

High-resolution analysis of the conformational transition of pro-apoptotic Bak at the lipid membrane

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DOI: [10.15252/embj.2020107159](https://doi.org/10.15252/embj.2020107159)

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Review Timeline:

Submission Date:	27th Oct 20
Editorial Decision:	4th Jan 21
Revision Received:	15th Jun 21
Editorial Decision:	28th Jul 21
Revision Received:	9th Aug 21
Accepted:	20th Aug 21

Editor: Stefanie Boehm/ Elisabetta Argenzio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript entitled "Pro-apoptotic Bak undergoes membrane-dependent unfolding to trigger pore formation" (EMBOJ-2020-107159) to The EMBO Journal. Please accept my sincerest apologies for the delay in getting back with our decision, also due to the recent holiday season. Three referees were assigned to your manuscript but one of them have not returned his/her comments even after repeated chasing. The two available reports are enclosed below for your information.

As you can see, the referees find your work potentially interesting, but also raise several major and minor issues that need to be fully 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 referees' points as well as strong support from the reviewers would be needed for publication here.

REFEREE REPORTS

Referee #1:

The work by Sperl et al. provides new data about the membrane-associated structure of BAK, a key protein effector of the intrinsic apoptotic pathway. The authors report the structure of BAKDC bound through a His tag to lipid nanodiscs and the TMH of BAK also inserted in lipid nanodiscs, both obtained by NMR with atomic resolution. The authors stated clearly in the abstract "Here, we

determined the structure of membrane-attached full-length Bak trapped in lipid nanodisc", which would be a breakthrough in the field of Bcl-2 proteins. However, they offer a computationally derived model connecting the NMR-solved structure of the TMH with the RDC-refined soluble domain of BAK. I think this statement is very misleading and inappropriate.

Based on extensive structural and biophysical characterization of the conformational changes that BAKDC undergoes in solution, as well as from complementary data in lipid nanodiscs, the authors conclude that the unfolding of helix 1 is key to trigger BAK activation in apoptosis. Overall, the results suggest that, upon binding a BH3 ligand, BAK, which is constitutively targeted to mitochondria, undergoes a profound rearrangement that is N-terminal dependent. Although the atomic resolution obtained by NMR provide valuable new information, this concept is not new, and as a consequence the conceptual advance provided by the study is limited.

Moreover, the authors conclude that BAK's TM is not required for its oligomerization. However, in my opinion they do not sufficiently prove this hypothesis and I have serious concerns about some of the experimental conditions. On the positive side, the structural differences with the N-terminus of Bcl-xL are interesting. Altogether, I would not consider it suitable for its publication in EMBO journal.

Major concerns.

1. There are several statements in the abstract and introduction that are inaccurate. They need to be better written to avoid misleading interpretations. Some examples:

A) "Despite a large set of data suggesting global conformational changes during pore formation, high-resolution structural details on this crucial step remain elusive. Here, we determined the structure of membrane-attached full-length Bak trapped in a lipid nanodisc and were able to monitor its structural transformation upon BH3-peptide-induced activation at the lipid bilayer surface at an unprecedented resolution. Once activated at the membrane, the N-terminal α -helix1 in BAK dissociates from the protein core and adopts a highly dynamic disordered conformation". In this work the authors used: BAKDC, the TM of BAK, FL BAK and BADC-targeted to the membrane by nickel lipids. Of note, many parts of the manuscripts lead to the misconception and englobe the conclusions to BAK FL, which could not be the case, as many other studies indicate. Indeed, BAK FL is only used for the determination of the inactive conformation of BAK at E coli membrane.

"The Bcl2 protein family consists of three subgroups, which interact with each other on the cytosolic side of the OMM"

The BCL2 family proteins is very heterogeneous as it is its interaction network, several studies reported that these proteins interact also in the cytosol, ER, nucleus as well as other mitochondrial compartments.

"All family members are either soluble or membrane-anchored proteins, mediated via a single transmembrane helix"

Only applies in the inactive conformation.

"Additionally, both pro and anti-apoptotic members bind so-called Bcl2-homology (BH)3-only proteins (Kuwana et al, 2005)."

The field classifies the 3 subgroups as anti-apoptotic, effectors and BH3-only proteins, with the last two being pro-apoptotic. Considering that the BH3 domain of the BH3 only proteins is the one inserted in the groove of both BAX-type and BCL2-type proteins I would say that are the former ones bind to the last ones. Czabotar P et al 2013 in Cell, Moldoveanu T et al 2013 etc

"Despite their structural homology, only pro-apoptotic Bcl2 proteins undergo distinct structural transformations upon the activation of pore formation by binding partners or a lipid bilayer membrane surface (O'Neill et al, 2016; Bleicken et al, 2017)."

There is an increasing amount of evidences suggesting that BCL2-type proteins can rearrange and promote rather than inhibit apoptosis. Hellmuth S et al 2020 in Nature, Cheng EH et al 1995 Science, Flores Romero H 2018 CDD etc. Also the work of David Andrews reports on the structural changes of anti-apoptotic BCL2 proteins.

"Concerning the state of the pore, it has long been discussed that both Bak and Bax most likely form lipidic rather than proteinaceous pores (Mandal et al, 2016; López et al, 2019; Uren et al, 2017a). A very recent study has confirmed this by showing that lipids are bound between dimeric Bak core domain interfaces (Cowan et al, 2020)"

The references are not adequate. The nature of BAX/BAK pore is definitely not confirmed in the study above mentioned. Cowan et al they provided important evidences about the role of lipids on BAX/BAK oligomerization, but not about pore nature. BAX/BAK proteolipidic was first proposed already in Basanez et al PNAS 1996; structural confirmation can be found in Salvador-Gallego et al. EMBO J 2016.

The authors finish the introduction with: "This study provides unprecedented structural insights into the initial steps of Bak activation where the lipid bilayer surface selectively triggers large conformational changes initiated by the dissociation and unfolding of its first α -helix."

The conformational changes that they observe are in solution mostly, in absence of a membrane, so even if they also observe them in the membrane, the lipid bilayer does not selectively trigger the changes. Indeed, the authors need to add BH3 peptides or have a very high membrane density of BAK for activation in the membrane.

In addition, the introduction does not include the large body of structural work based on EPR that has been done with full-length BAX and BAK also in real lipid membranes, and not just detergents as the authors claims. Specially the previous work by Oh and colleagues is neglected, as well as that of Kluck and coworkers, which is problematic because together they report many of the findings that are claimed by the authors in their manuscript. Of note, the studies by the Oh group use a version of BAK including helix 1 and missing only the first 16 amino acids.

There is also a mutant, full-length version of BAK reported by the Walensky group that can be handled in vitro and supposedly is better at mimicking BAK than the truncated protein at C-terminus. It does not autoactivate at concentrations typically used in liposome assays.

2. Concerns about BAK DC validation.

In figure 1. The membrane used in these assays is the one of *E. coli* supplemented with 10% of nickel lipids. This composition does not reconstitute mitochondrial outer membrane. According to authors it possesses, 67%PE, 23% PG and 10% of CL, and this amount of negative charge is particularly attractive to proteins like BAK, with cationic surfaces. Also 10% nickel lipids will drive binding of BAK with a very high density in the membrane, which is known to affect the auto-activation of these proteins. These issues could explain the i) high autoactivation of BAKDC, and ii) the moderated effect of cBID in BAK DC activation.

I strongly suggest to validate BAKDC with a MOM-like lipid composition and to include the effect of cBID in the membrane at the concentration tested, as well as to titrate down the concentration of Ni lipids. Moreover, I would suggest to explain with more detail the protein lipid ratios used in this experiment and to do dose-dependence analysis (with tBid and Bcl-xL too) to reach the current standards in the field.

Regarding CD experiments, a MOM-like lipid composition would be suitable to address the effect of the membrane on BAKDC.

3. The authors mutated several residues and two key regions on BAK to demonstrate the role of helix 1 of BAK but not of BCLXL on BAK pore forming activity. Importantly, the region 1 located at alpha1 in the latch domain, that reassembles the interaction found in BCLXL, had no significant effect on BAKDC pore forming activity. Differently, the mutation at region 2 including helix 1, the BH3 domain and the core domain has a more profound effect, that is further increases when both regions are combined.

Considering the position and the aminoacids substitutions, the authors could not exclude the possibility that the mutations at the region two are impairing BAK activity not due to a dissimilar arrangement of BAK helix1, but because the mutation is affecting to the BH3 into groove interaction with BH3 ligands and even between BAK monomers in order to form dimmers and undergo a pore formation, putting in to question their conclusions.

4. The authors claim in the conclusions of "These data confirm our assumption that the detachment and unfolding of helix 1 is the key step in the initial activation of Bak, which is absent in anti-apoptotic Bcl2 proteins." The last part of the sentence is not true according to Vasques-Montes V et al 2019 BBA. Regarding the first part of the sentence, the role of helix 1 dislodgement in the initial steps of BAK activation is already known. The new information provided here is that helix 1 unfolds in the process, but it is not shown here whether the unfolding itself is necessary for BAK activation or simply the dislodgement is sufficient, as already known.

5. Does the unfolded, active form of BAK in the membrane keep the secondary structure? Precisely the structural information about the membrane-interacting regions of BAK is not obtained from the experiments.

6. How many molecules of BAK are there per nanodisc? It is important to determine this aspect in order to interpret the structural data, including for example, protection to solvent.

Minor points

Figure 1a. The scale at the X-axes is not suitable to see differences between BAKDC in the presence absence of cBID.

Figure 1b, and S1c the authors label as % of pore opening to early stages of BAK activity. Another possibility is that BAKDC when interacts with the membrane, induces membrane perturbations that induce leakage but not a stable pore.

Figure 1c. Can the authors include cBID in BAXDC activation together with PUMA

Figure EV3 can the authors please include the molecular weight standards for the column used.

Figure.1 (a&b): Control of bid only is lacking (may be the difference in permeabilization upon bid addition is attributed to bid activity itself)

Referee #2:

EMBOJ-2020-107159 manuscript "Pro-apoptotic BAK undergoes membrane-dependent unfolding to trigger pore formation" by Sperl, Hang and colleagues presents biophysical data, including NMR, HDX-MS, and thermal and denaturant-based unfolding by CD and intrinsic tryptophan fluorescence,

for the pore-forming BCL-2 protein BAK in the presence and absence of bilayers in the forms of liposomes and nanodiscs. The authors adopt divide and conquer approach to piece together a model for the full-length BAK with its C-terminal transmembrane helix (TMH) embedded in a bilayer. To do this they: 1) determine the NMR structure of the TMH in detergent and a nanodisc, which shows an embedded helix spanning the bilayer resembling that of BCL-xL TMH; 2) using a previously reported construct, MEAS-BAK-LE-His6 (MEAS is a leader sequence important for expression), which contains the flexible N-terminal region (aa 1-22), they use RDCs correlative analysis to confirm that the structure in solution is similar to the originally reported crystal structure of BAK (PDB 2ims) while revealing that the N-terminus is indeed disordered; 3) using MD simulations they assemble a model of the full-length BAK at the membrane. The authors then look at the NMR-observed changes in MEAS-BAK-LE-His6 when it is recruited to nanodiscs or liposomes {plus minus} the BAK activator cBID and observe that the N-terminal helix alpha1 is partially unfolded in the absence of cBID and it becomes fully unfolded in its presence. Only the N-terminal part up to residue 66 is visible by NMR under these conditions. Denaturation analysis comparing BAK-deltaTM and BCL-xL-deltaTM is also presented suggesting an intermediate molten globule two step transition for the BAK consistent with similar observations for BAX, as well as single step transition for BCL-xL.

