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# WASH inhibits autophagy through suppression of Beclin1 ubiquitination

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## 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.)

*Editor: Anke Sparmann*

1st Editorial Decision 03 July 2013

Thank you for submitting your research manuscript entitled "WASH is a negative regulator of autophagy through suppression of lysine 437 ubiquitination of Beclin 1" (EMBOJ-2013-85905) to our editorial office. It has now been seen by two referees and their comments are provided below.

Both reviewers generally judge your findings as interesting, but stress that a significant revision will be required prior to potential publication at The EMBO Journal. In particular, the referees request additional insight into the mechanism by which WASH controls Beclin1 ubiquinitation. In addition, it will be crucial to disambiguate the function of WASH in the endosomal pathway versus its role in autophagy.

These issues would have to be addressed by a considerable amount of additional work. As this appears feasible based on the constructive suggestions made by the reviewers, we would be willing to grant the opportunity to significantly extend and revise the current manuscript. This will entail challenging and time-consuming experiments, and therefore we would understand if you might decide to seek rapid publication elsewhere.

However, in case you do embark on revisions for our journal, please take the specified demands into careful consideration to avoid disappointments later in the process. I should add that it is our policy

 

to allow only a single major round of revision and that it is therefore important to address the all raised concerns at this stage.

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

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

Thank you for the opportunity to consider your work for publication!

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REFEREE COMMENTS

Referee #1

In this manuscript, the authors show that depletion of the protein WASH strongly increases autophagy in mammalian cells and leads to embryonic lethality. Interestingly, WASH binds Beclin-1, and thereby suppresses its polyubiquitination at lysine 437. Beclin-1 polyubiquitination strengthens the interaction between Vps34 and Beclin-1 and thus stimulates Vps34 kinase activity. While the main findings of the paper are interesting and warrant publication in EMBO J., the experiments fails to explain how WASH is involved in the upregulation of Beclin-1 ubiquitination under physiological conditions. Moreover, some of the data is not convincing and needs better controls and quantifications. Finally, the manuscript would profit from a concise discussion.

Main points

1) The WASH Beclin-1 interaction should be probed under starvation conditions to see whether reduced WASH binding may account for the increase in Beclin-1 poly-ubiquitination. The authors should discuss further possibilities how WASH may regulate autophagy induction upon nutrient starvation.
2) The authors should show that the autophagosome-like structures in Fig. 1F and Fig. 2E are indeed autophagosomes or autolysosomes by immuno-electron microscopy. This is important to understand since WASH -/- embryonic cells also have problems in endosome sorting. The authors should also comment why the morphology is so different and label some cellular reference points in both panels.
3) In panel 3 of Fig. 3F there is a strong accumulation of electron dense particles in the autolysosome, which are not clearly distinguishable from the ones labeled with the red arrow in terms of intensity and size. Hence, the authors cannot claim that WASH does not localize to autolysosomes since the data rather imply the opposite. This would also be more consistent with their data that WASH resides both in forming and closed autophagosomes.
4) The authors need to show the Vps34 input for the Vps34 kinase activity assays in Fig. 4C, Fig. 6F and Fig. 7D. Otherwise the differences in kinase activity are meaningless.
5) Fig. 7A: The authors cannot conclude that the ubiquitin chain on Beclin-1 is exclusively K63-linked since they do not show any data, which disproves that K48-linked polyubiquitin chains (or other linkages) are also present.
6) Fig. 7B: This Figure cannot be used to show co-localisation of Beclin-1, K63-Ub and GFP-LC3 since the GFP-LC3 signal differs greatly between WASH transfected and control cells. Why is there such a strong GFP-LC3 signal in the nucleus upon WASH transfection? The authors should comment on this. Worryingly, the co-localisation between Beclin-1 and K63-Ub is even slightly more pronounced in WASH transfected cells, hence the sentence "However, WASH overexpressed HeLa cells did not exhibit poly-ubiquitinated Beclin 1" has to be removed from the manuscript. The authors need to further include the quantification of the co-localisation between Beclin-1 K63-Ub since there is visually no difference. The authors should furthermore quantify the number of GFP dots per cell (normalized to the cell area). This might be a better read out to check whether

