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BAX inhibitor-1 regulates autophagy by controlling the IRE1α branch of the unfolded protein response

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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 25 February 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by two referees whose comments are shown below. As you will see while both referees consider the study as interesting and while referee 1 is considerably more positive, referee 2 is not convinced that the study is strong and convincing enough to be publishable at this stage of analysis. He/she feels strongly that apart from sorting out issues with the conclusiveness of the data, both the mechanistic depth as well as the in vivo data need to be strengthened by further experimentation. All in all, we should thus be able to consider a revised manuscript that is strengthened along the lines suggested by referee 2. Please do not hesitate to get back to me if you have any questions regarding the revision.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees. We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on 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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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):

IRE1α is a master regulator of the unfolded protein response (UPR) and has been shown to play a role in the activation of autophagy through JNK signaling. The authors previously showed that Bax inhibitor 1 (BI-1) is a negative regulator for IRE1α . In this article, the authors discovered a link between BI-1 and autophagy.
The authors showed that BI-1 knockout cells presented a faster and stronger induction of autophagy and BI-1 knockout cells were resistant to cell death under nutrient starvation conditions. The authors revealed that repression of autophagy by BI-1 was dependent on IRE1α -mediated JNK activation. In addition, the authors demonstrated that BI-1 expression regulates autophagy in vivo using BI-1 knockdown flies and BI-1 knockout mice. The authors' model is supported by well-executed experiments. There are several minor points that should be addressed by the authors.

1. In Figure 2A, expression levels of both LC3-I and LC-3 II were increased. The authors should comment on this.
2. In Figure 4C, expression levels of total IRE1α protein were increased in BI-1 knockout cells. Is BI-1 involved in IRE1 protein degradation?
3. In Figure 7E, the authors should examine IRE1α protein expression levels. The expression levels of IRE1 α might be increased in tissues from BI-1 knockout mice.
4. Please check the color of bars in Figure 1 E
5. Please check the lanes shown in the upper panel of Figure 6A for accuracy.
6. The last sentence of the summary is not clear and should be more specific.

Referee #2 (Remarks to the Author):

In this paper by Castillo et al, the authors show a role for BI-1 as a negative regulator of autophagy, both basal and starvation induced. They propose a mechanism involving disruption of the IRE1a-TRAF2 interaction by BI-1, which leads to reduced JNK activation and consequently, reduced autophagy. Furthermore, they show increased autophagy upon loss of BI-1 in physiological models of autophagy in flies and mice. This is an interesting study yet many of the presented data are not convincing as detailed below. The depth of the research has been sacrificed in order to achieve a greater breadth by combining mechanistic studies with physiological models. In general terms, the most critical missing factors are a) more rigorous proof of the proposed mechanism involving the IRE1 α -TRAF2 --> JNK --> Bcl2/Beclin pathway; and b) improvements in their physiological models.

Major Specific Points:
1. Assays for autophagy: Although the authors use several parameters to measure autophagy to further assess the impact of BI-1 knock-out in MEFs, the assays used are not always appropriate to prove autophagy induction and some are technically problematic. A. The change in acidic vacuole accumulation is dramatic, but unfortunately is not necessarily specific for autophagosomes, but rather for lysosomal activity, and in itself is not an adequate readout for autophagy. Likewise, in the

