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Genome-wide siRNA screen reveals amino acid starvation-induced autophagy requires SCOC and WAC

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1st Editorial Decision 22 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while all three referees consider the study as interesting in principle, they all think that the depth of analysis is not sufficient at this point to support publication in The EMBO Journal. It becomes clear from their reports that the role of SCOC and its interaction partner FEZ1 in autophagy would need to be analysed in more depth both at the present level of conceptual understanding as well as by adding some deeper mechanistic insight. While these concerns thus preclude publication of the study here at this stage of analysis, we would like to give you the chance to strengthen and develop the study further by a major round of revision. However, the referees' concerns regarding the conclusiveness of part of the data need to be addressed in a convincing manner, and the referees' suggestions how to develop the study further mechanistically, in particular those of referee 2, should be followed. In addition, it would strengthen the study enormously if at least some further analysis of the functional link between SCOC and the Beclin 1 subnetwork could be included. I would further like to suggest focussing on the analysis of SCOC, rather than on taking the analysis of WAC further.

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. Now, I recognise that the additional work required is rather substantial, and we would thus be happy to extend the deadline for your revision if needed. Please do not hesitate to contact me at any time in case you would like to discuss any aspect of the revision further.

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

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This paper designed a siRNA-based screen to examine GFP-LC3 localization during treatment with starvation medium and leupeptin to identify new genes that modulate starvation-induced autophagy. The screen was designed to examine the early stages of autophagy induction and autophagosome formation. Since the stable cell line HEK293-GFP-LC3 was treated with the lysosome inhibitor leupeptin, this screen is not assaying for genes involved in the autolysosmal degradation step of autophagy. This paper claims identification of two novel autophagy proteins, SCOC and WAC, using genome-wide siRNA screen in the HEK293 cell line stably expressing GFP-LC3. The authors have proposed that SCOC is involved in autophagosome formation through an interaction with FEZ1, an ULK1-binding negative regulator of autophagy, and that WAC is a positive regulator of autophagy while negatively regulating the ubiquitin proteosome system. Despite the solid bioinformatical analyses to identify SCOC and WAC as regulators of autophagy from 21,141 siRNA pools, more in-depth biochemical and cell biological analyses will have to be provided to strengthen the hypothesis, including the following recommendations:

1. Fig.1A: It is difficult to distinguish the difference in GFP-LC3 localization in the normal vs serum starved medium, DAPI co-staining and a color picture of GFP-LC3 would help to show GFP-LC3 cytoplasmic localization.

2. Fig.1D: Based on the fact that Rubicon and VPS16 play opposing roles in negatively and positively regulating autophagosome maturation, respectively, it doesn't seem appropriate that VPS16 is included in the group with high SCPO A-scores.

3. Fig.2C: Based on the data from figure 2A, one would expect that siRNA against SCOC or WAC would result in a decreased level of autophagy. LC3 processing by Western blot does not support this conclusion.
(1) The LC3-II protein level in HeLa cells has been reported to increase upon
depletion of autophagy proteins because autophagic flux is blocked
(Noboru's MBC paper in 2008). For better explanations, p62 level needs to be included.
(2) Plot LC3-II/actin instead of LC3-II/LC3-I, which is more widely accepted in
the field and also for the consistency throughout the manuscript.

(3) The increasing effect of leupeptin seems minimal. Please provide improved blots using other inhibitors such as bafilomycin A1 or chloroquine.

4. Fig.3B and 3C: Please include p62 in addition to LC3 blot.

5. Fig.3D: Endogenous colocalization of SCOC, which is known to be primarily in golgi and alternatively at plasma membrane, seems incorrect (staining mainly appears in the nucleus and cytoplasm, unlike Atg9 colocalization pattern observed in Figure 3D). It is difficult to accept that the available antibody for SCOC can detect endogenous protein by immunofluorescence but not by western blotting. Please demonstrate colocalization of endogenous SCOC with golgi organelle markers. Alternatively, specific signal for SCOC can be shown in the overexpression system with appropriate controls (ex. difference between SCOC-mCherry vs. mCherry empty vector; observed colocalization of mCherry-SCOC and the endogenous SCOC antibody). In this image it is difficult to conclude whether GFP-LC3 colocalizes with SCOC. Colocalization of GFP-LC3 and SCOC may improve following treatment of cells with chloroquine to induce accumulation of autophagosomes.

6. Fig.4A and 4B: Please include p62 in addition to LC3 blot.

7. Fig.4C: please clarify whether the WB for SCOC levels was determined using an anti-myc antibody or to endogenous SCOC. Text in the manuscript mentioned that there is not an antibody available to endogenous SCOC, yet the figure 4C WB is labeled as SCOC. Is there a reason for using different tags for the reciprocal coIPs for FEZ1 and SCOC? Why not IP for GFP-FEZ1 using GFP antibody and WB for FLAG-SCOC and perform the reciprocal IP for FLAG antibody using FLAG-SCOC and WB for GFP-FEZ1. For the figure legend in 4C, the interaction of SCOC and FEZ1 may not be considered an "in vivo" interaction since the IP data presented is for overexpression of tagged protein instead of IP for endogenous protein.

8. Fig.4D: Please include negative controls, which do not bind to SCOC, to demonstrate specificity of the SCOC-FEZ1 interaction (ie. 35S-radiolabeled myc vector as a non-specific radiolabeled control). It is difficult to see SCOC in the input lane. It might be useful to use an arrow to point out the band for GST-SCOC.

9. Fig.5A: It seems to be mislabeled that "Flag-tagged SCOC was not detectable on western blots of lysates analysed by BN-PAGE when transfected alone, unless FEZ1-GFP was co-transfected", as Flag-SCOC protein expression was equivalent regardless of the presence or absence of FEZ1-GFP while complex formation was affected (the lane4 and 5 in the middle panel). Based on the lane 4 and 5 in the first panel, it looks more likely that "FEZ1-GFP is not detectable when transfected alone, unless Flag-SCOC is co-transfected".

10. Fig.6: The fact that WAC knockdown gave rise to accumulation of p62 and decrease of LC3-II protein level (in figure 6B and 6C) and decrease of HttQ25-GFP and the soluble fraction of HttQ103-CFP levels (in figure 6D and 6E) suggests the potential role of WAC in positive and negative regulation of autophagy and ubiquitin proteosome system (UPS), respectively. However, this does not necessarily imply that WAC may be involved in autophagy of ubiquitinated proteins by negatively regulating UPS. The degradation of UbG76V-YFP (in figure 6D) in full medium in WAC knockdown only supports the data from figure 6E, but does not address whether WAC has a direct effect on autophagy. A GFP-LC3 degradation assay may be a better experiment to address whether WAC plays a role in autophagy of ubiquitinated proteins by negatively regulating UPS.

11. On page 6 and 7 of the manuscript please refer to the figure, otherwise state "(data not shown)":
-Knockdown of RBM12 appeared to block maturation of autophagosomes, as the amount of LC3 lipidation in starvation medium without leupeptin was the same compared to starvation medium with leupeptin (include reference to Figure X or data not shown).

12. On page 10 of the manuscript please explain why you didn't see accumulation of UbG76V-YFP in WAC siRNA treated cells during starvation induced autophagy conditions. The manuscript only mentioned the decreased levels of UbG76V-YFP in WAC depleted cells in full medium.

Referee #2

In their manuscript entitled "Genome-wide siRNA screen reveals amino-acid starvation-induced autophagy requires SCOC and WAC", the authors performed a genome-wide siRNA screen and identified 20 "strong" candidates as autophagy regulators. Among these candidates, they further characterized two proteins SCOC and WAC. While identification of new autophagy regulators is interesting and should provide new insights into autophagy regulation, subsequent analyses of the two factors are preliminary, and somewhat confirmatory of previous findings.

