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BH3-only proteins are part of a regulatory network that control the sustained signaling of the Unfolded Protein Response sensor IRE1α

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

1st Editorial Decision 20 September 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. This was caused by problems with the availability of referees during the past summer holiday period.

In the meantime, your manuscript has been seen by three referees, and their comments are shown below. As you will see, while all three referees are positive in principle, it becomes clear that major revision will be required before the referees support publication. Essentially, there are three major issues. First, more statistically significant and convincing XBP-1 splicing data will be required as mentioned by all three referees. Furthermore, data on the interaction of endogenous Ire1 with endogenous PUMA, BIM and BNip3 will be needed as put forward by referee 3. Finally, it would be good to add at least some more mechanistic depth, e.g. with respect to the regulation of the interaction as suggested by referee 3. Taking together all these considerations we should be able to consider a revised version of the manuscripts that addresses the three points mentioned above in addition to the other points put forward by the referees.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees.

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When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Please do not hesitate to contact me at any time in case you would like to discuss any aspect of the revision further.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This article has identified the BCL-2 family members Bim and PUMA as novel IRE1α interactors. BIM and PUMA double-knockout cells failed to maintain sustained XBP-1 mRNA splicing after prolonged ER stress. This regulation required BCL-2 (although it is not clear why) and was antagonized by either BAD or the BH3 domain mimetic ABT-737. Overall, the data are convincing and well presented. But there is a lot of data presented and it is not always clear. It seems that in several figures (esp Fig. 7) important controls are missing. While the data in Figure 7 are the most intriguing in the paper the lack of controls makes it difficult to ascribe as much weight as I would like to. I have provided a list of more minor points for the authors to consider when revising the paper.

1. It is very strange that mRNA or protein levels of Bip are unchanged when the IRE-1 pathway is activated. I follow their reasoning when the authors state that "bip (a UPR-target gene independent of XBP-1s)" on page 8. However, Bip is the chaperone interacting with ER stress transducer IRE-1 and constitutively inhibits its oligomerization (Nature Cell Biology 2, 326 - 332). The text needs clarification regarding the interpretation of the Bip data as independent of XBP-1s.

2. In the top western blot of left panel in the Fig 1A, why is the size of IRE1α-HA so much bigger than IRE1α in the WT (lane 1 vs lane 3)? The difference in the size between IRE1α-HA and wild type IRE1α should be just the HA epitope which is small. The data in this blot need further explanation. More functional assays comparing IRE1α and IRE1α-HA (other than XBP-1 splicing assay) were presumably performed and could be summarized without adding all the data.

3. While the authors may have used the mass spectrometry data to identify Bim as a potential IRE1α binding partner, in the manuscript a lot of emphasis is placed on what appears to have been the identification of a single peptide.

4. The authors make extensive use of the XBP-1 splicing assay in the paper yet quantification of Xbp-1 splicing data often does not match the sample data. In Fig 2D, Xbp-1s% of WT at 16-24 h time points do not resemble what is shown in the graph. The same problem occurs in Fig 2B to a lesser extent. More important in the experiments with Noxa in Fig 4E there is more unspliced XBP in Noxa expressing cells than in GFP expressing cells. The graph shows % spliced but it does not match the data shown. I understand the graph is an average of multiple experiments but if the data shown were included then the error bars cannot be so small. SE for N=3 does not match the data shown.

5. There is no indication of transduction efficiency or expression level for the proteins expressed by the retroviral constructs. In one place they do mention that the transfection efficiency (not when using the retroviral constructs) was 30% but that is about it. The methods section is also not clear and includes information about constructs not used in the manuscript (ER localized Bim, cytochrome b5 Bak etc.).

6. The authors state that "Administration of ABT-737, or mutation of the BH3 domain of BIM, abrogated the activation of XBP-1 mRNA splicing. Moreover, the binding of BIM to IRE1α was mediated by the BH3 domain, as demonstrated by mutational analyses". Other data presented suggest that binding to IRE1α was via the BH3 domain of Bim and Puma. I don't understand why Bcl-2 augments the response. "The effects of BIM over IRE1ΔN-HIS RNAse activity were potentiated by the presence of recombinant BCL-2 and importantly reduced when BIMWT was replaced by BIML150E (Figure 5E)." Shouldn't Bcl-2 compete with IRE1α for binding to Bim and Puma? Please explain.

7. In fig 6, it would be good to confirm the results of the in vivo experiments using some other approach such as immunohistochemistry rather than relying on only the XBP-1 splicing assay.

Referee #2

Rodriguez and co-workers report on an extensive series of experiments suggesting a link between Bim (and Puma) expression and IRE1α activity associated with XBP-1 splicing. The findings are intriguing and potentially important but are quite variable in terms of supporting the authors' claims. Data from animal and cell based experiments in Figs 6 and 7 clearly document a potential relationship between Bim, XBP-1 splicing, and UPR outputs, whereas other results appear far less robust.

Overall there is a need to better articulate (speculate) on a potential underlying mechanism and rationale. Why does a pro-apoptotic maintain IRE1α activity? How does pro-survival Bcl-2 fit in?

Specifics

1. Utilizing KO and DKO cells run the risk of additional compensating differences compared to wt. Have the authors recapitulated the findings with sh or siRNA?

2. The assumption throughout is that the ER stessors are not causing toxic side effects. Since caspase activation is typically associated with these agents, have key experiments been conducted in the presence of zVAD?

3. A massive conc of ABT is used, putting at risk the on-target conclusions of these experiments (also putting at risk the cell viability status having an indirect effect).

4. As but one example, it is hard to reconcile the raw data shown in Fig 5A (middle panel) with the quantification? What are the internal controls?

