Differential retrotranslocation of mitochondrial Bax and Bak

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Editor: Andrea Leibfried

1st Editorial Decision 19 May 2014

Thank you for submitting your manuscript entitled 'Differential retrotranslocation of mitochondrial Bax and Bak'. I have now received the reports from all referees.

As you can see below, both referees appreciate that your work will be of interest to the apoptosis field, and referee #2 suggests only minor amendments to render the manuscript more accessible to a broader audience. However, referee #1 raises numerous concerns, and thinks that the conclusions are at this stage not sufficiently supported by the data presented. S/he further finds that the manuscript does not provide at this point sufficient insight to make it suitable for further consideration here.

Given the constructive comments provided by the referees, I could offer to consider a revised version should you be able to substantiate your analysis along the lines suggested by the referees. Importantly, we would need further insight into the mechanism underlying retrotranslocation (see also referee #1, point 5 and 8 of the general comments). This clearly demands a lot of work and time, as many of the experiments would have to be repeated and refined, and additional ones would have to be performed as well, with uncertain outcome. I can extend the revision time to 6 months, should that be helpful.

Please note that I would need strong support from referee #1 on the revised manuscript to consider publication here. Therefore, do consider your options carefully. If you see yourself in a position not to be able to address the concerns raised, then it is in your best interest to seek publication elsewhere at this stage.
I thank you in any case for the opportunity to consider your work for publication. Please contact me in case of further questions.

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Referee #1:

Bax and Bak are the key effectors of apoptosis, yet despite their importance and functional redundancy, aspects of their regulation such as why Bax is largely cytosolic as opposed to Bak that is mitochondrial, remain poorly understood. The authors and associated colleagues were the first to report that rather than being a cytosolic protein, that Bax actually targets mitochondria but is retrotranslocated to the cytosol by interaction with Bcl-xL. The current study now extends this to show that Bak retrotranslocates in a similar fashion, and involving a similar mechanism, but does so at a reduced rate thus explaining its constitutive mitochondrial association. Although authors provide data to suggest that the rate of retrotranslocation is determined by the relative hydrophobicity of the tail anchor, the mechanism involved in retrotranslocating these proteins remains largely unclear.

General comments
1. In Fig 1 C and D the levels of Bak in HM and cytosol in human tissues is shown. It is unclear why this data is shown in two different panels. For ease of comparison it is imperative to run the equivalent proportions of cytosol and heavy membrane fractions side by side on the same gel. Can the authors exclude that the Bak detected in the cytosol is not newly synthesised protein that is en route to mitochondria? Obviously not feasible to test in the human tissues, this could be confirmed in cultured cells by pretreating with cycloheximide.

2. Does retrotranslocation of endogenous Bak and Bak still occur in Bcl-xL-/- cells? i.e. can other pro-survivals compensate?

3. The justification that transient transfection limits protein over-expression compared with stable expression is not convincing as protein expression was assessed using a population-based assay (i.e. western). How many cells were transfected in this experiment (based on GFP positivity)? In Figure 4C, for example, this appears to be only approx. 50%. Thus the explanation that stable cells express more Bax than transiently transfected cells does not necessarily hold. Likewise the selection of cells with comparable mitochondrial wild-type Bax and BaxTBak. In each case were 100% of the cells transfected or only a proportion? If only a proportion then the ability/efficiency to mediate MOMP cannot be directly compared between the two variants. A better way to test would be to stably express each variant and select multiple clones on the basis of expression. Given that the mutations may affect the fold of the protein and so be potentially destabilising, an assessment of protein levels following stable expression would be informative.

4. The authors state that their finding that BakTBax (a chimera where the entire C-terminus of Bak is swapped for that of Bax) is significantly cytosolic is consistent with a previous study by Ferrer et al. However, as I see it the most similar mutant in that study (BakBaxCT chimera) was predominantly mitochondrial and integrated and so indistinguishable from wild-type Bak. These findings would suggest that the c-terminus is not the sole defining factor in determining subcellular localisation of Bak and Bax as claimed in this current study. Can the authors clarify this apparent discrepancy?

5. In Bak retrotranslocation the mitochondrial population of Bax is not integrated into the MOM. However, Bak that is resident at mitochondria is mostly integrated in the mitochondrial outer membrane protein likely via its C-terminal anchor. Thus it is difficult to envisage that the peripherally attached Bax and membrane-integrated Bak would be translocated to the cytosol by similar mechanisms. Can the authors provide some further insight into the mechanism?

6. I am unclear of the use of the 1-2/L-6 Cys variant as a control for a mutant that fails to bind Bcl-xL. Is this only when the mutant is oxidised to induce the disulphide-tethers? One would predict that in the reducing environment of the cytosol that the tethers would not be formed and so the variant would behave much like wild-type Bax in its interaction with Bcl-xL.

