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Autophagy proteins control goblet cell function by potentiating reactive oxygen species production

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

1st Editorial Decision 01 June 2013

Thank you for submitting your paper to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find your study important and suitable for publication in the EMBO Journal. The referees raise a number of issues that should be resolved for publication here. Most of the concerns are centered on the need to provide better support for a role of autophagy in mucin secretion. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of the reviewers. I should add that it is EMBO Journal policy to allow only a single major round of revision and it is therefore important to address the concerns raised at this stage.

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

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

REFEREE REPORTS:

Referee #1:

Patel and colleagues explore the relationship between autophagy and secretion, prompted by the observation that in a mouse model deficient in Atg5 in the intestinal epithelium the goblet cells have altered mucin-containing granules. This observation ties in with literature published which suggests there is a link between secretion in specialized secretory cells and autophagy, although so far there are little mechanistic details. The data provided support the phenotype described and show that deletion of Atg5 impairs mucin secretion, and a change in the distribution of LC3 in the Atg5VC mice. A microarray analysis of genes altered in the Atg5VC mice identified endocytosis as a possible target pathway. Using LC3 as a marker and EEA1 for endosomes, the authors explore the relationship between endocytosis and autophagy proteins in secretion of mucin. They conclude that autophagy (ie LC3-positive vesicles) do not require endocytosis to form, but that endocytosis does play a role in mucin secretion. Finally, they provide a molecular link by demonstrating that NADPH, ROS and calcium levels correlate with effects seen when autophagy and endocytosis is inhibited. Data from a mouse model lacking p22phox-/- support the link as they show an inhibition of mucin secretion upon altered ROS levels, which phenocopies the manipulation of endocytosis which inhibit the colocalization of LC3 and EEA1.
The manuscript is clear and consise, and the in vivo and vitro data is very well presented and on the whole convincing. While the data do suggest a potential mechanism for the regulation of secretion that involves both the endocytic and autophagic machinery, mediated by ROS, there are a few controls missing, and most importantly some important additional experiments. These additional experiments will provide further support for their hypothesis based on the "amphisome", however what is really lacking is an insight into how fusion and the fusion machinery involved in mucin granule fusion with the plasma membrane is affected by the NADPH oxidase on the amphisome. The indirect evidence they provide about calcium suggests the classical SNARE-synaptotagmin machinery may be involved.

Major points:

1. Related to the microarray data Fig. S3 is not well explained- what are the green squares, and the red stars meant to represent? Fig. S3A is the same as Fig. 3A and its not clear how they conclude the endocytic pathway is more enriched than for example the Golgi apparatus.

2. Fig. 4 The data support the conclusion that endocytosis is not needed for LC3 vesicle formation but the authors have not demonstated if autophagy is altered. To support the conclusion autophagsome formation is altered they have to include analysis of LC3 lipidation with and with Baf A in all their conditions.

3. The image in Fig. 5D is insufficient to support their comclusion that this LC3 positive structure is a small endosome. It could just as easily be part of an early autophagosome. The authors should include an endocytic tracer in the experiment (small gold conjugated to BSA for example), and adhere to the points below.

4. The immunogold labeling overall is very nice. However the images show are too selective and larger fields need to be presented to allow the reader to assess the distribution of the proteins, the specificity of the antibody and the signal to noise. In general a membrane not expected to have label on it should always be included, ie the nucleus or a mitochondria in this particular case.

5. In this referees opinion, the most important experiment is to correlate the data shown in Fig 6 and 7 with the LC3 data, in particular the DPI, H2O2 and NAC treatments, for example Fig 6A, 7B, E, G and I. This should provide additional information (for example the recovery of LC3 vesicles in 7e

or h) in support of their model.

6. The model is somewhat misleading as the authors don't include the target membrane for fusion, the plasma membrane, they just state mucin granule release. This also highlights the point that the target for fusion inhibition is not at all addressed in this manuscript.

7. Related to point 6, the discussion about the amphisome is somewhat naÔve and confusing. They start out by stating the LC3 positive structures are not conventional autophagosomes (without defining what definition they use-is it the morphology) but they then say they are amphisomes, which some researchers, for example Seglen, would include in the definition as conventional autophagosomes, they are certainly involved in the formation of autolysosomes and autophagy. Amphisomes have also been shown to be the fusion partner with lysosomes, see references included by Seglen. Finally, the link with the "LAP" compartment identified by D. Green (see references cited) is not at all discussed. How can the authors exclude the possibility that the LC3 structures they observe are not the LAP?