Overall, the extent of experimental approaches support the overall conclusions but the lack of robust XBP-1 splicing differences in a number of gels detracts from the manuscript. The authors would do well to consider removing some of these marginal data.

Referee #3

In this manuscript, Hetz and his colleagues report the identification of BH3-{degree sign}{copyright, serif}-only proteins, including PUMA, BIM, and BNip3, as Ire1α associating proteins via proteomic study using Mass Spectrophotometer. Following confirmation of their interactions with Ire1α by co-immunoprecipitation experiments in ectopically expressed and tagged

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components, the authors investigated the functional significance of the proteins' interactions with Ire1 using MEFs derived from PUMA and BIM double knockout mouse embryo.

The authors conclude that PUMA and BIM play roles in the ability of Ire1 RNase to sustain XBP1 mRNA splicing at the late stage of UPR time course. And they claim that the lack of both PUMA and BIM causes the premature termination of Ire1 activation. In the PUMA and BIM double knockout MEFs, the recovery of Ire1 measured by disappearance of the spliced XBP1 mRNA started significantly faster than in WT cells, while no effect was shown on the initial activation Ire1. After a subsequent series of experiments, the authors propose that Ire1 requires PUMA and BIM to sustain activation state of Ire1.

The manuscript deals with the recovery phase of Ire1 in response to re-{degree sign}{copyright, serif}- establishment of the functional capacity of the ER. The emerging importance of this subject is apparent from the recent burst of publications on this issue. Furthermore, if specific functions proposed for PUMA/BIM/BNip3 are clearly demonstrated, it will provide a significant step forward towards understanding of Ire1 biology. Unfortunately, the data presented in the current manuscript could lead to an equally likely alternative conclusion. Specifically, the following issues will need to be addressed.

(1) One of the major issues is whether or not the interaction between endogenous Ire1 with PUMA, BIM, and BNip3 takes place in cells. All the experiments-including the initial Mass Spec analyses that identified PUMA, BIM, and BNip3 as Ire1 interacting proteins and subsequent validation experiments-were performed with cells expressing the ectopically expressed tagged proteins.

While the authors attempt to address the specificities of the interactions again using the tagged components, the exact nature of the interactions is not clear. For example, does interaction between Ire1 and PUMA, BIM, and BNip3 take place in response to ER stress?

Also, no detailed description was given on how Mass Spec samples were prepared: What was the concentration of tunicamycin (Tm) used to treat cells to prepare extracts for the initial Mass Spec analyses?

This is an important issue specifically since defects seen in BIM/PUMA double knockout cells were only observable in cells treated at low concentration of Tm.

Furthermore, was the Ire1 immunoprecipitant from un-treated and Tm treated cells compared? Do the authors imply that all Ire1 interacts with all three proteins identified? Or does a small population of Ire1 interact with BIM while others interact with PUMA? If the latter is the case, what are the relationships between the different forms of Ire1?

(2) In Figure 2B, the authors state that there are "no significant differences in XBP1 mRNA splicing kinetics" in BIM/PUMA DKO cells when compared to those of the wild type cells and that any difference is statistically not significant.

However, based on the actual data shown, there is a significant difference in levels of spliced XBP1 mRNA at 4 or 8 hrs time points. In fact, splicing of XBP1 mRNA in BIM/PUMA DKO cells never reach the same extent as in wild type cells. One of the issues comes from the quantitation.

The quantitation shows that % splicing of XBP1 mRNA is 100% with both 4 and 8 hr time points for WT cells. However, the actual data shows that significant levels of un-spliced XBP1 mRNA is still left in the cells and, thus, the absolute level of XBP1 mRNA splicing was not 100%.

It appears that the quantitation was performed such that the splicing values for both 4 and 8 hr time points was defined as 100% and recalculated other values. Even if that were the case, this does not match the actual data as % splicing of WT cells is clearly much higher at the 8hr time point than the 4 hr time point.

In order to compare % splicing in two different strains, the method used by the authors here requires the assurance that the time point where the maximum level of XBP1 splicing is included. There is an extra time point (~6 hr) shown only for BIM/PUMA DKO cells, but not for WT cells. XBP1

splicing increased significantly at the 6 hr time point in DKO cells. What does the value for the 6 hr time point for WT cells look like? It is possible that % splicing of XBP1 mRNA is higher than either the 4 or 8 hr time points in WT cells. (If so, the value for 6hr time point should be defined as 100%.) Or alternatively, the maximum splicing might be reached at the 7 hr time point, and in such a case, inclusion of the 6hr time point is not sufficient.

Normally, in almost all other previous publications, % splicing of either HAC1 or XBP1 mRNA is calculated and presented as (spliced XBP1)/[(spliced) + (unspliced XBP1)] x 100%. The authors will need to re-plot % splicing of XBP1 [upon re-calculation as spliced XBP1/(spliced and unspliced XBP1) x 100%] and also include the value for the 6 hr time point for WT cells.

Based on the actual data (rather than quantitation), BIM/PUMA DKO cells were simply defective for their ability to induce ER stress when compared to WT cells. Regardless of the calculations, this is also clear from production of less XBP1-s protein in BIM/PUMA DKO cells (Figure 2C). Thus, the data presented here could yield an alternative explanation/model: In BIM/PUMA DKO cells, less spliced XBP1 mRNA was generated due to the lower activation level of Ire1. Furthermore, as the extent of Ire1 activation was lower, it is reasonable to expect the faster kinetics of Ire1 recovery to the basal level during the inactivation process in the recovery assays (Figures 2D & 2E). Results shown in Figure 3D that no observable difference was noted in Ire1 behaviors at high concentration of Tm also support the alternative explanation.

