Ubiquilin4 is an adaptor protein that recruits Ubiquilin1 to the autophagy machinery

Dong Yun Lee, David Arnott and Eric J Brown

Corresponding author: Eric J Brown, Genentech

Review timeline:

Submission date: 18 October 2012
Editorial Decision: 16 November 2012
Revision received: 19 January 2013
Editorial Decision: 07 February 2013
Revision received: 11 February 2013
Accepted: 12 February 2013

Editors: Alejandra Clark, Nonia Pariente

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 16 November 2012

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports on it, which I copy below.

As you will see, although all referees find the study potentially interesting referees #2 and #3 agree that further experimental evidence is needed to strengthen the main conclusions that Ubql4 is an autophagy cargo protein and that it is required for autophagosome-lysosome fusion. Both referees suggest that the study would be strengthened by the identification of the LC3-binding motif on Ubql4 and further characterization of the intra-cellular puncta detected by immunofluorescence to confirm these are autophagosomes. In addition, they indicate that further experimental evidence is needed support the proposed formation of Ubql1-Ubql4 and LC3 on the outer membrane of the mature autophagosomes. Referee #1 suggests that the observed specificity of Ubql4 in LC3II binding should be investigated. Furthermore, the referees raise additional points including technical issues, such as further statistical analysis of the data and additional controls, and clarifications in the text.

Given the potential interest of the novel findings and considering that all referees provide constructive suggestions on how to move the study forward, I would like to give you the opportunity to revise the manuscript, with the understanding that the main referees concerns have to be
addressed and that acceptance of the manuscript would entail a second round of review. I would like to point out that it is EMBO reports policy to allow a single round of revision and thus, acceptance or rejection of the manuscript will depend on the outcome of the next final round of peer-review.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:
---------------------------

Referee #1

Previous studies by this group and others showed that ubiquilin proteins, a family containing a ubiquitin-binding domain (UBL) and a ubiquitin-associated domain, (UBA) function in the autophagy pathway. The proteins were found to bind the autophagosomal protein LC3. However, how ubiquilin mediates interaction with LC3 was not known. Understanding how ubiquilin function in autophagy is important because ubiquilin proteins have been implicated in neurodegeneration. In this paper the authors have found that ubiquilin-4 protein binds directly to LC3, providing a key clue as to their method of recruitment to the autophagosome machinery. Interestingly, also they found that ubiquilin-1 and ubiquilin-4 bind each other and that their coexpression is required for ubiquilin function in autophagy. The authors have used co-immunoprecipitation of GFP-tagged constructs as well as deletion variants of ubiquilin to demonstrate these findings. The work is well done and the results are convincing. Also the interpretation and discussion is appropriate.

Main issue.
1. The authors show that of the two LC3 forms only the LC3II modified form is co-immunoprecipitated by ubiquilins, but not the LC3I form. This is surprising because recombinant LC3 binds ubiquilin-4. The authors should see if the converse is true does ubiquilin-4 bind the non-modified LC3I form (p62 could be used as a control), and if so they should discuss the specificity in binding only LC3II.

There are few minor issues that need to be addressed.

a) The first paper showing ubiquilin involvement with Alzheimer's was the Mah paper, which was omitted in the Discussion on page 11.
b) In the Material and Methods section, page 15, third line from the bottom... switch ....should be changed to ....switched.

---------------------------

Referee #2

In the manuscript "The interaction between Ubiquilin1 and Ubiquilin4 is important for their functions in autophagy" by Lee DY, et al., the authors found that UbqIn4 is necessary for UbqIn1 function during autophagy. In particular, they propose UbqIn4 as an adapter protein to mediate the interaction between UbqIn1 and LC3 during autophagy. In general, the data are clearly presented, the majority of the experiments are well performed and the
manuscript results to be interesting, proposing a novel role of Ubqln4 protein in autophagy regulation. However, in some cases the experimental data are not sufficient to support the conclusions. The authors should perform some more experiments to improve the most important parts of the paper: i.e., prove the role of Ubql4 as an autophagy cargo protein (by identifying the LC3-binding motif on Ubqln4) and its role in autophagosome-lysosome fusion.