Referee #2:

Patel and co-workers have explored the role of autophagy proteins in colonic goblet cells. They found that in these cells autophagy proteins are required for mucus secretion and mucin granules accumulate in their absence. LC3, an autophagome marker protein, however, does not localizes to the mucus-containing granules but rather to intracellular multi-vesicular structures that are often also positive for the early endosomal marker protein EEA1, leading the authors to propose that those copartments are amphisomes (autophagosomes fused with endosomal compartments). They also found that NADPH oxidases localized to and enhanced the formation of these LC3-positive organelles. Accordingly, the absence of autophagy proteins or inhibition of endosome formation impair the generation of reactive oxygen species by NADPH oxidases, which plays an important role in the regulation of mucin granule secretion in goblet cells, something also shown in this study.

This is a very interesting work where experiments have been very well planned and executed. It adds more to our understanding on the regulation of mucus secretion in goblet cells and implicate for the first time autophagy proteins in the control of this process.

MAJOR CRITICISM

In the title and abstract, in agreement with their data, the authors emphasize the role of autophagy proteins in controlling goblet cell function. In the result and discussion sections, however, the authors talk about the involvement of autophagy, in particular amphisomes, in goblet cell homeostasis, something that is not supported by experimental evidences. While their results clearly show that Atg5, Atg7 and LC3B play an important role in mucin-containing granule secretion, they are not showing that the contribution of these proteins is through their function in autophagy. Atg5, Atg7 and LC3B have been implicated in other pathways not linked to autophagy, including LC3-associated phagocytosis (LAP), a process that has recently also been shown to be involved in the establishment of a specialized endosomal compartment (Henault et al, 2012, Immunity, 37, 986-997). The authors have thus to clarify whether Atg5, Atg7 and LC3B contribute to mucus secretion regulation as components of either the autophagy machinery or another pathway. Two major aspects therefore have to be addressed:

1) The function of Atg5, Atg7 and LC3B is functionally linked during autophagy. These proteins are also involved in LAP. To prove that autophagosomes indeed contribute to the formation of the LC3B-positive multi-vesicular compartments, the author have to assess the involvement of other autophagy proteins that are not functionally linked with Atg5/Atg7/LC3B such as Atg14 and Fib200. The latter protein is not required for LAP. In addition, the authors have to analyze the LC3-positive structures observed in Dynasore-treated cells by immuno-electron microscopy. Under these conditions, the authors show that there is no fusion between LC3B-positive autophagosomes and EEA1-positive endosomes (which leads to the formation of LC3B- and EEA1-positive

amphisomes). If this is indeed the case, an ultrastructural examination must reveal the accumulation of double-membrane, LC3B-positive autophagosomes.
If autophagosomes are indeed accumulated, the discussion will have to address why cell treatment with Dynasore does not block autophagy (i.e. LC3 puncta formation; figures 4c and 4d) as shown by the laboratory of David Rubinsztein (Ravikumar et al, 2010, Nat Cell Biol, 12, 47-57). Moreover, amphisomes fuse with lysosomes during autophagy. Are the LC3B- and EEA1-positive organelles fusing with lysosomes? Are p22phox and and p47phox degraded? If not, possible interpretations have to be provided.

2) The authors indicate throughout the text that LC3B-positive autophagosomes fuse with endosomes. The term endosomes is quite vague because there are recycling, early and late endosomes, and EEA1 is a marker protein for the early endosomes and not for the recycling and late endosomes. To the best of my knowledge, late endosomes are the principal type of endosomes fusing with autophagosomes. To determine whether the LC3B- and EEA1-positive organelles studied in this work are bonafide amphisomes, the authors have also to study the distribution of at least one recycling endosome marker protein and one late endosome marker protein, for example the transferin receptor and CD63 or Rab7, respectively.

MINOR

Figure 3A: A magnification is needed to see that indeed the LC3 signal does not localize to the mucin-containing granules.

Figure 3B: An enlargement of the depicted LC3-positive structure has to be provided to clearly show the presence of internal vesicles. In addition, a quantification of LC3 subcellular distribution have to be provided (also for figures 5D and 5E). Moreover, to demonstrate the specific labeling of the used anti-LC3 antibody, the authors have to also label LC3B knockout goblet cells.

Figure 5D: The authors show this image and they affirm "we found that p22phox localized to LC3B-negative small vesicles, characteristic of endosomes". Unless the authors provide a double labeling with a specific marker, the depicted vesicle could also be derived from another compartments than an endosome.

The LC3B-positive structures are sometime called multi-vesicular compartments/organelles, sometime vesicles. These terms normally define structures of different size, with vesicles being carriers with a diameter ot 40-150 nm in most of the cases, unless being defined differently. This terminology is confusing and have to be consistent throughout the manuscript.

