EMBO reports

Phosphorylation switches Bax from promoting to inhibiting apoptosis thereby increasing drug resistance

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

1st Editorial Decision 18 October 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, while all referees in principle agree on the potential interest of your findings, they also raise a number of - often overlapping - issues that would need to be addressed before publication. In particular, it will be important to add further evidence for the phosphorylation of endogenous Bax at S184 and for its subcellular localization. The link between Akt and Bax phosphorylation needs to be strengthened and further evidence for the proposed mechanism, i.e., that Bax-S184P acts as sink for pro-apoptotic BH3-only proteins in the cytosol, has to be provided.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript becomes suitable for publication in EMBO reports. Yet, given the potential interest of your findings and the constructive comments, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Your article currently has 5 figures and should therefore be published as Scientific Report. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research

Article. In this case the Results and Discussion section can stay as it is now. If a Scientific Report is submitted, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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REFEREE REPORTS

Referee #1:

In this Manuscript, Kale et al conclude that phosphorylation of Bax on serine 184 by Akt converts it into an anti-apoptotic molecule, and that this modification contributs to oncogenesis. The data presented shows that Bax-S184P appears in ABT-737 resistant cells (and is phosphorylated in these cells by Akt), resides predominantly in the cytosol, sequesters BH3-only proteins in the cytosl, is unable to target membranes and release cytochrome c, and protects cells from apoptotic signals. Based on these observations, the conclusion of the authors is attractive, however additional important experiments are required to strengthen it.

Specific comments:

1) Fig 1C: The authors conclude that Bax-S184P appears in ABT-737 resistant cells but not in ABT-737 sensitive cells. The authors need to prove that the phosphorylated form of Bax that appears in

ABT-737 resistant cells is indeed phosphorylated on serine 184, since the antibody used in these experiments is not specific to S184P and can recognize all phospho-serines in Bax. Thus, they need to show that Bax WT is recognized by the antibody whereas Bax-S184A is not.

2) Fig 1D: The authors conclude that GFP-Bax-S184A is predominantly mitochondrial and GFP-Bax-S184E is predominantly cytosolic. In the pictures presented, the resolution of punctated Bax is not high enough and the pictures are not accompanied by statistics, and thus the conclusions reached by the authors are not strong enough. The authors should perform subcellular fractionations followed by Western blot analysis to strengthen their conclusions. Is GFP-Bax-WT not phosphorylated in ABT-737 sensitive cell lines? Is the phosphorylated form of endogenous Bax WT predominantly localized to the cytosol?

3) Fig 2: The authors elegantly show using liposome assays that recombinant Bax-S184E binds cBid but does not insert into membranes, does not homo-dimerize, and does not permeabilize liposomes. How does Bax-S184A behave in these assays? The authors should also perform in-vitro experiments with purified mitochondria (closer to the in-vivo setting), and demonstrate that Bax-S184E: 1) does not target mitochondria; 2) does not homo-dimerize/expose its N-terminus; 3) does not release cytochrome c; and 4) is not triggered by cBid/tBid to perform all the above. If indeed phosphorylation of Bax in the cytosol triggers its ability to bind activator BH3's and inhibits its homo-dimerization, then it is important that the authors show that the phosphorylated form of endogenous Bax behaves similarly.

4) Fig 3B: the authors show that stable expression of Bax-S184E in WT BMK cells protects from apoptotic signals. This protection is impressive with TNFα + CHX (shows the same behavior as the DKO BMK cells), but is much less impressive with either Staurosporine or Panobinostat. Why? How does stable expression of Bax-S184A effect the sensitivity of the cells to apoptotic signals? In addition, in Fig 3C,D the resolution of punctated Bax is not high enough (as like in Fig 1D), and the authors should perform subcellular fractionations followed by Western blot analysis to strengthen their conclusion that STS does not trigger the translocation of Bax-S184E to the mitochondria. If indeed phosphorylation of Bax in the cytosol inhibits its ability to translocate to the mitochondria in response to STS, then the authors should demonstrate that the phosphorylated form of endogenous Bax does not appear in the mitochondrial fraction following STS.

5) Fig 4: The authors conclude that Akt inhibits mitochondria priming by phosphorylating Bax, however they do not show direct evidence for this. Specifically, they demonstrate that: 1) pre-treatment of ABT-737 resistant cells with Akt pathway inhibitors (or immunodepletion of Akt from the S100 fraction) abrogates inhibition of priming by the S100 fraction (Fig 4B); 2) Bax phosphorylation in ABT-737 resistant cells was substantially reduced by pretreatment with Akt pathway inhibitors (Fig 4D). If Bax phosphorylation is indeed critical for Akt to inhibit mitochondrial priming, then immunodepletion of Bax from the S100 fraction and adding back Bax-S184A, should prevent Akt's ability to inhibit mitochondria priming. A similar approach can be taken in the setting presented in Fig 4E to determine whether replacement of recombinant Bax-WT with recombinant Bax-S184A prevents Akt from inhibiting tBid-induced cytochrome c release.

Referee #2:

Understanding the molecular control of Bax and Bak during apoptosis is key to targeting the apoptotic machinery in cancer. The current manuscript describes the phosphorylation of Bax in its C-terminal transmembrane anchor that prevents its localisation to the mitochondrial outer membrane. Additionally they report that phosphorylated Bax can act as a sink for activating BH3-only proteins to limit cell death. That Bax Ser184 in the TM domain is a target for phosphorylation and that this inhibits Bax apoptotic activity is not novel. However, the concept that phosphorylation not only impairs Bax apoptotic activity but converts Bax to an anti-apoptotic protein that can potentially cause resistance to chemotherapy is new and of interest. This finding aligns with the first characterisation by the Youle lab of the Bax S184E mutant suggesting it may limit cell death (Nechushtan et al EMBO 1999). Generally the manuscript is clear and the experiments on the whole well-performed. However, I feel that direct evidence of Bax phosphorylation is lacking and some of the experiments have alternative interpretations.