7. Can the authors clarify the statement that,"Both pro-apoptotic Bcl-2 proteins share similar apoptotic activity and subcellular localization, when the same MA is exposed." As Bax with the Bak C-terminus is seemingly constitutively active yet Bak is not. Also in the opening paragraph of the Discussion the authors state that their data indicate that no other region other than the C-termini govern subcellular localisation and thus exclude an influence of the putative "rear pocket" in Bax...
translocation. It is an important distinction that retrotranslocation controls steady-state localisation of Bax whereas the rear pocket is proposed as an activation mechanism downstream of an apoptotic stimulus. Additionally the final paragraph of the Discussion brings in mitochondrial dynamics. This link seems rather tenuous and in the interest of clarity I would suggest removing it.

8. The authors should address the mechanism involved in retrotranslocating Bax and Bak. For example, an interaction between the BH3 domain of Bax/Bak and Bcl-xL is implicated. Thus preventing BH3 domain exposure using the tethering approach or by mutagenesis should render both Bak and Bax refractory to retrotranslocation. An additional consequence of this mechanism is that BH3 domain exposure must be reversible. This should be tested.

Specific points
1. A number of the legends do not indicate how many times the experiments were repeated. In particularly the Western blotting of subcellular localisation and carbonate extraction studies. Indication of reproducibility should be provided for each experiment.
2. Can the authors explain why VDAC was detected as a doublet in Fig 4D but not in Figs 3C and D?
3. Amphotropic and not ecotropic Phoenix packaging cells were presumably used to infect HeLa cells.
4. Molecular weight markers should be included on Western blots.
5. Supp Figure E2e - Is this from one experiment? If so this should be ideally repeated.
6. Supp Figure E2f - again, to be able to gauge relative amounts of Bcl-xL in cytosol versus membrane, equivalent proportions should be run alongside on the same gel.
7. There is no legend to describe the data shown in Table 1 including what the variance represents and how many experiments the data is collated from.
8. Error bars (SEM or SD?) should be indicated in Figure E4a and b.

Additional

1. Page 10, "...contrasting (with) the regulation of...."
2. Page 18, Sp "Hela" and "....imaged prior (to) bleaching."
3. Some of the labelling of axes in the Supp Figures are corrupted (but this could just be my version)

Referee #2:

In this manuscript, the group of Frank Edlich compares the regulation of Bax and Bak by retrotranslocation from mitochondria to the cytosol. They described this mechanism for Bax in a study published in Cell in 2011, and they further elucidated the mechanism through which Bcl-xL can mediate this process, in another article published in Cell Death and Differentiation in 2013. Others also reported similar results in another system. However, it was really important to address whether such a regulatory mechanism could also be relevant to Bak. Indeed, Bak is functionally redundant with Bax but is mainly located at the mitochondria whereas Bax is mainly cytosolic in healthy cells. In the present study, the authors show that Bak is also retrotranslocated from the mitochondria to the cytosol but its low retrotranslocation rates cause predominant localization at the mitochondria. Moreover, comparison of different chimeric constructions of Bax and Bak interchanging their membrane anchors raises interesting conclusions about regulation of subcellular localization and pro-apoptotic activity of the two proteins. The results are very convincing and of potential importance for a wide audience. However, the clarity of the manuscript should be really improved so that a non specialised reader could understand it. Some points also require further discussion.

Major points:

1. The way experiments are described and results are presented is very confusing, maybe due to length restriction and to the complexity of the results. The hypothesis and conclusions are also difficult to follow. Here are some suggestions to improve the clarity of the manuscript:
- Some additional elements should be given to non specialized readers to understand the background and the principle of the experiments: a brief description of the Bcl-2 family in the introduction (the three groups, the BH domains, how they interact); what is already known about the mutants that are used (especially S184V but also G138A, Bax D68R and 1-2/L-6) and why they are used in the present study, before description of the results; at least in Materials and methods or Figure legend sections, what is DD Bcl-xL and why shield-1 reagent is necessary; talk about change of conformation instead of 6A7-positivity and explain what is 6A7 in Materials and methods section; talk about integral membrane protein instead of carbonate extractability and explain the principle of the experiment in Materials and methods section.

- Some sentences are not clear and should be rephrased: "the lack of Bak shuttling..." at the beginning of p 8; "the lack of Bcl-xL-mediated inhibition..." p10; "Bax, Bak and BaxTBak commit cells to apoptosis..." at the end of p10, beginning of p11; "However, Bax activation on the mitochondria..." at the end of p11 (should "but" be replaced by "because"?).

- Some figures presented as supplemental data should be in the main manuscript and vice versa to provide the reader with all significant data in the main article and to have only additional controls in the supplemental file. For example, Fig E2B and E2C should be added to Fig 3, Fig E4A and E4B should be in Fig 5 but Fig 5B and D could be in Fig E4; the quantification of Fig 5F should be in the same figure and not in Fig E4D.

