EMBO reports

BioID reveals an ATG9A interaction with ATG13-ATG101 in the degradation of p62/SQSTM1 clusters

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DOI: 10.15252/embr.202051136

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Review Timeline:

Submission Date:	22nd Jun 20
Editorial Decision:	31st Jul 20
Revision Received:	27th Apr 21
Editorial Decision:	19th May 21
Revision Received:	28th May 21
Author Correspondence:	11th Jun 21
Revision Received:	16th Jun 21
Editorial Decision:	9th Jul 21
Revision Received:	14th Jul 21
Accepted:	16th Jul 21

Editor: Martina Rembold / Achim Breiling

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 31st Jul 2020

Dear Dr. Andersen

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

I am sorry to say that the evaluation of your manuscript is not a positive one. As you will see, while all three referees acknowledge that the findings are potentially interesting, they also point out that the data appears rather preliminary and that the functional significance of the observed ATG9A puncta remains elusive. Moreover, the referees also raise a number of technical concerns and point out missing control experiments that will need to be provided.

Due to the nature of the criticisms, the amount of work likely to be required to address them, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from the referees upon initial assessment, I am afraid that we do not feel it would be productive to call for a revised version of your manuscript at this stage.

Given the potential interest of your findings, we would, however, have no objections to consider a resubmission of the manuscript in the future if you were able to address all main concerns of the reviewers as highlighted above and in their reports. In particular, the functional significance of the ATG13-ATG101-ATG9A interaction to autophagy and the nature and function of the ATG9A puncta would need to be resolved and strengthened to consider the manuscript again for EMBO reports. I would like to stress though that such a manuscript would be treated as a new submission and would be evaluated again, also with respect to the literature and the novelty of your findings at the time of resubmission.

I apologize that I cannot be more positive at this point. I hope, however, that the referee comments are going to be helpful in strengthening your indeed very interesting initial observations and I will be happy to discuss any additional data on this topic with you in the future.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

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Referee #1:

The manuscript by Kannagara et al provides evidence that a subset of the ULK complex autophagic proteins alters the trafficking of ATG9A (an essential autophagy protein).
The evidence is primarily that in the absence of ATG13 and ATG101 (but not of FIPI200 or ULK1/2), ATG9A is partially localised in large punctate structures which also contain P62. The specificity of this with respect to ATG13 is shown by employing various ATG13 mutants which are known to lack interacting domains for ULK1 or ATG101, and the data are very clean.
An overall impression on the manuscript is that it does not get to the bottom of this story: what are these ATG9A puncta, and what is their functional significance? What other proteins do they

contain in addition to P62? Are they stalled autophagy intermediates or something else that appears under the extreme condition of ATG13 absence? Without such extension the manuscript is too preliminary despite the fact that a lot of work has gone into the characterization of the phenotype.
In addition to the above, I had some specific comments:
1. The Introduction and Discussion provide some confusing information on basal vs selective autophagy. There is basal autophagy revealed by bafilomycin treatment of cells that still depends on mTOR activity. There is basal autophagy against selective targets which most likely is locally triggered and does not require TOR inactivation. These two processes are not equivalent.
2. The gel filtration experiment used detergent lysates as input. My understanding is that in these conditions ATG9A will be in detergent micelles and it is difficult to see how one can make conclusions about its size/behavior in comparison to cytosolic proteins.
3. Why does HA-ATG9A appear to be degraded in the presence of bafilomycin in the ATG13 and ATG101 deleted cells (Fig 5B)?
4. The ATG9 antibody from cell signaling ((D4O9D) Rabbit mAb, 13509S) works well for blots in human cells (and for iffl) and should be used in the experiments deleting and reconstituting ATG9.
5. I guess that this is a function of experimental timing, but in retrospect it would have been better to do the BIO-ID in cells deleted for ATG9A and rescued with the BIRA construct.

Referee #2:

In the manuscript by Kannangara et al., the authors examine the role of ATG13 in regulating ATG9 trafficking under basal conditions. Using a BIO-ID approach, components of the ULK1 complex are identified as interactors with ATG9. Loss of complex member ATG13 results in a change in ATG9 localization to large punctate structures that co-localize with SQSTM1/p62. The authors go on to characterize this step in greater detail and find that the phenotype can be rescued by an ATG13-ATG101 complex that does not interact with ULK1 or FIP200, both of which are seen to be key components of the ULK1 complex.
This is an interesting paper that potentially adds to the details of ATG9 trafficking and on the whole, the experiments are well performed and clear. However, I do not exactly understand what the specific role of this ATG13-ATG9 interaction is with respect to autophagy and authors need to better demonstrate that this complex exists independently of ULK1 and FIP200 in cells.

Main Points:
1. The authors are implying that ATG13 is present in a separate ULK1/FIP200-independent complex in cells, but they have not really demonstrated this (gel filtration alone is not enough). ULK1 was identified in the BIO-ID experiments, suggesting it may be in the complex but simply not required for this particular ATG9 trafficking step. The authors should perform some triple labelling immunofluorescence and show the presence of ATG13 puncta that colocalize with either ULK1 or ATG9.
2. Related to the above, as ULK1 is a kinase it may not have to interact strongly with the ATG13-ATG9 complex to exert an effect. To help rule this out, can the authors show in their KO-rescue experiments that the ATG13 mutant, which does not bind to ULK1, is no longer phosphorylated by ULK1 at the "Ser318" residue?
3. The authors have not sufficiently demonstrated an effect on autophagy. Block of the ATG13-ATG9 interaction leads to accumulation of p62 structures. What are these - ubiquitin aggregates? Is LC3 on these structures? Are they even destined to be degraded by autophagy (p62 can be degraded by the proteasome)? This latter point arises from the bafilomycin experiments shown in

Fig. 5, as there is no flux of p62 in WT after 24h of treatment. This suggests there is no basal autophagy of p62 occurring - which also may explain why there is no accumulation of p62 in the FIP200 KOs. FIP200 KOs should also display no autophagy and there is plenty of evidence in the literature that shows loss of FIP200 leads to p62 accumulation. What about LC3, is this fluxing under these conditions (and if so, can it be rescued by the ATG13 mutants)?
4. It appears that CRISPR KOs were only confirmed by western blot. This is not sufficient as depending on recombination events, truncated proteins with some "activity" can still be expressed, which are no longer recognized by antibodies. This could confound interpretation of results. Thus, the authors should confirm their KOs by genomic sequencing.
5. I appreciate that the authors are focussed on "basal" conditions but does this interaction have any bearing on classical starvation-induced autophagy? Are the ATG9-p62 structures (in ATG13 KO) exacerbated under starvation conditions and can they still be rescued (as well as LC3 flux) by the ATG13 ULK-interacting mutant?

Minor Points:
6. Molecular weight markers should be included on the blots.
7. Authors may wish to confirm they are using the correct statistical analyses. A t test is appropriate when just comparing two values, but it is not when multiple values are being compared (controls have been compared to multiple other conditions in many experiments). One-way Anova and a post hoc test will be more suitable.
7. In Figure 2E, why is there no endogenous ATG13 staining in the parental input?
8. The authors should discuss work from Sharon Tooze showing that ULK1 is required for ATG9 trafficking to autophagosomes and highlight why the authors have different observations (PMID:16940348).

