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BH3-in-groove dimerization initiates and helix 9 dimerization expands Bax pore assembly in membranes

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1st Editorial Decision 30 April 2015

Thank you for submitting your manuscript entitled 'BH3-in-groove dimerization initiates and helix 9 dimerization expands Bax pore assembly in membranes'. I have now received the reports from all referees.

As you can see below, all referees recognize that there is interest in better understanding the topology and oligomerization of activated Bax. However, they think that alternative models should be considered as well and that additional data and information are needed to support your conclusions and to make your manuscript a good candidate for publication in The EMBO Journal.

Given the very constructive comments provided, I can offer you to submit a revised version of the manuscript, addressing all concerns of the referees. Importantly,
- mutant Bax activity assays are required (referee #3, point 1)
- the proposed transitions need to be further supported (referee #3, point 2)
- all technical concerns need to be addressed
- controls need to be added to several of your assays (referee #1, points 1-2; referee #2, point 2; referee #3, point 1 and 5).
- previous findings must be better implemented into the presented work (referee #2 and #3).

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

REFEREE REPORTS

Referee #1:

In their manuscript, Zhang et al. investigate structural rearrangements associated with the oligomerization of Bax during mitochondrial outer membrane permeabilization (MOMP). Based on previous structural studies, the authors designed several cysteine mutants of Bax and demonstrate by disulfide crosslinking the interaction of Bax molecules through a previously described BH3-in-groove and a novel a2-a3-a4 conformation. By labeling of cysteines with the compound IASD, they furthermore demonstrate membrane embedding of helix a5. Using inhibitory mutants, the authors establish a line of events leading from Bax activation to pore formation, and present an attractive and detailed structural model for this event.

Even though Bax-mediated MOMP is a well-studied event, current knowledge on the molecular events occuring at the mitochondrial outer membrane between Bax activation and pore formation is rather limited. The experiments presented by Zhang et al. thus constitute a major advance regarding this question, even though several experiments confirm previous data by Bleicken et al (Mol Cell 2014). Overall, the work addresses the problem in great detail, and the quality of experiments is high. My concerns about the study are relatively minor:

1. Can the authors exclude that disulfide crosslinks that become possible after cysteine engineering might by themselves enhance dimer formation? In this respect, it might be interesting to test dimer formation in the absence of mitochondrial membranes, as was done for the L59C/M79C mutant, especially for those mutants that are autoactive in the absence of a BH3 activator.

2. A basal cytochrome c release of 20 % in the absence of tBid, as shown in Figs. 9 and 10, seems rather high. Can the authors show that MOM integrity is preserved in the mitochondria preparations they use throughout their experiments (before the addition of Bid/Bax), e.g. by a protease accessibility experiment?

Referee #2:

In this manuscript, Zhang et al. present research to extract details at the residue level of the protein, Bax, as it oligomerizes and causes mitochondrial membrane permeabilization. Bax is a pro-apoptotic member of the Bcl-2 family of proteins, which regulates apoptosis. This regulation is accomplished by dynamic interactions between pro-survival and pro-apoptotic members within this family.

The authors have provided promising results that have the potential to make a significant contribution to understanding of mitochondria membrane permeabilization that could be suitable for publication in EMBO. Among the very important questions still unresolved is the architecture of Bax in these oligomers. Presumably, by understanding the structure that Bax adopts, we could have a better understanding of how these membrane pores are built. Not only have these authors identified (previously) observed sites of contact, but have also determined a "hierarchy" of how these sites give structural integrity to these pores. This represents a breakthrough in how we understand apoptosis regulation. However, there are some technical issues that need to be resolved as well as in the presentation of the final results. If the authors can satisfactorily address these points, then I believe this would make a fine contribution to EMBO.

1) One of the interactions studied in this manuscript is between separate Bax molecules at their BH3 domains. This contact has been observed previously, as noted by the authors, and experiments were performed to address features of this contact observed in a high-resolution solution crystal structure. While such a comparison contributes to our understanding of these interactions, there are some issues with the interpretation of the results that could be resolved by straightforward experiments. The authors utilize an IASD labeling method to determine to what degree the membrane embeds a

particular residue. The degree the fluorescent dye, IASD, is protected from labeling a site, indicates the degree of shielding from the mitochondrial membrane. The authors also modified the protocol to determine "protein-only' shielding. A lot of information about the structure could be learned from these experiments, but most of this information is lost by assigning an "arbitrary cut-off" of 0.15 (page 13) to partition the labeling as exposed or buried in the membrane. A more informative experiment would be to observe the results of this IASD reaction on a completely buried site, T182 for example, and a site in the BH3 domain in the protein from 4BDU. Then report the degrees of shielding of IASD at various sites relative to these two extremes. According to Figure 2, the authors are judging the degree of shielding from a static structure and saying that it is equivalent to shielding in a membrane. More realistically, there could be dynamic fluctuations in both environments that could yield different degrees of shielding. These potential observations and discussions could be beneficial to how we understand interactions in solution and in a membrane environment.

2) In the cytochrome c release assays, I am confused by what is meant by "autoactive" (page 24) when Bax and variants are added to isolated mitochondria. I have not come across this term in the literature. In the cellular context, Bax is not constitutively active but must interact with other factors, including tBID, to induce membrane permeabilization. Where else is this phenomenon observed? Could this be an artifact of an in vitro assay? To help place the results into context, showing or mentioning if there is any cytochrome c is released before Bax or Bax/cBid is added might explain release before the addition of tBid. However, it should be mentioned that it is clear that there is an effect due to the addition of tBid, which is consistent with what is already known about this system.

3) The authors propose two orientations of the α9 helices when different Bax molecules form oligomers. The authors utilize molecular dynamics simulations incorporating observed cross-linking data as restraints to propose these two structures. What are conspicuously absent are references to lipid-lipid or lipid-protein interactions. Have the authors explored the possibility of incorporating lipid molecules into their simulations? In addition, could the effects of the deleterious mutations for this interaction, G179I and T182I, be explained with MD simulations? Addressing such issues would be very informative for this manuscript.

4) What would also be beneficial to the manuscript is mentioning how some published results from different papers bolster the authors' conclusions. A substantial contribution to this manuscript is the reporting of contacts between α9 helices of separate Bax molecules. While this contact has been reported previously (Bleicken et al., 2014) with some ambiguity in their orientation as well as between structurally homologous Bak molecules (Iyer et al., 2015), a parallel orientation of contact between the α9 helices has been observed in live cells after translocation and reported earlier (Gahl et al.,2014). Similar observation using a different technique reinforces the authors' conclusions about the requirement of this contact to release large proteins, like SMAC. Another feature in Bax that allows it to form pores to release large proteins is that it adopts an extended conformation. In the proposed model in Figure 11, there is no mention of intramolecular constraints even though the authors illustrated it as extended. There is previous published evidence that supports an extended conformation. Czabotar et al in 2013 propose a "hinge" motion to bring α5 and α6 away from the core. The DEER experiment (Bleiken et al., 2014) reports intra-molecular distances consistent with an extended conformation. Also, Gahl et al., 2014 report FRET measurements that place α9 away from the BH3 domain in order to form oligomers. The research presented in these other groups are complimentary to the conclusions presented in this manuscript and would be beneficial to include in its final form.

5) One minor comment. On page 20, in the middle of the second paragraph, the authors refer to residue Leu186. We assume the authors meant Leu185, as it is referred to in other parts of the text and figures.

Referee #3:

In response to cytotoxic stress, the pro-apoptotic Bcl-2 protein Bax commits mammalian cells to apoptosis. Thus, the nature of active Bax has been widely considered the holy grail of apoptosis research. In this manuscript, Lin and colleagues attempt to combine an early model of active Bax (helix 5 - helix 6 hairpin insertion, proposed by co-authors of this manuscript) with a model based

on recent structural data from Czabotar and colleagues. To this end, cysteine mutants have been generated and used in dimerization and labeling experiments to test distances between helices and surface accessibility of residues. The presented data could be potentially interesting to the Bcl-2 research field, if the authors can show that suggested interactions occur during Bax activation in cells.