fly, a more reliable assay for autophagy than lysotracker staining should be used to quantify reduction in autophagy in the gain of function model. B. To truly evaluate the impact of the lysosomal inhibitors on LC3-II levels, the +/- inhibitor data should be compared on the same blot, side by side so as to be able to assess the increase in LC3II upon lysosomal inhibition. C. The EM is not convincing in that classical autophagosomes are not shown, or at least cannot be distinguished at the resolution shown. D. The p62 data is counterintuitive; with enhanced autophagy, one expects to see increased p62 degradation and therefore fewer puncta, but their data shows the opposite. In the text (bottom pg 8), the authors state that p62 protein were lower in KO, in contrast to elevation in mRNA seen, yet Fig S2A and B suggest that basal levels of p62 protein are higher in KO as well. Since this model sees an increase in transcriptional levels of p62, its overall degradation levels cannot be used as an indicator of autophagy. E. The faster accumulation of GFP-LC3 in KD flies is not obvious; the authors should show quantitation of levels in many cells, with statistical comparison. F. The LC3 staining shown in Figs 1,4,5 is not convincing; it looks like aggregates and not LC3 puncta. G. In some of the immunoblots for LC3 detection it does not seen that the ratio LC3II/LC3I changes in the KO cells.
2.Other technical issues: The IPs in Fig6 are problematic. In Fig6A: more IRE1 was immunoprecipitated and that can explain the increased co-IP; in Fig6B- the authors do not show this important value at all.
3. The authors claim that JNK-mediated phosphorylation of Bcl2 is involved in BI-1 regulated autophagy, by showing that depletion of Beclin 1 blocks autophagy in BI-1 KD cells. Yet, as a critical regulator of Vps34, Beclin1 is required for autophagy in most cases, so it is not surprising that KD of this critical factor would block BI-1-KO-induced autophagy. To prove a more specific role, i.e. that Beclin is a downstream target of IRE1 α -JNK pathway, the authors should show enhanced Bcl-2 phosphorylation, and decreased interaction between Bcl-2 and Beclin1 in a JNK dependent manner. In fact fig 6 shows that BI-1 KO does not affect the Beclin/Bcl-XL interaction; what about Bcl-2?
4. The most novel finding regarding the mechanism by which BI-1 regulates autophagy is its influence on the IRE1 α -TRAF2 interaction. This point should be developed further. The authors show that BI-1 overexpression decreases the interaction, but since they show no functional data regarding BI-1 overexpression and autophagy, it is critical to show the reverse, that BI-1 KO leads to increased interaction compared to WT, and hence, increased JNK activation etc. The effect should also be shown with endogenous proteins, if antibodies for IP are available. Furthermore, is TRAF2 necessary for BI-1's effects on autophagy- does KD of TRAF2 in BI-1 KO cells block starvation induced autophagy, as the authors show for IRE1 α KD?
5. The physiological relevance of BI-1's role in the mouse model should be strengthened. The enhancement of basal autophagy in KO mice liver and kidney should be confirmed by observing the actual tissue for presence of autophagosomes by EM, or by crossing to GFP-LC3 mice and scoring for LC3 puncta in these tissue. What is the consequence of this upregulation of autophagy on tissue histology and organ function? Also, since most of the paper dealt with starvation-induced autophagy, the authors should determine the effect that BI-1 KO has in specific tissues during starvation in the intact mouse.
6. The authors discuss BI-1's role in regulating apoptosis during ER stress and ischemia reperfusion. Interestingly, these stimuli have been shown to involve autophagy as well. Considering the effects of BI-1 on IRE1 α , does BI-1 repress autophagy in these circumstances as well, or are its effects limited to starvation-induced autophagy? There is a conflict in opinion whether autophagy during ER stress and ischemia-reperfusion is protective or promotes cell death. Here, the authors show increased susceptibility (lower cell viability) in response to Tunicamycin in BI-1 KO vs WT, in contrast to starvation. Is this due to the anti-apoptotic effects of BI-1, or is this due to contribution of autophagy to cell death during ER stress? Furthermore, the KO mouse would be a good tool to use to test the role in BI-1 in the intact animal during ER stress or ischemia/reperfusion.

More minor points:
1. The authors state: "As shown in Figure 4C, knocking down IRE1 α decreased the basal levels of LC3-II in BI-1 deficient cells, in addition to reduce the induction of LC3-II after nutrient starvation (Figure 5A and B)." Yet, in Fig 5B, KD does not decrease the basal autophagy in non-treated cells when assessed by quantitating GFP-LC3 puncta, which presumably measures the same event as the western blot in a different manner. How is this discrepancy explained? Is the decrease in LC3II accumulation on western blot reproducible and statistically significant if quantified by densitometry?
2. What is the rationale for comparing starvation to glucose and ER stress in terms of IRE α

phosphorylation and XBP-1 splicing. There seems to be no particular increased phosphorylation in response to starvation, yet the authors see an increase WFS-1 transcription, which the authors say is regulated by such phosphorylation. What is the significance of this increase? Also, the increase in phospho-IRE1 α in KD untreated cells, when compared to glucose or tunicamycin treatment seems very small, especially when considering the large increase in total protein observed in KO cells. Phospho levels should be quantitated and normalized to total protein and also the loading control, which is not equal in all lanes. It is not clear what contribution this data has to their model.
3. The study of the putative yeast homologue is not very relevant and may be better placed in supplementary data, especially since only one assay is used to exclude a role for this protein in autophagy. Please note that the controls used are actually negative controls, not positive controls as stated.
4. English usage is sometimes awkward, and text should be carefully edited.
5. Additional technical problems in figures: A. The LC3 blot upon in Fig 4A is of poor quality, bands are fuzzy, esp in WT. Please replace. B. Fig. 4A seems mislabeled - should be a + under 16h WT. C. Fig 5b legend key- colors of boxes in legend does not match columns.

