THE EMBO JOURNAL

Manuscript EMBO-2009-72758

IBRDC2, an IBR-type E3 ubiquitin ligase, is a regulatory factor for Bax and apoptosis activation

Giovanni Benard, Albert Neutzner, Guihong Peng, Chunxin Wang, Ferenc Livac, Richard Youle, Mariusz Karbowski

Corresponding author: Mariusz Karbowski, University of Maryland, Baltimore

Review timeline:

Submission date:	02 October 2009
Editorial Decision:	23 October 2009
Revision received:	29 January 2010
Additional correspondence:	17 February 2010
Accepted:	18 February 2010

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 23 October 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, they all find your results identifying IBRDC2 as a novel Bax regulator interesting and potentially important. At the same time, they nevertheless raise a number of major issues that would need to be adequately addressed before publication in The EMBO Journal may be warranted. Among those, some of the more significant concerns pertain to the somewhat unclear mechanism of how IBRDC2 regulates (ubiquitinates?) Bax and its levels.

Given the overall interest and the fact that all referees seem to offer some detailed discussions and suggestions on how to improve on these issues, my conclusion is that we should be happy to consider a revised manuscript for publication if you should be able to satisfactorily address the main criticisms in the spirit of the reviewers' reports and comments. As it is EMBO Journal policy to allow a single round of major revision only, it will however be important to diligently answer to all the various experimental and editorial points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript identifies a little characterized ubiquitin E3 ligase as a modulator of Bax protein levels in cells. IBRDC2 is an in-between-ring domain containing protein that contains a hydrophobic domain at its carboxy-terminus which is required to drive its association with the mitochondria following an apoptotic stimulus. Its recruitment is Bax-dependent, and the protein is found within foci that are adjacent and partially overlapping with the previously described Bax/DRP1/Mfn2 foci studied by this group. Consistent with a role as a ubiquitin E3 ligase, silencing IBRDC2 leads to a selective upregulation of Bax protein levels in the absence of a death trigger and an increase in activated Bax precipitated with the 6A7 antibody. This silencing also leads to increased susceptibility to Bax-dependent death triggers, further promoting its role as an anti-apoptotic protein that may normally function to dampen Bax activity. However the overexpression of IBRDC2 does not significantly protect cells from death, and there is little effect on Bax behaviour in the presence of ectopic IBRDC2, If anything, there was an increase in death-triggered cytochrome c release upon expression of a TM-anchored form of IBRDC2 (Fig 3D). Overall, there are many merits to this study, and it brings a new player into the field of dynamic Bax regulation. However, there are some confusing elements that should be addressed.

1. There is little evidence that Bax is the target of IBRDC2 ubiquitination activity. The IP shown in Fig 5E is difficult to interpret since the loading of monomeric Bax is increased in the second lane. Generally the authors use indirect evidence to make this point, centering on experiments in Figure 5. A few additional experiments could help to clarify these data. In A, the authors should combine MG132 with G5 to show the appearance of ubiquitinated Bax ladders. Upon inhibition of the proteasome, the stabilized ubiquitinated Bax induced by the G5 drug should become clear, which would help to confirm the ubiquitination of Bax. This should also be done upon silencing of IBRDC2 to show that this ladder is dependent on this protein. If no ladder is observed, it could indicate a role for mono-ubiquitination, which may suggest alternative functions.
2. In Figure 5D, the different bands and changing levels of myc-IBRDC2 should be explained.
3. This study has excellent quantification of the confocal imaging experiments, but there are no quantifications of any biochemical experiments. The authors need to quantify the increase in Bax upon IBRDC2 silencing from different experiments to get some statistics, as well as the data in figures 5 and 9, including the IP, where the ubiquitin smear is normalized to the total Bax.
4. The recent identification of Bax suggests that ubiquitination must be inhibited during cell death in order to accumulate this pro-death splice variant. Although this is a variant of Bax, rather than the inhibition of an E3 ligase, we here we see the selective recruitment of IBRDC2 during death, which is proposed to down-regulate Bax protein levels. This may be true, but it is also possible that the steady state, non-mitochondrial IBRDC2 down-regulates Bax through ubiquitination and degradation, where the mitochondrial recruited IBRDC2 protein may have a different role during apoptosis. The sensitivity to death upon silencing of IBRDC2 could be due to the accumulation of Bax that occurred prior to the death stimuli, rather than reflecting an active role for IBRDC2 to block death on the membrane during an apoptotic signal. The binding to 6A7 was not accompanied by any data indicating that this form of Bax was ubiquitinated, either poly- or mono-, which could be a way to approach this question. This reviewer is looking for a clearer understanding of what IBRDC2 does specifically following its Bax-dependent, apoptotic recruitment to the mitochondria, which could help unify the observations from other recent studies on Bax turnover and stability in apoptosis.

