An amphipathic Bax core dimer forms part of the apoptotic pore wall in the mitochondrial membrane

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Thank you for submitting your manuscript for consideration by The EMBO Journal. I sincerely apologise for the protracted review process due to delays in review submission. We have now received three reviewer reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers appreciate the study, but also point out a number of issues that would have to be addressed and clarified before they can support publication of the manuscript. From my side, I find the reviewer comments generally reasonable. Therefore, based on the interest expressed in the referee reports, I would like to invite you to address the comments of all reviewers in a revised version of the manuscript.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

We have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

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Referee #1:

The manuscript from Lv et al. presents a solid NMR analysis of the bicelle-bound structure of a Bax alpha 2-5 dimer. The new structure reveals differences with respect to the previously crystallized Bax dimer in terms of curvature of the apolar surface created by helices alpha 4-5. Additionally, it provides robust experimental data to position the dimer with respect to the normal of the bicelle, which is an important step forward towards the understanding of the topology of active Bax dimers at the mitochondrial membrane. The authors conclude that both hydrophobic interactions between the center of the nonpolar alpha 4-5 surface with the lipid acyl chains and the ionic and polar interactions of the two positively charged arginines with the lipid headgroups are important driving forces for the Bax pore formation.

The NMR and PRE results are supported by cross-linking data and permeabilization assays in vitro. Additionally, the effects of point mutations in the formation of the active core dimer were investigated in the full length version of Bax in isolated mitochondria. Overall, this is an important study which advances the field, and provide a quantitative analysis which favors the clamp model of active Bax versus the in-plane model. I suggest publication with minor revision.

Comments:
- Please provide a detailed description of the method used to isolate the mitochondria. No
information is given in this manuscript and additional info cannot be found in the cited paper (Zhang et al., 2016).

- I have some questions about the structure of Bax alpha 2-5 in solution. The SEC-MALS shows a unique peak corresponding to a tetramer, but the cross-linking data shows co-existence of monomers, dimers, trimers and tetramers. Why no other peaks are visible in the SEC-MALS? The NMR spectra of the protein in solution shows very resolved peaks which could be assigned, but no structural analysis was performed. Are the active dimers already formed in solution, and maybe the non polar surfaces created by alpha 4-5 interact to form a stable tetramer? This oligomerization in solution is clearly due to the truncation because full length Bax is monomeric. Can the authors compare the NMR data of alpha 2-5 in solution with the data simulated with the cristallized Bax core dimers? It could be interesting to understand if the NMR changes observed upon bicelle addition can be ascribed to the transformation of a dimer of dimers with a concave surface into a dimer with a flat surface.

- The dimeric structure of Bak (alpha2-5, PDB 4U2V) shows a different curvature in the helices alpha 4-5 with respect to the analogous Bax structure (4BDU) and the new bicelle-bound Bax structure shows a flat surface. A comparison of the curvatures including Bak should be provided. The dynamic nature of this interface might enable different interactions with the membrane bilayers. There is the possibility that the curvature of the alpha 4-5 region could adapt to the type or composition of the membrane bilayers in vitro and in mitochondria. The authors should comment on that.

- Bicelles (made with DMPC, DHPC) provides a unique opportunity to perform the NMR studies in liquid state, however they are not mimicking the mitochondrial membrane bilayers, neither by lipid arrangement, nor by composition. Interestingly, Bax cores (monomers, dimers or tetramers?) interacts promptly with them in the absence of tBid and create the active dimeric structure. The properties of the bicelle and/or the tetrameric form of Bax in solution facilitate this interaction despite the absence of activators (as tBid) or cardiolipin. This should be addressed in the text.

- In the new dimer structure, alpha 5 was found to be helical up to residue 126. It should be mentioned that also in the EPR-derived clamp model (Bleicken et al., 2014), the helix alpha 5 from the crystallized core dimer was elongated up to residue 126, and this was in agreement with the EPR constraints.

- The topology of the core dimer with respect to the normal of the bicelle was obtained with NOEs between Bax and the deuterated DMPC lipids. It could be insightful to repeat the same experiments with deuterated DHPC to verify possible interactions of Bax with the edges of the bicelles. This could provide some information about the distribution of the lipids around the dimers.

- It is not clear if the authors propose that Bax dimers inserts in the bicelle directly in the central planar bilayer formed by long-chain lipids (DMPC), or at the edges stabilized by short-chain lipids. The interaction of Bax with the DHPC lipids at the edges of the bicelles and the subsequent displacement of the short-chain lipids could be thermodynamically favored.

**Referee #2:**

Manuscript EMBOJ-2020-106438 entitled "Amphipathic Bax core dimer forms part of apoptotic pore wall in the mitochondrial membrane" by Lv, Qi, Zhang, Wen, Lin, OuYang and colleagues presents the first high resolution membrane associated conformation of Bax α2-α5 core dimer. The Bax and Bak core dimers have been previously proposed to be the building blocks of the apoptotic pore, which is a heterogeneous poorly understood and highly controversial proteolipidic pore. The study describes the NOE-based structure of Bax core dimer in DMPC/DHPC biciples, which have been previously used successfully in elucidating NMR structure of a number of small membrane
proteins and domains. This structure is very similar to that of the Bax core dimer determined by crystallography with a noticeable difference. Instead of a concave shape the structure is flat, suggesting that the core lines the lumen of the pore. NOE- and PRE-based measurements help position the core relative to the bicelle bilayer. The authors validate the structure through mutagenesis. Mutations in the nonpolar region of the core dimer, which interacts with the acyl chains, inhibit the ability of Bax to permeabilize liposomes as well as dimerize in mitochondrial membranes. The authors also identify a polar region R89, E90 that interacts with the polar head group of lipids and may define the tilt of the core dimer to the lipid bilayer normal.

While many open questions remain regarding how Bax porates mitochondria to initiate apoptosis, this is a carefully executed and clearly presented study which provides insights into the interactions of the putative core dimer with the lipid bilayer. The study supports the clamp model rather than in-plane model (Figure 1A).

This is not a criticism to this study but rather meant to bring awareness of the difficulty in studying mitochondrial poration in the absence of high-resolution structures of intact effectors in the presence of membranes. Given that the core dimer does not exhibit activity alone it is unsatisfactory to make direct inference to the mechanism of poration by the full-length structure. Nonetheless, mechanistic insights from the isolated core are useful and the authors mention that this is a steppingstone towards elucidating structures of full-length proteins in membranes.

I have a few suggestions for straightforward experiments to strengthen the proposed model.