Potential reasons why lack of BIM/PUMA causes lower activation of Ire1 in DKO cells may be interesting. Somehow, lack of these components may make Ire1 to be less sensitive or effective to respond to ER stress. Alternatively, cells lacking BIM/PUMA are incapable of either generating ER stress or disrupting ER functional homeostasis at the concentration of Tm used. In the latter case, Ire1 in DKO cells functions just like Ire1 in WT cells, but the stimulating signal for Ire1 itself is altered. In order to distinguish these possibilities, the authors need to examine the functional state of the ER; specifically, levels of stress generated by Tm were similar in both WT and DKO cells and decrease in XBP1 splicing is correlated with recovery of ER stress.

(3) In order to assess the significance of identified interaction, the authors utilized PUMA and BIM double knockout cells. What about BNip3? Depending on how Ire1α interacts with PUMA, BIM, and BNip3, the use of double knockout cells may not be sufficient to deduce the functional significance of the interactions with each protein.

The authors need to characterize Ire1-BNip3 in PUMA & BIM double knockout (BIM/PUMA DKO) cells. For example, in BIM/PUMA DKO cells, is Ire1 interaction with BNip3 retained? Is the level of Ire1-BNiP 3 increased in double knockout cells or unchanged? Is there any change in BNiP3 protein levels in BIM/PUMA DKO cells? Are either PERK or ATF6 activities elevated at the basal level in BIM/PUMA DKO cells or upon ER stress induction?

(4) Does the interaction between Ire1 and PUMA, BIM, & BNiP3 change in response to ER stress induction? Since the proposed role of PUMA, BIM & BNip3 with Ire1 concerns the ability of Ire1α to sustain its activation at a later time point, is there any change at the later stage of ER stress induction?

(5) While the authors have found that Ire1α levels and its localization are similar in both WT and DKO cells (Supp. Fig. S4), the activation status of Ire1α (phosphorylation or oligomerization states) is the most important issue in their study and, thus, should be examined.

1st Revision - authors' response | 27 January 2012

Response to reviewers:

Reviewer 1

Comments:

This article has identified the BCL-2 family members Bim and PUMA as novel IRE1 interactors. BIM and PUMA double-knockout cells failed to maintain sustained XBP-1 mRNA splicing after prolonged ER stress. This regulation required BCL-2 (although it is not clear why) and was antagonized by either BAD or the BH3 domain mimetic ABT-737. Overall, the data are convincing and well presented. But there is a lot of data presented and it is not always clear. While the data in Figure 7 are the most intriguing in the paper the lack of controls makes it difficult to ascribe as much weight as I would like to. I have provided a list of more minor points for the authors to consider when revising the paper.

1. It is very strange that mRNA or protein levels of Bip are unchanged when the IRE-1 pathway is activated. I follow their reasoning when the authors state that "bip (a UPR-target gene independent of XBP-1s)" on page 8. However, Bip is the chaperone interacting with ER stress transducer IRE-1 and constitutively inhibits its oligomerization (Nature Cell Biology 2, 326 - 332). The text needs clarification regarding the interpretation of the Bip data as independent of XBP-1s.

Answer:
We apologize for this misunderstanding. As the reviewer mentioned, BiP is known to operate as an adjustor for UPR activation, modulating the initiation of IRE1, PERK and ATF6 responses. In this model, changes in BiP levels are not related to IRE1 activation, it is actually the dissociation (redistribution) of BiP from IRE1 which correlates with its activation. We only measured here basal or inducible levels of BiP as a measure of XBP1-independent UPR transcriptional responses.

2. In the top western blot of left panel in the Fig 1A, why is the size of IRE1α-HA so much bigger than IRE1α in the WT (lane 1 vs lane 3)? The difference in the size between IRE1α-HA and wild type IRE1α should be just the HA epitope which is small. The data in this blot need further explanation. More functional assays comparing IRE1α and IRE1α-HA (other than XBP-1 splicing assay) were presumably performed and could be summarized without adding all the data.

Answer:
The slight shift in the electrophoretic pattern is because this IRE1-HA construct was actually designed for the purification of native IRE1-containing complexes with a column and also contains two tandem HA sequences. This is why, it contains two tandem HA tags, and the construct has also a precision enzyme site for binding of IRE1 to an HA column and then the release by proteolytic cleave. As shown here and in a recent characterization of this line (Rojas et al, *Cell Death Diff,* 2012), this strategy does not alter the activity of IRE1. We have included this missing information in the revised text of methods.

3. While the authors may have used the mass spectrometry data to identify Bim as a potential IRE1; binding partner, in the manuscript a lot of emphasis is placed on what appears to have been the identification of a single peptide.

Answer:
We agree with this point. We have modified the text accordantly to avoid overstating the initial finding that opened the development of the full study. In addition, based on reviewer 2 and 3, we performed additional experiments to monitor the interaction with endogenous proteins, which in agreement with our *in vitro* RNA activity assay, further support the role of protein-protein interactions in the effects of BH3-only proteins on IRE1 signaling. In this new version protein interaction was verified by four different settings of IPs (HEK and MEFs), in addition to yeast two hybrid and pull down assays.

4. The authors make extensive use of the XBP-1 splicing assay in the paper yet quantification of Xbp-1 splicing data often does not match the sample data. In Fig 2D, Xbp-1s% of WT at 16-24 h time points do not resemble what is shown in the graph. The same problem occurs in Fig 2B to a lesser extent. More important in the experiments with Noxa in Fig 4E there is more unspliced XBP in Noxa expressing cells than in GFP expressing cells. The graph shows % spliced but it does not match the data shown. I understand the graph is an average of multiple experiments but if the data shown were included then the error bars cannot be so small. SE for N=3 does not match the data shown.