Specific comments:

1. The rationale for using different cell lines (293 and HeLa cells) throughout the study should be provided.
2. Figure 1C. Does the interaction between Ubqln1 and 4 increase after autophagy induction? Please, improve the image in Figure 1D: the authors should show the nuclei of the cells.
3. Figure 2A: the authors should perform the co-localization analysis at an endogenous levels (to rule out the presence of an overexpression artefact) and in the presence or not of autophagy inhibitors (bafilomycin or chloroquine). Does the interaction between LC3 and Ubqln4 increase after starvation? Moreover, the authors should increase the magnification of the figures to better appreciate the co-localisation between these two proteins.
4. In their previous work (N'Diaye et al., 2009) the authors show that also Ubqln2 co-localizes with LC3. Is Ubqln2-Lc3 interaction regulated by the same mechanism they propose for Ubqln1? Does Ubqln2 interact with Ubqln4?
5. The study will have been significantly strengthened if the authors were able to identify the LC3-binding motif in the central region of Ubqln4.
6. To confirm the data showed in Figure 3, the authors should perform a co-immunoprecipitation assay to check the interaction between Ubqln1 and LC3 after Ubqln4 down-regulation.
7. Since both the mutant constructs of Ubqln4 (ΔUBL and ΔUBA ) are compromised in their interaction with Ubqln1-UBA domain, the authors should better investigate this interaction.
8. The authors should improve the results shown in Figure 4, by checking Ubqln1-LC3 interaction in the presence of the ΔUBL Ubqln4 mutant construct.
9. In Figure 6, the authors show that Ubqln4 is important for Ubqln1’s role in autophagosome-lysosome fusion. However, the data are not sufficient to support the conclusion. If Ubqln1-4 are necessary for autophagosome-lysosome fusion, the authors should analyse the GFP-single positive LC3 puncta (not only the Cherry-single positive puncta) after Ubqln1 and Ubqln4 down-regulation, both in basal condition and after autophagy induction by starvation (and also in the presence of an autophagy inhibitor). GFP-single positive LC3 puncta and the number of GFP-mCherry double-positive puncta should also be measured. (Otherwise, why using this ocnstruct?) Moreover, to strengthen these results, the authors should monitor the cellular levels of LC3-II upon starvation after Ubqln1-4 down-regulation. This would help to conclude that there is a block in autophagy flux.
10. The authors should specify how did they obtain HeLa cells expressing GFP-LC3 or GFP-mCherry-LC3.

Answers to EMBO Rep. specific questions:

1) Does this manuscript report a single key finding?

Yes, the authors found that Ubqln4 is necessary for Ubqln1 function during autophagy.

2) Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (YES and NO)?

The role of Ubqln1 in regulating autophagosome-lysosome fusion by co-localizing with LC3 during autophagy is published. The novelty is the necessity of Ubqln4 in Ubqln1-LC3 interaction.

3) Is it of general interest to the molecular biology community?

YES. It could be interesting to identify new adapter proteins that act as cargoes during autophagy.

4) Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longerformat
article (NO)?

The authors need to perform some experiments to make data more convincing.

Referee #3

In the current manuscript Lee et al propose a role for ubiquilin 4 in autophagy. Indirect association between ubqln1 and LC3 has been recently reported, and the authors suggest that ubqln4 is the adaptor protein mediating this interaction. Ubqln4 co-precipitation with ubqln 1 and LC3 and the colocalization of these proteins in punctae prompted the authors to propose this model. The ubiquitin-associated (UBA) domain of ubqln 1 and the ubiquitin-like (UBL) domain of ubqln 4 facilitated their mutual interaction. Furthermore, the authors suggest that depletion of ubqln 4 inhibits autophagosome-lysosome fusion. Characterization the exact role of ubiquilin proteins in autophagy is rather important, and the current manuscript aims to provide new information on the role of ubqln 4 in this process. The overall data presented however, lack important controls providing only limited support to the authors' main conclusions. Interactions between ubqln 1 and ubqln 4, as well as direct interaction between ubqln 4 and LC3 were determined; however the association between these complexes and autophagosomes was not verified. Importantly, the nature of the intra-cellular punctae detected by immunofluorescence should be more carefully determined. Utilization of lysosomal inhibitors and lysosomal markers is needed to determine that these are indeed autophagosomes. The authors should provide more detailed information as for the interaction between LC3 and ubqln 4, such as the region(s) mediating these interactions. Of note, LC3 can be found on pre-autophagosomal membrane as well as on other subcellular compartments. The authors' conclusion that both ubqln 4 and ubqln 1 are essential for autophagosome-lysosome fusion should be therefore further established utilizing electron microscopy and lysosomal inhibitors and markers. Most importantly, the formation of ubqln1-ubqln4 and LC3 on the outer membrane of mature autophagosomes, suggested by the authors model, should be demonstrated.