1st Revision - authors' response 14 June 2011

Response to reviewers:

Referee #1:

IRE1α is a master regulator of the unfolded protein response (UPR) and has been shown to play a role in the activation of autophagy through JNK signaling. The authors previously showed that Bax inhibitor 1 (BI-1) is a negative regulator for IRE1 . In this article, the authors discovered a link between BI-1 and autophagy. The authors showed that BI-1 knockout cells presented a faster and stronger induction of autophagy and BI-1 knockout cells were resistant to cell death under nutrient starvation conditions. The authors revealed that repression of autophagy by BI-1 was dependent on IRE1 α -mediated JNK activation. In addition, the authors demonstrated that BI-1 expression regulates autophagy in vivo using BI-1 knockdown flies and BI-1 knockout mice. The authors' model is supported by well-executed experiments. There are several minor points that should be addressed by the authors.
1. In Figure 2A, expression levels of both LC3-I and LC-3 II were increased. The authors should comment on this.

Response:
We thank this reviewer for the helpful suggestions and commentaries. All these thoughts and observations clearly helped us improving the strength of the overall message and we have included all the points requested.
In many reports levels of LC3-I are induced concomitant to LC3-II form. In addition, recent evidence indicates that LC3 per se is upregulated under autophagy conditions. However little is know about possible regulatory events on LC3-I. In addition, the stability of both forms is very different (even in the test tube), which makes difficult the comparison between the levels of both LC3 forms (Klionskly et al, 2008 Autophagy). This is why the most accepted way of analyzing autophagy so far is measuring LC3-II levels with normalizations to a loading control in autophagy flux assays. We have included a sentence mentioning the increase in LC3-I in BI1- KO cells. In addition as control, we monitored lc3 mRNA levels under resting conditions in BI-1 WT and KO cells by real time PCR, and did not detect any significant differences between both genotypes (Fig. S1D).

2. In Figure 4C, expression levels of total IRE1 protein were increased in BI-1 knockout cells. Is BI-1 involved in IRE1 protein degradation? In Figure 7E, the authors should examine IRE1 protein expression levels. The expression levels of IRE1α; might be increased in tissues from BI-1 knockout mice.

Response:

As requested by this reviewer, we have now monitored and quantified the expression of total IRE1α in several experiments using MEFs and did not observe overall any significant differences at the protein or mRNA level (Fig. S1F). In addition, as requested we have also included a Western blot analysis of the liver and kidney protein extracts from of BI-1 deficient mice and litter mate control animals to corroborate this observation in vivo (Fig. 7A and B).

4. *Please check the color of bars in Figure 1 E*
5. *Please check the lanes shown in the upper panel of Figure 6A for accuracy.*
6. *The last sentence of the summary is not clear and should be more specific.*

Response:
All these great suggestions have been added to the new version of the manuscript. We deeply thank this reviewer for his/her efforts in improving this study.

Referee #2:
In this paper by Castillo et al, the authors show a role for BI-1 as a negative regulator of autophagy, both basal and starvation induced. They propose a mechanism involving disruption of the IRE1-TRAF2 interaction by BI-1, which leads to reduced JNK activation and consequently, reduced autophagy. Furthermore, they show increased autophagy upon loss of BI-1 in physiological models of autophagy in flies and mice. This is an interesting study yet many of the presented data are not convincing as detailed below...

Major Specific Points:
1. Assays for autophagy: Although the authors use several parameters to measure autophagy to further assess the impact of BI-1 knock-out in MEFs, the assays used are not always appropriate to prove autophagy induction and some are technically problematic.

A. The change in acidic vacuole accumulation is dramatic, but unfortunately is not necessarily specific for autophagosomes, but rather for lysosomal activity, and in itself is not an adequate readout for autophagy. Likewise, in the fly, a more reliable assay for autophagy than lysotracker staining should be used to quantify reduction in autophagy in the gain of function model.

Response:
We agree with this reviewer that lysosomal staining (content) is not a direct measurement of autophagy. Our intention with these assays was just to complement all the direct assays we used including EM and LC3-flux assays and survival experiments under nutrient starvation conditions. We used this information because this finding was the initial observation that moved us to the major discovery. We have now been more careful in the text to avoid over interpretation of these data. Interestingly, very recent reports indicate that lysosomal content and distribution is actually a key factor for efficient autophagy induction (Settembre et al., 2011 Science; Korolchuk et al., 2011 Nat. Cell Biol.). We have now included these references in the text, which clearly increased the relevance of Figure 1 in this new context in the field. We also performed quantifications of the number of cells presenting large lysosomal staining in BI-1 WT and KO cells in addition to cells overexpressing BI-1GFP (Fig. S1A).

As requested by this reviewer, to complement this study we have now performed new direct assays for autophagy activity by expressing the reporter LC3-RFP-GFP. This reporter construct allows directly visualizing autophagosomes (yellow dots) and autophagolysosomes (red dots) avoiding the use of lysosomal inhibitors (Klionsky et al., 2008). Using this system we confirmed augmented autophagy fluxes in BI-1 KO cells when compared with WT cells (Fig. 1E). Moreover, we performed new experiments to monitor by EM the presence of autophagosomes by co-localizing these images with LC3-RFP-GFP fluorescence using confocal microscopy (Fig. S1E).

