EMBO reports

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The deubiquitinase Usp27x stabilizes the BH3-only protein Bim and enhances apoptosis

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Transaction Report:

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

1st Editorial Decision 28 October 2015

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, the referees acknowledge that the findings are potentially interesting. However, referees 1 and 2 also point out several technical concerns and conflicting data and have a number of suggestions for how the study should be strengthened. As the reports are listed below I will not detail them here but I think that all of them should be addressed. All missing control experiments have to be provided. Both referees remark that the description of the SILAC experiments is confusing and suggest a better documentation of the Mass spec experiment.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns 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.

For a normal article there are no length limitations, but the results and discussion section must be separate and the entire materials and methods included in the main manuscript file.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

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.

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.

REFEREE REPORTS

Referee #1:

This manuscript (EMBOR-2015-41392V1) by Weber et al. is focused on the deubiquitinating enzyme Usp27X and its role in Bim stability and enhancement of apoptosis. The report is of potential interest and significance; however; there appears to be conflicting data in the manuscript and several concerns and issues warrant further attention.
- Though the paper is based on identification of enriched Usp27x by mass spec, no data is shown. It would be interesting to know the fold enrichment of Usp27x in the original BimEL IP versus control, and therefore it would be nice to see the profiles (at least in the supplemental). Also, the description of the SILAC experiment is confusing.
- In introduction (page 3, paragraph 2), the authors state that Bim is phosphorylated by Aurora kinase, then ubiquitinated by APC/CCdc20. This does not follow canonical APC/CCdc20 substrate recognition. Rather, this sounds more like the SCF, which recognizes phosphodegrons in its substrates. The APC/CCdc20 recognizes the D box sequence (RXXLXXXXN). The authors go on to analyze βTRCP interaction with Bim, which is a cofactor of the SCF. They never revisit APC/CCdc20.
- In discussion (page 11, paragraph 3), the authors suggest that the expression levels of Usp27x, Bim, and βTRCP regulate the activity of the complex formed by these 3 proteins. βTRCP activity is dictated by the kinase upstream of its substrates, so Aurora kinase activity would need to be considered in the case of Bim.
- Figure 1B: A mutant BimEL protein that supposedly cannot bind to other Bim interacting partners (namely Bcl-2 family proteins) is used to demonstrate Bim directly binding to Usp27x. Little conformation is shown that these proteins are absent from the complex (Bcl-2/Bcl-xL/Mcl-1).
- Figure 1C: The involvement of the E3-ligase is interesting. Is the ligase still active while in complex with Bim and Usp27x? Does the DUB inhibit the ligase or reduce its association with the complex? There is no reverse IP to show if overexpressing this DUB affects the quantity of ligase bound to Bim. These are probably out of the scope of the paper but it seemed counter intuitive that the ligase too remains associated with the complex.
- Figure 3A: In this figure, we see overexpression of Usp27x stabilizing Bim protein levels after treatment with PMA (PMA is used to induce ERK activation). The inability of the mutant Usp27x to stabilize Bim levels is adequate but I find several things in this panel concerning. First, overexpression of Usp27x in the presence of PMA brings Bim levels back to the level of the negative controls. Yet, it is stated that it has a large apoptotic effect on the cells. To counteract this, they treat with the apoptosis inhibitor QVD. If the increased apoptosis is due to Bim stabilization, why do they not have to treat their control cells with QVD to prevent death since they have similar Bim levels (either there must be another major target or stabilized (p)Bim after ubiquitination is more potent)? Minimally, does treatment with QVD and overexpression of Usp27x alone increase

the levels of Bim? This control is blatantly missing. Also, it suggests that the mutant overexpressing cells in the presence of PMA are not apoptotic. Is this true?
Secondly, early in the paper they determine the interaction between Bim and Usp27x to be exclusive to the BimEL isoform. However, in this experiment it seems obvious that the BimL isoform is also being stabilized by overexpression of Usp27x. This isn't addressed.
Finally, the level of overexpression in the experimental lane is much higher than the control (also has several other unmentioned bands). Therefore, I question whether higher levels of Usp27x overexpression may be able to increase Bim without PMA, or perhaps QVD is having an affect on Usp27x itself. Again, calls for the missing control.
In Figure 3B, they could validate whether the shifted band is phosphorylated Bim by addition of an ERK inhibitor.
- Figure 6. We are asked to compare exposures from what appears to be two separate membranes? Also, in panel A the control isn't even on the same blot. It would be more convincing if replicate experiments showed significance. Why is BimEL so high in the first lane? There is no Usp27x, so what is stabilizing its expression in 1205Lu cells? If Bim is being expressed at that level, why do the cells not apoptose? Why is QVD not required in this experiment?
- Figure 7. To me, this figure highlighted the existence of another major path being affected other than Bim (particularly panel B). Usp27x expression increases active caspase-3 alone in both cell types with no inhibition of prosurvival pathways, and this increase is not reversed by Bim protein reduction. Similarly, knocking down Bim levels appears to reduce the apoptotic population driven by gefitinib but to me it does not look like Bim protein reduction has any affect on Usp27x function. The rescue does not look additive when comparing Gefitinib and dox+Gefitinib after reducing Bim. They do consider alternative pathways in the discussion but this significantly weakens the paper.
- Supplemental: They attempt to show that the proteins could co-localize, which is a necessary addition to the paper. Obviously, there is no antibody effective for immunofluorescence for Usp27x, but in addition to the GFP tagged-Usp27x it would be nice to see Bim localization (instead of relying on mitotracker alone). Also, they state that Usp22 is exclusively found in the nucleus; however, several cells show cytosolic staining. Should this be interpreted to mean that Usp22 may additionally be found in the cytosol or that the tag makes localization determination unreliable?