1. The regulation of the core tilt relative the bilayer normal by helix 4 residues is intriguing, neat, and testable. Could the author perform the PRE analysis presented in Figure S5 with the R89E and F93E mutants. Two other mutants could be tested including R89F and R89A, which will eliminate the proposed polar interaction with the lipid polar head group and possibly abolish regulation of the tilt.
2. The authors present data for crosslinking by CuSO4 of engineered cysteines meant to capture the core dimer (Figure S9). Could such crosslinking happen in an asymmetric fashion? It would be expected that double disulfide dimers M79C/L59C' and M79C'/L59C will migrate faster than E69C'/E69C on SDS PAGE, but this does not seem to be the case. Related to this figure what is the concentration of Bax BH3 used, and why did the authors switch to a different modality of activating Bax from tBID, which was used in liposomes. The crosslinking analysis could be more revealing if combined with the MOMP assay although the experiment would have to be set up differently; adding CuPhe after a certain time has passed during a MOMP assay. What if the MOMP and liposome permeabilization assays show different results for the set of mutants? This may be done simply with purified proteins and using Western blotting.
3. If the concentration of tBID is increased in liposome permeabilization assay, would some of the weakly active mutants such as A117D and F114E induce more permeabilization. The authors need to comment on the possible effect of mutations on direct activation. Allosteric changes seem to perpetuate within the Bax structure in rather obscure ways as recently shown by Dengler et al Cell Reports. Bax activation continues to be controversial.
4. Can the authors estimate the molar ratio of Bax core dimers to bicelles. If this number is expected to be 1:1 it may explain why there are no intermolecular NOEs and why extensive crosslinking is not observed in bicelles (Figure S1C). It would be helpful to have a figure that shows the core dimer docked onto a bicelle for the readers to know that the core is likely displacing DHPC lipids to get to the DMPC layer (presumably 1 per bicelle?). Figure 4A may be improved and a zoom out show the core relative to the bicelle side and top view?
5. Consider adding this sentence at the end of first paragraph of introduction: "It is therefore expected that Bax assumes a number of different membrane-associated conformations that cooperate in regulating mitochondrial poration."
6. I would remove ", as expected from the dramatically different NMR spectra for the bicelle-bound and the soluble Bax (α2-α5)(Fig. 1)" since the crystal structure of the core is a dimer (Czabotar Cell 2013), so the spectra are different representing a soluble tetramer and bicelle-associated dimer.

Referee #3:

Lv and co-authors present the structure determination of the BAX core domain, α2-α5 helices region, within a model lipid bilayer and interaction studies using NMR spectroscopy. Specifically, they determined the NMR structure of the BAX core domain bound to the lipid bicelles. The structure of the soluble BAX core domain has been previously determined by X-ray crystallography (Czabotar et al., Cell, 2013). There are notable changes in the NMR spectra of the two BAX core conformations and authors present some changes on the structure upon interaction with the membrane. They also determined the topographic models of the BAX core domain in lipid bicelles using paramagnetic spin labels and showed evidence that the conformation of BAX in association with the membrane is more consistent with the clamp model than the in-plane model previously suggested by Bleicken and co-authors. Then the authors used the recently determined structure to design mutations on BAX which were evaluated on their ability to enable dimerization using cross-linking and pore forming activity in liposomal membranes. This study is novel as it presents one of the first studies investigating the interaction of BAX with the model membrane at atomic resolution. Besides the limitations and assumptions with studying only the truncated BAX core domain instead of the full length protein, the study provides some interesting structural insights in the ongoing attempt to understand how BAX acts on the mitochondrial membrane to induce membrane permeabilization. However, there are a few concerns and missing data to support the conclusions that need to be addressed.

- The authors have shown that in the absence and presence of lipid bicelles, BAX undergoes a conformation change that is evident in the TROSY-HSQC spectra Figure 1B. The authors should provide a chemical shift perturbations plot (bicelles bound BAX vs free BAX) so there is better understanding of the local and global structural changes upon addition of bicelles. They should also comment whether they see evidence of new peaks appearing in the spectra in the presence of bicelles. They should also highlight and map these residues on the BAX structure that show chemical shift perturbations upon membrane binding.
- Regarding the NMR structure determination, the authors should provide any reports and evaluation using structure validation databases.
- Please report the energies of the bicelles bound BAX structures and compare it with the crystal structure to know whether bicelles induce energetically more favorable conformations in solution.
- Why the authors chose to run NMR experiments (protein lipid NOE) at 32{degree sign} C as this introduce higher flexibility/mobility of protein that may disrupt local conformation or interaction of each protomer?
- The authors have used paramagnetic probe titration to identify the residues whose intensities are affected by paramagnetic relaxation. The paramagnetic effect is relative to the position. It is not mentioned where the probe (d-DOTA) is supposed to bind or if it is expected to move freely in the solution? In the former case, how it is ensured that the results obtained by d-DOTA are not biased.
For example the graph in S5A with high PRE doesn't indicate a particular region that is solvent protected? Have the authors calculated PRE in lower temperatures and with additional concentrations?

• The biochemical studies suggest that residues are involved somehow in the membrane interactions and the have effect on the pore-forming activity of BAX, which could be independent of their ability to dimerize within the membrane. Therefore, the authors should demonstrate that specific single points mutations or combinations of mutants can disrupt the dimer interaction with the lipid bilayer, preferably by NMR.

• Regarding the proposed structural model of oligomeric BAX in figure 5, can the authors show evidence of a specific interaction between BAX core dimers in the presence of the lipid bicelles?
The following are our point-to-point responses to the comments from the referees. The comments are in italic font.

Referee #1:

The manuscript from Lv et al. presents a solid NMR analysis of the bicelle-bound structure of a Bax alpha 2-5 dimer. The new structure reveals differences with respect to the previously crystallized Bax dimer in terms of curvature of the apolar surface created by helices alpha 4-5. Additionally, it provides robust experimental data to position the dimer with respect to the normal of the bicelle, which is an important step forward towards the understanding of the topology of active Bax dimers at the mitochondrial membrane. The authors conclude that both hydrophobic interactions between the center of the nonpolar alpha 4-5 surface with the lipid acyl chains and the ionic and polar interactions of the two positively charged arginines with the lipid headgroups are important driving forces for the Bax pore formation.

The NMR and PRE results are supported by cross-linking data and permeabilization assays in vitro. Additionally, the effects of point mutations in the formation of the active core dimer were investigated in the full length version of Bax in isolated mitochondria. Overall, this is an important study which advances the field, and provide a quantitative analysis which favors the clamp model of active Bax versus the in-plane model. I suggest publication with minor revision.