- It would be useful to add labels to figures, for example BaxTBak in Fig E3C and BakTBax in Fig E3D; write BaxTBak instead of Bak MA in Fig 6B; name the mutants plotted in Fig 6H.

2. The authors conclude that "the commitment of cells to apoptosis is determined by Bax/Bak shuttling rates", notably because Bax S184V and BakTBak S184V have a similar pro-apoptotic effect and a similar shuttling rate. However, Bak and BaxTBak also have a similar shuttling rate. Nonetheless, BaxTBak is much more toxic than Bak. The authors exclude the possibility that the Bak MA preactivates Bax by comparing BaxTBak and BaxTBakSS. I also agree that Bax S184V and BakTBax S184V have similar pro-apoptotic effect and similar shuttling rates, suggesting that Bax and Bak have similar pro-apoptotic effect provided comparable predominant cytosolic localization. However, this still does not explain why BaxTBak is much more toxic than Bak. This should be clearly discussed.

Minor points:

3. Fig 2A and 4C are very convincing. However, as confocal microscopy has been used, it means that Bak+Bcl-xL, Bax, and BakTBax are not only cytosolic but also nuclear. Could the authors comment on that?

4. The authors should take more caution when raising conclusion from the comparison of Bax vs. Bax184V with BakTBak vs. BakTBax S184V as it is very indirect.

5. Is there any evidence that the Bak MA would not bind the hydrophobic groove of Bak?

5. Supplemental figures are often mentioned as S instead of E in the text.

1st Revision - authors' response 05 August 2014

Referee #1:

We are pleased that our manuscript was positively received and thank the reviewer for her/his time and efforts in providing a constructive review.

Major comments:
1) The new Figure E1 shows equivalent amounts of cytosolic and HM protein loaded side-by-side for the three samples with a considerable cytosolic Bak pool according to spectrophotometric analysis. The high amount of cytosolic sample affects protein migration.

We have also incubated HeLa cells for 0-5 hours using 20 µg/ml cycloheximide (CHX) to test whether cytosolic Bak represents newly synthesized protein en route to mitochondria. The results of fractionations similar to Figure 3D of the revised manuscript show that the ratio between cytosolic and mitochondrial pools of endogenous Bak does not shift upon CHX treatment (new Figure E3E of the revised manuscript and Figure 1 of this letter). These results largely exclude the possibility that the cytosolic Bak pool is freshly synthesized protein on its way to mitochondria. In addition, this study demonstrates shuttling of wild-type Bak and Bak variants dependent on the presence of pro-survival Bcl-2 proteins as well as the hydrophobicity of the membrane anchor (MA; Figures 2, 3, 7, E3, E4 and E7 of the revised manuscript). Changes in the retrotranslocation rate shift Bak from the OMM into the cytosol (Figures 3, 4, E3 and E7). Bak exists in an equilibrium between cytosolic and mitochondrial pool.

Figure 1. Changes in the ratio between endogenous cytosolic and mitochondrial Bak in HeLa cells upon incubation with 20 µg/ml for the indicated time points. Data is normalized to the ratio at 0 h and depicted +/- SEM based on desitometric analysis of Western blot signal. n = 3.

2) We have addressed this important question by analyzing Bak retrotranslocation in the presence of different anti-apoptotic Bcl-2 proteins (new Figure 3C). Interestingly we find that Bcl-2 fails to accelerate Bak shuttling whereas Mcl-1 increases Bak shuttling more potently than Bcl-xL. This pattern differs from Bax retrotranslocation but shows redundant functions of pro-survival Bcl-2 proteins in the shuttling of Bak and Bax. In addition, Bcl-xL activity of Bak shuttling was inhibited by ABT-737. Together these results indicate that Mcl-1 plays a more important role in Bak shuttling than in Bax retrotranslocation (Figure 3C). The lack of increased Bak shuttling by Bcl-2 is in line with previous reports suggesting Bcl-2 is less effective in blocking Bak activity (Oltersdorf et al., 2005). However, Mcl-1 and Bcl-xL possess redundant activities in Bak retrotranslocation similar to Bax shuttling.