Major comments:

The role of SCOC remains largely unclear. Although SCOC interacts with FEZ1, these two proteins seem to have opposite functions; is this interaction indeed important? This question can be tested by overexpression of the SCOC1 L254P/L260P mutant, or more appropriately, by expressing the mutant in SCOC1 knockdown cells.

The authors hypothesize that ULK1 regulates formation of the SCOC and FEZ1 complex based on the overexpression of ULK1 KI (Fig. 5A). However, this conclusion is based solely on findings from the overexpression approach, and there is no evidence to show whether or not it is regulated by endogenous ULK1. Using siRNA to knockdown ULK1 is required to further validate their findings.

Do SCOC and FEZ1 function upstream or downstream of ULK1? Are they ULK1 substrates? Do the interactions between these proteins change under starvation conditions? Does SCOC regulate the FEZ1-ULK1 interaction? The authors provide a lot of attractive speculations in the Discussion, most of which can be experimentally addressed.

The studies on WAC is also preliminary; although it is interesting that WAC also affects proteasomal activity, its mechanism was not investigated in the present study.

The effects of knockdown of SCOC and WAC on LC3-II level are subtle (Fig. 3B and C, Fig. 6B). Additionally, LC3-I is rather increased in siFEZ1 cells, which may indicate reduced autophagic flux. What is the p62 expression level under non-starvation conditions in these knockdown cells? The authors show its level in starved WAC cells (Fig. 6C), but it should also accumulate in non-starved cells.

Minor comments:

The four SCOC isoforms are not described in Figure 3A.

The images in Figure 3D need to be replaced with higher magnification images. The authors previously showed that LC3 and Atg9 localize to numerous punctate structures in the cytoplasm under these conditions, but their peripheral structures are not visible in these images.

The high ULK1 expression level in SCOC-knockdown cells is intriguing (Fig. 3B), and should therefore be mentioned and discussed.

Referee #3

In search of novel proteins regulating starvation-induced autophagy, McKnight and coworkers performed a genome-wide siRNA screen, employing HEK cells stably expressing GFP-LC3. The readout for autophagy induction/reduction was the change in GFP-positive spots compared to control cells upon starvation treatment. Utilizing several steps of filtering, the authors eliminated most genes by setting statistical and technical thresholds, coming down to nine hits. While seven of these hits have potentially autophagy-

related relevance, the authors focus was set on two proteins - SCOC and WAC. SCOC, a Golgi protein was required for autophagosome formation and interacted with FEZ1, an ULK1-binding negative regulator of autophagy. WAC, a relatively uncharacterized protein, was required for starvation-induced autophagy but also acted as a potential negative regulator of the ubiquitin proteasome system.

The main goal of this work was to find novel players in the autophagic pathway, and specifically those controlling starvation-induced autophagy in mammalian cells. This is an important goal as it clear that additional yet unidentified autophagic factors are involved in the autophagic process. The authors applied stringent parameters to eliminate false positive results and ended up with nine hits, seven of which were neither characterized nor proven to participate in autophagy. The two remaining hits, SCOC and WAC, were further characterized. While attempts were done to prove their relevance in autophagy, and to investigate possible ways in which they control this pathway, the data in the manuscript remain too preliminary. Going down the long way from the screen to the autophagy-related hits, the authors should go the extra mile and bring stronger evidence for their hypotheses.

In general, the data lacks appropriate controls. LC3 lipidation, the main readout in this manuscript for autophagy activation, should be analyzed more carefully, i.e. by assessing autophagic flux. This should be demonstrated by fluorescence imaging in addition to WB analysis. In addition, the authors should take advantage of p62 (or any other autophagic receptor) as an autophagic marker throughout their work, as they did in the last part of this manuscript.

The authors tends to be over-conclusive on the one hand and to discard potentially interesting data on the other hand. For instance, the authors conclude that five proteins are required for starvation-induced autophagy based solely on the effect their knockdown had on LC3 lipidation. Conversely, referring to screen hits, the authors argue that knockdown of RBM12 appeared to block maturation of autophagosomes and therefore they seized to investigate this gene. SUPT5H, another candidate, was dismissed simply because it is a known subunit of the eukaryotic transcription elongation factor DSIF.
The screen and its preliminary results should take a considerably smaller portion of the manuscript, and the authors should focus on those hits that were further investigated. The other semi-validated hits leave much room for debate. These could be mentioned, briefly, in the discussion part.
While a big effort was evidently put into this work, the main findings are yet to be better established.

Specific comments

• The experimental procedure of the siRNA screen is not clear. Were the three parameters measured for each gene both with full medium and starvation medium+leupeptin, or just the latter?
• NRBP2 is used as an autophagy-suppressing control in this work, based on a previous work by the authors. Valid as it may be, it should at least be reinforced with a more common control.
• Figure 2B: The relevance of this figure to the manuscript is not clear and does not lead to any new insights.
• Figures 2C, 2D and 6D: LC3II levels are relatively high even in cells grown in full medium, which may indicate cells are in stress to begin with. This makes further conclusions hard to deduce.
• The authors provide statistical information on the comparison between LC3II levels in accumulated in EL. It is more important to compare the autophagic flux between the two samples. For example, knockdown of SCOC (Figure 3B) or its overexpression (Figure 3C) may have little or no effect on the autophagic flux.
• Figure 3B, 3C: The cells used in this experiment show a very low autophagic flux in response to starvation. Furthermore, knockdown of SCOC increased the flux, in contrast to its proposed role.
• Figure 3D: LC3 phenotype hardly resembles that of starved cells, indicating a possible error in the experiment. In addition, the localization between SCOC and LC3 and Atg9 is not convincing. A control experiment that includes lysosomal inhibitor is needed. Moreover, here to the authors should look for autophagic flux.
• Figure 4A. The level of LC3II in the control is very high. This exemplifies the need for additional experiments using other autophagy related markers (p62, nbr1 etc.) as well as fluorescence microscopy analysis. As mentioned above such analysis should be performed throughout this study.
• Figure 6A: The polyclonal antibody for WAC presumably recognizes two endogenous isoforms. This

claim should be favored over the possibility that the smaller fragment is a degradation or cleavage product of the full length protein.
• Figure 6D: based on LC3 and p62 levels, it seems that the starvation medium had no effect on the cells. The authors should relate to the inconsistency with the data presented in Figure 6C in which they observe accumulation of p62 upon WAC kD.
• Figure S4b: The interaction between WAC and Beclin is only demonstrated when both proteins were overexpressed. The authors should test the interaction of the endogenous proteins or explain why this is not possible.
• The authors should explain the inconsistency with regard to the way they analyze the WB results, i.e. LC3II/LC3I, LC3II/actin etc.