Referee #2:

The manuscript describes the identification of the deubiquitinating enzyme Usp27x as regulator of the stability of the pro-apoptotic Bcl2 family protein Bim. The authors by mass-spectrometry identified Usp27x as binding factor of Bim Further analysis shows that Usp27x by deubiquitinating Bim protects it from proteasomal degradation induced by activation of the Raf/ERK pathway. Finally, Usp27x induces apoptosis in a series of model cell lines upon inhibition of the Raf/ERK pathway.

The manuscript is interesting as no many functions for Usp27x have been reported and additionally our knowledge on the regulation of pro and anti apoptotic factors by the ubiquitin system is rapidly growing.

The manuscript is well presented and the data are overall convincing. The major issue is that with the exception of one experiment all data are based on overexpression of Usp27x. The reviewer acknowledges the difficulties in studying the biological function of deconjugating enzymes by knockdown experiments. Have the authors tried to make a knockout with CRISPR/CAS9? Or to study the apoptotic defects upon knockdown of Usp27x?

The description of the proteomics analysis is very confusing. Based on the manuscript by Frank et al. 2015 mitochondria enriched extracts from light and heavy media were used for IPs before mixing the precipitates and ms analysis. In the current manuscript it appears that control cells were labelled with light media and the HA-tagged with heavy, but in the study by Frank et al. 2015 it is the opposite. The list of Bim interactors in the study by Frank et al. 2015 is very limited and Usp27x is not present. These details should be provided in supplementary information with normalised SILAC ratios and number of identified and quantified peptides should be presented.

Fig.1 The model of interaction between Usp27x and Bim is not clear. Is the interaction of Usp27x to Bim regulated by PMA or other used stimulus? Does it depend on bTrCP? This is quite relevant for

the experiment in Fig 3C as overexpression of Usp27X promotes de-ubiquitination of Bim, which presumably is b-TrCP dependent. What is the effect of Usp27x on bTrCP levels? Many DUBs indirectly control the substrate through regulation of its E3-ligase stability. In 1B the authors should show that in these conditions the used Bim mutant (DD) is indeed deficient in binding to anti-apoptotic factors.

Fig3. In A, 48h panel 4th lane there is a band at 100kDa. Any idea of the nature of this species? In B the loading control is not good at all. In the ubiquitination assay in C, in the eluate the band at 35kD cannot be the ubiquitinated form of BimEL as it migrates identically to the unmodified form in the input. Does BimEL stick non-specifically to the column?

Fig.4 The authors should provide a control of overexpression of another similar to Usp27x DUB.

Fig.5 This is the only experiment addressing the effect of Usp27x knockdown. Has this approach been tested in the measurement of Bim half-life (Fig.6)?

Fig.6 The effects are not terribly convincing and statistics on the quantification should be provided. Have the authrors performed half-life experiments upon stimulus induced degradation of Bim? Additionally, 6hrs of CHX treatment is quite long and if possible pulse-chase experiments should be applied.

Fig7. These experiments clearly point towards a role of Usp27x for Bim function regulation but the Usp27x knockdown/knockout experiments should be tested (see above). Additionally, it is normally recommended that at least 2 different type of assays should be used for apoptotic related phenotypes.

1st Revision - authors' response 02 February 2016

Referee #1:

This manuscript (EMBOR-2015-41392V1) by Weber et al. is focused on the deubiquitinating enzyme Usp27X and its role in Bim stability and enhancement of apoptosis. The report is of potential interest and significance; however; there appears to be conflicting data in the manuscript and several concerns and issues warrant further attention.
- Though the paper is based on identification of enriched Usp27x by mass spec, no data is shown. It would be interesting to know the fold enrichment of Usp27x in the original BimEL IP versus control, and therefore it would be nice to see the profiles (at least in the supplemental). Also, the description of the SILAC experiment is confusing.

We now show the data (enrichment of isolated proteins) in Fig. S1 and supplemental table. Enrichment of Usp27x was about 183-fold. We have identified Usp27x alongside the experiment published earlier (Frank et al. 2015) and have made this now clearer in the text and suppl. FigS1A (legend). We have now also included a short material and method section of the SILAC experiment.

- In introduction (page 3, paragraph 2), the authors state that Bim is phosphorylated by Aurora kinase, then ubiquitinated by APC/CCdc20. This does not follow canonical APC/CCdc20 substrate recognition. Rather, this sounds more like the SCF, which recognizes phosphodegrons in its substrates. The APC/CCdc20 recognizes the D box sequence (RXXLXXXXN). The authors go on to analyze βTRCP interaction with Bim, which is a cofactor of the SCF. They never revisit APC/CCdc20.

We only quote APC/CCdc20 as one of the degradation-promoting pathways and machineries that have been proposed for the regulation of Bim (Wan et al., Dev. Cell 2014) but we have not ourselves worked on this. As the reviewer points out we have focused on the ERK-pathway, where the pathway from phosphorylation to ubiquitination for Bim has been well described. We are not in the position to say any more about Cdc20 and have now attempted to make this clearer in the introduction (p. 3, last paragraph).