Referee #2 (Remarks to the Author):

The manuscript by Benard and colleagues identifies IBRDC2, a E3 RING containing ligase as a regulator of Bax stability. Bax and IBRDC2 appear to interact upon apopotitic activation of Bax in response to genotoxic stress and co-translocate to mitochondiral outer membranes. RNAi against the E3 ligase causes accumualtion of Bax and increased cell death susceptibility.

I think the study is very interesting and well performed. I do have some minor comments and questions that should be addressed prior publication.

Minor:

there is a word missing in the intro - page 13rd line from the bottom ..poor prognosis in? CLL.

IBR should be explained when first mentioned (in between RING)

From the introduction it is not clear how the authors came about to work on IBRBC2 in the context of apoptosis and what other functions have been described for this protein before - such information should be included

Major
Does a c-term fusion of GFP change the localization/funciton of the protein. How do the authors explain that the IBRDC2-TM version of the protein actually promotes more efficient cyt. C release upon treatment . Is there evidence that this c-term aa exert an inhibitory role - is there even more.

Can the authors be sure that all Bax increase observed in IBRDC2 RNAi cells is due to reduced degradation - if so, cotreatment with CHX should have no effect.

The data in Fig. 5E is not so convincing, the observed effect could be indirect. Can the authors show the reciprocal experiment? i.e. IP ubiquitin and IB for Bax before and after treatment,

What is the region in IBRDC2 required for interaction with bax - does interaction with IBRDC2 interfere/influence interaction with other known partners such as Bcl-2. Is there any preliminary data available on this?

On page 11 the authors state that in Bax KO cells IBRDC2 does not translocate to mitos upon stress/apoptosis - as data not shown. I think this is a crucial experiment and immunofluorescence as well as biochemical data shall be provided.

In fig.8i the authors suggest that no interaction is detected in CHAPS - this refers to untreated cells, but upon apoptosis induction interaction should occur and also be detectable in CHAPS lysates. Is there evidence for such interactions.

IBRDC2 RNAi appears to sensitize to death while overexpression per se apparently does not prevent death. This is surprising and somewhat challenging their hypothesis. However, although in transient experiments little protection was noted, it would be interesting to see if Bak KO cells do show increased clonal survival when IBRDC2 is overexpressed or conversely, reduced clonal survival upon RNAi. This could be addressed experimentally by short term incubation with STS or ActD. Colony formation should be assessed under these conditions.

Referee #3 (Remarks to the Author):

General Remarks

This is a well written and detailed exploration of an association with IBRDC2 and Bax. The findings are novel and I believe they should be of interest to a broad readership such as EMBO J touching on both ubiquitylation and apoptosis.

The paper relies rather heavily on colocalisation data but figures 8 and 9 ameliorate this. My main criticism relates to figure 5. G5 seems a relatively unproven tool with which to draw significant conclusions. It also makes the manuscript far more complicated than need be, in my opinion. I also can't quite understand why G5 can detabilise Bax but MG132 can't stabilise it. Therefore I would suggest to improve Fig 5E and show that Bax does get ubiquitylated and this increases if you have you transfect IBRDC2, and remove the G5 data completely. HOIL-1/HOIP are other IBR RING fingers and they promote linear ubiquitylation which may not promote proteasomal degradation. Rather than go into all this, why not just confirm with a proper assay that Bax is a potential substrate

for IBRDC2 ubiquitylation.

I also think the association with p53 and the p53 inducible upregulation of Bax, a known p53 target suggests that, at the very least, the p53 inducible BH3 proteins puma and noxa should be looked at too. This would further support the specificity argument, if they are unaffected, and if they are affected suggests something even more interesting is going on.

Figure 1. Identification of IBRDC2, a novel mitochondria-associated RING finger protein
Two different localisations
Might be novel that it is associated with mitochondria but inaccurate to claim it is a novel protein, unnecessary.

Figure 2. Apoptosis induced mitochondrial accumulation of IBRDC2
Further correlation studies

Figure 3. Mechanism of mitochondrial translocation of IBRDC2
TM is 40 aa's and this looks sufficient to get mitochondrial localization.