This area of research is very important in our understanding of apoptosis initiation. Some of the data presented are very interesting and merit publishing but the paper needs extensive and careful revisions for further consideration. The major criticism is that the authors do not yet appreciate effector regulation, as evidenced in their very preliminary functional analysis, yet they overinterpret their data largely ignoring a large body of published studies related to how BAK is activated. A major problem related to observations presented in this manuscript is that above a certain threshold effectors BAK and BAX spontaneously activate and permeabilize membranes. This threshold is usually above 25 nM-50 nM and the levels of BAK in cells is usually < 25 nM-50 nM. The authors have performed all of the functional liposome permeabilization assays at 600 nM MEAS-BAK-LE-His6 (i.e. >10x over physiological levels), which exhibits spontaneous dye release from liposomes. Additionally, the authors likely performed NMR analysis in nanodiscs and liposomes at >100 μ M MEAS-BAK-LE-His6 (the concentration was not reported throughout the manuscript), which again predisposes to BAK autoactivation. Yet it is widely accepted that in many normal cells BAK and BAX are in a dormant conformation (please read Llambi et al Molecular Cell 2011 44: 517-31, which explains the unified model of BCL-2 protein interactions at the mitochondria). For instance, in MEFs and mitochondria extracted from mouse liver, BAK is not in a complex with pro-survival factors, although in certain tumor cell lines it is found engaged in such complexes even in the absence of known BH3-only proteins (as the authors are well aware). The authors need to fully consider their observations and limitations of using high protein concentrations at membrane bilayer and interpret their data in light of the unified model for mitochondrial poration by BAK.

To help the authors improve the manuscript I have the following comments going through the text and figures:

1. The Abstract needs to be toned down. It appears that everything one would like to know about BAK has been now done in this manuscript.

"Here, we determined the structure of membrane-attached full-length Bak trapped in a lipid nanodisc and were able to monitor its structural transformation upon BH3-peptide-induced activation at the lipid bilayer surface at an unprecedented resolution."

The paper presents a divide-and-conquer model for membrane attached full-length BAK that uses

low resolution RDC to confirm a crystal structure, the NOE NMR-derived structure of the TMH, and MD simulations to link the two while embedding the TMH in a bilayer. Moreover, the authors say the BH3 activation is unnecessary in the discussion, yet they clearly state in the abstract that there is a transformation upon BH3-peptide induced activation. There is nothing wrong with being objective vs subjective. Thinking about the unified model could help iron out the data interpretation.

2. The introduction needs to be tightened up throughout.

For instance, they introduce subgroups of BCL-2 family but don't properly define them (Effectors, BH3-only proteins, prosurvival BCL-2 proteins). "All family members are either soluble or membrane anchored via a single transmembrane helix." We do not know enough about membrane anchoring for all the BCL-2 proteins. In particular, the BH3-only proteins may have atypical anchoring to membranes (see work by David Andrews lab). The authors wished to ignore direct activation of effectors as a critical step in activation in the intro, but this should be described. Cowan et al. 2020 study did not confirm lipidic pores, it merely showed how lipids bind the $\alpha 2$ - $\alpha 5$ core. The statement "whereas the more C-terminal helices stably insert into the lipid bilayer" is speculation and needs to be removed.

3. Results section 1 should be titled "At high doses pore formation by BAK etc..."

The authors need to titrate down BAK to levels (~50 nM) at which it does not spontaneously permeabilize liposomes, and use cBID to convince themselves that BAK activation by cBID lowers the threshold for membrane permeabilization. That data needs to be presented. The paper by Llambi I mentioned above shows how a titration with cBID changes the BAK conformation from dormant to loaded onto BCL-xL before eventually fully active correlating with membrane permeabilization.

4. Results section 2 presents the MD simulation for the full-length BAK at a lipid bilayer. However, this model does not take into account the disorder in the GNG sequence observed in the NMR structure of the TMH. That flexibility may allow BAK to adopt multiple dynamic conformations at the membrane and not be stuck in one low energy MDS minima. The authors need to make this distinction in the text and final model.

The HDX data needs to be combined and presented in a separate figure (rather than Fig. 2a inset and Figure 5) showing the different states side by side. Also, there are different blue-to-red color shading ranges in the different structure cartoons. Should they be standardized to the same scale throughout. Difference in deuterium uptake bar graphs as shown in Figure 5a may be very useful to have throughout for easy comparisons.

The authors make the statement "We obtained complete backbone resonance assignments for Bak-TMH both in dodecylphosphocholine (DPC) micelles (Fig. EV2d) and DMPC/DMPG MSP1D1 Δ H5 nanodiscs" but the G184-189 assignments are not shown.

The authors can delete "Due to the high degree of sequence identity", as it is merely 40%.

The authors state "In order to establish a reasonable structural model" remove reasonable as one can argue that the globular domain is more flexible relative to the membrane than depicted in Figure 2e.

Related to the assay used to infer lack of oligomerization for the TMH in nanodiscs it seems a bit preliminary and not clearly described even though the assay was published previously. Do the authors see NOEs that would suggest intermolecular contacts? A related comment will also be

made in the related Figure below.

5. In results section 3 the authors state that "Furthermore, while BclxL Δ TM is produced as a monomer at high yields, the amount of protein is much lower for Bak Δ TM showing a much higher tendency to form larger oligomers, as assessed by size exclusion chromatography (Fig. EV3a)." MEAS-BAK-LE-His6 construct is a very well-behaved monomer when carefully purified from bacteria. The authors need to show the gels confirming that the oligomeric species are indeed BAK and describe the conditions used if they want to claim that BAK spontaneously oligomerizes. Also, it is very well known that BCL-xL high expression leads to spontaneous dimerization (possibly domain swapped) so the statement needs to be corrected and again gels need to be shown.

The authors should show the 2D-[1H,15N]-HSQC experiment with deuterated versus protonated Bak Δ TM and BCL-xL Δ TM. I'm not sure why there should be a correlation between the conformational changes or lack thereof, deuteration, and thermal stability.

6. In results section 4 authors present the most critical data in the manuscript. How many BAK monomers are present on each nanodisc? The authors need to perform Cu/Phe crosslinking that has the ability to show disulfide bond-mediated dimerization or intramolecular crosslinking. They claim that they have 4 Ni-binding lipids per nanodisc so there possible binding of up to 4 BAK proteins. The results should be included and discussed as it is important to know if the authors are studying a dimer or monomer or mixed species.

The authors state "the smaller and defined size of nanodiscs prevents premature membrane insertion of the soluble domain of Bak, a prerequisite for a specific investigation of the distinct structural states along the pore-forming trajectory by NMR". This is in contrast to the message that says that BAK spontaneously inserts, which is the message that the authors highlight as a main finding in the paper. They also believe that the active domain is actually inserted. So which is it? They go on to say "In nanodiscs, membrane insertion that leads to a decrease in thermal stability is most likely induced by heat during the melting process, in line with previous studies (Pagliari et al, 2005)."; and also "suggesting that even in this trapped and resting state, partial activation takes place (Fig. 4c)". The authors need to consider that the high protein levels are to blame for the spontaneous activation which does not happen below 50 nM (close to physiological levels).

This speculation should be saved for the discussion "In contrast, the remaining parts of Bak are most likely located in the membrane and therefore invisible due to line-broadening resulting from the slower tumbling time of the much larger nanodisc as well as possible sample inhomogeneities and intrinsic structural dynamics in the ms- μ s time scale."

The statement "The liposome assay data shown in Fig. 1a clearly show pore-forming activity of Bak Δ TM without the need for further activation." should also say "at high doses of protein".

There is no evidence supporting this statement "while the rest of the protein is partially or fully embedded" It could be that helices 4-7 are actually very stable without being embedded.

7. Results section 5 should be titled "Helix 1 inhibits BAK pore forming activity"

The functional assays should be done at lower BAK doses (50 nM) {plus minus} cBID. R156 has been mutated previously by Dewson group and others and it does not have an effect on BAK activity in cells or in mitochondria.

The triple substitutions are found in a critical region involved in the activation mechanism by BH3

ligands and are also participating in core α 2- α 5 dimerization. The triple substitution could affect both of these states with combined effect of lowering activity and therefore must be carefully tested.

8. The discussion needs to be adjusted in light of the changes related to the lower concentration of BAK used in the functional assays and the possibility of artifactual conformations induced spontaneously at high protein levels used in NMR

The authors state "we believe that our protein construct combined with a lipid blend that resembles the composition in mitochondria properly mimics the lipid-surface properties at the mitochondrial outer membrane." The authors used an E. coli lipid prep which is not exactly similar to the mitochondrial outer membrane used in typical studies with effectors. The E. coli lipid prep contains PG which is not typically used in the mito-like liposome preps.

The authors state "We could show that membrane interaction of the Bak soluble domain is mainly mediated by the latch region (α -helices 5-8), a structural element that is tightly interacting with α -helix 1 (Fig. 2a)." The latch is defined as helices 6-8.

The authors state "A more detailed 2DNMR-titration further shows that the regions most affected by the chemical denaturant were located in helix 1 and the BH3-domain of the soluble domain of Bak, pointing towards a role of this region in defining the pro-apoptotic activity of Bak as compared to BclxL." Without showing data on the BCL-xL titration with GuHCl. What if it binds to the same regions in xL yet the proteins unfold differently.

The authors need to mention that they speculate "the membrane incorporated conformation after activation" but there is no evidence in the study for this.

Also, they state "In addition, the latch region of Bak (α -helices 6-8) appear to be more solvent exposed in the active state. The remaining structural elements (α -helices 2-5) that most-likely are the key elements of a Bak pore (Cowan et al, 2020; Brouwer et al, 2014) and therefore need to be located inside the membrane, consequently show reduced HDX properties." Yet they showed the helices 4-7 are protected in active form by HDX so this suggests that not the entire latch is susceptible to HDX, which is in contrast with the models they show in Figure 5f.

The authors need to correct this statement "while the flexible N-terminal part before helix 2 was invisible in X-ray crystallographic studies". These parts were not present in the α 2- α 5 core dimer structure the authors refer to here.

Regarding HDX-MS data the authors need to mention the following possible pitfall: as BAK undergoes conformational changes the technique provides an ensemble-average view of BAK state (closed and opened monomer, possible dimer?) and therefore is quite low resolution.

9. in Materials and Methods the authors need to include the protein concentration used in almost every single subsection.

HDXMS - The authors mention that HDX data was collected at 6 different timepoints (0, 10, 60, 1800, 7200s) but they presented it for 10 s and 120 min. Was there anything interesting in between? I would guess the intermediate points are most interesting but possibly most heterogeneous.

NMR - What temperature and protein concentration was used to acquire the nanodisc targeted

MEAS-BAK-LE-His6 experiments?

Figures:

All figures need to mention the conditions used including protein concentration and temperature. It is difficult to interpret data when this information is missing in figures/figure legends, text, and materials and methods.

Figure 1a need to titrate down BAK to 50 nM

Figure 1b. What do the bar errors represent? How many times have these experiments been replicated?