- In discussion (page 11, paragraph 3), the authors suggest that the expression levels of Usp27x, Bim, and βTRCP regulate the activity of the complex formed by these 3 proteins. βTRCP activity is

dictated by the kinase upstream of its substrates, so Aurora kinase activity would need to be considered in the case of Bim.

We now mention this possibility in the context of such a tri-molecular complex in the discussion (p.14). However, as said above, Aurora kinase has been implicated in Bim-loss during mitosis while we focus on Bim-regulation by the ERK-pathway

- *Figure 1B: A mutant BimEL protein that supposedly cannot bind to other Bim interacting partners (namely Bcl-2 family proteins) is used to demonstrate Bim directly binding to Usp27x. Little conformation is shown that these proteins are absent from the complex (Bcl-2/Bcl-xL/Mcl-1).*

We had not done that since we have characterized this mutant in the past (Wilfling et al., 2012, as quoted in the manuscript). We now include an IP-experiment showing the loss (or very strong reduction) of binding of this mutant Bim to Mcl-1 and Bcl-X_L. There was no binding of Bcl-2 to even wt Bim in the cells used (new Fig. 1B, replacing the old figure that has now been moved to the supplement as Fig. S1C).

- *Figure 1C: The involvement of the E3-ligase is interesting. Is the ligase still active while in complex with Bim and Usp27x? Does the DUB inhibit the ligase or reduce its association with the complex? There is no reverse IP to show if overexpressing this DUB affects the quantity of ligase bound to Bim. These are probably out of the scope of the paper but it seemed counter intuitive that the ligase too remains associated with the complex*.

This is certainly an interesting aspect and we have been wondering about this ourselves. We have now done the reverse IP of Bim (rather than Usp27x) and can say that induction of Usp27x does not reduce (even appears to increase) the binding of b-TrCP to Bim (new Fig. 1D).

What we can also say is that the MEK/ERK-phosphorylation of Bim has an effect in recruiting Usp27x. This is indicated by the reduction of the binding of Bim to Usp27x when PMA-stimulated cells were treated with the MEK-inhibitor UO126 (new Fig. 1E and S2B). Since the same has been shown for the association of Bim with bTrCP (Dehan E. et al., Mol Cell 2009 as quoted in the manuscript) this is consistent with the model of DUB and E3-ligase having the same requirements (however b-TrCP is not required for Usp27x-binding to Bim, which is now shown by siRNA (anti-b-TrCP) experiments, new Fig. 1F). It is still possible that E3-ligase and DUB form a complex constitutively and are recruited together but recruitment of either protein is at least not exclusively regulated through the other.

We also have now analyzed the co-localisation of Bim and Usp27x by proximity ligation assay (new Fig. S2A). The results are also consistent with the recruitment of Usp27x to phosphorylated Bim (see discussion below regarding co-localisation).

- *Figure 3A: In this figure, we see overexpression of Usp27x stabilizing Bim protein levels after treatment with PMA (PMA is used to induce ERK activation). The inability of the mutant Usp27x to stabilize Bim levels is adequate but I find several things in this panel concerning. First, overexpression of Usp27x in the presence of PMA brings Bim levels back to the level of the negative controls. Yet, it is stated that it has a large apoptotic effect on the cells. To counteract this, they treat with the apoptosis inhibitor QVD. If the increased apoptosis is due to Bim stabilization, why do they not have to treat their control cells with QVD to prevent death since they have similar Bim levels (either there must be another major target or stabilized (p)Bim after ubiquitination is more potent)? Minimally, does treatment with QVD and overexpression of Usp27x alone increase the levels of Bim? This control is blatantly missing. Also, it suggests that the mutant overexpressing cells in the presence of PMA are not apoptotic. Is this true*?

It is correct that the levels of cells treated with PMA and expressing Usp27x, which are dying, have similar levels of Bim as untreated cells (which do not die). As we say in the manuscript this is very likely the result of the regulation of other proteins by PMA, which may or may not be directly related to the Bcl-2-family (both pro-apoptotic Noxa and anti-apoptotic Mcl-1 have also been suggested to be regulated through the ERK-pathway). We have now also generated data to the effect that PMA-induced apoptosis in the presence of Usp27x in 293FT cells at least partially relies on Bim (new Fig. 4). What the other targets of PMA-stimulation are we do not know, and we have now made this clearer in the revised manuscript (p. 10)

We have done the suggested control experiment and find that QVD and over-expression of Usp27x alone do not increase the levels of Bim in 293FT cells (this is shown in the new figures 3C and 3D), which do not have a high activity of the ERK-pathway normally, unlike the situation in the cell lines

from tumours where this pathway is constitutively strongly activated (as in the melanoma and NSCLC cells we used).

As the reviewer surmises, Usp27x, but not its inactive mutant (Usp27xC87A), kills the cells upon stimulation with PMA. We now include data to show this as the new Fig. 4A. We have further generated Bim-k.o. versions in these cells and have tested a polyclonal line as well as four individual clones for Bim-dependency of PMA/Usp27x-killing. As shown in Fig. 4, there was a statistically significant reduction of killing in the polyclonal line with targeted Bim-deletion. Of the four Bim-deficient clones three showed substantial, sometimes near-complete protection while one showed no protection at all (Fig. 4). While this is not as clear-cut as we would have wished, it does in our view suggest the involvement of Bim on one hand, but also Bim-independent effects on the other. The data are presented and discussed in this way on p. 10.