1. The mechanistic insight is revealed by experiments with the phosphomimetic mutation S184E. This is a reasonable approach, however without direct evidence of phosphorylation at S184, the consequences of this mutation could be just correlative with the charged residue inconsistent with integration of the TM into the lipid bilayer of the outer mitochondrial membrane. In my opinion the authors need to provide definitive evidence by mass spectrometry that Bax is phosphorylated at Ser184 and that this is different in their sensitive v resistant lines. Additionally it is unclear what percentage of Bax is phosphorylated to confer the proposed resistance in the cancer cell lines. The authors state 50% based on Figure EV4D, however this is an acellular assay with recombinant proteins and so is not relevant to the cancer cell lines. The authors could test this by immunodepleting phospho-Bax with an anti-phosphoserine IP or an alternative assay such as 2D gel electrophoresis to distinguish the phosphorylated form from the unphosphorylated form and compare between their sensitive and resistant cells.
2. The authors report Akt signalling amplification and high Bax correlate in cancer patient samples. This is interesting, but is purely correlative as they failed to detect S184-P with several commercial antibodies. Definitive evidence of Bax-P in patient samples by mass spectrometry would significantly strengthen this association and the conclusions.
Analysis of patient Ideally this should also be shown in patient samples.
3. The premise that the disconnect between the sensitivity of isolated mitochondria to Bad peptide and cells treated with ABT737 is due to the absence of post-translational modifications in the former setting is rather contrived without evidence to support it. The authors state that to rectify this difference they included phosphatases in their mitochondrial priming experiments but they do not then go on to show if this actually makes a difference indeed confers better correlation between mitochondrial and cell death assays. A more likely explanation for the difference is that a proportion of certain Bcl-2 proteins e.g. Bcl-xl are cytosolic and so are absent from the isolated mitochondrial assays. Additionally, as Bax is predominantly cytosolic and Bak is mitochondrial, MOMP in the mitochondrial assays is largely mediated by Bak not Bax. The authors should provide evidence in these cells of the subcellular localisations of Bax and Bak.
4. The proposal that Bax-P acts as a pro-survival protein by sequestering BH3-only proteins is a really interesting proposition and warrants further investigation. Based on this hypothesis and their recombinant protein data, one would predict that a stable association between Bax-P with BH3-only proteins should be detectable in the cytosol of resistant cells following ABT737. This should be tested and would strengthen their conclusions.
5. In Figure 4c, the authors supplement mitochondria isolated from sensitive cells with active Akt to render the mitochondria "unprimed". That commonly very little Bax is resident at mitochondria isolated from healthy cells suggests that the effect of Akt in this instance might be by another mechanism. Related to point #1, the authors should show that these mitochondria have significant Bax.
6. In the analysis of public databases, the authors report a correlation between high Bax and an amplified Akt pathway in cancers. They concede that an alternative explanation for this is that elevated Akt signalling permits high Bax levels. To distinguish between these possibilities they should repeat the analysis comparing Bak levels as there should be no correlation based on their hypothesis that Akt specifically limits Bax activity as opposed to generally providing a pro-survival advantage.
7. Can they exclude that NBD labelling is occluded by protein-protein interactions rather than lipid? Could the lack of NBD labelling in the a5 and a9 of the S184E mutant be explained by the tail not being displaced from the groove by the Bid? The detected interaction could be via the Walensky "rear site"? The lack of oligomerization of Bax S184E on liposomes could also be a consequence of its failure to activate and associate with membranes.

Specifics;
1. Cell-based assays are also "in vitro".
2. The Introduction states that "...since Bax is required for MOMP..". This is simplistic as Bak and Bax are redundant in most cells (Wei et al 2001).
3. The Introduction cites previous work reporting BaxS184 phosphorylation enhancing activity or having no effect. They should also cite the previous studies that reported that phosphorylation at S184 is inhibitory (such as Wang et al PlosONE 2010, Xin et al JBC 2005).
4. The authors make a statement regarding the mitochondrial dynamics in BaxS184 mutant expressing cells. However, the microscopy images are not of sufficient resolution to report this and there is no image analysis. As this is not referred to elsewhere and the relevance is unclear I would recommend removing the statement.

5. Pge 7, "...using fluorescence spectroscopy and (with) purified..."
6. Statistical analyses should be performed on the quantitative data in Figure 4. Is the Akt inhibitor MK2206 significantly different for example?
7. "...Akt levels makes logical sense..."
8. Figure 4E, the authors should confirm that Akt has no effect on non-phosphorylatable S184A to exclude that Akt is limiting MOMP/apoptosis in a Bax-independent fashion.
9. In EV4D, in order to compare the Bax/BaxP to the GSK standard curve, the Bax and GSK-3 should be run on and analysed on the same gel.
10. In their final model the authors depict Bax-P on mitochondria. However, they (and others) have shown that S184E phosphomimetic cannot target membranes?

Referee #3:

The authors report that phosphorylation of Bax at S184, or a phosphomimetic form of Bax in that position, changes the activity of Bax from pro-apoptotic to anti-apoptotic. They propose a molecular mechanism based on the binding and sequestration of BH3-only proteins in the cytosol, so that they cannot activate other non-phosphorylated Bax molecules, neither inhibit the prosurvival Bcl-2 members. The results are new and interesting for the field, and the work is elegantly done and clearly explained.

However, there are a number of issues that should be addressed:

The main novelty of the study regards the switch of Bax to an antiapoptotic proteins by sequestration of BH3 only proteins in solution. The authors show that phosphorylated or phosphomimetic Bax at S184 decrease the activity of wild type Bax in vitro and in cells. However an important control that is missing is a mutant version of Bax that is not able to bind specifically BH3 only proteins. Despite phosphorylation, or phosphomimetic mutation, this form of Bax should not be able to show this effect.

In addition, it would be interesting to show a quantitative correlation between Bax phosphorylation and ABT-737 sensitivity. This could be done by connecting the analysis in Figure 1B with quantification of Bax phosphorylation levels in the different cell lines.

The authors report an antiapoptotic function of soluble Bax, but do not take into account neither discuss recent reports on the inhibitory dimerization of Bax in the cytosol. How is this new antiapoptotic function of Bax related to its dimerization in the cytosol? Is phosphorylated Bax more or less dimerized than the non-phosphorylated form? Is this affected by binding to BH3-only proteins? Could the dimerization of Bax in the cytosol be part of the same mechanism for apoptosis inhibition that the binding to BH3 only proteins?

Related to this, does phosphomimetic Bax bind to Bcl-xL in solution.

In Figure 3B, a control experiment is missing for DKO + BaxS184E. Does this mutant form of Bax retain any prodeath activity?

Minor comments:

In general, the cells in the images are too small. They should be shown with a zoom in too. In figure 1D, why is the phosphomimetic Bax in the nucleus? In these images it is impossible to assess mitochondrial morphology. The authors should quantify all of this with proper image analysis. Lower panel: It is not clear what WT means and in the figure legend, it is not described.

Fig 2.B is it possible to include the Fret change of S184A in Liposomes and solution, which should give us a clear picture of its behavior as that we expect in cells shown in Fig 1. D?

Figure 2E: The % Liposome Permeabilization of Bax-S184E should be included in the figure.

Figure EV4B: How do you explain the localization of Akt in the cytosol and on mitochondria in all cell lines after treatment with A-443654?

Table S1: The table is missing.

Page 6, paragraph 1: We found that Bax is phosphorylated in ABT-737 resistant MDA-MB-468, and ZR-75-1cells, but not in ABT-737 sensitive MDA-MB-435 cells (Figure 1C, 4E). There are no results in the suggested figures showing data in the cells ZR-75-1. Figure 4E doesn´t support the statement that Bax is phosphorylated in ABT-737 resistant MDA-MB-468, and ZR-75-1cells, but not in ABT-737 sensitive MDA-MB-435 cells.

Page 7, paragraph 3: .... Bax S184E bound to both cBid (Figure 2B) and Bim (Figure EV2A) in the absence and in the presence of membranes. Figure EV2A shows only interaction on membranes and not in solution. This figure doesn´t suggest the statement.

Page 9, paragraph 3: Stable Bax-S184E expression in WT BMK cells had a protective effect against cell death induced by the pro-death cytokine TNF-α and cycloheximide, ... Are the cells treated in the same time with TNF-α and cycloheximide? If yes, the description in the legend should be included. If not, data has to be shown in the figure.

