 in STS-treated cells from three independent experiments. Data represent as averages ± SD.

To inspect the co-location of MFN2 or MFN1 with another pro-apoptotic Bak protein, cells were co-transfected with CFP-Bak and YFP-MFN2 or YFP-MFN1 in the presence of Z-VAD-FMK. 14 h after transfection, cells were stained with DilC1(5) for 30 min and subsequently were imaged using our system. Fluorescence images (Fig. 7A) showed that mitochondria were not stained by DilC1(5) in cells expressing CFP-Bak, but both YFP-MFN2 and YFP-MFN1 co-localized with Bak. Interestingly, Pearson correlation analysis showed that averaging *Rr* value (Fig. 7B) between CFP- Bak and YFP-MFN1 was 0.60 ± 0.17, higher than 0.39± 0.16 between CFP-Bak and YFP-MFN2, implying that relationship between MFN2 and Bak may different from that between MFN1 and Bak. Next, quantitative FRET measurement was further used to analyze relationship between MFN2/MFN1 and Bak/Mcl-1. Representative cells (Fig. 7C) co-expressing CFP-Bak and YFP- MFN1 showed higher *ED* and *EA* values than that of cells co-expressing CFP-Bak and YFP-MFN2. Statistical results (Fig. 7D) (60 cells) showed that *ED* or *EA* values of cells co-expressing CFP-Bak and YFP-MFN2 were all lower than 0.05 in 0–4 of *RC* or *1*/*RC*, while in cells co-expressing CFP- Bak and YFP-MFN1, *EA* value increased with 1/*RC*, tended to be over 0.15 when 1/*RC* was larger than 2. Therefore, FRET assay results also confirmed difference indeed existing between MFN2 and MFN1, and MFN1 but not MFN2 can bind with Bak. FRET assay also showed that MFN1 and

MFN2 can bind with Mcl-1 (Fig. 7 E and F).