Secondly, early in the paper they determine the interaction between Bim and Usp27x to be exclusive to the BimEL isoform. However, in this experiment it seems obvious that the BimL isoform is also being stabilized by overexpression of Usp27x. This isn't addressed.

The up-regulation of Bim_L is indeed an effect that we have not fully understood. We believe that the following explanation is the most likely: Bim does normally not exist as a monomer (Bim is C-terminally inserted in the outer mitochondrial membrane) but forms initially dimers by binding to dynein light chain 1 (DLC1), and this binding leads on to the formation of larger complexes (unpublished; see data below). Since both Bim_{EL} and Bim_L can be found in the same complex we speculate that Bim_L is indirectly 'co-rescued' by Usp27x targeting Bim_{EL}. This is speculation and not trivial to show. We have done substantial work on this but have not as yet brought it to a conclusion that we would publish. We therefore at this stage can only put forward this speculation; we show below data for the reviewer's scrutiny but would prefer to leave the discussion of this issue at the following admittedly vague level:

'Although Bim-binding of Usp27x was confined to the isoform Bim_{EL} (Fig. 1G), the splice variant Bim_L also appeared to be somewhat regulated by Usp27x (Fig. 3A). Since Bim is often involved in binding to Bcl-2-family proteins this may be indicative of protection against proteolysis through such varying complexes.' (p. 8/9)

We do think this is a likely explanation but we feel we cannot really fully discuss this at this stage. We hope that the reviewer can follow this reasoning.

[Data not included in the Peer Review Process File]

Left, co-IP-experiments showing association of tagged and untagged Bim. MEFs (which have very little endogenous Bim) were made stably to express untagged Bim, HA-tagged Bim or both. Anti-HA-IP also pulls down untagged Bim; this is the same for a BimEL-BimL-interaction, not shown. *Right*, on blue-native gels Bim assembles into higher order complexes. The right lane is from cells expression a mutant of Bim that cannot bind the dimerizer DLC1 (Bim here has a V5-anibody tag). We have also re-constituted this on liposomes with recombinant protein (not shown).

Finally, the level of overexpression in the experimental lane is much higher than the control (also has several other unmentioned bands). Therefore, I question whether higher levels of Usp27x overexpression may be able to increase Bim without PMA, or perhaps QVD is having an affect on Usp27x itself. Again, calls for the missing control.
In Figure 3B, they could validate whether the shifted band is phosphorylated Bim by addition of an ERK inhibitor.

The higher over-expression is certainly noticeable. A likely explanation appears to be that PMA has an effect on the viral promoter we used or additionally stabilized Usp27x by an unknown mechanism (post-translationally). The additional lanes are indeed unexplained. These are SDS-gels, so the higher species must contain a very tightly bound moiety, perhaps poly-ubiquitin, or could be an Usp27x dimer. The lower band may be a proteolytic fragment. It is however clear that the appearance of both bands requires activity of Usp27x since we never observe it in the mutant (see also Fig S5A). We now comment on this (although we are unable to explain it) in the legend to figure 3.

QVD does not have any effect on Usp27x itself (as already discussed above), but we here show an additional experiment demonstrating that in the absence of PMA Usp27x has no stabilizing effect on Bim. Here we transfected Usp27x or its inactive mutant into 293FT cells that resulted in high Usp27x levels. Again, we see the two bands mentioned above but, without PMA, no change in Bim-levels (new Fig. S5A). The missing QVD-control is included in the new Fig. 3C, D (and it has no effect). In this figure (3C) we also include the detection of phospho-(Ser69) Bim (this is the ERK-phosphorylation site). This is, as expected, strongly increased by PMA (although a faint band is always detectable). Again, PMA reduces Bim and Usp27x stabilizes it. The stabilization of the phosphorylated form by Usp27x-expression is very clear. The shift in the gel is seen, corresponding to the appearance of phosphorylated Bim and this shift is clearly reduced by the addition of the MEK-inhibitor UO126 (new Fig. 3D, S5D).

- Figure 6. We are asked to compare exposures from what appears to be two separate membranes? Also, in panel A the control isn't even on the same blot. It would be more convincing if replicate experiments showed significance. Why is BimEL so high in the first lane? There is no Usp27x, so what is stabilizing its expression in 1205Lu cells? If Bim is being expressed at that level, why do the cells not apoptose? Why is QVD not required in this experiment?

We are sorry not having been clearer in the description of the blots. Naturally the data shown are from the same blot and the very same exposure of the blot, it is only that we have cut out an area in between. This has now been made clear in the figure legend (now Fig. 7B).

The Bim levels are not actually high, this impression from the figure is only due to the higher sensitivity/longer exposure we used here. We now include a comparison of the Bim-levels of the melanoma cells and the 293FT cells as the new Fig. S6G. It is clear from that figure that the levels of Bim are actually much lower in the melanoma cells although they appear high when the conditions of detection are sensitive as in Fig. 7B.