In addition, as suggested by this reviewer, we have improved the in vivo studies in flies by quantifying the presence of LC3-GFP positive autophagosomes in dBI-1 RNAi flies in the salivary gland (a physiological model of autophagy) (Fig. 6C, information in the text). We also monitored the levels of LC3 and lysotraker staining in the fat tissue of fly larvae exposed to nutrient starvation (Fig. S4A). Furthermore, we performed flux assays in dBI-1 RNAi larvae after exposure to nutrient starvation and observed a dramatic increase in LC3 levels, which were partially reverted by inhibiting JNK (Fig. 6B). Together these experiments have improved the validation of dBI-1 in the

control of autophagy in vivo in our fly model.

B. To truly evaluate the impact of the lysosomal inhibitors on LC3-II levels, the +/- inhibitor data should be compared on the same blot, side by side so as to be able to assess the increase in LC3II upon lysosomal inhibition.

Response:
We apologize for this misunderstanding. All experiments comparing BI-1 WT and KO cells or treatments were always performed in the same gel and film exposure. For comparisons when samples did not fit on one gel, relevant controls were repeated and all quantifications were done in the same film with the same exposure time. The images were rearranged to maintain the symmetry of the figure. We now have stated this in the methods.

C. The EM is not convincing in that classical autophagosomes are not shown, or at least cannot be distinguished at the resolution shown.

Response:
We have replaced some of these images for new ones with higher magnification to better visualize autophagosome morphology. In addition, as mentioned in point A, we performed additional experiments using co-localization of EM and fluorescent images after expression of tandem monomeric LC3-RFP-GFP in BI-1 KO cells (Fig. 1E and S1E).

D. The p62 data is counterintuitive; with enhanced autophagy, one expects to see increased p62 degradation and therefore fewer puncta, but their data shows the opposite. In the text (bottom pg 8), the authors state that p62 protein were lower in KO, in contrast to elevation in mRNA seen, yet Fig S2A and B suggest that basal levels of p62 protein are higher in KO as well. Since this model sees an increase in transcriptional levels of p62, its overall degradation levels cannot be used as an indicator of autophagy.

Response:
We agree with this reviewer that p62 data is complex especially because this protein has cellular many functions beyond autophagy. We have now clarified in the text the interpretation of these experiments. Although p62 levels are higher at basal levels in BI-1 KO cells, when we induce nutrient starvation the extent (amplitude, area under the curve) of degradation are clearly higher in BI-1 KO cells than control cells (Western blot analysis), indicating faster p62 fluxes.
Since during autophagy p62 is first associated with autophagosomes and then is degraded in autophagolysosomes (autophagy flux), we have performed kinetic studies by immunofluorescence followed by quantifications. In agreement with our LC3 data, a differential kinetic of elimination of p62 was observed where it was more efficient in BI-1 KO cells (Fig. S2C). Finally, also realized that changes in p62 mRNA levels are observed late, with only minor changes during early phases of autophagy where we see changes on LC3 fluxes (Fig. S2D). During early time points (0-4h) no significant differences in p62 mRNA levels were observed between BI-1 WT and KO cells (Fig. S2D). We have now decided to move all p62 data to a new supplementary figure to center the main discussion of the paper on LC3, the gold standard in the field to monitor autophagy. As suggested by this reviewer another possible option could be to exclude p62 data from the article, which will not have a significant impact on the overall message.

E. The faster accumulation of GFP-LC3 in KD flies is not obvious; the authors should show quantitation of levels in many cells, with statistical comparison.

Response:
As mentioned, to improve these experiments we have now quantified LC3-GFP dots in these experiments and also monitored LC3-GFP dots and lysotracker staining in the fat tissue. We also performed additional assays in RNAi flies to monitor LC3 fluxes by exposing animals to nutrient starvation. In addition, the impact of JNK inhibitors in the system were now monitored, observing augmented accumulation of LC3-II in dBI-1 RNAi animals (Fig. 6B). More importantly, we have also performed new assays to assess the viability adult dBI-1 RNAi flies under starvation condition, which presented a marked protection over time when compared with control RNAi animals (Fig. 6E). This result will increase the physiological impact of this study.

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F. The LC3 staining shown in Figs 1,4,5 is not convincing; it looks like aggregates and not LC3 puncta. G. In some of the immunoblots for LC3 detection it does not seen that the ratio LC3II/LC3I changes in the KO cells.