The explanation of the different inhibition modes should be explained better, for the general reader.

1st Revision - authors' response 25 April 2018

Referee #1:

In this Manuscript, Kale et al conclude that phosphorylation of Bax on serine 184 by Akt converts it into an anti-apoptotic molecule, and that this modification contributs to oncogenesis. The data presented shows that Bax-S184P appears in ABT-737 resistant cells (and is phosphorylated in these cells by Akt), resides predominantly in the cytosol, sequesters BH3-only proteins in the cytosl, is unable to target membranes and release cytochrome c, and protects cells from apoptotic signals. Based on these observations, the conclusion of the authors is attractive, however additional important experiments are required to strengthen it.
We thank the referee for their positive comments.

Specific comments:

1) Fig 1C: The authors conclude that Bax-S184P appears in ABT-737 resistant cells but not in ABT-737 sensitive cells. The authors need to prove that the phosphorylated form of Bax that appears in ABT-737 resistant cells is indeed phosphorylated on serine 184, since the antibody used in these experiments is not specific to S184P and can recognize all phospho-serines in Bax. Thus, they need to show that Bax WT is recognized by the antibody whereas Bax-S184A is not.
This was a good suggestion. To address this we initially performed mass spectrometry analysis on samples of immunoprecipitated Bax from MDA-MB-468 and MDA-MB-435 cell lysates. We did this twice with independent samples. Both times peptides from the C-terminal region of Bax containing S184 were in low abundance. The first time 0 of 28 (for MDA-MB-435 samples) and 0 of 43 (for MDA-MB-468 samples) of the peptides were from the C-terminal region encompassing S184. The second time only 4 of 45 (for MDA-MB-435 samples) and 9 of 70 (for MDA-MB-468 samples) of the peptides were from the C-terminal region encompassing S184. While these peptides had no post-translational modifications they are clearly insufficient to draw any conclusions from. Phosphorylation of Bax at position S184 has been studied before, and we assume that like us some of these other groups have tried mass spectrometry and got the same answer we did – that the C-terminal region of Bax appears to be incompatible with mass spectrometry. Thankfully, we found a new antibody that in recent publications specifically detects Bax phosphorylated at position S184 (Li et al, 2017; Xin et al, 2014).

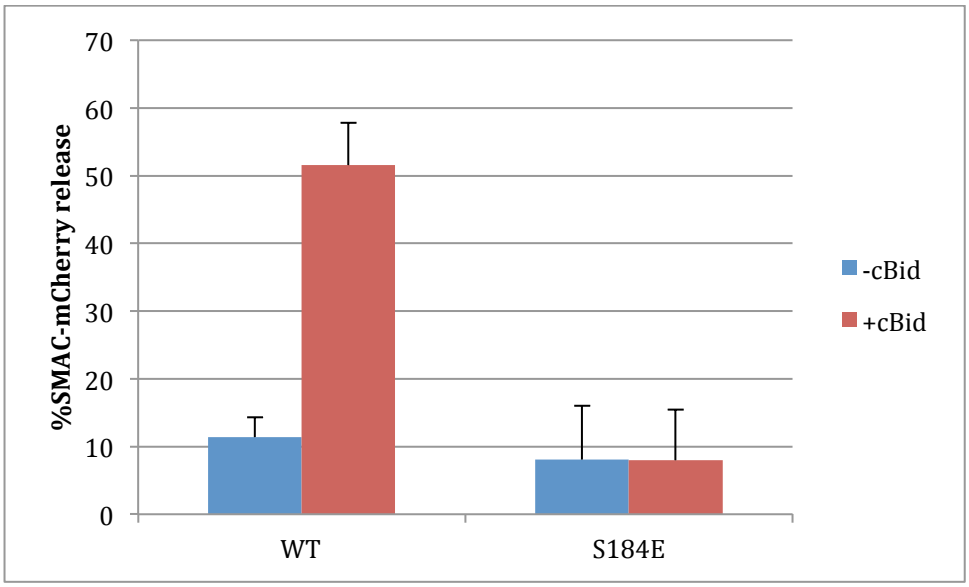
Using this antibody we now show that as predicted from our data, S184 phosphorylated Bax was detected in the MDA-MB-468 and ZR-75-1 cell lines but not in the MDA-MB-435 cell lines (Figures 2A and EV2A). GFP-Bax but not GFP-Bax S184A was also detected by this antibody indicating that, as published, it is specific for S184 and that S184 is being phosphorylated in our

system (Figure EV1B). Additionally, treatment of the ABT-737 resistant MDA-MB-468 and ZR-75-1 cell lines with Akt pathway inhibitors substantially reduced Bax phosphorylation at residue S184 (Figure EV2A). Importantly Bax was found to be phosphorylated at position S184 in a subset of ovarian cancer cells isolated from patients (Figure 6A). Moreover, the level of S184 phosphorylated Bax in the ovarian cancer cells, correlated with cellular resistance to ABT-737 (Figure 6B). These experiments provide substantial additional evidence that S184 is phosphorylated in cell lines and patient tissues.

2) Fig 1D: The authors conclude that GFP-Bax-S184A is predominantly mitochondrial and GFP-Bax-S184E is predominantly cytosolic. In the pictures presented, the resolution of punctated Bax is not high enough and the pictures are not accompanied by statistics, and thus the conclusions reached by the authors are not strong enough. The authors should perform subcellular fractionations followed by Western blot analysis to strengthen their conclusions. Is GFP-Bax-WT not phosphorylated in ABT-737 sensitive cell lines? Is the phosphorylated form of endogenous Bax WT predominantly localized to the cytosol?
We apologize for the low resolution images which resulted from conversion to PDF. In this version it is possible to zoom the individual image panels up to 1200%. As suggested we performed subcellular fractionation followed by western blot for GFP-Bax, GFP-Bax S184E and GFP-Bax S184A (Figure EV1C). We found that GFP-Bax is distributed equally between the cytoplasm and mitochondrial fractions, GFP-Bax S184A appears more mitochondrial and GFP-Bax S184E is predominately cytosolic. Statistics were performed on the images in figure (now Figure 2C) and Pearson's correlation coefficients between MitoTracker and GFP were added to the figure caption. The data suggest that in the live cells, GFP-Bax and GFP-Bax S184A co-localize more than GFP-Bax S184E with the mitochondria. By imaging, WT-Bax appears more mitochondrial than by subcellular fractionation because it is loosely bound as published previously (Gautier et al, 2011). Additionally, we performed more extensive co-localization experiments and analysis on the mCerulean3-Bax constructs used in the paper (Figure 5C and D) and found similar results.

3) Fig 2: The authors elegantly show using liposome assays that recombinant Bax-S184E binds cBid but does not insert into membranes, does not homo-dimerize, and does not permeabilize liposomes. How does Bax-S184A behave in these assays?
We thank you for suggesting this additional control. We performed similar mechanistic analysis to that of WT Bax and Bax S184E on Bax S184A (Figure EV3). We found that Bax S184A is functionally similar to WT Bax however, Bax S184A spontaneously targets liposomes in the absence of an activator, whereas WT Bax does not. This result is also consistent with the imaging and fractionation data described above.