Major concerns:
1) Are the suggested interactions relevant for Bax activation/regulation? The authors claim that finding perhaps mutant dimerization identifies a specific interaction occurring in the activation of Bax. If the authors intend to show the importance of the suggested interactions, the mutants must behave like Bax. Currently the authors present a poor assay (30 % cyt c release in the absence of tBid) testing the cyt c release of in vitro translated protein. However, expression at near endogenous levels in cells lacking endogenous Bax and characterization of localization and activity prior and following cytotoxic stress is essential. The authors clearly cannot properly characterize all generated mutants, but a pool of the 5-6 most interesting mutants must be thoroughly analyzed.

2) Is the 2-3-4 interface binding necessary for Bax activation or even occurring in cells? In Fig. 11 a model is presented, showing transitions between BH3-in-groove dimer and 2-3-4 dimer and then intersected/parallel dimers (tetramers). However, proof of a transition from one to the other state is lacking. The manuscript shows only prevention of BH3 symmetric dimerization and activity by G108E, which could be due to interference with dimerization or another reason, perhaps misfolding. The claims of the authors can only be substantiated by mutants discriminating between the different interactions. For instance, prevention of helix 9-9 interactions must lead to the 2-3-4 dimer, while interference with 2-3-4 dimerization must result in BH3-in-groove dimers. Based on their rich interaction data the authors can probably identify such discriminating residues and characterize the mutants in cells. Unfortunately, both helix 9 mutants generated to specifically interfere with one helix 9-9 interaction also disrupted the second potential interaction. Again, specific disruption is needed.

3) Can the data be interpreted alternatively? The idea of a 2-3-4 dimer is presented as the result of calculations, but the reader is left wondering, whether there were alternative models and why the authors are so sure about this one.

4) How can the authors distinguish between OMM-integral and residues buried in a protein dimer/oligomer? How can helix 5 be OMM-embedded when V121 and K119 are apparently surface exposed? Also, the analysis of helix 6 should distinguish between the two models. Are residues in helix 6 surface accessible?

5) Controls are lacking or not controlling for the experiment. Why was MOMP initially assayed with tBid, but then the Bax BH3 peptide was used producing an artificial dimer? The disappearance on reducing gels of dimers would significantly strengthen the manuscript. Especially since bands presented in Figure 8 as oligomers can be found on other gels. Why should we appreciate the marked band in fig. 3B lane 4 (for example), while similarly or more prominent bands are also in this lane? Controls for carbonate extraction need to be done with the samples and probably all gels but most importantly the MOMP assay should have loading controls.

6) How do the findings of the authors relate to the reversible OMM-association and the retrotranslocation of Bax and Bak in living cells? In the model tBid is included as a star, but mitochondrial Bax association in cells does not require tBid and the Bid KO mouse lacks a prominent phenotype.

Minor points
1) The authors cite preferably two groups of authors and not necessarily the appropriate papers. Especially the first paper suggesting helix9-9 interactions (Gahl et al., 2014) should be cited. Also what makes residues "Confirmed MOM-embedded"?
2) The depiction of protein structures is generally crowded and confusing. Figure 1A (for example) is perhaps only providing information to the reader that is used to look at Bax structures on a daily basis.
3) The data is compressed in too little space. It is impossible to judge whether mutants are shifting in Figure 2B. The reader is left with a "trust me". Reduction may be a solution.

Point-to-Point Responses

(1) *Editor: mutant Bax activity assays are required (referee #3, point 1).*

(1-a) *Referee #3, point 1: Are the suggested interactions relevant for Bax activation/regulation? The authors claim that finding perhaps mutant dimerization identifies a specific interaction occurring in the activation of Bax. If the authors intend to show the importance of the suggested interactions, the mutants must behave like Bax. Currently the authors present a poor assay (30 % cyt c release in the absence of tBid) testing the cyt c release of in vitro translated protein. However, expression at near endogenous levels in cells lacking endogenous Bax and characterization of localization and activity prior and following cytotoxic stress is essential. The authors clearly cannot properly characterize all generated mutants, but a pool of the 5-6 most interesting mutants must be thoroughly analyzed.*

Response: The referee brings up a very important point. We must test the apoptotic activity and localization of the Bax cysteine mutants in cells in order for us to relate our in vitro data to biological activity. Thus, we transiently expressed wild-type (WT) Bax and the most interesting cysteine mutants identified in the paper as Venus fusion proteins in *bax/bak* double knockout baby mouse kidney cells (*bax/bak* DKO BMK cells). We determined their intracellular location and apoptotic activity before and after Staurosporine (STS) treatment. Venus is fused to the N-terminus of the Bax constructs so it would not interfere with Bax activity since the C-terminal a9 helix inserts into membranes and aids in the ability of Bax to kill cells. Average Venus fluorescence per cell was measured and because it is fused to Bax correlated to expression. All mutant constructs were expressed at similar levels compared to Venus-WT Bax (Figure EV3A). Expression of Venus-WT Bax increased apoptosis compared to the Venus-only control, and the STS treatment further increased apoptosis (Figure EV3B). The cysteine-null (C0), the single-cysteine (A178C and A183C), and the double-cysteine (L59C,M79C and L59C,L76C) mutants behaved similarly to the WT protein significantly elevating apoptosis in response to STS. Therefore, the apoptotic activity data from the cells expressing the Bax cysteine mutants are consistent with the cytochrome c release data from the isolated mitochondria reconstituted with in vitro synthesized Bax proteins, demonstrating that the cysteine mutants used throughout the manuscript have similar activity compared to WT Bax. In addition, the intracellular localization of these cysteine mutants is similar to Venus-WT Bax, mostly in the cytoplasm but partially at the mitochondria in the untreated cells (Figure EV3C). The only exception is Venus-Bax L59C,L76C which is constitutively localized to the mitochondria. As expected, these intracellular localization data are in line with the mitochondrial binding data obtained in vitro (Figure EV2C, EV4F, EV7E and EV10E).

In addition, the following single-Cys Bax mutants that we used in our study were assayed by others in the $Bax^{-/-}/Bak^{-/-}$ mouse embryo fibroblast (MEF) cells and they induced apoptosis after etoposide treatment like WT Bax (Dewson et al, 2012; Iyer et al, 2015; Westphal et al, 2014): T56C, E69C, R94C, L122C, C126, I175C, V177C, A178C, G179C, and V180C. Thus, the cytochrome c release activity that we observed with these mutants in vitro is consistent with the apoptotic activity that the others observed in cells.

(2) *Editor: the proposed transitions need to be further supported (referee #3, point 2).*

(2-a) *Referee #3, point 2: Is the 2-3-4 interface binding necessary for Bax activation or even occurring in cells? In Fig. 11 a model is presented, showing transitions between BH3-in-groove dimer and 2-3-4 dimer and then intersected/parallel dimers (tetramers). However, proof of a transition from one to the other state is lacking. The manuscript shows only prevention of BH3 symmetric dimerization and activity by G108E, which could be due to interference with dimerization or another reason, perhaps misfolding. The claims of the authors can only be substantiated by mutants discriminating between the different interactions. For instance, prevention of helix 9-9 interactions must lead to the 2-3-4 dimer, while interference with 2-3-4 dimerization must result in BH3-in-groove dimers. Based on their rich interaction data the authors can probably identify such discriminating residues and characterize the mutants in cells. Unfortunately, both helix 9 mutants generated to specifically interfere with one helix 9-9 interaction also disrupted the second potential interaction. Again, specific disruption is needed.*

Response: The referee raises an interesting point and we agree with the referee's predictions if the binding interactions are independent. In our original paper we show that they are not independent but there is a hierarchy. The argument supporting this hierarchy was obviously unclear therefore we have revised the text and the figures to highlight our model. We assayed mutants that the crystal structure and the structural models predict would discriminate independent binding surfaces from a hierarchy. The crystal structure of BH3-in-groove dimer and the structural models of a2-a3-a4 dimer as well as a9 dimers predict the following "discriminating" mutations that would disrupt one but not the other interfaces if these interfaces can form independently. The G108E mutation in the BH3-binding groove would disrupt the BH3-in-groove dimer (Figure 4A) but not the a2-a3-a4 dimer and the a9 dimers. The G179I mutation in a9 would disrupt the intersected a9 dimer but not the parallel a9 dimer, whereas the T182I mutation would disrupt the parallel dimer but not the intersected dimer (Figure 7A). The crosslinking results are not consistent with independent binding surfaces based on these predictions. Thus, the G108E mutation not only inhibited the crosslinking specific to the BH3-in-groove dimer (Figure 4B), it also inhibited that specific to the a2-a3-a4 dimer (Figure 4C) and the a9 dimers (Figure 7C). Therefore, we proposed a model in Figure 11A that the BH3-in-groove interface is formed upstream of the other three interfaces, and the formation of the downstream interfaces depends on the formation of the upstream one.