Referee #3:

The authors examine the impact of autophagy protein deficiency on goblet cell function. They report that deletion of several autophagy proteins (Atg5, Atg7, LC3B) leads to mucin accumulation as a result of impaired secretion. This is an important insight into how autophagy protein deficiency might be impacting host-pathogen interactions in the intestine, and how it may predispose to inflammatory bowel disease. The paper benefits from mice models and a novel in vitro organoid model to study goblet cell function. Many of the observations are novel and important for the field, and deserve publication in a high impact journal like EMBO J. However, I feel the paper is a bit overspeculative in some areas, and the model proposed doesn't make sense. A simplification of the text and removal of some data for future studies would make the paper much stronger.

Major comments:

-Semantic issue: the authors suggest that autophagy is intersecting with the endocytic pathway. This is a muddy issue since they do not show evidence of actual autophagy anywhere in their paper. ImmunoTEMs indicate single membrane LC3+ structures that have EEA1. I suspect that something analagous to LC3-associated phagocytosis is occurring (Sanjuan et al, Nature, 2007) not autophagy, and that this somehow promotes mucin secretion. Text changes would help clarify the issue.

-Same as above: LC3 is used as a maker of autophagosomes throughout the text, but this protein

labels many structures including protein aggregates that are induced with LPS and stress.

-Model: how can endosomal calcium promote secretion of large mucin granules? This is the biggest speculation in the paper, and doesn't make sense to me. Clamping calcium is expected to block secretion, but what evidence is there that autophagy proteins or NADPH oxidases impact calcium in these cells?

-The results of the microarray experiments are not clear. The data indicates changes, but which direction? Which endocytic factors are upregulated vs downregulated?

-Important controls for endocytosis inhibition (Dynasore and Pitstop) are required. Do these impact LC3-II formation by western blotting? PI3P production (2xFYVE probe)? Dynamins and clathrin do many things at the plasma membrane and elsewhere in the cell, so it is hard to conclude where they are acting. Considering this, I would recommend removing the endocytosis aspect of the paper altogether since it is confusing and requires further study. The phenotypes shown and the novel link to NADPH oxidases in goblet cells make this paper quite strong already.

-Figure 6. DCF is used to measure ROS. Another assay such as cerium chloride should be used to validate the findings and localize where ROS production is occurring. The observation that Atg5 deficiency decreases ROS is surprising, since increased ROS has been observed in other cell types lacking autophagy proteins Atg5 (Tal et al, PNAS, 2009) or Atg16 (Saitoh et al, Nature, 2008).

Minor points:

-the title points to ROS production, but this is one of the weaker points of the paper

-page 12, last paragraph, should define p47phox as component of NOX2 NADPH oxidase, not all NADPH oxidases.

-Figure 6B, A series of difference conditions are shown. How do the data for different conditions relate to each other? Are the controls that far apart?

-Does hydrogen peroxide induce LC3-II formation?

1st Revision - authors' response 30 August 2013

Referee #1 (Remarks to the Author):

Patel and colleagues explore the relationship between autophagy and secretion, prompted by the observation that in a mouse model deficient in Atg5 in the intestinal epithelium the goblet cells have altered mucin-containing granules. This observation ties in with literature published which suggests there is a link between secretion in specialized secretory cells and autophagy, although so far there are little mechanistic details. The data provided support the phenotype described and show that deletion of Atg5 impairs mucin secretion, and a change in the distribution of LC3 in the Atg5VC mice. A microarray analysis of genes altered in the Atg5VC mice identified endocytosis as a possible target pathway. Using LC3 as a marker and EEA1 for endosomes, the authors explore the relationship between endocytosis and autophagy proteins in secretion of mucin. They conclude that autophagy (ie LC3-positive vesicles) do not require endocytosis to form, but that endocytosis does play a role inmucin secretion. Finally, they provide a molecular link by demonstrating that NADPH, ROS and calcium levels correlate with effects seen when autophagy and endocytosis is inhibited. Data from a mouse model lacking p22phox-/- support the link as they show an inhibition of mucin secretion upon altered ROS levels, which phenocopies the manipulation of endocytosis which inhibit the colocalization of LC3 and EEA1. The manuscript is clear and consise, and the in vivo and vitro data is very well presented and on the whole convincing. While the data do suggest a potential mechanism for the regulation of secretion that involves both the endocytic and autophagic machinery, mediated by ROS, there are a few controls missing, and most importantly some important additional experiments. These additional experiments will provide further support for their hypothesis based on the "amphisome", however what is really lacking is an insight into how fusion and the fusion machinery involved in mucin granule fusion with the plasma membrane is affected by the NADPH oxidase on the amphisome. The indirect evidence they provide about calcium suggests the classical SNARE-synaptotagmin machinery may be involved.