Specific comments
- The figures lack captions.
- Figure 1: These is no experimental evidence to support a direct interaction between ubqln1 and ubqln4.
- Figure 1A: The authors should relate to the fact that some of the identified peptides are not typical trypsin-digested products, namely C'-terminal residues that are not Lysine or Arginine.
- Figure 1D: The figure lacks statistical analysis.
- Figure 2A: The data is not convincing, statistical information is required.
- Figure 2C: GFP-expressing cells should be used as a negative control for the IP.
- Figure 2D: Positive and negative controls are missing. LC3 levels in the input and the IP should be presented.
- Figure 3A and 3C: Off-target effect of ubqln 4 siRNA on the expression of ubqln 1 should be excluded.
- Figure 4A and 4B: the increase in ubqln 4 interaction with delta-UBL ubqln1 should be addressed.
- Figure 4C: The explanation regarding the decrease in the interaction of delta UBA-ubqln 4 with ubqln 1 is not clear.
- Figure 5: The insertion of p62-LIR to ubqln 1 has limited relevance to the authors' hypothesis.
- Figure 6A: The IF images should be enlarged.

1st Revision - authors' response 19 January 2013

Our responses to the specific questions and concerns of the referees are detailed below.

Referee #1

We are pleased that this reviewer found our work well done, the results convincing, and the
interpretation appropriate.

1. The authors show that of the two LC3 forms only the LC3II modified form is co-immunoprecipitated by ubiquilins, but not the LC3I form. This is surprising because recombinant LC3 binds ubiquilin-4. The authors should see if the converse is true does ubiquilin-4 bind the non-modified LC3I form (p62 could be used as a control), and if so they should discuss the specificity in binding only LC3II.

As suggested by referee #1, we bought recombinant LC3I from Enzo Life Sciences and utilized p62 as a control to test Ubqln4’s ability to interact with LC3I. However, we were unable to find specific binding of recombinant LC3I to recombinant p62 or Ubqln4. Given that the control, p62, did not bind to LC3I, we are unable to dissect the binding specificity of Ubqln4 for LC3II vs. LC3I. Since Ubqln4 binds to an LC3 affinity column (Fig. 2D), it is possible that it can bind to LC3I, since the LC3 on the column, although exhibiting two forms (see WB below in response to point 5 of reviewer 3), was not palmitoylated. On the other hand, it is clear that LC3II is specifically co-immunoprecipitated in the context of a cell (Fig. 3D) under stress conditions that lead to autophagy. Because understanding specificity of the Ubqln4-LC3 interaction for LC3I or LC3II clearly will require significant investigation, we believe it is beyond the scope of this first report. Thus, we have not discussed this point in the revised manuscript. We would be happy to do so if you and the reviewers think this would be important.

a) The first paper showing ubiquilin involvement with Alzheimer’s was the Mah paper, which was omitted in the Discussion on page 11.

We apologize for this omission. We have included this reference in the discussion section.

b) In the Material and Methods section, page 15, third line from the bottom... switch ....should be changed to ....switched.

We have made the appropriate change.

Referee #2

Specific comments:

1. The rationale for using different cell lines (293 and HeLa cells) throughout the study should be provided.

We have mainly used 293 cells for biochemical experiments and Hela cells for imaging. However, we have shown that the biochemical data can be reproduced in Hela cells (for example Fig. 3D and S5B) and that the imaging data is reproducible in 293 cells (for example Fig. S4). Therefore, we believe that none of the major results of this work are cell-specific.