Consistent with our hierarchy interpretation, the G179I or T182I mutation in a9 inhibited the crosslinking specific to the intersected or parallel a9 dimer, respectively, as predicted (Figure 7B, lanes 1-8). Both mutations did not significantly alter the crosslinking specific to the BH3-in-groove dimer and the a2-a3-a4 dimer (Figure 7D and data not shown). Therefore, we placed the two a9 dimerizations downstream of the BH3-in-groove and the a2-a3-a4 dimerizations in Figure 11A, and suggested that the upstream dimerizations do not depend on the downstream ones.

Unexpected at then, each of the two a9 mutations also inhibited the crosslinking specific to the other a9 dimer (Figure 7B, lanes 9-16) that should not be inhibited according to the static and independent dimer structural models (Figure 7A). We now have some explanations for these observations, thanks to the referee #2's request for molecular dynamic (MD) simulations of a9 dimers in a lipid bilayer. The MD simulation results indicate that the G179I could disrupt both intersected and parallel dimers, but the T192I mutation would only disrupt the parallel dimer (Figure EV12). However, the simulation revealed a transition between the intersected and the parallel dimer conformations, and the free-energy of the transition indicated several intermediate states between the two extreme states (Figure EV11B). Therefore, the multiple conformations of the a9 dimers would be energetically linked, and destabilization of any particular one by a mutation might also destabilize the others. Particularly the intersected a9 dimer with the lowest free-energy may be evolved from the parallel dimer through the intermediate conformational states revealed by the simulation. And if so, blocking the upstream parallel dimerization by the T182I mutation may also block the downstream intersected dimerization. The crosslinking data from the G179I and T182I mutants are in line with this notion that is now discussed in the manuscript.

In addition, we explored the transition from the BH3-in-groove dimer conformation to the a2-a3-a4 dimer conformation in the lipid bilayer using MD simulation. The simulation started with the BH3-in-groove dimer structure on the bilayer surface except that the a5 helix from each monomer was moved into the bilayer to account for the IASD inaccessibility data shown in Figure 2. The simulation disclosed a dynamic nature of the dimer structure that is remained on the bilayer surface including the a2-a3-a4 helices (Figure EV6). Particularly, the residues that were initially distal became proximal during the simulation such that they would be linked by a disulfide if they were replaced by cysteines (Figure EV6B). Thus, the fluctuation of the a2-a3-a4 dimer structure on the lipid bilayer is enough to explain most of the observed disulfide linkages in Figure 3 that cannot be explained by the initial BH3-in-groove dimer structure. Although the final structure of the a2-a3-a4 dimer on the bilayer surface at the end of 135-ns simulation is different from the static a2-a3-a4 dimer structure generated by computational modeling that has the best packing and the most favorable energy score, It is likely that longer simulations are needed to sample these large-scale conformational changes.

The BH3-in-groove dimer structure and the static a2-a3-a4 dimer model predicted that L59D, D98E, and/or G103F would disrupt the latter but not the former. However, the triple mutations did not block the crosslinking specific to the a2-a3-a4 dimer (**Response Figure R1**). These data suggest that the a2-a3-a4 dimer interface may be flexible and hence able to tolerate the mutations. In accordance,

the MD simulation of the a2-a3-a4 dimer on the bilayer shows a flexible interface (Figure EV6). Nonetheless, since we have not found a mutation that could disrupt the a2-a3-a4 dimer interface and hence assess its effect on the other interfaces and on the Bax MOMP activity, we removed the arrows that represent the transitions from the a2-a3-a4 dimer to the other complexes from the model in Figure 11, and only kept the arrow from the BH3-in-groove dimer to the a2-a3-a4 dimer based on the effect of G108E mutation. We think that this more conservative interpretation of our data along with the new MD simulations provides the information requested by the referee.

To determine whether the model in Figure 11 is relevant to the biological activity of Bax in cells, we expressed WT Bax, and Bax G108E, S184V, G108E/S184V, G179I and T182I mutants as the N-terminal Venus fusion proteins in *bax/bak* DKO BMK cells, and assessed their intracellular localization and apoptotic activity in the absence and presence of STS as the referee requested. Expression levels of these mutants were similar to that of Venus-WT Bax, except for Venus-Bax G108E that had an increased expression that was likely tolerated by the cells since this mutant lacks apoptotic activity (Figure EV14A). Venus-Bax G108E induced a significantly lower level of apoptosis in the absence and presence of STS when compared to Venus-WT Bax (Figure EV14B), and the G108E mutant was mainly localized to the cytoplasm as the WT protein in the untreated cells (Figure EV14C). Addition of the S184V mutation to Venus-Bax G108E caused the double mutant to constitutively target to the mitochondria resulting in an apoptotic activity that was comparable to WT Bax. However, the apoptotic activity of the G108E/S184V double mutant was significantly lower than the S184V single mutant, although they both had constitutive mitochondrial targeting. Therefore, the G108E mutation inhibited an activation step downstream of mitochondrial targeting of Bax, such as the dimerizations that were detected by the crosslinking assay.

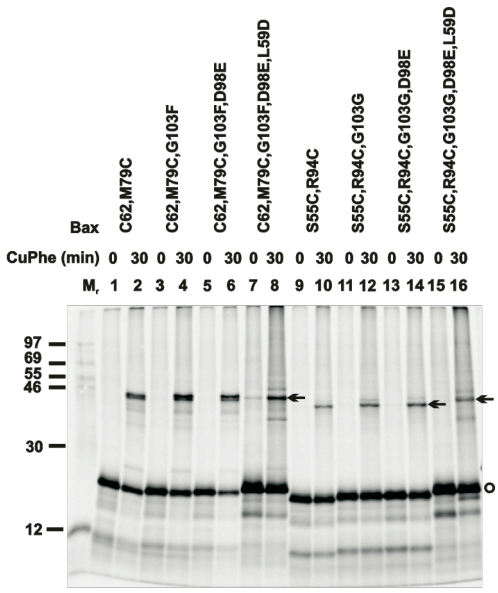

Figure R1. **Effect of mutations on α2-α3-α4 dimerization.** Oxidized mitochondria with the radioactive double-cysteine Bax proteins with the additional mutations as indicated were prepared and analyzed as in Figure 1B. Protein standards are indicated by their M_r, Bax monomers by circles, and disulfide-linked Bax dimers by arrows.

In contrast, the G179I mutant was constitutively active and spontaneously killed cells, an observation that is consistent with those from in vitro assays (Figure 10A-C). Due to the extensive cell death caused by the G179I mutant, accurate localization data was not obtainable. However, because the cells were dying, Venus-Bax G179I was likely targeted to the mitochondria constitutively, a notion supported by the in vitro mitochondrial binding data (Figure EV13C).