Answer:

As requested we have analyzed in detail this issue in the new version of the paper. In all experiments we presented standard error (not standard deviation) and proper statistical analysis of 3 or more independent experiments. We agree with this point since it was difficult to choose a particular experiment that matched exactly the quantification. We have now revised this issue and provided a gel that better represented the average result and also performed additional quantifications (see Figure 2B, 2E and 4D). Since the quality of some gels used for quantifications were not optimal and may interfere with this calculation, we re-run many gels of the same samples to perform new quantifications to improve the quality of the data in several experiments involving quantification of splicing.

Moreover, to give an example of the behavior of a full set of experiments, we have now presented the curve of Figure 2B now in supplementary S2A showing individual points of all three to five independent experiments and the average where the actual natural variability on the signaling kinetics is depicted. This example clearly illustrates that the quantification of single experiments was properly performed. The two genotypes shown always the same tendency, clear differences in splicing, but the actual absolute value specially in long term treatment as expected showed more variability. Thus, this supplementary information will clarify this point and will illustrate the readers exactly how the system behaves.

5. There is no indication of transduction efficiency or expression level for the proteins expressed by the retroviral constructs. In one place they do mention that the transfection efficiency (not when using the retroviral constructs) was 30% but that is about it. The methods section is also not clear and includes information about constructs not used in the manuscript (ER localized Bim, cytochrome b5 Bak etc.).

Answer:
We thank this reviewer for noticing this point. We always control the titters of the viruses and transduction efficiency by FACS analysis. As requested, we have now included one example in the main figure as control of transduction efficiency (Figure 4D) and mentioned this in methods. We have also eliminated the information in the methods that was not related to this paper (coming from early versions).

6. The authors state that "Administration of ABT-737, or mutation of the BH3 domain of BIM, abrogated the activation of XBP-1 mRNA splicing. Moreover, the binding of BIM to IRE1; was mediated by the BH3 domain, as demonstrated by mutational analyses". Other data presented suggest that binding to IRE1; was via the BH3 domain of Bim and Puma. I don't understand why Bcl-2 augments the response. "The effects of BIM over IRE1;N-HIS RNAse activity were potentiated by the presence of recombinant BCL-2 and importantly reduced when BIMWT was replaced by BIML150E (Figure 5E)." Shouldn't Bcl-2 compete with IRE1; for binding to Bim and Puma? Please explain.

Answer:
We apologize for this confusion. We are developing a follow-up story to describe the existence of a BCL2 complex with IRE1 that synergize with BIM and PUMA in the regulation of the UPR. All those experiments were excluded from this version to give a more direct message focusing on BIM and PUMA. The current version of the paper has already too much data for a 7 figures paper. This panel was a left over by mistake from a previous version! We are sorry about this issue. We have replaced this experiment for another one focusing on the effects of WT and mutant BIM in the activity of IRE1 *in vitro*. Interestingly, we have now included interesting experiments showing that actually BAK may compete with BIM for the binding to IRE1, in agreement with a rheostat model. We have now discussed in more detail our working model including in the new verison of the paper.

7. In fig 6, it would be good to confirm the results of the in vivo experiments using some other approach such as immunohistochemistry rather than relying on only the XBP-1 splicing assay.

Answer:
We tried hard to develop a histological analysis for XBP1s. We also discussed with the Laboratory of Laurie Glimcher at Harvard, a world leader on XBP1 biology, and they also confirmed that there are no good antibodies to detect XBP1 *in vivo* in IHC. Together we even tested four independent antibodies. However, we believe our *in vivo* data is solid since we validated the main results with a series of consistent assays including: (i) XBP1 mRNA splicing assays, (ii) we measured UPR target

genes y qPCR and Western blot, (iii) we monitored the levels of XBP1s protein in nuclear extracts, and (iv) performed several negative control to make sure the experiments were well performed and that the stress was triggered by the injection of tunicamycin.

We appreciate the overall enthusiasm and positive feedback of this reviewer since this study involved more than five years of work, the collaboration between six different laboratories in three continents and extensive cellular and animal experimentation with independent models, in addition to mechanistic studies in cell culture. We thank again this reviewer for giving us a positive response in the evaluation of the study and good ideas to improve the quality of the paper.

Reviewer 2

Rodriguez and co-workers report on an extensive series of experiments suggesting a link between Bim (and Puma) expression and IRE1; activity associated with XBP-1 splicing. The findings are intriguing and potentially important but are quite variable in terms of supporting the authors' claims. Data from animal and cell based experiments in Figs 6 and 7 clearly document a potential relationship between Bim, XBP-1 splicing, and UPR outputs, whereas other results appear far less robust.

Overall there is a need to better articulate (speculate) on a potential underlying mechanism and rationale. Why does a pro-apoptotic maintain IRE1; activity? How does pro-survival Bcl-2 fit in?

Answer:

We thank this reviewed for the overall feedback on the study. Regarding the role of BCL2, we have now speculated about this model in the discussion and provided an updated working model, and, based on new evidence included in this new version of the study, we can move forward to discuss a model where BCL2 and BIM-PUMA have synergic effects in the regulation of IRE1. We also included some new interesting data suggesting that this pathway is parallel to BAX-BAK (based on Co-IP data and the use of ABT-737 in BAX/BAK DKO cells) and may even compete for the binding to IRE1, increasing the novelty of the study. Finally, we have improved the mechanistic characterization of the effects of BIM/PUMA on IRE1, measuring clear changes on its phosphorylation and oligomerization state under prolonged ER stress (Figure 3E and supplementary S4A). These effects are well known to be essential to maintain IRE1 activity (Li et al *PNAS*, 2010) and will help explaining the phenotypes described here. Since we were able to (i) recapitulate interaction with purified components, (ii) we further confirmed the interaction with four different IP setting and yeast two hybrid, and (iii) modulated the activity of IRE1 *in vitro*, we propose that the effects of BH3-ony proteins on IRE1 are direct. Moreover, we now provide new evidence indicating that ATF6 and PERK activation are not regulated by BIM and PUMA, supporting the main findings in terms of specificity (Figure 3F).

