THE EMBO JOURNAL

Manuscript EMBO-2010-75763

Mitochondrial BCL-2 inhibits AMBRA1-induced autophagy

Flavie Strappazzon, Matteo Vietri-Rudan, Silvia Campello, Francesca Nazio, Fulvio Florenzano, Gian Maria Fimia, Mauro Piacentini, Beth Levine and Francesco Cecconi

Corresponding author: Francesco Cecconi, University of Rome Tor Vergata

Review timeline:

Submission date:	23 August 2010
Editorial Decision:	27 September 2010
Revision received:	24 December 2010
Editorial Decision:	25 January 2011
Revision received:	03 February 2011
Accepted:	04 February 2011

Transaction Report:

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

1st Editorial Decision 27 September 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. As you will see while all three referees consider the study as interesting in principle they all think that substantial revision would be required to increase the conclusiveness and completeness of the experimental evidence provided. One major concern is that a considerably stronger experimental case for the functional and physiological significance of your finding would be required and that the analysis of the endogenous proteins needs to be expanded significantly. On balance and given the overall support provided by the referees we would be able to consider a revised version of your manuscript. However, the referees' criticisms all need to be addressed in an adequate manner and to their full satisfaction. 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.

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 COMMENTS

Referee #1 (Remarks to the Author):

This study by Strappazzon et al. leads to the proposal that a pro-autophagic co-factor of the Beclin 1-containing Vps34 complex, Ambra1, is sequestered by the mitochondrial-localized pool of Bcl-2. Upon nutrient stress (or indeed induction of apoptosis by staurosporin), Ambra1 is released and now becomes available for its previously-identified role - interacting with Beclin 1 and stimulating autophagosome production. Thus, the proposed model has analogies to that of the Bcl-2 / Beclin 1 model for regulation of autophagy, although why a mitochondrial location would be required is not at all clear. Moreover, while the model is interesting considerably more experimentation is required.

The proposed interaction between Bcl-2 and Ambra1 does not benefit from being the result of an unbiased screen but rather from a let's look and see. I am not at all convinced by a single approach using mostly co-immunoprecipitation from ectopic expression vectors in highly over-expressing HEK293 cells. The limited analyses of endogenous proteins lacked adequate control: preimmune IgG at equivalent amounts as immune antibodies. As well confirmation must come from additional approaches (eg, FRET or chemical cross linking that target the mitochondrial population, possibly in vitro pull downs, in vitro targeting of Ambra1 IVT product to isolated mitochondria vs ER with and without Bcl-2, etc).

Co-localization by immunofluorescence lacked adequate resolution and quantification. The tests used for co-localization are complicated by the differential volumes of distribution of stains localized to mitochondria and ER. The analyses should be conducted in cell lines appropriate for resolution studies. The immunogold analysis was not quantitative, lacked any control, and indeed the image showed grains associated with the mitochondrial interior.

Finally, an Ambra1 mutant that does not bind to Bcl-2 but retains Beclin 1 interaction and function would be expected to evade autophagy regulation by Bcl-2, and confirm at least the model for inhibition by Bcl-2.

Referee #2 (Remarks to the Author):

The manuscript by Strappazzon et al reports on a preferential interaction of AMBRA1 with a pool of Bcl-2 on mitochondria, but not the ER localized-Bcl-2. This interaction is shown to be starvation sensitive, suggesting the availability of AMBRA1 (or Bcl-2) for other binding partners, in particular Beclin1 (which is required for autophagy and whose activity is regulated by Bcl-2) may be controlled by starvation. The authors could demonstrate that Beclin1 binding to AMBRA1 is modulated by starvation, and that increasing amounts of AMBRA1 disrupt the mito-Bcl-2 -Beclin1 interaction. Overall the individual experiments are clearly presented however a significant biological conclusion seems to be missing- is this interaction an essential regulatory mechanism or not? The answer perhaps does not matter as much as the clarification of what the authors data suggest is the answer. In addition to the points listed below there are some concerns about reproducibility (not emphasized at all in the manuscript) and overexpression effects. The final figure is somewhat confusing and it seems like it is addressing a separate, although related question.

Major points

1. The rationale which starts off the experiments is not entirely clear to this referee. They state that AMBRA1 is a positive regulator of Beclin1, Beclin1 is negatively regulated by Bcl-2 therefore they looked if AMBRA1 binds Bcl-2. Is there any data in the literature which would suggest AMBRA1

would bind Bcl-2? If so it would be helpful is this is made clear. It is confusing as well that the recently reported role of ULK1-dependent AMBRA1 binding to Dynein light chain is not really linked to the model. Is the AMBRA1 -mito-Bcl-2 interaction sensitive to microtubule depolymerization agents? The rationale is further shaken by the paprgraph in the Discussion (page21) about data from Zu et al which show AMBRA1 is not a stable part of the Beclin1 complex. How do the authors then think their data relate to the function of the Beclin1 complex and autophagy?

2. Figure 3a, b and other figures (Figure 4, 7c,d) should include a non-transfected controls (for Figure 3 AMBRA1 alone and Bcl-2 alone) to assess the effects on endogenous reporters.

3. Figure 3. According to the data shown in Figure 5 (AMBRA1 overexpression induces autophagy) one would expect to see effects on p62 levels when expressing ER-Bcl-2 versus mito-Bcl-2 after overexpression of AMBRA1 which they show induces autophagy. This is not reflected in the p62 assay.

4. Figure 4 should be accompanied by mitotracker staining to support localization of proteins.

5. Figure 5. It is not clear why the transfection of pcDNA3 induces autophagy and it is hard to understand why this level would be comparable to the conditions where AMBRA1 was co-transfected with mito-Bcl-2. Controls are really needed here such as Bcl-2 alone, ER-Bcl-2 and a comparison with starvation. In addition, a western blot of the overexpressed proteins to compare transfected protein levels. Is the "pcDNA3"induced autophagy an accumulation of basal would also be an important issue. Finally what do the error bars represent in the adjacent panel?