Response:
We have improved the quality of the images in Figure 1 to show a better example of what we consider as LC3 positive autophagosome by looking to the endogenous protein. As mentioned, we now used alternative assays to monitor LC3 dots. As described in point A, using the reporter LC3-RFP-GFP (monomeric GFP that do not aggregate) we corroborated the stronger induction of autophagy flux in BI-1 deficient cells (Fig. 1E and S1E). Also, based on our EM data, we now know that there are clusters of vesicles (a key event in autophagy; Korolchuk et al. 2011 Nat Cell Biol) that under low magnification may look like a large aggregate. However it is important to mention that we monitored by immunofluorescence endogenous LC3 levels. In general, the problem with LC3 aggregates is observed in the literature when LC3-GFP is overexpressed in cell culture.
To avoid wrong interpretations of LC3I/LC3II levels, we always quantified several independent experiments, in addition to specifically normalize LC3-II levels with a loading control (Hsp90) without measuring LC3-I as recommended by the guidance of the autophagy community (Klionsky et al., 2008 Autophagy).

2. The IPs in Fig6 are problematic. In Fig6A: more IRE1α was immunoprecipitated and that can explain the increased co-IP; in Fig6B- the authors do not show this important value at all.

Response:
Since we performed triple co-transient transfection for protein-protein interaction studies with different plasmids, there was some degree of variability on individual experiments in terms of total expression levels of the constructs. This is why we have now performed quantification of three independent experiments for the co-IP of TRAF2 with IRE1 in the presence or absence of BI-1 normalizing the IP data by the expression of the proteins in the inputs. This new quantitative data indicates a strong and significant effect of BI-1 on the binding of TRAF2 to BI-1 (~80% reduction of IRE1/TRAF2 interaction; Fig. 5F).

3. ...To prove a more specific role, i.e. that Beclin is a downstream target of IRE1a-JNK pathway, the authors should show enhanced Bcl-2 phosphorylation, and decreased interaction between Bcl-2 and Beclin1 in a JNK dependent manner. In fact fig 6 shows that BI-1 KO does not affect the Beclin/Bcl-XL interaction; what about Bcl-2?

Response:
We appreciate this great suggestion from the reviewer since this experiment could certainly strength the mechanistic aspects of the paper. We have now monitored BCL-2 phosphorylation in BI-1 WT and KO cells undergoing nutrient starvation. In agreement with our JNK-related data, a stronger BCL-2 electrophoretic shift (endogenous protein) was observed in BI-1 deficient cells after induction of autophagy when compared with control cells (Fig. 4C). This shift was partially reverted by treatments with JNK inhibitors (Fig. 4C and Fig. S3B).
The interaction data presented in the original version was designed to monitor possible physical interaction of BI-1 with Beclin-1. In our hands BCL-2 and BCL-XL show similar interactions with Beclin-1 as reported by Kroemer's group. In addition, both proteins are structurally very similar, they (i) present an unstructured loop, (ii) both show similar phosphorylation patterns that are modulated by JNK inhibitors, and (iii) both regulate Beclin-1 activity.
It is important to highlight that not always autophagy is dependent on Beclin-1 (see some examples in Scarlatti et al., 2008 Cell Death Diff; Smith et al., 2010 Cell Death Diff; Grishchuk et al., 2011 Autophagy). This is why our knockdown experiments are very important to define the functionality of Beclin-1 in the regulation of autophagy by BI-1 to define a mechanism. In addition, the only known mechanism to induce Beclin-1-dependent autophagy is to dissociate its repression by BCL-2/BCL-XL by competition or post-translational modifications. To strength this point, we have now monitored the stability of the Beclin-1/BCL-XL complex in BI-1 WT and KO cells. These experiments showed a higher dissociation of BCL-XL from Beclin-1 in the presence or absence of BI-1 under conditions of nutrient starvation (Fig. 4E). In combination, these experiments have improved the mechanistic aspects of this report.

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4. The most novel finding regarding the mechanism by which BI-1 regulates autophagy is its influence on the IRE1-TRAF2 interaction. This point should be developed further. The authors show that BI-1 overexpression decreases the interaction, but since they show no functional data regarding BI-1 overexpression and autophagy… The effect should also be shown with endogenous proteins, if antibodies for IP are available. Furthermore, is TRAF2 necessary for BI-1's effects on autophagy- does KD of TRAF2 in BI-1 KO cells block autophagy?

Response:
As requested by this reviewer, we have now performed overexpression experiments of BI-1-GFP in BI-1 deficient cells and repeated a few key experiments to validate the major findings obtained with BI-1 KO cells. We observed that overexpression of BI-1 repressed autophagy (LC3 fluxes) and also sensitized cells to cell death under conditions of nutrient starvation (Fig. 2F and 3E). As mentioned we also improved the TRAF2/IRE1 IP data by performing quantification of three independent experiments (Fig. 5F). As recommended, we have now expressed a TRAF2 dominant negative construct in BI-1 KO cells and monitored LC3-II levels. A clear reduction in LC3-II levels was observed in BI-1 deficient cells after blocking TRAF2 function with this strategy (Fig. 5E). In relation to the interaction point with endogenous proteins, we did try hard to perform immunoprecipitation of endogenous complexes with available commercial antibodies with no luck due to the poor immunoreactivity of these antibodies with native conformations. Since the new version of the paper has now many complementary experiments addressing the same mechanistic points, and the IRE1/TRAF2 complex is well validated in the literature, we think our experiments will be well received by the community.