The role of BCL2 was only studied in the context of BAD and ABT-737. We are currently performing a full follow-up story to investigate the possible formation of a regulatory complex between BCL2-IRE1-BH3-only proteins. We think that the current paper has already important complex data, and we prefer not to expand it to other complementary areas. Based on the new results and the request of this reviewer, we have now provided an updated model that is better discussed in the text.

Specifics

1. *Utilizing KO and DKO cells run the risk of additional compensating differences compared to wt. Have the authors recapitulated the findings with sh or siRNA?*

Answer:

We agree with this reviewer in this important point. We already controlled the possible issue of compensatory effects by reconstituting BIM and PUMA DKO cells with an expression vector for wild-type and BH3 mutant BIM, which confirmed the main findings of the study. As requested, to strength the main message we have now performed a knockdown of PUMA or BIM using shRNA and lentiviral-mediated delivery. As shown in Figure 2G, reducing the levels of BIM or PUMA decreased the levels of XBP1 mRNA splicing after prolonged ER stress.

2. The assumption throughout is that the ER stessors are not causing toxic side effects. Since

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caspase activation is typically associated with these agents, have key experiments been conducted in the presence of zVAD?

Answer:
Yes, all key experiments where apoptosis could be triggered by treatments, zVAD-fmk was employed to avoid non-specific effects. In all the experiments we used low concentrations of tunicamycin (100 ng/ml), and as shown in Supplementary Figure S2E, the kinetic of cell death were very different from the splicing effects and zVAD-fmk treatment never affected splicing. We also used caspase-9 KO cells to test possible effects of cells death on the splicing activity, and these cells did not show any UPR phenotype (not shown). More importantly, when the full data of the study and (data from the literature) is analyzed in terms of apoptosis regulatory activities (anti- and pro-apoptotic) versus their impact on XBP1 mRNA splicing (positive, negative or null effects), there is a clear dissociation between both variables. We have included a new supplementary table to discuss this important point that we believe clearly shows a dissociation between the effects of the BCL-2 family on apoptosis and UPR (Supplementary Figure S7C).

3. A massive conc of ABT is used, putting at risk the on-target conclusions of these experiments (also putting at risk the cell viability status having an indirect effect).

Answer:
We thank this reviewer for noticing this important point. As requested we performed a cell death curve with different doses of ABT-737 in WT and BAX/BAK DKO cells. We confirmed the cytotoxic effects of ABT-737 to MEF cells. This is why, we decided to perform the experiments in BAX and BAK DKO cells that were fully resistant to ABT-737 treatment, eliminating this negative issue. Data was recapitulated in these DKO cells, observing inhibition of the XBP1 mRNA splicing. We have moved to supplementary previous data in WT cells to avoid misinterpretation of the results, and replaced by these new experiments (Figure 4C). Data in WT cells was obtained after co-treatment with z-VAD-fmk to prevent apoptosis.

4. As but one example, it is hard to reconcile the raw data shown in Fig 5A (middle panel) with the quantification? What are the internal controls?

Answer:
The experiment was controlled in relation to the mock transfection. This is why we showed both the raw data and the normalization of splicing levels by discounting the basal effect of transient transfection. The experiment was performed three times and proper statistical analysis is presented to support the conclusions. We thought this graphic representation was more straightforward to give the message by taking into consideration the effects of the transfection. This setting of transient transfection was the only way to perform the experiments since prolonged overexpression of BIM WT MEFs triggers apoptosis.

5. Overall, the extent of experimental approaches support the overall conclusions but the lack of robust XBP-1 splicing differences in a number of gels detracts from the manuscript. The authors would do well to consider removing some of these marginal data.

Answer:
This is an important point also raised by reviewer 1. We have fully solved this problem in this revised version. In all experiments we presented standard error and proper statistical analysis of 3 or more independent experiments. We agree with this point since it was difficult to choose a particular experiment that matched exactly the quantification. We have now revised this issue and provided a gel that better represented the average result and also performed additional quantifications (Figure 2B, 2E and 4D).

Since the quality of some gels was not optimal and may interfere with this quantification, we re-run many gels to perform new quantifications to improve the quality of the data. Moreover, to give an example of the behavior of a full set of experiments, we have now presented the curve of experiment of Figure 2B in Supplementary Figure S2A showing individual points of all three to five independent experiments and the average where the actual natural variability on the signaling kinetics is depicted. We have revised all gels and quantification to provide a representative experiment that closely matches the splicing quantification. As the reviewer will notice, the phenotype observed in BIM and PUMA DKO cells is robust. In all experiments we observed the

same result. The absolute values of splicing levels after prolonged ER stress were more variable as expected, but it always showed the same tendency and striking differences, which is confirmed with the statistical analysis.

Furthermore, to complement our main splicing assay, in the new version of the paper we performed the measurements with two independent splicing assays, which confirmed the main findings of the study and showed clear effects in splicing attenuation in BIM and PUMA DKO cells (Figure 2B and 2F).

