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Bax assembly into rings and arcs in apoptotic mitochondria is linked to membrane pores

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 07 August 2015

Thank you for submitting your manuscript entitled 'Bax assembly into rings and arcs in apoptotic mitochondria is linked to membrane pore'. I have now received reports from all referees, which are enclosed below. I am afraid that their recommendation let us to conclude that we cannot offer publication in The EMBO Journal at this stage.

As you will see, the referees appreciate your analysis and find it potentially interesting. However, none of the referees endorses publication in the general evaluation of your manuscript. Furthermore, the referees note that your conclusions are not sufficiently supported at this stage and they raise several technical issues. I won't list all concerns here, as the reports are clearly written and constructive. Most importantly, your results rely on overexpression studies performed in a Bax/Bak positive background as pointed out by referee #1 and #3, and this could affect your observations and thus conclusions. Addressing this concern is feasible with experiments in Bax/Bak double knock-out cells, however, doing so would represent a significant amount of work with uncertain outcome. Therefore, I have no other choice but to return your manuscript to you at this stage. I nevertheless hope that you will find the referees' comments helpful.

REFEREE REPORTS

Referee #1:

How Bak and Bax form the putative apoptotic pore to cause cell death is one of most hotly debated

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research questions. Visualisation of the "pore" particularly in cells or isolated mitochondria has proven extremely difficult, but will undoubtedly reveal new insight into how Bak and Bax actually damage the mitochondrial outer membrane during apoptosis. The current manuscript describes super resolution imaging and atomic force microscopy of Bax in cells undergoing apoptosis. The authors describe interesting data indicating the detection of numerous distinct and heterogeneous structures including closed ring-like structure reminiscent of proteinaceous pores, but also lines and open arcs suggestive of torroidal lipid pores. They also show some evidence that these are not epiphenomenon using a mutant of Bax that targets mitochondria yet is not active and that both arcs and rings can permeabilize the MOM using AFM. Based on this data the authors conclude that Bax locates partially or completely to the circumference of the pore. The manuscript is clearly written and is of interest to the field. However, I have reservations regarding the experimental system that would question the conclusions.

Major points
1. The cell-based aspects of the study are all based on transient transfection of GFP-Bax in HeLa cells that already express endogenous Bax and Bax. This confounds the characterisation of the pore structures as they are likely to contain a heterogeneous mix of tagged ectopically expressed Bax, but also untagged Bax and Bak. Although their model membrane studies suggest a similar array of structures, the contribution of untagged Bax/Bak to the structures seriously undermines the cell-based studies. Also, it is difficult to gauge the generality of their findings as the study is limited to HeLa cells. Thus the study would be significantly strengthened by analysing Bak/Bax DKO HCT116 or MEFs that are readily available. Ideally this should also involve stable expression at close to physiological levels to avoid potential artefacts of significant over-expression following transient transfection.

2. The authors use a tethered mutant to confirm the relevance of their findings. Why not just use a BaxS184 mutant that constitutively locates to mitochondria? This would also negate the stated problem of minimizing the signal from cytosolic Bax. Also, several single point mutants of Bax have been identified that do not function. These may have provided cleaner data and been simpler to interpret than the tethered mutant which is reliant on efficient disulphide-tethering in the reducing environment of the cytosol. Incomplete linkage may possibly have contributed to aggregates observed with this mutant. To fully confirm that the strcutures are relevant the mutant form also needs to be tested following STS-treatment. However, again this would require the use of Bak/Bax DKO cells.

3. Are the line and arc structures distinct or just different stages in the nascent evolving pores? Is Bax fully activated and oligomerised as assessed by other approaches at the time points tested? This interpretation may be aided again by using limited expression in DKO cells and confirming that all of the Bax is oligomerised under the conditions tested.

Minor points
1. The authors concede that a limitation of the SMLM approach in cells is that only structures observed perpendicular to the membrane would be observed as rings, and consequently the rings may be under represented in their analyses. However, this would presumably also hold true for arcs that would appear as lines when observed in the plane of the MOM?

Referee #2:

Raquel Salvador-Gallego et al. present a study of the assembly and supramolecular organization of the apoptosis regulator Bax at the mitochondrial outer membrane, MOM. Bax mediates the MOM permeabilization responsible for cytochrome c release during apoptosis. The authors used superresolution microscopy for studying active GFP-Bax in HeLa cells at the single molecule level and AFM for assessing the Bax assembly in in supported lipid bilayers. For single molecule detection GFP-Bax was labeled with anti-GFP nanobodies coupled to Alexa Fluor 647or with other fluorophors with suitable properties.

This paper is certainly interesting. It reveals new information that goes beyond previously published work. The in vivo analysis suggests a significant variety of Bax oligomers, aggregates, rings, arcs,

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lines and double lines. AFM of supported bilayers incubated with Bax revealed the same structures. Importantly, the topographs showed conclusively that the bilayer is missing in pores (97%) and sometimes close to arcs (12%).

Comments:
It is simply amazing that a 21 kDa protein assembles to structures such as large as rings, arcs and linear assemblies observed in this work. Dimerization is the first step, but how do higher oligomers form? Here are data that would allow rough estimates of the number of Bax proteins involved to be made (quantitative single molecule microscopy, AFM). It is clear that the labeling methods (one GFP is about 30kDa, the additional nanobody adds the same order of mass) would distort the analysis. AFM data would be better, yet the resolution would limit the estimate. It should nevertheless be made, and properly discussed.