When expressed in cells, the T182I mutant had similar activity and localization to that of WT Bax. While the apoptosis data from the cells expressing the T182I mutant are mostly in line with the cytochrome c release data from the isolated mitochondria reconstituted with the in vitro synthesized

T182I mutant (Figure 10A), the defect of the T182I mutant detected in the in vitro Smac and Smac-mCherry release assays (Figure 10B-C) does not translate into a defect in the cell-based apoptosis assay. The simplest explanation is that the cytochrome c release caused by the T182I mutant in the cells is sufficient to induce apoptosis, and the reduction of the Smac release caused by the mutation in the cells is not sufficient to block the apoptosis. Taken all the data together, the model in Figure 11 adequately describes the roles of the BH3-in-groove and the a9 dimerizations as well as the a9 insertion in the Bax pore formation in a manner consistent with the crosslinking data and the topology data (see (3-a) below). The most speculative aspect is the role of pore expansion mediated by a9 insertion and dimerization in the execution of apoptosis. However, other published data is consistent with the model and we have attempted within text limitations to describe which aspects of the model are very well supported and which are somewhat more speculative.

Regarding the potential effect of G108E mutation on Bax folding, we agree with the referee that misfolding is always a potential problem with mutagenesis strategies. We have added to the manuscript a description indicating that the G108E mutant was most likely folded correctly as it still binds to tBid via its canonical groove when targeted to the mitochondria by the S184V mutation as shown by the tBid-Bax crosslinking data in Figure EV8. A previous paper (Kim et al, 2009) and our data in Figure EV14 showed that the G108E mutant was expressed in cells at a level higher than the WT Bax yet did not kill the cells, and targeted to the mitochondria by the S184V mutation, suggesting that the G108E mutation does not misfold the protein. Moreover, the Kim et al paper showed that the G108E,S184V double mutant failed to form oligomers at the mitochondria in cells, consistent with our findings from the in vitro crosslinking assay.

(3) ***Editor: all technical concerns need to be addressed.***

(3-a) *Referee #2, point 1: One of the interactions studied in this manuscript is between separate Bax molecules at their BH3 domains. This contact has been observed previously, as noted by the authors, and experiments were performed to address features of this contact observed in a high-resolution solution crystal structure. While such a comparison contributes to our understanding of these interactions, there are some issues with the interpretation of the results that could be resolved by straightforward experiments. The authors utilize an IASD labeling method to determine to what degree the membrane embeds a particular residue. The degree the fluorescent dye, IASD, is protected from labeling a site, indicates the degree of shielding from the mitochondrial membrane. The authors also modified the protocol to determine "protein-only' shielding. A lot of information about the structure could be learned from these experiments, but most of this information is lost by assigning an "arbitrary cut-off" of 0.15 (page 13) to partition the labeling as exposed or buried in the membrane. A more informative experiment would be to observe the results of this IASD reaction on a completely buried site, T182 for example, and a site in the BH3 domain in the protein from 4BDU. Then report the degrees of shielding of IASD at various sites relative to these two extremes. According to Figure 2, the authors are judging the degree of shielding from a static structure and saying that it is equivalent to shielding in a membrane. More realistically, there could be dynamic fluctuations in both environments that could yield different degrees of shielding. These potential observations and discussions could be beneficial to how we understand interactions in solution and in a membrane environment.*

Response: We followed the suggestion from the referee, and used G179 as the reference for membrane burial because it is in the a9 helix that is mostly embedded in the MOM as shown by the IASD inaccessibility data in Figure 5 and our previously study (Annis et al, 2005). We used the membrane burial index of G179C to normalize that of the other residues. The resulting relative membrane burial indices are now shown in Figure 2C along with the “absolute” values shown in Figure EV5B. We also used Y115 as the reference for protein burial because it is buried in the hydrophobic core of the BH3-in-groove dimer crystal structure (PDB entry 4BDU) and is the most protein-buried residue in the mitochondria-bound Bax protein as the IASD inaccessibility data in Figure 2B and EV5C indicated. We obtained the relative protein burial indices for the other residues by normalizing their absolute indices with that of Y115C, and showed both absolute and relative indices in Figure EV5C and 2D, respectively. Based on the relative membrane burial indices, four residues in the BH3/a2 helix, one residue in a4, and six residues in a5 interact with the lipid environment in the membrane at least for a fraction of Bax molecules at the mitochondria because their membrane burial is 40% or more compared to the reference G179 (100%). By this criterion seven residues in a9 helix interact with the lipid environment in the membrane (Figure 5 and EV9).

Using a similar criterion for protein burial (>/= 40% compared to Y115), three residues in the BH3/a2 helix, one in a3, four in a5, and one in a9 are buried in the protein or its complex.

We appreciate the comment from the referee as this new way of presenting the data makes it clearer that the interactions are occurred in dynamic environments, which is also suggested by the MD simulations. Nevertheless, the overall conclusion about the structure and topology of the membrane-bound Bax molecules is unchanged. Thus, a significant fraction of the Bax molecules have these helices buried either in the membrane, or the protein/complex, or both. While the static models presented in Figure 2A and 5D fit with most of the IASD labeling data, the dynamic fluctuations of the protein structures inside and outside the membrane easily account for the results being less than binary. The molecular dynamic simulations of the membrane-bound protein complexes that we described in (2-a) above and (3-b) below provide an explanation for the most likely structural fluctuations. We are sure that the referee will agree that adding this information has improved the manuscript significantly.

(3-b) *Referee #2, point 3: The authors propose two orientations of the α9 helices when different Bax molecules form oligomers. The authors utilize molecular dynamics simulations incorporating observed cross-linking data as restraints to propose these two structures. What are conspicuously absent are references to lipid-lipid or lipid-protein interactions. Have the authors explored the possibility of incorporating lipid molecules into their simulations? In addition, could the effects of the deleterious mutations for this interaction, G179I and T182I, be explained with MD simulations? Addressing such issues would be very informative for this manuscript.*

Response: We fully agree with referee #2 that simulations including lipids are worth doing to assess the effect of lipids and the mutations on the dimer structure and stability. We have now completed the molecular dynamic simulations for the two a9 dimers in the lipid bilayer comprised of the MOM characteristic phospholipids (Kuwana et al, 2002) and in the solution containing counterions. We compared the initial conformations of both intersected and parallel a9 dimers, which were generated by the computational modeling based on the crosslinking data, to the final conformations after 175-ns simulation. The results indicate that both intersected and parallel helical dimer conformations in the lipid bilayer remain stable given small fluctuations of backbone root-mean-square deviation (RMSD) ~1 Å (Figure EV11A). In contrast, both dimer conformations in the solution became unstable resulting in large backbone RMSD fluctuations ~5 Å and loss of helicity from both termini. Therefore, the MD simulations suggest that the membrane plays an important role in stabilizing the a9 dimer structures. Consistent with the MD simulations we observed a9 dimer-specific crosslinking in the mitochondrial fraction after Bax was activated by cBid and targeted to the mitochondria, but not in the soluble fraction with or without cBid (Figure EV10E).

To determine the stability of the intersected and parallel a9 dimer conformations, we have performed metadynamics simulations. The resulting free-energy of a9 dimer transition between intersected and parallel states shows that the free-energy of the intersected dimer is ~3.5 kcal/mol lower than the parallel one (Figure EV11B). In addition, several intermediate dimer conformations were revealed with different free-energy levels. Thus, the intersected a9 dimer is more stable than the parallel dimer, and they may coexist in equilibriums with other intermediate dimer conformations in the membrane.

We also did the MD simulation with the a9 dimers containing the G179I or T182I mutation to assess the mutational effect. Comparison of the initial and final structures of the dimers shows that the G179I mutation disrupts both intersected and parallel dimers, whereas the T182I mutation only disrupts the parallel dimer (Figure EV12). To compare with the crosslinking data from the A183C or A178C mutant that captures the intersected or parallel dimer, respectively, we monitored the b carbon distance between the two A183 residues in the intersected a9 dimer or between the two A178 residues in the parallel a9 dimer with or without the G179I or T182I mutation. The results indicate that the G179I mutation increases the b carbon distance for both residue pairs (Figure EV12), thereby explaining its inhibitory effect on the disulfide crosslinking of the A183C and A178C mutants. In contrast, the T182I mutation only increases the b carbon distance for the A178 residue pair, thereby only explaining its inhibition on the A178C crosslinking. While the inhibition of the A183C crosslinking or the intersected a9 dimerization by the T182I mutation cannot be explained by the MD simulation, it remains possible that the intersected a9 dimer with the lowest free-energy may be evolved from the parallel dimer with a higher free-energy through the intermediate free-

energy states revealed by the simulated conformational transition (Figure EV11B). And if so, blocking the upstream parallel a9 dimerization by the T182I mutation may also block the downstream intersected dimerization. Only long-term extensive experimentation would determine if this scenario is true, which is clearly beyond the scope of this manuscript.