Reviewer 3

In this manuscript, Hetz and his colleagues report the identification of BH3-only proteins, including PUMA, BIM, and BNip3, as Ire1; associating proteins via proteomic study using Mass Spectrophotometer. Following confirmation of their interactions with Ire1; by co-immunoprecipitation experiments in ectopically expressed and tagged components, the authors investigated the functional significance of the proteins' interactions with Ire1 using MEFs derived from PUMA and BIM double knockout mouse embryo.

The authors conclude that PUMA and BIM play roles in the ability of Ire1 RNase to sustain XBP1 mRNA splicing at the late stage of UPR time course. And they claim that the lack of both PUMA and BIM causes the premature termination of Ire1 activation. In the PUMA and BIM double knockout MEFs, the recovery of Ire1 measured by disappearance of the spliced XBP1 mRNA started significantly faster than in WT cells, while no effect was shown on the initial activation Ire1. After a subsequent series of experiments, the authors propose that Ire1 requires PUMA and BIM to sustain activation state of Ire1.

The manuscript deals with the recovery phase of Ire1 in response to re-establishment of the functional capacity of the ER. ***The emerging importance of this subject is apparent from the recent burst of publications on this issue****. Furthermore, if specific functions proposed for PUMA/BIM/BNip3 are clearly demonstrated, it will provide a significant step forward towards understanding of Ire1 biology.*

Comments

1a. One of the major issues is whether or not the interaction between endogenous Ire1 with PUMA, BIM, and BNip3 takes place in cells. All the experiments-including the initial Mass Spec analyses that identified PUMA, BIM, and BNip3 as Ire1 interacting proteins and subsequent validation experiments-were performed with cells expressing the ectopically expressed tagged proteins. While the authors attempt to address the specificities of the interactions again using the tagged components, the exact nature of the interactions is not clear. For example, does interaction between Ire1 and PUMA, BIM, and BNip3 take place in response to ER stress (same comment as point 4)?

Answer:

We agree with this reviewer that interaction of endogenous proteins should be reinforced with further experiments. We would like to start clarifying that our screening for the IRE1 interactome in MEFs using Mass Spec identified PUMA as one possible candidate. Then, through co-expression experiments of IRE1 tag with HA-tagged BH3-only proteins in HEK cells we found an association also with BIM, PUMA, and also Bnip3.

As requested by this reviewer, in the new version of the study we have tested the possible interaction of endogenous BIM/PUMA with IRE1. First of all, we used IRE1 KO cells reconstituted with physiological levels of IRE1-HA (virtually identical levels to endogenous IRE1) to perform studies and measure the possible binding to endogenous BIM and PUMA at basal levels or under ER stress conditions. This experimental system allowed us eliminating the technical problems of observing the immunoglobulin in the blot (we used agarose beads covalently bound to anti-HA and performed HA peptide elution). **This analysis revealed two interesting and important findings. The binding of BIM to IRE1 is constitutive and not altered by ER stress, whereas the association with PUMA is further induced by ER stress** (Figure 1E), consistent with the proteomic study. IPs with endogenous IRE1 was technically challenging. We were able to demonstrate an association of endogenous BIM with endogenous IRE1 at basal levels (Figure 1F). These experiments have clearly improved the mechanistic aspects of the study ad further supported the proteomic analysis.

Furthermore, to increase the characterization of the system, we also performed IPs for BIM using a construct directly targeted to the ER using a cytochrome b5 tag, and then assessed the association with endogenous IRE1. We confirmed the interaction and further demonstrated that BIM possibly competes with BAX/BAK for binding to IRE1 since the co-IP was enhanced in BAX and BAK DKO cells (Figure 1G). These effects were not modulated by ER stress, confirming our previous finding (Supplementary Figure S1B)

Finally, analysis of Bnip3 levels in MEFs cells revealed almost no expression in these cells and not induced by ER stress (Supplementary Figure S1C), when compared with a positive control like mouse brain cortex (Sassone et al, *Cell Death Dis,* 2010) were BNIP3 is abundant. We have included this information in the text to justify focusing on BIM and PUMA.

In summary, overall we postulate through multiple assays that BIM and PUMA directly regulates IRE1 based on key observations using: (i) Proteomic analysis, (ii) Four different setting of IPs in living cells, (iii) pull-down assays with purified components, (iv) yeast two hybrid, and (v) the in vitro activity assay. In addition, data provided with mutations of the BH3 domain in interaction assays and XBP1 mRNA splicing assays indicate a strong association between binding and the biological effects on UPR signaling.

1b. Also, no detailed description was given on how Mass Spec samples were prepared: What was the concentration of tunicamycin (Tm) used to treat cells to prepare extracts for the initial Mass Spec analyses? This is an important issue specifically since defects seen in BIM/PUMA double knockout cells were only observable in cells treated at low concentration of Tm. Furthermore, was the Ire1 immunoprecipitant from un-treated and Tm treated cells compared? Do the authors imply that all Ire1 interacts with all three proteins identified? Or does a small population of Ire1 interact with BIM while others interact with PUMA?

Answer:

We have now clarified these issues in the methods section to explain how the MS was performed. With current methodologies, we are not able to revolve single molecule interactions, however taken together with our genetic data in BIM and PUMA DKO cells we could state that both proteins synergize in the control of IRE1 inactivation.

2a. In Figure 2B, the authors state that there are "no significant differences in XBP1 mRNA splicing kinetics" in BIM/PUMA DKO cells when compared to those of the wild type cells and that any difference is statistically not significant. However, based on the actual data shown, there is a significant difference in levels of spliced XBP1 mRNA at 4 or 8 hrs time points. In fact, splicing of XBP1 mRNA in BIM/PUMA DKO cells never reach the same extent as in wild type cells. One of the issues comes from the quantitation.