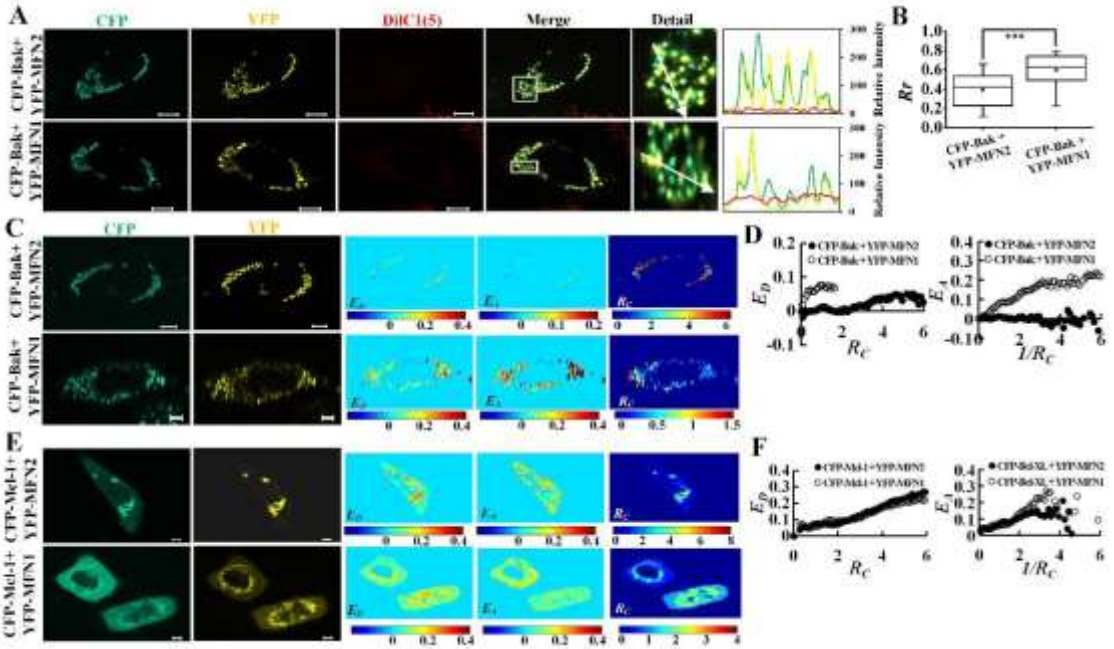

Fig. 7. FRET assay between MFN2/MFN1 and Bak/Mcl-1. **A** Fluorescence images of representative Hela cells co-expressing CFP-Bak and YFP-MFN2/MFN1, and stained with DilC1(5). Detail panel show information of white boxes in the merge images, and intensity line profiles show the fluorescence intensities of CFP-, YFP- and DilC1(5) along the selected white arrow line in the merge panels. **B** Statistical *Rr* values for the colocalization between Bak and MFN2/MFN1 in Hela cells expressing CFP-Bak and YFP-MFN2/MFN1, n = 20 cells. **C** Fluorescence images (DD and AA) of representative cells co-expressing CFP-Bak and YFP-MFN2/MFN1, and corresponding pixel-to-pixel pseudo-color *ED* and *EA* as well as *RC* images. Scale bar: 10 ¤m. **D** *ED-RC* and *EA*-1/*RC* plots (0.01 bin size of *RC*) from cells co-expressing CFP-Bak and YFP-MFN2/MFN1 (n = 60 cells each). **E** Fluorescence images (DD and AA) of representative cells co-expressing CFP-Mcl-1 and YFP-MFN2/MFN1, and corresponding pixel-to-pixel pseudo-color *ED* and *EA* as well as *RC* images. Scale bar: 10 ¤m. **F** *ED-RC* and *EA*-1/*RC* plots (0.01 bin size of *RC*) from cells co-expressing CFP-Mcl-1 and YFP-MFN2/MFN1 (n = 60 cells each).”

We also added some discussions to Discussion section as follow,

“Several studies focus on uncovering roles of Bax in regulating mitofusins to balance mitochondrial networks. The mitochondria in cells lacking Bax and Bak are shorter, have less network continuity and lower fusion rates (Karbowski et al., 2006). In vitro fusion assay demonstrated that soluble form of Bax, positively regulates mitochondrial fusion exclusively through homotypic MFN2 trans complexes (Hoppins et al., 2007) and yeast two-hybrid analysis indicates that Bax selectively interacts with MFN2 and not MFN1. co-IP experiments show that Bax bound both MFN2 and MFN1 in Hela cells (Cleland et al., 2011). In apoptosis cells, Bax and Bak colocalize with MFN2 (Karbowski et al., 2002; Youle and Karbowski, 2005), mitochondrial fusion is blocked by the accumulation of membrane-inserted, oligomerized Bax (Karbowski et al., 2004, Hoppins et al., 2007). The difference in these results may due to experimental environment. Our live-cell FRET results show no directly binding relation between Bax and MFN2 or MFN1 in healthy cells, but both Bax-MFN2 and Bax-MFN1 complexes exist in apoptosis cells. We reason that monomeric Bax may have two ways, including directly binding MFN2 and indirectly mediating another protein, to activate MFN2, and the second way dominates in living healthy Hela cells. In apoptosis cells, each Bax in Bax clusters can bind one MFN2 or MFN1 and oligomerized Bax seems to inhibit activation of MFN2 or MFN1 by directly binding with them.

In contrast to oligomerized Bax associating both MFN2 and MFN1 in apoptosis cells, Bak selectively associate with MFN1 in apoptosis Hela cells. Previous co-IP assay (Brooks et al., 2007) performed in Hela cells show that Bak interacts with MFN1 and MFN2 in healthy Hela cells, but Bak dissociates from MFN2 and enhances the association with MFN1 in apoptosis cells, which is consistent with our results. It is difficult to obtain cells with *RC* of MFN1 to Bak larger than 2, which further confirm the view that only oligomerized Bak can associate MFN1. Moreover, from *EA-1/RC* and *ED-RC* plots for cells co-expressing Bak and MFN1, *ED or EA* values both increased with *RC* or 1/*RC*, but *EA* do not tend to be a constant value when 1/*RC* was larger than 2 even 4, probably because of multiple binding