We sincerely thank the reviewer for the appraisal of our manuscript as new, solid, robust, cross supportive and important toward the understanding of Bax dimer’s topology in the membrane that drives the Bax pore formation.

Comments:

Comment 1-1: Please provide a detailed description of the method used to isolate the mitochondria. No information is given in this manuscript and additional info cannot be found in the cited paper (Zhang et al., 2016).

Response 1-1: We have included a detailed description of the method used to isolate the mitochondria, with the correct reference from the Andrews lab (Pogmore J.P., Pemberton J.M., Chi X., Andrews D.W. (2016) Using Förster-Resonance Energy Transfer to Measure Protein Interactions Between Bcl-2 Family Proteins on Mitochondrial Membranes. In: Puthalakath H., Hawkins C. (eds) Programmed Cell Death. Methods in Molecular Biology, Vol 1419. Humana Press, New York, NY. https://doi.org/10.1007/978-1-4939-3581-9_15).

Comment 1-2: I have some questions about the structure of Bax alpha 2-5 in solution. The SEC-MALS shows a unique peak corresponding to a tetramer, but the cross-linking data shows co-existence of monomers, dimers, trimers and tetramers. Why no other peaks are visible in the SEC-MALS?

Response 1-2: From the SEC-MALS data shown in Appendix Figure S1C and the complete data shown in Figure R1-left panel below, the average molecular weights determined for the only peak of eluted proteins does not fit to a straight line, indicating that Bax (α2-α5) proteins in solution
exist in various complexes, with a fraction as the tetramers. Thus, the estimated MW for the proteins in the front portion of the peak is 35.7 kDa (more clearly shown in Figure R1-right panel), close to the expected MW of a tetramer (36.5 kDa). Since the MW of a monomer is 9.13743 kDa, it is difficult to separate the monomer, dimer, trimer and tetramer using SEC. On the other hand, due to the efficiency of crosslinking reaction, not all the complexes can be covalently linked. To address this reviewer’s question, we modified the related text in the revised manuscript on page 4 as follows: “SEC-MALS analysis showed that the soluble Bax (α2-α5) is mainly a tetramer mixed with lower order complexes (Appendix Fig. S1C). Consistent with this observation, crosslinking with bis(sulfosuccinimidyl)-suberate (BS3) detected tetramers alongside dimers and trimers (Appendix Fig. S1D). The crosslinking efficiency was low for the bicelle-bound Bax (α2-α5) (Appendix Fig. S1D), possibly because the crosslinker is membrane impermeable. In addition, the efficiency (E) of each bimolecular cross-linking reaction is expected to be less than 1, and hence, the crosslinking efficiency of two or three crosslinking reactions that must occur to a trimer or tetramer to result in a covalently linked trimer or tetramer, respectively, is expected to be much lower since it equals to \(E^2\) or \(E^3\).”

Figure R1. SEC-MALS analysis of the purified Bax (α2-α5) protein. (Left) The normalized light scattering (LS), UV absorbance at 280 nm (UV) and differential reflective index (dRI) were analyzed to determine the molecular weight (MW). The black curve represents the determined MW of the peak fractions of the eluted Bax (α2-α5) protein. (Right) The magnified region of the SEC-MALS data for the peak fractions to show the MW for the proteins in the front portion of the peak is \(~35.7\) kDa, and for the back portion is ranged from \(~35.6\) to \(~28.7\) kDa. The theoretical MW for Bax (α2-α5) monomer is 9137.43 Da.

Comment 1-3: The NMR spectra of the protein in solution shows very resolved peaks which could be assigned, but no structural analysis was performed. Are the active dimers already formed in solution, and maybe the nonpolar surfaces created by alpha 4-5 interact to form a stable tetramer? This oligomerization in solution is clearly due to the truncation because full length Bax is monomeric. Can the authors compare the NMR data of alpha 2-5 in solution with the data simulated with the crystallized Bax core dimers? It could be interesting to understand if the NMR changes observed upon bicelle addition can be ascribed to the transformation of a dimer of dimers with a concave surface into a dimer with a flat surface.

Response 1-3: We agree that it would be interesting to understand the differences between the
NMR structure of Bax (α2-α5) in solution and the crystal structure. However, with only the NMR backbone assignment on soluble Bax (α2-α5), we cannot determine the NMR structure and compare to the crystal structure. To obtain the NMR structure of soluble Bax (α2-α5), we need to collect intramonomer and intermonomer distance restraints. However, it will be very difficult for NMR to distinguish the intermonomer distance restraints in the dimer from that in the tetramer of the soluble Bax (α2-α5). And, the co-existence of monomeric and other multimeric states in the same sample for NMR measurement as indicated by the SEC-MALS and crosslinking data from the sample that were discussed above make the task even more difficult. On the other hand, the soluble Bax (α2-α5) is mainly a tetramer in the NMR sample, but a dimer in the crystal structure when each monomer is fused to a dimerizable GFP that makes an additional contact in the crystal resulting in a tetramer of GFP-Bax (α2-α5) proteins. So, these two structures are not comparable, or the comparison may not be informative. Since the soluble structure is not our main focus for this manuscript, we did not invest substantial efforts to solve the structure of soluble Bax (α2-α5) by NMR.

Comment 1-4: The dimeric structure of Bak (alpha2-5, PDB 4U2V) shows a different curvature in the helices alpha 4-5 with respect to the analogous Bax structure (4BDU) and the new bicelle-bound Bax structure shows a flat surface. A comparison of the curvatures including Bak should be provided. The dynamic nature of this interface might enable different interactions with the membrane bilayers. There is the possibility that the curvature of the alpha 4-5 region could adapt to the type or composition of the membrane bilayers in vitro and in mitochondria. The authors should comment on that.

Response 1-4: Great point! We compared the crystal structure of Bak (α2-α5) dimer (4U2V) with the NMR structure of Bax (α2-α5) dimer and added the result to the revised Figure EV2B. We also noted recently published structures of Bak (α2-α5) dimers in complex with lipids or detergents that are different from the structure of free Bak (α2-α5) dimer. We revised the related text on page 5 as follows: “The crystal structure of Bak (α2-α5) dimer (PDB code: 4U2V) (Brouwer et al, 2014) shows a different curvature in the α4-α5 surface than the analogous Bax surface (PDB code: 4BDU) (Czabotar et al, 2013). The structural comparison between the Bak (α2-α5) dimer and the bicelle-bound Bax (α2-α5) dimer results in a 5.177 Å backbone RMSD and the α4-α5 surface is flatter in the latter than the former (Fig. EV2B). Recently published crystal structures of lipid- or detergent-bound Bak (α2-α5) dimers also showed flatter α4-α5 surfaces than the free Bak (α2-α5) dimer (Cowan et al, 2020). Overall, these structure comparisons suggest that the (α2-α5) dimers adjust their structures to better accommodate the membranes or lipids that they bind to.”