(3-c) *Referee #3, point 3: Can the data be interpreted alternatively? The idea of a 2-3-4 dimer is presented as the result of calculations, but the reader is left wondering, whether there were alternative models and why the authors are so sure about this one.*

Response: This is an excellent point raised by the referee. The structure we proposed was the best fit we could find using relatively static structures. However, we agree with the referee that it would be good to sample other potential models. Therefore we did molecular dynamic simulation to test the hypothesis that the BH3-in-groove dimer structure revealed by crystallography may change, particularly after the two a5 helices are released from the structure into the membrane as our IASD inaccessibility data suggested (Figure 2). The MD simulation was initiated with a structure modified from the BH3-in-groove structure in which the two a5 helices are moved into the lipid bilayer, and the structures of the two a2-a3-a4 regions is unchanged and remain on the bilayer surface. The simulation disclosed a dynamic nature of the a2-a3-a4 dimer structure on the bilayer (Figure EV6). Particularly, several residues that were initially distal became proximal during the simulation such that they would be linked by a disulfide if they were replaced by cysteine residues (Figure EV6B). Thus, the fluctuation of the a2-a3-a4 dimer structure is enough to explain most of the observed disulfide linkages in Figure 3A-C that cannot be explained by the initial BH3-in-groove dimer structure.

The dynamics of the a2-a3-a4 dimer may eventually lead to a stable structure that could not have been reached by the MD simulation due to the time scale (135 ns). Therefore, to predict a stable a2-a3-a4 dimer structure that can better accommodate all of the crosslinking data we performed a new computational structural modeling with minimal restraints and maximal flexibility. In this modeling, we allowed the three helices a2, a3, and a4 to rearrange freely in space driven by the optimization of distance constraints derived from the experimental crosslinking data. This calculation produced a dimer model in which helices a2 and a3 are merged into a single helix in a conformation that resembles an anti-parallel coiled coil, whereas a4 folds back after a loop, capping the end of the coil. The model has excellent packing interactions and satisfies all imposed experimental constraints, with the β carbon distances of the crosslinkable residue pairs near 5 Å.

Although the final structure of the a2-a3-a4 dimer on the bilayer surface at the end of 135-ns MD simulation is different from the static a2-a3-a4 dimer structure that was generated by computational modeling and had the best packing and the most favorable energy score, longer time scale simulations may reveal further conformational changes that may reach this most stable dimer state with the lowest energy. However that sort of modeling study is not necessary to interpret the data presented here and the modeling that we have done allowed us to examine other potential structures as requested by the referee. Both MD simulation and static remodeling have revealed alternative conformations for the a2-a3-a4 dimer, which are related to but distinct from the a2-a3-a4 part of the crystal structure for the BH3-in-groove dimer. We thus refined the working model to reflect these new findings that indicate the a2-a3-a4 dimer structure we show based on static modeling represents a cohort of different, yet related, conformations that likely coexist among the membrane-bound Bax molecules.

(3-d) *Referee #2, point 4) How can the authors distinguish between OMM-integral and residues buried in a protein dimer/oligomer? How can helix 5 be OMM-embedded when V121 and K119 are apparently surface exposed? Also, the analysis of helix 6 should distinguish between the two models. Are residues in helix 6 surface accessible?*

Response: Based on the questions raised by the referees here and above we reworded the manuscript to more clearly explain the IASD data. A cysteine residue in a protein domain that is integrated into the membrane is poorly labeled by IASD in the presence of urea that unfolds the protein, but can be labeled in the presence of both urea (to unfold or loosen protein folding) and CHAPS to solubilize the membrane. While we acknowledge that the CHAPS can also contribute to unfolding the protein, experimentally we and others find that a cysteine residue that is buried in a protein or complex of proteins at the membrane but that is not itself in the lipid tends to be poorly labeled by IASD in the

presence of CHAPS or other detergents, but can be labeled in the presence of urea alone and is usually more accessible with both urea and detergents (Annis et al, 2005; Kim et al, 2004; Westphal et al, 2014). Based on the IASD labeling data from multiple cysteine residues, we derived a relative membrane burial index (residues made accessible to labeling by CHAPS) and a relative protein burial index (residues made accessible by urea) for each cysteine residue to quantify the extent and nature of the burial relative to a reference residue that is known to bury in the membrane or the protein complex as detailed in (3-a) above. Based on this analysis, we concluded that a portion of helix a5 in a fraction of mitochondria-bound Bax molecules is embedded in the membrane, and another portion of helix a5 in another fraction of Bax molecules is buried in the structure of Bax protein or its complex such as the BH3-in-groove dimer. In particular, K119 is within the membrane-embedded portion in one Bax population, and within the protein-buried portion in the other population. V121 is outside the membrane-embedded portion but within the protein-buried portion.

The accessibility of helix a6 in mitochondrial Bax to aqueous milieu has been studied before using IASD labeling (Annis et al, 2005; Westphal et al, 2014). Although the models drawn are different, the data are similar in both studies and both are consistent with models in which a6 is partially embedded in the MOM. Another disulfide crosslinking study suggested that some of the a6 residues are located in a dimer interface (Dewson et al, 2012). Thus, many more experiments will be required to resolve the different environments that may be occupied by a6 under different conditions. Because our focus in this study is on the BH3-in-groove dimer formed by helices a2, a3, a4, and a5, and the helix a9 dimer, we did not study helix a6. However, our model in Figure 11 depicts a6 in multiple locations in various Bax monomers and multimers to accommodate the previous data.

(4) ***Editor: controls need to be added to several of your assays (referee #1, points 1-2; referee #2, point 2; referee #3, point 1 and 5).***

(4-a) *Referee #1, point 1: Can the authors exclude that disulfide crosslinks that become possible after cysteine engineering might by themselves enhance dimer formation? In this respect, it might be interesting to test dimer formation in the absence of mitochondrial membranes, as was done for the L59C/M79C mutant, especially for those mutants that are autoactive in the absence of a BH3 activator.*

Response: This is a legitimate concern as cysteines are prone to form disulfides that may enhance dimer formation or even activate Bax. For this reason we examined the issue carefully for all of the mutants prior to the first submission. Due to space limitations these controls were not discussed in the previous version. We have now added a brief discussion in the revised manuscript.

The fact that the Bax L76C or V110C mutants either fully or partially release cytochrome c from permeabilized mitochondria in the absence of tBid, respectively, prompted us to investigate the issue carefully. For these mutants MOMP activity is not correlated with disulfide formation as only the L76C mutant forms a disulfide (Figure 1B). We also tested the following Bax cysteine mutants that were used extensively in the manuscript, some of which are autoactive (permeabilized mitochondria without added tBid), for dimerization in the absence and presence of the mitochondria and a biological BH3 activator cBid. The results show that the L59C/M79C, L59C/L76C, A183C, and A178C mutants did not form any disulfide-linked dimer in the “mitochondrial” pellet fraction when the mitochondria were absent whether cBid was present or not (Figure EV4F, EV7E, and EV10E). Moreover none of these mutants efficiently formed dimers in the pellet fraction in the presence of the mitochondria but absence of cBid. Indeed only L59C/L76C formed a detectable trace of dimer. However, when both mitochondria and cBid were present, all of the mutants formed the disulfide-linked dimer that was clearly detectable in the pellet fraction. And the amount of the L59C/L76C dimer from the cBid-containing mitochondrial sample was significantly higher than that from the mitochondrial sample lacking cBid. Therefore, the formation of the disulfide-linked dimer in the pellet fraction requires the activation of Bax by cBid at the mitochondria. Thus we conclude that the engineered cysteines by themselves do not enhance the dimer formation, but serve as the tool to capture the dimers that resulted from the interactions between the active Bax molecules at mitochondria.