2. Figure 1C. Does the interaction between Ubqln1 and 4 increase after autophagy induction?

In 293 cells, the interaction between Ubqln1 and Ubqln4 does not increase after autophagy induction because the level of Ubqln4, already high, does not change. However, in Hela cells, during autophagy induction, there is an increase in Ubqln4 expression (Fig. S5A). This ~2-fold increase in Ubqln4 leads to a 2-fold increase in the interaction between Ubqln1 and Ubqln4 (Fig. S5B). Thus, at our current level of understanding, the interaction of Ubqln1 and Ubqln4 is dependent on the concentration of Ubqln4, and we are not aware of any other type of regulation that occurs as a consequence of autophagy induction.

Please, improve the image in Figure 1D: the authors should show the nuclei of the cells.

As suggested, we improved the image by showing the nuclei of the cells.

3. Figure 2A: the authors should perform the co-localization analysis at an endogenous levels (to rule out the presence of an overexpression artefact) and in the presence or not of
autophagy inhibitors (bafilomycin or chloroquine).

We have added a new figure showing the co-localization of endogenous LC3 and Ubqln4 (Fig. 2B).

Does the interaction between LC3 and Ubqln4 increase after starvation?

There is an increase in the interaction of Ubqln4 and LC3 after starvation. Cellular LC3II concentration and Ubqln4 concentration also both increase, and the localization of both proteins changes, as demonstrated in this paper. We agree that understanding the precise mechanism(s) by which LC3-Ubqln4 interaction is regulated will be very interesting, and it is a future goal of our work.

Moreover, the authors should increase the magnification of the figures to better appreciate the co-localisation between these two proteins.

We have added insets in Fig. 2A and Fig 2B to show magnification of an area where co-localization is observed.

3- In their previous work (N'Diaye et al., 2009) the authors show that also Ubqln2 co-localizes with LC3. Is Ubqln2-Lc3 interaction regulated by the same mechanism they propose for Ubqln1? Does Ubqln2 interact with Ubqln4?

Ubqln2 does interact with Ubqln4 and depends at least in part of the UBL domain of Ubqln4 (see WB below). However, we have not investigated the mechanism, extent, or biological significance of this interaction. This would need to be the subject of a separate investigation, as the functions of Ubqln1 and Ubqln2 are not identical. Therefore, we have not included the WB below in the revised manuscript. However, we have added a sentence to the Discussion in the revised manuscript (p 14) suggesting that the interaction between Ubqln4 and Ubqln2 exists and may be important.

<table>
<thead>
<tr>
<th>WCL</th>
<th>IP: GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+ -</td>
</tr>
<tr>
<td>-</td>
<td>+ +</td>
</tr>
<tr>
<td>+</td>
<td>- +</td>
</tr>
</tbody>
</table>

![GFP-S-Ubqln2](image)

4- The study will have been significantly strengthened if the authors were able to identify the LC3-binding motif in the central region of Ubqln4.

It has been difficult for all labs studying the central region of Ubqlns to further define its properties, largely because of the absence of structural information and the few predicted motifs in this region of the molecules. There are 4 STI1 motifs in the central region, but even these are poorly understood structurally and functionally. We have made significant strides in understanding how the central region of Ubqln4 interacts with LC3 by demonstrating that the two more N-terminal STI1 motifs are required for Ubqln4 interaction with LC3, but neither the two C-terminal STI1 motifs nor the intervening sequences are required. This result is included in the revised manuscript (Fig. 2E and text on pp. 6-7). The finding is intriguing because the one reported role for STI1 motifs is in protein-protein interactions. We have not been able to express the N-terminal STI1 motifs on their own, nor make smaller deletions, precluding a more refined analysis of the interaction.

5- To confirm the data showed in Figure 3, the authors should perform a co-immunoprecipitation assay to check the interaction between Ubqln1 and LC3 after Ubqln4 down-regulation.

We immunoprecipitated GFP-S-Ubqln1 and looked for its interaction with LC3 with and
without knockdown of Ubqln4. After knockdown of Ubqln4, we observed a decrease in LC3 association to Ubqln1. We have included this result as Fig 3D in the revised manuscript.

