THE EMBO JOURNAL

Dynamic re-configuration of pro-apoptotic BAK on membranes

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DOI: 10.15252/embj.2020107237

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Review Timeline:

Submission Date:	11th Nov 20
Editorial Decision:	4th Jan 21
Revision Received:	14th Jul 21
Editorial Decision:	1st Aug 21
Revision Received:	2nd Aug 21
Accepted:	6th Aug 21

Editor: Elisabetta Argenzio, Stefanie Boehm

Transaction Report:

1st Editorial Decision 4th Jan 2021

Dear Grant,

First of all, my best wishes for 2021. Thank you for submitting your manuscript entitled "Dynamic re-configuration of pro-apoptotic BAK on membranes" (EMBOJ-2020-107237) to The EMBO Journal. Please accept my apologies for the delay in getting back with our decision due to the recent holiday season. Your study has now been assessed by three reviewers, whose reports are enclosed below for your information.

As you can see, the referees find your work potentially interesting, but also raise several major issues that need to be addressed before they can support publication in The EMBO Journal.

Given the overall interest of your study, we have decided to invite you to submit a new version of the manuscript revised according to the referees' requests. I should add that it is The EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in the revised version. Please note that addressing all major and minor referees' points as well as strong support from the reviwers would be needed for publication here.

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content is published elsewhere.

I realize that addressing all the referees' criticisms will require time and additional efforts involving experiments that might be technically challenging. I would therefore understand if you were to choose not to undergo an extensive revision here and submit your manuscript elsewhere, in which case please inform us about your decision at your earliest convenience.

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 made available online. For more details on our Transparent Editorial Process, please visit our website:
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Before submitting your revised manuscript, deposit any primary datasets and computer code produced in this study in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Please remember to provide a reviewer password, in case such datasets are not yet public. The accession numbers and database names should be listed in a formal "Data Availability" section (placed after Materials & Method). Provide a "Data availability" section even if there are no primary datasets produced in the study.

Feel free to contact me if you have any questions about the submission of the revised manuscript to The EMBO Journal. I thank you again for the opportunity to consider this work for publication and look forward to your revision.

Best regards,

Elisabetta

Elisabetta Argenzio, PhD
Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Referee #1:

EMBOJ-2020-107237 manuscript "Dynamic re-configuration of pro-apoptotic BAK on membranes" by Sandow, Webb, Dewson and colleagues reveals conformational changes monitored by HDX MS as BAK transitions from a dormant protein to a pore-forming protein on liposomes. The authors recruit deltaN-BAK-deltaTM-His6 to liposomes containing a Ni-binding lipid and observe its deuteration over time in the absence of BID and after BID-treatment for 60 min, at which point the liposomes have been completely permeabilized according to dye dequenching assays. The data is consistent with the exposure of the N-terminal region of BAK from biochemical crosslinking, epitope accessibility, and proteolysis data suggesting that helix 1 containing the BH4 region is inhibitory to BAK-mediated poration. The authors identify two regions in helix 1 for mutagenesis to probe the conformational changes involving helix 1 dissociation and to destabilize contacts between this helix and the BH3 helix thereby spontaneously activating BAK. Additionally, the authors engineer a protease site in the loop connecting helices 1 and 2 and show that cleavage at this site enhances liposome and in vitro mitochondrial permeabilization. Moreover, using epitope capture using antibodies to the BH3 region, the authors have revealed the transient exposure of the BH3 at the level of the liposome, which has been postulated from studies using mitochondrial heavy membranes, supporting the role of the BH3 burial presumably within the putative BH3-in-groove α2-α5 core dimer. The study is straightforward to follow and well executed providing additional support and insights into the changes in BAK conformation during membrane permeabilization. This process is critically important in our mechanistic understanding of apoptosis initiation and may be exploited in targeting apoptosis.

The further clarify our understanding of this highly dynamic process in light of this study I have the following comments/suggestions:

1. BAK is a moving target and the current study seeks to follow some of its states using HDX MS. The authors have provided data in liposomes for BAK alone and after 60 min of incubation in the presence of cBID. At the later time point BH3 epitope exposure was no longer observed suggesting that the reaction mechanism was perhaps complete. One could argue that the most interesting BAK conformations occur during the transient state at 1 min, 5 min, and 15 min upon cBID activation in the liposome assays when there is hardly any dye release, partial release, and full release, respectively (Figure 1A). What does HDX MS teach us under such transient conditions? Or is it too low a resolution to distinguish between several BAK conformations? Can one detect the latch release from the core? The data suggests that helix 6 is not changing much although not all of it is "visible" based on peptide coverage. Related to Figure 1A, at 25 nM BAK there is significant dye release by BAK alone which can further hamper analysis. The authors should account for these possible pitfalls/issues in the text.