Major points:
1. Related to the microarray data Fig. S3 is not well explained- what are the green squares, and the red stars meant to represent? Fig. S3A is the same as Fig. 3A and it's not clear how they conclude the endocytic pathway is more enriched than for example the Golgi apparatus.
Comment: We apologize for the confusion and have changed the data presentation and text to clarify the microarray analysis. We had previously performed pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG). We now include a chart that shows the most significantly altered pathways. The most significant pathway by this analysis was endocytosis. We have clarified the KEGG pathway chart in the figure legend.
Action taken: We have included the KEGG pathway chart that includes significance (Fig 3C). This table shows that endocytosis is the most significantly altered pathway in Atg5-deficient crypt base epithelial cells. We clarified in the figure legend for Fig S5A what the green squares (genes) and red stars (altered genes) represent. Additionally, for clarity, we have moved the GO term heat map to Fig S5C. We made changes to the Results sections on page 10-11.

2. Fig. 4 The data support the conclusion that endocytosis is not needed for LC3 vesicle formation but the authors have not demonstrated if autophagy is altered. To support the conclusion autophagosome formation is altered they have to include analysis of LC3 lipidation with and with Baf A in all their conditions.
Comment: We agree with the reviewer and in order to address this issue, we analyzed LC3 lipidation and autophagic flux using lysosomal inhibitors as suggested.

Action taken: We have included immunoblots of LC3 with and without Dynasore and BafA1 (Fig 4 E).We found that Dynasore treatment did not alter LC3 lipidation either with or without BafA1 treatment, indicating that Dynasore treatment does not inhibit autophagosome formation. We made changes to the Results sections on page 12.

3. The image in Fig. 5D is insufficient to support their conclusion that this LC3 positive structure is a small endosome. It could just as easily be part of an early autophagosome. The authors should include an endocytic tracer in the experiment (small gold conjugated to BSA for example), and adhere to the points below.
Comment: A point of correction, the vesicle in the original figure 5D was only labeled for p22phox antigens and is not LC3 positive (we only observed co-labeling as in the original figure 5E in larger vacuolar structures). That said, we agree with the reviewer's comment that we did not prove that the p22phox-positive vesicles were endosomes. To address this, we have added a lower power image immunogold data to show that the small p22phox positive vesicle is not encompassed by a larger vesicle. Instead of using an endocytic tracer, we performed double label immunogold localization with p22phox and Rab5 (a marker for early endosomes) and found vesicles with co-labeling for both p22phox and Rab5.
Action taken: We have included new figures: (i) a lower power image (Fig. S7B), and (ii) immunogold co-labeling with Rab5 and p22phox (Fig 5E) to demonstrate the p22phox+ small single membrane vesicles are endosomes. We made changes to the Results sections on page 13.

4. The immunogold labeling overall is very nice. However the images show are too selective and larger fields need to be presented to allow the reader to assess the distribution of the proteins, the specificity of the antibody and the signal to noise. In general a membrane not expected to have label on it should always be included, ie the nucleus or a mitochondria in this particular case.
Comment: We agree with the reviewer and have included lower power images (Fig S4). We did not note any LC3 staining on nuclei and mitochondria. Importantly, LC3 was also not present on mucin granules.
Action taken: We have included an additional lower power image of Fig 3B (Fig S4) showing negative membranes (mucin granules). Note that the background is minimal which shows specificity.

5. In this referees opinion, the most important experiment is to correlate the data shown in Fig 6 and 7 with the LC3 data, in particular the DPI, H2O2 and NAC treatments, for example Fig 6A, 7B, E, G and I. This should provide additional information (for example the recovery of LC3 vesicles in 7e or h) in support of their model.
Comment: We agree and the reviewer brings up an important point. It is important to demonstrate that autophagy (or LC3 lipidation) is not altered when later steps (p22phox deficiency, addition of hydrogen peroxide, BAPTA and ionomycin) in the pathway are manipulated.
Action taken: We have checked for LC3 lipidation and performed flux assays by immunoblotting in p22 mut spheroids (Added Fig. 5D) and spheroids treated with hydrogen peroxide, BAPTA and ionomycin (Added Fig. S9 A, C and D). In all cases, we observed no change in LC3 lipidation or autophagic flux, suggesting that autophagy is not altered when later steps in the pathway (ROS production by NADPH oxidases and calcium release) are manipulated. We added this data on pages 13 and 16 of the Results section.

6. The model is somewhat misleading as the authors don't include the target membrane for fusion, the plasma membrane, they just state mucin granule release. This also highlights the point that the target for fusion inhibition is not at all addressed in this manuscript.
Comment: We apologize for the confusion created by the model. The mechanisms by which mucin granule release is stimulated by calcium are not well understood and we don’t know what the target is in goblet cells.