The statement 'On the one hand, in the case of complete rings, only those which are observed perpendicular to the ring plane would be detected as such in the two dimensional SMLM approach used here, while any other orientation would likely appear as a line' needs to be reformulated. First, rings would appear as ellipses, lines being less frequent. Second, because the mitochondria are outlined by specific labels (e.g., Fig. 1), it should be possible to exclude the interpretation of lines being projections of rings, making that part of the discussion obsolete. That arcs also induce pores and that they exist is an interesting observation, which should not be diluted.
Bax produces openings that allow 2 MDa dextran particles to pass - it might be worthwhile to include this in the discussion, and might put the estimates of Bax proteins involved into a perspective.

The comparison to perforin-like aggregates is difficult: recent papers show the truly regular organization of the arcs and rings formed. These proteins are different; the Bax assembly process is unlikely to follow the same principle. Therefore, the statement 'in general, toroidal pore-forming proteins, stabilize membrane pores is not by building a poly-peptide wall that buries the hydrophobic membrane core, but rather by releasing the curvature stress' needs to be modified.
The model in Fig. 7 and the comparison to the AFM data is difficult. Again, Bax is only 21 kDa, the dimer would be a blob of about 4-5nm. The smallest discernable blobs in the AFM images are about 15nm, they would not reflect the dimers. Moreover, structures available do not readily indicate how dimers assemble to higher oligomers.

Specific comments:
p5: It is difficult to see the cell shape in Figs. 2 a and b.
p7: It is not clear why STED is a suitable control concerning sample preparation or imaging technique; material& methods should make the preparation differences clear.
p7: why is mito-dsRED used - what is difference to mMAPLE-mito; please clarify.
p9: the AFM provides a much better than 25 to 45 nm. Membrane surfaces have been imaged at sub-nm resolution.

Minor comments:
Please rearrange the keywords, either alphabetically or thematically.
Figure reference: use either Fig. X a or Fig. X A in both figures and text.

Referee #3:

The authors discuss here the mechanisms by which Bax, a regulator of apoptosis, leads to mitochondrial damage. They use super-resolution microscopy to analyze the distribution of Bax in apoptotic cells, and find that many different types of Bax arrangements can be formed on mitochondria membranes, such as rings, arcs, and linear assemblies. In parallel experiments, they analyze the assemblies of recombinantly expressed Bax on supported lipid bilayers, by atomic force microscopy. They find that Bax appears to form and/or stabilize pores in the bilayers. They conclude that Bax "fully or partially delineates pores" that permeabilize the mitochondrial outer membrane.

While the manuscript is interesting, a few conceptual and experimental problems should be addressed:

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1) The distribution of Bax is measured in cells overexpressing this protein. Is there any possibility to check whether some of the structures observed are dependent on overexpression? For instance, it is conceivable that Bax normally forms rings, but that the protein accumulates in aggregates and lines when overexpressed.

2) There is little evidence that Bax forms membrane pores in real mitochondrial membranes. Atomic force microscopy experiments on pure lipid membranes have shown for almost a decade that aggregates of neurodegenerative proteins, such as Abeta or synuclein, can form pores. However, it is still unclear whether they do so in native membranes. Therefore, this type of experiment should be interpreted with care. Ideally, such pores may be visualized in correlative fluorescence and electron microscopy experiments, especially if the pore radius is as large as seen in Figure 6. I would encourage the authors to at least attempt to determine whether such pores exist in the mitochondria of Bax-expressing apoptotic cells, by simple transmission electron microscopy. A pore that is 50 nm or larger in size (Figure 6) should be visible.

3) The authors should analyze the frequency of the different types of Bax aggregates in the cells. This information seems to be missing in Figure 3, where various characteristics of these aggregates are shown, but not their proportions in the cell.

4) The use of size bars of variable length, as in Figure 2, is confusing.

Resubmission - authors' response 28 October 2015

ANSWERS TO REVIEWERS

Referee #1:

How Bak and Bax form the putative apoptotic pore to cause cell death is one of most hotly debated research questions. Visualisation of the "pore" particularly in cells or isolated mitochondria has proven extremely difficult, but will undoubtedly reveal new insight into how Bak and Bax actually damage the mitochondrial outer membrane during apoptosis. The current manuscript describes super resolution imaging and atomic force microscopy of Bax in cells undergoing apoptosis. The authors describe interesting data indicating the detection of numerous distinct and heterogeneous structures including closed ring-like structure reminiscent of proteinaceous pores, but also lines and open arcs suggestive of toroidal lipid pores. They also show some evidence that these are not epiphenomenon using a mutant of Bax that targets mitochondria yet is not active and that both arcs and rings can permeabilize the MOM using AFM. Based on this data the authors conclude that Bax locates partially or completely to the circumference of the pore. The manuscript is clearly written and is of interest to the field. However, I have reservations regarding the experimental system that would question the conclusions.

We thank the reviewer for the positive evaluation of our work.