Referee #3:

Manuscript summary:
Autophagy is a stepwise process resulting in the degradation of various cargos and subsequent recycling of components. Cargo targeted for degradation is engulfed by autophagosomes, which in turn fuse with degradative-active lysosomes. Dysregulated autophagy results in the accumulation of misfolded proteins or damaged organelles, thus being involved in various diseases. Additional to starvation-induced autophagy, mediated by inhibited mTORC1 and active ULK1-signaling, basal autophagy is important for maintaining cellular homeostasis. ATG9A, located on small vesicles, is essential for membrane supply to the nascent phagophore and associates with different compartments of vesicular trafficking pathways. Recent studies suggest that autophagosomes still form in the absence of ATG9A, yet ATG9A might be crucial for proper cargo-adaptor turnover under basal conditions. However, mechanisms regulating basal autophagy, especially via ATG9A trafficking, are poorly understood.
In this report, Kannangara et al. describe novel ATG9A interactions and establish an ULK1-independent ATG13-ATG101 subcomplex mediating ATG9A trafficking in basal autophagy. Having experienced difficulties in co-IP proteomics, the authors switched to a BioID-mediated proximity proteomics approach to identity potential new interactors of ATG9A. In doing so, both known and novel interactors could be identified, including different regulators of autophagy and general trafficking. Interestingly, all components of the ULK1-complex were enriched in the proximity of ATG9A. The manuscript additionally provides some data indicating that the C-terminal part of ATG9A could be crucial for its interactions. Being interested in the ULK1-complex, the authors decided to focus on the interaction between ULK1-complex components and ATG9A. To overcome

antibody challenges, endogenously HA-tagged ATG9A cell lines were generated. The manuscript demonstrates that ATG9A is interacting with ATG13, a component of the ULK1-complex. Interestingly, ATG9A associates with both, the canonical ULK1-complex and an ULK1-independent ATG13-ATG101 subcomplex. This interaction is mediated by the ATG13-HORMA domain. Investigating ATG9A trafficking, the manuscript shows that loss of ATG13 and ATG101 but not FIP200, another component of the canonical ULK1-complex, results in large ATG9A puncta. These accumulations almost exclusively colocalize with p62/SQSTM1. Furthermore, depletion of ATG9A, ATG13, and ATG101 but not FIP200 result in accumulation of p62/SQSTM1 itself.
This manuscript convincingly demonstrates that ATG9A is interacting with an ATG13-ATG101 sub-complex, independent of the canonical ULK1-complex. Loss of this non-canonical complex results in disturbed ATG9A trafficking and accumulation of p62/SQSTM1. The authors speculate that the intact ATG13-ATG101 complex interacts with ATG9A at p62/SQSTM1 clusters, thus promoting proper basal autophagy and recycling of ATG9A. Loss of ATG13-ATG101 might result in defective ULK1-independent basal autophagy, followed by an accumulation of ATG9A at non-degraded p62/SQSTM1 clusters. This work supports recent findings that some components of the autophagic machinery (here ATG9A, ATG13, and ATG101) might be crucial for basal autophagy while others mainly function upon induced autophagy.

Major points
1) Was the localization of overexpressed ATG9A-BirA investigated? If so, did ATG9A co-localize with known ATG9A interactors? If not, the authors should perform this analysis. Regarding the biotinylation pattern, is it specific and co-localizing with ATG9A? The authors should address this experimentally.

2) In Figure S1C, the effect of different ATG9A variants on LC3 level and procession is depicted. The same experiment should be performed with HA-ATG9A-BirA overexpressing cells in Figure 1A.

3) In Figure 1B, biotin signals vary between biological replicates of ATG9A-BirA overexpressing cells. Is this variance also present in the proteomic dataset? If so, were Pearson correlations calculated?

4) Why are more proteins enriched in the BirA-only cells (Figure 1D)? Do these fit with expected interactors of BirA-only? What is its subcellular localization and is it represented in a GO-term analysis? How do the authors ensure that potential interactors are not lost? The authors should use a different control, e.g. another membrane protein that shows a different localization than ATG9A.

5) Since the authors speculate that the C-terminus is important for ATG9A interactions, the authors should repeat the BioID-experiment with a truncated ATG9A variant. This would also be a essential control to the presented proximity proteome of full length ATG9A.

6) What are the upstream signals that lead to ATG9A recruitment to p62/SQSTM1 clusters? Given the capacity of proximity labeling approaches to capture transient interactions, should examine their proximity data for factors that could help understand this regulation.

7) Figure 1E lacks validation of HA-ATG9A-BirA in the pulldown.

8) Figure 3B, C, and D show ATG9A interactions with different components of the ULK1 complex. To support the claim that ATG9 is actually bound to the entire assembled ULK1 complex and not separately to its individual subunits, the authors should blot for i) ATG9A-BirA and HA-ATG13 in

Figure 3B, ii) ATG13, ATG9A and ULK1 in Figure 3C and iii) ATG9A and ULK1 in Figure 3D.

9) How do the authors explain low co-localization rates of wild-type ATG9A with cellular markers, even though they are known to co-localize with ATG9A (Figure 4)?

10) Figure 5 and 6 show increase / accumulation of p62/SQSTM1 upon depletion of ATG13, ATG101 and ATG9. Did the authors check if NBR1 levels are also affected, since NBR1 function is ATG9A but not ULK1 dependent?

11) The authors should perform live cell imaging to validate the proposed effects of ATG13-ATG101 loss on ATG9A retrograde trafficking.

Minor point
12) All western-blots lack information about the molecular weight of detected bands. The authors should provide this important information.

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We thank the reviewers for their thoughtful and constructive critiques of this study. We have added new data that we think significantly improve the manuscript. Below is a point-by-point response to the reviewer comments.

Reviewer 1

1. The Introduction and Discussion provide some confusing information on basal vs selective autophagy. There is basal autophagy revealed by bafilomycin treatment of cells that still depends on mTOR activity. There is basal autophagy against selective targets which most likely is locally triggered and does not require TOR inactivation. These two processes are not equivalent.

We appreciate this clarification. We have changed our descriptions of basal autophagy in the introduction and discussion to reflect this point.

2. The gel filtration experiment used detergent lysates as input. My understanding is that in these conditions ATG9A will be in detergent micelles and it is difficult to see how one can make conclusions about its size/behavior in comparison to cytosolic proteins.

Given the new data we've added, we decided to remove the old figure 2 (gel filtration) because it didn't add significant insight to manuscript. In regard to the comment on micelles, we kept the triton X-100 concentration below the critical micelle concentration while in the column, which we thought should allow for maintenance of ATG9A interactions. Nevertheless, the data have been removed, which we think has ultimately improved the manuscript.

3. Why does HA-ATG9A appear to be degraded in the presence of bafilomycin in the ATG13 and ATG101 deleted cells (Fig 5B)?

We asked ourselves the same question. We observed this mostly in HEK-293T cell lines. We also noted that the HEK-293T ATG13 and ATG101 KO cell lines show the strongest increase in ATG9A-P62 colocalization, which we interpret to be a result of defective autophagy (we think these stalled ubiqiutin/p62 clusters persistently signal for ATG9A recruitment). When these cells are treated with bafilomycin, any residual autophagic flux is also inhibited. Therefore, we reasoned that under bafilomycin treatment, more and more ATG9A would accumulate at these ubiquitin/p62 clusters. We suspected that the Triton X-100 lysis buffer used in these experiments didn't completely solubilize p62 aggregates, perhaps allowing a portion of ATG9A to fall out of the lysis in a Triton X-100 insoluble fraction. This would make the ATG9A level in the bafilomycin treated lanes appear artificially low. To test this idea, we performed cell lysis with SDS-containing RIPA buffer (new figure 4B), which helped—it reduced the loss of ATG9A levels in the + Baf samples, suggesting that some ATG9A was indeed in a Triton X-100 insoluble fraction in the baf-treated cells. However, this didn't completely *eliminate* the reduction in ATG9A, and we're not yet sure why.

4. The ATG9 antibody from cell signaling ((D4O9D) Rabbit mAb, 13509S) works well for

blots in human cells (and for iffl) and should be used in the experiments deleting and reconstituting ATG9.

We used the ATG9A rabbit monoclonal (Abcam, ab108338) antibody to validate ATG9A KOs in HEK-293T cells (figure S2B) as well as to measure ATG9A levels in ATG9A KOs reconstituted with HA ATG9A and ATG9A BL. We found that oddly the recognition of ATG9A by the CST ATG9A antibody is diminished when the ATG9A C-terminus is fused to a tag (the antibody recognizes an epitope around amino acid 780). We've seen this problem with multiple tags and different vectors, including BirA or fluorescent proteins, all of which we've validated as functional (and the full sequence is fine), but yet are compromised for detection by the CST antibody. Nevertheless, the Abcam ATG9A antibody works fine.

5. I guess that this is a function of experimental timing, but in retrospect it would have been better to do the BIO-ID in cells deleted for ATG9A and rescued with the BIRA construct.