2. In Figure 1D it would be useful to show the corresponding liposome permeabilization profiles. What was the protein concentration? What would the oligomerization profiles look like if one added 4B5 antibody at 1 min, 5 min, and 15 min?

3. The authors color code the HDX MS data obtained in Figure 2A for full-length BAK undergoing transitions {plus minus} cBID over the BAK monomer (Figure 2B) and BH3-in-groove α2-α5 core dimer (Figure 2C). It would be useful to test the HDX MS on the hexameric α2-α5 core (trimer of dimers stabilizes by lipids) reported by Cowan et al NSMB 2020, which may provide a better comparative analysis for accessibility of the α2-α5 core dimer for deuteration.

4. Can the authors speculate how helix 1 may be inhibitory once unfolded, if this is what they believe happening through the thrombin proteolysis experiments which releaves the inhibition. The authors should consider a cartoon summarizing the changes in BAK conformation.

Minor comments

5. The Thrombin assay shown in Figure 5F is not convincing likely because the mitochondria have been manipulated for too long (how long was this assay ongoing? 3 hrs for the thrombing incubation + cBID up to 60 min?). The authors could consider doing the thrombin and cBID addition at the same time. As the N-terminus is revealed thrombin will cleave and release it and the overall effect will hopefully be more obvious. As is it looks like the reaction is completed at 10 min with no further cyt c release subsequently.

6. the statement "and inhibited BAK-mediated membrane permeabilization" in the abstract is a bit confusing in the context of the full sentence. The authors should break the latter clause into a separate train of thought.

7. Pg. 4 "Co-incubation with recombinant BCL-XL blocked BAK oligomerization on liposomes driven by cBID as assessed by BN-PAGE (Fig 1D)." should be Fig 1C

8. The heading of results section reads better "BAK adopts an inactive conformation on liposomes revealed by HDX-MS"?

9. Pg. 6 "Our HDX-MS identified a potential salt bridge interaction between residues E24 at the N-terminal end of □1 and R169 in □7 (Fig 3A)." is misleading. The crystal structures identified the salt bridge and the HDX-MS suggested that a change in conformation in this area occurs upon activation.

10. Pg. 13 "Dead cells were were stained with propidium iodide uptake and quantified by flow cytometry." Remove 1x were

11. Fig 1B, C, D, E, legend, what are BAK protein concentrations in these assays.

Referee #2:

The study by Sandow et al. reports the structural changes that BAK undergoes during activation and membrane insertion analyzed by hydrogen-deuterium exchange mass spectrometry in liposomes. The HDX-MS profile obtained is in good agreement with the known structure of inactive soluble BAK and with the known reorganizations of the protein in the membrane. The authors show that the BH4 domain was resistant to deuteration in the inactive protein, but exposed in the active conformation, which suggests a role of BH4 in restraining BAK activity supported in the study by the use of mutant versions of BAK. The high exchange of the N-terminus of BAK activated in the membrane indirectly suggested that this part of the protein becomes unstructured, which adds information to the already known detachment of the N-terminus during BAK activation. The authors argue that the disordered conformation of the N-terminus of active BAK is functionally relevant and that it acts by restricting protein activity. However, this is not really demonstrated in the study as the higher activity of the N-terminal truncated BAK that the authors observe is likely due to the elimination of the first step of activation, this is, the N-terminus exposure. Although the work is

technically well executed and logically organized, it does not provide a significant advance over the previous knowledge, to which the authors have so elegantly contributed.

Comments:
- The authors should present the liposome pore assay as % of leakage and include the reduction in BAK activity in presence of BCL-xL, in agreement with the oligomerization analysis.
- The authors should control that the use of pH 2.5 to quench the deuteration does not release BAK from the liposomes, as it is attached to them by His tag. This is perhaps unlikely for the activated protein, but may be happening for the inactive, non-inserted form.
- How do the authors interpret the lower than expected exchange for helices 6/7/8 in inactive BAK?
- As mentioned above, the use of an N-terminal truncated BAK does not really address the functional relevance of the disordered state for this region in the active protein conformation. It could easily be, in agreement with previous work by the authors, that removing the N-terminus improves BAK activation and thereby pore activity, by eliminating one step of the activation process.

