Autophagy suppression by TORC1 maintains epithelial plasma membrane integrity and inhibits syncytium formation

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Dear Parisa,

Thank you for submitting your manuscript and your point-by-point response to The EMBO Journal. Your study was reviewed at Review Commons where the review process was carried out.

I have now had a chance to take a careful look at your manuscript and your response to the comments raised by the referees. I am sorry for the slight delay in getting back to you, but I wanted to discuss some of the issues further with referee #3 in order to give you more definitive input.

First, let me add that I find your analysis supporting that autophagy leads to the breakdown/ removal of lateral plasma membranes and that this promotes cell fusion and syncytium formation very interesting!

The points that I wanted to discuss further with referee #3 concerned 1) if more genetic support for the role of autophagy is needed and 2) what level of support do we need for the finding that autophagy promotes degradation/removal of lateral membranes.

I have now heard back from the referee and the referee finds that Figures D (Colocalization of Atg8a/FasIII) and F (EM showing autophagosomes with membranes) in your response provide OK support for autophagy of lateral membranes.

Referee #3 did note that adding more EM examples and another marker in addition to FasIII would be great. I presume that you have more EM images on hand. Is there another marker besides FasIII that you can use? I think it would strengthen the findings if you could add another marker. This part of the paper is an exciting and the more support that you can provide for this the stronger the paper will be.

Regarding the question if this process requires classical autophagy, an unconventional role, the entire whole autophagy machinery or autophagy completion is not entirely clear.

Referee #3 had originally suggested that you could resolve the claim that later steps of autophagy are not required for cell fusion by looking at genes that target autophagosome-lysosome fusion such as snap29, stx17 and vamp7.

I see the issue raised with obtaining the lines and have discussed this with the referee. While it would be nice to have this issue resolved, we also see your point. I would like to ask you to tone down the conclusion that later steps of autophagy are not required. Do keep the data in the paper and speculate on this in the discussion.

Regarding the other points raised by the referees, please go ahead and respond to them as discuss in your response.

I am happy to discuss the revisions further - we can also arrange for a video call if that is helpful.

You can use the link below to upload the revised version. I have attached a document with helpful tips on how to prepare the revision.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days. Let us know if you need an extension. Please click on the link below to submit the revision online before 8th Feb 2022:

Link Not Available

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Review #1

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In this study, the authors use the fruit fly as a model to understand the role and regulation of autophagy in epidermal integrity during development and wound healing. They discover that hyper activation of autophagy via overexpression of Atg1 leads to disruption of epithelial organization, junctional protein localization, and syncytium formation. In addition, these epidermal defects were found to be dependent on TORC1 as knockdown or inhibition of TORC1 antagonists resulted in similar epidermal defects which could be rescued by knockdown of Atg1 or Atg5. Wound healing in fruit fly epidermis is known to induce cell fusion and here the authors show that syncytium formation is dependent on autophagy. GFP-Atg8a autophagosomes were found to accumulate in cells adjacent to the wound site, but Atg1-induced syncytium formation was dispensable for wound repair. However, the authors found that hyper activation of autophagy prior to injury slowed wound closure. This may be due to defects in actomyosin organization or another developmental defect the authors observed in the epidermis. Overall, the key conclusions of this study are convincing, but the experiments would be strengthened by validation of all the RNAi strains used as well as demonstration that epidermal barrier remains intact as described.

**Major Comments**

1. This study uses a number of UAS-RNAi strains as well as dominant negative and overexpression transgenes. There is no validation that these genetic perturbations work as expected. In fact, the authors state on pg 5 that RNAi to Atg6, Atg7, and Atg12 may be less effective, but do not verify the knockdown efficiency to the gene of interest (i.e. Atg5 RNAi knock downs Atg5 transcript or protein). This is particularly important as authors use a single UAS-rictor RNAi strain to conclude that autophagy is dependent on TORC1 and not TORC2. If rictor RNAi is also weak or ineffective than this conclusion would be erroneous.

2. A major conclusion of this study is that autophagy remodels the lateral cell membranes and not the basal or apical, so the membrane integrity remains intact. This is described and shown in Fig S3a, but it is hard to see that the apical membrane is intact. It would be helpful if authors could show a true membrane marker, such as UAS-CD8mGFP to see if there is a continuous membrane. Alternatively, is there a barrier assay that could help demonstrate that syncytium formation does not disrupt epithelial integrity? This could be performed in the fly gut, using the smurf assay (Rera M et al. 2011), since the authors also describe (pg 9) a similar role for autophagy in disruption of epithelial lateral membranes.
3. Is autophagy dependent syncytium formation cell autonomous? The A58-Gal is not cell-type specific as authors describe (pg 9) similar effects in trachea, salivary glands, and intestine and it is unclear if effects are due to disruption of autophagy in epidermal cells or general disruption in fly's physiology. The authors should determine, using a more restrictive Gal driver, whether syncytium formation is due to activation of autophagy in the epidermal cells or another cell type (trachea, salivary glands, or intestine). Alternatively, if no other Gal4 is available for the larval epidermis then authors could at least show using enterocytes driver (NP1-Gal4) that overexpression of Atg1 is sufficient to induce syncytium formation and its effect on gut barrier integrity.

4. In Fig 8, authors nicely show that Atg1 RNAi can rescue Tor RNAi and raptor RNAi, but, what about the reverse. Is overexpression of Tor sufficient to inhibit the overexpression Atg1 and reduce autophagy-induced syncytium formation?

**Minor comments:**

1. Check spelling of abbreviations, Sqh is often misspelled Shq in figures
2. The order of images in Figure 3 should match the description in the text (pg. 6). AtgW is described in text, but not shown in Fig 3a-c. Also, upstream activators of TORC1 are described first, but shown last in this Figure making it difficult to follow.
3. Fig7a should show junctional effect of Atg1W alone and in combination with Atg5i which is used in 7b. It is unclear why authors switched to this weak overexpression for this photobleaching assay when Atg1S was predominantly used in the rest of the study.

3. **Significance:**

Significance (Required)

This study elucidates the role and regulation of TORC1 and autophagy in epithelial membrane remodeling. This is important work that is significant to both developmental and wound healing research. Many cell types become multinucleate during differentiation, aging, and wound healing and here the authors find a novel role for autophagy in remodeling lateral cellular junctions to facilitate syncytium formation.

Review #2

1. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month
2. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In their present manuscript Kakanj and colleagues show that during epithelial wound healing autophagy pathway controls plasma membrane integrity and homeostasis. Furthermore, elevated autophagic activity is sufficient to induce syncytium formation, which is essential for wound closure and healing. Authors used the epidermis of fruit fly larvae as model to study wound healing and video microscopy to examine this process. The methodology is well established, since authors already published a related study in 2016 using similar tools.

The findings presented here are interesting and promising, the quality of most experiments are satisfactory, the confocal images/videos are excellent and I truly appreciate that authors used electron microscopy to support some of their claims. Findings are well presented and the text is well written and easy to read.

Overall, my opinion is very positive about this manuscript.

I believe most of the findings are very well supported, but I have some suggestions, which may can help strengthen the authors' points.