We agree. It could have boosted our signal-to-noise ratio (this was indeed a function of experiment timing), but ultimately we think the BioID results turned out very well, aligning with what we would expect for ATG9A while also revealing some intriguing novel interactors.

Reviewer 2

1. The authors are implying that ATG13 is present in a separate ULK1/FIP200-independent complex in cells, but they have not really demonstrated this (gel filtration alone is not enough). ULK1 was identified in the BIO-ID experiments, suggesting it may be in the complex but simply not required for this particular ATG9 trafficking step. The authors should perform some triple labelling immunofluorescence and show the presence of ATG13 puncta that colocalize with either ULK1 or ATG9.

We are excited to report that we've addressed this concern in ways we think significantly improve the manuscript. First, we developed a split-Venus system for ATG13-ATG101 in which the N- and C-terminal halves of Venus are fused to ATG13 and ATG101, respectively. We verified that the addition of Venus halves to ATG13 and ATG101 didn't affect their autophagy function, as these constructs rescued the accumulation of p62 in an ATG13-ATG101 dKO line (figure S9C). Importantly, this system allowed us to visualize and capture the intact ATG9A ATG13-ATG101 complex (figures 7 and S9)—something (as the reviewer points out) we had only attempted by gel filtration before. We also introduced the ULK1-binding mutation (Δ2AA) into the ATG13-N-Venus construct, which allowed us to evaluate the ATG13-ATG101 dimer in the absence of direct physical interaction between ATG13 and ULK1. This approach worked remarkably well. Figure 7B shows that the WT ATG13-ATG101 dimer (captured by GFP-Trap resin) interacts with ATG9A and ULK1, while the dimer carrying the ATG13 Δ2AA mutant interacts with ATG9A, but not ULK1. This split-Venus GFP-trap capture approach illustrates its power over conventional binary co-IPs, as this is the first evidence (to our knowledge) of ATG9A and the ATG13-ATG101 dimer interacting in an ULK1-independent complex—in our opinion, a major improvement over our earlier data.

The ATG13-ATG101 split-Venus system also allowed us to visualize quadruple colocalization of ATG13, ATG101, ATG9A and ULK1 by confocal imaging, which revealed several interesting points. First, we see that ATG9A and the ATG13-ATG101 dimer (WT *or* Δ2AA) are frequently colocalized in small puncta that do not include ULK1 (Figure 7C). This is interesting in light of work from the Ktistakis group showing that ATG13 associates with clusters of ATG9A (https://www.nature.com/articles/ncomms12420). In this study, ATG13 was assumed a surrogate for the ULK1 complex, but our data indicate that the ATG13-ATG101 dimer interacts with ATG9A puncta independently of ULK1, suggesting the ATG13-ULK1 relationship is more dynamic than previously thought. Second, we do see colocalization of the ATG13-ATG101 dimer, ATG9A and ULK1 in the largest clusters (figure 7C), which our data suggest are sites of ubiquitin/p62/SQSTM1 accumulation (figure 6B and 7E). Importantly, this occurs even with the ATG13 Δ2AA mutant, suggesting that a direct physical interaction between ULK1 and ATG13 is not necessary for components of the ULK1 complex to coalesce at these clusters. This supports a small but growing body of evidence that different components of the ULK1 complex can arrive separately at autophagosome nucleation sites (e.g., Shi et al., 2020, JCB; Lin et al., MBoC; Itakura et al., 2012, JCS). Indeed, in addition to our split-Venus data, this idea is also supported by our BioID and coIP data (figures 2 and S5) showing that ATG13 doesn't require FIP200 or ULK1 to interact with ATG9A; and vice versa, ULK1 doesn't require ATG13 to interact with ATG9A .

2. Related to the above, as ULK1 is a kinase it may not have to interact strongly with the ATG13-ATG9 complex to exert an effect. To help rule this out, can the authors show in their KO-rescue experiments that the ATG13 mutant, which does not bind to ULK1, is no longer phosphorylated by ULK1 at the "Ser318" residue?

We agree that this is an important point. We have added new data in figure 7D showing that the phosphorylation at S318 of ATG13 is reduced in the ATG13 Δ2AA mutant.

3. The authors have not sufficiently demonstrated an effect on autophagy. Block of the ATG13-ATG9 interaction leads to accumulation of p62 structures. What are these - ubiquitin aggregates? Is LC3 on these structures? Are they even destined to be degraded by autophagy (p62 can be degraded by the proteasome)? This latter point arises from the bafilomycin experiments shown in Fig. 5, as there is no flux of p62 in WT after 24h of treatment. This suggests there is no basal autophagy of p62 occurring - which also may explain why there is no accumulation of p62 in the FIP200 KOs. FIP200 KOs should also display no autophagy and there is plenty of evidence in the literature that shows loss of FIP200 leads to p62 accumulation. What about LC3, is this fluxing under these conditions (and if so, can it be rescued by the ATG13 mutants)?

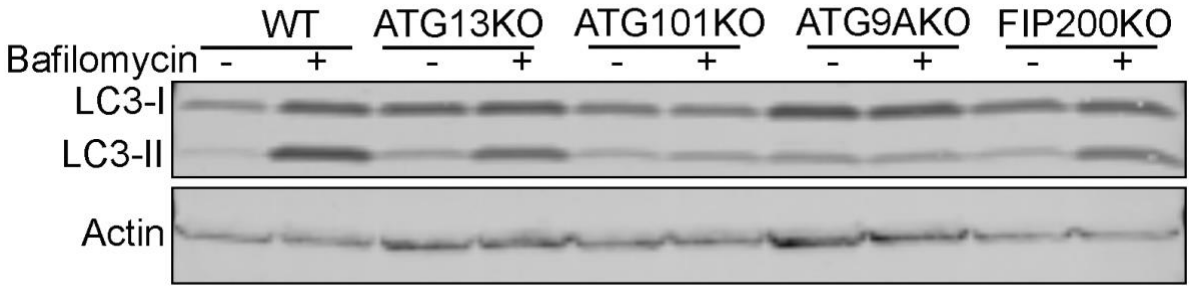
We were also puzzled by the fact that bafilomycin didn't increase p62 levels even in wild-type cells. However, we have now gone back and repeated multiple replicates of figures 4A and B with a new source of bafilomycin. Our new results show that P62 does indeed accumulate with 24-hour bafilomycin treatment, suggesting that p62 is being degraded through an autophagic/lysosomal pathway. We also looked at p62 accumulation in proteasome-inhibited cells, but didn't see significant p62 accumulation under these conditions (data not shown),

supporting the idea that the main route of p62 degradation in these cells is autophagy. Also, supporting these data, our confocal experiments show that ATG9A-positive p62 puncta are also positive for ubiquitin and LC3B (new data added in figures 6B and 3E). This suggests that p62 degradation occurs through autophagy.

We also took advantage of our ATG9A-BirA and designed a modified pulse chase experiment to ask whether these clusters of p62 that interact with ATG9A in the ATG13 KOs show stalled degradation (as opposed to an increased rate of synthesis, for example). For comparison here, Bjørkøy et al. (2005, JCB) show that the half-life of p62 is about 6 hours under fed conditions. In short, we pulsed ATG9A-BirA expressing cells with biotin, followed by a time course of streptavidin pull down and immunoblotting for p62. As shown in a new figure 6C, p62 shows delayed degradation in the ATG13 KO. All together with the new data mentioned above, these results suggest that the build-up of p62 in ATG13 KOs is caused by defective autophagy and can be rescued by the ULK1-binding defective ATG13.

Regarding our FIP200 KOs, we do see variation in p62 puncta size in FIP200 KOs trending toward larger puncta. We also see increased colocalization of ATG9A and p62 in the FIP200 KOs, which is consistent with the idea that build-up of p62/ubiquitin clusters sends a signal to recruit ATG9A. In addition, we see increased p62 in FIP200 KO cells by immunoblot, albeit less of an increase than in ATG9A, ATG101 or ATG13 KO lines. A recent biorxiv paper by Turco et al. (https://www.biorxiv.org/content/10.1101/2020.07.07.191189v1.full) also shows that ATG13 and p62 clusters are larger in ATG9A KOs compared to FIP200 KOs. Also, recent work by the Hurley lab (Shi et al., 2020) raise the question of whether TBK1 might compensate for the loss of FIP200, which could help explain our results.