Referee #3:

In this study, Sandow and colleagues investigate dynamic structural rearrangements of the pro-apoptotic BCL-2 family member BAK. As the authors state in their intro, exactly how BAK transitions from an inactive to active state is unclear, in large part due to the difficulty in examining this is a membrane (native) environment. Coupling an elegant in vitro membrane tethering approach with HDX/MS, the authors demonstrate that this largely can reproduce published structural analysis of inactive BAK (validating the system), they then proceed to demonstrate extensive structural rearrangements in BAK, notably defining an auto-inhibitory effect of the BH4 domain and an unexpected inhibitory effect of the flexible N terminal domain upon activation. The study is well performed, highly interesting and the data in my view largely support the authors conclusions. This study provides important, much needed insight into activation of pro-apoptotic BAK, and as the authors state repeatedly, beyond fundamental insight, such analysis is essential for rational development of inhibitors. I have a few comments that should be addressed, either textually or experimentally.

- figure 4, the authors claim an inhibitory effect of the alpha 1 domain based on point mutants V34C, S37C spontaneously activating cell death upon expression (4C). While I agree this is the case in the S37C mutant (comparing against the most appropriate delta C control), there doesn't appear to be any statistical difference between the V34C and delta Cys control in terms of spontaneous cell death - the authors should address this point through textual comment

- figure 4d, while there doesn't appear to be a difference in spontaneous cell killing discussed above, clear that the activated BAK antibody can detect more activated BAK comparing V34C, S37C vs delta Cys BAK, therefore there appears some disconnect between detection of activated BAK and cell killing potency, that should be commented on.

- the activated BAK antibody in flow (D) appears to detect to a degree non-actiavted BAK (after DOX treatment the staining intensity of BAK in both BAK wt and delta Cys is increased - however presumably these cells are not expressing active BAK, since they don't appreciably undergo death (C), this should be commented on.

- figure 5F, supports the hypothesis that the N terminus is inhibitory, however its important to include a similar control expt. using native BAK, to rule out (hopefully) any non-BAK sensitising effect of thombin treatment on the mitochondrial membrane.

We thank the reviewers for their helpful comments and suggestions, and we apologise for the delay in providing a revised manuscript due to repeated COVID restrictions and pausing of research activities. Where possible, we have attempted to address the issues raised with additional experiments as detailed below. In addition to addressing the reviewer comments, we have corrected an error in the original manuscript relating as recombinant mouse BAKC21-6H was used in Figs 1 and 2 and not mBAKΔNΔC-6H.

Referee #1:

EMBOJ-2020-107237 manuscript "Dynamic re-configuration of pro-apoptotic BAK on membranes" by Sandow, Webb, Dewson and colleagues reveals conformational changes monitored by HDX MS as BAK transitions from a dormant protein to a pore-forming protein on liposomes. The authors recruit deltaN-BAK-deltaTM-His6 to liposomes containing a Ni-binding lipid and observe its deuteration over time in the absence of BID and after BID-treatment for 60 min, at which point the liposomes have been completely permeabilized according to dye dequenching assays. The data is consistent with the exposure of the N-terminal region of BAK from biochemical crosslinking, epitope accessibility, and proteolysis data suggesting that helix 1 containing the BH4 region is inhibitory to BAK-mediated poration. The authors identify two regions in helix 1 for mutagenesis to probe the conformational changes involving helix 1 dissociation and to destabilize contacts between this helix and the BH3 helix thereby spontaneously activating BAK. Additionally, the authors engineer a protease site in the loop connecting helices 1 and 2 and show that cleavage at this site enhances liposome and in vitro mitochondrial permeabilization. Moreover, using epitope capture using antibodies to the BH3 region, the authors have revealed the transient exposure of the BH3 at the level of the liposome, which has been postulated from studies using mitochondrial heavy membranes, supporting the role of the BH3 burial presumably within the putative BH3-in-groove α2-α5 core dimer. The study is straightforward to follow and well executed providing additional support and insights into the changes in BAK conformation during membrane permeabilization. This process is critically important in our mechanistic understanding of apoptosis initiation and may be exploited in targeting apoptosis.
We thank the reviewer for their comments on our work and its importance to the field.

The further clarify our understanding of this highly dynamic process in light of this study I have the following comments/suggestions:

1. BAK is a moving target and the current study seeks to follow some of its states using HDX MS. The authors have provided data in liposomes for BAK alone and after 60 min of incubation in the presence of cBID. At the later time point BH3 epitope exposure was no longer observed suggesting that the reaction mechanism was perhaps complete. One could argue that the most interesting BAK conformations occur during the transient state at 1 min, 5 min, and 15 min upon cBID activation in the liposome assays when there is hardly any dye release, partial release, and full release, respectively (Figure 1A). What does HDX MS teach us under such transient conditions? Or is it too low a resolution to distinguish between several BAK conformations? Can one detect the latch release from the core? The data suggests that helix 6 is not changing much although not all of it is "visible" based on peptide coverage. Related to Figure 1A, at 25 nM BAK there is significant dye release by BAK alone which can further hamper analysis. The authors should account for these possible pitfalls/issues in the text.