1st Revision - authors' response 23 October 2011

Referee #1

This paper designed a siRNA-based screen to examine GFP-LC3 localization during treatment with starvation medium and leupeptin to identify new genes that modulate starvation-induced autophagy. The screen was designed to examine the early stages of autophagy induction and autophagosome formation. Since the stable cell line HEK293-GFP-LC3 was treated with the lysosome inhibitor leupeptin, this screen is not assaying for genes involved in the autolysosmal degradation step of autophagy. This paper claims identification of two novel autophagy proteins, SCOC and WAC, using genome-wide siRNA screen in the HEK293 cell line stably expressing GFP-LC3. The authors have proposed that SCOC is involved in autophagosome formation through an interaction with FEZ1, an ULK1-binding negative regulator of autophagy, and that WAC is a positive regulator of autophagy while negatively regulating the ubiquitin proteasome system. Despite the solid bioinformatical analyses to identify SCOC and WAC as regulators of autophagy from 21,141 siRNA pools, more in-depth biochemical and cell biological analyses will have to be provided to strengthen the hypothesis, including the following recommendations:

We have substantially improved the data to strengthen our hypothesis, in particular concerning the role of SCOC. We provide additional data using a variety of autophagy inducers to support the role of SCOC and WAC in autophagy (revised Fig, 2C-E). We now provide data which demonstrates SCOC is required for autophagosome formation, LC3 lipidation and p62 degradation (revised Fig. 4). In addition, we show that SCOC and FEZ1 are in a complex with ULK1, and SCOC is required for the stabilization of this complex (revised Fig. 6). Furthermore, we find that SCOC and FEZ1 are in a complex with UVARG, and that this complex is sensitive to starvation (new Fig. 7). This data supports the identification of SCOC as a novel regulator of autophagy, and suggests it may link the ULK1/2 complex and Beclin 1 complex to regulate autophagosome formation.

1. Fig.1A: It is difficult to distinguish the difference in GFP-LC3 localization in the normal vs serum starved medium, DAPI co-staining and a color picture of GFP-LC3 would help to show GFP-LC3 cytoplasmic localization.

In Fig. 1A we have added a panel below the low magnification images to illustrate the parameters that the cellomics measures, the definition of the nuclei delineated by the Hoechst labelling, the outline of the cell, and magnified images of the GFP-LC3 puncta formed in full medium versus EBSS plus leupeptin.

2. Fig.1D: Based on the fact that Rubicon and VPS16 play opposing roles in negatively and positively regulating autophagosome maturation, respectively, it doesn't seem

appropriate that VPS16 is included in the group with high SCPO A-scores.

We have removed VPS16 and used another protein, TBC1D25 (OATL1) recently established to be involved in autophagosome maturation, to illustrate what a high SCPO candidate may be.

3. Fig.2C: Based on the data from figure 2A, one would expect that siRNA against SCOC or WAC would result in a decreased level of autophagy. LC3 processing by Western blot does not support this conclusion.
(1) The LC3-II protein level in HeLa cells has been reported to increase upon depletion of autophagy proteins because autophagic flux is blocked (Noboru's MBC paper in 2008). For better explanations, p62 level needs to be included.
(2) Plot LC3-II/actin instead of LC3-II/LC3-I, which is more widely accepted in the field and also for the consistency throughout the manuscript.
(3) The increasing effect of leupeptin seems minimal. Please provide improved blots using other inhibitors such as bafilomycin A1 or chloroquine.

We have moved the HeLa data to the Supplementary figures. As acknowledged above HeLa cells are unique in their response to autophagy. For the HeLa cells we find that as reported by others they have a high level of basal autophagy and the alteration in LC3II levels is often not as informative as the ratio between LC3I/LC3II. Our original data does support the published data from Mizushima, and show that HeLa cells have altered formation as well as flux after depletion of SCOC and WAC.
The HeLa cell data has been replaced by a secondary screen we performed using different induction conditions and treatments to provide further support for the identification of WAC and SCOC (Fig. 2C-E). We have performed p62 degradation assays as requested that support the role of SCOC in formation.
We used leupeptin to validate and extend our screen findings because this lysosomal protease inhibitor was used in the siGenome screen. Our lab has found leupeptin treatment gives reproducible inhibition of flux and for our siGenome screen we chose to use leupeptin to minimize possible side effects caused by disruption of the proton ATPase by bafilomycin A treatment. We have performed experiments using BafA to investigate p62 degradation (see new Fig. 4B and 5A and B).

4. Fig.3B and 3C: Please include p62 in addition to LC3 blot.

We have included p62 degradation after siRNA depletion of SCOC (see Fig. 4A). Regarding overexpression of SCOC we have performed these experiments with FEZ1, which interacts with SCOC, as FEZ1 has a potent effect on the trimeric complex we observe between SCOC-FEZ1-ULK1 complex, and the SCOC-FEZ1-UVRAG complex and directly interacts with ULK1 and UVRAG (see below).

5. Fig.3D: Endogenous colocalization of SCOC, which is known to be primarily in golgi and alternatively at plasma membrane, seems incorrect (staining mainly appears in the nucleus and cytoplasm, unlike Atg9 colocalization pattern observed in Figure 3D). It is difficult to accept that the available antibody for SCOC can detect endogenous protein by immunofluorescence but not by western blotting. Please demonstrate colocalization of endogenous SCOC with golgi organelle markers. Alternatively, specific signal for SCOC can be shown in the overexpression system with appropriate controls (ex. difference between SCOC-mCherry vs. mCherry empty vector; observed colocalization of mCherry-SCOC and the endogenous SCOC antibody). In this image it is difficult to conclude whether GFP-LC3 colocalizes with SCOC. Colocalization of GFP-LC3 and SCOC may improve following treatment of cells with chloroquine to induce accumulation of autophagosomes.

We have revised this figure: see new Figure 3. The original data presented by Van

Valkenburgh et al., 2001 demonstrated that SCOC co-localized with ARL1 and was found in the Golgi and plasma membrane. We have repeated the experiments now using the fixation protocol detailed in the original paper, and we could reproduce the Golgi staining using TGN46, but could not detect staining on the plasma membrane. Using this fixation protocol we also confirmed that SCOC colocalizes with the juxta nuclear pool of Atg9 we originally described (Young et al., 2006). We have also repeated the LC3 and SCOC colocalization with both GFP-LC3 and endogenous LC3 which more clearly demonstrates the colocalization of SCOC with LC3 after starvation.
The antibody we obtained from R. Kahn's lab was the original one described in Van Valkenburgh et al., which they report only works by immunofluorescence (personal communication from R. Kahn) and we confirmed it does not recognize the endogenous protein by western blot, though it does recognize overexpressed SCOC-myc (see Fig. 5C, left-hand panel). We hypothesize that either the epitope recognized by the antibody has a low affinity towards SDS denatured protein (a common problem when soluble recombinant protein is used for immunization of rabbits) and can recognize the overespressed protein simply because of the large amount expressed, or the endogenous SCOC protein may be present in multiple isoforms all of which are expressed at too low a level to detect as individual bands in westerns but co-localize within cells to the same structure and give a detectable signal in immunofluorescence.

6. Fig.4A and 4B: Please include p62 in addition to LC3 blot.

We have performed these experiments and they are included in Fig. 5A and B.

7. Fig.4C: please clarify whether the WB for SCOC levels was determined using an antimyc antibody or to endogenous SCOC. Text in the manuscript mentioned that there is not an antibody available to endogenous SCOC, yet the figure 4C WB is labeled as SCOC. Is there a reason for using different tags for the reciprocal coIPs for FEZ1 and SCOC? Why not IP for GFP-FEZ1 using GFP antibody and WB for FLAG-SCOC and perform the reciprocal IP for FLAG antibody using FLAG-SCOC and WB for GFP-FEZ1. For the figure legend in 4C, the interaction of SCOC and FEZ1 may not be considered an "in vivo" interaction since the IP data presented is for overexpression of tagged protein instead of IP for endogenous protein.