In our experiments looking at the processing of LC3 under fed conditions (+/- Baf) in these KOs, we see only a very minor defect in basal LC3 processing (see blot below—ATG9A KO showing the most severe defect). Interestingly, the degree of defect in LC3 processing didn't correlate to the level of p62 build-up in any of our experiments (compare with figure 5A), suggesting that basal turnover of p62 and cargo may be at least partially independent of LC3. This is supported by a recent study from Christian Behrends' group, which found that autophagic engulfment of adapter cargo was partly independent of LC3 and GABARAP https://pubmed.ncbi.nlm.nih.gov/33545068/); this is also consistent with a study from the Shoemaker group (https://www.embopress.org/doi/full/10.15252/embj.2020104948)



Regarding whether the ATG13 mutants can rescue LC3 flux defects, Bjorn Stork's group has published that the ULK1-binding defective ATG13 rescues the defect in starvation-induced LC3 flux in ATG13 KO MEFs (Wallot-Hieke et al., 2018, Autophagy).

4. It appears that CRISPR KOs were only confirmed by western blot. This is not sufficient as depending on recombination events, truncated proteins with some "activity" can still be expressed, which are no longer recognized by antibodies. This could confound interpretation of results. Thus, the authors should confirm their KOs by genomic sequencing.

We appreciate this concern. We do sequence-validate CRISPR knock-ins, while our KOs are not only validated by western blot, but also by functional assays and gene rescue experiments (with the end points of p62 degradation, confocal imaging, etc.). Genomic sequencing was cost- and labor-prohibitive for us (exacerbated by the pandemic) and we respectfully point out that western blots coupled with functional assays and gene rescue experiments are accepted broadly in the literature to establish the validity of a CRISPR KO (this was also the consensus of a recent American Cancer Society zoom panel discussion on CRISPR).

5. I appreciate that the authors are focused on "basal" conditions but does this interaction have any bearing on classical starvation-induced autophagy? Are the ATG9-p62 structures (in ATG13 KO) exacerbated under starvation conditions and can they still be rescued (as well as LC3 flux) by the ATG13 ULK-interacting mutant?

Interestingly, we don't see any significant exacerbation of the ATG9A-p62 structures in starved conditions—in fact, they look essentially identical to nutrient replete conditions, perhaps reflecting that these ATG9A-p62 structures accumulate over long periods of time in fed conditions and aren't appreciably affected by a comparatively brief period of starvation. In addition, we see the same pattern of p62 accumulation by western blot after starvation (new figure 5B).

Minor Points:

6. Molecular weight markers should be included on the blots.

We have now added molecular weight markers to all the western blots

7. Authors may wish to confirm they are using the correct statistical analyses. A t test is appropriate when just comparing two values, but it is not when multiple values are being compared (controls have been compared to multiple other conditions in many experiments). One-way Anova and a post hoc test will be more suitable.

Thank you for the correction, and we have now changed our statistical methods. In short, we normalized all samples to the WT reference sample so that all other samples are represented as a fold-change to the WT (unless the variation between blots was not an issue as with figure S1C). We then analyzed the immunoblot data with more than one comparison using a repeated measures (RM) one-way ANOVA test followed by Fisher's LSD tests omitting the reference control (since it now has no standard deviation). RM was selected since each sample was linked

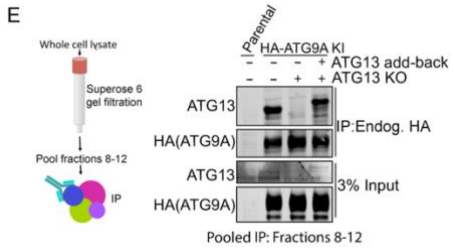
(paired) to the other samples on the same blot (same gel, transfer, antibody signal etc.) We then selected the Fisher's LSD post hoc test since each sample is also independent (ATG13 KO sample is not affected by ATG13 WT sample) and because we had very few replicated to correct for (n=3). Immunoblots with only two samples were normalized as with other samples (fold-change to WT) and then analyzed with a one sample t-test comparing to a hypothetical mean of 1 according to the recommendation of GraphPad Prism software since (once again) our reference control has no standard deviation.

Comparisons of confocal data are not limited by the reference sample problem. All image analysis yielded raw data with comparable variation, so no normalization was needed. Experiments with multiple comparisons were analyzed using an Ordinary one-way ANOVA test followed by Šídák's multiple comparisons tests. Ordinary one-way was used because each sample was treated separately (not paired) and the Šídák's multiple comparisons test was used as a more stringent measure to ensure that differences were significant despite observed variation and because we had a larger number of measurements to correct for (n=30 unless otherwise indicated).

Post hoc comparisons are shown as pairwise to facilitate data interpretation. We have now included these changes in our methods section.

7. In Figure 2E, why is there no endogenous ATG13 staining in the parental input?

In light of new data (in particular, the split mVenus data), we decided to remove the old figure 2 because we felt it didn't add significantly to the manuscript. However, we did go back and rerun the gel filtration lysates for ATG13 immunoblotting and you can see those data here. The input signal for ATG13 is there but faint.



8. The authors should discuss work from Sharon Tooze showing that ULK1 is required for ATG9 trafficking to autophagosomes and highlight why the authors have different observations (PMID:16940348).

We have added new discussion of this point in the discussion section. In short, Sharon's work shows that ATG9A redistribution is regulated by ULK1 under starvation conditions. We actually don't think our data disagree with her findings at all and would argue that differences in ULK1 involvement may simply reflect mechanistic differences in basal turnover of p62 versus starvation-induced autophagy. Furthermore, while our data suggest that the ATG13-ULK1 interaction is dispensable for basal p62 turnover, we do not rule out the possibility that ULK1, perhaps activated locally or through a yet uncharacterized mechanism, does play a role in this process. Notably, previous work by Richard Youle's group shows that ULK1 can be activated independently of mTOR and AMPK at sites of mitophagy (Vargas et al., 2019, Mol Cell) and it's entirely possible that this also occurs through some means at ubiquitin/p62 clusters. We have modified our discussion to make this point more clear.

Reviewer 3

1) Was the localization of overexpressed ATG9A-BirA investigated? If so, did ATG9A co-localize with known ATG9A interactors? If not, the authors should perform this analysis. Regarding the biotinylation pattern, is it specific and co-localizing with ATG9A? The authors should address this experimentally.

We have included new data in figure S2C, in which we evaluated ATG9A-BirA* by confocal imaging. First, ATG9A-BirA* shows a typical pattern of mostly peri-nuclear localization, like we see with WT ATG9A. Additionally, we find that ATG9A-BirA* clusters into large puncta in ATG13 KO cells, identical to what we see with WT ATG9A. In addition, we show that ATG9A-BirA* is able to rescue the accumulation of p62 (figure 1A) and defect in LC3-II processing (figure S2A) in an ATG9A KO cell line. Together, these data suggest that addition of the BirA* tag to the ATG9A C-terminus doesn't appreciably affect ATG9A function in autophagy. We have seen similar results in other ATG9A C-terminal fusions (e.g., ATG9A-GFP)

2) In Figure S1C, the effect of different ATG9A variants on LC3 level and procession is depicted. The same experiment should be performed with HA-ATG9A-BirA overexpressing cells in Figure 1A.

We have added new data in figure S2A: the increased LC3-I to actin ratio in ATG9A KO cells is rescued by expression of ATG9A-BirA*.

3) In Figure 1B, biotin signals vary between biological replicates of ATG9A-BirA overexpressing cells. Is this variance also present in the proteomic dataset? If so, were Pearson correlations calculated?

The experiment in figure 1B was done to validate whether biotinylation by ATG9A-BirA* occurred properly prior to submitting samples for mass spectrometry. We only devoted a small fraction of the streptavidin pulldowns to a single SDS PAGE coomassie. We noted immediately that the first sample of ATG9A-BirA* replicates was under loaded by error. Since the rest of the sample was used for mass spectrometry, we were unfortunately not able to repeat the gel.