1) Authors used GFP-Atg8a reporter to follow autophagy during wound healing. While I also believe that, the appearing GFP-Atg8a dots represent autophagic vesicles after wounding but GFP-Atg8a has some certain limitations. First: Atg8a (or LC3 in mammals) is removed from the outer surface of autophagosomes by Atg4 and the Atg8a trapped inside the autophagosomes will be degraded in the autolysosomal lumen. Thus Atg8a not always localizes to autolysosomes, actually Atg8a immunostaining mostly labels autophagosomes (and phagophores) but not autolysosomes in insect cells. Accordingly, GFP-Atg8a reporter is also subject of autolysosomal degradation and furthermore most of the GFP signal is quenched in the acidic lumen of autolysosomes, since at lower pH GFP loses fluorescence. Nevertheless, if lysosomal degradation proceeds normally, GFP-Atg8 will be degraded completely. Thus, some of the autolysosomes cannot be detected using this reporter, for this mCherry-Atg8a reporters can be used, since mCherry is more resistant than GFP and thus accumulate inside lysosomes, and retains its fluorescence in acidic environments. However, I still believe that for video microscopy GFP-Atg8a was a perfect choice, I just suggest to confirm the appearance of autophagosomes after wounding by other means: for instance, immunostaining of the epidermis after wounding (120 min) against Atg8a should confirm the presence of autophagosomes. There are a few specific available antibodies working in flies which are listed in the reviews of Nagy (PMID: 25481477) or more recently in Lorincz (PMID: 28704946)

2) One of the major claims of the authors is that elevated autophagy leads to the breakdown or removal of lateral plasma membranes to promote syncytium formation. It is clearly seen on the confocal or EM images that lateral membranes disappear after wounding. However, it is also suggested that the lateral plasma membrane material is incorporated into autophagosomes or plasma membrane is a potential membrane source of autophagosome formation. I believe this is the least supported claim of the manuscript since no direct evidence for this is presented. This is based on BodyPy staining only, that BodyPy positive vesicles accumulate inside the cells. If this is indeed the case plasma membrane components should be detected in autophagic vesicles. Thus, I recommend co-staining membrane components with autophagic markers. However if authors observe no colocalization of plasma membrane components with autophagy markers I still believe this study worth to be published. I would like to recommend the review of Ungermann and Reggiori (PMID: 29966469) in which the trafficking of Atg9 is discussed, since the source of autophagosomal Atg9 is in part the plasma membrane in mammalian cells. Therefore, these findings may
**Minor points:**

Figure 2A: I believe authors wanted to use the word ‘maintaining’ not mating in their scheme.

Discussion: Authors suggest that: another function of autophagy in the cells surrounding the wound may be to clear up debris as in planarian and other cell types autophagy is activated in healthy cells, which simultaneously phagocytose cell debris. Honestly, I do not believe that this is the case here. Some of the Atg proteins are indeed required for phagocytosis during LC3-associated phagocytosis (LAP) (see: PMID: 30787029), but LAP is independent form Atg1 and if LAP happened in the cells, surrounding the wound then GFP-Atg8a positive phagosomes would appear in those cells. However, it is clearly not the case here.

3. Significance:

**Significance (Required)**

I highly recommend this manuscript to be uploaded to a relevant journal and I believe the findings presented here will be interesting for biologists specialized in regeneration and readers from the autophagy fields alike.

Review #3

1. How much time do you estimate the authors will need to complete the suggested revisions:

**Estimated time to Complete Revisions (Required)**

(Decision Recommendation)

 Between 1 and 3 months

2. Evidence, reproducibility and clarity:

**Evidence, reproducibility and clarity (Required)**

**Summary:**

The larval epidermis of Drosophila is a prime model for studying wound healing by combining live imaging with cellular, genetic and molecular analysis of the processes involved. Autophagy is known to be activated and necessary for efficient wound healing in animal models through secretion of cytokines and clearance of bacteria. This manuscript implicates autophagy in cellular syncytium formation during wound healing. Live imaging demonstrates autophagy activation in cells surrounding the wound. Inhibition of autophagy by RNAi against atg1 or atg5, required for autophagy initiation and autophagosome formation had no effect on the rate
of constriction and closing of the wound site. However, elegant live imaging demonstrates that autophagy is required cell autonomously for cell fusion, a normal process during wound healing in flies. Autophagy can also be instructive for cell fusion. Strong induction of autophagy by TORC1 inhibition, TSC1/2 overexpression or Atg1 overexpression induce cell fusion that is genetically dependent on atg5, a gene acting downstream of atg1 in autophagosome formation. As Chloroquine treatment, a chemical inhibiting autophagosome fusion to the lysosome and lysosomal breakdown showed no effect, the authors suggest that later steps of autophagy are not involved. Live imaging with a selection of cellular fluorescently tagged markers of apical, lateral and basolateral membrane domains, combined with electron microscopy show clearly that lateral membrane are disrupted and removed within the epithelium. During this process, membranous large vesicles "drift" away from the plasma membrane. If these vesicles relate to autophagy is not addressed. In addition to the effect on cell fusion, strong autophagy induction also leads to autophagy within the nucleus, chromatin condensation and distortion of the nuclear membrane. The manuscript is well written and easy to follow. Figure panels and data are clearly presented. All experiments are well described throughout and skillfully executed with appropriate controls and statistical analysis. It remains unknown what induces autophagy in response to wounding. It also remains unclear whether autophagy deconstructs or engulfs parts of the plasma membrane, or if parts of the autophagy machinery has additional roles in plasma membrane fusion.

**Major comments:**

- Are the key conclusions convincing?
  - Conclusions are generally balanced and convincing.
  - I have seldom seen a paper so well written, presented and balanced by first pass. Hence my experimental suggestions are few.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
  - Claims are well founded,

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary to evaluate the paper as it is, and do not ask authors to open new lines of experimentation.
  - The inhibition of autophagy is performed using knockdown of two genes acting in autophagy initiation (atg1, a part of the ULK1 kinase complex) and atg5, required for autophagosome formation. Later acting genes in the autophagy process such as autophagosome closure, fusion with the lysosome or degradation were not analyzed. In the abstract, the authors state "Proper functioning of TORC1 is needed to prevent autophagy from destroying the larval epidermis which depends on membrane isolation and phagophore expansion, but not fusion of autophagosomes to lysosomes". As far as I can see, the last statement on fusion derives from experiments with Chloroquine. Although frequently used for qualitative experiments, CQ is not suited for conclusive experiments. Without genetic experiments targeting genes for autophagosome-lysosome fusion such as snap29, stx17, vamp7 this statement is in my mind not well supported.

- Are the suggested experiments realistic for the authors? It would help if you could add an estimated cost and time investment for substantial experiments.
  - Given the expertise of the authors, these experiments should be easy to perform within 3 months.

- Are the data and the methods presented in such a way that they can be reproduced?
  - The manuscript is well written and an excellent example of how how methods and experiments should be presented. Methods, tools and experiments are all well described.
• Are the experiments adequately replicated and statistical analysis adequate?
-Replicates and statistics are adequate and custom for the type of analysis performed.

**Minor comments:**

• Specific experimental issues that are easily addressable.

Figure 3 h. The live imaging documents the striking disappearance of lateral cell membranes using SRC-GFP. In 3h, large vesicle formation and movement towards the cell interior is shown. How frequent is this? Is this believed to be the mechanism of lateral membrane removal? If so, is it dependent on the autophagy machinery? Are these vesicle positive for autophagy markers? Resolving this issue may lift the conclusions of the paper. Using 3xCherry-Atg8 together with SRC-GFP, this should be possible.

Using CQ, the authors should be able to detect plasma membrane and junctional components in autophagosomes or autolysosomes (by confocal and electron microscopy) as degradation is inhibited. This should help to distinguish whether lateral membranes are engulfed and digested or if cells simply fuse, by using a part of the autophagy machinery.

The authors, state that strong autophagy activation also leads to syncytium formation of tracheal cells, salivary glands and gut EC cells. Representative images in a supplementary figure would be useful for future reference.

• Are prior studies referenced appropriately?
-Yes. Key literature and findings are cited and discussed.

• Are the text and figures clear and accurate?
-Yes

• Do you have suggestions that would help the authors improve the presentation of their data and conclusions?
-See suggested experiments above.

3. Significance:

Significance (Required)

• Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.