Major points
1. The cell-based aspects of the study are all based on transient transfection of GFP-Bax in HeLa cells that already express endogenous Bax and Bax. This confounds the characterisation of the pore structures as they are likely to contain a heterogeneous mix of tagged ectopically expressed Bax, but also untagged Bax and Bak. Although their model membrane studies suggest a similar array of structures, the contribution of untagged Bax/Bak to the structures seriously undermines the cell-based studies. Also, it is difficult to gauge the generality of their findings as the study is limited to HeLa cells. Thus the study would be significantly strengthened by analysing Bak/Bax DKO HCT116 or MEFs that are readily available. Ideally this should also involve stable expression at close to physiological levels to avoid potential artefacts of significant over-expression following transient transfection.

*We thank the reviewer for this suggestion. To avoid possible effects of endogenous Bax and Bak on the structures detected for GFP-Bax, we have now performed single molecule localization microscopy experiments in apoptotic HCT116 Bax/*Bak *-/- cells transfected with GFP-Bax. As shown in figure 4a-b, we could also identify a significant fraction of non-random GFP-Bax*

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*structures including lines, double lines, arcs and full rings. These structures are comparable to those found in HeLa cells. Therefore, these new data confirm that the GFP-Bax structures described in the studies with HeLa cells are not affected by the presence of endogenous Bax and Bak. Regarding the use of stably transfected cells, we had difficulties with the generation of the cell lines because the cells died before incorporation of the virus. Nevertheless, we have compared the expression levels of Bax and GFP-Bax in the transient transfected cells and in wild type HeLa cells by western blot. The results are now shown in extended figure EV1. Expression levels in HCT116 Bax/*Bak *-/- cells transfected with GFP-Bax were also comparable. Interestingly, we find that the levels of GFP-Bax and endogenous Bax together in the transfected HeLa cells are only slightly higher that those of endogenous protein in wild type cells, even considering that we usually get around 75% transfection efficiency. We have the impression that transfected HeLa cells down-regulate the levels of endogenous Bax so that the total amount of protein is not too high and allows survival. Most likely, those cells efficiently transfected and expressing very high levels of GFP-Bax die shortly after transfection. Altogether, these results indicate that potential artifacts due to significant overexpression of GFP-Bax are highly unlikely.*

2. The authors use a tethered mutant to confirm the relevance of their findings. Why not just use a BaxS184 mutant that constitutively locates to mitochondria? This would also negate the stated problem of minimizing the signal from cytosolic Bax. Also, several single point mutants of Bax have been identified that do not function. These may have provided cleaner data and been simpler to interpret than the tethered mutant which is reliant on efficient disulphide-tethering in the reducing environment of the cytosol. Incomplete linkage may possibly have contributed to aggregates observed with this mutant. To fully confirm that the structures are relevant the mutant form also needs to be tested following STS-treatment. However, again this would require the use of Bak/Bax DKO cells.

The main reason to use GFP-Bax 1-2/L6 is that it is a fairly characterized inactive mutant of Bax that localizes constitutively to mitochondria. The idea of having it tethered to mitochondria is to be able visualize inactive, monomeric Bax, because it is not possible to characterize the soluble, inactive Bax diffusely located at the cytosol with superresolution microscopy. We were interested in this as a reference of the signal for inactive Bax to be compared with the activated one.
In principle, using BaxS184 could have been a similar solution. However, this mutant has been reported to form clusters in non-apoptotic cells (Nechushtan A. et al., JCB 2001, 153:265-1276), to have already exposed the N-terminus (Kim H. et al., Mol Cell 2009, 36:487-499) and to have increased apoptotic activity with respect to wt Bax (Todt F. et al., EMBO J 2015, 34:67-80). Considering this, we thought it would be complicated to control the activity state of this mutant and it would be best to use the GFP-Bax 1-2/L6 mutant. Although several point mutants of Bax that do not function have been identified, we are not aware of any well-characterized mutant that will be fully tethered to mitochondria in monomeric form. If the reviewer has a good suggestion, we would be open to trying it.

We agree with the reviewer that the GFP-Bax 1-2/L6 mutant is reliant on efficient disulphide-tethering in the reducing environment of the cytosol. Indeed, Frank Edlich in a personal communication explained to us that the inactive mutant is not fully inactive and that it activates at long transfection times (longer than 12h). We corroborated these observations with independent experiments in our lab, and therefore always used this mutant at short transfection times, under which the mitochondrial potential was maintained, to ensure that we were imaging mostly inactive protein. But, as the reviewer points out, we cannot discard that some of the structures and aggregates observed with this mutant are due to incomplete linkage. We have now included this possibility in the results. We suspect that a similar result would be obtained with the S184 mutant. Following the reviewer's suggestion, we have analyzed GFP-Bax 1-2/L6 following STS-treatment in HeLa cells by confocal microscopy and observed that it leads to activation of at least a fraction of the mutant protein (see Figure R1, for review purposes only). As a result, the signal of this mutant in apoptotic cells is definitely more contaminated with activated protein than in healthy cells and does not provide a good reference for the signal of inactive Bax, so we think it is not worthy analyzing it with superresolution microscopy.