5. The physiological relevance of BI-1's role in the mouse model should be strengthened. The enhancement of basal autophagy in KO mice liver and kidney should be confirmed by observing the actual tissue for presence of autophagosomes by EM, or by crossing to GFP-LC3 mice and scoring for LC3 puncta in these tissue. What is the consequence of this upregulation of autophagy on tissue histology and organ function? Also, since most of the paper dealt with starvation-induced autophagy, the authors should determine the effect that BI-1 KO has in specific tissues during starvation in the intact mouse.
The authors discuss BI-1's role in regulating apoptosis during ER stress and ischemia reperfusion. Interestingly, these stimuli have been shown to involve autophagy as well... Is this due to the anti-apoptotic effects of BI-1, or is this due to contribution of autophagy to cell death during ER stress? Furthermore, the KO mouse would be a good tool to use to test the role in BI-1 in the intact animal during ER stress or ischemia/reperfusion.

Response:
We have performed additional experiments to address the impact of BI-1 in vivo. Since ER stress is a well accepted inducer of autophagy, we have moved forward and monitored the flux of LC3 in the liver of BI-1 WT and KO mice after injection of tunicamycin. Remarkably, after quantification of these experiments, we were able to confirm the occurrence of a very potent effect of BI-1 deficiency in the induction of autophagy, observing a strong and robust LC3 flux (kinetic and amplitude of LC3 clearance (Fig. 7C). We believe the data presented in terms of LC3-II levels in kidney and liver tissue is strong because it was complemented with the analysis of ATG5-ATG12 complex, another key step in the autophagy process. Since the estimated time for crossing BI-1 KO mice with transgenic mice for LC3 will take at least 10-12 month (importation, quarantine, and crossing for 3 generations to get a KO/transgenic mice), it is not feasible to perform this type of experiments in the 90 days period allowed by EMBO Journal to answer his reviewers request. Endogenous LC3 histology in liver in our hands gave also poor quality images/sensitivity for publication.
Since approval of an animal protocol in our institution to perform nutrient starvation in mice is very complex due to bioethical restrictions, we decided to further address the function of BI-1 in full animal survival under conditions of nutrient starvation on an alternative animal model. Flies are well suitable full organisms to answer this type of physiological questions. We have developed an assay using adult RNAi flies. Striking results were obtained when we exposed adult dBI-1 RNAi flies to nutrient starvation, observing a significant extension of life span when compared with control animals (3 independent experiments, 100 animals per conditions) (Fig. 6E and Fig. S4B). In sharp contrast, when the same model was exposed to ER stress, dBI-1 RNAi flies were highly susceptible to ER stress (Fig. 6F). This is a stinking result that overall validate a new function of dBI-1 in the control of survival against nutrient starvation conditions, increasing the impact and significant of the study. These experiments involved two new collaborators that are expert on fly models. Thus, we

provide strong evidence indicating an inhibitory effect of BI-1 on autophagy and survival under starvation condition.

In terms of using ischemia/reperfusion, we respectfully think that this particular model is very complex to analyze and interpret since known many factors, in addition to autophagy (i.e. ROS, necrosis, inflammation, HIF, and apoptosis) contribute to the pathological process and will not directly help solving the main question directly. Although investigating ischemia/reperfusion models is very interesting and certainly will have a great biomedical impact, we think that using this model properly will involve a full study (a new full paper) and is out of the scope of the current paper.
In summary, we have improved the in vivo validation of the role of BI-1 in autophagy using flies (LC3 levels in (i) salivary gland and (ii) fat tissue, (iii) LC3 flux assays in larvae, and (iv) adult fly survival under starvation conditions) in addition to monitor the dynamic of LC3 flux in the liver of BI-1 KO mice. In addition we improved the mechanistic insights to link the BI-1 regulatory pathway to JNK and Beclin-1.

More minor points:
1. The authors state: "As shown in Figure 4C, knocking down IRE1α; decreased the basal levels of LC3-II in BI-1 deficient cells, in addition to reduce the induction of LC3-II after nutrient starvation (Figure 5A and B)." Yet, in Fig 5B, KD does not decrease the basal autophagy in non-treated cells when assessed by quantitating GFP-LC3 puncta, which presumably measures the same event as the western blot in a different manner. How is this discrepancy explained? Is the decrease in LC3II accumulation on western blot reproducible and statistically significant if quantified by densitometry?