-The findings clearly documents a role of autophagy in syncytium formation in the physiological process of wounding. This has parallels to muscle syncytium formation, but has to my knowledge not been demonstrated in any other cell type to be performed by autophagy. Moreover, the authors show that strong autophagy induction can lead to fusion of epithelial cells. This may have relevance for processes and diseases where polyploidy are observed.

• Place the work in the context of the existing literature (provide references, where appropriate).

• State what audience might be interested in and influenced by the reported findings.
-The data are very strong and the demonstration that autophagy controls syncytium formation outside of muscle development is surprising and significant. It is of interest to the field of cell biology and development in general and the autophagy field in particular. It will also be of interest for the medical field that deals with multinuclear phenotypes, such as cancer.

- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

-Development, cell signaling, autophagy, vesicle trafficking.
Dear Sara,

We describe below our responses to the referees, whom we thank for their critique and suggestions.

We would be grateful if you could transfer the manuscript to The EMBO Journal for consideration.

We note in the responses below which of the suggested experiments we will be able to do within a reasonable time frame. We expect to be able to complete the experimental work, including imaging and analysis, around the end of November.

Some experiments, though desirable, are either not possible at all (e.g. all necessary constructs are on the same chromosome; necessary fluorophore combinations not available etc.) or would take too long because they would require very lengthy crossing schemes – if we were even able to obtain the necessary stocks in the first place. This is also explained in the responses.

However, we hope the editors at The EMBO Journal and the referees will find that our proposed changes and additional experiments will strengthen our manuscript and make it suitable for publication in EJ.

Best wishes

Parisa and Maria on behalf of all authors.
Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this study, the authors use the fruit fly as a model to understand the role and regulation of autophagy in epidermal integrity during development and wound healing. They discover that hyper activation of autophagy via overexpression of Atg1 leads to disruption of epithelial organization, junctional protein localization, and syncytium formation. In addition, these epidermal defects were found to be dependent on TORC1 as knockdown or inhibition of TORC1 antagonists resulted in similar epidermal defects which could be rescued by knockdown of Atg1 or Atg5. Wound healing in fruit fly epidermis is known to induce cell fusion and here the authors show that syncytium formation is dependent on autophagy. GFP-Atg8a autophagosomes were found to accumulate in cells adjacent to the wound site, but Atg1-induced syncytium formation was dispensable for wound repair. However, the authors found that hyper activation of autophagy prior to injury slowed wound closure. This may be due to defects in actomyosin organization or another developmental defect the authors observed in the epidermis. Overall, the key conclusions of this study are convincing, but the experiments would be strengthened by validation of all the RNAi strains used as well as demonstration that epidermal barrier remains intact as described.

**Major Comments**

1. This study uses a number of UAS-RNAi strains as well as dominant negative and overexpression transgenes. There is no validation that these genetic perturbations work as expected.

Almost all of the lines we use have been extensively used and validated by others as documented in the literature. We append a table (below, page 14) with these references. It would be close to impossible for us to show their tissue specific efficacy in the larval epidermis because it is extremely difficult to obtain clean dissections of epidermis without contamination from other tissues (muscles, nerves, etc.), and we believe we can rely on the known validation of most of the lines. It is true that some of the lines are less well characterised, and we comment on those below, and will eliminate our speculation on their effects in the manuscript.

In fact, the authors state on pg 5 that RNAi to Atg6, Atg7, and Atg12 may be less effective, but do not verify the knockdown efficiency to the gene of interest (i.e. Atg5 RNAi knock downs Atg5 transcript or protein).

Atg12 and Atg7 have been shown (PMID: 25882046) by quantitative RT-PCR to effectively reduce RNA levels in the midgut during larval to pupal transition. We will therefore have to eliminate our speculation that the weak effect in the epidermis may be due to ineffective knock-down. Rather, it seems that these components are accessory but not necessarily essential for the completion of autophagy, as also observed by others (PMID: 25882046, PMID: 1805642, PMID: 23599123, PMID: 15296714, PMID: 23873149, PMID: 23406899)

This is particularly important as authors use a single UAS-riCTOR RNAi strain to conclude that autophagy is dependent on TORC1 and not TORC2. If rictor RNAi is also weak or ineffective than this conclusion would be erroneous.

The function of rictor has been validated by classic genetics: Animals homozygous for deletions of rictor show no defects throughout their normal life cycle (Hietakangas and Cohen, 2007). We have also shown that epidermis of homozygous rictorΔ1 larvae (marked with Src-GFP, DsNuc-Red2) shows no abnormalities in cell shapes or cell membranes. We include an image here.
A major conclusion of this study is that autophagy remodels the lateral cell membranes and not the basal or apical, so the membrane integrity remains intact. This is described and shown in Fig S3a, but it is hard to see that the apical membrane is intact. It would be helpful if authors could show a true membrane marker, such as UAS-CD8mGFP to see if there is a continuous membrane.

We will include new experiments with this marker.

Alternatively, is there a barrier assay that could help demonstrate that syncytium formation does not disrupt epithelial integrity?

This follows from the fluorescence recovery we performed (Supplementary Video 13), where we observe rapid diffusion between areas in the epidermis, but never any leakage of fluorescence in the y-axis into the body cavity. We will emphasize this more clearly in the text.

This could be performed in the fly gut, using the smurf assay (Rera M et al. 2011), since the authors also describe (pg 9) a similar role for autophagy in disruption of epithelial lateral membranes.

We had done a smurf assay, and observed no leakage from the gut, but didn’t document this at the time because of difficulties during the period of Covid restrictions of accessing a dissecting scope/camera set up in a lab outside our own. We will try to repeat this now in the hope that with current reduced restrictions we can record the result.

3. Is autophagy dependent syncytium formation cell autonomous?

Our clonal analysis in wound healing addresses this point (Figure 2; text page 5 and 6). Clones of GFP-expressing cells neighbouring a wound share their cytoplasmic contents with
their neighbours during wound closure. However, a clonal cell that is Atg5-deficient in a wild-type background does not share its content with the neighbouring cells. This shows that for a cell to participate in syncytium formation, that every cell itself has to be competent to perform autophagy. We will expand the explanation of this point in the text.

The A58-Gal is not cell-type specific as authors describe (pg 9) similar effects in trachea, salivary glands, and intestine and it is unclear if effects are due to disruption of autophagy in epidermal cells or general disruption in fly's physiology. The authors should determine, using a more restrictive Gal driver, whether syncytium formation is due to activation of autophagy in the epidermal cells or another cell type (trachea, salivary glands, or intestine).

We apologize if our phrasing of ‘ectodermal’ led to the impression that A58-Gal4 is cell-type specific. A58 also drives expression in the tracheal system, as all other available epidermal drivers do. A58 expression in the salivary gland is presumably due to the origin of the Gla4 construct, which like many other Gal4 drivers (e.g. NP1-Gal4) includes salivary gland specific enhancers (PMID: 8223268 and PMID: 12324947). A58 is not active in the gut, and for the experiments in the gut we used the NP1 driver. We will rephrase the text in the paper to avoid confusion. There is no driver that is absolutely restricted to the epidermis.

Alternatively, if no other Gal4 is available for the larval epidermis then authors could at least show using enterocytes driver (NP1-Gal4) that overexpression of Atg1 is sufficient to induce syncytium formation and its effect on gut barrier integrity.

We did do this experiment but didn’t include the images because of the large number of figures we already had. We now show them here. As mentioned above, barrier integrity is not compromised. We can also provide images of the phenotype in tracheal cells.
**Figure B | Effect of uncontrolled autophagy on enterocytes and salivary glands.** Larval gut or salivary glands expressing the indicated markers and overexpression (Tsc1,2 or Atg1S) or RNAi (raptor) constructs using the NP1-Gal4 driver. Images are from live imaging of gut or salivary gland of 6 to 11 larvae for each genotype. Scale bars, 20 µm.