We apologize for the confusion. In Fig. 5C (old Fig. 4B) the blot was performed with the anti-SCOC antibody which recognizes the overexpressed protein.
There was no particular reason behind the use of the different tags for the IPs. The experiments were performed in our two labs (Tooze and Johansen). We show in new Fig. 7 the reciprocal IP using FLAG-SCOC and WB for GFP-FEZ1. All of the data from our two labs show the position of the tag, or the size of the tag has no effect on the interactions we describe.
Regarding figure legend Fig. 4C (now Fig. 5C) we have now removed "in vivo" and it is clear the proteins are tagged and overexpressed.

8. Fig.4D: Please include negative controls, which do not bind to SCOC, to demonstrate specificity of the SCOC-FEZ1 interaction (ie. 35S-radiolabeled myc vector as a nonspecific radiolabeled control). It is difficult to see SCOC in the input lane. It might be useful to use an arrow to point out the band for GST-SCOC.

We have included in new Fig. 5D the myc-tag alone control. The input lane refers to the 35S-labelled reaction mix that is programmed to express Myc or Myc-FEZ1, and we do not expect to find SCOC here. We have added an asterisk to indicate the GST-SCOC.

9. Fig.5A: It seems to be mislabeled that "Flag-tagged SCOC was not detectable on western blots of lysates analysed by BN-PAGE when transfected alone, unless FEZ1-GFP was co-transfected", as Flag-SCOC protein expression was equivalent regardless of

the presence or absence of FEZ1-GFP while complex formation was affected (the lane4 and 5 in the middle panel). Based on the lane 4 and 5 in the first panel, it looks more likely that "FEZ1-GFP is not detectable when transfected alone, unless Flag-SCOC is cotransfected".

The description of the figure is complex and we apologize if it was not clear. What we have said is correct, that on the BN-PAGE gels FLAG-SCOC is not detected (middle top gel, probed with anti-flag, lane 5) unless FEZ1 is co-transfected (compare middle top gel, probed with anti-flag, lanes 2-4). The expression of FLAG-SCOC as detected on normal SDS-PAGE is equivalent regardless of FEZ1 expression (see middle bottom gel, lanes 2-5). FEZ1 is always detectable in BN-PAGE, but its molecular weight changes upon expression of SCOC. We have rewritten the text to clarify this further and indicated FEZ1 in BN-PAGE in Fig. 6.

10. Fig.6: The fact that WAC knockdown gave rise to accumulation of p62 and decrease of LC3-II protein level (in figure 6B and 6C) and decrease of HttQ25-GFP and the soluble fraction of HttQ103-CFP levels (in figure 6D and 6E) suggests the potential role of WAC in positive and negative regulation of autophagy and ubiquitin proteasome system (UPS), respectively. However, this does not necessarily imply that WAC may be involved in autophagy of ubiquitinated proteins by negatively regulating UPS. The degradation of UbG76V-YFP (in figure 6D) in full medium in WAC knockdown only supports the data from figure 6E, but does not address whether WAC has a direct effect on autophagy. A GFP-LC3 degradation assay may be a better experiment to address whether WAC plays a role in autophagy of ubiquitinated proteins by negatively regulating UPS.

We planned our experiments using the GFP-LC3 processing assay as suggested by the referee, and first carried out a control experiment in HEK293-GFP-LC3 cells using amino acid starvation without or with 100 mM bafilomycin A1, or 4 hr Torin treatment. As can be seen in the Figure 1R (for referee only) we observed a normal flux of p62 but could not robustly detect processing of GFP-LC3 as a GFP band in ES or accumulation in the presence of BafA1, only after incubation with Torin1. Our attention was also brought to the paper by Ni et al., ("Dissecting the dynamic turnover of GFP-LC3 in the autolysosome, Autophagy 7:188-204, 2011) which explains our observations that during amino acid starvation the GFP-fragments are degraded in the absence of protease inhibitors, and 100mM BafA1 prevents any cleavage of GFP-LC3. We therefore were unable to use this assay. We agree it remains to be formally proven that WAC plays a role in autophagy of ubiquitinated proteins and this will be explored in future studies.

11. On page 6 and 7 of the manuscript please refer to the figure, otherwise state "(data not shown)":
-Knockdown of RBM12 appeared to block maturation of autophagosomes, as the amount of LC3 lipidation in starvation medium without leupeptin was the same compared to starvation medium with leupeptin (include reference to Figure X or data not shown).

To simplify the data we have for the 20 validated hits we prepared a new Table shown in in Fig. 2B which summarizes our results. We have removed the text about these candidates to reduce the discussion in the results about the preliminary screen data (see comment from Ref. No. 2), including RBM12, with which we did not see a correlation with the screen results. We have retained the qRTPCR results in Supplementary Figures 1 and 2, and the LC3 lipidation assays for the relevant validated hits (4 increasers and 5 decreasers) in Supplementary Figure 3.

12. On page 10 of the manuscript please explain why you didn't see accumulation of UbG76V-YFP in WAC siRNA treated cells during starvation induced autophagy conditions. The manuscript only mentioned the decreased levels of UbG76V-YFP in WAC depleted cells in full medium.

The literature would support the notion that UbG76V-YFP is a proteasomal substrate (Dantuma et al., 2000), and our data supports the idea that in full medium the proteasome would normally target UbG76V-YFP unless autophagy was impaired (Korolchuk et al., 2009). We show UbG76V-YFP degradation is increased in full medium after siRNA depletion of WAC which supports our hypothesis. We in fact found a decrease of the UbG76V-YFP in starvation which was comparable to the FM for all three conditions (siRNA Atg16, RISCfree, and siRNA WAC) which suggests that starvation per se does not affect the turnover of this substrate. We have added a sentence to the text.

Referee #2

In their manuscript entitled "Genome-wide siRNA screen reveals amino-acid starvationinduced autophagy requires SCOC and WAC", the authors performed a genome-wide siRNA screen and identified 20 "strong" candidates as autophagy regulators. Among these candidates, they further characterized two proteins SCOC and WAC. While identification of new autophagy regulators is interesting and should provide new insights into autophagy regulation, subsequent analyses of the two factors are preliminary, and somewhat confirmatory of previous findings.

As detailed in this response, we have extended our findings beyond the reported known interactions in particular for SCOC to reveal new data about SCOC and its potential function as a scaffolding-adaptor for both the ULK1/2 complex and the Beclin 1 complex. We believe that the new data we provide extends the existing data and more importantly identifies a starvation dependent role for one SCOC complex. In summary, the data we provide allows us to confirm the identification of SCOC and WAC as two new regulatory proteins of starvation-induced autophagy.

Major comments:

The role of SCOC remains largely unclear. Although SCOC interacts with FEZ1, these two proteins seem to have opposite functions; is this interaction indeed important? This question can be tested by overexpression of the SCOC1 L254P/L260P mutant, or more appropriately, by expressing the mutant in SCOC1 knockdown cells.

We have provided new data in Figs. 4, 6 and 7 which we believe allow us to hypothesize that the function of SCOC is to regulate the activity of the ULK1/2 complex and the Beclin 1 complex. In particular, we have shown that SCOC interacts with both complexes, and its interaction with the Beclin 1 complex member UVRAG is sensitive to starvation. The interaction of SCOC with FEZ1 is indeed important as the overexpression of FEZ1 inhibits this starvation response. Overexpression of FEZ1 also inhibits LC3 lipidation and p62 degradation, and we predict this is mediated in part by SCOC. We apologize if the text was not absolutely clear with regard to the binding mutant: the mutant L254P/L260P is a FEZ1 mutant not an SCOC mutant and so the suggested experiment is not feasible.