Action taken: We have modified the model in Fig. 8 to show that ROS can induce intracellular calcium release (possibly from ER stores), which in turn, can influence mucin granule release. We added question marks between ROS and calcium release as well as between calcium release and mucin granule release as the precise mechanisms are still unclear in these areas.

7. Related to point 6, the discussion about the amphisome is somewhat naïve and confusing. They start out by stating the LC3 positive structures are not conventional autophagosomes (without defining what definition they use-is it the morphology) but they then say they are amphisomes, which some researchers, for example Seglen, would include in the definition as conventional autophagosomes, they are certainly involved in the formation of autolysosomes and autophagy. Amphisomes have also been shown to be the fusion partner with lysosomes, see references included by Seglen. Finally, the link with the "LAP" compartment identified by D. Green (see references cited) is not at all discussed. How can the authors exclude the possibility that the LC3 structures they observe are not the LAP?

Comment: This thoughtful comment has two underlying questions: 1) distinction between autophagosomes, amphisomes and autolysosomes and 2) question of involvement of LAP. To address question 1, autophagosomes, by definition, can be identified by their double or multiple delimiting membrane and unaltered cytoplasmic contents, amphisomes by a single delimiting membrane and contents that differ (often moderately) from the surroundings and autolysosomes by a single membrane and strongly altered contents (Berg et al., JCB 1998; 34:21883-92). The presence of an endocytic marker eliminates autophagosomes conclusively, but its absence may be fortuitous, depending on the section plane. The distinction between amphisomes and autolysosomes is difficult, depending on a quantitatively higher level of lysosomal enzymes/markers in the latter. We addressed this issue by performing additional double label immunogold studies using markers of lysosomes (Lamp1) and late endosomes (Rab7).

For the second point, LAP is known to involve recruitment of LC3 to single membranes (Sanjuan et al., Nature, 2007; 7173:1253-7 and Henault et al., Immunity 2012; 37:986-997). The LC3+ vacuoles we observed in goblet cells are multi-vesicular. However, we agree that it is important to distinguish the role of autophagy initiation and autophagosome elongation factors in goblet cell function. We addressed this issue by examining the phenotype of colonic spheroids that lack expression of FIP200 which is not required for LAP (Florey et al., Nature Cell Biology 2011; 11: 1335-1343). We provided further evidence in this regard by analyzing the role of Atg14L1 which is part of the VPS34 containing class III PI3K complex that is required for the initiation of autophagy.

Actions taken: 1) We have added figures that show the colocalization of LC3 with additional markers of the endocytic pathway (Rab7, Rab11 and Lamp1; Fig 3 E-G). These findings show that the autophagosomes can fuse with both early and late endocytic compartments. We added this new data to pages 10-11 of the Results. On page 17 of the Discussion we altered the text to read:

"These processes appeared to intersect at vacuoles that carry both autophagic (LC3) and endocytic (EEA1, Rab7) markers. All three processes..."

"We observed... EEA1. The majority of the autophagic vacuoles in colonic epithelial cells would thus seem to be amphisomes, formed by fusion between LC3-positive (but, by definition, EEA1-negative) autophagosomes and EEA1-positive endosomes.

2) To address the LAP issue, we analyzed the involvement of autophagy genes involved in autophagosome nucleation (Atg14 and Fip200, which are not involved in LAP). We added new data (Fig 2 I-L, Fig S3 B, C) that shows that loss of Atg14 and Fip200 also results in goblet cell mucin accumulation. These results suggest that a role for conventional autophagy rather than non-canonical processes such as LAP. We added new figures and test to the Results on page 9 and the Discussion on page 18.

Referee #2 (Remarks to the Author):

Patel and co-workers have explored the role of autophagy proteins in colonic goblet cells. They found that in these cells autophagy proteins are required for mucus secretion and mucin granules accumulate in their absence. LC3, an autophagome marker protein, however, does not localizes to the mucus-containing granules but rather to intracellular multi-vesicular structures that are often also positive for the early endosomal marker protein EEA1, leading the authors to propose that those compartments are amphisomes (autophagosomes fused with endosomal compartments). They also found that NADPH oxidases localized to and enhanced the formation of these LC3-positive organelles. Accordingly, the absence of autophagy proteins or inhibition of endosome formation impair the generation of reactive oxygen species by NADPH oxidases, which plays an important role in the regulation of mucin granule secretion in goblet cells, something also shown in this study.

This is a very interesting work where experiments have been very well planned and executed. It adds more to our understanding on the regulation of mucus secretion in goblet cells and implicates for the first time autophagy proteins in the control of this process.