The authors should also perform in-vitro experiments with purified mitochondria (closer to the in-vivo setting), and demonstrate that Bax-S184E: 1) does not target mitochondria; 2) does not homo-dimerize/expose its N-terminus; 3) does not release cytochrome c; and 4) is not triggered by cBid/tBid to perform all the above.
Although these experiments seemed like they were a good idea, we elected not to add additional data with mitochondria because we have previously performed experiments testing the function of this point mutant with isolated mitochondria. We assessed whether WT Bax and Bax S184E can release a fluorescent fusion protein (SMAC-mCherry) from the mitochondrial intermembrane space in response to cBid (see below). As this step depends on the preceding ones it encompasses the questions raised by the referee.



We observed for mitochondria the same phenotype shown in the paper for liposomes and cells in which Bax S184E is inhibited in pore-formation. We assume from this experiment that Bax S184E behaves similarly in mitochondria-based experiments as it does in our liposome based system. Furthermore, Bax S184E is predominately cytosolic in cells (Figure EV1C, Figure 5C and D) and does not become punctate in response to STS (Figure EV4E) suggesting that BaxS184E does not bind mitochondria inside cells.

If indeed phosphorylation of Bax in the cytosol triggers its ability to bind activator BH3's and inhibits its homo-dimerization, then it is important that the authors show that the phosphorylated form of endogenous Bax behaves similarly.
Bax S184E is predominately cytosolic in cells (Figure EV1C, Figure 5C and D) and does not become punctate in response to STS (Figure EV4E) suggesting that Bax does not coalesce into large oligomers within cells. Showing that the phosphorylated form of endogenous Bax binds activator BH3s is technically challenging. Immunoprecipitation experiments with Bcl-2 family proteins are difficult to interpret since detergents alter the interactions between proteins as we and other have previously described (Hsu & Youle, 1997; Lovell et al, 2008). Non-ionic detergents, such as NP-40, promote the artefactual interaction of Bax and BH3-only proteins (Hsu & Youle, 1997) and zwitterionic detergents, such as CHAPS, inhibit authentic interactions between Bax and BH3-only proteins (Lovell et al, 2008). As a test for feasibility of the requested immunoprecipitation experiments we added recombinant Bax and Bim to modified RIPA cell lysis buffer, immunoprecipitated Bax and blotted for Bim. In these experiments Bim co-immunoprecipitated with Bax showing that the detergents cause Bim-Bax binding in solution, something that does not happen in non-apoptotic cells. Because data from immunoprecipitates from cells would be too difficult to interpret we have not done the requested experiments.

4) Fig 3B: the authors show that stable expression of Bax-S184E in WT BMK cells protects from apoptotic signals. This protection is impressive with TNF• + CHX (shows the same behavior as the DKO BMK cells), but is much less impressive with either Staurosporine or Panobinostat. Why? How does stable expression of Bax-S184A effect the sensitivity of the cells to apoptotic signals?
We reason that since TNF-alpha has been shown to specifically trigger apoptosis by the activation of Bid, Bax-S184E efficiently protects because a BH3 protein is the only apoptotic trigger. STS is a pan-kinase inhibitor and panobinostat is an HDAC inhibitor, these drugs do not kill exclusively by apoptosis as seen by higher concentrations killing Bax/Bak double knock-out cells. These drugs alter many cellular functions and may activate Bax and Bak indirectly by a mechanism that is BH3-protein independent (Brahmbhatt et al, 2016) as well as by activating non-apoptotic cell death pathways. As a result Bax-S184E cannot protect from these drugs as well as it protects from TNF-alpha.
To address this issue further, we generated BMK cell lines stably expressing mC3-Bax-S184A and re-assayed cell death in response to TNF-alpha, STS and panobinostat. Originally we scored apoptosis only as Annexin-V positive cells but we realized that for some cells neighbouring or

overlapping dead cells that were Annexinv-V positive could cause what appear to be growing cells to score as Annexin-V positive. To overcome this we co-stained cells with the mitochondrial membrane potential dye TMRE. As a more robust assay only cells that are both Annexin-V positive (exposed phosphatidylserine) and TMRE negative (lost mitochondrial membrane potential) were scored as apoptotic. The results of the new experiments are similar to that of the old ones but the error is lower. Using the improved assay we find that Bax-S184E protects cells from TNF-alpha, STS and panobinostat, whereas expression of Bax-S184A increases the amount of cell death in response to treatment with these drugs (Figure 5B, EV4C). These results are consistent with our conclusion that Bax-S184E inhibits apoptosis. We have added the appropriate text to the paper to indicate that this new data supports our model.

In addition, in Fig 3C,D the resolution of punctated Bax is not high enough (as like in Fig 1D), and the authors should perform subcellular fractionations followed by Western blot analysis to strengthen their conclusion that STS does not trigger the translocation of Bax-S184E to the mitochondria. If indeed phosphorylation of Bax in the cytosol inhibits its ability to translocate to the mitochondria in response to STS, then the authors should demonstrate that the phosphorylated form of endogenous Bax does not appear in the mitochondrial fraction following STS.
Higher resolution images are included with the revised manuscript. The lower resolution was a result of conversion to PDF in the initial submission. While the images are small they can be zoomed to 1200% larger in Acrobat Reader for viewing as mentioned in response to comment 2 above. In addition we have provided images highlighting single cells. We performed mitochondrial co-localization analysis on >1000 individual live cells in the presence and absence of STS, across two independent experiments for mC3-Bax, mC3-Bax S184E and mC3-Bax S184A (Figure 5D). The results clearly demonstrate that STS mediated translocation of Bax-S184E to mitochondria is greatly reduced. Together with the fractionation data provided for Figure 1 this result is now much better supported by quantitative data.

5) Fig 4: The authors conclude that Akt inhibits mitochondria priming by phosphorylating Bax, however they do not show direct evidence for this. Specifically, they demonstrate that: 1) pre-treatment of ABT-737 resistant cells with Akt pathway inhibitors (or immunodepletion of Akt from the S100 fraction) abrogates inhibition of priming by the S100 fraction (Fig 4B); 2) Bax phosphorylation in ABT-737 resistant cells was substantially reduced by pretreatment with Akt pathway inhibitors (Fig 4D). If Bax phosphorylation is indeed critical for Akt to inhibit mitochondrial priming, then immunodepletion of Bax from the S100 fraction and adding back Bax-S184A, should prevent Akt's ability to inhibit mitochondria priming. A similar approach can be taken in the setting presented in Fig 4E to determine whether replacement of recombinant Bax-WT with recombinant Bax-S184A prevents Akt from inhibiting tBid-induced cytochrome c release.
This is an excellent point. We performed the suggested experiment and showed that incubation of Bax S184A and AKT does not alter tBid induced cytochrome c release in the isolated mitochondria. The data is shown in Figure 3G Thank you for suggesting this additional control as the data strongly support our conclusion that Bax with a serine at position 184 is critical for Akt to inhibit mitochondrial priming.