We believe that our HDX data reveal new insights into the dynamic nature of BAK conformation change, importantly, as it occurs in a membrane environment. However, we agree with the reviewer that interrogating the very early structural transitions in BAK conformation would be of interest. To monitor these very early changes would require a completely different approach of continuous HDX-MS (as opposed to pulse HDX-MS used here) and, importantly, also a highly homogeneous response to the activating stimulus. We have amended the text to incorporate the reviewer's suggestions and to recognise the limitations of the pulse HDX-MS approach (page 7).

2. In Figure 1D it would be useful to show the corresponding liposome permeabilization profiles. What was the protein concentration? What would the oligomerization profiles look like if one added 4B5 antibody at 1 min, 5 min, and 15 min?

The purpose of these experiments was to define the conformation change of BAK on model liposomes compared with known activation events on mitochondria or in cells. We apologise for the omission of the protein concentrations used in these experiments, these are now detailed in the accompanying Figure legend. As suggested, we have now performed additional experiments to show the effect of blocking antibody and BCL-XL on liposome permeabilisation (new Fig 1B, C and D). These data support that the formation of higher order BAK complexes correlate with liposome permeabilisation.

Consistent with the detection of BAK oligomers on BN-PAGE, under these conditions of adding antibody at the outset prior to stimulation with cBID, co-incubation with the 4B5 antibody reduced, but did not completely block liposome permeabilisation. In contrast, 7D10 that had no effect on oligomer formation had no impact on membrane permeabilisation (new Fig 1D). We hypothesise that the incomplete inhibition of BAK oligomer formation and liposome permeabilisation by 4B5 is due to the relatively rapid kinetics of BAK activation and subsequent homodimerisation and that the local concentration of BAK tethered to the membrane exceeds the concentration of antibody in solution. Hence, the antibody cannot trap all activated BAK monomer prior to its engagement with another BAK molecule.

3. The authors color code the HDX MS data obtained in Figure 2A for full-length BAK undergoing transitions {plus minus} cBID over the BAK monomer (Figure 2B) and BH3-in-groove α2-α5 core dimer (Figure 2C). It would be useful to test the HDX MS on the hexameric α2-α5 core (trimer of dimers stabilizes by lipids) reported by Cowan et al NSMB 2020, which may provide a better comparative analysis for accessibility of the α2-α5 core dimer for deuteration.

This is an interesting proposal. We mapped our HDX-MS data to the BAK homodimer as evidence from various biochemical approaches supports the formation of the symmetric BH3:groove homodimeric form of BAK (and BAX) in cells and on mitochondria (PMID: 26702098, PMID: 20605789, PMID: 26271728, PMID: 18471982, PMID: 28182867). However, as suggested by the reviewer we have now also mapped our HDX-MS data onto the crystal structure of hexameric BAK stabilised by *E. coli* lipids (PDB:6UXM, new Figure EV3). Potentially consistent with their increased exchange, helices α2-α3 reside on the exterior of the hexameric form. In contrast helices α4-α5, that are orientated towards the centre of the hexameric structure where they interact with lipids, did not change significantly in their deuterium exchange profile compared with the inactive monomer. Given that helices α4-α5 do not exhibit greater exchange in the pre-activated form compared with activated BAK suggests that they have similar solvent exposure, and supports that α4-α5 do not line a pore in oligomeric BAK on a membrane, but rather are buried in the bilayer or protein interior. Overall, our HDX-MS profiling is consistent with the conclusions

of Cowan et al that the small surface area involved in the crystal contacts do not support biological relevance of the hexamer as an intermediate in a larger BAK pore-forming oligomer and also with reports that BAK forms disordered and heterogeneous complexes on mitochondria (PMID: 28182867, PMID: 26783362).

The crystal structure of the hexamer revealed phospholipids interacting with the N-terminal end of α5 that were found to stabilise dimer:dimer interactions. Whilst our HDX-MS profiling did not reveal a significant change in these α5 residues at endpoint (i.e pre and post full activation), whether a transient exposure of α5 residues followed by their reburial due to lipid interactions occurs during BAK activating transtions is unclear. To resolve this would require continuous HDX-MS profiling rather than pulse HDX-MS used in our study. We have now discussed this in the manuscript.

4. Can the authors speculate how helix 1 may be inhibitory once unfolded, if this is what they believe happening through the thrombin proteolysis experiments which releaves the inhibition. The authors should consider a cartoon summarizing the changes in BAK conformation.