6. Figure 6. The competition experiments would take on real significance if they could be done with the Flag-Beclin1 constructs mutated in the domain which they authors surmise bind AMBRA1 (141-150). This would entail a better characterization of this Beclin1 mutant and a fine mapping of the Bcl-2 binding domain in the regio of 141-150 to ask if this was required for Bcl-2 binding to Beclin-1 as well as AMBRA1.

Minor points

1. 5th sentence in abstract. It may not be obvious from previous sentences that "After autophagy induction, AMBRA1/Beclin1 interaction is therefore increased".

2. Figure 2d appears to show again what Figure 1d shows, and Figure 1 d should be removed.

Referee #3 (Remarks to the Author):

Strappazzon et al. report the roles of BCL-2 and AMBRA1 in Beclin 1-dependent autophagy. Previous studies demonstrated that the anti-apoptotic factor BCL-2 at the endoplasmic reticulum (ER-BCL-2) binds Beclin 1, an essential component of the Class III PI-3-kinase complex, to negatively regulate autophagy, and that AMBRA1 interacts with Beclin 1 to promote autophagosome formation. How AMBRA1 regulates Beclin 1-dependent process remains elusive. In this manuscript, the authors reveal that BCL-2 binds AMBRA1 in a Beclin 1-independent manner. They also found that BCL-2 at the mitochondrial surface (mito-BCL-2) preferentially associates with AMBRA1. Interestingly, this interaction is abrogated under starvation conditions. Concomitant with this process, the AMBRA1-Beclin 1 interaction is increased at both the ER and mitochondria. In addition, overexpression of AMBRA1 suppresses BCL-2-Beclin 1 interaction.

It is still unclear how AMBRA1 dissociates from mito-BCL-2, and how AMBRA1 activates the downstream autophagy events. However, this study provides new insights into the mechanism of Beclin 1-mediated autophagy induction. Data presented in this manuscript are mostly solid with appropriate controls. The significance of this paper would be more strengthened by addressing the points below.

Specific points:

1. Co-localization of myc-AMBRA1 and mito- or ER-BCL-2 in Figure 4 is not convincing. Endogenous AMBRA1, BCL-2, and mitochondria or ER resident marker in cells under nutrient-rich and starvation conditions should be examined.

2. In Figure 5C, the autophagic flux should be analyzed in cells treated with or without bafilomycin A1. In addition, cells expressing AMBRA1 and ER-BCL-2 should also be tested.

3. Similar to the data shown in Figure 6e, the effect of mito- or ER-BCL-2 overexpression on AMBRA1-Beclin 1 interaction should be investigated in order to fully support the idea that BCL-2 competes with AMBRA1 for Beclin-1 binding.

4. AMBRA1 siRNA should be performed to ask whether starvation-induced dissociation of BCL-2 from Beclin 1 requires AMBRA1.

1st Revision - authors' response 24 December 2010

POINT-BY-POINT Response:

Referee#1:

Comment: *This study by Strappazzon et al. leads to the proposal that a pro-autophagic co-factor of the Beclin 1-containing Vps34 complex, Ambra1, is sequestered by the mitochondrial-localized pool of Bcl-2. Upon nutrient stress (or indeed induction of apoptosis by staurosporin), Ambra1 is released andnow becomes available for its previously-identified role - interacting with Beclin 1 and stimulating autophagosome production. Thus, the proposed model has analogies to that of the Bcl-2 / Beclin 1 model for regulation of autophagy, although why a mitochondrial location would be required is not at all clear.*

Answer : We agree with the Reviewer that the "reasons" of the regulation we have found at the mitochondria may, in principle, sounds *obscure*. However, several speculations can be made (and most of them are reported in the Discussion paragraph) about this matter:

1) Mitochondria may well be considered as the rheostat of the death/survival switch in the cell; a number of autophagy-regulating molecules (Atg5, DRAM, p53 itself) play a role on mitochondria to induce cell death when the stress response overcomes a certain threshold; the fact that AMBRA1 may prevent cell death besides inducing autophagy could be accounted for, at least in part, by its presence at the mitochondria;

2) Hailey et al., 2010 demonstrated that, in starved cells, the outer membrane of mitochondria participates in autophagosome biogenesis. Since our results illustrate an AMBRA1-BCL-2 interaction at the mitochondria, we are tempted to speculate that a pool of AMBRA1 may participate to autophagosome formation at the mitochondrial membrane;

Finally, 3) Mitophagy, whose relevance is very high in biomedicine, as this process is involved in neurodegeneration and cancer prevention, may need autophagic molecules to co-regulate mitochondrial damage with autophagosome priming. AMBRA1, whose role in the nervous system has been clearly demonstrated, may have a crucial role in the mitophagy process as well.

Comment: *Moreover, while the model is interesting considerably more experimentation is required. The proposed interaction between Bcl-2 and Ambra1 does not benefit from being the result of an unbiased screen but rather from a let's look and see. I am not at all convinced by a single approach using mostly co-immunoprecipitation from ectopic expression vectors in highly over-expressing HEK293 cells. The limited analyses of endogenous proteins lacked adequate control: preimmune IgG at equivalent amounts as immune antibodies. As well confirmation must come from additional approaches (eg, FRET or chemical cross linking that target the mitochondrial population, possibly in vitro pull downs, in vitro targeting of Ambra1 IVT product to isolated mitochondria vs ER with and without Bcl-2, etc).*

Answer: We agree with these Referee's comments and we set up to establish several crucial experiments to reinforce our results concerning the AMBRA1-BCL-2 interaction:

First, we added adequate controls to the endogenous immunoprecipitation experiments:

- The new Figure 1d illustrates a co-immunoprecipitation between endogenous AMBRA1 and endogenous BCL-2 in total cells;
- The new Figures 3c and 3d illustrate the binding between endogenous AMBRA1 and endogenous BCL-2 in mitochondrial fractions, both in normal and starvation conditions.