 

autophagy is impaired in WASH transfected cells. The authors should treat the cells with BafA1 to prevent lysosomal degradation in order to accurately quantify the number of GFP dots. Depending on the results from the reanalysis the whole paragraph needs to be rewritten.
7) Fig. 4C and 7D: The authors need to show the samples, which were used for the kinase activity assays (similar to Fig. 4A). Why did the authors not use the samples from Figure 4A, which have a clean deletion of WASH, for Vps34 kinase activity assays and instead use shRNA treated samples? The authors should include the kinase activity results for the samples shown in Fig. 4A.
8) The discussion needs to be rewritten since it rather provides another introduction and lacks a critical discussion of the results and their physiological relevance. Moreover, it contains too many repetitive elements (for example on page 9 "Here, we found that WASH colocalizes with LC3 and Atg16, which might be implicated in autophagosome formation." and on page 10 "Interestingly, we demonstrated that WASH associates with Beclin 1 and regulates the Beclin 1-Vps34 complex. WASH colocalizes with LC3 and Atg16, suggesting that WASH might function in the early stage of autophagosome biosynthesis.")

Minor points

1) Fig. 3A should include an overlay with EEA1 as indicated in the text (data currently missing).
2) The authors should comment whether WASH expression levels vary between different tissues and during different developmental stages.
3) The quantification of the LC3-II to LC3-1 ratio does not reflect the visual impression from the selected experiment in Fig. 2A. Moreover, the Figure should also include the BafA1 treatment for the 1 and 2 hour time points.
4) The authors should comment why they do not include a DAPI signal for Fig. 2B. This is particularly problematic in the case of the 4 hour BafA1 treated WASH -/- sample.
5) The authors should remove Fig. 2C since quantification of GFP-Atg12 dots alone should not be used to conclude enhanced autophagy.
6) The authors need to include a proteasome inhibitor control experiment in Fig. 2D and specify the length of the EBSS/BafA1 treatment. The antibody used should be indicated in the Figure legend. If an anti-ubiquitin antibody was used the signal cannot be labeled with "Poly-Ub" since it recognizes both mono- and polyubiquitinated proteins.
7) The discrepancy between the drastically enhanced lysosomal degradation of ubiquitinated proteins and the rather subtle increase in autophagy in WASH -/- cells is remarkable. The authors should comment on this discrepancy in the text.
8) The co-localization of WASH with Atg16 and DFCP1 is not convincing from the Figures included in the manuscript. Can the authors also comment why an inhibitor would co-localize with forming autophagosomes?
9) "...especially after EBSS treatment (Fig. 4A)." The effect is rather subtle hence it should be phrased more carefully. Otherwise the authors should provide a proper quantification.
10) "By contrast, Flag-WASH overexpressed MEFs declined the Vps34 protein level and dots of DFCP1 (Fig. 4D, E)." - change to "decreased the amount of co-purifying Vps34" or something similar.
11) Fig. 4B: The authors need to provide images for both culture medium and EBSS along with the quantification.
12) Fig. 5H: The authors need to quantify the co-localisation in CM and EBSS and comment on the results.
13) Page 6: Most figures in brackets should be changed from Fig. 3 to Fig. 5.
14) The yeast two-hybrid analysis should also probe the interaction of WASH with other Vps34 and Ulk1/Ulk2 complex members to get an idea about other potential interactors and the specificity of the experiment.
15) Why did the authors not test lysine 117 in Beclin-1 for an ubiquitination defect since it has been reported to be required for Beclin-1 ubiquitination previously (Shi and Kehrl, 2010).
16) Can a WASH deletion on top of the Beclin-1 K437R mutation rescue the weakened Vps34 Beclin-1 interaction?
17) Fig. 6: The authors should provide the uncropped anti-Beclin-1 Western blots in the Supplementary information for all experiments shown in Fig. 6 and Fig. 7C.