Comment 1-5: Bicelles (made with DMPC, DHPC) provides a unique opportunity to perform the NMR studies in lipid state, however they are not mimicking the mitochondrial membrane bilayers, neither by lipid arrangement, nor by composition. Interestingly, Bax cores (monomers, dimers or tetramers?) interacts promptly with them in the absence of tBid and create the active dimeric structure. The properties of the bicelle and/or the tetrameric form of Bax in solution facilitate this interaction despite the absence of activators (as tBid) or cardiolipin. This should be addressed in
Response 1-5: Very good point! We performed an NMR experiment with cardiolipin in the bicelles. Based on the result, we added the following to the revised manuscript on page 4: “The same spectral changes were observed when cardiolipin was included in the lipid bicelles (Appendix Fig. S1B), suggesting that the conformational changes of Bax (α2-α5) are not dependent on this mitochondrial lipid. This is in contrast to Bax protein whose conformational changes are triggered by the mitochondrial membranes and activator proteins such as tBid that require cardiolipin to be fully active (Lovell et al, 2008; Shamas-Din et al, 2015).” We guess cardiolipin may play an important role during the membrane insertion of Bax, but once the Bax pore is formed, cardiolipin is not that critical.

Comment 1-6: In the new dimer structure, alpha 5 was found to be helical up to residue 126. It should be mentioned that also in the EPR-derived clamp model (Bleicken et al., 2014), the helix alpha 5 from the crystallized core dimer was elongated up to residue 126, and this was in agreement with the EPR constraints.

Response 1-6: We added this point in the text on page 5 and referenced (Bleicken et al., 2014).

Comment 1-7: The topology of the core dimer with respect to the normal of the bicelle was obtained with NOEs between Bax and the deuterated DMPC lipids. It could be insightful to repeat the same experiments with deuterated DHPC to verify possible interactions of Bax with the edges of the bicelles. This could provide some information about the distribution of the lipids around the dimers.

Response 1-7: Interesting point! However, there is a technical problem for the suggested NMR experiment, because for short lipids like DHPC with a high CMC (~15 mM), it exists as free lipids (up to 15 mM) as well as micellar or bicellar lipids in the NMR sample. Therefore, we will not be able to distinguish the DHPC in the bicelles from that in the micelles or free in the solution using NMR.

Comment 1-8: It is not clear if the authors propose that Bax dimers inserts in the bicelle directly in the central planar bilayer formed by long-chain lipids (DMPC), or at the edges stabilized by short-chain lipids. The interaction of Bax with the DHPC lipids at the edges of the bicelles and the subsequent displacement of the short-chain lipids could be thermodynamically favored.

Response 1-8: We regret that we did not make our model for Bax core dimer-membrane interaction clearer in the original manuscript. We have clarified in the revised manuscript that the most reasonable model based on all of the data is that a Bax core dimer interacts with the edge of a bicelle and is in direct contact with the central DMPC bilayer lipids after some of the DHPC micellar lipids surrounded the edge are displaced by the dimer. We made a three-dimension cartoon model to clearly illustrate this model in Fig. 3.

Referee #2:
Manuscript EMBOJ-2020-106438 entitled "Amphipathic Bax core dimer forms part of apoptotic pore wall in the mitochondrial membrane" by Lv, Qi, Zhang, Wen, Lin, OuYang and colleagues presents the first high resolution membrane associated conformation of Bax α2-α5 core dimer. The Bax and Bak core dimers have been previously proposed to be the building blocks of the apoptotic pore, which is a heterogeneous poorly understood and highly controversial proteolipidic pore. The study describes the NOE-based structure of Bax core dimer in DMPC/DHPC bicelles, which have been previously used successfully in elucidating NMR structure of a number of small membrane proteins and domains. This structure is very similar to that of the Bax core dimer determined by crystallography with a noticeable difference. Instead of a concave shape the structure is flat, suggesting that the core lines the lumen of the pore. NOE- and PRE-based measurements help position the core relative to the bicelle bilayer. The authors validate the structure through mutagenesis. Mutations in the nonpolar region of the core dimer, which interacts with the acyl chains, inhibit the ability of Bax to permeabilize liposomes as well as dimerize in mitochondrial membranes. The authors also identify a polar region R89, E90 that interacts with the polar head group of lipids and may define the tilt of the core dimer to the lipid bilayer normal.

While many open questions remain regarding how Bax porates mitochondria to initiate apoptosis, this is a carefully executed and clearly presented study which provides insights into the interactions of the putative core dimer with the lipid bilayer. The study supports the clamp model rather than in-plane model (Figure 1A).

We gratefully appreciate that the reviewer recognized our effort in making this a carefully executed and clearly presented study that described the first high-resolution conformation for the membrane-associated Bax core dimer whose most likely function among the multiple proposed functions is to line the lumen of the proteolipidic pore that has so far been poorly characterized, and hence, highly controversial.

This is not a criticism to this study but rather meant to bring awareness of the difficulty in studying mitochondrial poration in the absence of high-resolution structures of intact effectors in the presence of membranes. Given that the core dimer does not exhibit activity alone it is unsatisfactory to make direct inference to the mechanism of poration by the full-length structure. Nonetheless, mechanistic insights from the isolated core are useful and the authors mention that this is a steppingstone towards elucidating structures of full-length proteins in membranes.

We agree with the reviewer that the high-resolution structures of the pores which are formed by intact Bax proteins in the membranes are the ultimate goal of the field of basic apoptosis research, and our achievement is a steppingstone to reach this goal since it provides mechanistic insights by determining the isolated Bax core region structure that binds to a membrane disc. We have attempted to make this point clear in the revised manuscript.

I have a few suggestions for straightforward experiments to strengthen the proposed model.
Comment 2-1: The regulation of the core tilt relative the bilayer normal by helix 4 residues is intriguing, neat, and testable. Could the author perform the PRE analysis presented in Figure S5 with the R89E and F93E mutants. Two other mutants could be tested including R89F and R89A, which will eliminate the proposed polar interaction with the lipid polar head group and possibly abolish regulation of the tilt.