Figure 4. Downregulation of IBRDC2 induces an abnormal accumulation of Bax.
I think the association with p53 and the p53 inducible upregulation of Bax which is a p53 target suggests that at the very least the p53 inducible BH3 proteins puma and noxa should be looked at too.

Figure 5. Role of IBRDC2 in ubiquitination-dependent regulation of Bax.

Perhaps because G5 induces apoptosis, Fig. 5e looks bizarre. Mw markers should be present (in all figures). Because G5 increased ubiquitin in IP control and the increase caused by G5 is extremely modest, it makes it hard to interpret this figure. Furthermore unless this is performed with his-tagged ubiquitin and denaturing purification then one cannot conclude that the increase in ubiquitin is due to ubiquitylated Bax. It could be a bax associated protein.

Figure 6. Synchronous mitochondrial translocation of IBRDC2 and Bax
Figure 7. Submitochondrial localization of IBRDC2 and Bax
Figure 8. Activated Bax is required and sufficient for mitochondrial accumulation of IBRDC2
Figure 9. Role of IBRDC2 in the regulation of apoptosis
A and B could easily and profitably be combined. Rather than 1 and 2 I'd prefer labelled as IBRDC2 siRNA and control.

Specific Remarks

Fontanini ref not correctly cited in bibliography

Figures

Mw markers should be present in all Figures - it is always a courtesy to the reader and an aid to the reviewer to include Mw markers.

Size markers should be present in the cell and flourescent images

1st Revision - authors' response 29 January 2010

Manuscript: Benard et al. "IBRDC2, an IBR-type E3 ubiquitin ligase, is a regulatory factor for Bax and apoptosis activation" (EMBOJ-2009-72758)

Point-by-point response:

We would like to thank the Reviewers for their interest in our work and for their insightful comments on our manuscript: "IBRDC2, an IBR-type E3 ubiquitin ligase, is a regulatory factor for Bax and apoptosis activation" (EMBOJ-2009-72758). In the revised version of the manuscript we address the Reviewer's concerns and incorporate their suggestions. The major changes, comparing to the original version include:
1) New data demonstrating increased stability of Bax in cycloheximide (CHX)-treated IBRDC2 RNAi cells have been added (new Figure S4A).
2) The original Bax/ubiquitin immunopecipitation data have been replaced with new data that, we believe, more clearly demonstrate that Bax can be targeted by ubiquitination, and that G5-dependent deubiquitination of Bax also has a significant role in regulation of this protein (new Figure 5C).
3) New biochemical data confirming a central role for Bax in the regulation of subcellular distribution of IBRDC2 have been also added to the revised manuscript (new Figure 8H).
4) Quantifications of Western blot data from several independent experiments have been included (see new Figure 9B, text referring to Figure 4A, and new Figure S4A).
5) We examined levels of Puma and Noxa in Control RNAi and IBRDC2 RNAi cells (see new panels in Figure 4A).
6) We have also incorporated all suggestions regarding manuscript organization and did our best to address all other issues/concerns raised by the Reviewers.

We hope that changes/corrections and new data incorporated into a revised manuscript will make it acceptable for the publication in The EMBO Journal.
The detailed point-by-point answers to the Reviewer's comments follow:

Reviewer 1.
1. There is little evidence that Bax is the target of IBRDC2 ubiquitination activity. The IP shown in Fig 5E is difficult to interpret since the loading of monomeric Bax is increased in the second lane. Generally the authors use indirect evidence to make this point, centering on experiments in Figure 5. A few additional experiments could help to clarify these data. In A, the authors should combine MG132 with G5 to show the appearance of ubiquitinated Bax ladders. Upon inhibition of the proteasome, the stabilized ubiquitinated Bax induced by the G5 drug should become clear, which would help to confirm the¨ ubiquitination of Bax. This should also be done upon silencing of IBRDC2 to show that this ladder is dependent on this protein. If no ladder is observed, it could indicate a role for mono-ubiquitination, which may suggest alternative functions.