Figure 1d. How many times have these experiments been performed? Figure 1d black trace is the same as Figure 3b bottom left trace and this needs to be stated.

Figure 2a inset HDX data should be presented in a separate figure along with other HDX data for easy visualization.

Figure 2e Modeling does not reflect the disorder of the GNG sequence which may allow access of the domain to the bilayer in different orientations.

Figure 3c What are the black and green colors representing?

Figure 3d X-axis needs additional tick marks

Figure 4a How many BAK monomers are found in each nanodisc? Cu/Phe oxidation after {plus minus} cBID treatment

Figure 5a b could be combined with other HDX data in EV5 and Figure 2a as this data must be clearly presented and interpreted.

Figure 5f part 1 should indicated possibly flexibility by GNG linker; part 2 shows a model where the latch falls off from the core, yet the authors have evidence that helix 6 does not change in deuterium uptake in the inactive and active BAK. This is inconsistent with the current model and needs to be depicted and discussed. Part 3 is known from the literature and should be stated.

Figure EV1 a and b should possibly be shown on the same x-axis scale.

Figure EV1c Pore opening should be replaced with normalized liposome permeabilization

Figure EV2h The authors should show traces for other TMH:MSP combinations.

Figure EV3a - BAK does not spontaneously oligomerize, the authors need to show the SDS-PAGE profiles for the BAK fractions and describe the conditions for the SEC analysis.

Figure EV5a, b, c data combine in a single figure along with other data in main figure

Figure EVb increase font and symbol size and y-axis scale to 3

First of all, we would like to thank both referees for their constructive comments that helped us very much to improve the quality of the manuscript in the light of current literature and existing models of Bcl2 protein pore formation. We also used this opportunity to stress the novel aspects of this work for communication to a broad readership.

Referee #1:

The work by Sperl et al. provides new data about the membrane-associated structure of BAK, a key protein effector of the intrinsic apoptotic pathway. The authors report the structure of BAKDC bound through a His tag to lipid nanodiscs and the TMH of BAK also inserted in lipid nanodiscs, both obtained by NMR with atomic resolution. The authors stated clearly in the abstract "Here, we determined the structure of membrane-attached full-length Bak trapped in lipid nanodisc", which would be a breakthrough in the field of Bcl-2 proteins. However, they offer a computationally derived model connecting the NMR-solved structure of the TMH with the RDC-refined soluble domain of BAK. I think this statement is very misleading and inappropriate. Based on extensive structural and biophysical characterization of the conformational changes that BAKDC undergoes in solution, as well as from complementary data in lipid nanodiscs, the authors conclude that the unfolding of helix 1 is key to trigger BAK activation in apoptosis. Overall, the results suggest that, upon binding a BH3 ligand, BAK, which is constitutively targeted to mitochondria, undergoes a profound rearrangement that is N-terminal dependent. Although the atomic resolution obtained by NMR provide valuable new information, this concept is not new, and as a consequence the conceptual advance provided by the study is limited. Moreover, the authors conclude that BAK's TM is not required for its oligomerization. However, in my opinion they do not sufficiently prove this hypothesis and I have serious concerns about some of the experimental conditions. On the positive side, the structural differences with the N-terminus of Bcl-xL are interesting. Altogether, I would not consider it suitable for its publication in EMBO journal.

A: Thanks to this referee for their honest and clear opinion on our work. Actually, we very much believe that the presented work provides a multitude of novel insights on the structural basis of Bak pore formation. We agree that the idea of a more exposed N-terminus in Bak has been shown before, as clearly stated in the manuscript. However, in this study we use high-resolution methods to probe this structural transformation, and provide protein folding data suggesting that there are distinct differences in the folding landscape of a pore forming *versus* survival Bcl2 protein. In addition, we present an elegant way to control the structural transition of Bak in a lipid environment by using lipid nanodiscs as a membrane mimetic. This strategy should be widely applicable for (structural-) studies of pore-forming Bcl2 proteins where tight conformational control is required. Furthermore, we believe that the structural characterization of the transmembrane helix of Bak in different membrane mimetics represents an important piece of novel information, which will be helpful for the design of structural models of a Bak pore in the future. Thus, we disagree with the overall conclusion of this referee and hope that, together with the large body of new data added to the revised manuscript, we now did a better job in presenting the novelty and impact of this work.

Major concerns.

1. There are several statements in the abstract and introduction that are inaccurate. They need to be better written to avoid misleading interpretations. Some examples:

"Despite a large set of data suggesting global conformational changes during pore formation, high-resolution structural details on this crucial step remain elusive. Here, we determined the structure of membrane-attached full-length Bak trapped in a lipid nanodisc and were able to monitor its structural transformation upon BH3-peptide-induced activation at the lipid bilayer surface at an unprecedented resolution. Once activated at the membrane, the N-terminal α -helix in BAK dissociates from the protein core and adopts a highly dynamic disordered conformation". In this work the authors used: BAKDC, the TM of BAK, FL BAK and BADC-targeted to the membrane by nickel lipids. Of note, many parts of the manuscripts lead to the misconception and englobe the conclusions to BAK FL, which could not be the case, as many other studies indicate. Indeed, BAK FL is only used for the determination of the inactive conformation of BAK at E coli membrane.

A: We are sorry for the confusion. The constructs used in this work are: Bak-TMH and Bak Δ TM (lacking the TMH) as well as BclxL Δ TM, cBid and the peptides of Bid- and Puma-BH3. What we meant with "full length" at this point was that we used the full-length N-terminus of the Bak Δ TM construct, which was not the case in earlier studies which used various N-terminally truncated versions. In the revised manuscript, we more clearly indicate what constructs have been used for each experiment. The abstract is now toned down making it clear that we did not work with the full-length Bak protein.

*"The Bcl2 protein family consists of three subgroups, which interact with each other on the cytosolic side of the OMM"
The BCL2 family proteins is very heterogeneous as it is its interaction network, several studies reported that these proteins interact also in the cytosol, ER, nucleus as well as other mitochondrial compartments.
"All family members are either soluble or membrane-anchored proteins, mediated via a single transmembrane helix"
Only applies in the inactive conformation.*

Thank you to this reviewer for these specific suggestions. We changed this part of the introduction accordingly for more accuracy. See lines 31-33.

*"Additionally, both pro and anti-apoptotic members bind so-called Bcl2-homology (BH)₃-only proteins (Kuwana et al, 2005)."
The field classifies the 3 subgroups as anti-apoptotic, effectors and BH3-only proteins, with the last two being pro-apoptotic. Considering that the BH3 domain of the BH3 only proteins is the one inserted in the groove of both BAX-type and BCL2-type proteins I would say that are the former ones bind to the last ones. Czabotar P et al 2013 in Cell, Moldoveanu T et al 2013 etc*

A: Thank you. This passage was modified according to the reviewer's suggestion. See lines 33-42.

*"Despite their structural homology, only pro-apoptotic Bcl2 proteins undergo distinct structural transformations upon the activation of pore formation by binding partners or a lipid bilayer membrane surface (O'Neill et al, 2016; Bleicken et al, 2017)."
There is an increasing amount of evidences suggesting that BCL2-type proteins can rearrange*

and promote rather than inhibit apoptosis. Hellmuth S et al 2020 in Nature, Cheng EH et al 1995 Science, Flores Romero H 2018 CDD etc. Also the work of David Andrews reports on the structural changes of anti-apoptotic BCL2 proteins.

A: Thank you for these helpful comments. We replaced “only” by “predominantly”. We are aware that also anti-apoptotic proteins have been found to become pro-apoptotic under certain circumstances. Since this is not the primary topic of this manuscript and referee #2 asked us to tighten up the introduction, we did not explain the structural changes reported for anti-apoptotic BCL2 proteins at this point. See lines 45-48.

"Concerning the state of the pore, it has long been discussed that both Bak and Bax most likely form lipidic rather than proteinaceous pores (Mandal et al, 2016; López et al, 2019; Uren et al, 2017a). A very recent study has confirmed this by showing that lipids are bound between dimeric Bak core domain interfaces (Cowan et al, 2020)"

The references are not adequate. The nature of BAX/BAK pore is definitely not confirmed in the study above mentioned. Cowan et al they provided important evidences about the role of lipids on BAX/BAK oligomerization, but not about pore nature. BAX/BAK proteolipidic was first proposed already in Basanez et al PNAS 1996; structural confirmation can be found in Salvador-Gallego et al. EMBO J 2016.

A: Thank you for pointing out that the references were not adequate at this point. We adjusted the citations accordingly and deleted the second sentence. See lines 58-60.

The authors finish the introduction with: "This study provides unprecedented structural insights into the initial steps of Bak activation where the lipid bilayer surface selectively triggers large conformational changes initiated by the dissociation and unfolding of its first α -helix."

The conformational changes that they observe are in solution mostly, in absence of a membrane, so even if they also observe them in the membrane, the lipid bilayer does not selectively trigger the changes. Indeed, the authors need to add BH3 peptides or have a very high membrane density of BAK for activation in the membrane.

A: In our study, the described conformational changes were obtained with Bak-bound to lipid nanodiscs or liposomes. Indeed, we observe spontaneous, BH3-independent pore formation of Bak only at relatively high protein concentrations in liposomes. This is not the case at low concentrations <100 nM as we now show in detail in Figures 1 + EV1. We fully understand that the wording was a bit misleading and thus re-wrote the final part of the introduction for clarification. See lines 63-88.

In addition, the introduction does not include the large body of structural work based on EPR that has been done with full-length BAX and BAK also in real lipid membranes, and not just detergents as the authors claims. Specially the previous work by Oh and colleagues is neglected, as well as that of Kluck and coworkers, which is problematic because together they report many of the findings that are claimed by the authors in their manuscript. Of note, the studies by the Oh group use a version of BAK including helix 1 and missing only the first 16 amino acids.

A: Thank you. The EPR work by Oh, Kluck and their colleagues is now included in the introduction. See lines 52-58. We agree that these studies report changes in the structure and accessibility of helix 1 upon activation. It was not our intention to ignore these previous results as our data are in very good agreement with these studies. Yet, our high-resolution NMR work, as well as the HDX-MS data provide a large body of additional insights that have not been reported before, by resolving the helix 1 unfolded state at high resolution as

well as by probing various structural and biophysical features of Bak. Furthermore, the herein probed dynamics reveal a more constrained patch in the unfolded helix 1 indicating lipid binding. This offers an explanation to how a partially unfolded structure can be stabilized on the lipid surface. See discussion lines 537-539.

There is also a mutant, full-length version of BAK reported by the Walensky group that can be handled in vitro and supposedly is better at mimicking BAK than the truncated protein at C-terminus. It does not autoactivate at concentrations typically used in liposome assays.

A: Thank you for pointing this out. We are aware of the mentioned previous study, where mutation of the Bak TMH resulted in a soluble and thus easier to handle full-length protein. It is interesting that they do not see autoactivation at concentrations of 500 nM, in contrast to our studies reported here using Ni-lipid-mediated membrane attachment. However, it remains unclear how the mutations, making the TMH less hydrophobic, affect the membrane interaction and consequently Bak membrane location. The less pronounced tendency in such a Bak variant for autoactivation might simply be caused by a higher population of the soluble, non-membrane-attached state, as observed with Bax, where activation by activator BH3 proteins is essential for membrane residence, a critical feature for activation. We show that membrane-binding is absolutely necessary for activation in our assays with and without Ni-lipids (Fig. EV1a). Additionally, our studies on the TMH imply that it simply serves as a membrane anchor of the soluble domain and thus we believe that the Ni-NTA-mediated membrane attachment of Bak is able to mimic the native situation sufficiently well while facilitating the handling of the soluble Bak protein in a properly folded form without the need for mutations or the usage of detergents. To account for the reviewer's concern, we added a short statement into the Discussion section (lines 576-584).