Referee #2:

Understanding the molecular control of Bax and Bak during apoptosis is key to targeting the apoptotic machinery in cancer. The current manuscript describes the phosphorylation of Bax in its C-terminal transmembrane anchor that prevents its localisation to the mitochondrial outer membrane. Additionally they report that phosphorylated Bax can act as a sink for activating BH3-only proteins to limit cell death. That Bax Ser184 in the TM domain is a target for phosphorylation and that this inhibits Bax apoptotic activity is not novel. However, the concept that phosphorylation not only impairs Bax apoptotic activity but converts Bax to an anti-apoptotic protein that can potentially cause resistance to chemotherapy is new and of interest. This finding aligns with the first characterisation by the Youle lab of the Bax S184E mutant suggesting it may limit cell death (Nechushtan et al EMBO 1999). Generally the manuscript is clear and the experiments on the whole well-performed. However, I feel that

direct evidence of Bax phosphorylation is lacking and some of the experiments have alternative interpretations.
We thank the reviewer for the positive comments.

1. The mechanistic insight is revealed by experiments with the phosphomimetic mutation S184E. This is a reasonable approach, however without direct evidence of phosphorylation at S184, the consequences of this mutation could be just correlative with the charged residue inconsistent with integration of the TM into the lipid bilayer of the outer mitochondrial membrane. In my opinion the authors need to provide definitive evidence by mass spectrometry that Bax is phosphorylated at Ser184 and that this is different in their sensitive v resistant lines.
This is a great point. Please see our response to reviewer 1 comment 1 above about our 4 attempts at specifically detecting peptides containing S184 by mass spectroscopy to verify that Bax is phosphorylated. As this approach is simply not currently feasible we made use of a new antibody specific for Bax phosphorylated at S184. These results provide the most direct measure possible for phosphorylation of S184 and as pointed out above the result has now been replicated by two other groups.

Additionally it is unclear what percentage of Bax is phosphorylated to confer the proposed resistance in the cancer cell lines. The authors state 50% based on Figure EV4D, however this is an acellular assay with recombinant proteins and so is not relevant to the cancer cell lines. The authors could test this by immunodepleting phospho-Bax with an anti-phosphoserine IP or an alternative assay such as 2D gel electrophoresis to distinguish the phosphorylated form from the unphosphorylated form and compare between their sensitive and resistant cells.
We agree that quantification of Bax phosphorylation by Akt in an acellular assay is not satisfactory so we removed the quantification data. However as mentioned in response to reviewer 1 comment 1, we used the pBax S184 antibody to assay in a relatively quantitative way S184 Bax phosphorylation in ovarian cancer cells isolated from cancer patients. We show that differences in the levels of S184 phosphorylated Bax in the ovarian cancer cells correlate with cellular resistance to ABT-737 (Figure 6B).

2. The authors report Akt signalling amplification and high Bax correlate in cancer patient samples. This is interesting, but is purely correlative as they failed to detect S184-P with several commercial antibodies. Definitive evidence of Bax-P in patient samples by mass spectrometry would significantly strengthen this association and the conclusions. Ideally this should also be shown in patient samples.
Excellent point, this was completed using a pBax S184 antibody as stated in the previous response. Thank you, we feel this new data significantly strengthens our hypothesis.

3. The premise that the disconnect between the sensitivity of isolated mitochondria to Bad peptide and cells treated with ABT737 is due to the absence of post-translational modifications in the former setting is rather contrived without evidence to support it. The authors state that to rectify this difference they included phosphatases in their mitochondrial priming experiments but they do not then go on to show if this actually makes a difference indeed confers better correlation between mitochondrial and cell death assays. A more likely explanation for the difference is that a proportion of certain Bcl-2 proteins e.g. Bcl-xl are cytosolic and so are absent from the isolated mitochondrial assays. Additionally, as Bax is predominantly cytosolic and Bak is mitochondrial, MOMP in the mitochondrial assays is largely mediated by Bak not Bax. The authors should provide evidence in these cells of the subcellular localisations of Bax and Bak.
To address this we tested mitochondrial priming using BH3 profiling in the presence and absence of phosphatase inhibitors (Figure 1C). We found that phosphatase inhibitors decreased mitochondrial priming only in the ABT-737 resistant cell lines and had no effect on mitochondrial priming in the ABT-737 sensitive cell lines. We performed subcellular fractionation of GFP-Bax, GFP Bax S184E and GFP-Bax S184A (Figure EV1C) in the MDA-MB-468 cell lines and subcellular fractionation of Bax in the MDA-MB-435 cell lines (Figure EV2D). In all cases for these cell lines some Bax is found in the mitochondrial fractions. However, this result does not mean that the protein has inserted into the membrane as Bax is often found to be bound peripherally to membranes. We cannot rule out or account for the localization of all Bcl-2 family proteins in priming assays therefore, we

describe the limitations of the assay in the text (discussion paragraph 1) and have supported the conclusions with a variety of other data as described below.

4. The proposal that Bax-P acts as a pro-survival protein by sequestering BH3-only proteins is a really interesting proposition and warrants further investigation. Based on this hypothesis and their recombinant protein data, one would predict that a stable association between Bax-P with BH3-only proteins should be detectable in the cytosol of resistant cells following ABT737. This should be tested and would strengthen their conclusions.
This is an excellent suggestion and we attempted co-immunoprecipitations between Bax and Bim following ABT-737 treatment in the resistant and sensitive cell lines. But as mentioned above, in our response to reviewer 1 comment 3, these experiments proved difficult to interpret and the control described above highlighted important technical limitations. Therefore, we have not further pursued this line of investigation.

5. In Figure 4c, the authors supplement mitochondria isolated from sensitive cells with active Akt to render the mitochondria "unprimed". That commonly very little Bax is resident at mitochondria isolated from healthy cells suggests that the effect of Akt in this instance might be by another mechanism. Related to point #1, the authors should show that these mitochondria have significant Bax.
We performed the subcellular fractionation and found that indeed, Bax was present in the mitochondrial fraction (Figure EV2D). The amount of Bax that co-fractionates with mitochondria is highly variable between cell lines and types.

6. In the analysis of public databases, the authors report a correlation between high Bax and an amplified Akt pathway in cancers. They concede that an alternative explanation for this is that elevated Akt signalling permits high Bax levels. To distinguish between these possibilities they should repeat the analysis comparing Bak levels as there should be no correlation based on their hypothesis that Akt specifically limits Bax activity as opposed to generally providing a pro-survival advantage.
This is great control. We repeated an identical analysis with Bak (gene *BAK1*) and found that there are many less significant associations between Bak and the Akt pathway compared to Bax and the Akt pathway (Figure 6C, EV5B). Additionally, there was no difference between high or low BAK1 levels and Akt pathway activation in our analysis of the pan-cancer dataset (Figure 6D).

7. Can they exclude that NBD labelling is occluded by protein-protein interactions rather than lipid? Could the lack of NBD labelling in the a5 and a9 of the S184E mutant be explained by the tail not being displaced from the groove by the Bid.
In these experiments the Bax is already labelled with NBD. What we are tracking is the emission of the NBD dye which changes in response to the hydrophobicity of the environment. Upon re-reading our text we realized that the wording is not as clear as we had hoped and could be mis-interpreted. Therefore we changed the text to be clearer. Regardless, it is difficult to determine if the change in emission of the NBD dye is due to protein-protein interactions, rather than lipid interactions. However using chemical labeling we have shown that in cells residues 126 and 175 insert into the mitochondrial outer membrane (Annis et al, 2005). For these experiments protein-protein interactions were not confounding as the assay was performed in high concentrations of urea. Thus we interpret the increase in NBD emission of Bax labelled at residues 126 and 175 as the insertion into the bilayer.