The authors hypothesize that ULK1 regulates formation of the SCOC and FEZ1 complex based on the overexpression of ULK1 KI (Fig. 5A). However, this conclusion is based solely on findings from the overexpression approach, and there is no evidence to show whether or not it is regulated by endogenous ULK1. Using siRNA to knockdown ULK1 is required to further validate their findings.

We have performed siRNA knockdown followed by BN-PAGE analysis as suggested. We found when ULK1 was depleted that the ULK1-FEZ1 complex was lost, and therefore we asked if the FEZ1-GFP could be found in complex with endogenous ULK1 and if this was sensitive to depletion of SCOC (see new Fig. 6D). This demonstrates that

there is an endogenous complex formed with ULK1 and FEZ1 which requires SCOC and we believe that it is likely that ULK1 regulates this complex through phosphorylation of FEZ1 (see next comment). We have also provided data in which we have demonstrated that SCOC can complete with ULK1 for binding to FEZ1.

Do SCOC and FEZ1 function upstream or downstream of ULK1? Are they ULK1 substrates? Do the interactions between these proteins change under starvation conditions? Does SCOC regulate the FEZ1-ULK1 interaction? The authors provide a lot of attractive speculations in the Discussion, most of which can be experimentally addressed.

Based on our data we hypothesize that SCOC and FEZ1 may function downstream of ULK1. We have investigated if FEZ1 and SCOC are substrates of ULK1 using in vitro kinase assays. As shown in Fig. 2R (for referee only) we have evidence that FEZ1 is a substrate of ULK1, and this supports the data shown in Fig. 6C. In similar experiments using immunoprecipitated FLAG-SCOC we could not detect any ULK1-dependent phosphorylation of SCOC. These results are preliminary as the validation of a true substrate requires a substantial body of evidence, which we feel is beyond the scope of this manuscript. We have in addition examined if the interaction between FEZ1 and ULK1 is starvation sensitive and have seen no alteration during starvation (Fig. 6B) as is the case for the interaction between FEZ1 and SCOC (Fig. 7C). However, there is a starvation-sensitive interaction between SCOC and UVRAG (see Fig. 7C), which is regulated by FEZ1. In summary this new data provides a substantial amount of new information towards understanding these novel complexes and interactions.

The studies on WAC is also preliminary; although it is interesting that WAC also affects proteasomal activity, its mechanism was not investigated in the present study.

We have focused on SCOC as the main candidate for this manuscript. We agree WAC requires further investigation however we would like to point out a recent paper which shows WAC interacts with VCIP135, a deubiquitinase (Totsukawa et al., EMBOJ 2011). A second study demonstrates that WAC regulates the activity of RNF20/40, a protein that mediates Histone H2B ubiquitination (Zhang et al., 2011). These recent publications, along with the reported interaction with UBQLN4 support our preliminary data that WAC may be involved in the UPS and highlight the complexity of the ubiquitination pathways in which WAC may be involved.

The effects of knockdown of SCOC and WAC on LC3-II level are subtle (Fig. 3B and C, Fig. 6B). Additionally, LC3-I is rather increased in siFEZ1 cells, which may indicate reduced autophagic flux. What is the p62 expression level under non-starvation conditions in these knockdown cells? The authors show its level in starved WAC cells (Fig. 6C), but it should also accumulate in non-starved cells.

We agree that the effects may appear subtle but they are statistically robust and similar to our positive control. We have added an autophagic flux experiment with a measure of STIPO (spot total intensity per object) after siRNA depletion of SCOC and WAC in Fig. 2C using FM with leupeptin, ES and ES with leupeptin. Our analysis of greater than 1200 cells shows that SCOC and WAC are required for autophagic flux. The increase in LC3-I which we observed in the original Fig. 4C was not always observed and we have replaced the gel with a more representative gel (see Fig. 5A). We have included experiments with siRNA depletion of SCOC and FEZ1 and quantified p62 degradation to further support our LC3 data (see Figs. 4B and 5A and B). In addition, we examined p62 levels in WAC depleted cells and found they increased about 1.5 fold compared to RISCfree. This is now included in the manuscript.

Minor comments:

The four SCOC isoforms are not described in Figure 3A.

We have included a description in the text, page 8 and in the figure legend.

The images in Figure 3D need to be replaced with higher magnification images. The authors previously showed that LC3 and Atg9 localize to numerous punctate structures in the cytoplasm under these conditions, but their peripheral structures are not visible in these images.

We have revised the immunofluorescence data in a new Fig. 3 using the fixation protocol described by Van Valkenburgh et al., 2001. We have previously demonstrated and quantified the colocalization of Atg9 and LC3 in peripheral structures (Young et al., 2006), and now show that SCOC colocalizes with GFP-LC3 and endogenous LC3. Our recent data on Atg9 (manuscript under revision at Mol Biol Cell) suggest Atg9 is very transiently associating with LC3 positive structures. While we have detected a low but reproducible number of SCOC and Atg9-positive peripheral structures we would like to understand more about what these structures are relative to other Atg proteins before we make any conclusions about SCOC and Atg9 during starvation. These studies are challenging because of the fact SCOC is a polyclonal antibody and it works best after saponin permeabilization and quantification with multiple markers is required.

The high ULK1 expression level in SCOC-knockdown cells is intriguing (Fig. 3B), and should therefore be mentioned and discussed.

We have not explored this further as we are not convinced this is a robust change, and if it is affected by starvation. A complete understanding of this effect we feel is beyond the scope of this study.

Referee #3

In search of novel proteins regulating starvation-induced autophagy, McKnight and coworkers performed a genome-wide siRNA screen, employing HEK cells stably expressing GFP-LC3. The readout for autophagy induction/reduction was the change in GFP-positive spots compared to control cells upon starvation treatment. Utilizing several steps of filtering, the authors eliminated most genes by setting statistical and technical thresholds, coming down to nine hits. While seven of these hits have potentially autophagy-related relevance, the authors focus was set on two proteins - SCOC and WAC. SCOC, a Golgi protein was required for autophagosome formation and interacted with FEZ1, an ULK1-binding negative regulator of autophagy. WAC, a relatively uncharacterized protein, was required for starvation-induced autophagy but also acted as a potential negative regulator of the ubiquitin proteasome system.
The main goal of this work was to find novel players in the autophagic pathway, and specifically those controlling starvation-induced autophagy in mammalian cells. This is an important goal as it clear that additional yet unidentified autophagic factors are involved in the autophagic process. The authors applied stringent parameters to eliminate false positive results and ended up with nine hits, seven of which were neither characterized nor proven to participate in autophagy. The two remaining hits, SCOC and WAC, were further characterized. While attempts were done to prove their relevance in autophagy, and to investigate possible ways in which they control this pathway, the data in the manuscript remain too preliminary. Going down the long way from the screen to the autophagy-related hits, the authors should go the extra mile and bring stronger evidence for their hypotheses.

We have provided additional evidence in support of the role of SCOC in the regulation of autophagy through the ULK1/2 complex and Beclin 1 complex (see comments above).

In general, the data lacks appropriate controls. LC3 lipidation, the main readout in this manuscript for autophagy activation, should be analyzed more carefully, i.e. by assessing autophagic flux. This should be demonstrated by fluorescence imaging in addition to WB analysis. In addition, the authors should take advantage of p62 (or any other autophagic receptor) as an autophagic marker throughout their work, as they did in the last part of this manuscript.