4. In Fig 8, authors nicely show that Atg1 RNAi can rescue Tor RNAi and raptor RNAi, but, what about the reverse. Is overexpression of Tor sufficient to inhibit the overexpression Atg1 and reduce autophagy-induced syncytium formation?

Overexpression of Tor would affect both TORC1 and TORC2. We have done this experiment using UAS-TorWT construct but found that it leads to excessive autophagy rather than suppression, consistent with similar results reported by others (PMID: 12324961 and PMID: 15186745). This approach can therefore not be used to do the proposed experiment. Instead, one could use downregulation of the Tor inhibitor TSC1, which acts on TORC1, and we have shown to reduce autophagosome formation in wound healing (Fig. 1d). Another option is to overexpress the TORC1-specific activator Rheb (PMID: 12893813, PMID: 17208179 and PMID: 31422886). We will set up the experiments with these constructs in the hope that they will yield interpretable results.

**Minor comments:**

1. Check spelling of abbreviations, Sqh is often misspelled Shq in figures

   We will correct them. Thanks for alerting us.

2. The order of images in Figure 3 should match the description in the text (pg. 6).

   We would prefer to retain the current order because it is then consistent with all the other figures. Re-writing the text to reflect this order would make it less clear.

   AtgW is described in text, but not shown in Fig 3a-c. Also, upstream activators of TORC1 are described first, but shown last in this Figure making it difficult to follow.

   We will only mention Atg1W later in the text where we also show it in a figure.

3. Fig7a should show junctional effect of Atg1W alone and in combination with Atg5i which is used in 7b.

   We had left this out to save space, but we will now include these data.

It is unclear why authors switched to this weak overexpression for this photobleaching assay when Atg1S was predominantly used in the rest of the study.

The reason we used Atg1W in this particular experiment is that we had it on a chromosome where it was recombined with GFP which made it genetically much easier to use for FLIP experiments. However, perhaps these constructs merit some discussion. Atg1W and Atg1S were originally called “weak” and “strong” based on studies in other tissues and other stages (PMID: 33253201). However, we found that in the epidermis their effects are practically indistinguishable, as judged by TEM (Fig.3d,e) (Fig 5e,f) (Suppl. Fig. 5a,b and Suppl. Fig. 6b,c), and all markers we used in confocal analyses (which we will include them). Thus, to avoid confusion, we will change the nomenclature we use on our paper to the neutral Atg1GS and Atg1OS.
Reviewer #1 (Significance (Required)):

This study elucidates the role and regulation of TORC1 and autophagy in epithelial membrane remodeling. This is important work that is significant to both developmental and wound healing research. Many cell types become multinucleate during differentiation, aging, and wound healing and here the authors find a novel role for autophagy in remodeling lateral cellular junctions to facilitate syncytium formation.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In their present manuscript Kakanj and colleagues show that during epithelial wound healing autophagy pathway controls plasma membrane integrity and homeostasis. Furthermore, elevated autophagic activity is sufficient to induce syncytium formation, which is essential for wound closure and healing. Authors used the epidermis of fruit fly larvae as model to study wound healing and video microscopy to examine this process. The methodology is well established, since authors already published a related study in 2016 using similar tools.

The findings presented here are interesting and promising, the quality of most experiments are satisfactory, the confocal images/videos are excellent and I truly appreciate that authors used electron microscopy to support some of their claims. Findings are well presented and the text is well written and easy to read.

Overall, my opinion is very positive about this manuscript.

I believe most of the findings are very well supported, but I have some suggestions, which may can help strengthen the authors’ points.

1) Authors used GFP-Atg8a reporter to follow autophagy during wound healing. While I also believe that, the appearing GFP-Atg8a dots represent autophagic vesicles after wounding but GFP-Atg8a has some certain limitations. First: Atg8a (or LC3 in mammals) is removed from the outer surface of autophagosomes by Atg4 and the Atg8a trapped inside the autophagosomes will be degraded in the autolysosomal lumen. Thus Atg8a not always localizes to autolysosomes, actually Atg8a immunostaining mostly labels autophagosomes (and phagophores) but not autolysosomes in insect cells. Accordingly, GFP-Atg8a reporter is also subject of autolysosomal degradation and furthermore most of the GFP signal is quenched in the acidic lumen of autolysosomes, since at lower pH GFP loses fluorescence. Nevertheless, if lysosomal degradation proceeds normally, GFP-Atg8 will be degraded completely. Thus, some of the autolysosomes cannot be detected using this reporter, for this mCherry-Atg8a reporters can be used, since mCherry is more resistant than GFP and thus accumulate inside lysosomes, and retains its fluorescence in acidic environments.

This is a good suggestion and we had done these experiments. However, the red fluorophores have a serious problem in that they all tend to form small aggregates or puncta – not in all tissues and at all stages, but this is a very wide-spread phenomenon, and is even observed in in vitro experiments (own observations). This makes quantification of vesicles or other small structures such as autophagosomes complete impossible. Nevertheless, here are a few figures from our analyses. While some of the spots clearly appear to be autophagosomes, as judged by their positions, they cannot be objectively distinguished from the other spots.
**Figure C | Autophagy during epidermal wound healing.** Time-lapse series of single-cell wound healing in larva expressing mCherry-Atg8a (black) to mark autophagosomes and autolysosomes (A58>mCherry-Atg8a). a, z-projections of a time-lapse series. b, Higher magnification of the areas marked by magenta boxes in (a), n=11 larvae. Each frame is a merge of 57 planes spaced 0.28 μm apart. Scale bars: a 20 μm; b, 10 μm.

However, I still believe that for video microscopy GFP-Atg8a was a perfect choice, I just suggest to confirm the appearance of autophagosomes after wounding by other means: for instance, immunostaining of the epidermis after wounding (120 min) against Atg8a should confirm the presence of autophagosomes. There are a few specific available antibodies working in flies which are listed in the reviews of Nagy (PMID: 25481477) or more recently in Lorincz (PMID: 28704946)

This is technically a huge challenge. We would have to induce a single cell wound, then filet and fix the epidermis, during which it rolls up and often destroys the area of interest. If it doesn’t, then the prep can be flattened out, but it still can be very difficult to find the wound in the prep.

2) One of the major claims of the authors is that elevated autophagy leads to the breakdown or removal of lateral plasma membranes to promote syncytium formation. It is clearly seen on the confocal or EM images that lateral membranes disappear after wounding. However, it is also suggested that the lateral plasma membrane material is incorporated into autophagosomes or plasma membrane is a potential membrane source of autophagosome formation. I believe this is the least supported claim of the manuscript since no direct evidence for this is presented. This is based on BodyPy staining only, that BodyPy positive vesicles accumulate inside the cells. If this is indeed the case plasma membrane components should be detected in autophagic vesicles. Thus, I recommend co-staining membrane components with autophagic markers.

This is indeed the clear next step, and we did a number of experiments along those lines, but they were once again compromised by the problem with the mCherry aggregates. This made the interpretation in the unwounded epidermis with artificially upregulated autophagy impossible. However, experiments with naturally upregulated autophagy in wound healing yielded results that are consistent with plasma membrane components being associated with autophagosomes (with the caveat that not every red dot we see represents an autophagosome). We have just repeated some of these using the septate junction marker FasIII and have obtained some beautiful movies that show FasIII labelled membrane (green) being surrounded by mCherry spots, and as the membrane begins to dissociate, the mCherry spots turn from red to yellow. We have included stills from results of these analyses here and will include them in a new figure in the revised manuscript.
Figure D | Colocalization of Atg8a and the septate junction component FasIII during epidermal wound healing. a, Time-lapse series of single-cell wound healing in a larva expressing mCherry-Atg8a (red) (A58>mCherry-Atg8a) and endogenously tagged FasIII (GFP gene trap; green), a transmembrane component of septate junctions. b, Higher magnification of the time-lapse marked by magenta boxes in (a). n=11 larvae. a,b, Each frame is a merge of 68 planes spaced 0.28 μm apart. Scale bars: a,b 20 μm.