2. Concerns about BAK DC validation.

In figure 1. The membrane used in these assays is the one of E. coli supplemented with 10% of nickel lipids. This composition does not reconstitute mitochondrial outer membrane. According to authors it possesses, 67%PE, 23% PG and 10% of CL, and this amount of negative charge is particularly attractive to proteins like BAK, with cationic surfaces. Also 10% nickel lipids will drive binding of BAK with a very high density in the membrane, which is known to affect the auto-activation of these proteins. These issues could explain the i) high autoactivation of BAKDC, and ii) the moderated effect of cBID in BAK DC activation.

I strongly suggest to validate BAKDC with a MOM-like lipid composition and to include the effect of cBID in the membrane at the concentration tested, as well as to titrate down the concentration of Ni lipids. Moreover, I would suggest to explain with more detail the protein lipid ratios used in this experiment and to do dose-dependence analysis (with tBid and Bcl-xL too) to reach the current standards in the field.

A: Thanks to the reviewer for their specific and helpful comment. As suggested, the pore forming assay was repeated at low and high protein concentrations using a lipid composition representing mitochondrial outer membranes and the E. coli polar lipid blend used for our structural studies. In both lipid systems our constructs show the expected functionality with autoactivation taking place only at concentrations higher than 50 nM.

With these comparative experiments we could show that the *E. coli* polar lipids slightly facilitate autoactivation, presumably due to the higher amount of negatively charged lipids as mentioned by this reviewer. Furthermore, the Ni-lipids were titrated down, which also reduced autoactivation. Therefore, the assay at low protein concentrations was finally performed with 2% Ni-lipids. See Figures 1 and EV1 and the first result section, which was carefully re-written, for more details.

Regarding CD experiments, a MOM-like lipid composition would be suitable to address the effect of the membrane on BAKDC.

A: The CD experiments were now measured in MOM-like lipids and in *E.coli* polar lipids that have been used for the structural studies. See updated figures 1e,f and EV1e,f. The initially reported strong reduction of the secondary structure content of Bak upon activation turned out to be caused by problems in the determination of a reliable protein concentration in liposomes required for normalization of the CD spectra. We now repeated these experiments several times and can report with high confidence that the secondary structure content of Bak in the inactive *versus* active form does not markedly change. In addition, consistent with the tendency of Bak to spontaneously insert into the membrane, we do not see an additional change in secondary structure in presence of an activator BH3 peptide.

3. The authors mutated several residues and two key regions on BAK to demonstrate the role of helix 1 of BAK but not of BCLXL on BAK pore forming activity. Importantly, the region 1 located at alpha1 in the latch domain, that reassembles the interaction found in BCLXL, had no significant effect on BAKDC pore forming activity. Differently, the mutation at region 2 including helix 1, the BH3 domain and the core domain has a more profound effect, that is further increases when both regions are combined.

Considering the position and the aminoacids substitutions, the authors could not exclude the possibility that the mutations at the region two are impairing BAK activity not due to a dissimilar arrangement of BAK helix1, but because the mutation is affecting to the BH3 into groove interaction with BH3 ligands and even between BAK monomers in order to form dimmers and undergo a pore formation, putting in to question their conclusions.

A: We agree with the referee that mutations in Bak, especially at the functionally highly diverse BH3 region, might have a multitude of effects. However, looking at the available structures of Bak in complex with a BH3 ligand as well as the core dimer, we are very confident that the two mutations in region two do not disturb binding to BH3 ligands nor Bak homo-dimerization. This is further corroborated by our new data at 50 nM Bak concentrations in Fig. 6c,d, where pore formation of the two variants still can be activated by cBid and inhibited by BclxL. However, since the structure of a Bak pore is not known, we cannot exclude other effects. Thus, we now clearly state in the Discussion (lines 597 -602) that these initial mutagenesis experiments merely suggest that the helix 1-core interaction is important for Bak pore formation (as also probed by cystein-tethering experiments by others cited in the manuscript). A more detailed mutagenesis screening can definitely be done in future studies but is in our opinion beyond the scope of the present work.

4. The authors claim in the conclusions of "These data confirm our assumption that the detachment and unfolding of helix 1 is the key step in the initial activation of Bak, which is absent in anti-apoptotic Bcl2 proteins." The last part of the sentence is not true according to Vasques-Montes V et al 2019 BBA. Regarding the first part of the sentence, the role of helix 1 dislodgement in the initial steps of BAK activation is already known. The new information provided here is that helix 1 unfolds in the process, but it is not shown here whether the unfolding itself is necessary for BAK activation or simply the dislodgement is sufficient, as already known.

A: We removed the last part of the sentence since we do not cover the conformational changes in anti-apoptotic Bcl2 proteins. Regarding the first part of the sentence, we did not intend to ignore previous results on this topic and have carefully cited this work. However, we strongly believe that our data enable a better picture of the structural transformation of the N-terminal region occurring during pore formation. Our data clearly show that helix 1 dislodgement is coupled to its unfolding. In addition, this unfolded state is able to transiently interact with the lipid bilayer surface (Fig. 4d), presumably further stabilizing the activated structural state and preventing re-association of helix 1. Furthermore, our protein folding studies show that only Bak but not BclxL unfolds in a two-step fashion involving a molten globular intermediate state (Fig. 3c). Using NMR, we show that helix 1 in Bak is the most labile part of the protein (Fig. 3d), consistent with the fact that it needs to undergo a large structural transformation upon activation. Thus, we believe that our study does provide novel insights on various levels. The novel aspects of our study are now presented in more detail in the revised Discussion section.

5. Does the unfolded, active form of BAK in the membrane keep the secondary structure? Precisely the structural information about the membrane-interacting regions of BAK is not obtained from the experiments.

A: Structural information of the membrane-incorporated part of Bak could not be obtained by NMR due to disappearance of the corresponding signals. Using HDX-MS we could obtain information on solvent protection and existence of secondary structure. Additionally, our CD-data (Fig. 1e) show that the overall content of secondary structure does not significantly decrease. Taking these combined findings as well as previous structural insights into account, we can conclude that most membrane-incorporated parts of Bak still adopt an α -helical conformation. However, a high resolution picture of the structural state of a Bak (or Bax) pore remains to be determined and is certainly a valid and attractive goal for future studies.

6. How many molecules of BAK are there per nanodisc? It is important to determine this aspect in order to interpret the structural data, including for example, protection to solvent.

A: As referee #2 suggested, we now performed crosslinking experiments on activated Bak in lipid nanodiscs data (Fig. EV 4b). These data clearly indicate that Bak^{ATM} bound to nanodiscs is mainly monomeric prior to activation, which is one main reason why autoactivation does not take place in nanodiscs at high Bak protein concentrations. A minor band representing a dimer is most likely responsible for the minor population of active species visible in the NMR spectra prior to activation. After the Bid-BH3 peptide is added, dimers, trimers and also higher molecular species can be detected in this assay. Using size exclusion chromatography, we now show that the herein used small lipid nanodiscs have the capability to disassemble once the inserted protein cargo becomes too large, as is the case during Bak oligomerization (Fig. EV 4c & results lines 342-349). Thus, the chosen nanodisc

system offers the benefit of stabilizing the pre-pore state of Bak and at the same time does not inhibit Bak di/oligomerization, which is required for the investigated structural transformation and pore formation. Thus, the NMR and HDX experiments monitor the structural state of the Bak oligomer, consistent with the model that oligomerization is a key step for pore formation.

Minor points

Figure 1a. The scale at the X-axis is not suitable to see differences between BAKDC in the presence absence of cBID.

A: Figure 1a is now part of the new Figure EV1c. For the sake of comparability, we decided not to change the x-axis scale since all kinetic assay data were plotted the same way.

Figure 1b, and S1c the authors label as % of pore opening to early stages of BAK activity. Another possibility is that BAKDC when interacts with the membrane, induces membrane perturbations that induce leakage but not a stable pore.

A: Thank you. The axis label was changed to “relative fluorescence” to be more exact about what we was detected in the measurement.

Figure 1c. Can the authors include cBID in BAKDC activation together with PUMA

A: We added the trace with cBid by subtracting the data of cBid +liposomes from Bak Δ TM + cBid + liposomes, which does not differ from the other secondary structure profiles. See new Fig. 1e.

Figure EV3 can the authors please include the molecular weight standards for the column used.

A: In the course of the paper revision, we removed this figure since the contained data is not essential for our studies.

Figure.1 (a&b): Control of bid only is lacking (may be the difference in permeabilization upon bid addition is attributed to bid activity itself)

A: Figure 1a now includes a cBid reference, which shows no membrane permeabilization, consistent with earlier studies, e.g. Kale et al & Andrews, Meth Enzym, 2014.

Referee #2:

EMBOJ-2020-107159 manuscript "Pro-apoptotic BAK undergoes membrane-dependent unfolding to trigger pore formation" by Sperl, Hang and colleagues presents biophysical data, including NMR, HDX-MS, and thermal and denaturant-based unfolding by CD and intrinsic tryptophan fluorescence, for the pore-forming BCL-2 protein BAK in the presence and absence of bilayers in the forms of liposomes and nanodiscs. The authors adopt divide and conquer

approach to piece together a model for the full-length BAK with its C-terminal transmembrane helix (TMH) embedded in a bilayer. To do this they: 1) determine the NMR structure of the TMH in detergent and a nanodisc, which shows an embedded helix spanning the bilayer resembling that of BCL-xL TMH; 2) using a previously reported construct, MEAS-BAK-LE-His6 (MEAS is a leader sequence important for expression), which contains the flexible N-terminal region (aa 1-22), they use RDCs correlative analysis to confirm that the structure in solution is similar to the originally reported crystal structure of BAK (PDB 2ims) while revealing that the N-terminus is indeed disordered; 3) using MD simulations they assemble a model of the full-length BAK at the membrane. The authors then look at the NMR-observed changes in MEAS-BAK-LE-His6 when it is recruited to nanodiscs or liposomes {plus minus} the BAK activator cBID and observe that the N-terminal helix $\alpha 1$ is partially unfolded in the absence of cBID and it becomes fully unfolded in its presence. Only the N-terminal part up to residue 66 is visible by NMR under these conditions. Denaturation analysis comparing BAK-deltaTM and BCL-xL-deltaTM is also presented suggesting an intermediate molten globule two step transition for the BAK consistent with similar observations for BAX, as well as single step transition for BCL-xL.

A: Thanks to this referee for their positive overall assessment of our work and the very constructive comments that helped us very much to improve the quality of our manuscript.

This area of research is very important in our understanding of apoptosis initiation. Some of the data presented are very interesting and merit publishing but the paper needs extensive and careful revisions for further consideration.

The major criticism is that the authors do not yet appreciate effector regulation, as evidenced in their very preliminary functional analysis, yet they overinterpret their data largely ignoring a large body of published studies related to how BAK is activated.