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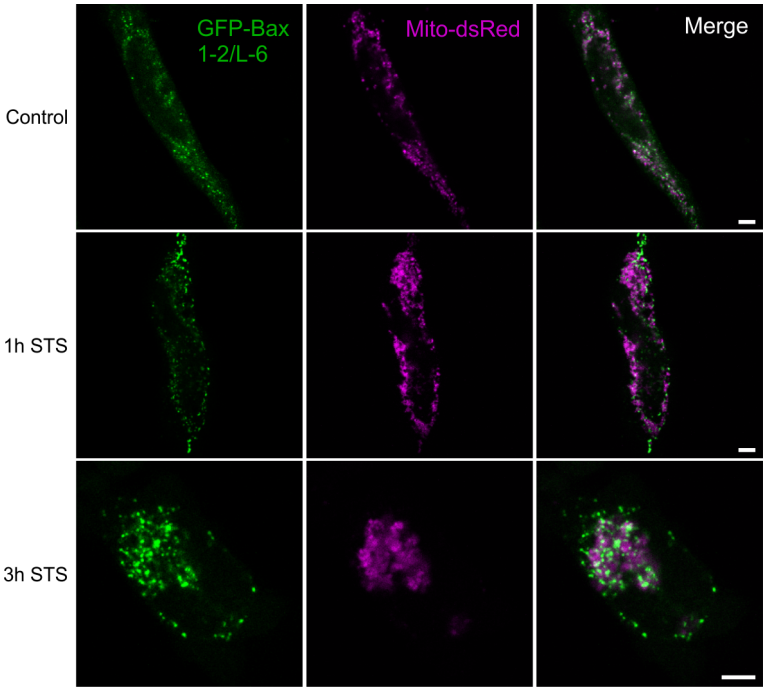

Figure R1. GFP-Bax 1-2/L6 forms large clusters during apoptosis. HeLa cells transiently transfected with GFP-Bax 1-2/L6 (green) and mito-dsRed (magenta) under control conditions and 1 or 3h after treatment with 1μM staurosporine.

3. Are the line and arc structures distinct or just different stages in the nascent evolving pores?

Unfortunately we do not know the answer to this. We find that both arc and line structures are coexisting at the same time point in apoptotic mitochondria. Given the heterogeneity in the structures identified, it could well be that they are indeed consecutive stages in the process of full ring assembly or that they are kinetically trapped intermediates. In addition, we cannot discard that lines and arcs correspond to the same type of supramolecular organization observed from different angles, although the AFM experiments do not support this. To improve clarity, we have now emphasized this issue in the discussion part of the text.
To answer this question properly, one should be able to follow the individual structures over time, which is technically not feasible at the moment.

Is Bax fully activated and oligomerised as assessed by other approaches at the time points tested? This interpretation may be aided again by using limited expression in DKO cells and confirming that all of the Bax is oligomerised under the conditions tested.

Following the reviewer's suggestion, as additional controls for Bax translocation and oligomerization under the experimental conditions used in the imaging experiments, we present now western blot analysis of Bax association to mitochondria and presence of oligomers (stabilized by cross-linking). These data are included in extended figure EV1 and confirm that Bax is activated/oligomerized in mitochondria under the conditions used.

Minor points

1. The authors concede that a limitation of the SMLM approach in cells is that only structures observed perpendicular to the membrane would be observed as rings, and consequently the rings may be under represented in their analyses. However, this would presumably also hold true for arcs that would appear as lines when observed in the plane of the MOM?

We thank the reviewer for this comment. This is true and we have now included this possibility in the discussion section.

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Referee #2:

Raquel Salvador-Gallego et al. present a study of the assembly and supramolecular organization of the apoptosis regulator Bax at the mitochondrial outer membrane, MOM. Bax mediates the MOM permeabilization responsible for cytochrome c release during apoptosis. The authors used superresolution microscopy for studying active GFP-Bax in HeLa cells at the single molecule level and AFM for assessing the Bax assembly in in supported lipid bilayers. For single molecule detection GFP-Bax was labeled with anti-GFP nanobodies coupled to Alexa Fluor 647or with other fluorophors with suitable properties.

This paper is certainly interesting. It reveals new information that goes beyond previously published work. The in vivo analysis suggests a significant variety of Bax oligomers, aggregates, rings, arcs, lines and double lines. AFM of supported bilayers incubated with Bax revealed the same structures. Importantly, the topographs showed conclusively that the bilayer is missing in pores (97%) and sometimes close to arcs (12%).
We thank the reviewer for remarking the novelty and interest of our work.

Comments:
It is simply amazing that a 21 kDa protein assembles to structures such as large as rings, arcs and linear assemblies observed in this work. Dimerization is the first step, but how do higher oligomers form? Here are data that would allow rough estimates of the number of Bax proteins involved to be made (quantitative single molecule microscopy, AFM). It is clear that the labeling methods (one GFP is about 30kDa, the additional nanobody adds the same order of mass) would distort the analysis. AFM data would be better, yet the resolution would limit the estimate. It should nevertheless be made, and properly discussed.

We are currently addressing this question using stoichiometry analysis of single particle microscopy images. Our initial findings have been recently published (Subburaj Y., et al., Nat Commun 2015, 6:8042). In this study, performed in the single molecule regime, we detect a mixture of oligomeric species including dimers, tetramers and hexamers. We believe that the distribution of oligomers depends on Bax density on the membrane, as we found that the size of individual Bax pores depends on it (Bleicken S., et al., J Biol Chem 2013, 288:33241). We are currently working to validate this hypothesis.