 

Referee #2

In this manuscript the authors show that the WASH protein (Wiskott-Aldrich syndrome protein (WASP) and SCAR homolog) that plays a critical role in endosomal sorting is a down-regulator of autophagy. Firstly, it is reported that WASH deficiency, which causes embryonic lethality, is characterized by extensive autophagy. Next they show that WASH is localized in the autophagosomes and interacts with Beclin 1. WASH down-regulates starvation-induced autophagy by suppressing Beclin 1 ubiquitination at position437, and repressing VPS34 activity.
Although the concept that a protein that controls endosomal fission also regulates autophagy is interesting, and ubiquitination of autophagic protein is an emerging post-translational control of autophagy, as it stands the study raises several concerns.
It is not clear that the effect observed is not a consequence of a WASH defect in the endosomal pathway. The mechanism by which WASH controls Beclin 1 ubiquitination is ill-defined. This is important, because other E3 ligases (NEDD4 and TRAF6) can also unbiquitinate Beclin 1 and modulate the activity of VPS34.

Major points
1. The authors suggest that WASH is associated with both unclosed and closed autophagosomes. This calls for greater analysis. The authors need to use an ATG4B mutant (C74A) that hampers LC3 lipidation and leads to defective autophagosome closure to find out whether WASH accumulates in unclosed autophagosomes. Alternatively they could knock down ATG5 or ATG7, both of which lead to the accumulation of ATG by acting upstream of the conjugation systems.
2. The data shown in Figures 4 and 6 do not make it clear whether the authors are analyzing the VPS34 activity associated with Beclin 1 or the overall activity of VPS34. An important aspect of the study is the effect of WASH on the Beclin 1 interactome. It looks as though WASH dissociates the Beclin 1: VSP34 complex. Does WASH influence the stability of Beclin 1?
3. It is essential to identify the E3 ligase that is engaged in the WASH-dependent ubiquitination of Beclin 1. As mentioned in the general comments, at least two E3 ligases are known to be involved in Beclin 1 polyubiquitination. This is very important here, because the authors suggest that WASH-dependent ubiquitination is central in regulating starvation-induced autophagy.
4. The authors need to show the effect of WASH mutants that are known to impair its endosomal function on autophagy. Alternatively they must also show the effect of mutants that affect autophagy on endosomal function.

Other points
It is not clear why the authors used DFCP1 as an early marker of autophagosome formation in this context. It would have been more appropriate to use WIPI1/2, a PI3P-binding protein associated with the phagophore. DFCP1 has been associated with the omegasome
In the section "WASH interacts with Beclin 1", the authors need to check the numbering of the figures (From 3B to 3H - probably 5B to 5H?).

1st Revision - authors' response 14 July 2013

**Referee #1:**
**Main points:**
1) The WASH Beclin-1 interaction should be probed under starvation conditions to see whether reduced WASH binding may account for the increase in Beclin-1 poly-ubiquitination. The authors should discuss further possibilities how WASH may regulate autophagy induction upon nutrient starvation.

**Answer:** This is a good point. We performed co-IP experiments using anti-WASH to immunoprecipitate Beclin1 from lysates of untreated and EBSS-treated conditions. The association of WASH with Beclin1 was dramatically decreased upon starvation stimulation (new Figure 5I, left panel). Consistently, anti-Beclin 1 antibody could precipitate less WASH under EBSS treatment (new Figure 5I, right panel). These data indicate that WASH disassociates Beclin 1 to liberate the inhibition of WASH, leading to Beclin 1 ubiquitination. We identified that Ambra1 is an E3 ligase for K63-linked ubiquitination of Beclin 1 (new Figure 7F-H). WASH and Ambra1 can

 

competitively bind to Beclin 1. Thus WASH could inhibit Beclin 1 ubiquitination through competitive binding inhibition. Other possibility is that WASH may be modified after receiving autophagic signals, whose modifications might be involved in the regulation of autophagy. We are further defining these issues. How WASH is modified in autophagy induction is beyond the scope of this paper.

2) The authors should show that the autophagosome-like structures in Fig. 1F and Fig. 2E are indeed autophagosomes or autolysosomes by immuno-electron microscopy. This is important to understand since WASH -/- embryonic cells also have problems in endosome sorting. The authors should also comment why the morphology is so different and label some cellular reference points in both panels.