3) We addressed the reviewer’s comment in several independent ways as follows. Figure E2 of the revised manuscript contains imaging data and quantification of GFP-positive HCT116 Bax/Bak DKO cells transfected with either GFP-Bax or GFP-Bak constructs (new Figure E2C, D). Approx. 90 % of the cells were transfected in both cases. Similar transfection efficiencies were achieved with all constructs (data not shown). Independently, the colony forming assays in Figure 5F-G reflect a 90 % transfection rate, with 10 % cell survival of BaxTBak-expressing cells. This result also excludes the suggested respective stable transfection experiment, as all cells expressing BaxTBak will die within 1 week. For other constructs the clonogenic assays indicate different levels of toxicity in accordance with measured apoptotic activities (Figure 5 of the revised manuscript). As the reviewer is aware, the advantage of our transient expression approach is that it allows analysis of the different variants at comparable protein levels, allowing direct comparisons to be drawn. The densitometric analysis of the Western blot in Figure E2B suggests that the GFP-Bax levels in stably expressing cells exceed those of transiently expressing cells by 700 %. Based on 90 % transfection efficiency, HCT116 cells with transient expression results in approximately the same amount of endogenous Bax that is present in wild-type cells and about 20-25 % less Bax than present in Bak
KO cells. As we have previously shown (Todt et al., 2013), an excess of protein produced after stable transfection can stress the cell and produce artificial results, whereas transient transfection under controlled conditions can result in near endogenous levels, avoiding artefacts and allowing appropriate comparisons.

4) The reviewer is correct that data presented by Ferrer and colleagues in Figure 3 suggests that the chimera most similar to BakTBax localizes predominantly to the mitochondria. However, the localization of another variant (BakBaxCS) with changes exclusively in the MA presented by Ferrer et al. indicates, in concordance to our findings, a strict dependence of the Bak localization on the C-terminal segment. Thus, both datasets agree on the importance of the C-terminus for Bak localization and exclude a major role of other segments. On the other hand, our results do argue against an important role of the “C segment” as suggested by Ferrer et al., because we measure similar shuttling rates and localization with different C-terminal segments of comparable hydrophobicity. This matter now is more carefully discussed in the revised manuscript. In this study, BakTBax localization demonstrated by confocal imaging and subcellular fractionation correlates with the measured retrotranslocation rate. Differences may occur due to the stable transfection, different constructs and different cells used in the study by Ferrer and colleagues.

5) Bak and Bcl-xL share a predominant mitochondrial localization (Figure 3A, 3D, E7B). Substantial pools of OMM-integral Bak and Bcl-xL can be detected in human cells despite their retrotranslocation into the cytosol (Figure 1E, 7E). Their localization pattern clearly differs from the largely cytosolic Bax. However, a small but detectable OMM-integral Bax pool is present in human cells (new Figures 7H, E7D). OMM-integral Bax can be gradually increased by decreasing Bax shuttling, for instance, using the S184V substitution (new Figures 7H, E7D, E). Conversely, this Bax pool can also be decreased by accelerated Bax shuttling in the presence of ectopically expressed Bcl-xL (new Figure E7D, E). OMM-integral Bax in proliferating cells is 6A7-negative and thus inactive (new Figure E7F, G).

The new Figure 7E and F shows that OMM-integral Bcl-xL is shifted into the OMM-associated and cytosolic forms (new Figure E7B) by retrotranslocation. This conclusion is based on the facts that (1) wild-type Bcl-xL is shifted from the mitochondria upon presence of Bax/Bak (Figure 7E) and that (2) Bax increases wild-type Bcl-xL retrotranslocation (Todt et al., 2013). On the other hand, the Bcl-xLTBax variant does not co-retrotranslocate Bax (Todt et al., 2013), but is capable of interacting with Bax similar to the wild-type (new Figure E7C). Bcl-xLTBax is not shifted by Bax or Bak, nor is the shuttling of this Bcl-xL variant increased in the presence of Bax (new Figure 7F, (Todt et al., 2013)). The observation that Bcl-xL is largely OMM-integral in the absence of Bax/Bak suggests initial interactions for retrotranslocation by the OMM-integral forms. The lack of a large OMM-integral Bax pool logically results from fast Bax shuttling preventing the toxicity of slow-shuttling Bax in proliferating cells. Thus, Bax and Bak exist in a dynamic equilibrium between cytosolic, OMM-associated and OMM-integrated forms.

Based on these findings two different scenarios are conceivable: (1) there could be two equilibriums between OMM-integral and OMM-associated protein and between OMM-associated and cytosolic protein and (2) OMM-integral protein is targeted by retrotranslocation. In the latter scenario OMM-associated protein would be an intermediate state en route to OMM-integral protein. It could be possible to distinguish both scenarios, if specific labeling of one protein pool could be achieved. Unfortunately, this is currently impossible since retrotranslocation measurements require intact cells. Our experimental insights into the common retrotranslocation process of Bax and Bak are now summarized in the new Figure 7I of the revised manuscript.