forms existing between Bak and MFN1. In addition, neither MFN2 nor MFN1 prevent apoptosis induced by oligomerized Bak (Fig. 7D) in our experiment, which is different from previous study that MFN2/MFN1 expression delay Bak activation (Sugioka et al., 2004). This difference may due to that overexpressed Bak have so strong ability to auto-oligomerize without apoptotic stimulus, but MFN2/MFN1 does not have the same ability as Bcl-XL to prevented mitochondrial Bak oligomerization (Willis et al., 2005; Hockings et al., 2018)."

Reference in this part:

Yang, F., Qu, W., Du, M., Mai, Z., Wang, B., Ma, Y., W, X., & Chen, T. (2019) Stoichiometry and regulation network of Bcl-2 family complexes quantified by live-cell FRET assay. Cell. Mol. Life Sci. https://xs.scihub.ltd/https://doi.org/10.1007/s00018-019-03286-z.
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Cleland, M. M., Norris, K. L., Karbowski, M., Wang, C., Suen, D. F., Jiao, S., George, N. M., Luo, X., Li, Z., & Youle, R. J. (2011). Bcl-2 family interaction with the mitochondrial morphogenesis machinery. Cell Death Differ. 18(2), 235.
Brooks, C., Wei, Q., Feng, L., Dong, G., Tao, Y., Mei, L., Xie, Z. J., & Dong, Z. (2007). Bak regulates mitochondrial morphology and pathology during apoptosis by interacting with mitofusins. Proc. Natl. Acad. Sci. 104(28), 11649-11654.
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Willis, S. N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J. I., Adams, J. M., & Huang, D. C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Gene. Dev. 19(11), 1294-1305.
Hockings, C., Alsop, A. E., Fennell, S. C., Lee, E. F., Fairlie, W. D., Dewson, G., & Kluck, R. M. (2018) Mcl-1 and Bcl-xL sequestration of Bak confers differential resistance to BH3-only proteins. Cell Death Differ. 25, 721-734.

Discussion :

D.1 Authors state : 'Similar to the increased Drp1 GTPase activity by Bcl-XL, Bcl-XL may increase the GTPase activity of MFN2 to induce MFN2-dependent mitochondrial aggregation.'Authors need to cite and discuss relevant literature in the field reporting that Bcl-xL activates Mfn2-mediated mitochondrial fusion (Hoppins et al 2011, PMID 21255726) and increases mitochondrial fission, fusion and biomass in neurons (Berman et al 2009, PMID 19255249).

Response: According to your comments, we have revised the fifth paragraph of Discussion section as follow,

"Interestingly, the GTPase domain mutants of MFN2 (MFN2(K109A), alanine replacement of lysine at residue 109 (K109A)) and MFN1 (MFN1(K88T), threonine replacement of lysine at residue 88 (K88T)) still have the ability of forming oligomers, but lose the ability for binding with Bcl-XL. The two mutated lysine here belong to the conserved G2 or G1 (P-loop) motif of GTPases, which coordinate the phosphate groups of the bound guanine nucleotide (Yan et al., 2018), and the two mutants will decrease activity of GTPases (Yan et al., 2018; Ishihara et al., 2004; Detmer et al., 2007). In fact, we performed quantitative FRET assay for cells co-expressing CFP-labeled MFN2(K109A) and YFP-labeled MFN2(K109A), CFP-labeled MFN1(K88T) and YFP-labeled MFN1(K88T), or CFP-MFN1(K88T) and YFP-MFN2(K109A), respectively, and our results showed that MFN2(K109A) and MFN1(K88T) can form homo-/hetero-oligomers (Fig. S3), which is contrast to the previous report that MFN1(K88T)-FLAG does not coprecipitate with MFN1(K88T)-HA (Ishihara et al., 2004). However, FRET assay demonstrated no binding between MFN2(K109A) and Bcl-XL (Fig. S4C), and between MFN1(K88T) and