**Answer:** We stained the sections with anti-LC3 antibody for immuno-electron microscopy and changed new figures. We found LC3 was localized exactly to these structures like to autophagosomes. We added cellular reference particles (asterisk) to the enlarged images. Atg4 is essential for formation of autophagosomes, and Atg4 mutant (C74A) that impairs LC3 lipidation to disrupt the closure of autophagosomes (Fujita et al, 2008). Importantly, in Atg4(C74A) expressed cells, WASH colocalized with Atg5 (new Figure 3C), suggesting that WASH is localized in forming autophagosomes. As shown in the new Figure 3G, WASH was not localized in the autolysosomes through detection of fractionation of organelles. We also showed that the autophagy-related WASH was independent of endosomal sorting function (new Figure 3B, new Supplementary Figure S2, S5).

As to the different morphologies in embryos and in MEFs, over-activated extensive autophagy in WASH KO embryos might disrupt many structures of the E7.5 embryo and finally caused severely enlarged autophagosomes. The enlarged vacuole structures in WASH KO embryos may be caused by other unknown mechanisms. However, MEFs were cultured in vitro, whose vacuole structures appeared to be normal morphology. We commented these in the text.

3) In panel 3 of Fig. 3F there is a strong accumulation of electron dense particles in the autolysosome, which are not clearly distinguishable from the ones labeled with the red arrow in terms of intensity and size. Hence, the authors cannot claim that WASH does not localize to autolysosomes since the data rather imply the opposite. This would also be more consistent with their data that WASH resides both in forming and closed autophagosomes.

**Answer:** This is a very good point. For our immuno-electron experiments, we found that autolysosomes contained densed materials that appeared to be quite dark under microscopy. It is therefore difficult to distinguish from WASH labeled gold particles. To exclude that WASH localizes in autolysosomes, we separated autolysosomes from HeLa cells through fractionation of organelles and immunoblotted WASH for its expression. As shown in new Figure 3G, WASH was not localized in autolysosomes. Moreover, LAMP1 did not colocalized with WASH (Figure 3D). We also did additional experiments as shown in new Figure 3C. In Atg4(C74A) expressed cells, WASH colocalized with Atg5, which further confirmed that WASH is localized in forming autophagosomes.

4) The authors need to show the Vps34 input for the Vps34 kinase activity assays in Fig. 4C, Fig. 6F and Fig. 7D. Otherwise the differences in kinase activity are meaningless.

**Answer:** We added all the inputs in suggested figures.

5) Fig. 7A: The authors cannot conclude that the ubiquitin chain on Beclin-1 is exclusively K63-linked since they do not show any data, which disproves that K48-linked polyubiquitin chains (or other linkages) are also present.

**Answer:** We stripped the same membrane and probed with K48-ubiquitin specific antibody (new Figure 7A, right panel). No signal was detected for K48-ubiquitin chains.

6) Fig. 7B: This Figure cannot be used to show co-localisation of Beclin-1, K63-Ub and GFP-LC3 since the GFP-LC3 signal differs greatly between WASH transfected and control cells. Why is there such a strong GFP-LC3 signal in the nucleus upon WASH transfection? The authors should comment on this. Worryingly, the co-localisation between Beclin-1 and K63-Ub is even slightly more pronounced in WASH transfected cells, hence the sentence "However, WASH overexpressed HeLa cells did not exhibit poly-ubiquitinated Beclin 1" has to be removed from the manuscript. The authors need to further include the quantification of the co-localisation between Beclin-1 K63-Ub since there is visually no difference. The authors should furthermore quantify the number of GFP dots per cell (normalized to the cell area). This might be a better read out to check whether autophagy is impaired in WASH transfected cells. The authors should treat the cells with BafA1 to

 

prevent lysosomal degradation in order to accurately quantify the number of GFP dots. Depending on the results from the reanalysis the whole paragraph needs to be rewritten.

**Answer:** This is very good suggestion. We stained Beclin1 and K63-Ub in Vector or Flag-WASH overexpressed cells and calculated the colocalization efficiency between Beclin1 and K63-Ub (new Figure 7B). We found the colocalization rate between Beclin1 and K63-Ub dramatically decreased in Flag-WASH overexpressed cells under EBSS treatment. In our GFP-LC3 transfection experiments, we found that GFP-LC3 transient overexpression led to nuclear localization in normal conditions. Therefore, we stained endogenous LC3 in cells with vector and Flag-WASH overexpression upon starvation with or without BafA1 (new Figure 7C). We found that Flag-WASH overexpression suppressed the lipidation of LC3. We rewrote this paragraph according to our new data.