The mechanism by which the unfolded and exposed α1 inhibits BAK pore forming ability is unclear, but potentially its flexible nature impairs dimer formation and higher order oligomerisation. We now speculate that this may be an explanation in the Discussion text.

Minor comments

5. The Thrombin assay shown in Figure 5F is not convincing likely because the mitochondria have been manipulated for too long (how long was this assay ongoing? 3 hrs for the thrombing incubation + cBID up to 60 min?). The authors could consider doing the thrombin and cBID addition at the same time. As the N-terminus is revealed thrombin will cleave and release it and the overall effect will hopefully be more obvious. As is it looks like the reaction is completed at 10 min with no further cyt c release subsequently.

We thank the reviewer for this suggestion. We adopted a sequential approach to cleave the inactive form of BAK prior to its activation with cBID as we found that thrombin took 3h to cleave sufficient BAK. And, as noted, cBID induced BAK activation and cytochrome c release occurred much more rapidly over 10 mins. Hence, co-incubation was not a feasible strategy. However, we agree with the reviewer that the lengthy incubation of mitochondria was not ideal. So, following the reviewer's suggestion we revisited a BAK variant with an extended thrombin cleavage site incorporating a GG flanking the cleavage site (as was used in the recombinant protein experiments in Fig 5C), with the aim of increasing thrombin cleavage efficiency. In order to utilise this variant in mitochondrial assays, we selected cells with low/intermediate expression to avoid potential activity issues of high expression of this variant. This mutant was readily expressed, was functional yet showed no constitutive activity (new Fig EV6C). In contrast with the original minimal thrombin site variant, the extended thrombin site variant was almost completely cleaved within 30 mins and cleavage was potentiated by cBID-induced activation. This further supported the rationale and feasibility of co-incubating with thrombin and cBID to test the effect of thrombin cleavage on BAK activity whilst limiting the timeframe of mitochondrial incubation. In these new co-incubation experiments, consistent with our previous data, we saw a modest but reproducible increase in the release of cytochrome c upon thrombin-treatment, importantly only when the BAKthrombin mutant was expressed and not wt Bak that lacked the thrombin site (new Figs 5E and EV6C). This supports that the thrombin cleavage effect was specific to cleaved BAK and supports that the unfolded N-terminus when exposed is not required for BAK pore formation and may potentially restrain it.

6. the statement "and inhibited BAK-mediated membrane permeabilization" in the abstract is a bit confusing in the context of the full sentence. The authors should break the latter clause into a separate train of thought.
As suggested, we have modified the text as follows, “Moreover, the entire BAK N-terminus preceding the BAK oligomerisation domains became disordered post-activation and remained disordered in the activated oligomer. Removal of the disordered N-terminus did not impair, but slightly potentiated, BAK-mediated membrane permeabilisation of liposomes and mitochondria.”

7. Pg. 4 "Co-incubation with recombinant BCL-XL blocked BAK oligomerization on liposomes driven by cBID as assessed by BN-PAGE (Fig 1D)." should be Fig 1C
Corrected.

8. The heading of results section reads better "BAK adopts an inactive conformation on liposomes revealed by HDX-MS"?
Altered as suggested.

9. Pg. 6 "Our HDX-MS identified a potential salt bridge interaction between residues E24 at the N-terminal end of α1 and R169 in α7 (Fig 3A)." is misleading. The crystal structures identified the salt bridge and the HDX-MS suggested that a change in conformation in this area occurs upon activation.
We agree with the reviewer and apologise for this confusion. We have modified the text accordingly to read, “Our HDX-MS identified a potential interaction between residues E24 at the N-terminal end of a1 and R169 in a7 (Fig 3A) consistent with a salt-bridge identified between these residues in a crystal structure of truncated BAK in solution”

10. Pg. 13 "Dead cells were were stained with propidium iodide uptake and quantified by flow cytometry." Remove 1x were
Corrected.

11. Fig 1B, C, D, E, legend, what are BAK protein concentrations in these assays.
We apologise for this omission. The concentration is now stated in the Figure legend.

Referee #2:

The study by Sandow et al. reports the structural changes that BAK undergoes during activation and membrane insertion analyzed by hydrogen-deuterium exchange mass spectrometry in liposomes. The HDX-MS profile obtained is in good agreement with the known structure of inactive soluble BAK and with the known reorganizations of the protein in the membrane. The authors show that the BH4 domain was resistant to deuteration in the inactive protein, but exposed in the active conformation, which suggests a role of BH4 in restraining BAK activity supported in the study by the use of mutant versions of BAK. The high exchange of the N-terminus of BAK activated in the membrane indirectly suggested that this part of the protein becomes unstructured, which adds information to the already known detachment of the N-terminus during BAK activation. The authors argue that the disordered conformation of the N-terminus of active BAK is functionally relevant and that it acts by

restricting protein activity. However, this is not really demonstrated in the study as the higher activity of the N-terminal truncated BAK that the authors observe is likely due to the elimination of the first step of activation, this is, the N-terminus exposure. Although the work is technically well executed and logically organized, it does not provide a significant advance over the previous knowledge, to which the authors have so elegantly contributed.