MAJOR CRITICISM

In the title and abstract, in agreement with their data, the authors emphasize the role of autophagy proteins in controlling goblet cell function. In the result and discussion sections, however, the authors talk about the involvement of autophagy, in particular amphisomes, in goblet cell homeostasis, something that is not supported by experimental evidences. While their results clearly show that Atg5, Atg7 and LC3B play an important role in mucin-containing granule secretion, they are not showing that the contribution of these proteins is through their function in autophagy. Atg5, Atg7 and LC3B have been implicated in other pathways not linked to autophagy, including LC3-associated phagocytosis (LAP), a process that has recently also been shown to be involved in the establishment of a specialized endosomal compartment (Henault et al, 2012, Immunity, 37, 986-997). The authors have thus to clarify whether Atg5, Atg7 and LC3B contribute to mucus secretion regulation as components of either the autophagy machinery or another pathway.

Two major aspects therefore have to be addressed:
1) The function of Atg5, Atg7 and LC3B is functionally linked during autophagy. These proteins are also involved in LAP. To prove that autophagosomes indeed contribute to the formation of the LC3B-positive multi-vesicular compartments, the author have to assess the involvement of other autophagy proteins that are not functionally linked with Atg5/Atg7/LC3B such as Atg14 and Fip200. The latter protein is not required for LAP.
Comment: We agree that it is important to distinguish the role of conventional autophagy versus non-canonical processes that involve autophagy proteins. We agree that FIP200 has been shown to be not required for LAP (Florey et al., Nature Cell Biology 2011; 11: 1335-1343). We addressed this issue by examining the phenotype of colonic spheroids that lack expression of FIP200. We also provided further evidence in this regard by analyzing the role of Atg14 which is part of the VPS34 containing class III PI3K complex that is required for the initiation of autophagy.
Action: We have analyzed the involvement of autophagy genes involved in autophagosome nucleation (Atg14 and Fip200, which are not involved in LAP). We found that loss of Atg14 and Fip200 also resulted in goblet cell mucin accumulation (Fig 2 I-L), suggesting a role for conventional autophagy and not LAP. We added figures showing this new data and added text to the Results section on page 9 and the Discussion on page 18.

In addition, the authors have to analyze the LC3-positive structures observed in Dynasore-treated cells by immuno-electron microscopy. Under these conditions, the authors show that there is no fusion between LC3B-positive autophagosomes and EEA1-positive endosomes (which leads to the formation of LC3B- and EEA1-positive amphisomes). If this is indeed the case, an ultrastructural examination must reveal the accumulation of

double-membrane, LC3B-positive autophagosomes.
If autophagosomes are indeed accumulated, the discussion will have to address why cell treatment with Dynasore does not block autophagy (i.e. LC3 puncta formation; figures 4c and 4d) as shown by the laboratory of David Rubinsztein (Ravikumar et al, 2010, Nat Cell Biol, 12, 47-57). Moreover, amphisomes fuse with lysosomes during autophagy. Are the LC3B- and EEA1-positive organelles fusing with lysosomes? Are p22phox and and p47phox degraded? If not, possible interpretations have to be provided.
Comment: It would be difficult to quantify LC3-positive structures by immunogold as the sample size would be very small. We have quantified LC3+ structures by immunofluorescence data, which shows no significant difference in LC3 puncta with Dynasore treatment (Fig. 4C). We found that block of endosome formation does not block autophagy. We are aware the work by Rubinsztein and colleagues that elegantly shows that endosomal membranes are a source of autophagosomes. There are other potential sources of membranes that could be the source of autophagosome initiation (Levine et al., Nature 2011; 7330: 323-35). As we did not examine this question in this study, we did not speculate on this point in the discussion.
Action: We have included new data of LC3 immunoblots with and without Dynasore and BafA1 (Fig 4 E). We observed no change in LC3 lipidation or autophagic flux upon treatment with Dynasore, indicating that Dynasore does not block autophagy in our cell culture system. We made changes to the Discussion on page 18. Additionally, we have included new data of additional markers of the endocytic pathway (Rab7, Rab11 and Lamp1Fig 3 E-G). Co-labeling of LC3 with these markers suggests fusion of autophagosomes with lysosomes and late endosomes; however, not recycling endosomes. We made changes to the Results on page 11. We added new data showing that p22phox was not degraded by autophagy (Fig S7A).

2) The authors indicate throughout the text that LC3B-positive autophagosomes fuse with endosomes. The term endosomes is quite vague because there are recycling, early and late endosomes, and EEA1 is a marker protein for the early endosomes and not for the recycling and late endosomes. To the best of my knowledge, late endosomes are the principal type of endosomes fusing with autophagosomes. To determine whether the LC3B- and EEA1-positive organelles studied in this work are bonafide amphisomes, the authors have also to study the distribution of at least one recycling endosome marker protein and one late endosome marker protein, for example the transferin receptor and CD63 or Rab7, respectively.
Comment: The presence of EEA1 and MPR in amphisomes has been demonstrated in hepatocytes (Berg et al., JCB 1998; 34:21883-92), indicating that they may be formed by fusion of autophagosomes with EEA1-positive early endosomes as well as with MPR-positive late endosomes.
Action: We have added new data that included additional markers of the endocytic pathway (Rab7, Rab11 and Lamp1Fig 3 E-G). Co-labeling with these markers suggests fusion with lysosomes and late endosomes; however, not recycling endosomes. We made changes to the Results section on page 11.