...In order to compare % splicing in two different strains, the method used by the authors here requires the assurance that the time point where the maximum level of XBP1 splicing is included. There is an extra time point (~6 hr) shown only for BIM/PUMA DKO cells, but not for WT cells. XBP1 splicing increased significantly at the 6 hr time point in DKO cells. What does the value for the 6 hr time point for WT cells look like? It is possible that % splicing of XBP1 mRNA is higher than either the 4 or 8 hr time points in WT cells. (If so, the value for 6hr time point should be defined as 100%.) Or alternatively, the maximum splicing might be reached at the 7 hr time point, and in such a case, inclusion of the 6hr time point is not sufficient.

....Based on the actual data (rather than quantitation), BIM/PUMA DKO cells were simply defective for their ability to induce ER stress when compared to WT cells. Regardless of the calculations, this is also clear from production of less XBP1-s protein in BIM/PUMA DKO cells (Figure 2C). Thus, the data presented here could yield an alternative explanation/model: In BIM/PUMA DKO cells, less spliced XBP1 mRNA was generated due to the lower activation level of Ire1.

Answer:

We apologize for generating this confusion. We agree with the reviewer that not all quantifications exactly matched the representative splicing gel presented. We thank for providing all this comments, which are very important and we have considered seriously. We have now fully solved this issue with multiple strategies summarized here:

- In all experiments we showed standard error (not standard deviation) and proper statistical analysis of 3 or more independent experiments. We agree with the point addressed by the reviewer since it was difficult to choose a particular experiment that matched exactly the quantification. We have

now revised this issue and provided a gel that better represented the average results and performed additional quantifications (Figures 2B, 2E and 4D). We also repeated most PCRs for the kinetic analysis to make sure all PCRs have the same rate of amplification with similar quality for proper quantification. Moreover, to give an example of the behavior of a full set of experiments, we have now presented the curve of Figure 2B now in Supplementary Figure S2A showing individual points of three to five independent experiments and the average where the actual natural variability on the signaling kinetics can be depicted. As the reviewer will notice, all results showed the same tendency with robust phenotypes in BIM and PUMA DKO cells. As expected there was more variability after prolonged ER stress, however ALL experiments showed the same tendency as proven by the statistical analysis. All together, the new data is more straightforward to illustrate future readers the observation indicating that the inactivation phase of IRE1 signaling is significantly altered in BIM and PUMA DKO cells.

-As requested, we performed an additional experiment that not only included the 6h time point, but also 1, 2, 4 and 6h. **The results provided now in** Figure 2C **clearly demonstrate that early activation for XBP1 mRNA splicing is not affected by BIM and PUMA double deficiency.** We wanted to make sure the answer obtained is clear to avoid alternative interpretations by including all these time points. We thank this reviewer for requesting this experiment that will help illustrating the major findings.

-To further confirm our main results, we also performed additional and independent XBP1 mRNA splicing assays using specific primers for *xbp1s* mRNA detection, and also a Pst1 digestion assay to better separate spliced and unspliced XBP1 mRNA forms. These new experiments are provided in Figure 2 and further support the main finding of the paper. In addition, they illustrate that the absolute effect measured on the splicing obtained with 3 assays is not exactly the same, but the tendency and effects depicted in BIM and PUMA DKO cells are virtually identical. These results have strengthened the main findings of this study. Also they illustrate that each assay has its own problem on sensitivity (absolute values of measurements are not identical for each assay), the same samples were analyzed for comparison. This information will be relevant to the field because not many labs perform this type of multiple confirmation.

- We would like to reinforce the fact that results obtained with a pulse of thapsigargine were black and white showing a specific defect on inactivation of XBP1 mRNA splicing (Figure 2F).

- Just to clarify the methodology, in all experiments the amount of unspliced + spliced XBP1 was counted as 100% splicing for each time point and genotype. This is the most accepted way for quantifying XBP1 mRNA splicing with this RT-PCR assay. We have now explained the assays in the methods.

2b. ...Potential reasons why lack of BIM/PUMA causes lower activation of Ire1 in DKO cells may be interesting. Somehow, lack of these components may make Ire1 to be less sensitive or effective to respond to ER stress. Alternatively, cells lacking BIM/PUMA are incapable of either generating ER stress or disrupting ER functional homeostasis at the concentration of Tm used. In the latter case, Ire1 in DKO cells functions just like Ire1 in WT cells, but the stimulating signal for Ire1 itself is altered. In order to distinguish these possibilities, the authors need to examine the functional state of the ER; specifically, levels of stress generated by Tm were similar in both WT and DKO cells and decrease in XBP1 splicing is correlated with recovery of ER stress.

Answer:
We agree with this points that was partially addressed in the original version of the manuscript.

-For example in Figure 3D we monitored the basal and inducible levels of general ER foldases not regulated by XBP1 including: the disulfide isomerases ERp72, ERp57 and PDI and the ER chaperone BiP. The expression of all these markers was not altered in BIM and PUMA DKO cells indicating that possibly (i) basal ER physiology is normal and (ii) inducible levels after Tm treatment are similar in DKO cells. In addition we monitored ATF4 levels and BiP in the liver of BIM KO mice injected with Tm (Figure 6D), which showed normal inducible levels.

-Moreover we performed a full set of experiments demonstrating that basal ER calcium levels are normal in BIM and PUMA DKO cells, a parameter known to be altered in ER stressed cells (Rojas et al., *Cell Death Diff*, 2012).