We appreciate the reviewer's consideration of our data and manuscript. We believe that these are interesting and novel findings that add to our understanding of BAK killing activity in a membrane environment.

Comments:

- The authors should present the liposome pore assay as % of leakage and include the reduction in BAK activity in presence of BCL-xL, in agreement with the oligomerization analysis.

We thank the reviewer for this suggestion and we now show the liposome permeabilisation under similar conditions as new Figs 1B-D and present this new data as % permeabilisation of a detergent-lysed maximum. These data indicate that liposome permeabilisation correlates with the formation of BAK oligomers as detected by BN-PAGE. Together, these data indicate that blockade of BAK oligomer formation with BCL-XL (or to a lesser extent 4B5 antibody) confirmed the critical role of the BH3 domain consistent with previously published data on isolated mitochondria and in cells thereby supporting the relevance of BAK structural transitions on liposomes. In the context of BCL-XL inhibition, as stated in the text, BCL-XL can block cBID-induced permeabilisation by either sequestering cBID (Mode 1) or activated BAK (Mode 2), whereas BCL-X_L predominantly blocks cBID M97A by Mode 2 and so is informative from the perspective of resolving BAK transitions on liposomes. Interestingly, we found that whilst cBID M97A was able to induce greater BAK-mediated liposome permeabilization than cBID WT, its affect was likewise inhibited by BCL-X_L (new Fig 1D).

- The authors should control that the use of pH 2.5 to quench the deuteration does not release BAK from the liposomes, as it is attached to them by His tag. This is perhaps unlikely for the activated protein, but may be happening for the inactive, non-inserted form.

Quenching the reaction at pH2.5 is an important and necessary step in all HDX-MS protocols as it limits further exchange and also back-exchange of hydrogen/deuterium. Hence, quenching at pH2.5 will not alter the HDX-MS profile of BAK resident in the liposome membrane.

- How do the authors interpret the lower than expected exchange for helices 6/7/8 in inactive BAK?

This is an interesting question. Time-dependent exchange occurred in inactive BAK at α6/7/8 suggesting that it is solvent exposed in contrast to the buried α5 helix where exchange was not detected consistent with its burial in the core of the BAK protein. However, as noted, the extent of exchange was somewhat limited compared with other exposed helical elements such as α2 and α3. This may tentatively suggest that in inactive BAK the α6/7/8 residues are orientated toward a protein interface with known interactors such as VDAC2 or interface with lipids thereby constraining deuterium exchange. Hence, although speculative at this stage, we have modified the text to postulate this as follows, "Additionally, residues in the α6/7/8 were relatively resistant to exchange despite their predicted solvent exposure in the structure of soluble BAK (Fig 1E,F) (Moldoveanu *et al.*, 2006), possibly suggesting that these residues may interface with interacting proteins such as VDAC2 (Cheng et al., 2003) or lipids of the mitochondrial outer membrane."

- As mentioned above, the use of an N-terminal truncated BAK does not really address the functional relevance of the disordered state for this region in the active protein conformation. It could easily be, in agreement with previous work by the authors, that removing the N-terminus improves BAK activation and thereby pore activity, by eliminating one step of the activation process.