Considering the quantification of the Bax protein numbers from the single molecule localization microscopy, we are afraid that this is currently not feasible in a precise and reliable manner.
As for the AFM data, and following the reviewer's suggestion, we have now included a rough estimation of the number of Bax dimers that would fit with the size of the high structures detected at the pore rims. This is now included in the discussion and as a cartoon in extended figure EV3. As mentioned in the manuscript text, one should be extremely cautious with the over-interpretation of the data (as they do not have sufficient quality for any proper fitting) and there is no scientific ground behind the scaled 3D structures of the Bax dimers inserted the AFM images, other than reasonable overlap. Nevertheless, this superficial analysis suggests that a relative small amount of Bax dimers (in the order of 8-10) could be enough to stabilize large membrane pores. Such protein-lined pore structures would also contain lipid molecules, in agreement with the toroidal model for Bax pore formation.

The statement 'On the one hand, in the case of complete rings, only those which are observed perpendicular to the ring plane would be detected as such in the two dimensional SMLM approach used here, while any other orientation would likely appear as a line' needs to be reformulated. First, rings would appear as ellipses, lines being less frequent. Second, because the mitochondria are outlined by specific labels (e.g., Fig. 1), it should be possible to exclude the interpretation of lines being projections of rings, making that part of the discussion obsolete. That arcs also induce pores and that they exist is an interesting observation, which should not be diluted.

Although the reviewer is right that a circle observed from an orientation that is not perpendicular to the plane would most likely appear as an ellipse, we think that due to off-focus blurring (and considering the noise we have in our images), it is quite difficult that we would be able to distinguish these ellipses. We think that we would detect the portion of the ellipse that is in focus as

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a line, in some cases as an arc. In addition, from the two color superresolution experiments we cannot discard that lines are the objects we detect corresponding to an underlying structure that is wrapping mitochondria, which would in this case be an arc or ring.

To clarify this issue, we have now reformulated the statement, as suggested by the reviewer and included this line of reasoning also for arcs (see comments from reviewer 1). We have also emphasized that, as the AFM data indicate, all structures can be detected in the same membrane plane, make the most likely situation that they indeed correspond to distinct Bax assemblies.

Bax produces openings that allow 2 MDa dextran particles to pass - it might be worthwhile to include this in the discussion, and might put the estimates of Bax proteins involved into a perspective.

Following the reviewer's suggestion, we have now included this in the discussion and linked it to the estimates of Bax dimers involved in pore formation.

The comparison to perforin-like aggregates is difficult: recent papers show the truly regular organization of the arcs and rings formed. These proteins are different; the Bax assembly process is unlikely to follow the same principle. Therefore, the statement 'in general, toroidal pore-forming proteins, stabilize membrane pores is not by building a poly-peptide wall that buries the hydrophobic membrane core, but rather by releasing the curvature stress' needs to be modified.

Following the reviewer's suggestion, we have now removed the generalization to all toroidal pore forming proteins from the discussion.

The model in Fig. 7 and the comparison to the AFM data is difficult. Again, Bax is only 21 kDa, the dimer would be a blob of about 4-5nm. The smallest discernable blobs in the AFM images are about 15nm, they would not reflect the dimers. Moreover, structures available do not readily indicate how dimers assemble to higher oligomers.

For clarity, we have now included a sentence in the figure caption that indicates that the number of Bax spheres is illustrative and does not need to correlate with the actual number of dimers present in the structures. Speculation about the number of Bax dimers in the structures has been included elsewhere in the discussion, in extended figure EV3 and above.

Specific comments:

p5: It is difficult to see the cell shape in Figs. 2 a and b.
We thank the reviewer for pointing this out. We show now the cell shape with dotted lines.

p7: It is not clear why STED is a suitable control concerning sample preparation or imaging technique; material& methods should make the preparation differences clear.

To make this clearer, we have now extended the explanations in the results section. Briefly, in STED, the fluorescence signal of GFP is directly measured, while in SMLM we performed immunostaining of GFP with nanobodies and dected the fluorophore attached to the nanobody (which is suitable for SMLM, but not GFP). In addition, the working principle of STED is different to SMLM: it is based on the overlay of a diffraction-limited spot with a doughnut-shaped laser, which depletes the fluorescence everywhere in the focal region but in the very center of the spot. As a result, the fluorescence emission is confined to a very small region.

p7: why is mito-dsRED used - what is difference to mMAPLE-mito; please clarify.

Mito-dsRED is a standard marker for mitochondria based on the fluorescent protein dansyl red. We used it as a second, compatible color in confocal imaging of GFP-Bax. For single molecule localization microscopy, special fluorophore characteristics (photoswitching) are desired. In this sense, we used mMAPLE-mito, which is a marker for mitochondria based on the fluorescent protein mMAPLE, a photoswitchable fluorescence protein that makes it suitable for single molecule localization microscopy. We have now briefly extended this explanation in the results section.

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p9: the AFM provides a much better than 25 to 45 nm. Membrane surfaces have been imaged at sub-nm resolution.

This is true and in the case of membranes, it strongly depends on the phase of the lipids forming the membrane. To our best knowledge, higher resolution is obtained only when using 2D crystal membranes or lipid bilayes in the gel phase. To work under more physiological conditions, we performed all of our experiments with a fluid lipid membrane.

Minor comments:

Please rearrange the keywords, either alphabetically or thematically.
Following the reviewer's suggestion, we have now rearranged the keywords alphabetically.

Figure reference: use either Fig. X a or Fig. X A in both figures and text.
We have now corrected this.