7) Fig. 4C and 7D: The authors need to show the samples, which were used for the kinase activity assays (similar to Fig. 4A). Why did the authors not use the samples from Figure 4A, which have a clean deletion of WASH, for Vps34 kinase activity assays and instead use shRNA treated samples? The authors should include the kinase activity results for the samples shown in Fig. 4A.

**Answer:** We added the input along with the kinase assays in new Figure 4D and Figure 7E. We also performed kinase assays with WASH KO cells as shown in new Figure 4B.

8) The discussion needs to be rewritten since it rather provides another introduction and lacks a critical discussion of the results and their physiological relevance. Moreover, it contains too many repetitive elements (for example on page 9 "Here, we found that WASH colocalizes with LC3 and Atg16, which might be implicated in autophagosome formation." and on page 10 "Interestingly, we demonstrated that WASH associates with Beclin 1 and regulates the Beclin 1-Vps34 complex. WASH colocalizes with LC3 and Atg16, suggesting that WASH might function in the early stage of autophagosome biosynthesis.")

**Answer:** We revised the discussion section as suggested.

**Minor points:**

1) Fig. 3A should include an overlay with EEA1 as indicated in the text (data currently missing).

**Answer:** We added the images as shown in the new Figure 3B.

2) The authors should comment whether WASH expression levels vary between different tissues and during different developmental stages.

**Answer:** We checked WASH expression in several tissues and found WASH was constitutively expressed in these detected tissues (Supplementary Figure S1A). We also checked the expression level of WASH in mouse embryos from embryo day 7.5 to 19.5 (Supplementary Figure S1B). We found WASH was also constitutively expressed in embryos with various expression levels. We stated these in the text.

3) The quantification of the LC3-II to LC3-1 ratio does not reflect the visual impression from the selected experiment in Fig. 2A. Moreover, the Figure should also include the BafA1 treatment for the 1 and 2 hour time points.

**Answer:** We quantified the LC3 II to β-actin to show more directly the change of LC3II during autophagy process (new Figure 2A). We also performed LC3 conversion experiments by adding BafA1 for the 1 and 2 h time points as shown in the same Figure.

4) The authors should comment why they do not include a DAPI signal for Fig. 2B. This is particularly problematic in the case of the 4 hour BafA1 treated WASH -/- sample.

**Answer:** This is a good suggestion. We changed these images with new ones as shown in the new Figure 2B. We used DAPI for counterstaining.

5) The authors should remove Fig. 2C since quantification of GFP-Atg12 dots alone should not be used to conclude enhanced autophagy.

**Answer:** We removed it.

6) The authors need to include a proteasome inhibitor control experiment in Fig. 2D and specify the length of the EBSS/BafA1 treatment. The antibody used should be indicated in the Figure legend. If an anti-ubiquitin antibody was used the signal cannot be labeled with "Poly-Ub" since it recognizes both mono- and polyubiquitinated proteins.

 

**Answer:** This is a very good suggestion. We treated cells with EBSS/BafA1 for 8 h in our previous experiments in order to observe polyubiquitinated proteins, which was longer time than the starvation time we used in other experiments. We replaced this Figure with new experimental data. In the new experiments, we treated cells with EBSS/BafA1 for 2 h with or without a proteasome inhibitor MG132 (new Figure 2C). We found MG132 did not restore the polyubiquitinated protein level compared with BafA1 treatment, indicating that the decreased polyubiquitinated proteins are degraded through the autophagy-mediated lysosomal pathway. We specified that anti-ubiquitin antibody in the legend.

7) The discrepancy between the drastically enhanced lysosomal degradation of ubiquitinated proteins and the rather subtle increase in autophagy in WASH -/- cells is remarkable. The authors should comment on this discrepancy in the text.

**Answer:** The discrepancy might be caused by longer EBSS treatment in our previous experiments. As there are large amounts of polyubiquitianted proteins inside the cell and the degradation of proteins through a lysosomal pathway takes hours to accomplish. Thus, 8 h treatment of EBSS/BafA1 was performed to see distinguishable decrease of polyubiquitinated proteins in WASH KO cells. We changed this Figure with new experiments for 2 h starvation treatment (new Figure 2C).