The detected interaction could be via the Walensky "rear site"?
As we understand it, Bim peptides are able to bind the rear pocket whereas Bid peptides cannot, but both can bind the canonical pocket. Thus, the experiments with Bid should be limited to the canonical pocket. Although it would be interesting to determine whether phosphorylated Bax binds BH3 proteins in the rear or canonical pocket the effort involved is not justified for the current paper as the answer would not change the conclusions of this study.

The lack of oligomerization of Bax S184E on liposomes could also be a consequence of its failure to activate and associate with membranes.
The lack of oligomerization of BaxS184E is indeed a consequence of its failure to integrate into membranes, as outlined in figure 4 and described in the text. Bax S184E cannot insert into the bilayer and thus cannot oligomerize as oligomerization is downstream of Bax insertion into

membranes. We realized that the concluding sentence discussing these results was unclear. It now reads:
"However, FRET was not detected for Bax S184E or between Bax and Bax S184E supporting that phosphorylation prevents the upstream step Bax insertion into membranes therefore also inhibiting Bax oligomerization"

Specifics;

1. Cell-based assays are also "in vitro".
Correct, we changed the text to clarify this.

2. The Introduction states that "...since Bax is required for MOMP..". This is simplistic as Bak and Bax are redundant in most cells (Wei et al 2001).
The reviewer is quite correct. "since Bax is required for MOMP" has been removed as it didn't add anything to the sentence.

3. **The Introduction cites previous work reporting BaxS184 phosphorylation enhancing activity or having no effect. They should also cite the previous studies that reported that phosphorylation at S184 is inhibitory (such as Wang et al PlosONE 2010, Xin et al JBC 2005).**
We apologize for the oversight. We have included the suggested citations (they were inadvertently removed when shortening an earlier version of the text). We also changed the text to "inhibited" instead of "completely non-functional" as inhibited is more correct.

4. The authors make a statement regarding the mitochondrial dynamics in BaxS184 mutant expressing cells. However, the microscopy images are not of sufficient resolution to report this and there is no image analysis. As this is not referred to elsewhere and the relevance is unclear I would recommend removing the statement.
Resolution was reduced when converted to a PDF of the size required for upload. In the new version the resolution should be improved and co-localization analysis was performed. The statement referring to mitochondrial dynamics was removed as we agree that it was not relevant.

5. Pge 7, "...using fluorescence spectroscopy and (with) purified..."
This has been corrected

6. Statistical analyses should be performed on the quantitative data in Figure 4. Is the Akt inhibitor MK2206 significantly different for example?
This was done. Stats are now listed in the figure captions and in table EV1 and table EV2 where indicated in the text.

7. "...Akt levels makes logical sense..."
This portion of the sentence was removed.

8. Figure 4E, the authors should confirm that Akt has no effect on non-phosphorylatable S184A to exclude that Akt is limiting MOMP/apoptosis in a Bax-independent fashion.
This is a great suggestion. We observed, as expected, that Akt has no effect on Bax S184A (Figure 3G)

9. In EV4D, in order to compare the Bax/BaxP to the GSK standard curve, the Bax and GSK-3 should be run on and analysed on the same gel.
We were not satisfied with the quantification of pBax so this was removed. Quantification of immunoprecipitated products by western blotting is problematic – even with the newer antibody. We left in the blots for Bax to show qualitative data indicating that Bax is phosphorylated.

10. In their final model the authors depict Bax-P on mitochondria. However, they (and others) have shown that S184E phosphomimetic cannot target membranes?
We show here that under apoptotic conditions a fraction of Bax-S184E is at the mitochondria and that a fraction of Bax is located at the mitochondria in the ABT-737 resistant cells. We predict that the majority of phosphorylated Bax would inhibit BH3 proteins in the cytosol but if Bax binds to BH3 proteins that are anchored on mitochondria (Bim and Bid independently bind to mitochondria) then we would expect to find phosphorylated Bax on mitochondria. In this situation phosphorylated

Bax would be peripherally bound to the bilayer via interaction with membrane bound BH3 proteins. The text in the caption for figure 7 was changed to make this clear.

Referee #3:

The authors report that phosphorylation of Bax at S184, or a phosphomimetic form of Bax in that position, changes the activity of Bax from pro-apoptotic to anti-apoptotic. They propose a molecular mechanism based on the binding and sequestration of BH3-only proteins in the cytosol, so that they cannot activate other non-phosphorylated Bax molecules, neither inhibit the prosurvival Bcl-2 members. The results are new and interesting for the field, and the work is elegantly done and clearly explained.
We thank the reviewer for their positive comments.

However, there are a number of issues that should be addressed:

1) The main novelty of the study regards the switch of Bax to an antiapoptotic proteins by sequestration of BH3 only proteins in solution. The authors show that phosphorylated or phosphomimetic Bax at S184 decrease the activity of wild type Bax in vitro and in cells. However an important control that is missing is a mutant version of Bax that is not able to bind specifically BH3 only proteins. Despite phosphorylation, or phosphomimetic mutation, this form of Bax should not be able to show this effect.
Initially this seemed like a good control however we think the data would be hard to interpret. If we used a mutant Bax that could not bind BH3 proteins and the data showed that there is still protection it could be that the phosphorylated form of Bax or the phosphomimetic mutation is still binding BH3 proteins, either differently or at another location on Bax such as the rear pocket proposed by Gavathiotis and Walensky. If the data showed that there was no longer protection then it could suggest that this is a Bax specific effect. Or that the combination of mutants resulted in a non-functional protein. Due to the number of extra experiments required to generate what would likely be an ambiguous result we felt the alternate control of using Bax S184A as suggested by both reviewer 1 and 2 would be sufficient. We show that this mutant can no longer be phosphorylated (Figure 2B, EV1B), that it cannot prevent Bim or cBid mediated liposome permeabilization (Figure EV4A) and it cannot protect cells from apoptosis (Figure 5B, EV4B). Importantly when Bax S184A is incubated with mitochondria, tBid and Akt there is no protective effect observed suggesting that Akt acts specifically on Bax S184 (Figure 3G).

In addition, it would be interesting to show a quantitative correlation between Bax phosphorylation and ABT-737 sensitivity. This could be done by connecting the analysis in Figure 1B with quantification of Bax phosphorylation levels in the different cell lines.
This is a very good idea particularly as this requires only relative as opposed to absolute quantification. Therefore, we extended our experiments to include analyzing cancer cells isolated from ovarian cancer patients. We found that pBax S184 levels correlate with cellular resistance to ABT-737 in primary patient samples (Figure 6A, 6B). This seems the most directly relevant approach to test the query raised.