The experiments suggested by the Reviewer have been performed. Although, we did not detect any increase in the Bax ladders in Control RNAi cells, upon co-treatment with MG132 and G5, this treatment revealed a surprising accumulation of a dimer-size (~45kD) SDS-resistant form of Bax in IBRDC2 RNAi cells (see Figure below). We found that this form of Bax was barely detectable in Control RNAi cells, indicating that inhibition of IBRDC2 together with G5-dependent inhibition of Ub peptidase activities likely resulted in an abnormal accumulation of Bax dimers. Since changes in Bax conformation can initiate oligomerization of this protein, these data support the notion that IBRDC2 specifically targets an activated form of Bax. Thus, it is plausible that IBRDC2 targets oligomerized or oligomerizing Bax that accumulates when IBRDC2 is depleted. Significantly, our data also show that prolonged treatment with MG132, a proteasome inhibitor, induces accumulation of the ~45kD molecular species of Bax, but not the monomeric form of Bax. Therefore, it appears that the Bax dimer might be specifically prone to proteasomal degradation.

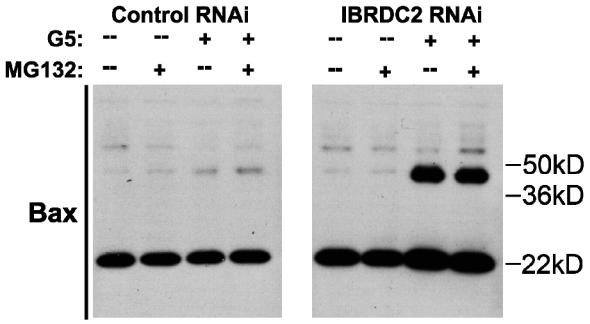

Figure: Control RNAi and IBRDC2 RNAi cells were treated with DMSO (solvent), G5 or MG132 or co-treated with these compounds and then analyzed by Western blot for Bax.

We are currently unable to explain why Ub peptidase inhibition accentuates formation of the ~45kD Bax species specifically in IBRDC2 RNAi cells. One possibility is that another protein is also implicated in the IBRDC2 containing molecular complex and that its regulation depends on its ubiquitination status. We are planning to carry out new experiments to specify the molecular mechanism of Bax ubiquitination/degradation cycles, including the role of mitochondria localized IBRDC2, in more detail.

2. In Figure 5D, the different bands and changing levels of myc-IBRDC2 should be explained.

We found that levels of overexpressed IBRDC2 are decreased in STS- or ActD-treated cells. Furthermore, since this decrease is less pronounced in IBRDC2 RING mutant expressing cells, we anticipate that self-ubiquitination of IBRDC2 might play a role in the regulation of the stability of this protein. The top band can also be detected by Western blot of endogenous IBRDC2 (though it requires more protein loading). We do not know the nature of this band. Yet, since exogenous MYC IBRDC2 shows this pattern the likely explanation is that this is a modified form of IBRDC2 (e.g. ubiquitinated or phosphorylated), but not non-specific protein. This information has been included in the legend to Figure 5E.

3. This study has excellent quantification of the confocal imaging experiments, but there are no quantifications of any biochemical experiments. The authors need to quantify the increase in Bax upon IBRDC2 silencing from different experiments to get some statistics, as well as the data in figures 5 and 9, including the IP, where the ubiquitin smear is normalized to the total Bax.

Thank you for this suggestion. We have quantified changes in protein levels and added these data to the revised manuscript.
1) Quantification of Bax levels in IBRDC2 RNAi HeLa cells obtained on five separate occasions results in 1.93±0.49 average increases of Bax, as compared to Control RNAi cells. This information has been added to the revised manuscript.
2) Furthermore, we have also quantified cleavage of PARP in Control RNAi and IBRDC2 RNAi cells (from 3 independent experiments). These data have been included in the revised manuscript as a new Figure 9B.
We believe that these two sets of experiments are critical for the conclusions reached in this manuscript and that the new extended data corroborate our original statements.
3) We have also quantified changes of Bax levels (normalized to levels of Tom20) in CHX-treated Control and IBRDC2 RNAi cells. These data, included in the revised manuscript as Figure S4A, indicate that Bax is more stable in IBRDC2 RNAi cells. The data show that there were only slight differences in Bax levels in cells treated with CHX for up to 16hr. Yet at 24 and 32hr of CHX treatment, Bax levels in Control RNAi decreased to 59% and 29% of initial values at 24 and 32hr, respectively. Since in IBRDC2 RNAi cells this decrease was much smaller (84% and 77% of initial values, at 24 and 32hr, respectively), these data indicate that Bax stability is increased in IBRDC2 RNAi cells.