-To strengthen this important point addressed by this reviewer, we have now measured the

levels of PERK activation (phosphorylation shift) and also measured the processing of ATF6 in WT and BIM/PUMA DKO cells. The activation of these two sensors was not drastically affected in BIM and PUMA DKO cells. In addition to indicate that stress sensitivity is normal in DKO cells, these new experiments also clarified the specificity of BIM/PUMA in the specific control of IRE1 (Figure 3E). We thank again this reviewer for prompting us performing these important determinations.

3. In order to assess the significance of identified interaction, the authors utilized PUMA and BIM double knockout cells. What about BNip3? Depending on how Ire1; interacts with PUMA, BIM, and BNip3, the use of double knockout cells may not be sufficient to deduce the functional significance of the interactions with each protein. ...For example, in BIM/PUMA DKO cells, is Ire1 interaction with BNip3 retained? Is the level of Ire1-BNiP 3 increased in double knockout cells or unchanged? Is there any change in BNiP3 protein levels in BIM/PUMA DKO cells?

Answer:
As clarified in point 1a, the interaction of BNip3 with IRE1 delta N was found only in an overexpression system in HEK cells. We believe Bnip3 is not a relevant player in BIM and PUMA DKO cells since this protein levels were almost not detected at basal or ER stress conditions (Supplementary Figure S1C). We have used this information to state in the text why this protein was not studied. We believe that addressing the impact of BNip3 may involve a full study.

With the data provided regarding BIM, PUMA and BAD in vitro, in vivo and in a physiological model of ER stress. We believe in this revised version the main message of the paper is hopefully clear. In the literature there are more than 20 different BH3-only proteins described that could be investigated in the context of the UPR and could be the subject of future systematic analysis. We have now reinforced the main results by more interaction assays, controls and also knockdown experiments for BIM and PUMA using shRNA (i.e. Figure 2G)

5. While the authors have found that Ire1α levels and its localization are similar in both WT and DKO cells, the activation status of Ire1α (phosphorylation or oligomerization states) is the most important issue in their study and, thus, should be examined.

Answer:
We also agree with this reviewer that many other important aspects of IRE1 biology were not directly investigated in this study that will give important mechanistic insights to the proposed model. However, it is important to mention that most recent studies make more difficult the interpretation of IRE1 phosphorylation data since (i) activation involves several steps of phosphorylation (Kaufman group 2006 PNAS), and (ii) further phosphorylation events may be even needed to inactivate yeast IRE1p (Walters and Niwa´s studies *J Cell Biol*, 2011).

As requested by this reviewer, we have implemented two new assays to determine the rate of IRE1 phosphorylation using recently described PhosTag™ assays. These experiments were technically challenging and took us very long to solve (dozens of experiments!), this is why we resubmitted this paper after three months of work instead of two months. These new experiments indicate that high state IRE1 phosphorylation is altered in BIM and PUMA DKO cells undergoing ER stress, with a dramatic effect in the inactivation phase (Figure 3E). Then, we also monitored the appearance of IRE1 clusters/oligomers in non-denaturing gels associated with the appearance of a smear in the electrophoresis pattern of IRE1 triggered by ER stress. This smear was drastically decreased in DKO cells after prolonged ER stress, suggesting faster inactivation (Supplementary Figure S4A). We tried to monitor clusters by immunofluorescence but this never worked at the low doses of stress used here (not shown). It is important to mention that the only study about clusters in mammalian cells was performed using overexpression, not endogenous proteins (Lin et al., *PNAS*, 2010). Only few labs in the word perform these type of experiments, with are technically challenging and give poor quality images. Importantly, we also showed that activation of ATF6 or phosphorylation of PERK was not drastically altered in BIM and PUMA DKO cells (Figure 3F), reinforcing the interaction data.

A new coauthor was included in the current version of the paper, Hery Urra, who performed all these new experiments. Together, these two assays gave deeper insights about the molecular regulation of IRE1 by BIM and PUMA. Together with on our yeast two hybrid data, in vitro interaction assays, co-immunoprecipitations, and the cell free RNAse activity assay, we postulate a model where BH3-only proteins regulate IRE1 directly through the formation of a protein complex.

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Overall with a multidisciplinary approach we have provided independent evidence supporting a role of BIM and PUMA in the regulation of IRE1 with strategies ranging from: in vitro, in cell culture, in vivo and in a physiological model of ER stress.

We appreciate the deep feedback from this reviewer and his/her constructive ideas helped us to improve the clarity and quality of the data presented here. We believe that the new splicing data, co-IP experiments, and P-IRE1 have improved the quality of the main message of the paper, suggesting a novel role of BIM and PUMA in the inactivation of IRE1 signaling.

Thanks very much for your time and effort in handling this manuscript- we realize that this letter is overly long and detailed but we wanted to be thorough in our response. We hope that you will find that the revised manuscript merits publication in *EMBO Journal.*

2nd Editorial Decision 22 February 2012

Thank you for sending us your revised manuscript. Referee 2 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner and that the paper will be publishable in The EMBO Journal.

Prior to formal acceptance, there are a number of editorial issues that need further attention:

* Please add an author contributions section and a conflict of interest statement into the main body of the manuscript text after the acknowledgements section.

* Please add scale bars and explanations to figures 1G, S4B, S7A.

* Please add the statistical details including the number of independent repeats to figures 1A, 2B, 3B/C, 4C, 5A, 7A/C/D, S5.

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

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

The authors have addressed the majority of the deficiencies that were cited in my original review of this manuscript. While many questions remain, the reported observations on the relationship

between Bcl-2 family members and the IRE1alpha scaffold, and their influence on IRE1alpha activitie, justifies publication in EMBO J.

2nd Revision - authors' response 07 March 2012

We just submitted the final version of the paper with all the changes requested. We also included all raw images for blots as a separate file.

Thank again for your interest in our work.

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