Second, since we were not able –even though after a number of laborious attempts, to generate a recombinant AMBRA1, we decided to perform two new experiments using both the different technical approaches suggested by the Referee:

- A series of **FRET experiments** were carried out according to Albertazzi *et al.* (2009) to confirm the interaction by fluorescence, in HEK293 cells overexpressing both AMBRA1-mCherry and BCL-2-GFP. This experiment clearly indicates a very high proximity of AMBRA1 and BCL-2 within the cell (see novel Figure 1e).
- A **mitochondrial cross-linking experiment** in HEK293 cells grown in normal and starvation conditions, further demonstrates the endogenous binding between AMBRA1 and BCL-2 in mitochondrial fractions of HEK293 cells (see novel Figure 3d).

We hope that the Referee will be now satisfied by our *comprehensive* approach to this issue.

Comment: *Co-localization by immunofluorescence lacked adequate resolution and quantification. The tests used for co-localization are complicated by the differential volumes of distribution of stains localized to mitochondria and ER. The analyses should be conducted in cell lines appropriate for resolution studies.*

Answer: In agreement with the Referee's desiderata, we performed some pilot immunofluorescence analyses using HeLa cells, which posses a larger cytoplasm than HEK293. The Referee will find, as a representative example, a mito-BCL-2/AMBRA1 double staining in HeLa cells in the attached Figure REF.1. However, since 1) the results were *always and strictly* reproducible in both cell types, HeLa and HEK293, 2) no significant morphological differences were observed between the two cell types, and 3) the vast majority of the key experiments were performed in HEK293 cells, we preferred to omit this repetition in the manuscript.

In addition, this Referee will see in the novel Figure 4 a triple staining for AMBRA1, BCL-2 and the mitochondria, that reports once again of the interaction of these two proteins on the same organel. In sum, this co-localization is supported by: 1) co-immunoprecipitation data both on endogenous and transfected proteins; 2) imaging data: double or triple staining in two different cell types with appropriate magnification ; 3) FRET analysis; 4) Mitochondrial cross-linking experiment besides subcellular fractionation assays; 5) Immuno gold and ultrastructural analysis on both whole cells and purified mitochondria.

Comment: *The immunogold analysis was not quantitative, lacked any control, and indeed the image showed grains associated with the mitochondrial interior.*

Answer: We must admit that the choice of the previous image was not appropriate. We changed the image (which, admittedly, was showing grains associated with the mitochondrial interior due to the breaking of the mitochondrial membrane in a very specific mitochondrion), with a new image, which better represents the vast majority of the samples analysed, showing grains on the outer mitochondrial membrane (see new Figure 5b, purified mitochondria). We also added on Supplementary Figure S5, negative controls using only the secondary antibody on whole cells and purified mitochondria. In addition, a quantitative immunogold analysis showed the percentage of mitochondria with AMBRA1 which is now indicated in the text.

Comment: *Finally, an AMBRA1 mutant that does not bind BCL-2 but retains Beclin 1 interaction and function would be expected to evade autophagy regulation by BCL-2, and confirm at least the model for inhibition by BCL-2.*

Answer: We are grateful with the Referee for this wondeful suggestion. In order to answer this interesting issue, we analysed the induction of autophagy using overexpression of the F2 fragment of AMBRA1 (illustrated on Figure 1a). This fragment that, indeed, has been demonstrated to induce autophagy (Fimia et al., 2007), is able to bind BECLIN 1 but is not able to bind BCL-2 (see results on Figure 1b). We are glad to conclude that our results, illustrated on the new Figure 6, show that F2 evades autophagy regulation by mito-BCL-2, confirming the model of the present work.

Referee#2:

Comment: *The manuscript by Strappazzon et al reports on a preferential interaction of AMBRA1 with a pool of Bcl-2 on mitochondria, but not the ER localized-Bcl-2. This interaction is shown to be starvation sensitive, suggesting the availability of AMBRA1 (or Bcl-2) for other binding partners, in particular Beclin1 (which is required for autophagy and whose activity is regulated by Bcl-2) may be controlled by starvation.*

The authors could demonstrate that Beclin1 binding to AMBRA1 is modulated by starvation, and that increasing amounts of AMBRA1 disrupt the mito-Bcl-2 -Beclin1 interaction.

Overall the individual experiments are clearly presented however a significant biological conclusion seems to be missing- is this interaction an essential regulatory mechanism or not? The answer perhaps does not matter as much as the clarification of what the authors data suggest is the answer. In addition to the points listed below there are some concerns about reproducibility (not emphasized at all in the manuscript) and overexpression effects. The final figure is somewhat confusing and it seems like it is addressing a separate, although related question

Answer: We strongly believe that this is an important regulation in the context of the autophagy/apoptosis cross-talk. However, we cannot certainly conclude (and we do not pretend) that this is a "*sine qua non*" mechanism. The hypothesis we present in our model is that mitochondrial AMBRA1 can *enhance* the autophagic response by freeing Beclin 1 from its BCL-2 bondage, and *vice versa*. Although this finding may sound marginal, we are aware (and we hope to share this awareness with the Referee) that most researchers in the field are now seeking for subtle regulation processes, which might hopefully be manipulated by specific drugs in a number of critical human diseases.

As regards the issue of reproducibility and overexpression effects, we apologize for the lack of clarity. We now added a long series of information regarding those issues all over the paper. As regards the final figure, we believe that (since mito-BCL-2 is a well known anti-apototic factor), it is important to include in this study the evaluation of the binding between AMBRA1 and mito-BCL-2 under apoptosis condition, too. We agree with this Referee that these results are opening a new area of research, and we will explore it in a near future.

Comment:

The rationale which starts off the experiments is not entirely clear to this referee. They state that AMBRA1 is a positive regulator of Beclin1, Beclin1 is negatively regulated by Bcl-2 therefore they looked if AMBRA1 binds Bcl-2. Is there any data in the literature which would suggest AMBRA1 would bind Bcl-2?