We thank the reviewer for this interesting point. As the cleavage occurs in the flexible α1- α2 loop, we hypothesised that the N-terminus would not dissociate from cleaved BAK in its inactive form, but that it would remain associated with the BAK core due to intramolecular interactions including those of the BH4 domain. Hence, as N-terminal exposure must occur for BAK to adopt its membrane-permeabilising conformation, the steps in BAK activation are retained and unaffected by the cleavage and so the thrombin cleaved mutant would be expected to still undergo the same structural transitions as uncleaved BAK. We now confirm this using combined cleavage and disulphide-linkage to show that following 30 min of thrombin cleavage of the inactive form an intramolecular disulphide-link could still form between Cys14 and Cys166 that is diagnostic of the inactive conformer of BAK (Cheng et al., 2003) (new Fig 5D), indicating that the N and C-termini remain associated post-loop cleavage (new Fig 5D). In addition, we also show that cleavage of the BAK α1/2 loop is significantly potentiated by activation by cBID consistent with the induced exposure and misfolding of the N-terminus upon BAK activation. As cleavage is potentiated by BAK activation, and in light of the suggestion of reviewer 1, we adopted a new strategy to co-incubate isolated mitochondria with thrombin and cBID. This approach was not previously possible as cleavage with thrombin was too slow, and so we tested an optimised BAKthrombin variant with a modified thrombin cleavage site that significantly enhanced the efficiency of thrombin cleavage. This variant could be efficiently and specifically cleaved by thrombin within 30 mins. Whilst cleavage alone did not significantly induce cytochrome *c* release from isolated mitochondria, the cleaved variant was able to mediate cytochrome c release induced by cBID (new Fig 5E). In these experiments, thrombin cleavage occurs either concurrently or post activation with cBID, suggesting that the potentiating effect on cytochrome c release by loop cleavage was not due to bypassing an early step in BAK activating conformation change. However, we agree with the reviewer that loop cleavage may enhance the kinetics of conformation change and we now also discuss this interpretation.

Our previous work on truncation of the BAK N-terminus (Dewson et al 2009) indicated that deletion to the start of α1 was tolerated but did not have a significant impact on BAK apoptotic activity, whilst truncations encroaching on the α1 resulted in lack of stable protein expression and so were not informative of the role of the α1 following its exposure during activation.

Referee #3:

In this study, Sandow and colleagues investigate dynamic structural rearrangements of the pro-apoptotic BCL-2 family member BAK. As the authors state in their intro, exactly how BAK transitions from an inactive to active state is unclear, in large part due to the difficulty in examining this is a membrane (native) environment. Coupling an elegant in vitro membrane tethering approach with HDX/MS, the authors demonstrate that this largely can reproduce published structural analysis of inactive BAK (validating the system), they then

proceed to demonstrate extensive structural rearrangements in BAK, notably defining an auto-inhibitory effect of the BH4 domain and an unexpected inhibitory effect of the flexible N terminal domain upon activation. The study is well performed, highly interesting and the data in my view largely support the authors conclusions. This study provides important, much needed insight into activation of pro-apoptotic BAK, and as the authors state repeatedly, beyond fundamental insight, such analysis is essential for rational development of inhibitors. I have a few comments that should be addressed, either textually or experimentally.
We thank the reviewer for their helpful suggestions and their positive comments on the study.

- figure 4, the authors claim an inhibitory effect of the alpha 1 domain based on point mutants V34C, S37C spontaneously activating cell death upon expression (4C). While I agree this is the case in the S37C mutant (comparing against the most appropriate delta C control), there doesn't appear to be any statistical difference between the V34C and delta Cys control in terms of spontaneous cell death - the authors should address this point through textual comment
Whilst the V34C variant did exhibit enhanced killing activity following induction, the reviewer is correct that the influence of the V34C mutation was less pronounced than the S37C mutation in these experiments. We speculate that lower total expression of V34C compared to S37C and the wild-type and Cys-null controls as assessed by Western blot (original Fig 4B) contributed to the lower cell death observed upon induced expression of V34C. To address this, we regenerated these cell lines from *Bax/Bak* DKO MEFs to normalise protein expression (new Fig 4B), and repeated the cell death and BAK activation assays (new Fig 4C and D). In repeated experiments with these new lines, consistent with previous findings, the V34C and S37C variants clearly adopted an activated conformation upon induction and the induced expression of both was able to promote significantly more cell death than either of the control lines in the absence of exogenous apoptotic stimulus (new Fig 4C). Whilst the death induced by V34C compared with controls when expressed at comparable levels was statistically significant, consistent with our previous data, the V34C variant did not induce as much cell death as the S37C variant.

- figure 4d, while there doesn't appear to be a difference in spontaneous cell killing discussed above, clear that the activated BAK antibody can detect more activated BAK comparing V34C, S37C vs delta Cys BAK, therefore there appears some disconnect between detection of activated BAK and cell killing potency, that should be commented on.
Please also see the response above. We repeated the BAK activation assays in our newly generated lines, and now present the new matching FACS data as collated from 3 experiments (new Fig 4D) and the individual FACS plots (new Fig EV4) to support the spontaneous activated conformation of both V34S and S37C consistent with their constitutive cell death activity (new Fig 4C-E).