4. The recent identification of Bax suggests that ubiquitination must be inhibited during cell death in order to accumulate this pro-death splice variant. Although this is a variant of Bax, rather than the inhibition of an E3 ligase, we here we see the selective recruitment of IBRDC2 during death, which is proposed to down-regulate Bax protein levels. This may be true, but it is also possible that the steady state, non-mitochondrial IBRDC2 down-regulates Bax through ubiquitination and degradation, where the mitochondrial recruited IBRDC2 protein may have a different role during apoptosis. The sensitivity to death upon silencing of IBRDC2 could be due to the accumulation of Bax that occurred prior to the death stimuli, rather than reflecting an active role for IBRDC2 to block death on the membrane during an apoptotic signal. The binding to 6A7 was not accompanied by any data indicating that this form of Bax was ubiquitinated, either poly- or mono-, which could be a way to approach this question.

The Reviewers comments completely support our original model of IBRDC2 mechanism. We believe that the data support the notion that IBRDC2 acts to downregulate Bax primarily in non-

apoptotic cells. Consequently, in cells with reduced levels of IBRDC2 an abnormal accumulation of Bax sensitizes these cells to apoptosis. In addition, taking into consideration the lack of antiapoptotic activity of IBRDC2 in ActD and STS-treated cells, we proposed a role for IBRDC2 prior to apoptosis induction (see Discussion). Our results indicate mitochondrial translocation of IBRDC2 might not have any specific role in the apoptotic cascade (e.g. inhibition of Bax activation). However, our data also indicate that apoptotically active conformation of Bax is a primary target for IBRDC2 activity. Since, as shown by many laboratories, the activated Bax is likely to accumulate on the mitochondria, we believe that mitochondrial translocation of IBRDC2 merely mimics the "non-apoptotic" housekeeping role of IBRDC2 in the regulation of Bax degradation. The major difference being a massive apoptotic activation of Bax in apoptosis inducer treated cells, as opposed to likely much less pronounced events of Bax activation in "healthy cells". Our observations support a feedback inhibition model in which IBRDC2 safeguards healthy cells from unwanted activation of Bax. To prevent a self-accelerating Bax activation cascade, active Bax needs to be neutralized. In addition, the removal of active Bax may also be relevant when a sub-threshold BH3-only protein-dependent apoptotic signal is overcome by a strong anti-apoptotic response. In addition to the role of anti-apoptotic Bcl-2 family proteins in this process, IBRDC2-dependent elimination of activated Bax would serve this purpose.

Reviewer 2

1. There is a word missing in the intro - page 13rd line from the bottom ..poor prognosis in? CLL. IBR should be explained when first mentioned (in between RING)
From the introduction it is not clear how the authors came about to work on IBRBC2 in the context of apoptosis and what other functions have been described for this protein before - such information should be included.

We have corrected this sentence. The introduction has been also modified to include the requested information (see page 4, last paragraph).

2. Does a c-term fusion of GFP change the localization/function of the protein. How do the authors explain that the IBRDC2-TM version of the protein actually promotes more efficient cyt. C release upon treatment. Is there evidence that this c-term exerts an inhibitory role - is there even more.

We have tested the subcellular localization of IBRDC2-YFP construct and rather surprisingly found it to resemble the YFP-IBRDC2 construct in its subcellular localization in healthy and apoptotic cells. We found cytosolic localization of IBRDC2-YFP in majority of untreated cells and mostly mitochondrial localization in apoptotic cells. Therefore, it appears that YFP does not interfere with the subcellular dynamics of IBRDC2. Results showing subcellular distribution of IBRDC2-YFP were added to the revised version of our manuscript as a new Figure S2.

3. Can the authors be sure that all Bax increase observed in IBRDC2 RNAi cells is due to reduced degradation - if so, cotreatment with CHX should have no effect.

Thank you for this suggestion. We have analyzed the effect of CHX on Bax in Control RNAi and IBRDC2 RNAi cells. Control RNAi and IBRDC2 RNAi HeLa cells were treated with CHX for up to 32hr followed by Western blot analyses with anti-Bax monoclonal antibody. Since CHX induces cell death, we also applied zVAD-fmk simultaneously with CHX to inhibit caspase activation. The data show that there were only slight differences in Bax levels in cells treated with CHX for up to 16hr, confirming the notion that in HeLa cells Bax is a very stable protein. Yet at 24 and 32hr of CHX treatment, Bax levels in Control RNAi decreased to 59% and 29% of initial values at 24 and 32hr, respectively. Since in IBRDC2 RNAi cells this decrease was much smaller (84% and 77% of initial values, at 24 and 32hr, respectively), these data indicate that Bax stability is increased in IBRDC2 RNAi cells. These important new data have been added to the revised manuscript as Supplemental Figure 4A, and referred to in the text.