Response 2-1: Good point. We performed the PRE analysis with the R89E and F93E mutants of the Bax (α2-α5) protein. However, as the membrane association was disrupted by the mutations, the fitting of any structural models to the residue-specific PREamp data had failed for many residues, especially those in the α4 region, and thereby the tilt angle of each mutant structure relative to the bilayer normal could not be estimated.

We made the R89F or R89A mutation in the Bax (α2-α5). The resulting R89F protein is not stable in solution. In particular, the elution peak of this mutant protein is broader than the wild-type protein and is eluted earlier, as shown by the SEC profile in Figure R2A, indicating that the mutant protein forms larger complexes which are more heterogenous in size. The NMR spectrum obtained from the R89F mutant in solution showed much fewer peaks even at high concentration (see Figure R2B). The addition of bicelles to the R89F mutant in solution improved the NMR spectrum initially (Fig. R2C). However, precipitation occurred in the sample with both R89F and bicelles within two days, preventing collection of enough NMR-PRE data that are necessary for further analyses to reach a solid conclusion about the orientation of the mutant structure in the bicelle. Thus, we prefer not to add the NMR data for the R89F mutant to the revised manuscript but will follow the direction from the reviewers about this data. In contrast, the R89A mutant gave high quality NMR spectra in both solution and bicelles, and the spectrum of the protein in bicelle was altered by the lipophilic PRE probe titration. Thus, we added the data to the revised Figure EV4 together with the data from R89E and F93E, and revised the related text in page 7.

Since the NMR-PRE data from the R89A mutant in bicelles clearly show that the mutation reduces the core region interaction with the membrane discs, and that the NMR spectrum from the R89F mutant was altered by the addition of bicelles indicates that the mutated core region is capable to interact with the membrane discs, we made the same mutations individually in the full-length Bax protein and performed the liposomal membrane permeabilization assay. As shown in the revised Figure EV4A, the effect of R89A mutation on the dye release is less than the R89E mutation, which is expected since the R89A does not introduce the repulsive negative charge like the R89E, and hence would be less detrimental to the membrane interaction. The effect of R89F on the dye release is similar to the R89A (Fig. EV4A), further testifying the importance of the positive charge of R89 to the membrane interaction since this mutation eliminates the positive charge by may retain some of the nonpolar interaction of the aliphatic portion of the R89 side chain with the lipid acyl tails. In addition, R89F also reduced the crosslinking but to a less degree than R89E, whereas R89A slightly increased the crosslinking as shown in the revised Figure EV5D-E. All the data together support the conclusion that the core dimer-membrane interaction and the core dimer configuration are important to the Bax pore formation.
**Figure R2.** The purification and NMR characterization of Bax (α2-α5) mutants. (A) SEC chromatography of Bax (α2-α5) (black), Bax (α2-α5) R89A (red) and Bax (α2-α5) R89F (blue) from a HiLoad® 16/600 Superdex 200. (B) 2D $^1$H-$^{15}$N TROSY-HSQC spectra of soluble Bax (α2-α5) R89F at 700 MHz. (C) 2D $^1$H-$^{15}$N TROSY-HSQC spectra of bicelle-bound Bax (α2-α5) R89F at 700 MHz.

Comment 2-2: The authors present data for crosslinking by CuSO$_4$ of engineered cysteines meant to capture the core dimer (Figure S9). Could such crosslinking happen in an asymmetric fashion? It would be expected that double disulfide dimers M79C/L59C' and M79C'/L59C will migrate faster than E69C'/E69C on SDS PAGE, but this does not seem to be the case. Related to this figure what is the concentration of Bax BH3 used, and why did the authors switch to a different modality of activating Bax from tBID, which was used in liposomes. The crosslinking analysis could be more revealing if combined with the MOMP assay although the experiment would have to be set up differently; adding CuPhe after a certain time has passed during a MOMP assay. What if the MOMP and liposome permeabilization assays show different results for the set of mutants? This may be done simply with purified proteins and using Western blotting.

Response 2-2: The crosslinking data for Bax M79C/L59C, and for E69C shown in Figure EV5B-C were obtained from two different gels. When we run the two crosslinked samples from other two independent experiments through the same SDS-PAGE gel, the migration of the crosslinked Bax dimer protein band from the double-Cys L59C/M79C mutant was actually faster that from the single-Cys E69C mutant as shown in Figure R3A, as this reviewer expected. Since this data is a derivative from the focus of this experiment, i.e., whether the mutations that disrupt the core dimer interaction with the membrane affect the core dimerization itself, we decided not including this data in the manuscript. If the reviewer thinks it is important to show that the crosslinking occurs symmetrically, we will include this data.

The reason that we used a BH3 peptide (at 2 μM) to activate Bax in the mitochondria for the crosslinking assay is that most of the peptides did not associate with Bax in the membranes after they interact and activate Bax, instead, they remained in the soluble fraction as we showed previously (Tan et al, 2006). In contrast, most of the tBid proteins associated with the membranes, and a significant fraction of which associates with the Bax in the membranes. Thus, although both BH3 peptide and tBid can activate Bax, the tBid interaction with the Bax in the membranes competes with the homotypic interaction between the activated Bax proteins in the membranes, thereby reducing the yield of the crosslinked Bax homodimer as shown in Figure R3B-C. However,
the comparison between the ratio the crosslinked dimer to the monomer of the wild type Bax to that of the mutants whose crosslinked dimers were still detectable suggests that the dimerization of the tBid-activated Bax was affected similarly as that of the BH3 peptide-activated Bax.

Figure R3. The disulfide crosslinking of Bax mutants. (A) Disulfide crosslinking of Bax E69C or L59C/M79C mutant analyzed on the same SDS-PAGE gel. The disulfide-linked dimer of L59C/M79C or E69C is indicated by an arrowhead in lane 2 or 4, respectively. It is clear that the migration of the crosslinked dimer band from the double-Cys mutant was faster than that from the single-Cys mutant. (B-C) Comparison of disulfide crosslinking of Bax proteins that were activated by tBid (lanes 3 to 12 and 14 to 18 in each panel) or BH3 peptide (lanes 1 to 2 and 13 in each panel), and targeted to the mitochondria (lanes 1 to 12 in each panel). By comparing the arrowhead-indicated disulfide-linked dimer bands in lanes 2 and 4 in each panel, and the dimer:monomer ratios that were determined as described in Figure EV5 except that the ratio from the BH3 peptide-activated Bax was normalized by that from the tBid-activated Bax, it is clear that more crosslinked dimers were generated from the BH3 peptide-activate Bax than from the tBid-activated Bax. As a result, some of the Bax mutants did not form detectable crosslinked dimers when tBid was used to activate them. For the mutants that formed detectable dimers, the additional mutations that reduced the core dimer interaction with membranes also reduced the core dimer formation as indicated by the reduced dimer:monomer ratios relative to the single or double-Cys Bax without the additional mutations that were determined as described in Figure EV5, similar to what was detected when the BH3 peptide was used to activate Bax. In addition,
the reduction of Bax dimer formation cannot be attributed to the reduction of Bax concentration in the mitochondria since most of the mutations did not reduce the mitochondrial association of Bax that was determined as described in Figure EV5 and shown below the gel images.