In the presence of the G179I mutation that activates Bax such that it induced MOMP in the absence of a BH3 protein (Figure 10A-C) and killed cells before Staurosporine treatment (Figure EV14;

detailed in (2-a) above), the L59C/M79C mutant formed the dimer at the mitochondria in the absence of cBid. The G179I mutation not only increased the dimer formation by the L59C/L76C mutant in the mitochondrial fraction, but also induced the dimer formation in the pellet fraction when the mitochondria were not present, indicating that the mutation may induce Bax aggregation in the absence of membranes. Because the G179I mutation that promotes Bax activation in the absence of membranes has a bigger more consistent effect on crosslinking than the introduction of cysteines, it suggests that crosslinking augmented by the introduced cysteines such as L59C/L76C is not contributing significantly to the final model presented in Figure 11. Furthermore, if the introduced cysteines really promoted dimerization, they would likely also lead to autoactivation. The autoactivating cysteines have already been noted in the paper (Figure EV2). Therefore, although a few autoactivating cysteines may promote the formation of dimers, the majority of functionally inert cysteines are unlikely to affect dimerization.

Finally, we also monitored the dimer formation in the supernatant fraction with soluble proteins. Only the L59C/L76C mutant formed a detectable amount of the dimer, which was further enhanced by the G179I mutation. Because the focus of our manuscript is on the Bax activation and oligomerization at the mitochondria, this data does not impact the final model that was supported by the overwhelming data from the mitochondria fraction.

(4-b) *Referee #1, point 2: A basal cytochrome c release of 20 % in the absence of tBid, as shown in Figs. 9 and 10, seems rather high. Can the authors show that MOM integrity is preserved in the mitochondria preparations they use throughout their experiments (before the addition of Bid/Bax), e.g. by a protease accessibility experiment?*

(4-c) *Referee #2, point 2: In the cytochrome c release assays, I am confused by what is meant by "autoactive" (page 24) when Bax and variants are added to isolated mitochondria. I have not come across this term in the literature. In the cellular context, Bax is not constitutively active but must interact with other factors, including tBID, to induce membrane permeabilization. Where else is this phenomenon observed? Could this be an artifact of an in vitro assay? To help place the results into context, showing or mentioning if there is any cytochrome c is released before Bax or Bax/cBid is added might explain release before the addition of tBid. However, it should be mentioned that it is clear that there is an effect due to the addition of tBid, which is consistent with what is already known about this system.*

(4-d) *Referee #3, point 1: Currently the authors present a poor assay (30 % cyt c release in the absence of tBid) testing the cyt c release of in vitro translated protein*.

Response: The points (4-b, c, and d) from the three referees are related to the cytochrome c release assay, particularly the high background release by Bax mutants in the absence of tBid. We have done the controls that the referees asked, and the data are shown in Figure EV2A. Clearly most of the background release occurred in the mitochondria only sample (mito only; open bar; ~20%). This may be due to the mitochondria that were isolated from *bak* knockout mouse livers, which also lack Bax as it is in the cytoplasm, and then frozen in a trehalose buffer, shipped from Andrews' lab to Lin's lab, thawed and used in the cytochrome c release assay. The protocol for this mitochondria preparation and usage was established in Newmeyer's lab (Yamaguchi et al, 2007), and was used to allow us to compare results between two international labs. We do not understand all the nuances of this assay, but what we have observed are that addition of bacterial expressed and purified tBid protein (+tBid; open bar) increased the release slightly to ~30%.

Nevertheless, addition of both the tBid protein and the TNT mixture that produced the WT Bax protein showed a synergistic increase (+Bax WT, +tBid; open bar; ~80%). After the "Raw" release (open bar) of the "+Vector" control was subtracted from the "Raw" releases (open bars) of the "+Vector, +tBid", the "+Bax WT", and the "+Bax WT, +tBid" samples, which all contained the vector (with or without the coding region for WT Bax) and the TNT mixture, the resulting "Corrected" releases (hatched bars) showed the net releases of cytochrome c by the tBid protein (~20%), the Bax protein (~0%), and both (~50%).

With the background release common to these samples subtracted, the "Corrected" cytochrome c release data are a more direct and clear way to quantify the tBid-induced and Bax-mediated MOMP activity. We therefore decided to show the cytochrome c release data from all of the Bax mutants as

the "Corrected" data in Figure EV2B, 9, and 10A. It is clear from the "Corrected" data, the cytochrome c release by most of the Bax mutants in the absence of tBid are ~10% or lower, except of the "autoactive" mutants. And, addition of tBid to the "non-autoactive" Bax mutants increased of the release to ~30-60%, in line with the data from the wild-type Bax. In contrast, the loss-of-function mutation G108E reduced the cytochrome c release by at least 30% (Figure 9, compare the G108E/S184V mutants to the S184V mutants). Importantly, the apoptosis activity of the ten representative mutants assayed in bax/bak DKO BMK cells mirrors their cytochrome c release activity measured in this in vitro assay, validating the usefulness of this simple and quick in vitro assay even though the background is high.

Cytochrome c release was observed from the mitochondria only control (Figure E2A, mito only; open bar), already suggesting that the MOM integrity was compromised in a fraction (~20%) of the mitochondria that we have used. Furthermore, protease accessibility would not add significantly to the conclusions of the paper as there is no evidence that Bax inserts into intact and damaged membranes differently. Therefore, we did not do the protease accessibility experiment suggested by referee #1 in (4-b).

We have attempted to clarify the word "autoactive" that was used on page 24 (and 8), and that confused referee #2 in (4-c). We now state in the revision first in page 8, "…L76C and V110C. These two mutants are "autoactive" as they released ~30-60% of cytochrome c in the absence of tBid", and then in page 31, "Some of the Bax mutants (e.g., A112C,S184V and L59C,L76C,S184V) were autoactive, as they released cytochrome c in the absence of tBid." Our intention is to indicate that "autoactive" meant that a mutant Bax protein, permeabilized the MOM without activation by tBid. This phenomenon was previously reported after expression of certain Bax mutants in cells (Zhou et al, 2007). And, now we also have the data from the cells expressing the "autoactive" mutant G179I that kills the cells in the absence of Staurosporine (see (2-a) above). If another term would be more appropriate, we are open to a suggestion from the referee or editor.

(4-e) *Referee #3, point 5: Controls are lacking or not controlling for the experiment. Why was MOMP initially assayed with tBid, but then the Bax BH3 peptide was used producing an artificial dimer? The disappearance on reducing gels of dimers would significantly strengthen the manuscript. Especially since bands presented in Figure 8 as oligomers can be found on other gels. Why should we appreciate the marked band in fig. 3B lane 4 (for example), while similarly or more prominent bands are also in this lane? Controls for carbonate extraction need to be done with the samples and probably all gels but most importantly the MOMP assay should have loading controls.*

Response: We fully understand the referee's concern about the critical controls. We did the controls but omitted the data solely because the space limit. Now we include the data in the revision.

The justification for using the Bax BH3 peptide to activate Bax in the crosslinking assay is that the BH3 peptide can activate Bax like tBid as we showed before (Ding et al, 2014; Tan et al, 2006). Unlike tBid that is localized to the mitochondria and interacts with Bax and hence may compete with Bax homodimerization particularly through the BH3-in-groove interface as the crosslinking in Figure EV8 shows, the BH3 peptide mostly remained in the soluble fraction and thereby does not interfere with Bax homodimerization at the mitochondria. Therefore we found it useful to perform some of the experiments with the BH3 peptide.

Based on the queries of the referee we have added data to Figure EV4E showing that the disulfide-linked Bax L59C/M79C dimer band formed in the BH3 peptide-containing sample is more intense than that formed in the tBid or cBid-containing sample. Furthermore, to ascertain that the major Bax homodimers detected after the BH3 peptide activation are not artifacts, we used cBid to activate four Bax cysteine mutants, one for each interface, and monitored the dimer formation by disulfide crosslinking. Without exception the dimer of each of the mutants induced by the BH3 peptide was also induced by cBid at the mitochondria (Figure EV4F, EV7E, and EV10E).