Response:
We believe the sensitive of both assays is different. The Western blot assay is more accurate, where even signals of 10% LC3 II can be carefully measured. The standard deviation observed after analyzing several independent experiments illustrates this point. Overall, LC3 flux assays with both methods confirmed the same result.

3. The study of the putative yeast homologue is not very relevant and may be better placed in supplementary data, especially since only one assay is used to exclude a role for this protein in autophagy. Please note that the controls used are actually negative controls, not positive controls as stated.

Response:
As suggested, we have moved this data to supplementary material. However, to confirm this important finding, we have now presented a new yeast assay of autophagy using ATG8-GFP degradation by Western blot (Fig. S4D).

4. English usage is sometimes awkward, and text should be carefully edited.
5. Additional technical problems in figures: A. The LC3 blot upon in Fig 4A is of poor quality, bands are fuzzy, esp in WT. Please replace. B. Fig. 4A seems mislabeled - should be a + under 16h WT. C. Fig 5b legend key- colors of boxes in legend does not match columns.

We have improved these images. We have carefully proofread the revised version for typos and errors.

2nd Editorial Decision 08 July 2011

Thank you for sending us your revised manuscript. In the meantime, our original referees have seen it again and both now support publication of the study here. Still, referee 2 raises a number of points that still need to be addressed before we can ultimately accept your manuscript.

In addition, there are a number of editorial issues that need further attention. First, please include an author contribution section after the acknowledgement section. Second, please add and explain scale

bars for all microscopic images shown, including those in the supplementary material. Furthermore, from the legend of figure 3D it appears that mean and standard deviations are based on two data points (n=2). If this is the case we need to ask you to change the way the data are presented. You could either plot the individual data points for each condition in a vertical arrangement or show one individual repeat without mean and error bars together with an explanation in the figure legend.

Finally, prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication and explanation in the figure legend in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) to ask for the original scans (for our records). In the case of the present submission there are a number of panels that do not appear to fully meet these requirements: Figure 4C third panel (both parts); 5B

I therefore like to kindly ask you to send us a new version of the manuscript that addresses the points mentioned above and that contains suitably amended versions of these figures with explanations in the figure legends. Please be reminded that according to our editorial policies we also need to see the original scans for the figures in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors sufficiently addressed the points raised in the initial review. This article can be published in its current form.

Referee #2 (Remarks to the Author):

The authors have significantly improved their manuscript in this revised version. However, several points still need to be addressed:
1. The authors seem not to have understood reviewer's Comment 1B - the intent was that they need to show WT or BI-1 KO samples +/- inhibitors in side by side lanes to assess that there is indeed a further increase in LC3-II upon inhibition of lysosome in BI-1 KO- fig 2C, D, E. The point is not necessarily to compare WT and KO in this case, but to compare LC3-II accumulation in the presence and absence of the inhibitors- the authors only show the inhibitors, so it is not possible to determine if there is an increase.
2. The authors have added Fig 1E- LC3-GFP-RFP expression to evaluate autophagy flux. This is an excellent assay, but the authors should show quantitative data and not just one representative cell.
3. In most of the paper (cell work and in flies), the authors show that BI-1 KO enhances autophagy and increases cell survival under starvation, but actually leads to decreased survival during ER stress (the implication is that this is due to BI-1's role in apoptosis, although this is not proven here). Yet the authors use this stimulus in vivo in mice, to show that BI-1 KO enhances autophagy. This seems to be a contradiction- why does ER stress involve BI-regulated autophagy in the latter model but not the earlier ones?

2nd Revision - authors' response 22 July 2011

Thank you very much for giving us a positive decision on our manuscript "BAX inhibitor-1 regulates autophagy by controlling the IRE1 /JNK branch of the unfolded protein response" by Castillo et al.

We have addressed all the final points that were requested prior to final acceptance of the manuscript.

In the revised version of the article we have:

- Included an author contribution section after the acknowledgement section.
- We have added the missing scale bars for all microscopic images shown, including those in the supplementary material.
- We corrected the information in legend of figure 3D for the statistical analysis. Basically the graph shows the average of three determinations of a experiment that represents a total of two independent experiments with similar outcome.
- For Figure 5B and 4C, we have stated in the legend that the image containing lanes of one gel that was assembled from cropped lanes of the same film and Western blot analysis. As requested, here we are also attaching for your records the full scan of those films.
- We have reduced the length of the manuscript (originally 64.408 characters) to reach the limit of 55,000 characters with spaces (excluding references, tables and supplementary material).

We appreciate the positive outcome of both reviewers. We have prepared a point by point response to the minor comments of reviewer 2 along with a revised version of the manuscript with the new quantification requested.