A: Thank you for this clear statement. We addressed this point in the revised version of our manuscript carefully which we will describe in more detail in the sections below.

A major problem related to observations presented in this manuscript is that above a certain threshold effectors BAK and BAX spontaneously activate and permeabilize membranes. This threshold is usually above 25 nM-50 nM and the levels of BAK in cells is usually < 25 nM-50 nM. The authors have performed all of the functional liposome permeabilization assays at 600 nM MEAS-BAK-LE-His6 (i.e. >10x over physiological levels), which exhibits spontaneous dye release from liposomes.

A: Thanks to this reviewer for this comment. We now provide assay data at the suggested much lower Bak concentration and could systematically evaluate the concentration-dependent autoactivation properties of Bak (Fig. 1 & EV1).

Additionally, the authors likely performed NMR analysis in nanodiscs and liposomes at >100 μ M MEAS-BAK-LE-His6 (the concentration was not reported throughout the manuscript), which again predisposes to BAK autoactivation. Yet it is widely accepted that in many normal cells BAK and BAX are in a dormant conformation (please read Llambi et al Molecular Cell 2011 44: 517-31, which explains the unified model of BCL-2 protein interactions at the mitochondria). For instance, in MEFs and mitochondria extracted from mouse liver, BAK is not in a complex with pro-survival factors, although in certain tumor cell lines it is found engaged in such complexes even in the absence of known BH3-only proteins (as the authors are well aware). The authors need to fully consider their observations and limitations of using high protein concentrations at

membrane bilayer and interpret their data in light of the unified model for mitochondrial poration by BAK.

A: We highly appreciate this helpful comment. We now added the protein concentrations used in NMR experiments to the methods part and provide new data on the concentration-, lipid- and membrane-attachment density-dependent autoactivation of Bak in great detail (Fig. 1c,d and first paragraph of the Results section). We can now conclude that Bak autoactivation is strongly concentration-dependent. Thus, the high protein concentrations used for NMR (~ 100 μ M) most likely drive the spontaneous conformational transformation at least in liposomes. In nanodiscs, the Bak molecules are separated in individual particles and thus protected from autoactivation. Therefore, the use of nanodiscs is very helpful to enable structural investigations of the pre-pore state in a native lipid environment. We agree with this reviewer that a more detailed presentation of the obtained observations and limitations, as well as a discussion in the light of existing models for pore formation is important for the reader. This is now included in the Discussion section (pages 15-20).

To help the authors improve the manuscript I have the following comments going through the text and figures:

A: We very much appreciate the great effort of this reviewer to help us improve the manuscript.

1. The Abstract needs to be toned down. It appears that everything one would like to know about BAK has been now done in this manuscript.

"Here, we determined the structure of membrane-attached full-length Bak trapped in a lipid nanodisc and were able to monitor its structural transformation upon BH3-peptide-induced activation at the lipid bilayer surface at an unprecedented resolution."

A: Fair point. The abstract was toned down by being more precise about the actual experimental work done. The indicated sentence was modified to be more precise about our results. See lines 19-22.

The paper presents a divide-and-conquer model for membrane attached full-length BAK that uses low resolution RDC to confirm a crystal structure, the NOE NMR-derived structure of the TMH, and MD simulations to link the two while embedding the TMH in a bilayer. Moreover, the authors say the BH3 activation is unnecessary in the discussion, yet they clearly state in the abstract that there is a transformation upon BH3-peptide induced activation. There is nothing wrong with being objective vs subjective. Thinking about the unified model could help iron out the data interpretation.

A: The mentioned discrepancy in the initial paper was actually caused by the observed inhibiting effect of lipid nanodiscs, used for structural investigations, where autoactivation did not take place. We agree that this appeared to be a bit confusing. However, since we now included all suggested experiments (at lower Bak concentration) in the manuscript, we come up with very much improved and refined dataset that enables us to draw conclusions that are in line with the current models of Bak pore formation, such as the unified model. This is now discussed in detail in the updated Discussion section.

2. The introduction needs to be tightened up throughout.

For instance, they introduce subgroups of BCL-2 family but don't properly define them (Effectors, BH3-only proteins, prosurvival BCL-2 proteins). "All family members are either soluble or membrane anchored via a single transmembrane helix." We do not know enough about membrane anchoring for all the BCL-2 proteins. In particular, the BH3-only proteins may have atypical anchoring to membranes (see work by David Andrews lab). The authors wished to ignore direct activation of effectors as a critical step in activation in the intro, but this should be described. Cowan et al. 2020 study did not confirm lipidic pores, it merely showed how lipids bind the α 2- α 5 core. The statement "whereas the more C-terminal helices stably insert into the lipid bilayer" is speculation and needs to be removed.

A: Thank you. As suggested by this referee, we removed/modified parts of the introduction that are not directly relevant for this study and now better describe the role of BH3-only proteins in the direct activation of the effectors.

3. Results section 1 should be titled "At high doses pore formation by BAK etc..."

The authors need to titrate down BAK to levels (~50 nM) at which it does not spontaneously permeabilize liposomes, and use cBID to convince themselves that BAK activation by cBID lowers the threshold for membrane permeabilization. That data needs to be presented. The paper by Llambi I mentioned above shows how a titration with cBID changes the BAK conformation from dormant to loaded onto BCL-xL before eventually fully active correlating with membrane permeabilization.

A: Thank you. The title was adjusted to highlight the concentration dependence. Our data at low concentrations now reflects the expected behavior. See Figures 1 & EV1

4. Results section 2 presents the MD simulation for the full-length BAK at a lipid bilayer.

However, this model does not take into account the disorder in the GNG sequence observed in the NMR structure of the TMH. That flexibility may allow BAK to adopt multiple dynamic conformations at the membrane and not be stuck in one low energy MDS minima. The authors need to make this distinction in the text and final model.

A: The figure was adapted to reflect the flexible nature of the region between the TMH and the soluble domain (Fig. 2e). Additionally, we added figure S2 showing the varying distances between the centers of mass of the soluble and TM domains during a 200 ns MD simulation, which represents the fluctuations between the two domains. See the explanation in lines 200-204 in the results section.

The HDX data needs to be combined and presented in a separate figure (rather than Fig. 2a inset and Figure 5) showing the different states side by side. Also, there are different blue-to-red color shading ranges in the different structure cartoons. Should they be standardized to the same scale throughout. Difference in deuterium uptake bar graphs as shown in Figure 5a may be very useful to have throughout for easy comparisons.

A: Thank you for this suggestion. We combined the HDX data in a separate figure (Fig. 5 and EV5). A bar graph showing the difference in deuterium uptake also for lipid surface binding was added (Fig. 5a). The y-axis in the bar graphs (Fig. 5a,d) were aligned for comparability of the effects making clear that the difference upon lipid binding is much smaller than upon activation. The color shading was not standardized because the effects

would not be clearly visible in such a representation, especially for the lipid binding effects. However, the ranges used for color-coding are indicated under each graph and also marked in the respective bar graphs for clarity.

The authors make the statement "We obtained complete backbone resonance assignments for Bak-TMH both in dodecylphosphocholine (DPC) micelles (Fig. EV2d) and DMPC/DMPG MSP1D1ΔH5 nanodiscs" but the G184-189 assignments are not shown.

A: Thank you for pointing out that this is unprecise. Actually, we meant that the TMH is completely assigned, but of course it makes more sense to refer to the entire construct used for the assignment, which also contains the unassigned linker. The respective sentence was modified accordingly. See lines 177-180.

The authors can delete "Due to the high degree of sequence identity", as it is merely 40%.

A: "Identity" was exchanged for "similarity" (line 187). As shown in the corresponding figure (Fig. EV2d) besides the 40% identity, the rest of the amino acids have similar physical properties. Polar/unpolar and basic amino acids are found in the same positions, as shown by the color coding. The legend was modified to highlight the similarity.

The authors state "In order to establish a reasonable structural model" remove reasonable as one can argue that the globular domain is more flexible relative to the membrane than depicted in Figure 2e.

A: We deleted "reasonable" and also made clearer that the globular domain is flexible relative to the membrane with an additional figure (Fig. S2), which depicts the various different distances between the COMs of the TMH and the soluble domains as the soluble domain moves outside the membrane. See line 191 and lines 200-204.

Related to the assay used to infer lack of oligomerization for the TMH in nanodiscs it seems a bit preliminary and not clearly described even though the assay was published previously. Do the authors see NOEs that would suggest intermolecular contacts? A related comment will also be made in the related Figure below.

A: Since the Bak-TMH sample was fully deuterated for NMR triple resonance and NOESY experiments, we could not observe any intermolecular contacts between protein side chains. However, all backbone amides showed strong NOEs to the surrounding detergent or lipid chains (Fig. 2c), suggesting that the TMH is not shielded at any side by inter-monomer contacts with a second TMH. We now describe the assay in more detail (lines 208-228) and additionally provide data on the dimeric TMH of Glycophorin A for comparison. It shows the expected tendency to assemble as a dimer even when it is added to the assembly at ratios which would favor a monomeric assembly. See also Fig. EV2e.

5. In results section 3 the authors state that "Furthermore, while BclxLΔTM is produced as a monomer at high yields, the amount of protein is much lower for BakΔTM showing a much higher tendency to form larger oligomers, as assessed by size exclusion chromatography (Fig. EV3a)." MEAS-BAK-LE-His6 construct is a very well-behaved monomer when carefully purified from bacteria. The authors need to show the gels confirming that the oligomeric species are indeed BAK and describe the conditions used if they want to claim that BAK spontaneously oligomerizes.

Also, it is very well known that BCL-xL high expression leads to spontaneous dimerization (possibly domain swapped) so the statement needs to be corrected and again gels need to be shown.

A: Thank you for pointing out this inaccuracy. As described by this referee, the first peak of Bak Δ TM is not an oligomeric species but an aggregate formed by sample impurities. We also observe a dimeric species by SEC if BclxL Δ TM is purified at high concentrations. We do not intend to claim that Bak spontaneously forms oligomers in solution and since the purification of Bak-solu for NMR has been described previously (Moldoveanu et al, 2006) we removed this figure from the manuscript.

The authors should show the 2D-[1H,15N]-HSQC experiment with deuterated versus protonated Bak Δ TM and BCL-xLdeltaTM. I'm not sure why there should be a correlation between the conformational changes or lack thereof, deuteration, and thermal stability.

A: We now added the spectra of both Bak Δ TM and BclxL Δ TM +/- deuteration (Fig. EV3a) which are almost identical for BclxL Δ TM, but display pronounced differences in the signal intensity for Bak Δ TM. This clearly observed solvent protection for Bak (helices 1, 2,5, 6 & 8; Fig. 3b) does not necessarily have to correlate with the ability to undergo structural changes but fits very well to the higher thermal stability of Bak versus BclxL, as described in the manuscript, and highlights the solvent protected nature of the central helix 5 in Bak, as described in lines 248-256.

6. In results section 4 authors present the most critical data in the manuscript. How many BAK monomers are present on each nanodisc? The authors need to perform Cu/Phe crosslinking that has the ability to show disulfide bond-mediated dimerization or intramolecular crosslinking. They claim that they have 4 Ni-binding lipids per nanodisc so there possible binding of up to 4 BAK proteins. The results should be included and discussed as it is important to know if the authors are studying a dimer or monomer or mixed species.