As requested we have added the reducing gel data in Figure EV4A, EV7A, and EV10A that are respectively related to Figure 1, 3, and 6 showing the corresponding non-reducing gel data. We have also added the reducing gel data to Figure 8A to compare with the non-reducing gel data directly. These reducing gel data, as the referee anticipated, clearly demonstrate that the dimers and oligomers seen on the non-reducing gels are disulfide-linked products. The only exception is the

open triangle indicated band formed by the A183C and A178C containing mutants because it cannot be reduced. We have noted this in the legend.

The referee is correct that there are other bands on the gels. Due to space limitations and to keep the focus of the manuscript we have not described these bands in detail. The more prominent unmarked band in the original Figure 3B, lane 4 (now marked in revised Figure 3A, lane 4) is the disulfide-linked dimer of Bax S72C and an unknown mitochondrial protein. We demonstrated this to be the case by pretreating the mitochondria with NEM to block the sulfhydryls of the mitochondrial proteins before adding the single-cysteine Bax mutant (Figure EV7B, lane 4). In contrast, the less prominent marked band is the disulfide-linked Bax S72C homodimer because it was still formed in the NEM pretreated mitochondria (Figure EV7B, lane 4, the arrow-marked band). Similar disulfide-linked bands between other Bax mutants and mitochondrial proteins were also detected, e.g., comparing the products from E69C at the untreated mitochondria in lane 8 of Figure 3A to those from the same mutant at the NEM-pretreated mitochondria in lane 4 of Figure 3C. We have indicated these bands with open triangles and explain their nature in the legend. Similar phenomena were reported before for other Bax and Bcl-XL cysteine mutants (Ding et al, 2014). While it will be of interest to determine what these other Bax interacting proteins are, such identification is clearly outside of the scope of the current manuscript.

As requested we have done carbonate extraction with other cysteine mutant(s) for each of the four dimer interfaces, C62+L76C for the BH3-in-groove, E69C for helices a2-a3-a4, I175C for helix a9 intersected, and L185C for a9 parallel. The data are entirely consistent with the previous data and were added to Figure 1D, 3C, 6C, and 6E, respectively, alongside the previous carbonate extraction data.

We have also now shown the “loading controls” as Figure EV2C, as part of the phosphor-images showing the amount of radioactive Bax proteins associated with the mitochondria in the MOMP assay that were responsible to the observed cytochrome c release.

(5) ***Editor: previous findings must be better implemented into the presented work (referee #2 and #3).***

(5-a) Referee #2, point 4: What would also be beneficial to the manuscript is mentioning how some published results from different papers bolster the authors' conclusions. A substantial contribution to this manuscript is the reporting of contacts between α9 helices of separate Bax molecules. While this contact has been reported previously (Bleicken et al., 2014) with some ambiguity in their orientation as well as between structurally homologous Bak molecules (Iyer et al., 2015), a parallel orientation of contact between the α9 helices has been observed in live cells after translocation and reported earlier (Gahl et al.,2014). Similar observation using a different technique reinforces the authors' conclusions about the requirement of this contact to release large proteins, like SMAC. Another feature in Bax that allows it to form pores to release large proteins is that it adopts an extended conformation. In the proposed model in Figure 11, there is no mention of intramolecular constraints even though the authors illustrated it as extended. There is previous published evidence that supports an extended conformation. Czabotar et al in 2013 propose a "hinge" motion to bring α5 and α6 away from the core. The DEER experiment (Bleiken et al., 2014) reports intra-molecular distances consistent with an extended conformation. Also, Gahl et al., 2014 report FRET measurements that place α9 away from the BH3 domain in order to form oligomers. The research presented in these other groups are complimentary to the conclusions presented in this manuscript and would be beneficial to include in its final form.

Response: We greatly appreciate this referee’s point that previous findings with different techniques reinforce our conclusions and model. We referenced the FRET data for Bax activation and the model from (Gahl et al, 2014) and compared with the DEER data and model from (Bleicken et al, 2014) and the crosslinking data and model from (Iyer et al, 2015) in the revised Introduction on page 6-7. We also discussed different models in the revised Discussion alongside our model in Figure 11.

(5-b) Referee #3, point 6: How do the findings of the authors relate to the reversible OMM-association and the retrotranslocation of Bax and Bak in living cells? In the model tBid is included

as a star, but mitochondrial Bax association in cells does not require tBid and the Bid KO mouse lacks a prominent phenotype.

Response: This referee made an interesting point about our model regarding the reversible association between Bax and mitochondria in the absence of tBid. Although we did not study the reversible Bax-mitochondria association and the retrotranslocation of Bax by antiapoptotic family members in this project, there are several findings that may be related to these events observed for Bax (and Bak) in cells (Edlich et al, 2011; Schellenberg et al, 2013; Todt et al, 2015; Todt et al, 2013). First, there is a small amount of Bax proteins associated with the isolated mitochondria in the absence of BH3 peptide or cBid (Figure EV4D & F, EV7C & E, and EV10C & E), consistent with the observation that the Bax-mitochondria association in cells does not require tBid. Second, dimerization of these mitochondria-associated Bax molecules were either not or barely detectable unless they were activated by BH3 activators or by mutations in themselves (e.g., G179I; Figure EV13C), suggesting they are in an inactive conformation. Third, the G179I mutation in a9 localized Bax to the mitochondria in cells and induced apoptosis in the absence of STS treatment. Another mutation in a9, S184V, also constitutively targeted Bax to the mitochondria but did not significantly increase apoptosis until STS was added. Similar observations were previously reported for the S184V mutant (Kim et al, 2009; Nechushtan et al, 1999; Suzuki et al, 2000). We have included some of these discussion points in the revised manuscript within the limited space that we have. The data showing that tBid is not essential are well known and in cells many other proteins can substitute for tBid. The best studied is Bim but Puma, Noxa, p53 and other proteins have all been shown to activate Bax similarly. The data in this paper use only tBid strictly to simplify what is already a very complicated manuscript.

(6) ***Minor concerns***

(6-a) *Referee #2, point 5: One minor comment. On page 20, in the middle of the second paragraph, the authors refer to residue Leu186. We assume the authors meant Leu185, as it is referred to in other parts of the text and figures.*

Response: We have corrected this typo, changing Leu186 to Leu185.

(6-b) *Referee #3, Minor points*
1) The authors cite preferably two groups of authors and not necessarily the appropriate papers. Especially the first paper suggesting helix9-9 interactions (Gahl et al., 2014) should be cited. Also what makes residues "Confirmed MOM-embedded"?

Response: We have cited (Gahl et al, 2014) (see (5-a) above). The “Confirmed MOM-embedded …residues” in Figure 2A are those their predicted localization to the hydrophobic core of the MOM was confirmed by the IASD labeling data and the relative membrane burial indices shown in the Figure 2B-C (see (3-a) and (3-d) above). We now state this in the legend.

2) The depiction of protein structures is generally crowded and confusing. Figure 1A (for example) is perhaps only providing information to the reader that is used to look at Bax structures on a daily basis.

Response: The goal for these structural depictions is to provide a view of the dimers with the interfacial residues that are proximal and thereby able to form a disulfide after cysteine substitution indicated. We agree with this referee that a more simplified view would be more readable, and have simplified the depictions by indicating the color codes for the four helices in monomer #1, and that for monomer #2 on top of the dimer structure instead of in the structure. We also deleted the numbers that indicate the distance between the dashed line-linked residues from the structure, but noted the distance range in the legend.

3) The data is compressed in too little space. It is impossible to judge whether mutants are shifting in Figure 2B. The reader is left with a "trust me". Reduction may be a solution.