However if authors observe no colocalization of plasma membrane components with autophagy markers I still believe this study worth to be published. I would like to recommend
the review of Ungermann and Reggiori (PMID: 29966469) in which the trafficking of Atg9 is discussed,

Yes, indeed. And there is in fact now a further paper that goes in a similar direction (PMID: 34257406). We had left this out because we did not have direct data on Atg9, but will be happy to include it in the discussion in which we cite the paper that shows that Drosophila Atg9 is localized on the lateral plasma membrane in nurse cells, and loss of it leads to syncytium formation.

since the source of autophagosomal Atg9 is in part the plasma membrane in mammalian cells. Therefore, these findings may strengthen the authors' claims.

**Minor points:**

Figure 2A: I believe authors wanted to use the word 'maintaining' not mating in their scheme.

Indeed. Thanks for alerting us.

Discussion: Authors suggest that: another function of autophagy in the cells surrounding the wound may be to clear up debris as in planarian and other cell types autophagy is activated in healthy cells, which simultaneously phagocyte cell debris. Honestly, I do not believe that this is the case here. Some of the Atg proteins are indeed required for phagocytosis during LC3-associated phagocytosis (LAP) (see: PMID: 30787029), but LAP is independent form Atg1

Good point, we will include this in the discussion.

and if LAP happened in the cells, surrounding the wound then GFP-Atg8a positive phagosomes would appear in those cells. However, it is clearly not the case here.

Reviewer #2 (Significance (Required)):

I highly recommend this manuscript to be uploaded to a relevant journal and I believe the findings presented here will be interesting for biologists specialized in regeneration and readers from the autophagy fields alike.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

**Summary:**

The larval epidermis of Drosophila is a prime model for studying wound healing by combining live imaging with cellular, genetic and molecular analysis of the processes involved. Autophagy is known to be activated and necessary for efficient wound healing in animal models through secretion of cytokines and clearance of bacteria. This manuscript implicates autophagy in cellular syncytium formation during wound healing. Live imaging demonstrates autophagy activation in cells surrounding the wound. Inhibition of autophagy by RNAi against atg1 or atg5, required for autophagy initiation and autophagosome formation had no effect on the rate of constriction and closing of the wound site. However, elegant live imaging demonstrates that autophagy is required cell autonomously for cell fusion, a normal process during wound healing in flies. Autophagy can also be instructive for cell fusion. Strong induction of autophagy by TORC1 inhibition, TSC1/2 overexpression or Atg1 overexpression induce cell fusion that is genetically dependent on atg5, a gene acting downstream of atg1 in autophagosome formation. As Chloroquine treatment, a chemical inhibiting autophagosome fusion to the lysosome and lysosomal breakdown showed no
effect, the authors suggest that later steps of autophagy are not involved. Live imaging with a selection of cellular fluorescently tagged markers of apical, lateral and basolateral membrane domains, combined with electron microscopy show clearly that lateral membrane are disrupted and removed within the epithelium. During this process, membranous large vesicles "drift" away from the plasma membrane. If these vesicles relate to autophagy is not addressed. In addition to the effect on cell fusion, strong autophagy induction also leads to autophagy within the nucleus, chromatin condensation and distortion of the nuclear membrane. The manuscript is well written and easy to follow. Figure panels and data are clearly presented. All experiments are well described throughout and skillfully executed with appropriate controls and statistical analysis. It remains unknown what induces autophagy in response to wounding. It also remains unclear whether autophagy deconstructs or engulfs parts of the plasma membrane, or if parts of the autophagy machinery has additional roles in plasma membrane fusion.

**Major comments:**

• Are the key conclusions convincing?
  - Conclusions are generally balanced and convincing.
  - I have seldom seen a paper so well written, presented and balanced by first pass. Hence my experimental suggestions are few.

• Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
  - Claims are well founded.

• Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary to evaluate the paper as it is, and do not ask authors to open new lines of experimentation.

  - The inhibition of autophagy is performed using knockdown of two genes acting in autophagy initiation (atg1, a part of the ULK1 kinase complex) and atg5, required for autophagosome formation. Later acting genes in the autophagy process such as autophagosome closure, fusion with the lysosome or degradation were not analyzed. In the abstract, the authors state "Proper functioning of TORC1 is needed to prevent autophagy from destroying the larval epidermis which depends on membrane isolation and phagophore expansion, but not fusion of autophagosomes to lysosomes". As far as I can see, the last statement on fusion derives from experiments with Chloroquine. Although frequently used for qualitative experiments, CQ is not suited for conclusive experiments. Without genetic experiments targeting genes for autophagosome-lysosome fusion such as snap29, stx17, vamp7 this statement is in my mind not well supported.

We agree this would strengthen our findings, and we had indeed ordered these strains from the Bloomington stock collection. However, they were dead on arrival and both our labs in Heidelberg and Cologne currently have major problems with shipments from Bloomington and German customs. Other colleagues whom we asked did not have them available either. We will continue to search for appropriate constructs, but even if we find them and they arrive alive, and are processed by customs within a reasonable time, it will take many weeks to establish and then expand them and subsequently do the multi-generation crosses to obtain the stocks with all the relevant drivers and markers to set up the experiment. Three months is the absolute lower limit provided everything works according to plan, and first time round 6 months is a more realistic assumption. We hope that the referees and the editors agree that while this is a desirable experiment, it is not essential for the publication of the other results we present.
• Are the suggested experiments realistic for the authors? It would help if you could add an estimated cost and time investment for substantial experiments.
  - Given the expertise of the authors, these experiments should be easy to perform within 3 months.

• Are the data and the methods presented in such a way that they can be reproduced?
  - The manuscript is well written and an excellent example of how methods and experiments should be presented. Methods, tools and experiments are all well described.

• Are the experiments adequately replicated and statistical analysis adequate?
  - Replicates and statistics are adequate and custom for the type of analysis performed.

**Minor comments:**

• Specific experimental issues that are easily addressable.
  Figure 3 h. The live imaging documents the striking disappearance of lateral cell membranes using SRC-GFP. In 3h, large vesicle formation and movement towards the cell interior is shown. How frequent is this?

  This can only be seen clearly in experiments with time-controlled (Gal80ts) induction of autophagy where we can observe the process unfolding. We see these structures very frequently, but great variability in morphology and the structures are not always captured clearly in the plane of imaging. We here provide further examples.
Figure E | Autophagy in unwounded epidermis. a-c, Three additional examples showing apparent extrusions from lateral membranes after induction of autophagy (same experiment as Figure 3h). Time-lapse series of epidermal cells expressing Src-GFP and Atg1S. Transgene expression is induced at the end of the second larval instar, live imaging started 6 h later (t=0) and continued for an additional 6 hours. a-c, Src-GFP containing material appears to be taken out of and eventually detached from lateral cell membranes (arrows).

Is this believed to be the mechanism of lateral membrane removal?

We would of course like to believe that, but we have no proof, and would therefore only be able to speculate.

If so, is it dependent on the autophagy machinery. Are these vesicle positive for autophagy markers?

Some autophagy markers have indeed been reported to be associated with the plasma membrane (e.g. Atg9, Atg16), but a conclusive study, while highly desirable, in our view goes beyond the scope of this study.