8) The co-localization of WASH with Atg16 and DFCP1 is not convincing from the Figures included in the manuscript. Can the authors also comment why an inhibitor would co-localize with forming autophagosomes?

**Answer:** We deleted these two images and added the new Figure 3B and 3C. We used Atg4B mutant (C74A) expressed cells to show the colocalization of WASH with Atg5 (new Figure 3C). As to the localization of WASH in forming autophagosomes, WASH may negatively regulate this process in the process of autophagy. As an inhibitor, WASH dissociates with Beclin 1 for efficient binding to its E3 ligase Ambra1, which catalyzes K63-linked ubiquitination of Beclin 1 to promote autophagosome formation.

9) "...especially after EBSS treatment (Fig. 4A)." The effect is rather subtle hence it should be phrased more carefully. Otherwise the authors should provide a proper quantification.

**Answer:** We quantified the blots (Figure 4A, right panel).

10) "By contrast, Flag-WASH overexpressed MEFs declined the Vps34 protein level and dots of DFCP1 (Fig. 4D, E)." - change to "decreased the amount of co-purifying Vps34" or something similar.

**Answer:** We changed these wording.

11) Fig. 4B: The authors need to provide images for both culture medium and EBSS along with the quantification.

**Answer:** We deleted this figure with new experiments as shown in the new Figure 4C. To monitor the Vps34 activity (autophagy-related) more exactly, we used WIPI1 to determine the activity of Vps34. WIPI1 is a PI3P-binding protein that is involved in the formation of phagophores (Proikas-Cezanne et al, 2007).

12) Fig. 5H: The authors need to quantify the co-localisation in CM and EBSS and comment on the results.

**Answer:** We quantified the co-localization of Beclin1 and WASH in CM and EBSS conditions and stated these results in the text (Figure 5H, lower panel). We found the colocalization rate of WASH with Beclin 1 was decreased upon autophagy induction. Moreover, with EBSS treatment, the association between WASH and Beclin 1 was dramatically decreased (new Figure 5I), indicating that the association of Beclin 1 with WASH is reduced during the process of autophagy.

13) Page 6: Most figures in brackets should be changed from Fig. 3 to Fig. 5.

**Answer:** We are sorry for these mistakes. We corrected them.

14) The yeast two-hybrid analysis should also probe the interaction of WASH with other Vps34 and Ulk1/Ulk2 complex members to get an idea about other potential interactors and the specificity of the experiment.

 

**Answer:** We performed yeast two hybrid analyses to determine the interactions of WASH with Vps34, Vps15, Ambra1, Atg14L, Ulk1, Ulk2, Atg13, or FIP200 (Supplementary Figure S4). We found that WASH did not interact with other components of the Vps34 or Ulk1/2 complex besides Beclin 1, indicating that the association of WASH with Beclin1 was specific.

15) Why did the authors not test lysine 117 in Beclin-1 for an ubiquitination defect since it has been reported to be required for Beclin-1 ubiquitination previously (Shi and Kehrl, 2010).

**Answer:** As shown in the Supplementary Figure S7, K117R-Beclin 1 could still be still ubiquitinated during starvation-induced autophagy. We stated these in the text.

16) Can a WASH deletion on top of the Beclin-1 K437R mutation rescue the weakened Vps34 Beclin-1 interaction?

**Answer:** We performed these experiments in WASH KO MEFs and found that WASH deletion could not rescue the weakened Vps34-Beclin 1 interaction in K437R-Beclin 1 transfected cells (data not shown). This may be because that WASH exerted its inhibitory role through suppressing ubiquitination of Beclin1, while K437R-Beclin 1 failed to be ubiquitinated even in the absence of WASH. Therefore, the weakened Vps34-Beclin 1 interaction could not be rescued by WASH deletion.

17) Fig. 6: The authors should provide the uncropped anti-Beclin-1 Western blots in the Supplementary information for all experiments shown in Fig. 6 and Fig. 7C.