If so it would be helpful is this is made clear.

Answer: The logical flaw we had followed is very simple: since we did notice that a pool of AMBRA1 was co-localizing with the mitochondria (see Di Bartolomeo et al., 2010), we hypothesised that this protein may play a role in this organel. Then, we considered that every protein interacting with the main components of the autophagy core-complex (which is formed by AMBRA1, BECLIN 1 and VPS34, see He and Levine, 2010) could potentially bind AMBRA1 (since this protein binds both BECLIN 1 and VPS34; see Fimia et al., 2007). Last, prompted by the described functional interaction between BCL-2 and BECLIN 1 at the ER, we wanted to explore whether both pools of BCL-2, that at the ER and that at the mitochondria, were also partners of AMBRA1. We found this of interest also because AMBRA1, as well as other pro-autophagic factors, may play a role as oncosuppressor gene; Therefore, its interaction with other proteins of the BCL2 family could turn out to be of the highest importance in biomedicine.

Comment*: It is confusing as well that the recently reported role of ULK1-dependent AMBRA1 binding to Dynein light chain is not really linked to the model. Is the AMBRA1 -mito-Bcl-2 interaction sensitive to microtubule depolymerization agents?*

Answer: What we report in this paper (see our model in Supplementary Figure S8) is the regulation of a *specific mitochondrial-resident* AMBRA1. However, prompted by the Referee's question, we tested whether AMBRA1-BCL-2 interaction was affected by microtubule depolymerization agents. To this aim, we treated AMBRA1/Mito-BCL-2-overexpressing HEK293 cells with 1 μM or 10 μM of nocodazole for 10 minutes, in order to destabilize unstable microtubules or stable microtubules, respectively. However, these treatments were not able to affect AMBRA1-Mito-BCL-2 interaction, indicating that this binding is not related to cytoskeleton dynamics (see Figure REF.2a).

Comment: *The rationale is further shaken by the paragraph in the Discussion (page21) about data from Zu et al which show AMBRA1 is not a stable part of the Beclin1 complex. How do the authors then think their data relate to the function of the Beclin1 complex and autophagy?*

Answer: We thank the Referee for this nice catch. The sentence has been changed accordingly. In fact, all approached used so far, aimed at identifying additional members of the autophagy core complex, did not consider proteins expressed at low levels (as is AMBRA1) in the cell system studied (the authors simply ignored weak bands!).

Comment: *Figure 3a, b and other figures (Figure 4, 7c,d) should include a non-transfected controls (for Figure 3 AMBRA1 alone and Bcl-2 alone) to assess the effects on endogenous reporters.*

Answer: According to the Referee's request, we added in the new version of the work non-transfected controls regarding Figure 3a (see new Supplementary Figure SD2b-d), concerning Figure 3b (see new Figure 3b), concerning Figure 4 (see new Supplementary Figure SD4), and concerning figure 7c-d (see new Figures 7c-d).

Comment*: Figure 3. According to the data shown in Figure 5 (AMBRA1 overexpression induces autophagy) one would expect to see effects on p62 levels when expressing ER-Bcl-2 versus mito-Bcl-2 after overexpression of AMBRA1 which they show induces autophagy. This is not reflected in the p62 assay.*

Answer: We agree with the Referee: in lanes 1 and 3 of Figure 3a, a p62 decrease cannot be observed. The fact that p62 does not vary in these conditions may also be due to the transfection of both ER- and Mito-BCL-2, which downregulates AMBRA1-induced autophagy (see previous Figure 5) counteracting its effect on p62 (by contrast, p62 decrease is very evident upon starvation). Of note, a strong decrement of p62 was *never* observed in these conditions; we did not include the analysis of p62 in untransfected cells since, upon *transient* transfection of AMBRA1, we were not able to appreciate its effect on p62 degradation, at variance with what observed with viral transduction of the same construct (see Di Bartolomeo et al., 2010). Komatsu et al. (2010) indicate that p62 can disrupt Nrf2/Keap1 interaction, binding Keap1 and unleashing Nrf2 that can go to the nucleus and activate the transcription of cytoprotective genes. We can speculate that transient transfection of AMBRA1 into cells induces a sub-lethal stress that can, in turn, prime a cytoprotective response which deregulate p62 turn-over.

Comment: *Figure 4 should be accompanied by mitotracker staining to support localization of proteins.*

Answer: We completely re-drawn Figure 4 with new images containing mitotracker staining (as a third reagent) with more clear magnification panels, as suggested by this Referee. We hope that he/she will appreciate the significant modification of this Figure.

Comment: *Figure 5. It is not clear why the transfection of pcDNA3 induces autophagy and it is hard to understand why this level would be comparable to the conditions where AMBRA1 was co-transfected with mito-Bcl-2. Controls are really needed here such as Bcl-2 alone, ER-Bcl-2 and a comparison with starvation. In addition, a western blot of the overexpressed proteins to compare transfected protein levels.*

Answer: The fact that pcDNA3 alone induces autophagy is probably due to a sub-lethal stress induced by the *transient* transfection approach, as repeatedly published in the autophagy literature (see also response to the point above). However, in line with Referee's comments, we performed conpletely new experiments adding controls such as WT-, mito- and ER-BCL-2 overexpressed alone and in comparison with an untransfected but 2h-starved condition (see novel Figure 6). Also, we added a sample western blot of the overexpressed proteins in order to help the Referee comparing transfected protein levels, as requested. Finally, to render even more clear the result, we changed the way to present our results. In the new version of the graph (see Figure 6a), we illustrate the densitometric analysis of LC3-II/LC3-I ratio as % of the control (pcDNA3 alone). We strongly believe that these results are now more convincing.