We did not notice any significant variance in the proteomics data set. To confirm this point, we asked Dr. Erik Soderblom at the Duke Proteomics core (where these data were collected) to go back and reanalyze the raw MS data. He confirmed that there was no decrease in signal in that sample as detected by LC-MS/MS. Here is his response email, which also explains some of his approach in normalizing signals between samples:

Hi Josh,
I went back and looked at the data and we didn't observe that lower signal for that one sample in our raw, non-normalized data. Either way, we treated all samples assuming they have equal amounts of material eluted off the resins. After the data is collected, I look to make sure the raw signal was fairly consistent across all samples – which it was with the exception of the first BirA sample which had higher signal. We then deployed a global normalization across all samples to reduce any differences in sample loading by summing all of the signals (after peptide imputation for missing data) and making that number the same for all samples. This is an appropriate strategy if the majority of the protein expression across all samples is relatively similar… which was the case for these samples. The variance within the groups is also very low for this dataset; <12% for our pooled samples and ~20% for each test group following normalization. These are both very, very good for this type of analysis.

Hope that helps,
Erik

4) Why are more proteins enriched in the BirA-only cells (Figure 1D)? Do these fit with expected interactors of BirA-only? What is its subcellular localization and is it represented in a GO-term analysis? How do the authors ensure that potential interactors are not lost? The authors should use a different control, e.g. another membrane protein that shows a different localization than ATG9A.

BirA* is localized to the cytosol. It's a bacterial protein so we're not aware of a canonical interactome in human cells, but we expected a high degree of promiscuous biotinylation, which is why it was selected as a control for background biotinylation.

After multiple rounds of discussions on the question of controls with Drs. Will Thompson and Erik Soderblom in the Duke proteomics core (they have years of experience with BioID), we decided to use BirA* alone as the control for this experiment for several reasons: BirA* expression is stable and, as a soluble (non-membrane bound) cytosolic protein, will have a different distribution than ATG9A. This is exactly what we wanted as a control, given that we're making a relative comparison of non-specific background biotinylation versus signal from a tagged protein. We also reasoned that fusing BirA* to a membrane protein would lead to biotinylation of many overlapping proteins with ATG9A (e.g., endosomal sorting complexes, etc.), which could erroneously eliminate those as ATG9A interactors in the final analysis. For these reasons, we decided that BirA* alone was the best available choice for the experiment and we think the data back that up. The BirA* control has also been accepted in similar published BioID experiments (for example, Uezu et al., 2016, Science; Spence et al., 2019, Nature Communications; Pedley et al., 2019, Cell Death & Disease; and many others).

5) Since the authors speculate that the C-terminus is important for ATG9A interactions, the authors should repeat the BioID-experiment with a truncated ATG9A variant. This would also be a essential control to the presented proximity proteome of full length ATG9A.

Our rationale for appending BirA* to the C-terminus was based on the C-terminus bearing features of a protein-protein interaction domain, which made it a good location for BirA*, but our goal was simply to identify ATG9A-interacting proteins—regardless of whether they physically interact via the C- or N-terminus. We had considered this experiment early on (fusing BirA* to a truncated ATG9A), but didn't pursue it because we argued that truncating off such a large unstructured segment of ATG9A (the entire C-terminus) then appending BirA* immediately after the last transmembrane domain would lead to artifactual changes in interactions that would be hard to interpret or would simply reflect changes in trafficking of a C-terminally deleted ATG9A—this latter question would be interesting, but was not within the scope of what we wanted to accomplish for this study. We did, however, do some experiments (unpublished) with BirA fused to the N-terminus of ATG9A, but this fusion interfered with ATG9A expression/stability.

6) What are the upstream signals that lead to ATG9A recruitment to p62/SQSTM1 clusters? Given the capacity of proximity labeling approaches to capture transient interactions, should examine their proximity data for factors that could help understand this regulation.

We agree—this question of upstream signals that recruit ATG9A to p62 structures is very compelling. We see a variety of kinases and other potential signaling proteins in the BioID. For example, here is a list of kinases from the ATG9A BioID interactor list:

Gene Report

Help and Manual

Current Gene List: List_1
Current Background: Homo sapiens
283 DAVID IDs
16 record(s)

Download File

UNIPROT_ACCESSION	GENE NAME	Related Genes	Species
Q9NSY1	BMP2 inducible kinase(BMP2K)	RG	Homo sapiens
O14976	cyclin G associated kinase(GAK)	RG	Homo sapiens
P51570	galactokinase 1(GALK1)	RG	Homo sapiens
Q9ULH0	kinase D-interacting substrate 220kDa(KIDINS220)	RG	Homo sapiens
Q7KZI7	microtubule affinity regulating kinase 2(MARK2)	RG	Homo sapiens
Q16584	mitogen-activated protein kinase kinase kinase 11(MAP3K11)	RG	Homo sapiens
Q5TCX8	mixed lineage kinase 4(MLK4)	RG	Homo sapiens
O00443	phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha(PIK3C2A)	RG	Homo sapiens
Q9Y2I7	phosphoinositide kinase, FYVE-type zinc finger containing(PIKFYVE)	RG	Homo sapiens
Q9H792	pseudopodium enriched atypical kinase 1(PEAK1)	RG	Homo sapiens
Q01974	receptor tyrosine kinase like orphan receptor 2(ROR2)	RG	Homo sapiens
Q96S38	ribosomal protein S6 kinase C1(RPS6KC1)	RG	Homo sapiens
Q15569	testis-specific kinase 1(TESK1)	RG	Homo sapiens
Q07912	tyrosine kinase non receptor 2(TNK2)	RG	Homo sapiens
O75385	unc-51 like autophagy activating kinase 1(ULK1)	RG	Homo sapiens
Q86Y07	vaccinia related kinase 2(VRK2)	RG	Homo sapiens

One possibility that we're interested in is that p62 itself may act as a platform to recruit signaling molecules, which in turn may recruit ATG9A, etc.—so as it builds up, the strength of the signal increases, which essentially raises the threat level and increases recruitment of autophagy machinery in an attempt to dispose of the ubiquitinated aggregates, etc. We have added new text to the discussion addressing this and another possibility. As a supplementary data file, we have also provided the entire ATG9A BioID interactome for the ATG9A/autophagy community to investigate these types of questions.

7) Figure 1E lacks validation of HA-ATG9A-BirA in the pulldown.

In figure 1E, we're looking at proteins biotinylated by ATG9A-BirA* (not a conventional affinity tag pulldown), which are captured on streptavidin resin. For this reason, blotting for HA-ATG9A-BirA* in the pulldown would only show self biotynlation of ATG9A-BirA*. However, we have added a blot of HA-ATG9A-BirA* in the lysates that shows ATG9A-BirA* levels in the assay.

8) Figure 3B, C, and D show ATG9A interactions with different components of the ULK1 complex. To support the claim that ATG9 is actually bound to the entire assembled ULK1 complex and not separately to its individual subunits, the authors should blot for i) ATG9A-BirA and HA-ATG13 in Figure 3B, ii) ATG13, ATG9A and ULK1 in Figure 3C and iii) ATG9A and ULK1 in Figure 3D.

As mentioned above, for these BioID streptavidin pulldows, probing for ATG9A in the pulldown will only show the level of self-biotinylation, which is plentiful, but not pertinent to the result. For this reason, we probe for ATG9A-BirA* in the lysate as a loading control. As far as assessing whether ATG9A is bound to the entire assembled ULK1 complex of to its individual subunits, we point the reviewer to co-IPs of endogenous (HA knock-in) ATG9A.