4. The data in Fig. 5E is not so convincing, the observed effect could be indirect. Can the authors show the reciprocal experiment? i.e. IP ubiquitin and IB for Bax before and after treatment.

Since all reviewers were not very enthusiastic about this figure we have removed it from the current version of the manuscript. As suggested by this Reviewer (as well as Reviewer#2), we have performed HA tagged ubiquitin (HA-Ub) IP under denaturing conditions. The data show a noticeable increase in a high molecular species of Bax co-immunoprecipitating with HA-Ub. Furthermore, treatment with G5 led to further stabilization/accumulation of the high molecular weight Bax species, further confirming a role for deubiquitination in Bax regulation. Significantly, these data also indicate that Bax is indeed targeted by Ub. These new data have been added to the revised manuscript as Figure 5C.

5. What is the region in IBRDC2 required for interaction with bax - does interaction with IBRDC2 interfere/influence interaction with other known partners such as Bcl-2. Is there any preliminary data available on this?

We have no preliminary data on this issue. However, since vMIA blocks IBRDC2 translocation to the mitochondria, and interacts with Bax, one possibility is that the Bax region blocked by vMIA is required for this interaction. Furthermore, since the C-terminal part of IBRDC2 cotranslocates to the mitochondria in a similar time frame as wild type IBRDC2 (and Bax), we anticipate that this part of IBRDC2 might also interact with Bax. We have already initiated studies aimed at the determination of the amino acid residues that are required for mitochondrial translocation of IBRDC2. We also intend to analyze Bax binding of all mutants that are being generated in our lab.

6. On page 11 the authors state that in Bax KO cells IBRDC2 does not translocate to mitos upon stress/apoptosis - as data not shown. I think this is a crucial experiment and immunofluorescence as well as biochemical data shall be provided.

We have included cell fractionation data obtained from ActD-treated HCT116 cells (new Figure 8H). Since these data confirms that Bax but not Bak is required for mitochondrial accumulation of IBRDC, a new Figure 8H replaced a similar figure from the original manuscript (the original figure showed effect of Bax, but data on Bax deficient cells were not included).

7. In fig.8i the authors suggest that no interaction is detected in CHAPS - this refers to untreated cells, but upon apoptosis induction interaction should occur and also be detectable in CHAPS lysates. Is there evidence for such interactions.

We have performed immunoprecipitation experiments using CHAPS lysates of STS- or ActD-treated cells. However, no interaction of IBRDC2 with Bax has been detected under these conditions. Anticipating asynchronous apoptosis induction in the populations of STS- or ActD-treated cells, as well as the known transient nature of E3 Ub ligases interactions with their substrates, we concluded that at a specific time point only in a very limited number of cells IBRDC2 might bind Bax. Therefore, this interaction could be extremely difficult to detect in CHAPS lysates. Conversely, Triton-X100 likely induces and stabilizes apoptotic conformation of Bax in a whole population of cells in highly synchronous manner. Therefore, the amount of Bax with "IBRDC2-interacting conformation" is also likely to be much more abundant in Triton-X100 lysates, than in CHAPS lysates obtained from apoptotic cells.

8. IBRDC2 RNAi appears to sensitize to death while overexpression per se apparently does not prevent death. This is surprising and somewhat challenging their hypothesis. However, although in transient experiments little protection was noted, it would be interesting to see if Bak KO cells do show increased clonal survival when IBRDC2 is overexpressed or conversely, reduced clonal survival upon RNAi. This could be addressed experimentally by short term incubation with STS or ActD. Colony formation should be assessed under these conditions.

We have performed the suggested clonal survival experiments in cells transfected with IBRDC2 and control YFP vector expressing cells treated with STS to induce apoptosis. However, as in the case of the apoptosis assays, we did not see any significant changes in cell survival. These data indicate that, as we discussed in the original version of the manuscript, overexpressed IBRDC2 has none/or little

effect on cell survival. This result further confirms the notion that IBRDC2 is not a strong antiapoptotic protein that acts through inhibition of proapoptotic factors under considerable stress-inducing stimuli.