Comment: *Is the "pcDNA3"induced autophagy an accumulation of basal would also be an important issue. Finally what do the error bars represent in the adjacent panel?*

Answer: We believe that, given our response to the two previous answers, the Referee could better appreciate the significance of pcDNA3-induced autophagy, which is –indeed, quantitatively very

variable among experiments. Values are the means ±SD of three experiments performed (as now clearly indicated). We apologize for this omission.

Comment: *Figure 6. The competition experiments would take on real significance if they could be done with the Flag-Beclin1 constructs mutated in the domain which they authors surmise bind AMBRA1 (141-150). This would entail a better characterization of this Beclin1 mutant and a fine mapping of the Bcl-2 binding domain in the regio of 141-150 to ask if this was required for Bcl-2 binding to Beclin-1 as well as AMBRA1*

Answer: We highly appreciated this interesting comment. As a consequence, we decided to generate a BECLIN 1 Δ141-150 mutant construct (these being **essential** for the binding). Unfortunately, as illustrated in the attached Figure REF.2b, this new mutant of BECLIN 1 can bind AMBRA1 in any conditions. It can be hypothesized that another sequence of aminoacids, present in the Coil-Coiled Domain of BECLIN 1, is **required** for the binding between the two proteins.

Comment: minor points

1. *5th sentence in abstract. It may not be obvious from previous sentences that "After autophagy induction, AMBRA1/Beclin1 interaction is therefore increased".*
2. *Figure 2d appears to show again what Figure 1d shows, and Figure 1 d should be removed.*

Answer:

1- We apologize for this unclear *consecutio* in the abstract. We re-phrased it as suggested into a more linear "[…] ;Moreover, after autophagy induction, AMBRA1 seems to be recruited on Beclin 1 […]".

2- Since Referee#1 asked us to re-perform the experiment shown in panel 1d with a Pre-Immune IgG control, we decided to place a new Western blot image in Figure 1d (containing the adequate negative control) and to remove Figure 2d which, admittedly, was redundant with Figure 1d and 2a.

Referee#3:

Comment: *Strappazzon et al. report the roles of BCL-2 and AMBRA1 in Beclin 1-dependent autophagy. Previous studies demonstrated that the anti-apoptotic factor BCL-2 at the endoplasmic reticulum (ER-BCL-2) binds Beclin 1, an essential component of the Class III PI-3-kinase complex, to negatively regulate autophagy, and that AMBRA1 interacts with Beclin 1 to promote autophagosome formation. How AMBRA1 regulates Beclin 1-dependent process remains elusive. In this manuscript, the authors reveal that BCL-2 binds AMBRA1 in a Beclin 1-independent manner. They also found that BCL-2 at the mitochondrial surface (mito-BCL-2) preferentially associates with AMBRA1. Interestingly, this interaction is abrogated under starvation conditions.*
Concomitant with this process, the AMBRA1-Beclin 1 interaction is increased at both the ER and mitochondria. In addition, overexpression of AMBRA1 suppresses BCL-2-Beclin 1 interaction. It is still unclear how AMBRA1 dissociates from mito-BCL-2, and how AMBRA1 activates the downstream autophagy events. However, this study provides new insights into the mechanism of Beclin 1-mediated autophagy induction. Data presented in this manuscript are mostly solid with appropriate controls.

Answer: We thank referee 3 for these kind comments. It is correct that the content of the present work does not inform on how AMBRA1 dissociates from Mito-BCL-2 and how AMBRA1 activates the downstream autophagy events. However, while revising this work in agrrement with his/her request, we tried to answer this interesting question. Since we recently published that AMBRA1 can be phosphorylated by the ULK1 kinase (Di Bartolomeo et al., 2010), we thought that post-translational modification of mito-AMBRA1 can also occur after autophagy induction. To test this hypothesis, which is presently only discussed in the text, we performed an electromobility mono-dimentional gel shift assay. As reported on the attached Figure REF.3, it seems that a slight shift of the mitochondrial AMBRA1 protein is observed after autophagy induction. This very preliminary finding suggests the existence of a potential modification (phosphorylation) of AMBRA1 in the mitochondrial cell compartment following autophagy induction. Prompted by this finding, we analysed the disruption of AMBRA1/Mito-BCL-2 binding following autophagy induction in the presence of a dominant negative (DN) form of the ULK1 kinase and, as illustrated on the attached

Figure REF.3, the disruption of AMBRA1/Mito-BCL-2 interaction is blocked after overexpression of DN-ULK1. This result suggests that also Mito-AMBRA1 is subjected to ULK1-dependent phosphorylation during autophagy induction, this regulating its binding to BCL-2. Although these data are quite promising, they open more questions, rather than assessing issues. The identification of the significance of this interaction, the characterization of the exact site on AMBRA1 (who possesses more than 90 Ser residues!), and its biological relevance would definitely need months of additional work. We hope that this Referee will be satisfied by this *appetizer-results* and will convene with us that any additional findings about this issue would be beyond the scope of this very first publication on the topic.

Comment:

The significance of this paper would be more strengthened by addressing the points below. 1. Co-localization of myc-AMBRA1 and mito- or ER-BCL-2 in Figure 4 is not convincing. Endogenous AMBRA1, BCL-2, and mitochondria or ER resident marker in cells under nutrient-rich and starvation conditions should be examined.

Answer: In agreement with the Referee's desiderata, we have completely re-drawn Figure 4 with new images containing mitotracker staining (as a third reagent) with more clear magnification panels. We hope that he/she will appreciate the significant modification of this Figure. As regards the ER localization, is now moved on a Supplementary Figure (S3). Due to the *negligibility* of the AMBRA1/BCL-2 interaction at the ER, we prefer to omit the triple staining AMBRA1/BCL-2/ER, which –being mostly negative, would have further overloaded the paper.

Comment: *2. In Figure 5C, the autophagic flux should be analyzed in cells treated with or without bafilomycin A1. In addition, cells expressing AMBRA1 and ER-BCL-2 should also be tested.*

Answer: According to this Referee's request, we added on the new Figure 6, an analysis of the autophagic flux using chloroquine A on HEK293 cells. We also analyze in the new Figure cells expressing AMBRA1 and ER-BCL-2. The autophagosome *off-rate* does not seem to be impaired upon this regulation.