6) It is correct that most disulfide bonds are reduced in the cytosol due to the presence of GSH. However, an ideal distance between the cysteine residues can lead to disulfide bonds that are stable in the cytosol (Bessette et al., 1999; Locker and Griffiths, 1999; Østergaard et al., 2004; Schouten et al., 2002). In a previous publication we have characterized the tethers in Bax 1-2/L-6 to be oxidized in the cytosol of HCT116 cells (Edlich et al., 2011, Figure S1B, C). In fact, 10 mM GSH or DTT are not sufficient to reduce the tethers of recombinant Bax 1-2/L-6 (Edlich et al., 2011, Figure S1D). The Bax 1-2/L-6 tethers interfere with conformational changes required for Bax/Bcl-xL heterodimerization and prevent Bax shuttling (Edlich et al., 2011, Figure S2C). We introduce this variant now also in response to comment #1 of reviewer 2 in the figure legend describing Figure E5C.
7) In response to this reviewer comment and comment #2 of reviewer 2, we have discussed more clearly the similar regulation of Bax and Bak at high shuttling rates as well as the activation of Bax specifically at low retrotranslocation rates. Furthermore, we added a schematic view on the common regulation of Bax and Bak in the new Figure 7I of the revised manuscript. We also fully agree with the reviewer that retrotranslocation determines the localization of Bax and Bak within a cell, but discrimination between the regulation of Bax localization and activity is perhaps artificial. Diminished Bax retrotranslocation leads to Bax activation. Thus, mitochondrial Bax accumulation and Bak integration into the OMM, hallmarks of Bax activation following apoptotic stimuli, could simply result from inhibited and thus slower Bax shuttling. Our results show a correlation between the hydrophobicity of the MA of Bak or Bax and shuttling rates, emphasizing the importance of the MA on the translocation and retrotranslocation of both pro-apoptotic Bcl-2 proteins. However, the experimental evidence based on MA variants cannot excluded that other segments and their interactions play a role of lower importance in the regulation in the wild-type proteins. Therefore, we will follow the reviewer’s advice and do not exclude the possibility that other segments than BH3 domain and MA play perhaps a role in the regulation of Bax and Bak by retrotranslocation. We also follow the advice of the reviewer and remove the discussion of implications of different forms of Bak on mitochondrial dynamics.

8) We made use of the D83R variant, containing the corresponding substitution to Bax D68R (Kvansakul et al., 2007). Similar to the effect of Bax D68R, the D83R substitution interferes with Bak retrotranslocation. We have included the measured Bak D83R retrotranslocation rates in the new Figure 3C. Thus, the interaction between Bak BH3 and Bcl-xL hydrophobic groove is as essential for Bak shuttling as it is for Bax retrotranslocation.

In order to assess a potential conformational change, we intended to introduce tethers into the Bak structure, corresponding to the Bax 1-2/L-6 variant. Figure 2 of this letter shows that the generated Bak 1-2 variant, containing cysteine substitutions in helices 1 and 2, showed an unexpected localization pattern indicating a problem with protein folding. Other Bak variants containing cysteine residues within the helices 1 and 2 are unlikely to form structural disulfide bonds based on the Bak structure (2IMS). Therefore, there are no experimental means available to analyze the importance of a conformational change in this region of Bak for retrotranslocation.

Figure 2. Confocal images of HCT116 Bax/Bak DKO cells expressing Bak 1-2. GFP fluorescence is depicted in the left panel and in green in the merged image and detail. MitoTracker-stained mitochondria are depicted in red in the merged images. Scale 5 μm.

The reviewer also suggested to test whether Bak and Bax might expose their BH3 domains during retrotranslocation. We attempted to identify Bax or Bak exposing the BH3 domains by IP using BH3-specific antibodies. Unfortunately we found only one commercially available antibody with the potential to bind to Bak BH3 specifically but none for Bax BH3. We performed the IP of Bax expressed in HCT116 Bax/Bak DKO cells in comparison to a polyclonal Bax antibody. Figure 3 of this letter shows that the BH3-specific Bax antibody did not detect Bax under any circumstances, making it impossible to identify Bax with the BH3 domain exposed.

Figure 3. IP of endogenous Bax from cell extracts of HCT116 precipitated by polyclonal anti-Bax (rabbit polyclonal, Abcam) and BH3-specific anti-Bax (rabbit...
polyclonal, Thermo Sci.) in the presence of 0.2% Triton X100 (TX) or CHAPS (CH). The input is indicated on the left. Actin serves as loading control. n = 3.

However, our results show that Bax/Bak retrotranslocation can be abolished by a point mutation in the BH3 domain, is accelerated by Bcl-xL and other pro-survival Bcl-2 proteins in accordance to their ability to inhibit Bax and Bak, respectively. Bcl-xL-mediated shuttling of Bax and Bak can be blocked by the G138A substitution or the BH3-mimetic ABT-737, but wild-type Bcl-xL shifts both proteins from the OMM-integral to the cytosolic form.