6- Since both the mutant constructs of Ubqln4 (ΔUBL and ΔUBA) are compromised in their interaction with Ubqln1-UBA domain, the authors should better investigate this interaction. We have investigated this further with several experiments. First, we show that Ubqln4 homo-oligomerizes (probably dimer) through its UBA domain (Fig S7A). This is different from Ubqln1, which homo-oligomerizes in the absence of its UBA domain (Ford & Monteiro, 2006). We believe that this UBA-dependent homo-oligomerization of Ubqln4 is necessary for stable interaction with Ubqlin1, perhaps through effects on avidity, since both Ubqln1 and Ubqln4 are dimers. We spent many months attempting to measure interaction of monomeric Ubqln1 and Ubqln4 and their domains both by Octet and surface plasmon resonance and failed to obtain recombinant protein sufficiently monomeric for these studies. Therefore, we took a different approach to understanding the interaction. Based on the known dependence of the interaction between UBA domains and Ubiquitin on I44 of Ubiquitin (Hicke et al, 2005), we mutated the equivalent amino acid (I55) in the UBL domain of Ubqln4. When I55 is mutated to alanine, the interaction of Ubqln1 and Ubqln4 is disrupted (Fig 4E). However, when the equivalent I79 in Ubqln1 is mutated to alanine, the interaction of Ubqln1 and Ubqln4 is not disrupted (Fig S7B). Together with the data that the UBA domain of Ubqln1 is required for the interaction, this experiment supports our model that there is an interaction between the UBL of Ubqln4 and the UBA of Ubqln1.

7- The authors should improve the results shown in Figure 4, by checking Ubqln1-LC3 interaction in the presence of the ΔUBL Ubqln4 mutant construct. The new panels Fig. 3E and F shows that ΔUBL Ubqln4 can’t act as a dominant negative and decrease the basal level of Ubqln1-LC3 interaction. We can show the same information biochemically, as shown in the WB below. We have not included this WB in the revised manuscript because we believe it is largely duplicative of Fig. 3E and F, but would be happy to do so if you and the reviewers believe it important.

8- In Figure 6, the authors show that Ubqln4 is important for Ubqln1’s role in autophagosome-lysosome fusion. However, the data are not sufficient to support the conclusion. If Ubqln1-4 are necessary for autophagosome-lysosome fusion, the authors should analyse the GFP-single positive LC3 puncta (not only the Cherry-single positive puncta) after Ubqln1 and Ubqln4 down-regulation, both in basal condition and after autophagy induction by starvation (and also in the presence of an autophagy inhibitor). GFP-single positive LC3 puncta and the number of GFP-mCherry double-positive puncta should also be measured. (Otherwise, why using this construct?); Moreover, to strengthen these results, the authors should monitor the cellular levels of LC3-II upon starvation after Ubqln1-4 down-regulation. This would help to conclude that there is a block in autophagy flux. We have now included the quantification for double positive and single mCherry positive puncta in Fig 6B. We have also quantified the number of puncta in basal condition and we only find on average 1-3 double positive puncta in all conditions. We have added this information in the result section.
The authors should specify how did they obtain HeLa cells expressing GFP-LC3 or GFP-mCherry-LC3. Hela cells were transiently transfected with GFP-LC3 or mCherry-GFP-LC3 to obtains cells that contain these constructs. This information is included in the figure legends of the appropriate figures.

Referee #3

The referee suggests in his general comments that more careful examination of the puncta containing LC3, Ubqln1, and Ubqln4 should be made by electron microscopy. While we agree that this certainly will be important for establishing the molecular mechanism by which the Ubqlns are involved in autophagosome trafficking, we believe that this is a separate study, beyond the scope of our present work as it will require the development and characterization of several new reagents. Moreover, we believe these data are not essential to establish the veracity of our conclusions. In N'Diaye (2009) EM and other colocalization data were provided that demonstrated Ubqln1 association with autophagosomes, including demonstration that Ubqln1 immunofluorescent puncta were autophagosomes. That study also provided functional data for the role of Ubqln1 in autophagosomal degradation. The focus of the current study is to demonstrate that Ubqln4 has an essential role in recruiting Ubqln1 to this function, and we have provided a multitude of biochemical, immunofluorescent, and functional data to support this hypothesis. We have tried to be very careful in the wording of our manuscript to confine our conclusions to the role for Ubqln4 in recruitment of Ubqln1 to autophagosomes (where its presence has already been established) via interaction with LC3, rather than to be specific about the precise step in autophagosome formation and maturation at which this occurs.