Comment: *3. Similar to the data shown in Figure 6e, the effect of mito- or ER-BCL-2 overexpression on AMBRA1-Beclin 1 interaction should be investigated in order to fully support the idea that BCL-2 competes with AMBRA1 for Beclin-1 binding.*

Answer: We thank the Referee for having raised this interesting point. We investigated the effect of Mito- and ER-BCL-2 overexpression on AMBRA1/BECLIN 1 interaction, and we found that also increasing concentrations of BCL-2 are able to distrupt the AMBRA1-BECLIN 1 interaction (see novel Supplementary Figure S6).

Comment: *4. AMBRA1 siRNA should be performed to ask whether starvation-induced dissociation of BCL-2 from Beclin 1 requires AMBRA1.*

Answer: We fully agree with the Referee about the potential importance of this experiment. However, as illustrated in Figure REF.3, we were unable to see an inhibition of the dissociation between BECLIN 1 and BCL-2 upon AMBRA1 down-regulation. Interestingly, in the absence of autophagy induction, BCL-2 seems to be more tightly associated to BECLIN 1 after AMBRA1 depletion. This would confirm the existence of a balance between AMBRA1 and BCL-2 for binding BECLIN 1 in non-autophagic conditions (see also our model in Supplementary Figure S8). However, it is difficult to conclude on this experiment, since we were not able to *totally* downregulate AMBRA1. It is highly possible that the phosphorylation of BCL-2 by the JNK1 Kinase, which occurs upon starvation, is sufficient to promote the disruption of the complex. For this reason we do not favor the inclusion of these data in the manuscript.

2nd Editorial Decision 25 January 2011

Thank you for sending us your revised manuscript. Our original referees have now seen it again. In general, the referees are now positive about publication of your paper. Still, all three referees feel that there are a few remaining issues that need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised in an amended version of the manuscript.

In addition there are a number of editorial issues that need further attention. First I would like to ask you to format the amended version of the manuscript in The EMBO Journal format: Title page, Abstract, Introduction, Results, Discussion, Materials and methods, Acknowledgements, Figure

legends, References, Figures, Tables, Supplementary information (in this order). Furthermore, 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 in all cases where such processing has been performed as well as an explanation in the figure legend 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) or explanation to ask for the original scans. In the case of the present submission there is one panel that does not fully meet these requirements: figure 7e, upper panel (WB AMBRA1). I therefore like to kindly ask you to include an explanation into the figure legend. Please be reminded that according to our editorial policies we also need to see the original scans for the figure in question. Furthermore, for statistical reasons we need to ask you to include more than two independent experiments, minimally three, into the quantification of figure 6b.

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.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have gone a long way toward addressing the major criticisms by the reviewers and the present manuscript is much improved. Nevertheless, there still needs to be some changes.

For one, there is too much speculation and statements that are not validated by the data. The authors need to carefully review the manuscript and make factual statements.

Examples include-
i) abstract "a pool of ambra1 is docked by Bcl-2...to inhibit its function". Also, "...and Bcl-2 responsive apoptosis." at the end of the abstract.

ii) P.16 l. the stated band decrease is not obvious from the figure (middle panel). At best the cross-linking followed by co-ip is supportive of an ambra1/Ccl-2 complex.

iii) Fig 8e and the corresponding text (p20) do not belong in this paper and should be removed.

iv) The Discussion p.22 bottom and p.23 top is weak and speculative.

v) Discussion p.23 middle. Based on the isolation employed for mitochondria, this heavy membrane mitochondria fraction is heavily contaminated with ER, which makes much of this discussion too speculative.

RECOMMENDATION

The key observation here is that mt Bcl-2 for some reason (an mt restricted co-factor?)preferentially binds ambra1, a known beclin 1 associated protein. Induction of autophagy releases ambra1, consistent with its ability to promote Beclin 1 activity. The authors should streamline the writing of the paper, with careful editing, otherwise they risk skepticism which could override the main conclusion.

Minor point - citation to Naf-1 (p.4) not provided.

Referee #2 (Remarks to the Author):

The previous comments have been attentively addressed and the revisions have improved the overall quality of the data and fully support their model. The authors have made a very good effort to address all the concerns. There are however two issues which have arisen due the to the new data provided.
1. The FRET experiment is very hard to understand. It is not clear what structure the panel is showing being labelled, or bleached. The scale bar is of similar size to that in the panel 1c, but the magnification looks very different, unless we are looking at two highly overexpressing cells slightly out of focus. The authors should show also the scale of the intensity of FRET in the right most panel. The efficiency should be compared with a control mCherry-EGFP fusion protein to determine the maximum FRET and compared with transfection of EGFP alone to determine the minimum FRET efficiency, otherwise the figure of 11.14% isn't placed into any context. Other controls (such as non-interacting tagged molecules) would also be acceptable.
2. Figure 6, again significant work has gone into this new data which is recognized and appreciated, however the effects on LC3-II are small, and not seen robustly in the western blots. While LC3-II/LC3-I quantification can be appropriate in certain cell lines (but not widely accepted) in the panels shown the LC3-I and -II levels vary dramatically for just the controls (starvation and pcDNA3.1 + Ambra1 in (a) top and bottom panel). In addition, in panel C the data is from two independent experiments done presumably in single replicates as that is what is shown in the western blot and the accumulation of LC3-II/I for ambra1+pcDNA3 is not very visible or rather comparable to the control starvation even with chloroquine. As error bars are shown in the bar graph the text may not be clear. Thus the raw data does not allow the reader to be fully convinced by the conclusions that the LC3-II/I ratio and the experiments are designed to provide.