We have validated the role of SCOC and FEZ1 in autophagy by examining levels of p62 in FM, EBSS and in EBSS with the addition of BafilomycinA. These results are statistically significant and support our original LC3 flux data which was also statistically significant. We chose to use leupeptin to analyse autophagic flux as it was the drug we used in the original siGenome screen (see Referee No. 1, point 3) and we agree the effects are milder but still statistically significant. In addition, we have included an autophagic flux experiment with a measure of STIPO (spot total intensity per object) after siRNA depletion of SCOC and WAC in Fig. 2C using FM with the addition of leupeptin, ES and ES with leupeptin. Our spot count and analysis of greater than 1200 cells shows that SCOC and WAC are required for flux.

The authors tends to be over-conclusive on the one hand and to discard potentially interesting data on the other hand. For instance, the authors conclude that five proteins are required for starvation-induced autophagy based solely on the effect their knockdown had on LC3 lipidation. Conversely, referring to screen hits, the authors argue that knockdown of RBM12 appeared to block maturation of autophagosomes and therefore they seized to investigate this gene. SUPT5H, another candidate, was dismissed simply because it is a known subunit of the eukaryotic transcription elongation factor DSIF. The screen and its preliminary results should take a considerably smaller portion of the manuscript, and the authors should focus on those hits that were further investigated. The other semi-validated hits leave much room for debate. These could be mentioned, briefly, in the discussion part.

We agree we have not fully considered all the potential roles of the other validated hits, including SUPT5H. As we reasoned in the Results, given the limitation of time and resources we chose to investigate the candidates for which the evidence in the literature supported an obvious link to autophagy, and thus investigated SCOC and WAC. We have decreased the space devoted to the screen and its preliminary results by summarizing the data we obtained in a table in Fig. 2B. However, given the restriction in character count we do not have enough space to include a discussion of the validated but not furtherinvestigated candidates. To provide more support and information about SCOC, SUPT5H and WAC and their role in autophagy we have performed an induction screen using different conditions in a new Fig. 2C-E.

While a big effort was evidently put into this work, the main findings are yet to be better established.

We have provided additional evidence to support our major findings in revised Figs. 4, 5, 6 and new Fig. 7.

Specific comments

The experimental procedure of the siRNA screen is not clear. Were the three parameters measured for each gene both with full medium and starvation medium+leupeptin, or just the latter?

We have clarified this in the text and added the conditions to the flow chart in Fig. 1C.

NRBP2 is used as an autophagy-suppressing control in this work, based on a previous work by the authors. Valid as it may be, it should at least be reinforced with a more common control.

We used NRBP2 to control for the range of response we could obtain after the siRNA knockdown as our best autophagy-suppressing control. At the time the siGenome screen was performed (4 years ago) there was nothing in the literature to use as a more common control fitting the criteria which NRBP2 fulfilled (significant induction of LC3II lipidation, robust depletion of the mRNA using a single duplex with no off target effects on the levels of any other Atg proteins). We feel our previous results (Chan et al, JBC, 2007) and those in the screen (Fig 2B) validate the use of this control.

Figure 2B: The relevance of this figure to the manuscript is not clear and does not lead to any new insights.

We have removed this figure from the main manuscript. It is, however, still included in Supplemental Table S4 as it provides an overview how the 51 hits map onto the cellular component and protein class classifications.

Figures 2C, 2D and 6D: LC3II levels are relatively high even in cells grown in full medium, which may indicate cells are in stress to begin with. This makes further conclusions hard to deduce.

We have replaced Fig. 2C and 2D which are HeLa cells with data obtained on HEK293 cells which in general have lower levels of LC3-II in FM. In Fig. 8D (old Fig. 6D) the cells we used (MelJoSo which are derived form a malignant melanoma) have very high levels of LC3-II even when untreated. This is a recognized characteristic of many tumor cell lines. Unfortunately for the experiment shown in Fig. 8D there was no suitable replacement cell line.

The authors provide statistical information on the comparison between LC3II levels in accumulated in EL. It is more important to compare the autophagic flux between the two samples. For example, knockdown of SCOC (Figure 3B) or its overexpression (Figure 3C) may have little or no effect on the autophagic flux.

We have included an autophagic flux experiment with a measure of GFP-LC3 STIPO (spot total intensity per object) after siRNA depletion of SCOC in Fig. 2C using FM with the addition of leupeptin, ES and ES with leupeptin. Our analysis of greater than 1200 cells shows that SCOC is required for flux.

Figure 3B, 3C: The cells used in this experiment show a very low autophagic flux in response to starvation. Furthermore, knockdown of SCOC increased the flux, in contrast to its proposed role.

In the original Figure 3B and C we agree that the effects may appear subtle but they are statistically robust and similar to our positive control. We used leupeptin to validate and extend our screen findings because this lysosomal protease inhibitor was used in the siGenome screen. Our lab has found leupeptin treatment does give reproducible inhibition of flux and for our siGenome screen we chose to use leupeptin to minimize possible side effects caused by disruption of the proton ATPase by bafilomycin A treatment. We disagree that siRNA of SCOC increased flux. We have the data in Fig. 2C and have performed experiments using BafA to investigate p62 degradation (see new Fig. 4B and 5A and B), all of which support the data that SCOC inhibits flux.

Figure 3D: LC3 phenotype hardly resembles that of starved cells, indicating a possible error in the experiment. In addition, the localization between SCOC and LC3 and Atg9 is

not convincing. A control experiment that includes lysosomal inhibitor is needed. Moreover, here to the authors should look for autophagic flux.

We apologize if the original image in Figure 3D was not clear enough after the creation of the pdf. There were detectable GFP-LC3 puncta in the original image. We have replaced the original image with one taken with a better signal to noise ratio. We have also replaced the colocalization images between Atg9 and SCOC and included a Golgi marker, TGN46 to allow visualization of the Golgi population. In addition, we now include colocalization of both GFP-LC3 and endogenous LC3 in starved cells in which the vesicles are clearly detectable. We have included an autophagic flux experiment with a measure of STIPO (spot total intensity per object) after siRNA depletion of SCOC in Fig. 2C using FM with the addition of leupeptin, ES and ES with leupeptin. Our analysis of greater than 1200 cells shows that SCOC is required for flux.

Figure 4A. The level of LC3II in the control is very high. This exemplifies the need for additional experiments using other autophagy related markers (p62, nbr1 etc.) as well as fluorescence microscopy analysis. As mentioned above such analysis should be performed throughout this study.

We have replaced the blot in the original Fig. 4A (now 5A) with a more representative blot. We have used p62 degradation to further study the role of FEZ1. Our new additional data supports the notion that for FEZ1 maybe a negative regulator of autophagy.

Figure 6A: The polyclonal antibody for WAC presumably recognizes two endogenous isoforms. This claim should be favored over the possibility that the smaller fragment is a degradation or cleavage product of the full length protein.

We agree and have amended the text.

Figure 6D: based on LC3 and p62 levels, it seems that the starvation medium had no effect on the cells. The authors should relate to the inconsistency with the data presented in Figure 6C in which they observe accumulation of p62 upon WAC kD.

We believe the inconsistency relates to the high level of basal autophagy these cells have, however we were restricted to using this cell model for these experiments. There are however detected changes in LC3I and II levels after siRNA KD of Atg16 and WAC compared to RISCfree.

Figure S4b: The interaction between WAC and Beclin is only demonstrated when both proteins were overexpressed. The authors should test the interaction of the endogenous proteins or explain why this is not possible.

The purpose of the experiments were performed to reproduce original findings of Behrends et al. in particular as the interaction between Beclin 1 and WAC was not classified as a HCIP (high confidence interaction). While it would be very interesting to explore the endogenous interaction, we feel it is beyond the scope of this manuscript and will be the subject of future experiments.