Resolving this issue may lift the conclusions of the paper. Using 3xCherry-Atg8 together with SRC-GFP, this should be possible.
We are intrigued by this suggestion and will be setting up the necessary crosses to do the experiments. However, it will take a long time to generate the necessary stocks (see genetics described below), and we will then again encounter the problem with the mCherry aggregates (see response to referees # 2). We are curious about the outcome, but we do not think it will be reasonable to promise as part of this revision that we will be able to provide conclusive results in the foreseeable future. Along with the many other things to do, this may just have to become part of a future paper, especially if there turn out to be other problems to be solved along the way. Like, for example, having to make an infrared (like mIFP or mKate, with which we have had much better experience in other contexts) Atg8 construct.

Using CQ, the authors should be able to detect plasma membrane and junctional components in autophagosomes or autolysosomes (by confocal and electron microscopy) as degradation is inhibited. This should help to distinguish whether lateral membranes are engulfed and digested or if cells simply fuse, by using a part of the autophagy machinery.

We have many interesting EM images on which we have had extensive discussions with the Paolo Ronchi and Yannick Schwab at the EMBL (whom we embarrassingly forgot to acknowledge in our manuscript, which will now be corrected), and one of the authors of this paper (BM) is an expert in EM imaging of the larval epidermis. It was agreed that some structures could indeed be interpreted as autophagosomes with content resembling junctional material. However, in the absence of absolute proof, we did not include them in the paper. We now show them here. [Figure for referees not shown.]

The authors, state that strong autophagy activation also leads to syncytium formation of tracheal cells, salivary glands and gut EC cells. Representative images in a supplementary figure would be useful for future reference.
See response to other comments above (response to referees #1). We have added some images in this document (Figure B) and will be happy to add additional ones in the revised manuscript.

- Are prior studies referenced appropriately?
  - Yes. Key literature and findings are cited and discussed.

- Are the text and figures clear and accurate?
  - Yes

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?
  - See suggested experiments above.

Reviewer #3 (Significance (Required)):

- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.
  - The findings clearly documents a role of autophagy in syncytium formation in the physiological process of wounding. This has parallels to muscle syncytium formation, but has to my knowledge not been demonstrated in any other cell type to be performed by autophagy. Moreover, the authors show that strong autophagy induction can lead to fusion of epithelial cells. This may have relevance for processes and diseases where polyploidy are observed.

- Place the work in the context of the existing literature (provide references, where appropriate).

- State what audience might be interested in and influenced by the reported findings.
  - The data are very strong and the demonstration that autophagy controls syncytium formation outside of muscle development is surprising and significant. It is of interest to the field of cell biology and development in general and the autophagy field in particular. It will also be of interest for the medical field that deals with multinuclear phenotypes, such as cancer.

- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.
  - Development, cell signaling, autophagy, vesicle trafficking.

| Transgenes          | Stock ID | Source       | Publications using this construct | Reference |
|---------------------|----------|--------------|------------------------------------|-----------|
| UAS-GFP-Kuk         | Jörg Großhans | PMID: 16421189 | https://flybase.org/reports/FBal0161312 | 29        |
| (UAS-GFP-Kuk<sup>EV17066W1</sup>) | | | | |
| UAS-Atg1<sup>r</sup> | V # 16133 (GD7149) | PMID: 19363474 | PMID: 31995752 | PMID: 32032548 | PMID: 32915229 | https://flybase.org/reports/FBtp0034071.html |
| Gene       | Description               | V#       | PMIDs                                  | Additional Information                                                                 |
|------------|---------------------------|----------|----------------------------------------|----------------------------------------------------------------------------------------|
| UAS-Atg5i  | (UAS-Atg5RNAi)            | V # 104461 (KK108904) | PMID: 31995752  
PMID: 32032548  
https://flybase.org/reports/FBtp0046851.html | Dissertations of Philipp Trachte, Abb. 23.  
http://refubium.fu-berlin.de/handle/fub188/27709  
Dissertation of Sirena Soriano Rodriguez.  
https://roderic.uv.es/bitstream/handle/10550/50749/Tesis%20SSoriano.pdf?sequence=1 |
| UAS-Atg6i  | (UAS-Atg6RNAi)            | V # 110197 (KK102460) | PMID: 28581519  
PMID: 23599123  
PMID: 27542914  
PMID: 25644700 | Dissertations of Philipp Trachte, Abb. 23.  
http://refubium.fu-berlin.de/handle/fub188/27709  
Dissertation of Sirena Soriano Rodriguez.  
https://roderic.uv.es/bitstream/handle/10550/50749/Tesis%20SSoriano.pdf?sequence=1 |
| UAS-Atg7i  | (UAS-Atg7RNAi)            | V # 45558 (GD11671) | PMID: 25882046  
PMID: 31995752  
PMID: 32032548  
PMID: 23599123 | https://flybase.org/reports/FBtp0025106.html |
| UAS-Atg12i | (UAS-Atg12RNAi)           | V # 29791 (GD15230) | PMID: 25882046  
PMID: 17568747  
PMID: 31995752 | https://flybase.org/reports/FBtp0027770.html |
| UAS-TSC1,2 | (UAS-TSC1, AUS-TSC2)      |          | PMID: 15296714  
PMID: 11348592 | 64 |
| UAS-TSC1i  | (UAS-TSC1RNAi)            | V # 22252 (GD11836) | PMID: 23144831  
PMID: 29144896  
PMID: 29456138 | https://flybase.org/reports/FBtp0025266.html |
| UAS-Tor'   | (UAS-TorRNAi)             | BL # 33951 | PMID: 25882046  
PMID: 26395483 | https://flybase.org/reports/FBtp0065159.html |
| UAS-TORRN  | (UAS-TORRT2G)             | BL # 7013 | PMID: 15296714  
PMID: 29144896 | https://flybase.org/reports/FBtp0016360.html |
| UAS-raptor' | (UAS-raptorRNAi)          | BL # 34814 | PMID: 25882046  
PMID: 31048465 | https://flybase.org/reports/FBtp0068814.html |
| UAS-raptor1 | (UAS-raptorRNAi)          | BL # 41912 | PMID: 32097403 | https://flybase.org/reports/FBtp0081336.html |
| UAS-rictor' | (UAS-rictorRNAi)          | BL # 36699 | PMID: 25882046 | https://flybase.org/reports/FBtp0070835.html |
| UAS-Atg1S  | (UAS-Atg1S1G)             |          | PMID: 33253201 | https://flybase.org/reports/FBtp0041043.html |
| UAS-Atg1W, UAS-GFP | (UAS-Atg1W, UAS-GFP) |          | PMID: 33253201 | https://flybase.org/reports/FBtp00216676.html |
| UAS-S6K    | (UAS-S6KRNAi)             | BL # 41895 | PMID: 25284370 | https://flybase.org/reports/FBtp0080798.html |
| Gene    | Description | Expresser | Curator | PubMed 1 | PubMed 2 | Flybase Report Link |
|---------|-------------|-----------|---------|----------|----------|---------------------|
| UAS-Sqa<sup>x4</sup> (UAS-Sqa<sup>T279A/Cyo</sup>) | Guang-Chao Chen | PMID: 21169990 | https://flybase.org/reports/FBtp0071419 |
| UAS-RhoA<sup>i</sup> (UAS-RhoA<sup>RNAi</sup>) | PMID: 23853710 | PMID: 33789114 | https://flybase.org/reports/FBtp0031970.html |
| UAS-Rck<sup>i</sup> (UAS-Rck<sup>RNAi</sup>) | PMID: 24995985 | PMID: 33789114 | https://flybase.org/reports/FBtp0046110.html |
| UAS-Rheb<sup>AV4</sup> | PMID: 19009714 | PMID: 28829944 | https://flybase.org/reports/FBal0141561.html |
Dear Karin,

We enclose our revisions in response the referees' comments on our revision plan and your suggestions.

You will see that we have added even more experiments than we thought initially be able to do, in part because reagents became available that we were unable to get hold of when we wrote the revision plan.

We hope that with the extensive additional new data and the edits we have made the manuscript will now be suitable for publication in The EMBO Journal.