Specific comments

• The figures lack captions.
We have re-written the figure legends to include a more detailed description of the figures.

• Figure 1 - These is no experimental evidence to support a direct interaction between ubqln1 and ubqln4.
We have tried using recombinant protein in a variety of assays to show direct interaction between recombinant Ubqln1 and Ubqln4. However, we have been unsuccessful. In part, this is because the recombinant proteins are poorly behaved in solution, and we don’t trust results obtained with heterogeneous aggregates. We believe that Ubqln1 and/or Ubqln4 need to be in a dimer or even higher order multimer to form a strong interaction, but we have not yet been able to form physiologic multimers without getting heterogeneous aggregation. Instead, we have performed the experiment described above in response to reviewer 2, making a point mutant (I55A) in the UBL domain of Ubqln4 that abolishes interaction with Ubqln1 in cells (Fig 4E). This point mutant, I55A, is the equivalent of I44 found in ubiquitin, which has been show to be an important contact between Ubiquitin and UBA domains. Together with the requirement for the Ubqln1 UBA domain for interaction with Ubqln4, this experiment supports the hypothesis of an interaction between the UBL of Ubqln4 and UBA of Ubqln1.

• Figure 1A: The authors should relate to the fact that some of the identified peptides are not typical trypsin-digested products, namely C'-terminal residues that are not Lysine or Arginine.
There are indeed several peptides identified that are "semi-tryptic" in their sequences, being cleaved at one end c-terminal to lysine or arginine, but with a non-tryptic or ragged cleavage at the other. Our experience is that such cleavages are not uncommon when in-gel digestion is performed, and our database search was therefore done with allowance for semi-tryptic cleavage as one of its parameters. The spectra in question are of high quality and match the assigned sequences very well, by both numerical scoring and manual interpretation. The allowance for semi-tryptic cleavage as one of the parameters for database search is mentioned in the materials and method section.

• Figure 1D: The figure lacks statistical analysis.
We have counted at least 300 GFP-Ubqln1 punctate structures in each of 2 independent experiments. 89 ± 6% of GFP-Ubqln1 puncta also contain RFP-Ubqln4. We have included these data in the result section (p.5) of the revised manuscript.

• Figure 2A: The data is not convincing, statistical information is required. We have counted at least 150 RFP-Ubqln4 punctate structures in each of 3 independent experiments. 58 ± 7 % of RFP-Ubqln4 puncta also contain GFP-LC3. We have included these data in the result section (p.6).

• Figure 2C: GFP-expressing cells should be used as a negative control for the IP. We repeated the experiment using GFP-expressing cells as negative control. The figure has been updated.

• Figure 2D: Positive and negative controls are missing. LC3 levels in the input and the IP should be presented. We repeated the experiment using p62 as a positive control and using s5a as negative control. The figure has been updated. The LC3 used on the affinity column in this experiment was purchased already covalently coupled to agarose beads, and we added equal amounts of beads to each reaction. We have loaded the covalently coupled LC3 agarose beads onto the gel to show the input of LC3 was equivalent in each lane (see accompanying WB).

• Figure 3A and 3C: Off-target effect of ubqln 4 siRNA on the expression of ubqln 1 should be excluded. We repeated the experiment and included a western blot for Ubqln1 to show that there is no off-target effect of Ubqln4 siRNA on the expression of Ubqln1. Fig 3C has been updated to include the western blot for Ubqln1.

• Figure 4A and 4B: the increase in ubqln 4 interaction with delta UBL ubqln1 should be addressed. The increase in Ubqln4 interaction with Ubqln1 delta UBL is not statistically significant. The P value = 0.1118 using unpaired, two-tailed t-test.