- In figure S5C-D, we show that ATG9A co-IPs with ULK1 in ATG13 KO cells.
- In figure S5F, we show that ATG9A co-IPs with ATG13 in FIP200 KOs.
- Conversely, although we were unable to KO ULK1 in our cell lines, we showed that ATG9A can biotinylate ATG13 perfectly fine in ULK1/2 dKO MEFs (figure 2D)

We have also addressed the overarching question here in a way that we think significantly improves the manuscript: As stated above, we developed a split-mVenus system for ATG13-ATG101 in which the N- and C-terminal halves of Venus are fused to ATG13 and ATG101, respectively. We verified that the addition of Venus halves to ATG13 and ATG101 didn't affect their autophagy function, as these constructs rescued the accumulation of p62 in an ATG13-ATG101 dKO line (figure S9C). Importantly, this system allowed us to visualize and capture the intact ATG13-ATG101 dimer (figures 7 and S9). We also introduced the ULK1-binding mutation (Δ2AA) into the ATG13-N-Venus construct, which allowed us to evaluate the ATG13-ATG101 dimer in the absence of direct physical interaction between ATG13 and ULK1. In figure 7B, we show that the WT ATG13-ATG101 dimer (captured by GFP-Trap resin) interacts with ATG9A and ULK1, while the dimer carrying the ATG13 Δ2AA mutant interacts with ATG9A, *but not* ULK1.

The ATG13-ATG101 split-Venus system also allowed us to visualize quadruple colocalization of ATG13, ATG101, ATG9A and ULK1 by confocal imaging, which revealed several interesting points. First, we see that ATG9A and the ATG13-ATG101 dimer (WT *or* Δ2AA) are frequently colocalized in small puncta that do not include ULK1 (Figure 7C). This is interesting in light of work from the Ktistakis group showing that ATG13 associates with clusters of ATG9 (https://www.nature.com/articles/ncomms12420). In this study, ATG13 was assumed a surrogate for the ULK1 complex, but our data indicate that the ATG13-ATG101 dimer interacts with these ATG9A clusters independently of ULK1. Second, we do see colocalization of the ATG13-ATG101 dimer, ATG9A and ULK1 in the largest clusters (figure 7C), which our data suggest are sites of ubiquitin/p62 accumulation (figure 6B and 7E). Importantly, this occurs even with the ATG13 Δ2AA mutant, suggesting that a direct physical interaction between ULK1 and ATG13 is not necessary for components of the ULK1 complex to coalesce at these clusters. This supports a small but growing body of evidence that different components of the ULK1 complex can arrive separately to these autophagosome nucleation sites (e.g., Shi et al., 2020, JCB; Lin et al., MBoC; Itakura et al., 2012, JCS). Indeed, in addition to our split-Venus data, this idea is also supported by our BioID and coIP data (figures 2 and S5) showing that ATG13 doesn't require FIP200 or ULK1 to interact with ATG9A; and vice versa, ULK1 doesn't require ATG13 to interact with ATG9A.

9) How do the authors explain low co-localization rates of wild-type ATG9A with cellular markers, even though they are known to co-localize with ATG9A (Figure 4)?

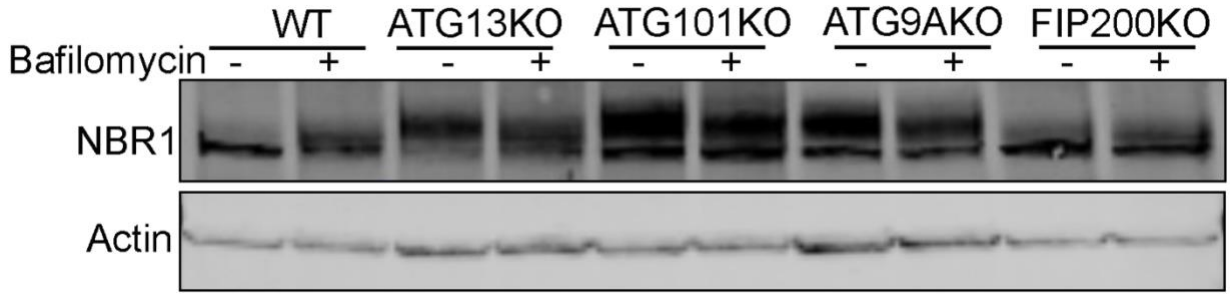
Perhaps the definitive analysis of ATG9A colocalization with cellular markers is from Sharon Tooze's group (Orsi et al., 2012, MBoC). They reported the percent of total ATG9A that colocalizes with various markers. For autophagosomal markers, the percent colocalization was quite low in their study. For example, only about 2% of total ATG9A colocalized with WIPI2, 0.52 with ULK1, 4% with DFCP1, 1.08% with ATG16 and 4.50% with LC3.

They did however see higher colocalization of ATG9A with some organelle markers. For example, they found 26.3% of ATG9A colocalized with TGN46 and 18.9% with EEA1. Our data are reported as a Pearson's coefficient derived from software analysis of deconvolved images so it's not directly translatable to their percentage numbers. However, we do see good colocalization with golgi markers (e.g., Golgin97), WIPI2, and retromer (VPS26A). In general, we also see a very similar gross distribution of ATG9A in the cell, with the bulk of ATG9A in a perinuclear area.

Differences in colocalization between studies may also reflect differences in analyses and resolution: automated versus manual counting (note that some of their analysis of "total ATG9A" colocalization was manually counted), deconvolution, etc. In addition, their analysis was done under starvation and ours was done under nutrient replete conditions, which could account for differences in localization. Differences may also be due to cell-to-cell variation. We also point out that we were careful to rigorously validate our HA knock-in ATG9A confocal imaging signal by knocking out ATG9A, so we are confident that the signal we're seeing by confocal is ATG9A.

10) Figure 5 and 6 show increase / accumulation of p62/SQSTM1 upon depletion of ATG13, ATG101 and ATG9. Did the authors check if NBR1 levels are also affected, since NBR1 function is ATG9A but not ULK1 dependent?

We went back and probed our samples for NBR1 and we do indeed see a similar pattern for NBR1 as p62/SQSTM1 in ATG13 KO, ATG101 KO, ATG9A KO and FIP200 KOs (see blot below)



11) The authors should perform live cell imaging to validate the proposed effects of ATG13-ATG101 loss on ATG9A retrograde trafficking.

We have added new data in figure 6 to address this question. In short, we generated WT and ATG13 KO cells stably expressing GFP-p62/SQSTM1 and ATG9A-mRuby. Still frames of these images are added in figure 6A. What we observed was quite striking—It appears that recruitment of ATG9A to the p62 clusters is increased in the ATG13 KO, suggesting that these large clusters somehow signal to upregulate recruitment of ATG9A. The videos are attached as supplementary files. We were unable to see any significant level of retrograde trafficking away from these clusters, suggesting the possibility of increased ATG9A recruitment and decreased trafficking away from the clusters—a sort of terminal recruitment of ATG9A We have modified the model to reflect these data.

Minor point

12) All western-blots lack information about the molecular weight of detected bands. The authors should provide this important information.

We have added molecular weights to all the blots.

1st Revision - Editorial Decision 19th May 2021

Dear Josh,

Thank you for the re-submission of your revised manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, all three referees acknowledge that the revision has resulted in a substantially improved manuscript and support publication in EMBO reports pending some minor revision.

Given this positive evaluation, we invite you to further revise your manuscript for publication in EMBO reports. Please address the remaining referee concerns in the manuscript and in a point-by-point response.

In addition to addressing these remaining concerns, we also ask you to address a number of editorial points. Please note that once your manuscript has been submitted in the correct format (see below), we will perform an initial quality control before proceeding with its handling. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to

link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>)

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Specifically, we would kindly ask you to provide public access to the following datasets:
- LC-MS/MS data

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below (see also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

11) Regarding data quantification
The following points MUST be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify if these are technical or biological replicates!) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends must contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

12) Other specific issues:

- Please add a Conflict of interest and an Author Contribution section.
- I noticed that your scale bars are rather thin and might be difficult to see in the final typeset version.
- The arrows in Fig. 7C are very thin and difficult to see.
- Supplementary data set: Please upload it as 'Dataset EV1' in the format of an .xls file. The legend can be part of the .xls file, e.g. in the first row or as separate tab.
- Movies: we need individual files for each movie. The legend is provided as README.txt file and zipped together with the movie. Then the .zip file is uploaded (one file per movie).

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

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

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

The authors did not get to the bottom of all questions asked, but they did so to a sufficiently high degree so that their paper can now be accepted, and any further delay would not be useful.

Referee #2:

The authors have done a good job in addressing my concerns - an intriguing manuscript that furthers our understanding of "basal" autophagy!