Best wishes

Parisa and Maria on behalf of all authors.
Our response to the referees, whom we thank for their critique and suggestions, as well as all changes in the revised manuscript are shown in blue font.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

In this study, the authors use the fruit fly as a model to understand the role and regulation of autophagy in epidermal integrity during development and wound healing. They discover that hyper activation of autophagy via overexpression of Atg1 leads to disruption of epithelial organization, junctional protein localization, and syncytium formation. In addition, these epidermal defects were found to be dependent on TORC1 as knockdown or inhibition of TORC1 antagonists resulted in similar epidermal defects which could be rescued by knockdown of Atg1 or Atg5. Wound healing in fruit fly epidermis is known to induce cell fusion and here the authors show that syncytium formation is dependent on autophagy. GFP-Atg8a autophagosomes were found to accumulate in cells adjacent to the wound site, but Atg1-induced syncytium formation was dispensable for wound repair. However, the authors found that hyper activation of autophagy prior to injury slowed wound closure. This may be due to defects in actomyosin organization or another developmental defect the authors observed in the epidermis. Overall, the key conclusions of this study are convincing, but the experiments would be strengthened by validation of all the RNAi strains used as well as demonstration that epidermal barrier remains intact as described.

**Major Comments**

1. This study uses a number of UAS-RNAi strains as well as dominant negative and overexpression transgenes. There is no validation that these genetic perturbations work as expected.

Almost all of the lines we use have been extensively used and validated by others as documented in the literature. We append a table (below, page 13) with these references. It would be close to impossible for us to show their tissue specific efficacy in the larval epidermis because it is extremely difficult to obtain clean dissections of epidermis without contamination from other tissues (muscles, nerves, etc.), and we believe we can rely on the known validation of most of the lines. It is true that some of the lines are less well characterised, and we comment on those below, and have eliminated our speculation on their effects in the manuscript.

In fact, the authors state on pg 5 that RNAi to Atg6, Atg7, and Atg12 may be less effective, but do not verify the knockdown efficiency to the gene of interest (i.e. Atg5 RNAi knock downs Atg5 transcript or protein).

Atg12\(^1\) and Atg7\(^1\) we used here have been shown (PMID: 25882046) by quantitative RT-PCR to effectively reduce RNA levels in the midgut during larval to pupal transition. We therefore agree that our speculation that the weak effect in the epidermis may be due to ineffective knock-down is not fully justified. Rather, it seems that these components are accessory but not necessarily essential for the completion of autophagy, as also observed by others (PMID: 25882046, PMID: 1805642, PMID: 23599123, PMID: 15296714, PMID: 23873149, PMID: 23406899), and we now include a statement to that effect.
This is particularly important as authors use a single UAS-riktor RNAi strain to conclude that autophagy is dependent on TORC1 and not TORC2. If rictor RNAi is also weak or ineffective than this conclusion would be erroneous.

The function of rictor has been validated by classic genetics: Animals homozygous for deletions of rictor show no defects throughout their normal life cycle (Hietakangas and Cohen, 2007). We have also shown that epidermis of homozygous rictor\(\Delta1\) larvae (marked with Src-GFP, DsNuc-Red2) shows no abnormalities in cell shapes or cell membranes. We include an image here.

A major conclusion of this study is that autophagy remodels the lateral cell membranes and not the basal or apical, so the membrane integrity remains intact. This is described and shown in Fig S3a, but it is hard to see that the apical membrane is intact. It would be helpful if authors could show a true membrane marker, such as UAS-CD8mGFP to see if there is a continuous membrane.

We have included new experiments with this marker.

Alternatively, is there a barrier assay that could help demonstrate that syncytium formation does not disrupt epithelial integrity?

This follows from the fluorescence recovery we performed (Movie EV14), where we observe rapid diffusion between areas in the epidermis, but never any leakage of fluorescence in the y-axis into the body cavity. We have emphasized this more clearly in the text.

This could be performed in the fly gut, using the smurf assay (Rera M et al. 2011), since the authors also describe (pg 9) a similar role for autophagy in disruption of epithelial lateral membranes.
We had done a smurf assay, and observed no leakage from the gut, but didn’t document this at the time because of difficulties during the period of Covid restrictions of accessing a dissecting scope/camera set up in a lab outside our own. We have now repeated this and include the results.

3. Is autophagy dependent syncytium formation cell autonomous?

Our clonal analysis in wound healing addresses this point (Figure 2). Clones of GFP-expressing cells neighbouring a wound share their cytoplasmic contents with their neighbours during wound closure. However, a clonal cell that is Atg5-deficient in a wild-type background does not share its content with the neighbouring cells. This shows that for a cell to participate in syncytium formation, that every cell itself has to be competent to perform autophagy. We have expanded the explanation of this point in the text.

The A58-Gal is not cell-type specific as authors describe (pg 9) similar effects in trachea, salivary glands, and intestine and it is unclear if effects are due to disruption of autophagy in epidermal cells or general disruption in fly’s physiology. The authors should determine, using a more restrictive Gal driver, whether syncytium formation is due to activation of autophagy in the epidermal cells or another cell type (trachea, salivary glands, or intestine).

We apologize if our phrasing of ‘ectodermal’ led to the impression that A58-Gal4 is cell-type specific. A58 also drives expression in the tracheal system, as all other available epidermal drivers do. A58 expression in the salivary gland is presumably due to the origin of the Gla4 construct, which like many other Gal4 drivers (e.g. NP1-Gal4) includes salivary gland specific enhancers (PMID: 8223268 and PMID: 12324947). A58 is not active in the gut, and for the experiments in the gut we used the NP1 driver. We have rephrased the text in the paper to avoid confusion. There is no driver that is absolutely restricted to the epidermis.

Alternatively, if no other Gal4 is available for the larval epidermis then authors could at least show using enterocytes driver (NP1-Gal4) that overexpression of Atg1 is sufficient to induce syncytium formation and its effect on gut barrier integrity.

We did do this experiment but didn’t include the images because of the large number of figures we already had. We have now added these data, included in new figures and reported in the text. As mentioned above, barrier integrity is not compromised.

4. In Fig 8, authors nicely show that Atg1 RNAi can rescue Tor RNAi and raptor RNAi, but, what about the reverse. Is overexpression of Tor sufficient to inhibit the overexpression Atg1 and reduce autophagy-induced syncytium formation?

Overexpression of Tor would affect both TORC1 and TORC2. We have done this experiment using UAS-Torwt construct but found that it leads to excessive autophagy rather than suppression, consistent with similar results reported by others (PMID: 12324961 and PMID: 15186745). This approach can therefore not be used to do the proposed experiment. Instead, one could use downregulation of the Tor inhibitor TSC1, which acts on TORC1, and we have shown to reduce autophagosome formation in wound healing (Fig. 1D). Another option is to overexpress the TORC1-specific activator Rheb (PMID: 12893813, PMID: 17208179 and PMID: 31422886). We have done the experiments with these constructs and found some suppression (though slightly confounded by the broad effects of upregulating TORC1 activity in the first place) and have added figures and text.

**Minor comments:**

1. Check spelling of abbreviations, Sqh is often misspelled Shq in figures
We have corrected them. Thanks for alerting us.

2. The order of images in Figure 3 should match the description in the text (pg. 6).

We prefer to retain the current order because it is then consistent with all the other figures. Re-writing the text to reflect this order would make it less clear.

AtgW is described in text, but not shown in Fig 3a-c. Also, upstream activators of TORC1 are described first, but shown last in this Figure making it difficult to follow.

We have adapted the text and have also added a note on these constructs.

3. Fig7a should show junctional effect of Atg1W alone and in combination with Atg5i which is used in 7b.

We had left this out to save space, but have now included these data.