• Figure 4C: The explanation regarding the decrease in the interaction of delta UBA-ubqln 4 with ubqln 1 is not clear. We have added additional experiments in Fig 4E and S7 to better explain the decrease in interaction of Ubqln1 with delta UBA Ubqln4. We show that Ubqln4 homo-oligomerizes through its UBA domain (Fig S7A). Oligomerization of Ubqln4 seems to be necessary for a stable interaction with Ubqln1. We confirmed that the interaction of Ubqln1 and Ubqln4 occurs through the UBA domain of Ubqln1 and UBL domain of Ubqln4 by creating a point mutation in the UBL domain of Ubqln4. All of the UBAs that have been characterized so far contact an overlapping face on ubiquitin that includes I44 (Hicke et al, 2005). The equivalent of I44 in Ubqln4’s UBL is I55. When I55 is mutated to alanine, the interaction of Ubqln1 and Ubqln4 is disrupted (Fig 4E). The point mutation was created in Ubqln4 lacking the UBA (can’t homo-oligomerizes) so that there would be no wild type I55 present and thus be able to get a greater signal to noise ratio. When the equivalent I79 in Ubqln1 is mutated to alanine, the interaction of Ubqln1 and Ubqln4 is not disrupted (Fig S7B).
• Figure 5: The insertion of p62-LIR to ubqln 1 has limited relevance to the authors' hypothesis. This figure shows that Ubqln4 can be bypassed if Ubqln1 gains the ability to bind LC3 directly. It provides a different experimental approach to support our hypothesis that Ubqln1 needs to interact with Ubqln4 for its function in autophagy.

• Figure 6A: The IF images should be enlarged. We have enlarged the IF images.

Ford DL, Monteiro MJ (2006) Dimerization of ubiquilin is dependent upon the central region of the protein: evidence that the monomer, but not the dimer, is involved in binding presenilins. The Biochemical journal 399: 397-404


2nd Editorial Decision 07 February 2013

Many thanks for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the three referees that assessed the initial version. As you will see, they all support the publication of your study. Referees 2 and 3 have no further comments, but referee 1 asks that you include some discussion about the specificity for LC3II in the text.

Browsing through the manuscript myself in preparation for acceptance, I have also come across a few things that need addressing in a last, minor revision. These are as follows:

- at over 40,000 characters (including spaces), your manuscript considerably exceeds our maximum length of 30,000, so I must ask you to shorten the text. Shortening will be made easier by adopting EMBO reports reference style, which is described in our instructions to authors online, and by combining the Results and Discussion into a single section, which we require. This will help eliminate the redundancy that is inevitable when discussing the same experiments twice. In addition, although basic Materials and Methods required for understanding the experiments performed must remain in the main text, additional detailed information may be included as Supplementary Material.

- we can publish a maximum of 5 figures in the main text and you currently have 6. In this case, I would suggest to move figure 5 to the supplementary material.

- In figures 4F and 5B, you indicate that the experiments were performed twice independently, yet the graphs show error bars. Please note that it is incorrect to present error bars when the sample size is less than three independent experiments (for guidance, you can refer to Cumming et al. JCB 2007). In addition, the number of experiments performed and identity of the error bars seems to be missing from the legend to supplementary figure 1C.

I look forward to seeing the final version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

The authors performed the experiment I suggested and the result was inconclusive. The positive
control did not work, so they could not evaluate the binding of ubiquilin and LC3. I think there should be some explanation in the text of the manuscript, so that readers might understand why ubiquilin only binds LC3II in their experiments. Otherwise the authors have addressed my other issues.

Referee #2:
The authors have assessed all issues raised by this Reviewer, and the manuscript has been significantly improved.

Referee #3:
The authors addressed most of the comments raised in the first review and the manuscript is now greatly improved.

2nd Revision - authors’ response 11 February 2013

Thank you very much for your letter of Feb. 7, concerning our manuscript, “Ubiquilin4 is an adaptor protein that recruits Ubiquilin1 to the autophagy machinery.” We are pleased that you and the reviewers support the publication of this study. We have made all the minor revisions that you and reviewer 1 suggested, including decreasing the length to 29,976 characters.

We hope that the revised manuscript is now ready for publication in EMBO Reports.

3rd Editorial Decision 12 February 2013

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

As part of the EMBO publication’s Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.