A: Thank you for this very helpful comment. We performed Cu/Phe crosslinking experiments, as suggested by this reviewer. In these experiments (Fig. EV4b), we could clearly show that Bak is mainly present as a monomer in the nanodisc attached form prior to activation, even though 4 Ni-NTA-lipids are present in each nanodisc. However, once activated by the addition of Bid, Bak oligomers were detected in this assay. Bak activation is accompanied by nanodisc opening due to the larger size of the oligomers. Thus, the nanodisc system is facilitating the stabilization of a stable monomeric Bak preparation but still allows for Bak oligomerization once activated by Bid. Therefore, the use of lipid nanodiscs is very convenient to study the conformational species that occur at each step, even at the high protein concentrations that are used for NMR and other structural techniques. See lines 342-349 in the Results section and lines 496-501 in the Discussion section.

The authors state "the smaller and defined size of nanodiscs prevents premature membrane insertion of the soluble domain of Bak, a prerequisite for a specific investigation of the distinct structural states along the pore-forming trajectory by NMR". This is in contrast to the message that says that BAK spontaneously inserts, which is the message that the authors highlight as a main finding in the paper. They also believe that the active domain is actually inserted. So which is it? They go on to say "In nanodiscs, membrane insertion that leads to a decrease in thermal

stability is most likely induced by heat during the melting process, in line with previous studies (Pagliari et al, 2005)."; and also "suggesting that even in this trapped and resting state, partial activation takes place (Fig. 4c)". The authors need to consider that the high protein levels are to blame for the spontaneous activation which does not happen below 50 nM (close to physiological levels).

A: Sorry for the somewhat confusing statements at different parts of the manuscript. The new liposome assays show that Bak Δ TM spontaneously inserts into the liposome membrane only at high concentrations as used in our NMR studies (Fig. EV1b). However, for the NMR studies, we use nanodiscs instead of liposomes (except for Fig. 4e, where again we see spontaneous activation even in smaller liposomes). The nanodiscs are smaller and, most importantly, constrained by the surrounding membrane scaffold protein (MSP). The NMR measurements show that the high concentration of Bak Δ TM alone is not enough to overcome the activation barrier in nanodiscs. However, upon exposure to high temperatures as present during thermal unfolding measurements, heat-induced activation is possible even in a nanodisc setting (Fig. EV4a). Likewise, the activator Bid-BH3 peptide also leads to selective Bak activation in nanodiscs. We modified the corresponding section in the results section (lines 328-335) and hope that this point is now clearly presented.

This speculation should be saved for the discussion "In contrast, the remaining parts of Bak are most likely located in the membrane and therefore invisible due to line-broadening resulting from the slower tumbling time of the much larger nanodisc as well as possible sample inhomogeneities and intrinsic structural dynamics in the ms- μ s time scale."

A: Thank you. This sentence was moved to the discussion and can now be found in lines 510-513.

The statement "The liposome assay data shown in Fig. 1a clearly show pore-forming activity of Bak Δ TM without the need for further activation." should also say "at high doses of protein".

A: This part of the manuscript is now heavily revised. See statements above.

There is no evidence supporting this statement "while the rest of the protein is partially or fully embedded" It could be that helices 4-7 are actually very stable without being embedded.

A: Thank you. This sentence was rewritten to "while the rest of the protein is partially or fully protected from the solvent." (line 410)

7. Results section 5 should be titled "Helix 1 inhibits BAK pore forming activity"

A: Thanks for the short and very accurate title suggestion. We changed it accordingly.

The functional assays should be done at lower BAK doses (50 nM) {plus minus} cBID.

R156 has been mutated previously by Dewson group and others and it does not have an effect on BAK activity in cells or in mitochondria.

The triple substitutions are found in a critical region involved in the activation mechanism by BH3 ligands and are also participating in core α 2- α 5 dimerization. The triple substitution could affect both of these states with combined effect of lowering activity and therefore must be carefully tested.

A: Thank you. The functional assay was now done at 50 nM +/- cBid and BclxL Δ TM to mimic physiological conditions. After revising our mutation sites, we removed the mutation D90N, as it was shown to form a salt bridge with R137 (Moldoveanu *et al*, 2006). By disrupting this salt bridge we also weaken the interaction between helices 3 and 5 with the Bak monomer, which might create unwanted effects.

As mentioned by this referee, R156 has been mutated previously without a reduction of activity (Li *et al*, 2017; Westphal *et al*, 2014; Dewson *et al*, 2009; Weber *et al*, 2013). There, a cysteine residue at this position was added while we here chose a glutamine residue, which could explain the differences seen in the functional assay. Nonetheless, this mutation alone only leads to a small reduction of the pore forming activity, in line with these previous studies. The triple, now double mutation in the BH3-binding/dimerization site is indeed a critical area (Brouwer *et al*, 2014). However, we are very confident that the two mutations in the BH3 region (aa 80 and 86) do not disturb the interaction with BH3 ligands or with BclxL nor Bak homo-dimerization, since these positions are not directly participating in the interactions as estimated from the existing NMR and crystal structures. This is further corroborated by our new data at a 50 nM Bak concentration shown in Fig. 6c,d, where pore formation of the two variants can still be activated by cBid and inhibited by BclxL. See updated results section "Helix 1 inhibits Bak pore forming activity", lines 419-453) and discussion section (590-602).

8. The discussion needs to be adjusted in light of the changes related to the lower concentration of BAK used in the functional assays and the possibility of artifactual conformations induced spontaneously at high protein levels used in NMR

A: The discussion was adjusted accordingly (lines 505-507). We think it is unlikely that high protein concentrations induce artefactual conformations in the conducted NMR experiments for various reasons: 1.) The pore forming assay data at increasing protein concentrations support the assumption that the structural transformation of Bak and its interaction pattern, e.g. with BclxL, is not fundamentally dependent on the protein concentration (Fig. 1 and Fig EV1). 2.) The NMR spectra of BH3-activated Bak in lipid nanodisc as well as in autoactivated Bak in liposomes are very similar, giving rise to the notion that the final structural state in both cases is alike (Fig. 4e), arguing for a conserved activation mechanism. 3.) The herein detected structural changes are in excellent agreement with previous studies obtained at lower resolution and lower protein concentrations.

The authors state "we believe that our protein construct combined with a lipid blend that resembles the composition in mitochondria properly mimics the lipid-surface properties at the mitochondrial outer membrane." The authors used an E. coli lipid prep which is not exactly similar to the mitochondrial outer membrane used in typical studies with effectors. The E. coli lipid prep contains PG which is not typically used in the mito-like liposome preps.

A: Both the liposome assay and the CD measurements were repeated with a MOM like lipid blend (Kale *et al*, 2014) and compared to the E.coli lipid extract which we used for our structural studies in nanodiscs. In these experiments we can now confirm/conclude that the negative charge density of the lipids favors pore formation, while at 50 nM Bak concentration, both lipids behave very similar. See Figures 1 and EV1.

The authors state "We could show that membrane interaction of the Bak soluble domain is mainly

mediated by the latch region (α-helices 5-8), a structural element that is tightly interacting with α-helix 1 (Fig. 2a)." The latch is defined as helices 6-8.

A: Thanks. We corrected this typo.

The authors state "A more detailed 2DNMR-titration further shows that the regions most affected by the chemical denaturant were located in helix 1 and the BH3-domain of the soluble domain of Bak, pointing towards a role of this region in defining the pro-apoptotic activity of Bak as compared to BclxL." Without showing data on the BCL-xL titration with GuHCl. What if it binds to the same regions in xL yet the proteins unfold differently.

A: Thank you for this suggestion. We added the same bar graph for BclxLΔTM in Fig. 3d, which is characterized by an overall less pronounced effect of the chemical denaturant and, most importantly, no specific effect on helix 1. Additionally, we added HSQC spectra of BclxLΔTM with increasing concentrations of GuHCl in Fig. S3. This clearly visualizes the distinct unfolding behavior for the two Bcl-2 proteins. BakΔTM already displays an unfolded tertiary structure at the first unfolding step at ~ 2.5 M GuHCl (see Fig. EV3e), at which most secondary structure is still stable (see Fig. 3c, black trace), which, together with high fluorescence of an extrinsic fluorescence dye (Fig. EV3b), defines a molten globule state. In contrast, BclxLΔTM shows a two-state equilibrium between the folded and unfolded states, without indications for the occurrence of an intermediate state. Only when the final plateau is reached (see Fig. 3c, around 5-5.5 M GuHCl) a completely unfolded spectrum is seen with NMR (Fig. S3). See also Results section (lines 269-305).

The authors need to mention that they speculate "the membrane incorporated conformation after activation" but there is no evidence in the study for this.

A: This sentence was removed. In lines 510-513 of the discussion, we write "The remaining parts of Bak are probably located in the membrane...", clarifying that this is an interpretation.

Also, they state "In addition, the latch region of Bak (α-helices 6-8) appear to be more solvent exposed in the active state. The remaining structural elements (α-helices 2-5) that most-likely are the key elements of a Bak pore (Cowan et al, 2020; Brouwer et al, 2014) and therefore need to be located inside the membrane, consequently show reduced HDX properties." Yet they showed the helices 4-7 are protected in active form by HDX so this suggests that not the entire latch is susceptible to HDX, which is in contrast with the models they show in Figure 5f.

A: Thank you for pointing out, that this is unclear. In the updated model in Fig. 6e we now show the inactive state, as well as the possible steps that lead to a Bak core dimer, involving the transient membrane interaction of the N-terminal tail. In this model, the structural state of the latch domain is not further visualized since we do not see this pore of Bak in our NMR studies and, as pointed out by this referee, cannot detect a large change in solvent accessibility by HDX-MS. However, we can detect differences between the core and latch domain, with the core domain becoming less accessible and the latch domain slightly more accessible after activation. See figure 5d and lines 412-417.

The authors need to correct this statement "while the flexible N-terminal part before helix 2 was

invisible in X-ray crystallographic studies". These parts were not present in the $\alpha 2$ - $\alpha 5$ core dimer structure the authors refer to here.

A: Thank you for pointing this inaccuracy out. We rewrote the entire discussion and didn't include this statement anymore.

Regarding HDX-MS data the authors need to mention the following possible pitfall: as BAK undergoes conformational changes the technique provides an ensemble-average view of BAK state (closed and opened monomer, possible dimer?) and therefore is quite low resolution.

A: A statement on this possible problem with HDX is now included in the Discussion section (lines 548-551).

9. in Materials and Methods the authors need to include the protein concentration used in almost every single subsection.

A: The concentrations were added to all parts of the methods section.

HDXMS - The authors mention that HDX data was collected at 6 different timepoints (0, 10, 60, 1800, 7200s) but they presented it for 10 s and 120 min. Was there anything interesting in between? I would guess the intermediate points are most interesting but possibly most heterogenous.

A: Actually, it was a gradual transition between the two endpoints shown in Fig. EV 5c and the heat maps now shown in Fig. S4.

NMR - What temperature and protein concentration was used to acquire the nanodisc targeted MEAS-BAK-LE-His6 experiments?

A: The experiments were conducted at 303 K and protein concentrations ranging from 50-150 μ M. This was also added to the methods section as mentioned above.