Second revision, reviewer #2:
1. The authors seem not to have understood reviewer's Comment 1B - the intent was that they need to show WT or BI-1 KO samples +/- inhibitors in side by side lanes to assess that there is indeed a further increase in LC3-II upon inhibition of lysosome in BI-1 KO- fig 2C, D, E. The point is not necessarily to compare WT and KO in this case, but to compare LC3-II accumulation in the presence and absence of the inhibitors- the authors only show the inhibitors, so it is not possible to determine if there is an increase.

Experiments presented in figure 2C and 2D were analyzed in many ways in the laboratory (>3 years project). We have three variables in these experiments: BI-1 WT and KO genotype, EBSS stimulation in time course experiments, and then plus/minus inhibitors. This is why, in general it is not possible to run all samples to test all variables in the same gel on a kinetic experiment. However, as the reviewer noticed we have a number of independent experiments for each quantification, and comparisons were made in different ways to ensure a proper interpretation of results.
First of all, the most important point is to clarify the way how we did the normalization and measurement of LC3-II levels. In all cases, for each experiment, the non-treated condition of WT cells (ratio of LC3 with Hsp90 levels) was normalized to 1 to obtain the fold change. This control was always run in every gel. Then, this normalization allowed us to pull all independent experiments for quantification of the fold change and perform proper statistical analysis. We have now stated this in the figure legend and also could be appreciated by the fact that the number of independent experiments for non-treated BI-1 WT and KO cells is 8-10. We believe this is the best way to compare this type of experiments to assess relative autophagy fluctuations since we have comparable values normalized always with the same condition.

Secondly, the conclusions of Figure 2 are built moving from one panel to the other. First, we show there is a clear basal difference in WT and KO cells after autophagy stimulation with proper statistical analysis (Western blots performed in the same gel (Fig. 2A and B). Then, we ask about the effects of autophagy inhibitors comparing both genotypes (Fig. 2C and D). In general, we actually compared the full kinetic of EBSS stimulation plus/minus inhibitor in cells of the same genotype (BI1 WT or KO), so normalization and pulling data is easier. In fact, this analysis confirmed the prediction because as the reviewer could notice by the quantification of fold changes,

there is an increment (amplitude) in LC3-II induction when lysosomal inhibitors are present in the experiments (Compare plots for panels B and D), which confirms that the normalization was done properly.

For Figure 2E, the two genotypes and the effects of the inhibitors are shown in the same gel. This analysis did not required starvation stimulation because it measures basal autophagy fluxes.

2. The authors have added Fig 1E- LC3-GFP-RFP expression to evaluate autophagy flux. This is an excellent assay, but the authors should show quantitative data and not just one representative cell.

We agree with this reviewer that the LC3-RFP-GFP reporter is an excellent assay. As requested, we have now quantified the ratio of red and yellow dots in several cells of independent experiments, and included a new quantification which reflects the augmentation of autophagy fluxes in BI-1 deficient cells, confirming the major finding of the article.

3. In most of the paper (cell work and in flies), the authors show that BI-1 KO enhances autophagy and increases cell survival under starvation, but actually leads to decreased survival during ER stress (the implication is that this is due to BI-1's role in apoptosis, although this is not proven here). Yet the authors use this stimulus in vivo in mice, to show that BI-1 KO enhances autophagy. This seems to be a contradiction- why does ER stress involve BI-regulated autophagy in the latter model but not the earlier ones?

We apologize for not clarifying properly the use of this model in the response letter. The reviewer is right and we fully agree with the point. The important concept to strength is the fact that cell survival upon nutrient starvation is fully dependent on autophagy induction. This is why looking at cell death after nutrient starvation in BI-1 deficient cells is a valid read out to monitor the physiological impact of BI-1 on macroautophagy.

In the case of ER stress, cell viability is well known to be controlled by complementary and parallel processes. ER stress-mediated apoptosis depends, for example, on the activation of several independent sensors and mechanisms including, gene expression of targets encoding dozens of chaperones, foldases, and proteins related to quality control; in addition to the regulation of protein translation and upregulation of key apoptosis genes of the BCL-2 protein family (that are also modulated by BI-1). Then, autophagy is just a small contributor to survival upon ER stress. This is why we only use tunicamycin in vivo to engage IRE1-dependent autophagy as an animal model to validate the autophagy flux effects in mice, and thus increase the impact of the paper. We did not measure apoptosis because as mentioned we are aware that the outcome is not direct and could lead to misinterpretations. The impact of autophagy in full animal survival upon starvation was nicely demonstrated here in the fly model. Technically and ethically, performing extreme starvation experiments as mentioned in mice is very complex.

We would like to sincerely thank this reviewer for helping us improving the quality and impact of the study.

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