Response: In Figure 2B, all of the mutants show two or more bands in at least one of the lanes. By compared the two bands with the major band in lane 1, the 0-min labeling control, readers will know which band is the unlabeled protein and which is the labeled one. Using these reference bands readers will be able to judge whether the single band in another lane is the unlabeled or the labeled protein. The only exception is the E69C mutant, for which all the lanes have only one band. However, by comparing the single band in lane 1 (the 0-min labeling control) with the single band in the adjacent lane 2 and in the lanes thereafter, readers will see that the band in lane 2 and beyond are the labeled protein. This conclusion is corroborated by the data from an independent replicate with the E69C mutant, which is provided side-by-side with the data shown in Figure 2B as **Response Figure R2** below for the referee to inspect. Of note, an IASD labeled protein cannot be reduced to an unlabeled one, because IASD uses its iodoacetyl group to react with the sulfhydryl group of a cysteine forming a stable thioether bond that cannot be reduced by reducing agents. In fact the samples were heated in the b-mercaptoethanol-containing reducing sample buffer before loading to gels. While the shifts are small when a modifying a single cysteine in a protein, and sometimes have to be detected by isoelectric focusing the IASD labeling protocol is well established and has been used successfully by multiple groups. Indeed the data published for the Bcl-2 proteins is remarkably consistent between independent groups (e.g., (Annis et al, 2005; Westphal et al, 2014)).

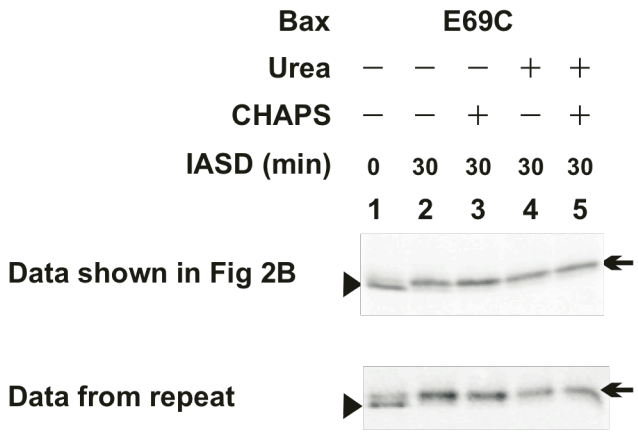

Figure R2. IASD labeling of Bax E69C mutant at mitochondria. The *in vitro* synthesized radioactive single-cysteine Bax protein was activated and targeted to the mitochondria. The resulting mitochondria were isolated and treated with IASD in the absence or presence of CHAPS, urea, or both. After 30 min, the labeling reactions were stopped by mercaptoethanol. For the “0 min” controls, the samples were pretreated with mercaptoethanol before addition of IASD. The IASD-labeled radioactive Bax proteins were resolved from the unlabeled ones using gradient SDS-PAGE and detected by phosphor-imaging. The data from two independent replicates are shown. Triangles and arrows indicate the unlabeled and IASD-labeled Bax proteins, respectively.

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2nd Editorial Decision 19 October 2015

Thank you for submitting your revised manuscript for our consideration. Your manuscript has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that they are both broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #3's suggestion on how to further discuss your data, and to provide a final version of your manuscript.

I am therefore formally returning the manuscript to you for a final round of minor revision. Once we should have received the revised version, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

REFEREE REPORTS

Referee #2:

The authors have addressed all of my concerns. I recommend that the manuscript be accepted for publication in EMBO journal.

Referee #3:

The authors have addressed most concerns adequately and clearly strengthened their manuscript. Their results now represent an important contribution to the discussion on Bax activation. However, the cyt c release assay is still substandard. cyt c release in the absence of Bax/Bak demonstrates the presence of co-purified endogenous Bax/Bak, obscuring the analysis of Bax mutants. This weakness is now compensated to some extend by the ectopic expression of mutant Bax in cells, but the authors should point to differences, such as the Smac release of Bax T182I. Their hierarchical model is one possible explanation of most results. However, the activation of G108E in combination S184V argues against it. According to the authors' model this mutation should prevent Bax activity all together. The position of G108 on the edge of the hydrophobic groove could suggest H bond formation with helix 9 and thus simply reduce interactions between Bax and the OMM. This rationale is supported by regained activity with the S184V substitution and thus interference with S184 interactions with the hydrophobic groove. A similar explanation could apply to G179I toxicity. In fact, the results could emphasize the importance of Bax contact to the membrane rather than the suggested active forms. The authors should at least state that G108E/S184V activity either indicates alternative Bax activation or incomplete block of Bax activation. With adequate discussion this manuscript is valuable input to the insight into Bax activation.

2nd Revision - authors' response 16 November 2015

Referee #3:
The authors have addressed most concerns adequately and clearly strengthened their manuscript. Their results now represent an important contribution to the discussion on Bax activation. However, the cyt c release assay is still substandard. cyt c release in the absence of Bax/Bak demonstrates the presence of co-purified endogenous Bax/Bak, obscuring the analysis of Bax mutants. This weakness is now compensated to some extend by the ectopic expression of mutant Bax in cells, but the authors should point to differences, such as the Smac release of Bax T182I. Their hierarchical model is one possible explanation of most results. However, the activation of G108E in combination S184V argues against it. According to the authors' model this mutation should prevent Bax activity all together. The position of G108 on the edge of the hydrophobic groove could suggest H bond formation with helix 9 and thus simply reduce interactions between Bax and the OMM. This rationale is supported by regained activity with the S184V substitution and thus interference with S184 interactions with the hydrophobic groove. A similar explanation could apply to G179I toxicity. In fact, the results could emphasize the importance of Bax contact to the membrane rather than the suggested active forms. The authors should at least state that G108E/S184V activity either indicates alternative Bax activation or incomplete block of Bax activation. With adequate discussion this manuscript is valuable input to the insight into Bax activation.

Response:
(1) We discussed the difference between the data from Bax T182I mutant in the cell-free and cell-based assays in the last paragraph on page 36:

"When expressed in cells, the T182I mutant had similar localization and slightly higher apoptotic activity compared to that of WT Bax (Figure EV2B-D). While the apoptosis data from the cells expressing the T182I mutant are mostly in line with the cytochrome c release data from the isolated mitochondria reconstituted with the *in vitro* synthesized or purified T182I mutant (Figure 10A-B), the defect of the T182I mutant detected in the *in vitro* Smac and Smac-mCherry release assays (Figure 10B-C) does not translate into a defect in the cell-based apoptosis assay. The most simple explanation is that the cytochrome c release caused by the T182I mutant in the cells is sufficient to induce apoptosis, and the reduction of the Smac release caused by the mutation in the cells, is not sufficient to block apoptosis. Therefore, the small pore-forming activity of Bax, which was largely inhibited by the G108E, and enhanced by the G179I, but not altered by the T182I mutation, mirrors the cellular apoptotic activity. The large pore-forming activity, which was inhibited by the T182I mutation, is not required at least for Bax to kill the *bax/bak* DKO BMK cells."

(2) We added the following discussions to the cell-free and cell data from Bax G108E, G108E/S184V and G179I mutants.

First paragraph on page 36: "Furthermore, the residual apoptotic activity of the G108E mutant that could be amplified by the S184V mutation is consistent with the residual dimerizations and cytochrome c release observed *in vitro* (Figure 4 and 9). Together the results from these mutants in our cell-free and cell-based systems suggest that the G108E mutation incompletely blocks Bax dimerization, MOMP and apoptotic activities especially in the presence of the S184V mutation that targets Bax to the mitochondria."

Last paragraph on page 37: "In line with this scenario disruption of a9-groove interaction by the S184V mutation (Suzuki et al, 2000) or increase of a9 hydrophobicity by the G179I mutation enhances Bax mitochondrial targeting, MOMP and apoptotic activities (Figures 9, 10, EV2 and EV5E)."

On page 38: "The G108E mutation adds a strong hydrogen donor to the edge of the groove, which increases the propensity for hydrogen bonding with the a9 residues in vicinity, and hence could stabilize the a9 in the groove thereby keeping Bax soluble in the cytosol (Suzuki et al, 2000). The S184V mutation apparently overwrites the inhibitory effect of G108E on the mitochondrial targeting reactivating the MOMP and apoptotic activity (Figures 9 and EV2)(Kim et al, 2009)."