Figures:

All figures need to mention the conditions used including protein concentration and temperature. It is difficult to interpret data when this information is missing in figures/figure legends, text, and materials and methods.

A: The conditions were added to the methods part. Additionally, the concentrations were added to some figures, where this is essential (Fig.1 and Fig. EV1)

Figure 1a need to titrate down BAK to 50 nM

A: Figure 1a now shows the relationship Bak-cBid-BclxL at low concentrations (50 nM Bak), a titration up to 1 μ M Bak can be found in Figure 1c,d.

Figure 1b. What do the bar errors represent? How many times have these experiments been replicated?

A: All liposome permeabilization measurements were replicated three times. All kinetics show averaged kinetics of 3 independent measurements and bar graphs with error bars show the error of 3 measurements as now indicated in the methods and the figure legends.

Figure 1d. How many times have these experiments been performed? Figure 1d black trace is the same as Figure 3b bottom left trace and this needs to be stated.

A: Figures now 1f and EV1f show representative traces derived from at least 3 experiments. The melting temperatures were averaged and are now summarized in a separate table S1 (mean value and standard deviation).

Figure 2a inset HDX data should be presented in a separate figure along with other HDX data for easy visualization.

A: Thanks. As suggested by this referee, we now present all HDX data in the separate Figures 5 and EV5 for allowing a more straightforward comparison.

Figure 2e Modeling does not reflect the disorder of the GNG sequence which may allow access of the domain to the bilayer in different orientations.

A: We added an arrow indicating the movement to Figure 2e. Additionally we added Figure S2 which visualizes the movement taking place in the MD simulation between the centers of mass (COMs) of the TMH and the soluble domain.

Figure 3c What are the black and green colors representing?

A: Thank you for noticing our missing legend. We now added the explanation in the figure legend. Black shows the unfolding of the secondary structure monitored by Far-UV CD, while green shows the unfolding of the tertiary structure monitored by Trp-fluorescence.

Figure 3d X-axis needs additional tick marks

A: The additional tick marks were added. Now we indicate every 25 residues.

Figure 4a How many BAK monomers are found in each nanodisc? Cu/Phe oxidation after {plus minus} cBID treatment

A: Cu/Phe oxidation reveals that there are different higher oligomeric species (dimers, trimers and also some higher) after treatment with the Bid-BH3 peptide, while Bak is mainly monomeric in the inactive state bound to nanodiscs. See figure EV4b and updated results and discussion sections (lines 342-349 and lines 496-501).

Figure 5a b could be combined with other HDX data in EV5 and Figure 2a as this data must be clearly presented and interpreted.

A: Thank you for this excellent idea. We combined the data of 2a, 5a, 5b and added the bar graph also for 2a. This can all now be found in the revised Fig. 5. We left EV5, as Fig. 5 would have become too crowded otherwise.

Figure 5f part 1 should indicated possibly flexibility by GNG linker; part 2 shows a model where the latch falls off from the core, yet the authors have evidence that helix 6 does not change in deuterium uptake in the inactive and active BAK. This is inconsistent with the current model and needs to be depicted and discussed. Part 3 is known from the literature and should be stated.

A: Figure 6e: The flexibility is now described in the figure legend. Additionally, Fig. S2 visualizes the flexibility by displaying the varying distances of the COMs between the TMH and the soluble domain. The flexibility was not additionally depicted in the model for clarity. Actually, the HDX-data in Fig. 5d shows that helix 6 does become slightly more accessible upon activation. This is indeed consistent with the current models. This feature is now described in the discussion section (lines 548-549).

Figure EV1 a and b should possibly be shown on the same x-axis scale.

A: The x-axis scale is now identical for all liposome permeabilization kinetics. See figures 1, EV1.

Figure EV1c Pore opening should be replaced with normalized liposome permeabilization

A: We replaced “pore opening” by “relative fluorescence” to be more precise about what we are monitoring. To account for this reviewer’s comment, we used the suggested terminology in the corresponding figure legend.

Figure EV2h The authors should show traces for other TMH:MSP combinations.

A: Figure EV2e now shows the suggested SEC traces for all BakTMH:MSP combinations.

Figure EV3a - BAK does not spontaneously oligomerize, the authors need to show the SDS-PAGE profiles for the BAK fractions and describe the conditions for the SEC analysis.

A: Thank you. Our SDS-PAGE confirms that the first Bak peak is not an oligomer but impurities. Thus, we removed this figure since it does not provide additional insights.

Figure EV5a, b, c data combine in a single figure along with other data in main figure

A: Thanks for this suggestion. We tried to rearrange and combine the HDX data figures and did so with Figs. 2 and 5. However, the inclusion of the data in Fig. EV5a-c would have caused a very complex and dense Fig. 5, which would be rather confusing to the (non-specialist) reader. Thus, we decided to keep the HDX-MS data in Fig. EV5.

Figure EVb increase font and symbol size and y-axis scale to 3

A: Figure EV5b is now shown with increased font and symbol size and the y-axis consistently scaled to 3.

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- Dewson G, Kratina T, Czabotar P, Day CL, Adams JM & Kluck RM (2009) Bak Activation for Apoptosis Involves Oligomerization of Dimers via Their $\alpha 6$ Helices. *Mol Cell* 36: 696–703
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Thank you for submitting your revised manuscript, which has been assessed by the original referees. I have now discussed the reviewers' reports and your point-by-point rebuttal letter with the other members of the editorial team. In addition, I have asked referee #2 to cross-comment referee #1's report.

The outcome of these discussions is that your plan to address referee #1's points appears to be reasonable. Therefore, I would like to invite you to address the remaining requests as indicated in your point-by-point rebuttal letter. Please add all available new data to the manuscript: you may decide to place them in the main or in the EV figures, or in the Appendix file. Also, you should cite and discuss the recent results by Birkinshaw et al. 2021 Mol. Cell.

In addition, I need you to address few editorial issues concerning the text and the figures as follows.

REFEREE REPORTS

Referee #1:

The authors study the structure of BAK fragments in the membrane using nanodiscs. They show that helix 1 of BAK unfolds into a random coil upon dislodgement during activation. The

dislodgement and its functional relevance were already known, so this part is rather confirmatory. Besides unfolding of helix 1, the activation pathway goes through a molten globule intermediate of BAK, which is not found in BCL-xL. In addition, unfolded helix 1 interacts with the membrane upon dislodgement, which likely facilitates the conformational transition to the molten globule and pore-forming structures.

The authors have done an excellent work at implementing the extensive lists of suggestions by the reviewers and the manuscript has improved in quality and clarity. At this point the manuscript has now potential to provide interesting new insight into different aspects of the structural organization of BAK in membranes. My opinion of the manuscript is now positive, but there are important issues that the authors need to address in another round of revision with experiments.

Major concerns:

-One key finding in the manuscript would be the interaction of helix 1 with the membrane upon dislodgement. This could facilitate the transition into the active pore-forming conformation, thereby providing an explanation for the role of the membrane in promoting the activation of BAX and BAK. However, this key point of the manuscript is still preliminary and should be further developed. The authors argue that helix 1 interacts with the membrane based on the lower dynamics of this segment in the NMR data and the presence of positively charged residues. To strengthen this finding, the authors should provide direct evidence of interaction with the membrane - for example by testing the membrane binding properties of a peptide derived from helix 1, or by other means.

-And importantly, they should also establish that the interaction with the membrane promotes the conformational change or molten globule transition. For this, they could design mutants in this helix that do not interact with the membrane, perhaps by changing the positive amino acids, and see if this affects the conformational change. Alternatively, and perhaps simpler if it works, they could use lipid compositions in the nanodiscs with no net negative charges, so that the interaction of the helix with the membrane is reduced and see if this affects the conformational change.

-The new results with the BAK mutants at reasonable concentrations in Figure 6 show that there is no significant difference in membrane permeabilizing activity compared with the wild type protein. These results do not add to the manuscript and only question the conclusions of the authors. Since the role of helix 1 dislodgement is in any case already well established, I would suggest to remove them and to add instead experiments with mutants that provide a functional role for the unfolding of helix 1 (not the dislodgement) or with the experiments proposed in the point above, aimed at demonstrating the role of the interaction of helix 1 with the membrane on the conformational transitions during activation.

-Calorimetry of BAK- Δ TM in solution shows aggregation of the protein with increased temperature, which limits the further analysis of the structural changes above that temperature. The authors should also confirm with DLS that the unfolding with GuHCl does not involve aggregation, to make sure that their analysis is correct.

-How are the CD experiments not showing any change in secondary structure upon membrane insertion compatible with the random coil transition of helix 1?

Minor comments:

-What do the authors mean when they state that BAK oligomerization opens the nanodiscs? It would be nice if they could provide some data explaining this membrane change. Perhaps EM of the

nanodiscs would be informative.

-On page 6, the authors still refer to their "non-truncated protein construct...", which is not yet accurate. They should correct with "protein construct non-truncated at the N-terminus".

-The authors conclude that the C-terminal of BAK is not involved in oligomerization. This is at odds with previous data from different groups and should be put in context with literature relating the C-terminus of BAX/BAK with oligomerization (Andreu-Fernandez et al PNAS 2017; Zhang et al. EMBO J 2015).

-The discussion is quite lengthy. I would recommend to remove the entire first paragraph. It is presented as new findings, but these results are rather for validation of the sample quality.

Referee #2:

The authors have done a good job addressing my comments (and those by reviewer #1) by performing new experiments and revising their conclusions and presentation. Even though probing helix 1 stabilization through mutagenesis remains a weak point of the study, I believe that the revised manuscript provides important insights into the conformational changes of BAK in the presence of membranes, while supporting the need for direct activation by BH3-only proteins to efficiently induce these changes. I suggest that the authors include this important conclusion in their abstract (and discussion). In the last section of the results the authors mislabeled R42 to R45.

A: We would like to express our gratitude to both referees that helped us tremendously to improve the quality of the manuscript and critically discuss our findings in the light of the current literature. Below, please find our answers to the general and specific comments raised by both referees.

Referee #1

The authors study the structure of BAK fragments in the membrane using nanodiscs. They show that helix 1 of BAK unfolds into a random coil upon dislodgement during activation. The dislodgement and its functional relevance were already known, so this part is rather confirmatory. Besides unfolding of helix 1, the activation pathway goes through a molten globule intermediate of BAK, which is not found in BCL-xL. In addition, unfolded helix 1 interacts with the membrane upon dislodgement, which likely facilitates the conformational transition to the molten globule and pore-forming structures.

The authors have done an excellent work at implementing the extensive lists of suggestions by the reviewers and the manuscript has improved in quality and clarity. At this point the manuscript has now potential to provide interesting new insight into different aspects of the structural organization of BAK in membranes. My opinion of the manuscript is now positive, but there are important issues that the authors need to address in another round of revision with experiments.

A: We thank this referee for their positive comments and are grateful for any suggestions to further improve the quality of the manuscript. As can be seen below, we have investigated the issues identified by this referee but, due to technical and sample issues, these experiments did not lead to a clear conclusion and thus do not provide additional insights that would further strengthen the manuscript. Undoubtedly, the investigation of the membrane interaction of the Bak N-terminal region would be very interesting. However, this would require quite intensive additional efforts (see below). Therefore, we strongly believe that such rather extensive studies are beyond the scope of the current manuscript but will be addressed in a later study. Furthermore, establishing a clear correlation between membrane binding of helix 1 and the formation of a molten globe of the Bak soluble domain would be fabulous. However, as explained below, the mutation of just positive surface charges in Bak did not lead to a clear effect. Thus, we think that the situation is by far more complicated and requires a more detailed screening for suitable mutations in the protein, as well as comparative protein folding studies, which would be a very nice paper on its own. In any case, we are grateful for this comment because such experiments are in the center of our expertise and interest, and will be addressed in the future.