Minor comments:

1) The revised manuscript provides now the number of repeats in the figure legends of each panel.
2) The used anti-VDAC antibody (Calbiochem, mouse anti-Porin 31HL (Ab-3)) recognizes all three isoforms of VDAC (VDAC1-3). The appearance as single or double band in the Western analysis depends solely on the migration during SDS-PAGE.
3) Indeed, we used amphotropic Phoenix packaging cells. We have corrected this mistake in the methods section.
4) We have included MW markers in most figures depicting Western blots except data sets containing PARP cleavage to avoid confusion.
5) We repeated this experiment according to the reviewer’s suggestion. Unfortunately, the original UV source is no longer available. Thus, the apoptotic stimulus is now ActD (new Figure E3D).
6) We followed the advice of the reviewer and present the data in the new Figure E7B.
7) We have included a legend for table 1 in the revised manuscript. Figure legends describing the FLIP data include the number of ROIs that data has been collected from for each data set.
8) Panels E4A and B have been added to Figure 5 (C, D) of the revised version of the manuscript (comment #1 of reviewer 2) and the figure legend now states that error bars represent SEM of both data sets.

Additional comments:
1+2) We have corrected these mistakes in the revised manuscript.
3) We hope this problem is resolved in the revised version of the manuscript.

Referee #2:

Major concerns:
1) We thank the reviewer for her/his efforts to improve our manuscript. As suggested, the revised introduction contains a brief description of the Bcl-2 family. We also provide a schematic summary of the main results in Figure 7I. We now explain the Bax S184V, Bcl-xLTBax and Bcl-xL G138A substitutions at their first appearance in the text and Bax D68R and Bax 1-2/L-6 are introduced in the figure legend, as they have been used as controls in one experiment of this manuscript. We now also describe in more detail the use of DD Bcl-xL and use conformational change and membrane-integral instead of 6A7-positive and carbonate-inextractable, respectively, as suggested. Likewise, the mentioned sentences have been rephrased to improve the readability of the manuscript.

We thank the reviewer for her/his advice on data presentation in the figures and extended material and now present the data accordingly. Only panel E2C is still included in the extended material, because the results have been included in the new Figure 3C.

2) As suggested, the revised manuscript now emphasizes more clearly that Bax and Bak are similarly regulated in their retrotranslocation rates and localization. We also stress that fast shuttling of Bax and Bak results in similar pro-apoptotic activity, but Bax is activated at low retrotranslocation rates in contrast to Bak. A schematic summary in the new Figure 7I now points out this difference to make it clear to the reader.

Bax specific cell death can be observed in human cells and could potentially occur due to low shuttling rates (Figure 4 of this letter). (Not shown in this Review Process file.) Interestingly, this cell death can be observed following ABT-737 treatment that interferes with the BH3 binding of pro-survival Bcl-2 proteins and thus directly with their retrotranslocation activity. However, the mechanism of Bax activation at low retrotranslocation rates remains to be elucidated. We will
certainly follow up on this process but refrain here from further discussion in the absence of conclusive insight.

Minor points:

3) We agree with the reviewer’s assessment and find this localization pattern not only with Bax constructs, but also with other GFP-fusion constructs of largely cytosolic Bcl-2 proteins, including Bcl-xL variants with increased cytosolic pools due to C-terminal truncations or substitutions (Todt et al., 2013). Similar nuclear/cytosolic localization pattern have been reported for GFP-Bax constructs (Brooks et al., 2007; Wolter et al., 1997; Zhou et al., 2007). FLIP measurements show that GFP-Bax, GFP-Bcl-xL or GFP-Bak variants localized to the nucleus migrate without restrictions into the cytosol. Thus, we believe that this altered localization is caused by the GFP-fusion but has no functional implications, as GFP-tagged and untagged Bax/Bak behave similarly in activity measurements.

4) We thank the reviewer for drawing our attention to this point. While we can compare retrotranslocation rates, localization and toxicity, structural insight is lacking. In the revised manuscript, we stress the point that Bax S184V and BakTBax S184V behave similarly in all assayed parameters and discuss other implications with more care (pages 11, 12).

5) We do not have structural insight into the protein fold of BakTBax. However, in the absence of direct evidence for the lack of interactions between hydrophobic Bak groove and the C-terminal MA of Bax, two lines of evidence suggest that an interaction as observed in wild-type Bax is unlikely: i) The sequence alignment of both C-terminal segments reveals large differences in the amino acid composition (Figure 5 of this letter). The S to Q exchange in the Bak sequence corresponding to S184 in Bax (box) is of special interest. Although the S184Q substitution has not been analyzed to our knowledge, the S184A, S184V, S184L, S184E, S184D, S184K and S184H substitutions all impair Bax localization and regulation probably in part by disrupting interactions between MA and hydrophobic groove (Nechushtan et al., 1999; Suzuki et al., 2000). Based on these data the S184Q substitution likely disrupts MA binding to the hydrophobic groove.

ii) The superimposition of the 3D structures of Bak (2IMS, Figure 5 of this letter, cyan) and Bax (1F16, green) suggests large differences in the surfaces of both hydrophobic grooves; these differences can be expected based on different MA sequences. However, the extent of structural differences even indicates a possible spatial overlap of Bak MA (orange) and residues forming the hydrophobic groove of Bak (cyan). Despite the lack of experimental evidence, the structural information suggests disturbed interactions between Bak hydrophobic groove and Bax MA and probably vice versa.