**Answer:** We provided these uncropped anti-Beclin1 blots as shown in the Supplementary Figure S6.

**Referee #2:**
**Major points:**

1. The authors suggest that WASH is associated with both unclosed and closed autophagosomes. This calls for greater analysis. The authors need to use an ATG4B mutant (C74A) that hampers LC3 lipidation and leads to defective autophagosome closure to find out whether WASH accumulates in unclosed autophagosomes. Alternatively they could knock down ATG5 or ATG7, both of which lead to the accumulation of ATG by acting upstream of the conjugation systems.

**Answer:** This is a good suggestion. We used Atg4B(C74A) mutant expressed cells to determine the colocalization of WASH with Atg5 (new Figure 3C). We found that in Atg4B (C74A) mutant transfected cells, GFP-Atg5 accumulated and co-localized with WASH.

2. The data shown in Figures 4 and 6 do not make it clear whether the authors are analyzing the VPS34 activity associated with Beclin 1 or the overall activity of VPS34. An important aspect of the study is the effect of WASH on the Beclin 1 interactome. It looks as though WASH dissociates the Beclin 1: VSP34 complex. Does WASH influence the stability of Beclin 1?

**Answer:** We performed these experiments by using anti-Beclin 1 antibody to precipitate the Beclin1 associated Vps34. Immunoprecipitates were separated for two equal parts, one for kinase assays and the other for input detection. We added these input blots in the corresponding figures (Figure 4B, 4D, and Figure 6F). We added the description in these legends. WASH did not influence the stability of Beclin1 (Supplementary Figure S3). The expression level of Beclin 1 was unchanged in normal or starved conditions in WASH KO cells.

3. It is essential to identify the E3 ligase that is engaged in the WASH-dependent ubiquitination of Beclin 1. As mentioned in the general comments, at least two E3 ligases are known to be involved in Beclin 1 polyubiquitination. This is very important here, because the authors suggest that WASH-dependent ubiquitination is central in regulating starvation-induced autophagy.

**Answer:** This is a very good suggestion. We identified that Ambra1 was an E3 ligase for K63-linked ubiquitination of Beclin 1 in starvation-induced autophagy (new Figure 7F-H). We found that knockdown of either NEDD4 or TRAF6 did not affect the K63-linked ubiquitination of Beclin 1 in starvation-induced autophagy (Supplementary Figure S8). Additionally, we also found that WASH hindered the association between Ambra1 and Beclin 1 (new Figure 7I, J), which indicates that WASH suppresses Beclin 1 ubiquitination by competitive binding inhibition. We addressed these data in the text.

 

4. The authors need to show the effect of WASH mutants that are known to impair its endosomal function on autophagy. Alternatively they must also show the effect of mutants that affect autophagy on endosomal function.

**Answer:** This is a very good point. The VCA domain of WASH is required for the endosomal sorting function (Derivery et al, 2009; Jia et al, 2010). Importantly, the VCA domain-truncated WASH (ΔVCA) mutant could still rescue the accelerated autophagy induction in WASH KO MEFs comparable to that of the full length WASH (FL) restoration (Supplementary Figure S2C). These observations indicate that WASH suppresses autophagy independently of its endosomal sorting function. We showed that aa 121-221 of WASH was responsible for binding Beclin1 (Figure 5D). Additionally, the deletion of aa121-221 (D121-221) of WASH failed to restore the accelerated autophagy process in WASH KO MEFs (Supplementary Figure S5A). However, the deletion of aa121-221 (D121-221) of WASH was able to rescue the enhanced EGFR degradation in WASH KO MEFs, while the WASH (ΔVCA) mutant had no such activity (Supplementary Figure S5B). These data confirmed that WASH exerts its autophagy regulation independently of its endosomal sorting role. We addressed these findings in the text.

**Other points:**

1) It is not clear why the authors used DFCP1 as an early marker of autophagosome formation in this context. It would have been more appropriate to use WIPI1/2, a PI3P-binding protein associated with the phagophore. DFCP1 has been associated with the omegasome

**Answer:** This is a good suggestion. We used WIPI1 to test the Vps34 activity as shown in the new Figure 4C, 4F, and Figure 6G.

2) In the section "WASH interacts with Beclin 1", the authors need to check the numbering of the figures (From 3B to 3H - probably 5B to 5H?).