Referee #3 (Remarks to the Author):

In this revised manuscript, Strappazzon et al. extensively reorganized the text and figures, and provided additional results on AMBRA1-BCL-2 interaction assessed by FRET (Figure 1e), chemical cross-linking (Figure 3d), and in vivo imaging (Figure 4a and b), and AMBRA1-mediated enhancement of autophagosome formation monitored by LC3-II generation (Figure 6a and b). Together, these data further support the model proposed by the authors that mitochondria-localized BCL-2 competes with AMBRA1 for Beclin 1 binding to negatively regulate autophagy.

Although the authors satisfactorily addressed most of my questions, there are still problems that must be clarified.

1. In the text (page 13, line 12-16), the authors describe as follows: "On a morphological ground, BCL-2-GFP and AMBRA1-mCherry appeared to be co-expressed in structures mainly resembling mitochondria and endoplasmic reticulum. The co-localization of the two proteins was almost complete. Although some BCL-2-GFP positive structures were devoid of AMBRA1-mCherry-positivity, all AMBRA1-mCherry positive structures were BCL-2-GFP positive." I do not understand these descriptions for Figure 1e.

2. It is still unclear to me how the authors obtained the quantitative data shown in Figure 4c with the localization patterns of AMBRA1, mito-BCL-2, and ER-BCL-2 (Figure 4a and b, and Supplementary Figure S3). Are there cells that do not exhibit AMBRA1-BCL-2 co-localization at all? A representative image of cells that display no co-localization of both proteins should be provided as supplemental data. Nevertheless, I would prefer the localizations of endogenous AMBRA1 and BCL-2 shown in Supplementary Figure S4 to support the model (Supplementary Figure S8) that, under starvation conditions, a fraction of AMBRA1 may dissociate from mitochondria and bind Beclin 1 at the ER to enhance autophagy.

POINT-BY-POINT Response:

Referee #1:

The authors have gone a long way toward addressing the major criticisms by the reviewers and the present manuscript is much improved. Nevertheless, there still needs to be some changes. For one, there is too much speculation and statements that are not validated by the data. The authors need to carefully review the manuscript and make factual statements.

RECOMMENDATION

The key observation here is that mt Bcl-2 for some reason (an mt restricted co-factor?) preferentially binds ambra1, a known beclin 1 associated protein. Induction of autophagy releases ambra1, consistent with its ability to promote Beclin 1 activity. The authors should streamline the writing of the paper, with careful editing, otherwise they risk skepticism which could override the main conclusion.

Answer: We thank the Referee for these recommendations. We have reviewed the manuscript in order to eliminate speculation and statements that were, admittedly, not validated by the data. In more details,

i) Abstract "a pool of ambra1 is docked by Bcl-2...to inhibit its function". Also, "...and Bcl-2 responsive apoptosis." at the end of the abstract.

Answer: The first sentence has been changed, accordingly, into a milder: "[…]a pool of AMBRA1 binds preferentially mito-BCL-2; after autophagy induction, AMBRA1 is released from BCL-2, consistent with its ability to promote BECLIN 1 activity […]". In the second sentence, the adjective "Bcl-2 responsive", which was—indeed, not supported, has been omitted.

ii) P.16 l. the stated band decrease is not obvious from the figure (middle panel). At best the cross-linking followed by co-ip is supportive of an ambra1/Ccl-2 comp

Answer: We modified the text, by omitting the description of the band decrease. We are still covinced that a decrease could be seen. However, we now state in the text that the cross-linking followed by co-ip is only supportive of an AMBRA1/BCL-2 complex, as suggested by the Reviewer.

iii) Fig 8e and the corresponding text (p20) do not belong in this paper and should be removed.

Answer: We removed Figure 8e and the corresponding text, in agreement with the Referee's desiderata.

iv) The Discussion p.22 bottom and p.23 top is weak and speculative.

Answer: We removed the paragraphs in agreement with the Referee's desiderata.

v) Discussion p.23 middle. Based on the isolation employed for mitochondria, this heavy membrane mitochondria fraction is heavily contaminated with ER, which makes much of this discussion too speculative.

Answer: We agree with the referee that mitochondria isolation cannot be so neat to completely exclude the ER from the sample (also considering the connections existing between the two organels). Thus, we modified this part by adding the following staetement:

"Although isolation of mitochondria is likely to be contaminated with ER, in line with these these results, we can spculate that AMBRA1 is present at the mitochondria to participate to the production of "mitochondrial autophagosomes"...

Minor point - citation to Naf-1 (p.4) not provided.

Answer: We thank the Reviewer for this nice catch. We now provide the citation of Naf-1 work in page 4 (see "Chang et al. 2010").

Referee #2:

The previous comments have been attentively addressed and the revisions have improved the overall quality of the data and fully support their model. The authors have made a very good effort to address all the concerns.
There are however two issues which have arisen due to the new data provided.

1.The FRET experiment is very hard to understand. It is not clear what structure the panel is showing being labelled, or bleached.

Answer: As mentioned in the figure legend, "A bleached region of the cytosol is indicated by the white rectangle". With this we wanted to indicate an area of the cytosol enriched by mitochondria. A close-by nuclear area is now labelled with „N" in order to facilitate the image interpretation by the reader.

The scale bar is of similar size to that in the panel 1c, but the magnification looks very different, unless we are looking at two highly overexpressing cells slightly out of focus.

Answer: Fig. 1c and Fig. 1e report two images taken by two different investigators with two different microscopes from different cells (HeLa and HEK293) used in different approaches. The scale bar of Fig. 1c is about one half shorter than that of Fig. 1e. For all these reasons the magnification is (and looks) different.

The authors should show also the scale of the intensity of FRET in the right most panel.

Answer: According to the Referee's request, the FRET intensity scale has been added to Fig. 1eIII.

The efficiency should be compared with a control mCherry-EGFP fusion protein to determine the maximum FRET and compared with transfection of EGFP alone to determine the minimum FRET efficiency, otherwise the figure of 11.14% isn't placed into any context. Other controls (such as non-interacting tagged molecules) would also be acceptable.