MINOR

Figure 3A: A magnification is needed to see that indeed the LC3 signal does not localize to the mucin-containing granules.
Action taken: We have included additional lower power image of Fig 3B (Fig S4) showing negative membranes (mucin granules).

Figure 3B: An enlargement of the depicted LC3-positive structure has to be provided to clearly show the presence of internal vesicles. In addition, a quantification of LC3 subcellular distribution have to be provided (also for figures 5D and 5E). Moreover, to demonstrate the specific labeling of the used anti-LC3 antibody, the authors have to also label LC3B knockout goblet cells.

Action taken: We have added new data with the quantification of p22phox co-localization with LC3 (panel to Fig 5F). We found approximately 60% of LC3 co-localized with p22phox puncta, suggesting that a majority of LC3 (and EEA1) + structures contain p22phox. We added a comment in the Results on page 13.

We have performed immunogold labeling of colons from Atg5VC mice with the anti-LC3 antibody and we did not see any signal on membranes. This is a negative result that is difficult to portray, so we did not include any image in the manuscript. We stated on page 10 of the Results that we did this control.

Figure 5D: The authors show this image and they affirm "we found that p22phox localized to LC3B-negative small vesicles, characteristic of endosomes". Unless the authors provide a double labeling with a specific marker, the depicted vesicle could also be derived from another compartments than an endosome.

Action taken: We have added new data to Fig S7B to include a low power image of what is shown in Fig 5E. Also, we added a double label of p22phox and Rab5 to Fig 5E to support the conclusion that the small single membrane vesicles are endosomes. We added a comment in the Results on page 13.

The LC3B-positive structures are sometime called multi-vesicular compartments/organelles, sometime vesicles. These terms normally define structures of different size, with vesicles being carriers with a diameter to 40-150 nm in most of the cases, unless being defined differently. This terminology is confusing and has to be consistent throughout the manuscript.

Action taken: We agree with the reviewer and apologize for the confusion. Since the structures we observe are larger than 200 nm, we have changed wording throughout the text to refer to these structures as vacuoles.

Referee #3 (Remarks to the Author):

The authors examine the impact of autophagy protein deficiency on goblet cell function. They report that deletion of several autophagy proteins (Atg5, Atg7, LC3B) leads to mucin accumulation as a result of impaired secretion. This is an important insight into how autophagy protein deficiency might be impacting host-pathogen interactions in the intestine, and how it may predispose to inflammatory bowel disease. The paper benefits from mice models and a novel in vitro organoid model to study goblet cell function. Many of the observations are novel and important for the field, and deserve publication in a high impact journal like EMBO J. However, I feel the paper is a bit overspeculative in some areas, and the model proposed doesn't make sense. A simplification of the text and removal of some data for future studies would make the paper much stronger.

Major comments:

-Semantic issue: the authors suggest that autophagy is intersecting with the endocytic pathway. This is a muddy issue since they do not show evidence of actual autophagy anywhere in their paper. ImmunoTEMs indicate single membrane LC3+ structures that have EEA1. I suspect that something analogous to LC3-associated phagocytosis is occurring (Sanjuan et al, Nature, 2007) not autophagy, and that this somehow promotes mucin secretion.

Comment: We agree that it is important to distinguish the role of conventional autophagy vs. non canonical processes that involve autophagy proteins.

Action taken: To address the LAP question, we have analyzed the involvement of autophagy genes involved in autophagosome nucleation (Atg14 and Fip200, which are not involved in LAP). We found that loss of Atg14 and Fip200 also resulted in goblet cell mucin accumulation (Fig 2 I-L), suggesting a role for conventional autophagy and not LAP. We added new figures and test to the Results on page 9 and the Discussion on page 18.

-LC3 is used as a maker of autophagosomes throughout the text, but this protein labels many structures including protein aggregates that are induced with LPS and stress.

Comment: It is certainly possible that LC3 localized with other structures such as protein aggregates. Given that we have shown that majority of the LC3 puncta co-localize with EEA1 (Fig 4D), we propose that this is a significant proportion of the LC3 signal.

Action taken: We have included lower power immunogold EM images (Fig S4 and S7B) to show that most LC3 localizes to membranes.