- the activated BAK antibody in flow (D) appears to detect to a degree non-activated BAK (after DOX treatment the staining intensity of BAK in both BAK wt and delta Cys is increased - however presumably these cells are not expressing active BAK, since they don't appreciably undergo death (C), this should be commented on.
The reviewer is correct that there is low level fluorescence with the conformation-specific G3172 antibody following Dox-induced expression of wt BAK and BAK ΔCys even in the absence of an apoptotic stimulus. This immunodetection with G3172 is consistent with the low levels of activated human BAK that can be immunoprecipitated from untreated cells with this antibody (Alsop et al NComms 2017). Whilst it is possible that this low level of G3172 immunoreactivity is due to the antibody recognising a non-activated conformer of BAK to a

limited extent, we believe a more likely explanation is that following Dox-induced expression a subpopulation of BAK in these cells adopts an activated conformation which presumably in individual cells is insufficient to provoke significant cell death. Regardless, exposure of the G3172 epitope significantly increases following BAK activation and the BH4 domain mutants S37C and V34C more readily adopt this G3172 activated conformer than the wt and ΔCys controls in the absence of exogenous apoptotic stimuli.

- *figure 5F, supports the hypothesis that the N terminus is inhibitory, however its important to include a similar control expt. using native BAK, to rule out (hopefully) any non-BAK sensitising effect of thombin treatment on the mitochondrial membrane.*

We thank the reviewer for this recommendation. In line with the reviewer's comment, and also to address the concerns raised by reviewer #1 and #2, we have now modified these thrombin cleavage experiments to co-incubate with thrombin and cBID. We now also include mitochondria from cells expressing wild-type BAK and confirm that thrombin treatment of wild-type BAK does not alter its ability to mediate cytochrome *c* release, whereas the cleavage of $Bak^{thrombin}$ modestly, but reproducibly, potentiated cytochrome *c* release (new Fig 5E and EV6C).

1st Revision - Editorial Decision 1st Aug 2021

Dear Grant,

Thank you for submitting your revised work. The manuscript has been sent back to referee #2 and #3 and we have now obtained their reports, which are appended below for your information.

As you can see, the referees find that their criticisms have been adequately addressed and recommend the study for publication. However, there are some editorial issues concerning the text and the figures that I need you to address before we can officially accept your manuscript.

-> List authors' full names in the manuscript text.

-> Reduce to 5 the number of EV figures.

-> Make the mass spectrometry dataset publicly available.

-> Update the funding information in our electronic system.

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

-> Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

-> Papers published in The EMBO Journal include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst and 2-5 one-sentence bullet points provided by the authors and should summarise the paper. I would therefore ask you to include your suggestions for bullet points. Please note that the synopsis blurb and bullet points can be edited by the editors.

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

I thank you again for giving us the chance to consider your manuscript for publication in The EMBO Journal and look forward to your revision.

Kind regards,

Elisabetta

Elisabetta Argenzio, PhD
Editor
The EMBO Journal

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Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 30th Oct 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #2:

The authors have done an excellent job to address the reviewers' concerns. This is specially the case in their efforts to demonstrate the functional relevance of the unfolding of the N-terminus in BAK with respect to the opening, which was one of my main concerns. This matter is extremely complicated to prove, but the new data with the optimized thrombin cleavage site and the cross-

linking provide a strong support to the author's model.

Referee #3:

Authors have comprehensively addressed all the points that I raised

2nd Revision - Editorial Decision 6th Aug 2021

Dr. Grant Dewson
Walter and Eliza Hall Institute of Medical Research
Ubiquitin Signalling Division
Walter & Eliza Hall Inst. of Medical Research
Parkville, Victoria 3052
Australia

6th Aug 2021

Re: EMBOJ-2020-107237R1
Dynamic re-configuration of pro-apoptotic BAK on membranes

Dear Dr. Dewson,

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

Congratulations on your successful publication, and thank you again for this contribution to The EMBO Journal! Please continue to consider EMBO Journal for your work in the future.

Kind regards,

Stefanie Boehm

Stefanie Boehm
Editor
The EMBO Journal

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Grant Dewson
Journal Submitted to: EMBO Journal
Manuscript Number: 107237R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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5. For every figure, are statistical tests justified as appropriate?	Yes. Statistical analysis was only perfromed when n=>3 and all data points from independent expeirments are shown. As described in the Methods, statistical analysis was perfromed using an unpaired, Students t-test where appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
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Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers and source for all commercial antibodies used are provided in the Methods. All in-house generated antibodies have been published and characterised previously, previous reports are cited.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used in this study have been authenticated by STR profiling (https://www.garvan.org.au/research/capabilities/molecular-genetics/cell-line-identification). Cell lines were routinely screened and confirmed to be Mycoplasma negative using MycoAlert kit, Lonza, Basel.

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13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Mass spectromtery data has been deposited at ProteomeXchange Consortium via the PRIDE partner repository with identifier PXD027288. A data availability statement is included in the Methods.
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