The authors should explain the inconsistency with regard to the way they analyze the WB results, i.e. LC3II/LC3I, LC3II/actin etc.

We have analysed all the data with the HEK293 cells, and the stable Ub- expressing cells using LC3II/actin. For the HeLa cells we find that as reported by others they have a high level of basal autophagy and the alteration in LC3II levels is often not as informative as the ratio between LC3I/LC3II. The HeLa cell data is now in the Supplementary Fig. S3.

Data for Referees only McKnight et al.

Figure 1R. GFP-LC3 processing assay is not a robust measure of starvation-induced autophagy

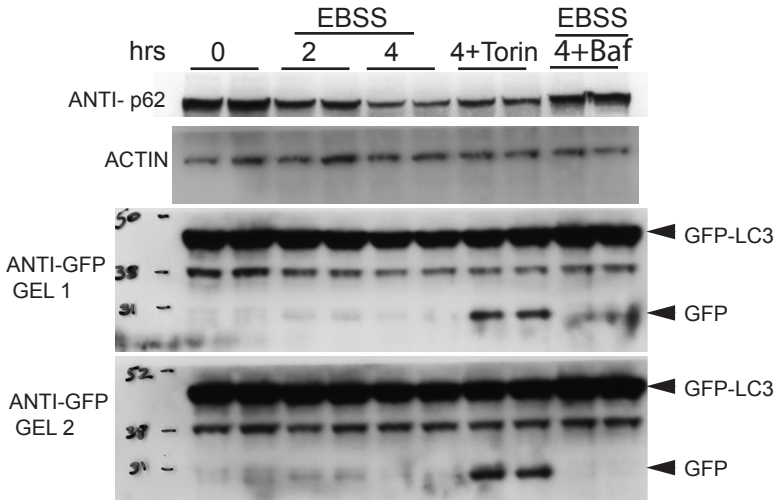


Figure 2R. UbG67V-YFP turnover in EBSS is affected in a similar way in FM.

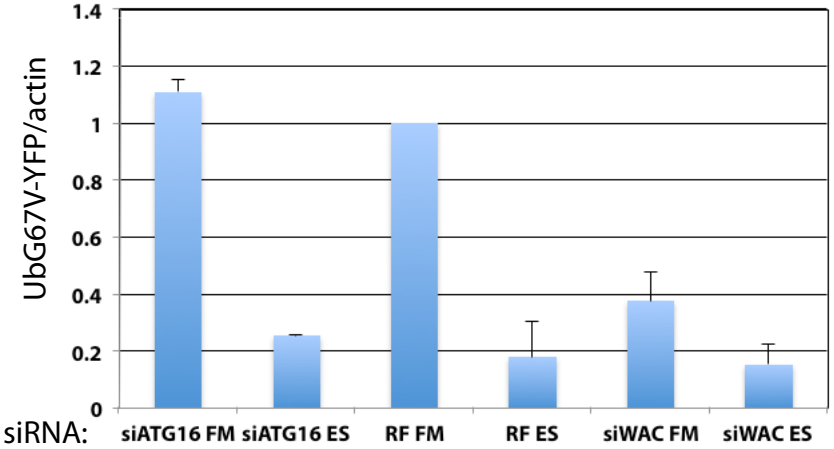
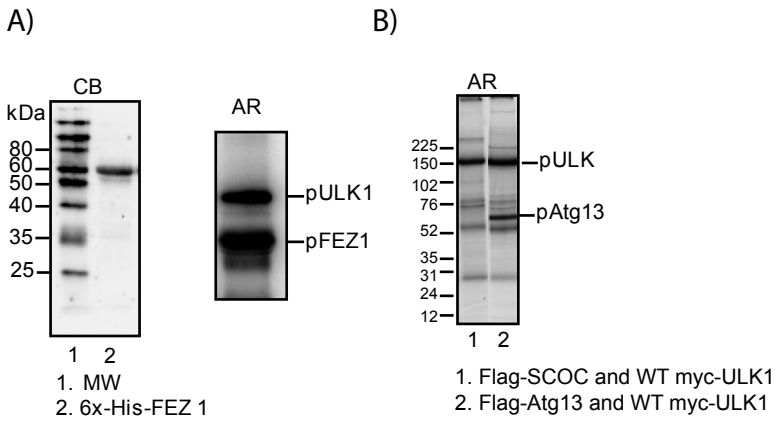


Figure 3R. Fez1 but not SCOC is an in vitro substrate of ULK1



1. MW
2. 6x-His-FEZ 1

1. Flag-SCOC and WT myc-ULK1
2. Flag-Atg13 and WT myc-ULK1

Supplementary figures for referees:
Figure 1R.
GFP-LC3 processing assay. We planned on using the GFP-LC3 processing assay as suggested by the referee, and first carried out a control experiment in HEK293-GFPLC3 cells using amino acid starvation without or with 100 mM bafilomycin A1, or 4 hr Torin treatment. As can be seen in the Figure 1R for referee we observed a normal flux of p62 but could not robustly detect processing of GFP-LC3 or accumulation in the presence of BafA1. Our attention was then brought to the paper by Ni et al., ("Dissecting the dynamic turnover of GFP-LC3 in the autolysosome, Autophagy 7:188-204, 2011) which explain our observations that amino acid starvation the GFPfragments are degraded in the absence of protease inhibitors, and 100mM BafA1 prevents any cleavage of GFP-LC3. We therefore were unable to use this assay to explore flux.

Figure 2R.
UbG67V-YFP turnover in EBSS is affected in a similar way in FM. Please see experiment in Figure 8D.

Figure 3R.
FEZ1 but not SCOC is an in vitro substrate of ULK1.
A) Purified recombinant 6x-his-FEZ1 was incubated with purified GFP-ULK1 in the presence of ATP and 32PγATP at 30°C for 30 min. Autoradiography of the reaction following SDS-PAGE revealed phosphorylation of both ULK1 and FEZ1 in the reaction.
B) Myc-ULK1 was immunoprecipitated from lysates and mixed with flag-SCOC (lane1) or flag-Atg13 (lane2) immunoprecipitated from cell lysates in an in vitro reaction containing ATP and 32PγATP. Both ULK1 and Atg13 are phosphorylated whereas SCOC is not.

2nd Editorial Decision 30 November 2011

Thank you for sending us your revised manuscript. In the meantime, our original referees 2 and 3 have seen it again. Referee 1 was not available to look at the revision. As you will see, referee 2 feels strongly that substantial additional experimentation will be required before he/she can support publication of the manuscript here. Referee 3 is more positive. In addition, I have now consulted with referee 3 on the points put forward by referee 2. He/she suggests concentrating on addressing points 3-6 as far as possible, and I would like to ask you to follow this suggestion. Also, including a scheme would be very useful to the reader. Points 1 and 2 may go beyond the scope of this study, and we will not require further experiments in this direction. Please feel free to contact me at any time if you would like to discuss any aspect of this additional revision further.

I look forward to your re-revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2

The authors have added a substantial amount of new data, but the overall picture of these factors remains vague. These data are fragmented and not convincing. Both SCOC and WAC seem to play several (more than one) complicated roles in the autophagy pathway. Although the authors have done a great job to identify these new autophagy factors, their functions and significance are not clearly demonstrated in the present paper.

1. It is still unclear whether the interaction between SCOC and FEZ1 is essential for autophagosome formation. The effect of overexpression of wild-type FEZ1 is not necessarily through SCOC. I apologize for the confusion in the previous comments. What I wanted to suggest is to test whether autophagy is indeed suppressed when the SCOC-FEZ1 interaction is broken. To this end, it would be important to test whether either expression of siRNA-resistant wild-type FEZ1, but not FEZ1(L254P/L260P), can rescue the autophagy-defective phenotype of FEZ1 knockdown cells, or overexpression of the FEZ1(L254P/L260P) mutant breaks the SCOC-FEZ1 interaction and shows a dominant-negative effect on autophagy.