Major concerns:

-One key finding in the manuscript would be the interaction of helix 1 with the membrane upon dislodgement. This could facilitate the transition into the active pore-forming conformation, thereby providing an explanation for the role of the membrane in promoting the activation of BAX and BAK. However, this key point of the manuscript is still preliminary and should be further developed. The authors argue that helix 1 interacts with the membrane based on the lower dynamics of this segment in the NMR data and the presence of positively charged residues. To strengthen this finding, the authors should provide direct evidence of interaction with the membrane - for example by testing the membrane binding properties of a peptide derived from helix 1, or by other means.

A: We purchased a peptide derived from helix 1 (EEQVAQDTEEVFRSYVFRHQQ) but found out that it is not soluble due to its unexpectedly strong hydrophobic properties. Thus, due to these technical difficulties caused by the inherent nature of helix 1 in isolation, we were unable to detect a direct interaction of helix 1 with the membrane. Since it would require a thorough optimization of the solvent conditions, the use of detergents or a screening procedure to insert/attach helix 1 into nanodiscs, it is unfortunately not foreseeable if and how such experiments would quickly lead to the desired outcome. However, we appreciate this comment and will continue working on this topic with the aim to obtain a complex structure of the Bak helix 1 bound to a lipid bilayer membrane.

-And importantly, they should also establish that the interaction with the membrane promotes the conformational change or molten globule transition. For this, they could design mutants in this helix that do not interact with the membrane, perhaps by changing the positive amino acids, and see if this affects the conformational change. Alternatively, and perhaps simpler if it works, they could use lipid compositions in the nanodiscs with no net negative charges, so that the interaction of the helix with the membrane is reduced and see if this affects the conformational change.

A: The role of the membrane on the Bak/Bax conformational change has been described in a vast amount of previous literature. In addition, we also see that Bak does not undergo a conformational change without a membrane. Thus, in our opinion, the general effect of a membrane environment has been sufficiently demonstrated in previous literature and in our large set of assay and structural data.

However, we have designed a Bak variant where charges were removed from residues R11, R169 and R174 located at the N-terminus and regions that, based on our HDX-MS data, are interacting with the membrane surface. This variant did not show a clear reduction in the pore forming activity and thus, has not been included in the manuscript. It can now be found in Figure S5 in the Appendix and is described lines 451-453 and 603-605. Therefore, we conclude that the charges are not the sole driving force but that additional factors such as membrane curvature induced by e.g. cardiolipin and most likely also hydrophobic membrane interactions play a role. We assume that the protein stretch interacting with the membrane also dips into the membrane and forms hydrophobic interactions. Thus, we removed the statement in the manuscript that the positive charge in helix 1 is a main factor for membrane binding.

The effect of lipid charges on Bax/Bak pore formation has already been investigated in detail in previous literature. The stimulating effect of cardiolipin on Bak activation in a membrane has been shown before (Landeta, 2011; Kuwana, 2002). The presence of just negative charge (PE + PG versus PE) was shown not to be the main factor for pore formation, as shown with the Bak homologue Bax (Yethon, 2003). In another paper on Bax, the amount of negative charge in the lipid blend could be correlated with higher pore forming activity (Lai, Y. C et al BBA - Biomembranes, 2019).

However, in these data, it is very difficult to separate the effects of individual lipids (like cardiolipin) and the net charge of the membrane. We think that a more systematic evaluation of the effects of lipid structure and charges would be very helpful. However, we feel that this is not the focus of the study here. Since we have new data confirming that charge removal in Bak does not have a strong effect, the use of different lipid compositions appears not suitable to extract further specific information on the role of the membrane interaction of helix 1.

-The new results with the BAK mutants at reasonable concentrations in Figure 6 show that there is no significant difference in membrane permeabilizing activity compared with the wild type protein. These

results do not add to the manuscript and only question the conclusions of the authors. Since the role of helix 1 dislodgement is in any case already well established, I would suggest to remove them and to add instead experiments with mutants that provide a functional role for the unfolding of helix 1 (not the dislodgement) or with the experiments proposed in the point above, aimed at demonstrating the role of the interaction of helix 1 with the membrane on the conformational transitions during activation.

A: We disagree with the referee that there is no significant difference in the presented assay data. There is indeed a very clear effect, but apparently, we did not point out clearly enough that the difference can be seen in the slower pore forming kinetics, presumably caused by a higher energy barrier for the initial activation step. We have modified the corresponding sections in the manuscript to better emphasize this point. (See lines 447-448 and line 597)

-Calorimetry of BAK-deltaTM in solution shows aggregation of the protein with increased temperature, which limits the further analysis of the structural changes above that temperature. The authors should also confirm with DLS that the unfolding with GuHCl does not involve aggregation, to make sure that their analysis is correct.

A: It is correct that we see the formation of aggregates of Bak^ΔTM both in the DSC measurements and in DLS in solution. We can exclude this from being relevant during GuHCl-induced unfolding as the conducted NMR experiments during the unfolding transition of Bak do not show any signs of aggregation. Since NMR is a very sensitive tool to detect the existence of larger species, which would immediately lead to very broad lines, we can conclude with great certainty that no aggregates built up during the GuHCl induced unfolding, rendering DLS experiments obsolete. Our DLS experiments at elevated temperatures (42°C) were merely conducted to emphasize the large differences in the aggregation behavior between a pro and anti-apoptotic Bcl2 protein.

-How are the CD experiments not showing any change in secondary structure upon membrane insertion compatible with the random coil transition of helix 1?

A: As explained in the manuscript, the lack of a pronounced change in the secondary structure content can be caused by a slight increase in the helical content in other parts of Bak once incorporated in the membrane, in line with the HDX MS data, where a stronger HDX protection can be observed in the Bak core. A possible gain in secondary structure in the Bak pore state is also suggested by the continuous helical structure of the latch domain in detergent-activated Bak (α2-8) (Birkinshaw et al, Mol Cell, 2021). An explanation for this is now presented in the results section (see lines 141-144).

We have also performed HDX measurements of the autoactivated form of Bak in liposomes, which proved identical to the species detected after BH3-induced activation in nanodiscs. Thus, we can conclude with certainty that the species gained by both methods are very similar and that the N-terminus is also unfolded in liposomes even if the CD measurements at their very limited resolution do not show a difference in the secondary structure content. The new HDX data are now included in Fig. EV4 (see lines 390-392).

Minor comments:

-What do the authors mean when they state that BAK oligomerization opens the nanodiscs? It would be nice if they could provide some data explaining this membrane change. Perhaps EM of the nanodiscs would be informative.

A: Bak oligomerization appears to be energetically favorable leading to the disruption of the preformed nanodisc structure that initially harbors only one Bak protomer giving the forming oligomer more space. The resulting oligomeric species is too large to be accommodated by a nanodisc, which was confirmed by chemical cross-linking experiments (as suggested by reviewer #2, shown in Fig. EV4b) and size exclusion chromatography in the first round of the revisions. We believe that the oligomerization is an essential driving force for pore formation. However, helix 1 needs to dislodge and stay in a state that hinders re-binding to the core. Using EM to characterize the structural state of Bak in the membrane is a great suggestion. However, this would be a massive effort aimed at solving one of the major remaining questions in the field, and therefore we believe that such an endeavor would be clearly beyond the scope of this manuscript.

-On page 6, the authors still refer to their "non-truncated protein construct...", which is not yet accurate. They should correct with "protein construct non-truncated at the N-terminus".

A: The statement on page 6 was changed accordingly.

-The authors conclude that the C-terminal of BAK is not involved in oligomerization. This is at odds with previous data from different groups and should be put in context with literature relating the C-terminus of BAX/BAK with oligomerization (Andreu-Fernandez et al PNAS 2017; Zhang et al. EMBO J 2015).

A: It is correct that helix 9 has been shown to be in close contact in the final pore state. This is also the case in the two models cited in our discussion (Mandal, 2016 and Bleicken 2018). However, what we were aiming at was to determine the features of the functional differences between pro- and antiapoptotic Bcl2 proteins. Our data show that the helix does not drive oligomerization by itself in Bak and BclxL. We did not intend to suggest that helix 9 is detached from the rest of the protein in the pore state, nor did we investigate this topic. This was perhaps not clearly stated in the manuscript so far but is now explicitly described in the discussion (see lines 523-526).

-The discussion is quite lengthy. I would recommend to remove the remove the entire first paragraph. It is presented as new findings, but these results are rather for validation of the sample quality.

A: The lengthy discussion is actually a direct consequence of the very detailed comments by both reviewers in the first round of revisions, which cannot be easily shortened without losing the requested content. However, to address this concern, we shortened the first paragraph to some extent.

Referee #2:

The authors have done a good job addressing my comments (and those by reviewer #1) by performing new experiments and revising their conclusions and presentation. Even though probing helix 1 stabilization through mutagenesis remains a weak point of the study, I believe that the revised manuscript provides important insights into the conformational changes of BAK in the presence of membranes, while supporting the need for direct activation by BH3-only proteins to efficiently induce

these changes. I suggest that the authors include this important conclusion in their abstract (and discussion). In the last section of the results the authors mislabeled R42 to R45.

A: Thanks to this referee for their very positive assessment of our revised manuscript, and in particular for the statement that we have done a good job in addressing his/her comments, as well as the comments of referee #1. We agree with the referee that further studies are required to investigate the role of helix 1 interaction with the helical core of Bak. However, this appeared to be a quite elaborate question that cannot be answered by just a few point mutations but will be addressed in future studies.

We updated the abstract and the last paragraph of the discussion according to this referee's suggestions and corrected the mentioned typo.

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

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Journal Submitted to: EMBO J

Manuscript Number: EMBOJ-2020-107159

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 justified.
- 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 χ^2 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?	n/a
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	n/a
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	n/a
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.	n/a
For animal studies, include a statement about randomization even if no randomization was used.	n/a
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.	no
4.b. For animal studies, include a statement about blinding even if no blinding was done	n/a
5. For every figure, are statistical tests justified as appropriate?	n/a
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	n/a
Is there an estimate of variation within each group of data?	no

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>
<http://1degreebio.org>
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor>

<http://grants.nih.gov/grants/olaw/olaw.htm>
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>
<http://ClinicalTrials.gov>
<http://www.consort-statement.org>
<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum>

<http://datadryad.org>

<http://figshare.com>

<http://www.ncbi.nlm.nih.gov/gap>

<http://www.ebi.ac.uk/ega>

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<http://biomodels.net/miriam/>
<http://jij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

Is the variance similar between the groups that are being statistically compared?	n/a
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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).	n/a
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	n/a

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	n/a
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	n/a
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	n/a

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	n/a
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n/a
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n/a

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	YES, a section on "Data Availability" is included in the manuscript with deposition codes.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All data in this manuscript are either available via public databanks or contained in the Extended View Section or Appendix.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n/a
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biocompare (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n/a

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	no
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