Referee #3:

The authors discuss here the mechanisms by which Bax, a regulator of apoptosis, leads to mitochondrial damage. They use super-resolution microscopy to analyze the distribution of Bax in apoptotic cells, and find that many different types of Bax arrangements can be formed on mitochondria membranes, such as rings, arcs, and linear assemblies. In parallel experiments, they analyze the assemblies of recombinantly expressed Bax on supported lipid bilayers, by atomic force microscopy. They find that Bax appears to form and/or stabilize pores in the bilayers. They conclude that Bax "fully or partially delineates pores" that permeabilize the mitochondrial outer membrane.

While the manuscript is interesting, a few conceptual and experimental problems should be addressed:
We thank the reviewer for acknowledging the interest of the study.

1) The distribution of Bax is measured in cells overexpressing this protein. Is there any possibility to check whether some of the structures observed are dependent on overexpression? For instance, it is conceivable that Bax normally forms rings, but that the protein accumulates in aggregates and lines when overexpressed.

The reviewer has raised a valid point. Please see the answer to comment 1 from reviewer 1. We have compared the expression levels of Bax and GFP-Bax in the transient transfected cells and in wild type HeLa cells by western blot. The results are now shown in extended figure EV1. Interestingly, we find that the levels of GFP-Bax and endogenous Bax together in the transfected HeLa cells are only slightly higher that those of endogenous protein in wild type HeLa cells, even considering that we usually get around 75% transfection efficiency. We have the impression that transfected HeLa cells down-regulate the levels of endogenous Bax so that the total amount of protein is not too high and allows survival. Most likely, those cells efficiently transfected and expressing very high levels of GFP-Bax die shortly after transfection.

In addition, comparable structures to those found in GFP-Bax transfected HeLa cells, this is, lines, arcs and rings, were found in the AFM images. We could detect those structures adding as little as 50 or 100 nM Bax to the liposome samples. Although both situations are difficult to compare, we can make some rough calculations. If one considers that one HeLa cell (volume ~2450 μm^3, bionumbers.hms.harvard.edu ID 103725) contains ~323.000 molecules of Bax (Kulak NA et al., Nat Methods 2014, 11:319), the concentration of Bax protein molecules in HeLa cell would be around 220 μM. Considering that 70% of a cell volume corresponds to cytosol volume, and that there is around 1.1 μm^2 MOM area per μm^3 cytosol, this gives 1886 μm^2 MOM area per cell (bionumbers.hms.harvard.edu ID 109409). If all of Bax is translocated to the MOM, the membrane density is in the order of ~171.3 Bax molecules / μm^2 MOM. Now, if we consider all molecules in the 50 nM sample of Bax bind to the total membrane of the liposomes (0.1 mg total lipid is $1.23*10^{-7}$ moles of lipids, with an average area of 0.71 μm^2 per lipid and considering a membrane as two lipid leaflets), this gives an estimation of ~171.1 molecules of Bax / μm^2 supported bilayer, which is in

very good agreement with the calculated surface density at the MOM. Having double the amount of Bax molecules (experiments with 100 nM) did not significantly change the results.
Altogether, these results strongly suggest that accumulation of aggregates and lines due to an overexpression artifact of GFP-Bax is unlikely.

2) There is little evidence that Bax forms membrane pores in real mitochondrial membranes. Atomic force microscopy experiments on pure lipid membranes have shown for almost a decade that aggregates of neurodegenerative proteins, such as Abeta or synuclein, can form pores. However, it is still unclear whether they do so in native membranes. Therefore, this type of experiment should be interpreted with care. Ideally, such pores may be visualized in correlative fluorescence and electron microscopy experiments, especially if the pore radius is as large as seen in Figure 6. I would encourage the authors to at least attempt to determine whether such pores exist in the mitochondria of Bax-expressing apoptotic cells, by simple transmission electron microscopy. A pore that is 50 nm or larger in size (Figure 6) should be visible.

We thank the reviewer for this comment. Following the reviewer's suggestion, we have imaged the mitochondria of Bax-expressing apoptotic cells by transmission electron microscopy. The results, presented in the new Figure 5, show that mitochondria of apoptotic HeLa cells, both wild type and transiently transfected with GFP-Bax, exhibit defects or disruptions in the outer mitochondrial membrane that are absent from control mitochondrial in healthy HeLa cells. The size of these disruptions is in good agreement with the size of the rings and arcs identified with single molecule localization microscopy and with the membrane pores visualized with atomic force microscopy. These results support the role of Bax in forming pores in the outer mitochondrial membrane.

3) The authors should analyze the frequency of the different types of Bax aggregates in the cells. This information seems to be missing in Figure 3, where various characteristics of these aggregates are shown, but not their proportions in the cell.

Following the reviewer's suggestion, we have now calculated the frequency of the different types of Bax aggregates in the cells. This is now shown in Figure EV2.

4) The use of size bars of variable length, as in Figure 2, is confusing.
We have now corrected this.

2nd Editorial Decision 11 November 2015

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

I would therefore like to ask you to address the remaining concerns 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.

REFEREE REPORTS
Referee #1:

I am satisfied with the authors' replies, and I am especially happy with the inclusion of the new figure 5. However, one more aspect needs to be covered before publication: the authors should not leave figure 5 as a collection of typical pictures. They should convince the reader by including a simple analysis, such as a bar graph of the number of mitochondrial defects seen per mitochondrion, for the different cell phenotypes (e.g. wild-type, Bax expression). This should be performed in a blinded fashion.