We thank the reviewer for the idea of conducting a combined crosslinking and MOMP assay to further determine the relationship of these two critical events of active Bax played in the membranes. However, this assay is not feasible with our systems because the crosslinking reaction was done with radioactive Bax proteins that were synthesized in vitro using a transcription and translation coupled systems from Bax-coding DNA plasmids, and thus, the concentration of the Bax proteins in the fraction in the mitochondria isolated from Bak knockout mouse liver can only be estimated at the range of nM to μM in the crosslinking reaction. In contrast, the MOMP reaction as described below using recombinant Bax proteins expressed in and purified from E. coli cells, and thus, of known concentrations at 10’s to 100’s nM for the total proteins with a targeting efficiency to all cellular membranes of the digitonin-permeabilized Bax and Bak double knockout BMK cells that can be estimated. Although the MOMP extent can be quantified with the fluorescent Smac-mCherry proteins expressed in the mitochondrial intermembrane space of these cells, it is rather difficult to correlate the extent of the MOMP to the extent of the Bax dimerization that occur in different mitochondria with different and uncertain amount of Bax proteins that are activated by different activators (cBid in the MOMP assay versus BH3 peptide or tBid in the crosslinking assay). In addition, while the extent of MOMP can be determined at certain window during a “real time” period since the centrifugation that separate the released Smac-mCherry proteins from the mitochondria localized proteins takes 60 minutes, the crosslinking reaction takes 30 minutes. Thus, the chronologies both events cannot be directly and accurately compared or correlated, and we decided not to conduct this suggested experiment. However, we will follow the direction of the reviewer and the editor.

Following the request of the reviewer to compare the liposomal membrane permeabilization activity to the mitochondrial permeabilization activity of the same set of Bax mutants, we performed a mitochondrial permeabilization assay with digitonin permeabilized cells expressing SMAC-mCherry that is localized to the mitochondria membrane space. The new data is located in Figure 4 and Appendix Figure S4. The detailed protocol is found in the ‘Materials and Methods’ section under ‘Mitochondrial Permeabilization Assay’. We measured the percent release of SMAC-mCherry from mitochondria in digitonin permeabilized cells due the activity of the Bax mutants. The relative activity of the mutants compared to WT Bax matches the liposome data quite well. A117F was the most active mutant followed by R89E, F93E, F114E, S118D and A117D. A117D and S118D did not show substantial activity at 25 nM therefore we titrated these two mutants up to 100 nM (Appendix Fig. S4). Bax S118D is less active than WT Bax at lower concentrations but shows similar activity to WT Bax at 100 nM, whereas Bax A117D is mostly inactive at the concentrations tested. These data further corroborate the conclusions from the liposome permeabilization data.

Comment 2-3: If the concentration of tBID is increased in liposome permeabilization assay, would some of the weakly active mutants such as A117D and F114E induce more permeabilization. The authors need to comment on the possible effect of mutations on direct activation. Allosteric
changes seem to perpetuate within the Bax structure in rather obscure ways as recently shown by Dengler et al Cell Reports. Bax activation continues to be controversial.

Response 2-3: We performed the liposome permeabilization with a fixed concentration of Bax protein (25 nM) and increasing concentrations of tBid protein (0, 4, 8, and 16 nM). The data is shown in the revised Appendix Figure S4, and the related text is in page 7 as follows: “In the absence of tBid, all the proteins released less than 10% of the dyes (Fig. 4C), demonstrating as expected that this low nM concentration of Bax requires activation by tBid to form pores in the membranes. On the other side, increasing tBid concentration did not restore the dye release by the Bax mutants (Appendix Figure S4), suggesting that the defect is at a step after tBid activation of Bax. These results clearly show that the mutations which are predicted by our structure-based model and/or confirmed by our PRE data (Fig. EV3) to reduce the core dimer interaction with the lipid bilayer reduced the pore formation by the Bax protein.”

Comment 2-4: Can the authors estimate the molar ratio of Bax core dimers to bicelles. If this number is expected to be 1:1 it may explain why there are no intermolecular NOEs and why extensive crosslinking is not observed in bicelles (Figure S1C). It would be helpful to have a figure that shows the core dimer docked onto a bicelle for the readers to know that the core is likely displacing DHPC lipids to get to the DMPC layer (presumably 1 per bicelle?). Figure 4A may be improved and a zoom out show the core relative to the bicelle side and top view?

Response 2-4: To make sure that in the NMR sample all Bax core dimers are bound to bicelles, we used more bicelles than the protein molecules (molar ratio of protein molecule to bicelle =1:1.5-1:2) so that proteins would largely bind to the bicelles in which we could reasonably assume one protein dimer per bicelle. This is consistent with our results that no intermolecular (inter-dimer) NOEs and no extensive crosslinking were observed in bicelle-bound samples. We added more proteins to the bicelles and the mixture was not stable since precipitations were observed in a couple of hours (Fig. R4). Thus, we were unable to determine if more than one core dimer can bind to a bicelle. However, after centrifugation the NMR spectrum of the supernatant is the same as bicelle-bound NMR spectrum (Fig. R4), suggesting that the supernatant contains the bicelle-bound Bax (α2-α5).

The suggestion from this reviewer about making a figure to show how a core dimer binds to a bicelle and adding a zoom out to Figure 4A to show the top and side views of the protein-membrane complex is great to more clearly present the model to readers including the reviewer 1 (see Response 1-8 above). We hence added a three-dimension cartoon model to the revised Figure 3 to show both side and top views of a core dimer bound to the edge of a bicelle.
**Figure R4. The characterization on the ratio of Bax (α2-α5) to bicelle.** (A) 2D \(^{1}H-{\^{15}}N\) TROSY-HSQC spectra obtained at 600 MHz from a sample with Bax (α2-α5) to bicelle ratio of 0.5 (red), and from the supernatant of a sample with Bax (α2-α5) to bicelle ratio of 1.5 (blue) after centrifugation. The spectra are very similar but the sample with the higher protein to bicelle ratio was not stable as precipitants were seen in the NMR sample tube, and both proteins and lipids were detected in the pellet after centrifugation by SDS-PAGE.