The authors report an antiapoptotic function of soluble Bax, but do not take into account neither discuss recent reports on the inhibitory dimerization of Bax in the cytosol. How is this new antiapoptotic function of Bax related to its dimerization in the cytosol? Is phosphorylated Bax more or less dimerized than the non-phosphorylated form? Is this affected by binding to BH3-only proteins? Could the dimerization of Bax in the cytosol be part of the same mechanism for apoptosis inhibition that the binding to BH3 only proteins?
This would be very interesting to determine however we did not assess Bax dimerization status from cells or in our liposome based experiments. Results of these experiments would not change our conclusions in this paper since regardless of the dimerization status of Bax, our results suggest that the protein sequesters BH3 proteins thus protecting cells from apoptosis. In Garner et al they show that purified recombinant Bax is mainly monomeric but can be forced into dimers via concentrating the protein or by point mutations (Garner et al, 2016). Further, they state that the affinity for Bax dimerization is 32 μM, however in our assays the Bax concentration is 100 nM such that Bax would exclusively be monomeric. The concentration of Bax in cells is estimated to be in the nanomolar range as well (Polster et al, 2003) and overexpression of fluorescent protein fusion constructs of Bax

are estimated to be 3 μM (Dussmann et al, 2010) – both well below the affinity for dimers. Although it is a possibility that Bax dimerization plays a role in the mechanism we think that the full examination of the issue required for the results to be interpretable extends well beyond the conclusions of the current paper.

Related to this, does phosphomimetic Bax bind to Bcl-xL in solution.
This is another point that would be interesting to determine however irrespective of the outcome the net effect of expression of phosphomimetic Bax is inhibiting apoptosis. Therefore, this data would require extensive further experimentation to provide interpretation that extends beyond the current paper. For this reason we did not assay Bax binding to Bcl-XL due to time constraints and the number of experiments required for revision.

In Figure 3B, a control experiment is missing for DKO + BaxS184E. Does this mutant form of Bax retain any prodeath activity?
This is a great point. This important control has now been included (Figure 5B, EV4C). The mutant of Bax does not retain pro-death activity when cells are treated with TNF-alpha or panobinostat but interestingly does have a small amount of pro-apoptotic activity when cells are treated with STS. Since STS is a pan-kinase inhibitor, many unknown changes occur within the cell. These alterations may result in the possibility that Bax-S184E can in response to some stimuli be slightly pro-apoptotic. This may relate to non-BH3 mediated activation of Bax as previously reported (Brahmbhatt et al, 2016). However, when expressed in cells containing endogenous Bax and Bak, this construct still protects cells from apoptosis enough that the anti-apoptotic activity outweighed any pro-apoptotic activity.

Minor comments:

In general, the cells in the images are too small. They should be shown with a zoom in too. In figure 1D, why is the phosphomimetic Bax in the nucleus? In these images it is impossible to assess mitochondrial morphology. The authors should quantify all of this with proper image analysis. Lower panel: It is not clear what WT means and in the figure legend, it is not described.
Thank you for pointing this out. Images were cropped and zoomed in to individual cells to aid in visual inspection of Bax localization. The resolution for these images has been improved. We performed co-localization analysis on the images and calculated Pearson's correlation coefficients (Figure 2C and 2D). For Figure 2D, WT was replaced with untransfected and further clarification was included in the figure caption. The statement on mitochondrial morphology has been removed. It is unclear why Bax is in the nucleus however it has been observed before in numerous publications. We performed subcellular fractionation experiments (Figure EV1C and 4D) and observed that both exogenously expressed GFP-Bax and endogenous Bax are present in nuclear fractions. Interestingly a recent publication showed that Bax can play a role in the nucleus of healthy cells where it can influence cell growth and differentiation (Brayer et al, 2017)

Fig 2.B is it possible to include the Fret change of S184A in Liposomes and solution, which should give us a clear picture of its behavior as that we expect in cells shown in Fig 1. D?
We included this important control both in our liposome based (Figure EV3) experiments and cell based experiments (Figure 5, EV4). These experiments show that Bax-S184A is functional and promotes membrane permeabilization/apoptosis.

Figure 2E: The % Liposome Permeabilization of Bax-S184E should be included in the figure.
We agree that this data needed to be clearer. The figure has been changed to include the WT Bax and WT-Bax with the S184E mutation (Figure 4E). Data for additional control experiments where the function of the single cysteine mutant and NBD-labeled mutants are assayed have been moved to the supplementary (figure EV3E).

Figure EV4B: How do you explain the localization of Akt in the cytosol and on mitochondria in all cell lines after treatment with A-443654?
A-443654 paradoxically promotes the phosphorylation of AKT but also inhibits the protein. The original demonstration of this phenomenon has been referenced as follows:

"In contrast, A-443654, promoted Akt localization to mitochondria, however this drug is known to promote paradoxical Akt phosphorylation, indicative of active Akt, with concomitant Akt kinase inhibition (Figure EV2C) (Luo et al., 2005)."
Our interpretation of the localization data is that phosphorylation is important for mitochondrial localization of AKT.

Table S1: The table is missing.
The table (now EV3) is located on the last page of the manuscript, as required by EMBO Reports, and not with the figures.

Page 6, paragraph 1: We found that Bax is phosphorylated in ABT-737 resistant MDA-MB-468, and ZR-75-1cells, but not in ABT-737 sensitive MDA-MB-435 cells (Figure 1C, 4E). There are no results in the suggested figures showing data in the cells ZR-75-1. Figure 4E doesn´t support the statement that Bax is phosphorylated in ABT-737 resistant MDA-MB-468, and ZR-75-1cells, but not in ABT-737 sensitive MDA-MB-435 cells.
We apologize for this. There was a typographical error in the main text that resulted in our referring to the wrong figure. The data was always included and we now reference the correct figure.

Page 7, paragraph 3: .... Bax S184E bound to both cBid (Figure 2B) and Bim (Figure EV2A) in the absence and in the presence of membranes. Figure EV2A shows only interaction on membranes and not in solution. This figure doesn´t suggest the statement.
This was a typographical error. The text has been altered to "Unexpectedly, Bax S184E bound to both cBid (Figure 4B) and Bim (Figure EV3B)."

Page 9, paragraph 3: Stable Bax-S184E expression in WT BMK cells had a protective effect against cell death induced by the pro-death cytokine TNF-α and cycloheximide, ... Are the cells treated in the same time with TNF-α and cycloheximide? If yes, the description in the legend should be included. If not, data has to be shown in the figure.
Yes they were treated at the same time. This has been clarified in the figure caption.

The explanation of the different inhibition modes should be explained better, for the general reader.
The sentence explaining the modes of inhibition has been changed to "Anti-apoptotic proteins have two modes of inhibition of MOMP (Llambi et al, 2011). Mode1 inhibition occurs when anti-apoptotic proteins sequester BH3-proteins, thus preventing BH3-mediated activation of Bax. Mode 2 inhibition occurs when anti-apoptotic proteins bind to active Bax and Bak, thus inhibiting their oligomerization. Both modes result in inhibition of MOMP and subsequent cell death."

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2nd Editorial Decision 1 June 2018

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see all three referees are positive about the study and support publication in EMBO reports after minor revision. Referee 3 further suggested testing the interaction between cytosolic Bax-S184E and Bid in solution using FRET measurements in cells. I have discussed this suggestion further with the referees. Both referee 2 and 3 indicated that this cell-based experiment would strengthen the manuscript, yet none of the referees considers it essential for publication, also since you have done similar experiments using recombinant proteins. I therefore suggest to include this particular experiment only if the plasmids are available and if the experiments are doable within short time.