The point about stabilization in the presence of cycloheximide is now made stronger by including additional experiments and quantifying the levels of Bim in all experiments (the figure is now Fig. 7). New Fig. 7A shows stabilization of Bim in 293FT cells overexpressing Usp27x and stimulated with PMA; Fig. 7B shows results with 1205Lu melanoma cells (one new experiment added; active BRAF) and Fig. 7C HCC827 cells (active EGFR). In all three cell lines Usp27x stabilized Bim.

We have further now made a CRISPR/Cas9-k.o. of Usp27x in 293FT cells. This cell line did show a faster loss of Bim upon treatment with PMA; the effect was not dramatic but clear in three separate experiments after 16 and 24 h (Fig. 6B) and again after 24 h (Fig. S6F).

- Figure 7. To me, this figure highlighted the existence of another major path being affected other than Bim (particularly panel B). Usp27x expression increases active caspase-3 alone in both cell types with no inhibition of prosurvival pathways, and this increase is not reversed by Bim protein reduction. Similarly, knocking down Bim levels appears to reduce the apoptotic population driven by gefitinib but to me it does not look like Bim protein reduction has any affect on Usp27x function. The rescue does not look additive when comparing Gefitinib and dox+Gefitinib after reducing Bim. They do consider alternative pathways in the discussion but this significantly weakens the paper.

We acknowledge the validity of this concern and have done a number of experiments to address this problem. The data describing apoptosis-induction in 293FT cells (showing an effect of Bim in some but not all cells) were discussed above (Fig. 4). We have also now added data using a Bim-k.o. in 1205Lu melanoma cells (new Fig. 8A). We have to add that the UO-dependent killing we got in these new experiments is much higher than in the previous set. This coincides with buying a new batch of UO126 (new formulation). UO126 is notoriously unstable, so it may be that the old one was off.

In these new conditions we get reduction of killing upon Bim-k.o. not only for UO126 but also clearly for Usp27x/UO126. The killing by Usp27x-expression in the presence of UO126 is clearly reduced in Bim-deficient cells. We believe that this does make the case stronger. There is no getting away from the fact that Bim is not the only factor that drives Usp27x-mediated killing, as also discussed above for 293FT cells. However, the data seem clear now that part of the effect is due to Bim.

Lastly, we targeted Usp27x-k.o. in the NSCLC cells using CRISPR/Cas9 and established polyclonal lines with two separate gRNAs. Both cell lines were significantly protected against killing through gefitinib (Fig. 9), which is Bim-dependent (see for instance Cragg et al. as quoted in the manuscript)).

- Supplemental: They attempt to show that the proteins could co-localize, which is a necessary addition to the paper. Obviously, there is no antibody effective for immunofluorescence for Usp27x, but in addition to the GFP tagged-Usp27x it would be nice to see Bim localization (instead of relying on mitotracker alone). Also, they state that Usp22 is exclusively found in the nucleus; however, several cells show cytosolic staining. Should this be interpreted to mean that Usp22 may additionally be found in the cytosol or that the tag makes localization determination unreliable?

The specific question is difficult to answer, and we have now discussed this in the manuscript (p. 8 upper paragraph); either possibility may be valid. We refer to literature data when we say we expect it in the nucleus. We now have toned this down and say 'almost exclusively' and suggest that the cytosolic localization may be due to the GFP-tag or to the high levels of expression.

It is difficult to be sure about co-localization with microscopy alone. We have therefore, as already mentioned above, done a proximity ligation assay (PLA). This assay detects proximity of two proteins (meaning they are no more than 30 nm apart). There was a small signal in untreated, Usp27x-over-expressing 293FT cells, which was strongly enhanced by PMA, suggesting recruitment to Bim upon its ERK-dependent phosphorylation (Fig. S2A). We also add a stain for Bim as suggested (Fig. S4B).

Referee #2:

The manuscript describes the identification of the deubiquitinating enzyme Usp27x as regulator of the stability of the pro-apoptotic Bcl2 family protein Bim. The authors by mass-spectrometry identified Usp27x as binding factor of Bim Further analysis shows that Usp27x by deubiquitinating Bim protects it from proteasomal degradation induced by activation of the Raf/ERK pathway. Finally, Usp27x induces apoptosis in a series of model cell lines upon inhibition of the Raf/ERK pathway.

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The manuscript is well presented and the data are overall convincing. The major issue is that with the exception of one experiment all data are based on overexpression of Usp27x. The reviewer acknowledges the difficulties in studying the biological function of deconjugating enzymes by knockdown experiments. Have the authors tried to make a knockout with CRISPR/CAS9? Or to study the apoptotic defects upon knockdown of Usp27x?

This is clearly a valid point, which we had not considered sufficiently earlier. We have now done a CRISPR/Cas9 knock-out in 293FT and in HCC827 cells. In 293FT cells we obtained one clone with a mutation in Usp27x (new Fig. S6H). In these cells the PMA-induced loss of Bim was somewhat more pronounced than in the maternal line (we show three separate experiments in Fig. 6B and Fig. S6F). Since 293FT cells die only when we over-express Usp27x in the presence of PMA (new Fig. 4) we cannot test apoptosis in this system. We therefore generated two polyclonal lines of the NSCLC line HCC827, using two different gRNAs to target Usp27x. In both lines cell death induced by inhibition of the ERK-pathway (by the EGFR-inhibitor gefitinib) was substantially reduced (Fig. 9). This form of cell death strongly depends on Bim, and the pro-apoptotic effect of Usp27x in this situation supports the model that a main target is Bim.