Referee #3:

The authors adequately addressed the majority of concerns and the manuscript has improved significantly. However, some improvements still need to be done.

Regarding the initial major point #1 (ATG9A-BirA expression validation):
The authors convincingly assured proper biological function of ATG9A-BirA fusions. However, they should also assess the biotinylation pattern by immunofluorescence to verify a colocalization of biotin with the construct and to exclude high levels of unspecific biotinylation or mislocalization.

Regarding the initial major point #5 (C-term ATG9A interactions):
It is likely that the C-terminal domain of ATG9A is responsible for its interaction and association with intracellular trafficking components. However, as the authors already pointed out in their response, the presented paper does not confirm the statement that the BioID approach "reveals proximity-based interactions between the ATG9A C-terminus and a network of trafficking proteins and complexes" (title of results part 1). The authors should either back up this claim by additional experiments showing that truncated ATG9A variants lack these interactions (full C-term, partial C-term, ...) or -if not possible or beyond the intended scope - extenuate this claim to "[...] between ATG9A and a network [...]" and rewrite respective phrases.

Minor point 1 (western blots):
Some western blots still lack molecular weight information (Figure 1E, Figure S1C and Figure S4B).

Overall, the presented manuscript conclusively shows an ULK1-independent interaction between ATG9A and ATG13-ATG101 with involvement of p62/SQSTM1 clusters. Upon addressing the remaining minor concerns outline above, I am happy to recommend this manuscript for publication.

Dear Erica, Martina:
We've looked through the data again and just want to give a quick summary before we submit the changes and ask one question:

- For 1), we will provide different images for the controls (these images were taken from one big experiment)
- For 2), we will clarify this point in the figure legend
- For 3), I went back and looked and was totally embarrassed to see that these do appear to be different exposures of the same blot (just the p62 blot, not the others). This was not a student mistake or anything intentional—we have all the correct source images of those blots. This was my mistake when I gathered the data from students and made the figure (mistakenly included the wrong image file). I'll correct the figure and send all the source blots with it.
- For the last bullet point about technical versus biological replicates in confocal images. Thank you for the clarification as we were confused on this issue. I've gone back with my students and looked at this in detail. We have cells counted from 2 different stainings of each experiment for some (e.g., figure 5) but cells were counted from one staining for others. Many of these confocal experiments either corroborate the blot data or essentially tell the same story across multiple figures that ATG9A accumulates at the large clusters of p62/ubiquitin, which is rescued by the ULK1 binding deficient ATG13. So my question is this: Should we go back and repeat for more replicates (would take a month or so) or leave it as is but of course clarify the biological versus technical replicate issue and remove p-values?

Thanks,
Josh

Dear EMBO Reports editorial staff and reviewers,
Below is a merged point-by-point response to the quality check email from June 11 and the remaining reviewer critiques:

Requests from the June 11th initial quality check email from Erica Wilfon Boxheimer

1) We noticed that you reuse the same control images in Figure 4C and 5C (Hct116 WT), in Fig. EV4 and EV5 (Hct116 WT, ATG13 KO) and in Fig. S3A and S3B (Hct116 WT). In principle, this could be OK if the data shown originate from the exact same experiment and the control shown is thus the correct control for both figure panels. However, I strongly recommend using different control images in these figure panels. Showing a variation of stainings and thus the variability or reproducibility of control IF images is of more value than showing the same image twice. Therefore, please replace these images with different examples.

We have replaced these with a variety of control images as requested.

2) Regarding the quantification in Fig. EV4 and EV5 and in Fig. S3A and S3B: Please state in the figure legend that the quantification for HCT116 WT and ATG13KO in EV5 is based on the same data/experiment shown in EV4. The same holds true for Hct116 WT in S3. You need to state that these are not independent experiments and that the control quantification is based on the same data (I assume it does).

We have clarified this point in the figure legends as suggested.

3) The Western blots for p62 in Fig. 5A and 5B appear very similar. Please double-check this figure panel, whether the correct blots have been assembled. Please also supply source data for this figure.

This was completely my fault. My student had sent me the correct image files but I simply confused an alternate exposure of the same p62 blot for the wrong file, as they look quite similar. I apologize for this error. I have fixed the problem in the current uploaded figure 5 file and I have also provided the source data for panels 5A and 5B.

In addition to these requests please also
- Change the nomenclature of the Appendix figures to "Appendix Fig. Sx"

We have replaced all references to appendix figures with this nomenclature.

- provide up to 5 keywords

I have added 5 keywords under the abstract each separated by a slash.

- address the comments of our data editors in the figure legends of the manuscript

I believe we have addressed all the comments (I do not see any remaining comments in the document or unaddressed critiques in emails from the editorial staff).

- Regarding Fig. 3H, Fig. 4D, E and Fig. 5D, E: you specify 30 biological replicates. Are these true biological replicates or do the data rest on 30 cells from one experiment? If the latter applies, please change the description to state the number of puncta quantified in the number of cells and how many replicates of the experiment were done (i.e., independent stainings). Please note that if you counted puncta in 30 cells from one dish, the number of indpendent experiments is 1 and the use of statistics not justified.

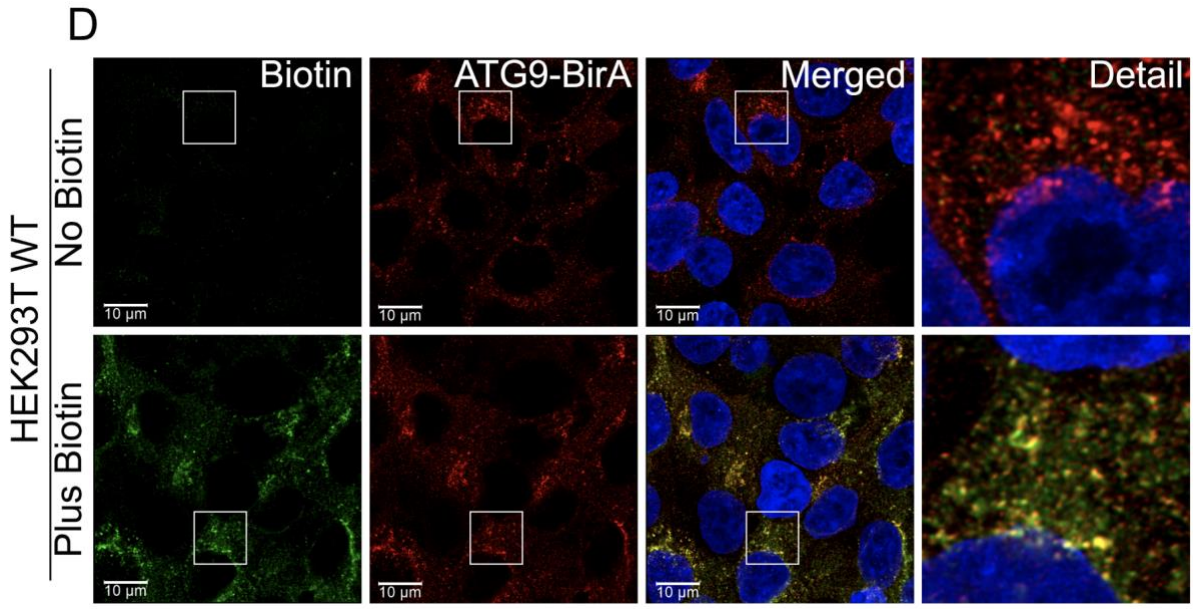
Thank you for the clarification on this point. Our confocal imaging experiments (showing ATG9A accumulating at p62 puncta, p62 puncta volume size, etc.) have been done many times (biological replicates), but the quantifications come from imaging multiple cells within a single representative experiment to allow for consistency in laser power and staining. Thus, we removed the p-values and clarified that the quantifications come from technical replicates (n=1). We point out that measurement of p62 puncta volume size is corroborated by western blots with quantifications and p-values, and the conclusion that ATG9A accumulates at large p62/ubiquitin clusters in basal autophagy-deficient/inhibited cells is repeated throughout the manuscript from various angles (e.g., ATG13 KOs, ATG101 KOs, wortmannin treatment).