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Referee #2:

Cell death is induced by destruction of mitochondria as result of large apoptotic pores formed by BAX or BAK proteins. This leads to a progressive loss of mitochondrial function, and the release of cytochrome c, which activates an apoptopic cycle. Salvador-Gallego et al. show that a significant fraction of activated GFP-Bax locates at discrete sites along mitochondria in the form of rings, arcs and lines 2 to 6 hours after apoptosis induction.

The authors achieved their results by using confocal microscopy, single molecule localization microscopy and STED, atomic force microscopy, thin section electron microscopy, and biochemical methods. They show that BAX clustering correlates with mitochondrial depolarization, that BAX is arranged in rings, arcs and lines, and that membrane pores exists in rings and arcs. These observations result in the hypothesis that BAX does not form pores but stabilizes them by releasing the curvature stress at the membrane edge.

The paper is well written and documented. The text marked in yellow adds clarity. The paper provides a wealth of information and could be suitable for the EMBO J.

Comments

Method descriptions given in the results section (e.g., STED, AFM) could be omitted or moved to the discussion section where appropriate.

The statement concerning resolution, 'around 20 nm in SMLM and 25 to 45 nm in AFM depending on the tip characteristics', is not correct. AFM has definitely a better resolution - as documented by the images (taking the 100nm scale bar for granted) and as reported in the literature.

It is difficult to reconcile the 8-10 BAX dimer model proposed. The protrusions seen on the AFM topographs are larger than what is expected from BAX dimer dimensions (e.g. ref 18). Could estimates be obtained from localization micrographs? (see last comment).

The model shown in Fig. 9 is different to that in ref 18. What is the reason?

Figures contain a lot of information. Figure legends are too short: e.g. in Fig. 1a the inserts are not described, the difference in Fig. 1b could be emphasized to guide the reader.

Fig. 2: How was the cell shape identified? Data could be shown in supportive information. (Consider this also for Fig. 4). The difference of cluster appearance in the insets bottom left (2b) and bottom right is not striking.

Fig. 6: In a) GFP-Bax (green) and mito-dsRed (magenta) signals are shown in different boxes and overlays. On the right, overlays alone are presented. Describe this in the caption. b) What should CoxIV cf680 antibody mark? The overlap with the AF647-anti-GFP-nanobody signal is not clear.

Please refer to the paper 'Bax monomers form dimer units in the membrane that further self-assemble into multiple oligomeric species'

Referee #3:

The authors have updated the text of their manuscript and importantly now provide new data, including analysis of Bak/Bax DKO cells and EM to support their findings, that significantly strengthens the manuscript.
The authors have addressed each of my concerns either experimentally or with sufficient and appropriate text amendments.

Some minor points:

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The new data in EV1 shows the relative expression levels of Bax/GFP-Bax in HeLa nad HCT116 cells. It is unclear what t adjacent quantitation of this data refers to. The y-axis states "%". Presumably this should this read "Total Bax relative to actin"? Also in EV1b as requested the authors show BMH cross-linking of mitochondrial fractions to report Bax oligomerization. The data presented suggest that not all of Bax is oligomerized under the conditions tested (notwithstanding that the BMH linkage may not be complete). This may provide rationale that the "arcs" they observe are just partially formed or newly forming "rings". Although I do not suggest it as a requirement for the current manuscript, it may be interesting to assess Bax oligomerisation by gel-filtration or BNPAGE and assess under conditions of complete oligomerization to test whether this alters the distribution of arcs v rings.

The rationale for using the tethered Bax mutant rather than a LOF mutant is sufficient. Although for a monomeric Bax that is targeted to the membrane they could use the double mutant L63A/S184L.

For completeness, when discussing the symmetrical dimerization of Bax, the authors should also reference the work of the Kluck and Walters labs (Dewson et al Mol Cell 2008, Dewson et al CD&D 2011, Oh et al JBC 2010), who have reported symmetrical homodimers of Bax and Bak.

2nd Revision - authors' response 30 November 2015

Referee #1:

I am satisfied with the authors' replies, and I am especially happy with the inclusion of the new figure 5. However, one more aspect needs to be covered before publication: the authors should not leave figure 5 as a collection of typical pictures. They should convince the reader by including a simple analysis, such as a bar graph of the number of mitochondrial defects seen per mitochondrion, for the different cell phenotypes (e.g. wild-type, Bax expression). This should be performed in a blinded fashion.

We have thought about possible meaningful analysis of the EM images, however, we are convinced that with the 2D experiments performed here, we cannot really estimate the number of defects per mitochondrion or, for example as an alternative, their size. Regarding the number of mitochondrial defects per mitochondrion, we believe this analysis is complicated, because it should be done with 3D EM tomograms. Due to the thin sectioning in the 2D EM images acquired, the most likely situation is that most mitochondria contain cero or one defect because only those in plane are detected, but we do not know if additional defects are present in other planes. As for a size analysis, we cannot discard that the defects observed are actually part of a larger structure if the sections imaged do not correspond with the equator of for example, a round structure.

Referee #2:
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Comments

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Method descriptions given in the results section (e.g., STED, AFM) could be omitted or moved to the discussion section where appropriate.