The description of the proteomics analysis is very confusing. Based on the manuscript by Frank et al. 2015 mitochondria enriched extracts from light and heavy media were used for IPs before mixing the precipitates and ms analysis. In the current manuscript it appears that control cells were labelled with light media and the HA-tagged with heavy, but in the study by Frank et al. 2015 it is the opposite. The list of Bim interactors in the study by Frank et al. 2015 is very limited and Usp27x is not present. These details should be provided in supplementary information with normalised SILAC ratios and number of identified and quantified peptides should be presented.

We apologize for possible confusion. Upon re-reading this passage we realize that it may have been confusing that we used the term 'heavy membranes' for the mitochondrial fractions, at the same time as the 'heavy labeling'. We have endeavoured to make this clear now both in the text and in the figure legend. Again, the Usp27x protein was identified alongside the same experiment published

earlier (Frank et al., 2015) and 3xHA-Bim_{EL} was light labeled, whereas the untagged Bim_{EL} was heavily labeled.

The normalized SILAC-ratios are now provided as Fig. S1A. The supplemental table shows the number of peptides identified with their score and PEP values. The list of co-purified, SILAC-identified peptides is of course much longer. However, we prefer to show only the ones (suppl. table) that we have indeed validated by further studies, and this we have not done systematically.

Fig.1 The model of interaction between Usp27x and Bim is not clear. Is the interaction of Usp27x to Bim regulated by PMA or other used stimulus? Does it depend on bTrCP? This is quite relevant for the experiment in Fig 3C as overexpression of Usp27X promotes de-ubiquitination of Bim, which presumably is b-TrCP dependent. What is the effect of Usp27x on bTrCP levels? Many DUBs indirectly control the substrate through regulation of its E3-ligase stability. In 1B the authors should show that in these conditions the used Bim mutant (DD) is indeed deficient in binding to anti-apoptotic factors.

We have done a number of additional experiments, and in the sum of the data we believe the model is now much clearer. The requested control is now shown in the new Fig. 1B (Bim-mutant shows no binding to Mcl-1 and strongly reduced binding to Bcl-XL; there is no binding of even wt Bim to Bcl-2 in these cells).

We had already shown that Bim-Usp27x binding is independent of deubiquitinase activity of Usp27x. We now add data to show that phosphorylation of Bim is a major stimulus for the recruitment of Usp27x. Upon stimulation of 293FT cells with PMA, Usp27x binds but this binding is strongly reduced when the MEK-inhibitor UO126 is added (new Fig. 1E, and new S2B). As shown in new Fig. 3C, an antibody against phospho-(Ser69)-Bim detects a weak band at steady state, and this band is strongly increased upon PMA-stimulation in the presence of Usp27x expression under Bim-degrading conditions (PMA).

We further did a proximity ligation assay (PLA), which detects proximity of two proteins (distance under about 30 nm). Using the PLA we find that there is a small amount of Usp27x associated with Bim at steady state, and the signal is strongly increased when PMA is added (new Fig. S2A). A similar pattern is seen by IP (pull-down of Usp27x, detection of Bim (new Fig. S2B).

bTrCP associated with Bim in the absence of Usp27x although the association appeared somewhat stronger in its presence (new Fig. 1D). The same was the case in the reverse direction (RNAi against bTrCP did not reduce Usp27x binding to Bim, new Fig. 1F). The recruitment of bTrCP to Bim has been reported to be dependent on phosphorylation of Bim in the ERK-pathway (Dehan et al., Mol Cell 2009). There was no effect of Usp27x-expression on bTrCP-levels (new Fig. 3C). The likely model thus now appears to be that Bim-phophorylation is a signal for recruitment of both bTrCP and Usp27x, and the expression levels of these two proteins may determine the level of Bim. There is however no indication that Usp27x regulates levels of bTrCP (at least there is no reduction in total bTrCP levels upon over-expression of Usp27x, new Fig. 3C).

Fig3. In A, 48h panel 4th lane there is a band at 100kDa. Any idea of the nature of this species? In B the loading control is not good at all. In the ubiquitination assay in C, in the eluate the band at 35kD cannot be the ubiquitinated form of BimEL as it migrates identically to the unmodified form in the input. Does BimEL stick non-specifically to the column?

The nature of the higher band is unclear. It is an SDS-gel, so it must be something bound very tightly; poly-ubiquitination could perhaps be a possibility but we can only speculate. What we do know is that the band only appears with the proteolytically active enzyme, the Usp27x-mutant never shows this band (we now also show a transient transfection of the two proteins (Fig. S5A). We refer to these bands now in the legend.

We have re-probed the blot in Fig. 3B with another antibody (anti-tubulin); the loading control now looks much better.

Thank you for observing the size problems with Bim in the ubiquitination assay, that had escaped us. We have now done the proper control and also done the assay with lysates from control (GFP)-transfected cells. We see the same band, so it is indeed Bim sticking to the beads as suggested. We now show this new experiment in the main paper (Fig. 3E) and have moved the old experiment (which also shows the Coomassie control) to the supplement (Fig. S3C).

Fig.4 The authors should provide a control of overexpression of another similar to Usp27x DUB.