In addition, the subcellular localizations of Bax S184V and BakTBax S184V appear similar, while less Bax localizes to the HM in comparison to BakTBax supporting an additional effect of the S184V substitution in Bax compared to BakTBax (Figure 6 of this letter). While this discussion is perhaps confusing to a broader audience, we have included the statement that our assumption of impaired binding is based on substantial differences in the amino acid sequence of both C-terminal MA and referred to Figure E5A.

5) We have corrected this problem.
Figure 5. A) Sequence alignment of the hydrophobic membrane anchor (MA) segments of Bax (168-192) and Bak (187-211). Identical residues (*), highly similar residues (:), and less similar residues (.) are indicated. S184 and the corresponding Bak residue are marked (box). B) Secondary structure elements of the 3D structures of Bax (1F16, green) and Bak 21MS, (cyan) are shown in superimposition. C) Superimposition of Bax (green) and Bak (cyan) 3D structures as shown in B rotated by 90° on the x axis. D) Surface of the superimposition of the 3D structures of Bax (green) and Bak (cyan) as depicted in C, showing the hydrophobic binding pocket of the MA of Bax (orange helix). E) Surface of the superimposition of the 3D structures of Bax (green) and Bak (cyan), showing the hydrophobic binding pocket of the MA of Bax (orange helix) as depicted in D. The structures have been rotated on x and y axis to show the potential collision of the Bax MA with the hydrophobic groove of Bak.
Figure 6. A) Comparison of the subcellular distribution of wild-type Bax and Bax S184V in cytosol (C) and heavy membrane fraction (HM). GAPDH and Tom20 serve as loading control for cytosol and mitochondria-containing HM. B) Cell fractionation of HCT116 Bak/Bax DKO cells expressing BakTBax and BakTBax S184V in cytosol (C) and heavy membrane fraction (HM) as in A).

References
Thank you for submitting the revised version of your manuscript to us. I have now received the reports from all referees, which I enclose below.

As you will see, both referees appreciate the introduced changes. However, they still have some issues that need to be addressed prior to publication here. I would therefore like to invite you to submit a revised version of the manuscript, addressing the comments of all reviewers. Please let me know in case you have further questions regarding this final revision.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

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Referee #1:

The authors have provided additional data to support the hypothesis that Bak, like Bax, retro-translocates from the mitochondria to the cytosol, including some mutagenesis data to provide some indication of mechanism (i.e. to involve the Bak BH3 into the pro-survival groove). They have addressed, or at least made attempts to address (see tethering and antibody blockade experiments), my comments and now the data (and how it is presented) is more convincing and is of interest to the apoptosis community. However some questions still remain relating to the new data that I feel should be clarified/discussed.

1. Given the key role for the D83 in the Bak BH3 domain for its apoptotic function, is the Bak D83R mutant functional? No death assay data for this mutant is shown. If not functional then its subcellular localisation (i.e. lack of retro-translocation) must be interpreted with caution.
2. The new mutagenesis data implies that the BakBH3 must interact with the Bcl-xL hydrophobic groove in order to translocate to the cytosol, as they have previously shown for Bax. However, they state that the Bax at mitochondria that interacts with Bcl-xL is "inactive", based on exposure of the N-terminal 6A7 epitope, and in the Discussion state that, "...retrotrotranslocation shuttles only inactive OMM-integral-Bax". The use of the term "inactive" needs to be addressed as the BH3 into groove interaction requires a conformation change as the BH3 domain, and in particular the implicated D68/D83 residues, are buried in the inactive structures of Bax and Bak respectively. So although Bax (and potentially Bak) may not have exposed their N-terminal epitopes they presumably are not in their "inactive" configuration when interacting with Bcl-xL. This is not merely a semantic issue, but has important mechanistic implications, as it suggests that in order for Bak and Bax to retrotranslocate that some activating conformation change is necessary (presumably promoted by a stimulus such as BH3-only interaction or interaction with mitochondrial lipid), and also implies that the BH3 domain exposure is reversible.
3. That ABT-737 inhibited retrotranslocation would support the Bak BH3 into pro-survival groove mechanism. However this data is not actually shown in the new Fig 3C as stated in the text and figure legend.

4. Although I do not necessarily agree that transient transfection is preferable to the generation of stable cell lines to assess the function of Bak/Bax mutants, I do concede that stable cell lines are not suitable to assess constitutively active mutants such as BaxTBax. Together with the indication of the % cells transfected in each experiment support the transient transfection as a interpretable approach.