Browsing through the manuscript myself, I also noticed a few things that we need before we can proceed with the official acceptance of your manuscript:

- Please reformat the references according to the numbered style of EMBO reports. You can download the respective EndNote file from our Guide to Authors https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

- Please note that the title may not exceed 100 characters incl. spaces.

- Please remove the Expanded View tables from the main manuscript file and either upload them as Excel file (Table EVx) with the legend in the first line of the file or alternatively, provide them as Appendix in a single pdf with a title page and a table of content including page numbers. I think the latter option might be better.

- Our data editors from Wiley have inspected the figure legends for completeness and accuracy.

Please see their suggestions as "track changes" in the attached Word file.

I look forward to seeing a final version of your manuscript as soon as possible.

****
REFEREE REPORTS

Referee #1:

The authors have performed an excellent revision of their MS and have adequately addressed all my comments

Referee #2:

Overall, the authors have addressed, or attempted to address, the issues raised.
The detection of Bax P-S184 is key to the manuscript. As they could not detect this modification by mass spec, the new data with a published Bax P-S184-specific Ab is very important. A specific point, the authors should show total Bax IP'd and in lysates in EV1A as they have in 6A for the ovarian cancer lines and in EV1B. It is unclear what EV1B adds beyond what is shown in new Figure 2B?

Referee #3:

The authors have addressed adequately the reviewers' concerns.
Two points:
-The Pearson's coefficient analysis and image quantification supposedly belonging to figure 2 are not there.
-Could the authors detect an increased interaction between cytosolic Bax-S184E and Bid in solution by using FRET measurements in cells as they have done before? This should help solve one of reviewer 2 concerns.

2nd Revision - authors' response 11 June 2018

Response to Reviews 6/10/2018

Editorial comments:

"As you will see all three referees are positive about the study and support publication in EMBO reports after minor revision. "
We are glad that only minor revisions were requested and have made the changes as suggested.

"Referee 3 further suggested testing the interaction between cytosolic Bax-S184E and Bid in solution using FRET measurements in cells. I have discussed this suggestion further with the referees. Both referee 2 and 3 indicated that this cell-based experiment would strengthen the manuscript, yet none of the referees considers it essential for publication, also since you have done similar experiments using recombinant proteins. I therefore suggest to include this particular experiment only if the plasmids are available and if the experiments are doable within short time."
Thank you. Below, under our response to reviewer 3 we explain why, although this is a great idea, we cannot perform this experiment in a short time.

"Browsing through the manuscript myself, I also noticed a few things that we need before we can proceed with the official acceptance of your manuscript:
- Please reformat the references according to the numbered style of EMBO reports. "
The references have been changed as required.

"- Please note that the title may not exceed 100 characters incl. spaces. "
We have changed the title to "Phosphorylation switches Bax from promoting to inhibiting apoptosis increasing drug resistance"

"- Please remove the Expanded View tables from the main manuscript file and either upload them as Excel file (Table EVx) with the legend in the first line of the file or alternatively, provide them as Appendix in a single pdf with a title page and a table of content including page numbers. I think the latter option might be better. "
We have moved the tables to the appendix and saved as a single pdf as suggested.

"- Our data editors from Wiley have inspected the figure legends for completeness and accuracy. Please see their suggestions as "track changes" in the attached Word file. "
All of the comments were addressed and changes made as appropriate
****

Referee #1:

The authors have performed an excellent revision of their MS and have adequately addressed all my comments
Thank you

Referee #2:

Overall, the authors have addressed, or attempted to address, the issues raised.
Thank you

The detection of Bax P-S184 is key to the manuscript. As they could not detect this modification by mass spec, the new data with a published Bax P-S184-specific Ab is very important. A specific point, the authors should show total Bax IP'd and in lysates in EV1A as they have in 6A for the ovarian cancer lines and in EV1B. It is unclear what EV1B adds beyond what is shown in new Figure 2B?
We agree this is an important control. We have added the control blots for total Bax IP'd and the input control for Bax and Actin to Figure EV1A and EV1B. EV1B adds 1) that we also cannot detect the specific pS184 modification on Bax when residue S184 is mutated and 2) that GFP-Bax can be specifically phosphorylated at S184 versus some other serine residue. We opted to include these blots as further confirmation for the specificity of the new P-S184 antibody.

Referee #3:

The authors have addressed adequately the reviewers' concerns.
Thank you

Two points:

-The Pearson's coefficient analysis and image quantification supposedly belonging to figure 2 are not there.
The Pearson's correlation analysis performed on the images shown in Figure 2C has been added to Appendix Figure S1. Additionally, the Pearson's coefficients are listed in the figure legend. We have changed the figure caption to clarify this further: "Whole image Pearson's correlation coefficients between MitoTracker and GFP for the single images shown were 0.644, 0.607 and 0.206 for GFP-Bax, GFP-Bax S184A, and GFP-Bax S184E respectively".
The manuscript includes a much more extensive and robust analysis of the localization of the fluorescent protein fused Bax constructs in Figure 5D.

-Could the authors detect an increased interaction between cytosolic Bax-S184E and Bid in solution by using FRET measurements in cells as they have done before? This should help solve one of reviewer 2 concerns.

This is an excellent suggestion and FLIM FRET for Bax is something we have been working on. However, since BaxS184E is poorly localized to mitochondria the FLIM data is hard to segment into defined regions of interest. As a result the exponential decays that have to be fit are not monoexponential as they are when we can segment interactions at the mitochondria, as we have done in the past for Bcl-XL. We are working on solving both the segmentation the interpretation of data fit to a double exponential. Until we do more control experiments we cannot be completely confident in our interpretation of the data. Therefore, doing the requested experiment while very interesting is still months away.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: David Andrews
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45235V2

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 $\chi 2$ 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?	For most experiments we arbitrarily decided on 3 independent experiments with a minimum of two technical replicates.
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?	Data was not excluded from analysis.
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Yes but only in the case of image data. Image acquisition and analysis was automated using Harmony software for figures 5B, 5C, 5D and EV4C, EV4D, EV4E.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	See methods under statistical analysis for further details.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We assume normal distributions - the number of independent experiments (n=3) is too small to assess normal distributions. We did not assume equal variances. Equal variance was tested in GraphPad prism using the Brown-Forsythe test. See methods under statistical analysis for further details.
Is there an estimate of variation within each group of data?	Yes, where indicated standard error of the mean was used, otherwise individual data points for each independent replicate are shown
Is the variance similar between the groups that are being statistically compared?	Equal variance was tested in GraphPad prism using the Brown-Forsythe test. All data being compared passed this test with the exception of data in Figure 5D. In figure 5D we used Welch's ANOVA and Welch's t-tests which do not assume equal variances. See methods under statistical analysis for further details.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tun

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

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).	All antibodies, with catalog numbers, are listed in the methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cancer cell lines were STR profiled and all cell lines were routinely tested for mycoplasma contamination

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The ethics committee of Baskent University School of Medicine (KA15/230)
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.	The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from all participants.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	NA
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).	NA
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).	NA
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 Biomodels (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.	NA

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.	NA