We have done this and have expressed the DUB most similar to Usp27x, Usp22 (identity score 75 %). Expression of Usp22 had no effect on Bim-levels (Fig. 4 is now Fig. 5A, see the added panel). Another 1205Lu cell line expressing 3xFlag-Usp22 already shown in the first version of the manuscript showed no induction of Bim (old Fig. S5A is now S6A).

Fig.5 This is the only experiment addressing the effect of Usp27x knockdown. Has this approach been tested in the measurement of Bim half-life (Fig.6)?

We have not tested half-life directly but we now add data to show that at 16 h and 24 h of PMA-stimulation less Bim is seen in 293FT cells carrying the CRISPR/Cas9-k.o. of Usp27x (Fig. 6B, S6F). In all three experiments there was a stronger loss of Bim by PMA-treatment when Usp27x was genomically absent. Although the effect was not dramatic, it was reproducible and clear.

Fig.6 The effects are not terribly convincing and statistics on the quantification should be provided. Have the authrors performed half-life experiments upon stimulus induced degradation of Bim? Additionally, 6hrs of CHX treatment is quite long and if possible pulse-chase experiments should be applied.

(now Fig. 7) We had shown a time course experiment with 1205Lu melanoma cells and one with HCC827 NSCLC cells. We have added another experiment with the melanoma cells (new Fig 7B) and one experiment with 293FT cells (Fig. 7A) and have done the quantification on all experiments. In all four experiments the effect of Usp27x-expression in stabilizing Bim is clear (new Fig. 7).

Fig7. These experiments clearly point towards a role of Usp27x for Bim function regulation but the Usp27x knockdown/knockout experiments should be tested (see above). Additionally, it is normally recommended that at least 2 different type of assays should be used for apoptotic related phenotypes.

We have made two polyclonal lines of the NSCLC line HCC827 where we have targeted the Usp27x-locus with CRISPR/Cas9, using two separate gRNAs. Both polyclonal lines were substantially protected against treatment with the EGFR-inhibitor gefitinib, which is known (we confirm these data in Fig. 8C) to kill Bim-dependently (new Fig. 9). At least in this context Usp27x has a pro-apoptotic function, which is likely through the regulation of Bim.

We have always found staining for caspase-3 an extremely reliable assay since it directly shows the activity of the apoptotic pathway. We have now however also done experiments using the detection of active Bax with a conformation-specific antibody. We have done this for 293FT cells, including Bim-k.o. (Fig. 4), for 1205Lu melanoma cells (Fig. 8) and for HCC827 NSCLC cells, including Usp27x-k.o. (Fig. 9). All the data confirm the effects seen. Bax is a direct effector of mitochondrial cytochrome *c*-release, so these results demonstrate the involvement of the Bcl-2-family of proteins.

2nd Editorial Decision 15 February 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the enclosed reports on it. As you will see, both referees find the manuscript suitable for publication in EMBO reports. Nevertheless, both referees have raised some points that should be addressed. I would therefore like to ask you for further minor revisions, addressing all points of the referees, before we can proceed with the formal acceptance of your manuscript.

Our policy at EMBO reports is that manuscripts should be accepted 6 months after the first decision (scooping protection period) otherwise revised versions will be treated as new submissions. In your case the first decision was made in October 2015, therefore it would be nice to have back the revised manuscript within the next couple of weeks.
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.

REFEREE REPORTS
Referee #1:

The authors have addressed the majority of the concerns raised. The following minor corrections need to be addressed prior to publication.

Figure 6: The reduction in BimEL expression observed in the 24h BimEL immunoblot presented in Figure 6B is not convincing, the reduction is clearly seen in the immunoblot panel presented in Supplemental Figure 6F and this panel should be added to Figure 6B.

Figure 8: In the results section, the description of figure 8 should be corrected. The authors state that inhibition of ERK-signaling in 1205Lu melanoma cells over-expressing usp27x had a substantial pro-apoptotic effect while the usp27xc87A mutant had no such activity (Figure 8A, 8B). The data presented in figure 8 no longer includes the negative control (GFP) and the usp27x mutant, only usp27x and usp27x/Bim2KO. I feel that the negative control (GFP) and the GFP-usp27x mutant should be added back to this panel as it strengthens the conclusions of the study.

Figure 8C presents data showing the effect of GFP-Usp27x on gefitinib-induced apoptosis in HCC827+ cells. The data should include a GFP negative control and should then be presented alongside Fig. 9A&B as the data presented in these panels is consistent with a pro-apoptotic role of Cdc27x.

The BIM KO data in panel 8A and C (% active caspase activity) for both 1205Lu and HCC827 cells should then be moved to supplemental as the inhibitory effect of Bim KO on apoptosis induction has been shown in Figure 4 (in different cell lines), also the increase in apoptosis observed when Usp27x is expressed in U0126 or gefitinib-treated Bim KO cells suggests that cdc27x is acting to promote apoptosis through mechanisms other than Bim.

Referee #2:

The revised manuscript has addressed all raised concerns. The authors have included many additional experiments that strengthen their original conclusions. Overall a good solid study.

due to the increased size of the manuscript, i feel the data in Fig2A/B, even if important, could be moved in the supplementary information

2nd Revision - authors' response 19 February 2016

We are delighted that both referees find our manuscript suitable for publication and only suggest a number of minor revisions. Please find below our reply to the points raised.

Referee #1:

The authors have addressed the majority of the concerns raised. The following minor corrections need to be addressed prior to publication.