Bcl-XL (Fig. S4C), which is contrast to our results of Co-IP assay (Fig. S4D) between CFP-Bcl-XL and MFN2(K109A)-Myc/ MFN1(K88T)-Myc. One reason is that different environments between living and apoptotic cells lead to different binding capacities between Bcl-XL and MFN2(K109A)/ MFN1(K88T) for explaining the controversial results. It is possible for that MFN2(K109A)/ MFN1(K88T) combined with Bcl-XL in the form of kiss and run in living cells, and our FRET assay based on averages of large amounts of data will weaken their combination but Co-IP assay may not detect this difference. These results confirm a notion that the GTPase domains of MFN2 and MFN1 play key roles in binding with Bcl-XL. Mutation of this residue normally won't affect the overall folding of the protein, but binding of the nucleotide (Yan et al., 2018). Is this residue directly involved in the interface or this interaction dependent on the loading of GTP/GDP to MFNs? It may be worthwhile to explore. FRAP analysis and direct observation of fission/fusion all showed that Bcl-XL overexpression significantly increased the rate of mitochondrial fusion (Berman et al., 2009; Delivani et al., 2006). Addition of purified recombinant full-length monomeric soluble Bcl-XL also stimulated mitochondrial fusion in a dose-dependent manner (Hoppins et al 2011). In vitro GTPase assay performed with purified recombinant full-length Bcl-XL showed that Bcl-XL increased Drp1 GTPase activity (Li et al., 2008), indicating Bcl-XL may induce mitochondrial fragment by activating Drp1. Similar to the increased Drp1 GTPase activity by Bcl-XL, Bcl-XL may increase the GTPase activity of MFN2 to induce MFN2-dependent mitochondrial aggregation."

References in this part:

Berman, S. B., Chen, Y. B., Qi, B., Mccaffery, J. M., Rucker, E. B., & Goebbels, S., Nave, K. A., Arnold,. K. A. Jonas, E. A., Pineda, F. J., & Hardwick, J. M., et al. (2009) Bcl-xl increases mitochondrial fission, fusion, and biomass in neurons. J. Cell Biol. 184, 707-719.

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Detmer, S. A., & Chan, D. C. (2007). Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. J. Cell Biol. 176, 405-414.

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Ishihara, N., Eura, Y. & Mihara, K. (2004) Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J. Cell Sci. 117, 6535-6546.

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Second decision letter

MS ID#: JOCES/2020/245001

MS TITLE: Mitofusin 2 but not mitofusin 1 mediates Bcl-XL-induced mitochondrial aggregation

AUTHORS: Mengyan Du, Si Yu, Wenhua Su, Mengxin Zhao, Fangfang Yang, Yangpei Liu, Zihao Mai, Yong Wang, Xiaoping Wang, and Tongsheng Chen
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks. Please make the minor changes suggested by reviewer #1 in your final editing of the paper.

Reviewer 1

Advance summary and potential significance to field

In the revised manuscript, the authors have well addressed my concerns on the original version of the submission. Overall, this work represents an suitable application and exhibits great value of a high-end biophysical technology in the field of cell biology.

Comments for the author

I have only some minor comments:

1. Line 536: Not G1 or G2, but both G1. Also the references should be Qi et al 2016 and Cao et al 2017.

2. Line 550, in a kiss-and-run. Similar typos and grammatical errors need to be corrected throughout the manuscript.

3. Figure 4A and 4B (I assume? The fourth page of the figures, regarding the si effect of MFN1 and MFN2), the WB results of the first row look a bit weird: the background of the blots seems not consistent with the surroundings. Why the blot of MFN2 (below 80 Kd) seems smaller than MFN1 (above 80 kD), while MFN2 actually has a larger size. Please double check.

4. Figure S1, an old and inaccurate topology of MFNs was illustrated here. Please refer to the recently reported crystal structures of truncated MFN1 and MFN2 (Qi et al 2016, Cao et al 2017, Li et al 2019) and update the topology here. The N-terminus of MFNs starts with the helix from the HD1 domain but not the G domain.