Minor:

Comment 2-5: Consider adding this sentence at the end of first paragraph of introduction: "It is therefore expected that Bax assumes a number of different membrane-associated conformations that cooperate in regulating mitochondrial poration."

Response 2-5: We decide not to add this sentence to the end of the first paragraph of Introduction because we have already detailed a series of conformational changes of Bax that culminate in the mitochondrial membrane poration in the second paragraph.

Comment 2-1: I would remove ", as expected from the dramatically different NMR spectra for the bicelle-bound and the soluble Bax (α2-α5)(Fig. 1)" since the crystal structure of the core is a dimer (Czabotar Cell 2013), so the spectra are different representing a soluble tetramer and bicelle-associated dimer.

Response 2-6: The sentence is removed from the revised manuscript.

Referee #3:

Lv and co-authors present the structure determination of the BAX core domain, α2-α5 helices region, within a model lipid bilayer and interaction studies using NMR spectroscopy. Specifically, they determined the NMR structure of the BAX core domain bound to the lipid bicelles. The structure of the soluble BAX core domain has been previously determined by X-ray crystallography (Czabotar et al., Cell, 2013). There are notable changes in the NMR spectra of the
two BAX core conformations and authors present some changes on the structure upon interaction with the membrane. They also determined the topographic models of the BAX core domain in lipid bicelles using paramagnetic spin labels and showed evidence that the conformation of BAX in association with the membrane is more consistent with the clamp model than the in-plane model previously suggested by Bleicken and co-authors. Then the authors used the recently determined structure to design mutations on BAX which were evaluated on their ability to enable dimerization using cross-linking and pore forming activity in liposomal membranes. This study is novel as it presents one of the first studies investigating the interaction of BAX with the model membrane at atomic resolution. Besides the limitations and assumptions with studying only the truncated BAX core domain instead of the full length protein, the study provides some interesting structural insights in the ongoing attempt to understand how BAX acts on the mitochondrial membrane to induce membrane permeabilization. However, there are a few concerns and missing data to support the conclusions that need to be addressed.

We were happy to note that the reviewer assessed that our study is one of the first studies of the Bax-membrane interaction at atomic resolution presenting interesting structural insights for how Bax induce mitochondrial membrane permeabilization.

Comment 3-1: The authors have shown that in the absence and presence of lipid bicelles, BAX undergoes a conformation change that is evident in the TROSY-HSQC spectra Figure 1B. The authors should provide a chemical shift perturbations plot (bicelles bound BAX vs free BAX) so there is better understanding of the local and global structural changes upon addition of bicelles. They should also comment whether they see evidence of new peaks appearing in the spectra in the presence of bicelles. They should also highlight and map these residues on the BAX structure that show chemical shift perturbations upon membrane binding.

Response 3-1: The chemical shift perturbations (Δδ) between bicelles bound BAX vs free BAX are now added in Figure EV1. We highlighted the residues on the BAX structure according to Δδ values. Nonetheless, this comparison indicates that the change of the protein structure is global. Also, new peaks in the spectra obtained from the bicelle-bound protein include F114, Y115 and F116, which is added in Page 4.

Comment 3-2: Regarding the NMR structure determination, the authors should provide any reports and evaluation using structure validation databases.

Response 3-2: The validation reports are provided.

Comment 3-3: Please report the energies of the bicelles bound BAX structures and compare it with the crystal structure to know whether bicelles induce energetically more favorable conformations in solution.

Response 3-3: The free energy comparison between the two structures is now reported in page 4. The energy of the bicelle-bound Bax structure is lower than that of the crystal structure, suggesting that the membrane binding appears inducing an energetically more favorable
Comment 3-4: Why the authors chose to run NMR experiments (protein lipid NOE) at 32 °C as this introduce higher flexibility/mobility of protein that may disrupt local conformation or interaction of each protomer?

Response 3-4: Reconstitution Bax core dimers into bicelles will yield a large system for NMR, which usually produces weak signals. Increasing the temperature will enhance the tumbling rate of the protein-lipid particles, thus provide better NMR signals. In response to this reviewer’s question, we performed the NMR experiments at 25 °C and found that the spectrum is very similar to the spectrum at 32 °C (Fig. R5). To reduce redundancy and shorten the manuscript that already exceeds the limit of the EMBO journal, we would not include this low temperature NMR spectrum in the manuscript unless the reviewer insists it is necessary.

Figure R5. The influence of temperature on the NMR spectrum of bicelle-bound Bax (α2-α5). 2D $^1$H-$^{15}$N TROSY-HSQC spectra of bicelle-bound Bax (α2-α5) recorded at 32 °C (pink) and 25 °C (blue) at 600 MHz, respectively.

Comment 3-5: The authors have used paramagnetic probe titration to identify the residues whose intensities are affected by paramagnetic relaxation. The paramagnetic effect is relative to the position. It is not mentioned where the probe (Gd-DOTA) is supposed to bind or if it is expected to move freely in the solution? In the former case, how it is ensured that the results obtained by Gd-DOTA are not biased. For example, the graph in Figure S5A with high PRE doesn’t indicate a particular region that is solvent protected? Have the authors calculated PRE in lower temperatures and with additional concentrations?

Response 3-5: Gd-DOTA is a water-soluble PRE reagent, so it moves freely in solution. At 40 mM Gd-DOTA (Fig. R6A), the NMR signals from most of the residues exposed to the solvent disappeared, indicating the left residues are more solvent protected. We presented the differences in Appendix Fig. S3B, page 6. However, the PREamp derived from the Bax (α4-α5) didn’t give a typical solvent protected pattern as an integral membrane protein, suggesting that residues in Bax (α4-α5) is not deeply inserted in the membrane, which we showed in Appendix Figure S3A, page 6. We titrated this PRE agent to 50 mM (Fig. R6B), at this concentration only a
few residues mainly in α5 including L113, F114, Y115, A117, S118, L120 and etc. remained weak signals, no additional concentrations were titrated. We kept the temperature at 32 °C with the expectation to obtain a high-quality spectrum for these experiments as explained above.

**Figure R6. Gd-DOTA titrations of bicelle-bound Bax (α2–α5).** (A) Superimposed 2D ¹H–¹⁵N TROSY-HSQC spectra of bicelle-bound Bax (α2–α5) at 0 mM Gd-DOTA and 40 mM Gd-DOTA recorded at 32 °C at 600 MHz, respectively. (B) Superimposed 2D ¹H–¹⁵N TROSY-HSQC spectra of bicelle-bound Bax (α2–α5) at 0 mM Gd-DOTA and 50 mM Gd-DOTA recorded at 32 °C at 600 MHz, respectively.