5. I did not raise this question in my original review so I am hesitant to raise it here and I do not necessarily expect the authors to amend their manuscript in light, but I have a potentially naïve question regarding the interpretation of their FLIP data. Clearly from the FLIP experiments Bcl-xL alters the rate of shuttling of Bak between the mitochondria and cytosol. However, can the authors exclude that the decrease in mitochondrial fluorescence observed is not due to INCREASED shuttling of the bleached cytosolic population TO mitochondria rather than increased shuttling of GFP-Bak to the cytosol? The net effect would be the same .ie. reduced fluorescence at mitochondria. Wouldn't FRAP experiments be necessary to show increased shuttling of GFP-Bak from mitochondria to the photobleached cytosol or would this be below the level of detection?

6. The authors now discuss the apparent discrepancy between their findings and those previously reported by the Kluck lab for a similar mutant. However, as it stands the sentence, "However, different localization observed for different BakTBax chimeras...", reads as though different BakTBax chimeras were tested in the current manuscript rather than differences observed compared with the previous study. To clarify, I would recommend rewording to, "However, the different localization observed for similar BakTBax chimeras (Ferrer et al) could be caused by...."

Referee #2:

In this manuscript, the group of Frank Edlich compares the regulation of Bax and Bak by retrotranslocation from mitochondria to the cytosol and I do believe that it was really important to address this question.

In the revised version of the manuscript, the authors improved many points and notably increased the clarity for a broad audience by better explaining the protocols and the nature of the mutants that they used.

However, the results are very complicated and the way they are presented and discussed is still confusing. The two paragraphs "increased mitochondrial residence time induces Bax activation" and "membrane-integral Bax and Bak are shuttled dependent on the MA hydrophobicity" both mix data about hydrophobicity, shuttling and apoptosis. Maybe, it would be clearer if one paragraph would deal with hydrophobicity and shuttling, and another one with shuttling and apoptosis. As they are written for the moment, it is very difficult to follow the arguments and understand the link between them. The authors should also clearly state that "the mechanisms of Bax activation at low translocation rates remains to be elucidated".

2nd Revision - authors' response 02 September 2014

Referee #1:

We thank the reviewer for her/his time and are pleased that s/he finds our manuscript convincing. 1) We have analyzed the localization and apoptotic activity of Bak D83R using caspase 3/7 activity measurements and PARP cleavage. Figure 1 of this letter shows that Bak D83R is shifted to the HM fraction compared to wild-type Bak, similar to Bax D68R in comparison with wild-type Bax. Activity measurements show a decreased apoptotic activity of Bak D83R compared to the wild-type but also show commitment to apoptosis in response to Bak D83R activity (Figure 1 of this letter).
Figure 1. A) Staurosporine (STS, 1 μM)-induced caspase 3/7 activity of Bax/Bak DKO cells overexpressing Bak or Bak D83R. Data represent averages ± SEM. pcDNA 3.1-transfected cells serve as control. n = 5. p-values according to One Way ANOVA. B) Western blot analysis of PARP cleavage of representative samples depicted in A. Similar sample loading is controlled by actin. n = 5. C) Western blot analysis of Bak and Bak D83R localization in cytosol (C) and heavy membrane fraction (HM) of HCT116 Bax/Bak DKO cells. GAPDH and Tom20 serve as fractionation controls.

2) We agree with the reviewer and avoid the term “inactive” when referring to Bax that is not in the active conformation to prevent potential confusion of the reader. The sentence in the discussion now reads: “…active Bax is not retrotranslocated, as Bax activation blocks shuttling into the cytosol…”

3) We thank the referee for drawing our attention to this mistake. We now present the data in Figure E3C.

4) The materials and methods section contains the statement that all constructs were transfected with similar efficiency and we now also refer to Figure E2 in this section.

5) The reviewer raises an important question. We have addressed this problem in a previous publication by determining the Bax translocation rate with a series of bleachings (in Figure 4G, H of Edlich et al., 2011). The results show that Bax translocation is not influenced by ectopic Bcl-xL expression. Thus, Bcl-xL-mediated differences in Bax shuttling are caused by accelerated retrotranslocation. Based on the small cytosolic Bak pool especially in the absence of ectopic Bcl-xL expression similar measurements of Bak translocation might prove impossible. However, the regulation of Bax and Bak by the same retrotranslocation process suggests that mitochondrial Bak association is also Bcl-xL-independent.

6) We followed the advice of the reviewer.

Referee #2:

We thank the reviewer for her/his advice and follow her/his suggestions.
Thank you for submitting the revised version of your manuscript to us. I am pleased with the introduced changes and am happy to accept your paper in principle for publication here.