Following the reviewer's suggestion, method descriptions have now been moved to the discussion section.

The statement concerning resolution, 'around 20 nm in SMLM and 25 to 45 nm in AFM depending on the tip characteristics', is not correct. AFM has definitely a better resolution - as documented by the images (taking the 100nm scale bar for granted) and as reported in the literature.

We thank the reviewer for this comment. Indeed, we realized that the sentence was misleading. We are aware that AFM can achieve better resolution, but we were refereeing to the resolution obtained with the specific tips used in our experiments (Bruker DNP-10, nominal tip radius 20nm). We have now clarified this in the text.

It is difficult to reconcile the 8-10 BAX dimer model proposed. The protrusions seen on the AFM topographs are larger than what is expected from BAX dimer dimensions (e.g. ref 18). Could estimates be obtained from localization micrographs? (see last comment).

We agree with the reviewer that the resolution of our AFM experiments does not allow for a reliable correspondence of the protrusions visualized with the Bax dimer units (represented according to the model proposed in our publication "Model of Active Bax at the Membrane. Molecular Cell ***56:*** *496-505") and we have insisted in the text that it is a very rough estimation without scientific background. However, the fact that the protrusions in the AFM images are larger than the dimers does not mean that higher oligomeric structures are responsible for them, mostly if one takes into account the lateral resolution in our experiments. Therefore, based on current understanding of Bax structure, we find that the most reasonable interpretation is that the protrusions correspond to dimers.*

On the other hand, the single molecule experiments performed in our publication "Bax monomers form dimer units in the membrane that further self-assemble into multiple oligomeric species" were performed in the single molecule regimen and in addition, the analysis of higher order oligomers was limited for technical reasons. Taking all our studies together, we think that Bax oligomers are variable in size, and that the number of molecules in the oligomer depends on the density of Bax in the membrane, as we discuss in the manuscript. In this scenario, the experiments from the single molecule studies do not disagree with the results shown here, neither with the estimations from the AFM images.

The model shown in Fig. 9 is different to that in ref 18. What is the reason?

We assume that the referee means that the model in Figure 8 does not correspond to the Bax model proposed in out publication "Model of Active Bax at the Membrane. Molecular Cell ***56:*** *496-505". It is indeed the same structure, but shown from a different perspective. We show here how we think that Bax oligomers would delineate the pore edge in a "clamp" conformation.*

Figures contain a lot of information. Figure legends are too short: e.g. in Fig. 1a the inserts are not described, the difference in Fig. 1b could be emphasized to guide the reader.

Figure legends have been now extended and figures are now better described.

Fig. 2: How was the cell shape identified? Data could be shown in supportive information. (Consider this also for Fig. 4). The difference of cluster appearance in the insets bottom left (2b) and bottom right is not striking.

The cell shape was identified by taking bright field images before de SMLM measurements, for GFP and the AF647. If the images are reconstructed without any cutoff, we observe that the signal of the localizations corresponds only to the shape of the cell. We have now explained this in the corresponding figure caption.

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Fig. 6: In a) GFP-Bax (green) and mito-dsRed (magenta) signals are shown in different boxes and overlays. On the right, overlays alone are presented. Describe this in the caption. b) What should CoxIV cf680 antibody mark? The overlap with the AF647-anti-GFP-nanobody signal is not clear.

a) *The single channels and overlays are now better explained in the caption.*
b) *CoxIV antibody is used to mark the mitochondria and cf680 is the fluorescent secondary antibody for the immunostaining. AF647-anti-GFP-nanobodies mark GFP-Bax signal, which is heterogeneously distributed in discrete foci along mitochondrial tubules as well as on the ends. Therefore, GFP-Bax does not have to completely colocalize or overlay with CoxIV staining.*

Please refer to the paper 'Bax monomers form dimer units in the membrane that further self-assemble into multiple oligomeric species'.

This reference has now been included.

Referee #3:
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Some minor points:

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We thank the reviewer for this comment. We have simplified the analysis. The quantitation of the western data was made as follows: all bands were first corrected to the expression of the correspondent loading control (actin). Then, the expression of endogenous Bax from WT cells was normalized to 100% and compared with the total amount Bax (arising from endogenous protein and GFP-Bax) in transfected cells. This has now been clarified in the figure caption.

Regarding the full oligomerization of Bax, we indeed cannot discard that the BMH linkage is not complete. Nevertheless, independently of Bax being partially or fully oligomerized, we would always expect a fraction of arc and line assemblies, even in the case that both structures are intermediates of full rings. The reason is that if one understands Bax oligomerization as a kinetic process with multiple seeding points (that could also start at different times) along the mitochondria tubules, at a certain point all Bax molecules in the cell will be depleted.

The rationale for using the tethered Bax mutant rather than a LOF mutant is sufficient. Although for a monomeric Bax that is targeted to the membrane they could use the double mutant L63A/S184L.

For completeness, when discussing the symmetrical dimerization of Bax, the authors should also reference the work of the Kluck and Walters labs (Dewson et al Mol Cell 2008, Dewson et al CD&D 2011, Oh et al JBC 2010), who have reported symmetrical homodimers of Bax and Bak.

These references have now been included.

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3rd Editorial Decision 01 December 2015

Thank you for sending your revised manuscript to us. I appreciate the introduced changes, and I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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