Comment 3-6: The biochemical studies suggest that residues are involved somehow in the membrane interactions and have effect on the pore-forming activity of BAX, which could be independent of their ability to dimerize within the membrane. Therefore, the authors should demonstrate that specific single points mutations or combinations of mutants can disrupt the dimer interaction with the lipid bilayer, preferably by NMR.

Response 3-6: We have tried PRE analysis on Bax (α2–α5) with the R89E, S118D or A117D mutation. We were not able to purify the A117D mutant protein, and most of the resonances from the α4–α5 residues of the S118D mutant disappeared due to the signal broadening, possibly caused by the structure disruption of S118D or the fast exchange between S118D and bicelles. Only the R89E mutant clearly showed a slower PRE intensity decay. This was already shown and discussed in the original manuscript (Fig. EV3 and in page 7). Now we added the NMR-PRE data from R89A and F93E to the revised manuscript (Fig. EV3 in page 7) showing that this new mutation also reduces the interaction with the bilayer discs.

Comment 3-7: Regarding the proposed structural model of oligomeric BAX in figure 5, can the authors show evidence of a specific interaction between BAX core dimers in the presence of the lipid bicelles?

Response 3-7: We used a sample in which the number of lipid bicelles is more than the number of the BAX core dimers for the NMR experiments, which simplified the data interpretation but prevented the detection of potential inter-dimer interactions in the same bicelle. As mentioned above in response to reviewer #2, we titrated the bicelles with excess proteins, the NMR results obtained show that this sample was not stable preventing a further analysis of the potential inter-dimer interaction in this system (see Figure R4). As evidences that support the model in
Figure 5, in page 5 of the revised manuscript, we discussed the previously published crosslinking and DEER data from Bax and Bak proteins in membranes that could be explained by this model.

References:
Brouwer JM, Westphal D, Dewson G, Robin AY, Uren RT, Bartolo R, Thompson GV, Colman PM, Kluck RM, Czabotar PE (2014) Bak core and latch domains separate during activation, and freed core domains form symmetric homodimers. Molecular cell 55: 938-946
Cowan AD, Smith NA, Sandow JJ, Kapp EA, Rustam YH, Murphy JM, Brouwer JM, Bernardini JP, Roy MJ, Wardak AZ, Tan IK, Webb AI, Gulbis JM, Smith BJ, Reid GE, Dewson G, Colman PM, Czabotar PE (2020) BAK core dimers bind lipids and can be bridged by them. Nature structural & molecular biology 27: 1024-1031
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Lovell JF, Billen LP, Bindner S, Shamas-Din A, Fradin C, Leber B, Andrews DW (2008) Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax. Cell 135: 1074-1084
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Tan C, Dlugosz PJ, Peng J, Zhang Z, Lapolla SM, Pfafker SM, Andrews DW, Lin J (2006) Auto-activation of the apoptosis protein Bax increases mitochondrial membrane permeability and is inhibited by Bcl-2. The Journal of biological chemistry 281: 14764-14775
Thank you for submitting a revised version of your manuscript. I apologise for the unusually protracted review process due to delayed submission of referee reports. Your study has now been seen by two of the original reviewers, who find that their main concerns have been addressed and now recommend publication of the manuscript. Therefore, I would like to invite you to address the remaining editorial issues before I can extend the official acceptance of the manuscript.

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised files.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

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Referee #1:

The revised manuscript from Lv et al. addresses all the minor issues I had. This is a solid paper, which investigates the bicelle-bound structure of a Bax alpha 2-5 dimer via NMR and cross-linking data. The new structure reveals some intriguing differences with respect to the previously crystallized Bax dimer and the robust experimental data shed light on the topology of active Bax dimers at the membrane. Additionally, the effects of point mutations in the formation of the active core dimer are corroborated with full length Bax in isolated mitochondria. Overall, this is an important study which advances the field, and put forward the clamp model of active Bax at the MOM.

I suggest publication in its present form.

Referee #3:

The authors have addressed satisfactorily previous comments and supported their conclusions with additional data. This study provides new insights of the BAX a2-a5 dimer in a membrane environment that advances the field. I recommend publication.
The authors performed the requested editorial changes.
Editor accepted the revised manuscript.
**B- Statistics and general methods**

| Question                                                                 | Answer |
|--------------------------------------------------------------------------|--------|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | No sample size calculation was performed and we didn’t do the statistical analysis. We repeated the measurements from the functional assays with independent experiments as indicated in the Methods and Material section. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No data were excluded from the analysis. |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g., randomization procedure)? If yes, please describe. | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or and when assessing results (e.g., blinding of the investigator)? If yes please describe. | Control experiments were carefully designed and multiple methods were applied to draw the conclusions and avoid the subjective bias. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | NA |
| 6. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | NA |
| 7. Is there an estimate of variation within each group of data? | NA |

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22. Could your study fall under dual use research restrictions? Please check biosecurity documents in a public repository or included in supplementary information.

19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal’s data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript text. cDNA and antibody data: Antibodypedia (see link list at top right), AntibodyOnline (see link list at top right).

16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. Please follow author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WHA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

11. Identify the committee(s) approving the study protocol.

10. We recommend consulting the ARRIVE guidelines (see link list at top right) (Furutani, R. et al. (2010) to ensure that other relevant aspects of animal studies are adequately reported. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.

9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

8. Report species, strain, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), AntibodyOnline (see link list at top right).

5. Report species, strain, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

4. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

3. Report any restrictions on the availability (and/or on the use) of human data or samples.

2. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WHA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

1. Identify the committee(s) approving the study protocol.

This section is provided in the manuscript text.

The structure information was deposited with Protein Data Bank (PDB) and Biological Magnetic Resonance Bank (BMRB). Data are available and can be obtained from DuFang’s lab.

The BMK Bax/Bak DKO cells were a gift from Dr. Eileen White. There is no STR profiling test available at present for the BMK Bax/Bak DKO cells used. The BMK Bax/Bak DKO cells routinely checked for mycoplasma contamination.

The following data sets are available from GenBank (Accession: GCA_002828045.1).Data are available at present for the BMK Bax/Bak DKO cells used.  The BMK Bax/Bak DKO cells were a gift from Dr. Eileen White. There is no STR profiling test available at present for the BMK Bax/Bak DKO cells used. The BMK Bax/Bak DKO cells routinely checked for mycoplasma contamination.

The variance similar between the groups that are being statistically compared?