Answer: We agree that the comparison with a control mCherry-EGFP fusion protein to determine the maximum FRET would help to place our FRET value into a biochemical context. This work has been made in a benchmark paper by Albertazzi et al. (Albertazzi L, Arosio D, Marchetti L, Ricci F, Beltram F (2009) Quantitative FRET analysis with the EGFP-mCherry fluorescent protein pair. *Photochem Photobiol.* **85**:287-97), which has been cited in our previous submission as validation paper in the use of mCherry-EGFP pair for FRET technique. In this work the authors investigated by FRET acceptor photobleaching techniques the FRET behavior of two tandem mCherry-EGFP fusion proteins which differed for the distance (short linker and long linker) between the two fluorescent proteins. In this new version of the manuscript we added a comparison among our FRET value and the values reported by Albertazzi and coworkers for two tandem mCherry-EGFP proteins in results section (see also the revised text). For the experiments with EGFP alone, we performed FRET measurements on additional groups, containing cells expressing only one of the fluorescent fusion proteins, GFP-BCL-2 or mCherry-AMBRA1, (see material and methods, our previous submission). These two groups were called external controls and did not display FRET effects.

2. Figure 6, again significant work has gone into this new data which is recognized and

appreciated, however the effects on LC3-II are small, and not seen robustly in the western blots. While LC3-II/LC3-I quantification can be appropriate in certain cell lines (but not widely accepted) in the panels shown the LC3-I and -II levels vary dramatically for just the controls (starvation and pcDNA3.1 + Ambra1 in (a) top and bottom panel).
In addition, in panel C the data is from two independent experiments done presumably in single replicates as that is what is shown in the western blot and the accumulation of LC3-II/I for ambra1+pcDNA3 is not very visible or rather comparable to the control starvation even with chloroquine. As error bars are shown in the bar graph the text may not be clear. Thus the raw data does not allow the reader to be fully convinced by the conclusions that the LC3-II/I ratio and the experiments are designed to provide.

Answer: We agree with this Referee. It is not easy to obtain, by overexpressing AMBRA1+pcDNA3 (which may lead to various amounts of transcribed proteins) the same quantitative accumulation of LC3II/I from one experiment to another. However, by performing quantification in each experiment, we are able to obtain always the same tendency and, thus, to compare the different conditions.
Concerning figure 6C, we performed a new experiment using chloroquine in order to do a quantification based on 3 experiments rather than 2. In this last case, we obtained the best looking and technically-sound experiment, which we then chose as the representative gel to be shown in Fig. 6C.

Referee #3:

In this revised manuscript, Strappazzon et al. extensively reorganized the text and figures, and provided additional results on AMBRA1-BCL-2 interaction assessed by FRET (Figure 1e), chemical cross-linking (Figure 3d), and in vivo imaging (Figure 4a and b), and AMBRA1-mediated enhancement of autophagosome formation monitored by LC3-II generation (Figure 6a and b). Together, these data further support the model proposed by the authors that mitochondrialocalized
BCL-2 competes with AMBRA1 for Beclin 1 binding to negatively regulate autophagy.
Although the authors satisfactorily addressed most of my questions, there are still problems that must be clarified.

1. In the text (page 13, line 12-16), the authors describe as follows: "On a morphological ground, BCL-2-GFP and AMBRA1-mCherry appeared to be co-expressed in structures mainly resembling mitochondria and endoplasmic reticulum. The co-localization of the two proteins was almost complete. Although some BCL-2-GFP positive structures were devoid of AMBRA1-mCherry-positivity, all AMBRA1-mCherry positive structures were BCL-2-GFP positive." I do not understand these descriptions for Figure 1e.

Answer: As the Referee has correctly pointed out, this description does not specifically refer to Figure 1e, but it is a general description of the fluorescence expressions pattern we have observed. To clarify this issue, the text has been changed, accordingly, into: „On a morphological ground, visual inspection of the fluorescence expression patterns in the histological material showed that BCL-2-GFP and AMBRA1-mCherry appeared to be co-expressed in structures mainly resembling mitochondria and endoplasmic reticulum. The co-localization of the two proteins was almost complete.“

2. It is still unclear to me how the authors obtained the quantitative data shown in Figure 4c with the localization patterns of AMBRA1, mito-BCL-2, and ER-BCL-2 (Figure 4a and b, and Supplementary Figure S3). Are there cells that do not exhibit AMBRA1-BCL-2 co-localization at all? A representative image of cells that display no co-localization of both proteins should be provided as supplemental data.

Answer: We obtained the quantitative data shown in figure 4c by counting the number of cells showing co-localization of AMBRA1 and mito- or ER-BCL-2. In some cases, we were not able to see co-localization at all (we counted 3 fields of cells obtained by 3 different experiments). Also, we now provide the Referee with an example of cells which do not display co-localization of both proteins.

Nevertheless, I would prefer the localizations of endogenous AMBRA1 and BCL-2 shown in Supplementary Figure S4 to support the model (Supplementary Figure S8) that, under starvation conditions, a fraction of AMBRA1 may dissociate from mitochondria and bind Beclin 1 at the ER to enhance autophagy.

Answer: In principle, we agree with this Referee's observation. However, it would have been almost impossible to quantify, at the endogenous level, cells showing co-localization of AMBRA1 and BCL-2 at the mitochondria or at the ER; this due to the signals coming from the two proteins (which partially overlap) and, at the same time, being on two organels (that are often in contact). We preferred to discern the ER- form the mito-localized Bcl-2 in order to give a more detailed quantification by two different experiments. By using overexpressing-cells, indeed, it has been easier to count cells reducing the error, since we had just 2 colors to take into consideration: AMBRA1 in red and mito- or ER-BCL-2 in green.

(See Supplementary file "Figure for Referee 3)

(See Supplementary file "Original scans for figure 7e - Upper western-blot" and "Explanation of Figure 7 mounting")