Figure 6: The reduction in BimEL expression observed in the 24h BimEL immunoblot presented in Figure 6B is not convincing, the reduction is clearly seen in the immunoblot panel presented in Supplemental Figure 6F and this panel should be added to Figure 6B.

We have done as suggested.

Figure 8: In the results section, the description of figure 8 should be corrected. The authors state that inhibition of ERK-signaling in 1205Lu melanoma cells over-expressing usp27x had a substantial pro-apoptotic effect while the usp27xc87A mutant had no such activity (Figure 8A, 8B). The data presented in figure 8 no longer includes the negative control (GFP) and the usp27x mutant, only usp27x and usp27x/Bim2KO. I feel that the negative control (GFP) and the GFP-usp27x mutant should be added back to this panel as it strengthens the conclusions of the study.

We agree with the validity of this and have added back the data for both negative controls (GFP and GFP-Usp27xC87A mutant). This figure is now Fig. 7A because, as suggested by reviewer #2 (below), we have moved Fig. 2 to the appendix (now appendix Fig. S3A, C). We have also moved data for the comparison between wt and BimKO for 1205Lu cells in the presence of UO126/Usp27x (old Fig. 8A) as suggested (below) to the appendix Fig. (now S7A). We have further moved Fig. 8B

to appendix Fig. S7B (active Bax stain) and add now also a Bax stain for the Usp27xC87A inactive mutant (shows no difference, appendix Fig. S7B).

The reviewer appears to give somewhat contradictory advice in this paragraph. In the first sentence he/she suggests to correct the description on the basis that the description was left from the first version of the manuscript but no longer in the revised version. He/she then suggests adding back the data (and then the old description will again be correct). Since we have added back the data we have not corrected the description.

Figure 8C presents data showing the effect of GFP-Usp27x on gefitinib-induced apoptosis in HCC827+ cells. The data should include a GFP negative control and should then be presented alongside Fig. 9A&B as the data presented in these panels is consistent with a pro-apoptotic role of Cdc27x.

We have not made GFP-only expressing HCC827 cells. We could of course make the cells and do the experiments (although not within the time frame of a couple of weeks). We have however made HCC827 cells expressing GFP-Usp27x mutant (see Western blot Fig. 4C, new label). We now include data from experiments with these cells (Fig. 7B). As in the case of the 1205Lu melanoma cells the induction of GFP-Usp27x mutant alone or in combination with gefitinib has no pro-apoptotic effect (please note that what was Fig. 8C is now Fig. 7B since Fig. 2 has been moved to the appendix Fig. S3). We are confident that the referee will agree with our view that since the GFP-Usp27x mutant shows no pro-apoptotic effect (unlike Usp27x wt) this is the better control than GFP on its own, and that by making the GFP cells and testing them nothing in addition could be gained.

The BIM KO data in panel 8A and C (% active caspase activity) for both 1205Lu and HCC827 cells should then be moved to supplemental as the inhibitory effect of Bim KO on apoptosis induction has been shown in Figure 4 (in different cell lines), also the increase in apoptosis observed when Usp27x is expressed in U0126 or gefitinib-treated Bim KO cells suggests that cdc27x is acting to promote apoptosis through mechanisms other than Bim.

We have done as suggested and have moved the data with 1205Lu Bim-k.o. cells (previous Fig. 8A) to appendix Fig. S7A (see above). We would prefer to keep the Bim-k.o. data for the HCC827 cells as it is (old Fig. 8A, now new Fig. 7B) and would like to show the Usp27xKO data for the HCC827 cells still in a separate figure (old Fig. 9A, B is now labelled Fig. 8A, B). It is correct that we have shown the effect of Bim-loss on apoptosis induction in Fig. 4 (now Fig. 3). However, this was a different situation with different cells. In Fig. 3 we are stimulating 293T cells with PMA to activate the ERK-pathway. In Fig. 7 we analyze tumour cells with constitutively active ERK, a potentially clinically important situation. We would prefer to keep these data in the main manuscript. We have moved substantial material to the appendix so we hope this is not a problem.

Referee #2:

The revised manuscript has addressed all raised concerns. The authors have included many additional experiments that strengthen their original conclusions. Overall a good solid study.

due to the increased size of the manuscript, i feel the data in Fig2A/B, even if important, could be moved in the supplementary information

Thank you for your kind words. We have moved the data as suggested and have integrated Fig. 2A, B completely into appendix Fig. S3 (A, C).

3rd Editorial Decision 25 February 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you very much for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Arnim Weber, Georg Häcker
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2015-41392V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	A number of experiments were performed according to the standards of the field
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.	N/A
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?	Every figure indicates the numer of experiments done. Statistics are described in the material and methods section (p. 24): for statistical analysis, 2-tailed Student's t-test was used to assess the significance of mean values. Bars show mean values of all experiments (±SEM). Differences were considered significant at a p-value of 0.05 or less.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The methods used are standard in the field. It is not possible to test for normal distribution in these experimental conditions.
Is there an estimate of variation within each group of data?	We provide the standard error of the mean for all numerical data.
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

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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).	We have done that.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We have done that.

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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
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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 accession codes for deposited data. See author guidelines, under '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	N/A
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).	Proteomic data for interaction of Bim are shown in Appendix Table
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
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22. 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.	N/A

G- Dual use research of concern

23. 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.	N/A