-Model: how can endosomal calcium promote secretion of large mucin granules? This is the biggest speculation in the paper, and doesn't make sense to me. Clamping calcium is expected to block secretion, but what evidence is there that autophagy proteins or NADPH oxidases impact calcium in these cells?

Comment: It is not clear in the field how calcium release from intracellular stores is related to mucin secretion. In addition, the cellular source of the calcium is not defined. Our study shows that autophagy proteins acting through NADPH oxidases control this process.

Action taken: We have modified the model (Fig 8) to indicate that ROS increases intracellular calcium, which results in mucin granule release. We have included question marks where the mechanism is unclear.

We have also added Fig S 9B to show that intracellular diminished calcium levels correlate with mucin granule accumulation phenotype. We added text to the Results on page 16.

-The results of the microarray experiments are not clear. The data indicates changes, but which direction Which endocytic factors are upregulated vs downregulated?
Comment: We apologize for the confusion and have changed the data presentation and text to clarify the microarray analysis.
Action taken: We have included KEGG pathway chart (Fig 3C) and have clarified in the figure legend. Additionally, we have moved the GO term heat map to Fig S5B. We also included a supplemental table that includes the fold differences of endocytic factors in Atg5-deficient epithelial cells vs. controls. We clarified the text in the Results on pages 10-11.

-Important controls for endocytosis inhibition (Dynasore and Pitstop) are required. Do these impact LC3-II formation by western blotting? PI3P production (2xFYVE probe)? Dynamins and clathrin do many things at the plasma membrane and elsewhere in the cell, so it is hard to conclude where they are acting. Considering this, I would recommend removing the endocytosis aspect of the paper altogether since it is confusing and requires further study. The phenotypes shown and the novel link to NADPH oxidases in goblet cells make this paper quite strong already.
Comment: We agree that LC3-II formation needs to be assessed with Dynasore treatment.
Action taken: We have included immunoblots of LC3 with and without Dynasore and BafA1 (Fig 4 E). Dynasore treatment does not alter LC3 lipidation or autophagic flux, suggesting that Dynasore does not inhibit autophagy. We added text to page 11 of the Results and Discussion on page 18.

-Figure 6. DCF is used to measure ROS. Another assay such as cerium chloride should be used to validate the findings and localize where ROS production is occurring. The observation that Atg5 deficiency decreases ROS is surprising, since increased ROS has been observed in other cell types lacking autophagy proteins Atg5 (Tal et al, PNAS, 2009) or Atg16 (Saitoh et al, Nature, 2008).
Comment: This is an excellent point and we carried out cerium chloride experiments in colonic epithelial cells grown in monolayer.
Action taken: We have added Fig 6D to show that ROS production is localized to complex vacuoles, characteristic of amphisomes. We added this experiment to the Results on pages 14-15.

Minor points:

-the title points to ROS production, but this is one of the weaker points of the paper
Comment: see major point above regarding cerium chloride experiments.

-page 12, last paragraph, should define p47phox as component of NOX2 NADPH oxidase, not all NADPH oxidases.
Action taken: We apologize for this error and have corrected the text accordingly.

-Figure 6B, A series of difference conditions are shown. How do the data for different conditions relate to each other? Are the controls that far apart?
Comment: These values are not that far apart. They are plotted on different graphs because these experiments were done separately with paired controls (in some instances treated with vehicle).

-Does hydrogen peroxide induce LC3-II formation?

Action taken: We have checked for LC3 lipidation and performed flux assays by immunoblotting in p22 mut spheroids (Fig. 5D) and spheroids treated with hydrogen peroxide, BAPTA and ionomycin (Fig. S9 A, C and D).Our results suggest that manipulating downstream events (ROS and calcium) does not alter autophagy (LC3 II formation). We added text to page 15 of the Results.

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Thank you for submitting your revised manuscript to The EMBO Journal. Referees #1 and 2 have now reviewed the manuscript and their comments are provided below.

As you can see both referees appreciate the introduced changes and support publication here. I am therefore very pleased to accept your paper for publication here. Referee #1 suggests adding one more reference, but that is it! You can send me a modified word document by email.

Please also see below for important information on how to proceed and return necessary forms to us as soon as possible. We also a need Conflict of Interest, and Author Contribution statements before we can transfer your manuscript to the Publisher. Please see our Author Instructions under the section Manuscript Preparation - Manuscript Format for further information.

Thank you for contributing to the EMBO Journal.

Referee #1:

The authors have done an excellent job in clarifying and addressing the points raised, in particular with the additional experiments.

One minor point. The reference to Razi et al., J Cell Biology 185:305 2009 should be added as these authors demonstrated that LC3 and EEA1 colocalize and early endosomes contribute to autophagy.

Referee #2:

The authors appropriately addressed my criticisms and I also believe those of the other referees. This manuscript can now be published in EMBO J.