<u>Reviewer critiques</u>

Reviewer 3

1) Regarding the initial major point #1 (ATG9A-BirA expression validation):
The authors convincingly assured proper biological function of ATG9A-BirA fusions. However, they should also assess the biotinylation pattern by immunofluorescence to verify a colocalization of biotin with the construct and to exclude high levels of unspecific biotinylation or mislocalization.

We have addressed the reviewer's comment by imaging biotin signal (detected by an Alexa-fluor 488 streptavidin conjugate) and HA-ATG9A-BirA in our stable HA-ATG9A-BirA cells treated +/- biotin. We were initially unsure of this experiment given that some level of biotin signal not colocalizing with HA-ATG9A-BirA is perhaps expected because the biotin incubation is 12 hours (so protein labeled early in the experiment may not track with ATG9A for the duration of the incubation). Nevertheless, in our opinion, this experiment turned out beautifully. As you can see below, there is strong biotin signal that colocalizes well with HA-ATG9A-BirA, while the biotin signal is gone in the no biotin control. These data are included in the new figure EV2 panel D.



2) Regarding the initial major point #5 (C-term ATG9A interactions):
It is likely that the C-terminal domain of ATG9A is responsible for its interaction and association with intracellular trafficking components. However, as the authors already pointed out in their response, the presented paper does not confirm the statement that the BioID approach "reveals proximity-based interactions between the ATG9A C-terminus and a network of trafficking proteins and complexes" (title of results part 1). The authors should either back up this claim by additional experiments showing that truncated ATG9A variants lack these interactions (full C-term, partial C-term, ...) or -if not possible or beyond the intended scope - extenuate this claim to "[...] between ATG9A and a network [...]" and rewrite respective phrases.

We agree that the BioID data do not prove that any interactor is docking to the ATG9A C-terminus. We have edited our phrasing accordingly to make that point clear. For example, the first header in the results section now reads "BioID reveals proximity-based interactions between ATG9A and a network of trafficking proteins and complexes".

Minor point 1 (western blots):
Some western blots still lack molecular weight information (Figure 1E, Figure S1C and Figure S4B).

We apologize for this mistake—we accidentally uploaded older versions of those blots for our initial submission of the revision. We have now uploaded the correct blots with MW markers.

***We have also gone through Dr. Rembold's checklist of updates/files to include and everything should be current according to those instructions. These updates include the following:**

1. Conflict of interest, author contribution, data availability sections (with link to LC-MS/MS data in public database) were added to the manuscript.
2. Scale bars and arrows were replaced with thicker versions in the figures
3. We have labeled supplementary figures as either EV or appendix figures
4. We added a legend to the data set EV1 (legend in separate tab in excel file)
5. We zip filed the live-cell movies with plain text legends
6. We specified biological or technical replicates in figure legends
7. We added a new coauthor (Erik Soderblom)

2nd Revision - Editorial Decision 9th Jul 2021

Manuscript number: EMBOR-2020-51136V3
Title: BioID reveals an ATG9A interaction with ATG13-ATG101 in the degradation of p62/SQSTM1 clusters
Author(s): Joshua Andersen, Ashari Kannangara, Daniel Poole, Colten McEwan, Vajira Weerasekara, Alex Thornock, Misael Lazaro, Eranga Balasooriya, Laura Oh, Erik Soderblom, JJ Lee, and Daniel Simmons

Dear Josh

Thank you for your patience while we have editorially reviewed your revised manuscript. I am now writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

1) For all figures with n = 1 please also show the individual data points as scatter blots (Fig. 3H, Fig. 4D, E, Fig. 5D, E, 6B, 7C).

2) Author checklist: please revisit the sections on statistical analysis in the checklist since it describes the statistical analysis of 30 datapoints that has now been removed (see above).
The respective description in the methods section might also have to be updated (confocal image data analysis).

3) Data availability section: please add a link that resolves to the data in the database.

4) We noticed that co-author Joushua Youngs is now missing from the relevant author field in the online submission system. He is still listed on the cover page of the manuscript as author, but not in the Author Contributions. Could you please clarify this?

5) Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Once you have made these minor revisions, please use the following link to submit your corrected manuscript:

Link Not Available

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

Kind regards,
Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

Dear Martina and editors,
We have now addressed the recent editorial comments. Below is our point-by-point response.

1) *For all figures with n = 1 please also show the individual data points as scatter blots (Fig. 3H, Fig. 4D, E, Fig. 5D, E, 6B, 7C).*

We have replaced these panels with scatter plots.

2) *Author checklist: please revisit the sections on statistical analysis in the checklist since it describes the statistical analysis of 30 datapoints that has now been removed (see above).*
The respective description in the methods section might also have to be updated (confocal image data analysis).

We have updated the author checklist accordingly and made changes to the materials and methods confocal analysis section.

3) *Data availability section: please add a link that resolves to the data in the database.*

I have added a link that resolves to the data in the uploaded manuscript. I wasn't sure if it should be hyperlinked. If it should be hyperlinked, the current final sentence in the data availability section can be replaced with the following:
"These data can be directly accessed here."

4) *We noticed that co-author Joushua Youngs is now missing from the relevant author field in the online submission system. He is still listed on the cover page of the manuscript as author, but not in the Author Contributions. Could you please clarify this?*

I apologize, I'm not sure how that happened. He is still an author. I have added him back to the online submission system author page. I also moved him up in author position.

5) *Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.*

I uploaded a word doc file with the short summary and bullets and a separate png file of the synopsis image.

Also, I wanted to make sure that the first two authors (Kannangara and Poole) are listed as co-first authors. I have it that way in the manuscript, but I didn't see an option in the online submission system.

Thank you,

-Josh

3rd Revision - Editorial Decision 16th Jul 2021

Dr. Joshua Andersen
Brigham Young University
Chemistry and Biochemistry
C203 BNSN
685 E University Pkwy
Provo, UT 84602
United States

Dear Dr. Andersen,

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

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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

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

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Joshua L. Andersen
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51136V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Multiple replicates of each immunoblot experiment were completed (at least 3 per experiment). For confocal imaging, technical replicates from a single staining were used to ensure consistency in staining and laser power. SEM was calculated after processing multiple (typically 30) technical replicates.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample was discarded from the analysis unless there was a technical error that ruined the experiment (e.g., faulty transfer of a western blot, or failed secondary antibody in an imaging experiment)
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Analysis of confocal imaging was done with software (rather than subjective by-eye analysis)
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. These are biological samples, so normal distribution is assumed. Experiments were not modified in any way to nullify this assumption. For western blots we normalized all samples to the WT reference sample so that all other samples are represented as a fold-change to the WT (unless the variation between blots was not an issue as with figure S1C). We then analyzed the immunoblot data with more than one comparison using a repeated measures (RM) one-way ANOVA test followed by Fisher's LSD tests omitting the reference control (since it now has no standard deviation and therefore not normally distributed). RM was selected since each sample was linked (paired) to the other samples on the same blot (same gel, transfer, antibody signal etc.) We then selected the Fisher's LSD post hoc test since each sample is also independent (ATG13 KO sample is not affected by ATG13 WT sample) and because we had very few replicates to correct for (n=3). Immunoblots with only two samples were normalized as with other samples (fold-change to WT) and then analyzed with a one sample t-test comparing to a hypothetical mean of 1 according to the recommendation of GraphPad Prism software since (once again) our reference control has no standard deviation. Confocal analysis is based on a single biological replicate with 30 cells. Thus statistical comparison is not shown. Each sample is shown with all data points collected, the mean, and the standard error of the mean.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

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http://ClinicalTrials.gov
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http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Variance was calculated and accounted for by One-way ANOVA tests for all experiments with more than 2 comparisons. Variance between western blots was minimized by normalizing all samples to the control lane.
Is the variance similar between the groups that are being statistically compared?	Yes. Post hoc statistical comparisons were only made after One-way ANOVA was performed to assure that variance was similar enough to make statistical comparisons.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used are listed in Materials and methods section with catalog numbers for the suppliers. All antibodies were used for their appropriate, pre-evaluated/verified application.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were purchased from ATCC, authenticated via genotyping, and shown to be mycoplasma free

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Done
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We have posted our proteomics data to a public database as described in the Data Availability statement
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No