**Answer:** We are really sorry for these mistakes. We corrected them.

2nd Editorial Decision 25 July 2013

Thank you for submitting your revised manuscript for our consideration. Your study has now been seen once more by one of the original referees, whose comments are provided below. Overall, the reviewer finds that all major concerns have been addressed. However, s/he stresses that a direct demonstration of the E3 ligase activity of AMBRA is essential to substantiate your conclusions and should be included prior to publication in The EMBO Journal.

In addition, I would like to mention that we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

I will now return your manuscript to you for one additional round of revision. Once the remaining issue has been addressed, we should be able to proceed swiftly with formal acceptance and production of the manuscript!

If you have any questions, please do not hesitate to contact me directly.

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REFEREE COMMENTS

Referee #2

The authors have addressed most of the concerns raised by the first version of their manuscript.

However, one major problem remains regarding the identity of the E3 ligase involved in the ubiquitination of Beclin 1. Based on the results obtained after the knock down of 2 E3 ligases, which are known to ubiquitinate Beclin 1 (TRAF6 and NEED4), the authors concluded that AMBRA1 is an E3 ligase involved in the K63-linked ubiquitination of Becln 1. Recently, AMBRA1 has been shown to interact with E3 ligases in various different settings (TRAF6 and Parkin). However, so far AMBRA1 has not been demonstrated to be an E3 ligase. The discovery that AMBRA1 is an E3 ligase would establish an important new function for this protein. The authors should design experiments that will provide a direct demonstration of the AMBRA1 E3 ligase activity using in vitro E3 ligase activity. Without such robust experimental support, the authors' unsubstantiated claim weakens their manuscript.

2nd Revision - authors' response 26 July 2013

We would like to thank you again for allowing us one additional round to revise our manuscript entitled "WASH is a negative regulator of autophagy through suppression of lysine 437 ubiquitination of Beclin 1" (EMBOJ-2013-85905R). We also thank the Reviewer #2 for his/her further suggestions. We already did these experiments to verify our conclusions by an in vitro ubiquitination reconstitution assay, and we are exploring the functions and mechanisms of the Ambra1-DDB1-Cullin4 complex on autophagy induction. Because of the scope limitation of this journal, we did not put these data in our previous version. We have added these data in our current version and carefully interpreted our data in the text. We strongly believe that these data have greatly strengthened our conclusions, and addressed the concerns of the Reviewer #2.

Additionally, we provided our all original and uncropped gel/blot panels per figure as a supplementary "Source Data" file, and submitted all the Production Forms to your office. A detailed response to theReviewer #2 comments follows.

Please let me know if you need any additional information. Thank you in advance for your consideration and we look forward to your decision.

**Referee #2:**

The authors have addressed most of the concerns raised by the first version of their manuscript. However, one major problem remains regarding the identity of the E3 ligase involved in the ubiquitination of Beclin 1. Based on the results obtained after the knock down of 2 E3 ligases, which are known to ubiquitinate Beclin 1 (TRAF6 and NEED4), the authors concluded that AMBRA1 is an E3 ligase involved in the K63-linked ubiquitination of Becln 1. Recently, AMBRA1 has been shown to interact with E3 ligases in various different settings (TRAF6 and Parkin). However, so far AMBRA1 has not been demonstrated to be an E3 ligase. The discovery that AMBRA1 is an E3 ligase would establish an important new function for this protein. The authors should design experiments that will provide a direct demonstration of the AMBRA1 E3 ligase activity using in vitro E3 ligase activity. Without such robust experimental support, the authors' unsubstantiated claim weakens their manuscript.

**Answer:** This is a very good point. We already did these experiments to verify our conclusions by an in vitro ubiquitination reconstitution assay, and we are exploring the functions and mechanisms of the Ambra1-DDB1-Cullin4 complex on autophagy induction. Because of the scope limitation of this journal, we did not put these data in our previous version. As shown in the new Figure 7I, Ambra1 could directly ubiquitinate Beclin 1 in the *in vitro* ubiquitination reconstitution system. Importantly, Beclin 1 could be polyubiquitinated by WT and K48 mutated ubiquitin (K48R), but not by K63 mutated ubiquitin (K63R), suggesting that Ambra1 contributes to the K63-linked ubiquitination of Beclin 1. We addressed these data in the text.

 