2. Although the authors found that SCOC interacts with UVRAG, the role of this new finding is not well characterized. UVRAG is thought to function at a late step of autophagosome formation or autophagy maturation, rather than at an initial step where the ULK1 complex functions. Unfortunately, how SCOC integrates these two distinct steps is unclear. Does UVRAG function as an SCOC reservoir? Addition of the UVRAG part makes this paper more preliminary.

3. The paper still largely depends on overexpression experiments and the relationship between SCOC, FEZ1 and ULK1 is not yet clear. Does siRNA knockdown of ULK1 affect the FEZ1-SCOC interaction (this should be a simple immunoprecipitation experiment)? Does endogenous ULK1 interact with either endogenous FEZ1 or SCOC?

4. The authors provide new IF data in Figure 3C. Unfortunately, these new data rather suggest that SCOC and LC3 do NOT colocalize. The staining patterns of these two proteins are very different and I do not think that they biologically overlap. However, even if SCOC does not colocalize, it is still possible that SCOC colocalizes with ULK1 and/or UVRAG. The authors should reexamine the localization of SCOC along with its binding partners they found.

5. The authors should show the raw data of p62 in Fig. 4B, 5A and 5B. This is especially important in Fig. 5A, because the authors paradoxically observed accumulation of p62 in FEZ1 siRNA cells as well as FEZ1-overexpressed cells.

6. In Fig. 8C, it is essential to demonstrate the p62 level in non-starved WAC-siRNA cells to show whether the starvation-induced reduction of p62 is blocked or not.

7. In page 9, the authors state that the coiled-coil region is important for the interaction. Is the data shown?

8. As this paper is very complicated, a schematic model would be helpful.

Referee #3

In the revised manuscript the authors addressed all my comments, thus significantly improving the manuscript.

Response to Referee No. 2

3. The paper still largely depends on overexpression experiments and the relationship between SCOC, FEZ1 and ULK1 is not yet clear. **(A)** *Does siRNA knockdown of ULK1 affect the FEZ1-SCOC interaction (this should be a simple immunoprecipitation experiment)?* **(B)** *Does endogenous ULK1 interact with either endogenous FEZ1 or SCOC?*

A. We have done this with tagged-FEZ1 and tagged-SCOC and asked if the coimmunoprecipitation of Fez1 and SCOC is altered in the absence of ULK1. This data is added as a panel in Figure 6, panel E. The loss of ULK1 did not affect the interaction between overexpressed tagged-SCOC and FEZ1 although it does affect the stability of SCOC. This result is not entirely unexpected as we know that SCOC and FEZ1 bind directly to each other and we have no evidence that ULK1 levels affect this binding (which also doesn't change during starvation), only that overexpression of ULK1 alters the mobility of each protein.

B. Using HEK293 cells we have been able to immunoprecipitate endogenous ULK1 and were successfully able to detect endogenous FEZ1 in the immunoprecipitate by western blotting (see new panel B in Figure 6). We were unable to immunoprecipitate endogenous FEZ1 with the existing antibodies. We cannot do the same experiment for endogenous SCOC to see if it binds endogenous FEZ1 because the SCOC antibody does not work by western.

4. The authors provide new IF data in Figure 3C. Unfortunately, these new data rather suggest that SCOC and LC3 do NOT colocalize. The staining patterns of these two proteins are very different and I do not think that they biologically overlap. However, even if SCOC does not colocalize, it is still possible that SCOC colocalizes with ULK1 and/or UVRAG. The authors should reexamine the localization of SCOC along with its binding partners they found.

We may have overstated our conclusion about the colocalization of SCOC and LC3, but we do feel our data supports the conclusion that a population of SCOC colocalizes with LC3. We did not mean to imply there was a complete colocalization but only that there was a partial colocalization of a population of SCOC with a population of LC3. Our data does support this and importantly, we find it to be the case with endogenous SCOC and endogenous LC3. In fact, partial colocalization is expected as we have no evidence to suggest SCOC is found on autophagosomes but our data demonstrates it is required for autophagosome formation. We have revised the text to reflect this qualified conclusion that a population of SCOC colocalizes with a population of LC3.

We have addressed the colocalization of endogenous binding partners. The SCOC antibody is rabbit, so we were limited to colocalization experiments with monoclonal antibodies. We purchased new commercial monoclonal antibodies to UVRAG and FEZ1, but unfortunately the monoclonal to UVRAG did not work in our hands. We could use the monoclonal FEZ1 with polyclonal SCOC and polyclonal UVRAG but we could not look at SCOC together with UVRAG. Therefore, we provide 1) new data about SCOC and FEZ1 showing they partially colocalize in new Figure panels 4E and 2) UVRAG partially colocalizes with FEZ1, in new Figure 7 panel F. This data confirms the interaction experiments using tagged proteins and substantially strengthens the paper. We thank the referee for this suggestion.

5. The authors should show the raw data of p62 in Fig. 4B, 5A and 5B. This is

especially important in Fig. 5A, because the authors paradoxically observed accumulation of p62 in FEZ1 siRNA cells as well as FEZ1-overexpressed cells.

We have included the data for Fig. 4A (siSCOC) and 5A (siFEZ1) as a Supplemental figure (Fig. S5) as the data was from three experiments all done together and the actin levels vary depending on the siRNA, which is why we quantified over actin. We have now included the western blot data for p62 after FEZ1 overexpression in Fig. 5B.

6. In Fig. 8C, it is essential to demonstrate the p62 level in non-starved WAC-siRNA cells to show whether the starvation-induced reduction of p62 is blocked or not.

We had previously added a sentence to the revised manuscript in response to this comment from previous revisions, but have now revised the figure (Fig. 8C) to include the western blots with the non-starved samples.

And finally, as Referee 3 and you suggested,
8. As this paper is very complicated, a schematic model would be helpful.

We have prepared a model, shown in Supplemental Figure S8. We are thankful for this suggestion as it was very helpful and makes the Discussion much easier to follow.

3rd Editorial Decision 19 January 2012

Thank you for sending us you re-revised manuscript and for all the effort you undertook with this manuscript. Referee 3 has now seen it again, and you will be pleased to learn that the manuscript will now be publishable in The EMBO Journal.

Prior to formal acceptance there are a number of editorial issues that should be looked into:

* I think that it would be better include the scheme (supplemental figure S8) into the main body of the manuscript as the last figure or panel.

* Could you clarify, please, for all cases where you indicate the number of independent experiments as n=2 (i.e. figure 8 and a number of the supplementary figures) whether these were performed in duplicates or triplicates. If the total number of data points included into the plots is below 2, it will be better to show one representative experiment without error bars and to indicate that in the figure legend.

I am looking forward to the final version of the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3

The authors has made significant progress addressing most of the referee's comments and the manuscript is now greatly improved.

3rd Revision - authors' response 23 January 2012

To address the points in your letter, we have made the final changes to the manuscript that are:

1. The model (formerly Fig. S8) is now Fig. 9. We have reduced the word count by judicious editing to accommodate this change and the additional text.

2. The have gone through the data and checked the n=2 experiments in Fig. 8 and S6. In Figure 8 we have removed the error bars from the Atg16 data set in panel D. In Fig. S6 we have shown a representative experiment and removed the error bars.