Reviewer 3

1. My main criticism relates to figure 5. G5 seems a relatively unproven tool with which to draw significant conclusions. It also makes the manuscript far more complicated than need be, in my opinion. I also can't quite understand why G5 can destabilize Bax but MG132 can't stabilize it. Therefore I would suggest to improve Fig 5E and show that Bax does get ubiquitylated and this increases if you have you transfect IBRDC2, and remove the G5 data completely. HOIL-1/HOIP are other IBR RING fingers and they promote linear ubiquitylation which may not promote proteasomal degradation. Rather than go into all this, why not just confirm with a proper assay that Bax is a potential substrate for IBRDC2 ubiquitylation.

Thank you for these comments. After careful consideration we have decided to leave the G5 data in the revised manuscript. The reasons for this decision are described below.

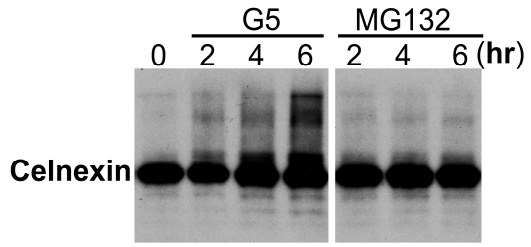

Figure A. HeLa cells were treated with G5 or MG132 for the indicated time, followed by Western blot with anti-Calnexin antibody.

We have applied G5 based on our earlier observations that this compound specifically affects the ubiquitination status of several proteins known to be under control of Ub/proteasome system (e.g. an ERAD substrate calnexin). Importantly, we found that the polyubiquitination pattern of calnexin was identical in G5- or MG132-treated cells; with a major difference being the much more pronounced effects of G5 (see Figure A). As shown in the manuscript, using anti-Ub antibody, we have confirmed a strong increase of polyubiquitinated proteins in G5-treted cells, suggesting its role in the regulation of protein deubiquitination (treatment with MG132 had lesser effects; see Figure 5A). Furthermore, since this compound increases Bax ubiquitination level and at the same time destabilizes Bax, we believe that it is plausible to conclude that G5 affects Bax ubiquitination levels through inhibition of Ub peptidase activity (a mechanism described in the original work describing this compound; Aleo et al. 2006; Cancer Research 66, 9235-9244). Furthermore, this original work also revealed G5 specificity towards ubiquitin COOH-terminal isopeptide linkage.
Why G5 can stabilize Bax but MG132 can't stabilize it? Our data suggest that normally in HeLa cells, Bax is a very stable protein (see new Figure S4A), but Bax ubiquitination and deubiquitination cycles might be very fast and this in turn might reflect degradation rates of Bax (e.g. fast deubiquitination of Bax should make it less prone to proteasomal degradation). Under the experimental conditions we used (G5-treatment), an inhibition of Ub isopeptidases would lead to abnormal ubiquitination of Bax, and make this protein much more prone to degradation than in untreated cells. The data also show that while MG132 alone does not induce Bax stabilization, G5-induced destabilization of Bax is noticeably inhibited by MG132 (see Figure B). Thus, it is likely that in the presence of G5, Bax is degraded more efficiently.
Given that IBRDC2 RNAi cells were analyzed ~4 days after transfection with IBRDC2 silencing vectors, Bax accumulation in these cells likely reflect a long term inhibition of IBRDC2, without general changes in Bax synthesis rates. The fact that MG132 treatment of IBRDC2 RNAi cells does not lead to further accumulation of Bax supports this possibility.

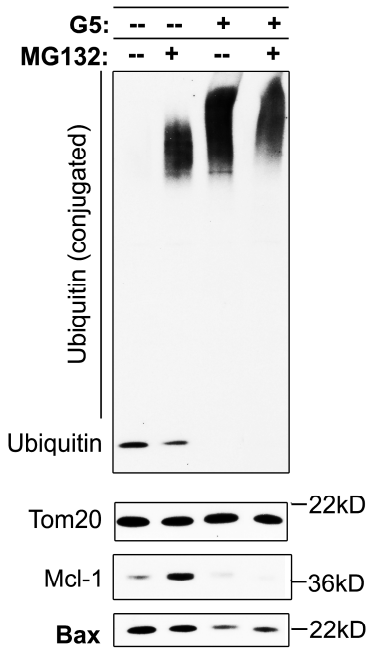

Figure B. HeLa cells were treated with G5, MG132, or co-treated with these compounds for 4hr followed by Western blot as indicated in the figure.

2. I also think the association with p53 and the p53 inducible upregulation of Bax, a known p53 target suggests that, at the very least, the p53 inducible BH3 proteins puma and noxa should be looked at too. This would further support the specificity argument, if they are unaffected, and if they are affected suggests something even more interesting is going on.

Thank you for this suggestion. We have looked at levels of Noxa and Puma in IBRDC2 RNAi cells. The data show that these proteins are unaffected by IBRDC2 depletion. We have confirmed that in these experiments Bax is stabilized as in all other studies described in our manuscript. We believe that this result further confirms a specific role for IBRDC2 in Bax regulation. These data have been added to the Figure 4A as two new panels.

3. Figure 1. Identification of IBRDC2, a novel mitochondria-associated RING finger protein.Two different localisations:Might be novel that it is associated with mitochondria but inaccurate to claim it is a novel protein, unnecessary.

We agree with the Reviewer and this statement has been corrected.

4. Figure 4. Downregulation of IBRDC2 induces an abnormal accumulation of Bax. I think the association with p53 and the p53 inducible upregulation of Bax which is a p53 target suggests that at the very least the p53 inducible BH3 proteins puma and noxa should be looked at too.

Please see #2.

5. Figure 5. Role of IBRDC2 in ubiquitination-dependent regulation of Bax. Perhaps because G5 induces apoptosis, Fig. 5e looks bizarre. Mw markers should be present (in all figures). Because G5 increased ubiquitin in IP control and the increase caused by G5 is extremely modest, it makes it hard to interpret this figure. Furthermore unless this is performed with his-tagged ubiquitin and denaturing purification then one cannot conclude that the increase in ubiquitin is due to ubiquitylated Bax. It could be a bax associated protein.

Since all reviewers were not very enthusiastic about this figure, we have removed it from the current version of the manuscript. As suggested by this Reviewer (and the Reviewer#2), we have performed HA tagged ubiquitin (HA-Ub) IP under denaturing conditions. The data show a notable increase in high molecular species of Bax coimmunoprecipitating with HA-Ub. Furthermore, treatment with G5 led to further stabilization/accumulation of high molecular weight Bax species, further confirming a role for deubiquitination in Bax regulation. Significantly, these data also indicate that Bax is indeed targeted by Ub. These new data have been added to the revised manuscript as Figure 5C.

6. Figure 9. Role of IBRDC2 in the regulation of apoptosis A and B could easily and profitably be combined. Rather than 1 and 2 I'd prefer labelled as IBRDC2 siRNA and control.
Figures 9A and 9B have been combined. Figure 9 have been also relabeled according to the Reviewer's suggestion.

7. Fontanini ref not correctly cited in bibliography.
This has been corrected.

8. Figures Mw markers should be present in all Figures - it is always a courtesy to the reader and an aid to the reviewer to include Mw markers. Size markers should be present in the cell and fluorescent images.

We have added protein MW markers in Western blot data and size bars in fluorescent microscopy images, as suggested.

Additional correspondence 17 February 2010

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees 1 and 2, and I am happy to inform you that both of them consider it now suitable for publication in The EMBO Journal. The only minor changes still required would be additional references for MULAN in the introduction (as asked for by referee 1) and the desired correction to Figure 5 and its legend that you mentioned. Could you therefore please send us (via email) modified files for the text and this figure at your earliest convenience. As soon as we will have received these modified final versions, we would replace them in the manuscript tracking system and should then be able to swiftly proceed with formal acceptance of the paper.

Thank you again for considering The EMBO Journal for publication of this interesting study!

Yours sincerely,

Editor
The EMBO Journal

Referee 1 (comments to the authors):

This revised manuscript provides compelling evidence for a novel regulatory axis for the selective targeting and degradation of activated Bax oligomers by a RING-finger protein IBRCD2. The primary challenge in the previous submission related to the model whereby this apparent ubiquitin E3 ligase would degrade activated Bax in steady state, but its recruitment during cell death may be rather a side-reaction of its function in healthy cells. Their model suggests that too much activated Bax during apoptosis cannot be reversed by IBRCD2 mediated ubiquitination. I still

wonder whether there isn't an apoptotic role for this IBRCD2 once its fully recruited. However the authors have shown a clear effect on Bax protein levels in response to this protein, and they have shown a direct interaction with the activated forms, and shown its colocalization to Bax on the mitochondria. They have also carefully quantified the biochemical data that I suggested, and the evidence presented is all statistically significant. Therefore, I think the study would be important for the field even if there is more work to be done before we fully understand the function of IBRCD2.

Minor point:
-in the introduction, MULAN should be referred to as MULAN/MAPL/GIDE with references.
-figure 6 could be simplified to just show the panel of insets, rather than also including the whole cells.

Referee 2 (no further comments to the authors)