It is unclear why authors switched to this weak overexpression for this photobleaching assay when Atg1S was predominantly used in the rest of the study.

The reason we used Atg1W in this particular experiment is that we had it on a chromosome where it was recombined with GFP which made it genetically much easier to use for FLIP experiments. However, perhaps these constructs merit some discussion. Atg1W and Atg1S were originally called “weak” and “strong” based on studies in other tissues and other stages (PMID: 33253201). However, we found that in the epidermis their effects are practically indistinguishable, as judged by TEM (Fig.3D; E) (Fig 5E, F) (Appendix Fig S6A, B and 8B, C), and all markers we used in confocal analyses (which we have included now). Thus, to avoid confusion, we will change the nomenclature we use on our paper to the neutral Atg1GS and Atg16B.

Reviewer #1 (Significance (Required)):

This study elucidates the role and regulation of TORC1 and autophagy in epithelial membrane remodeling. This is important work that is significant to both developmental and wound healing research. Many cell types become multinucleate during differentiation, aging, and wound healing and here the authors find a novel role for autophagy in remodeling lateral cellular junctions to facilitate syncytium formation.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

In their present manuscript Kakanj and colleagues show that during epithelial wound healing autophagy pathway controls plasma membrane integrity and homeostasis. Furthermore, elevated autophagic activity is sufficient to induce syncytium formation, which is essential for wound closure and healing. Authors used the epidemis of fruit fly larvae as model to study wound healing and video microscopy to examine this process. The methodology is well established, since authors already published a related study in 2016 using similar tools.

The findings presented here are interesting and promising, the quality of most experiments are satisfactory, the confocal images/videos are excellent and I truly appreciate that authors used electron microscopy to support some of their claims. Findings are well presented and the text is well written and easy to read.
Overall, my opinion is very positive about this manuscript.

I believe most of the findings are very well supported, but I have some suggestions, which may help strengthen the authors’ points.

1) Authors used GFP-Atg8a reporter to follow autophagy during wound healing. While I also believe that, the appearing GFP-Atg8a dots represent autophagic vesicles after wounding but GFP-Atg8a has some certain limitations. First: Atg8a (or LC3 in mammals) is removed from the outer surface of autophagosomes by Atg4 and the Atg8a trapped inside the autophagosomes will be degraded in the autolysosomal lumen. Thus Atg8a not always localizes to autolysosomes, actually Atg8a immunostaining mostly labels autophagosomes (and phagophores) but not autolysosomes in insect cells. Accordingly, GFP-Atg8a reporter is also subject of autolysosomal degradation and furthermore most of the GFP signal is quenched in the acidic lumen of autolysosomes, since at lower pH GFP loses fluorescence. Nevertheless, if lysosomal degradation proceeds normally, GFP-Atg8 will be degraded completely. Thus, some of the autolysosomes cannot be detected using this reporter, for this mCherry-Atg8a reporters can be used, since mCherry is more resistant than GFP and thus accumulate inside lysosomes, and retains its fluorescence in acidic environments.

This is a good suggestion and we had done these experiments. However, the red fluorophores have a serious problem in that they all tend to form small aggregates or puncta – not in all tissues and at all stages, but this is a very wide-spread phenomenon, and is even observed in in vitro experiments (own observations). It has now been described and analysed in a recent publication (bioRxiv: doi.org/10.1101/2021.12.07.471677). This makes a reliable quantification of vesicles or other small structures such as autophagosomes impossible. Nevertheless, here are a few figures from our analyses. While some of the spots clearly appear to be autophagosomes, as judged by their positions, they cannot be objectively distinguished from the other spots. We have added this figure in the paper.

![Figure B](image)

**Figure B | Autophagy during epidermal wound healing.** Time-lapse series of single-cell wound healing in larva expressing mCherry-Atg8a (black) to mark autophagosomes and autolysosomes (A58>mCherry-Atg8a). a, z-projections of a time-lapse series. b, Higher magnification of the areas marked by magenta boxes in (a). n=11 larvae. Each frame is a merge of 57 planes spaced 0.28 μm apart. Scale bars: a 20 μm; b, 10 μm.

However, I still believe that for video microscopy GFP-Atg8a was a perfect choice, I just suggest to confirm the appearance of autophagosomes after wounding by other means: for instance, immunostaining of the epidermis after wounding (120 min) against Atg8a should
confirm the presence of autophagosomes. There are a few specific available antibodies working in flies which are listed in the reviews of Nagy (PMID: 25481477) or more recently in Lorincz (PMID: 28704946)

This is technically a huge challenge. We would have to induce a single cell wound, then filet and fix the epidermis, during which it rolls up and often destroys the area of interest. If it doesn’t, then the prep can be flattened out, but it still can be very difficult to find the wound in the prep.

2) One of the major claims of the authors is that elevated autophagy leads to the breakdown or removal of lateral plasma membranes to promote syncytium formation. It is clearly seen on the confocal or EM images that lateral membranes disappear after wounding. However, it is also suggested that the lateral plasma membrane material is incorporated into autophagosomes or plasma membrane is a potential membrane source of autophagosome formation. I believe this is the least supported claim of the manuscript since no direct evidence for this is presented. This is based on BodyPy staining only, that BodyPy positive vesicles accumulate inside the cells. If this is indeed the case plasma membrane components should be detected in autophagic vesicles. Thus, I recommend co-staining membrane components with autophagic markers.

This is indeed the clear next step, and we did a number of experiments along those lines, but they were once again compromised by the problem with the mCherry aggregates. This made the interpretation in the unwounded epidermis with artificially upregulated autophagy impossible. However, experiments with naturally upregulated autophagy in wound healing yielded results that are consistent with plasma membrane components being associated with autophagosomes (with the caveat that not every red dot we see represents an autophagosome). We have repeated some of these using the septate junction marker FasIII and have obtained some beautiful movies that show FasIII-labelled membrane (green) being surrounded by mCherry spots, and as the membrane begins to dissociate, the mCherry spots turn from red to yellow. We include stills from results of these analyses below and have included a new figure and suppl. figure as well as videos (Fig S10A, B; Appendix Fig S13A and Movie EV18 and 19) in the revised manuscript. We have also included further results where we used a transmembrane marker, mCD8-GFP (Appendix Fig S13B, C and Movie EV20).
Figure C | Colocalization of Atg8a and the septate junction component FasIII during epidermal wound healing. 

a, Time-lapse series of single-cell wound healing in a larva expressing mCherry-Atg8a (red) (A58>mCherry-Atg8a) and endogenously tagged FasIII (GFP gene trap; green), a transmembrane component of septate junctions. b, Higher magnification of the time-lapse marked by magenta boxes in (a). n=11 larvae. a,b, Each frame is a merge of 68 planes spaced 0.28 μm apart. Scale bars: a,b 20 μm.

However if authors observe no colocalization of plasma membrane components with autophagy markers I still believe this study worth to be published. I would like to recommend
the review of Ungermann and Reggiori (PMID: 29966469) in which the trafficking of Atg9 is
discussed,

Yes, indeed. And there is in fact now a further paper that goes in a similar direction
(PMID: 34257406). We had left this out because we did not have direct data on Atg9, but
have included it in the discussion in which we cite the paper that shows that Drosophila Atg9
is localized on the lateral plasma membrane in nurse cells, and loss of it leads to syncytium
formation.

since the source of autophagosomal Atg9 is in part the plasma membrane in mammalian
cells. Therefore, these findings may strengthen the authors' claims.

**Minor points:**

Figure 2A: I believe authors wanted to use the word 'maintaining' not mating in their scheme.

Indeed. Thanks for alerting us.

Discussion: Authors suggest that: another function of autophagy in the cells surrounding the
wound may be to clear up debris as in planarian and other cell types autophagy is activated
in healthy cells, which simultaneously phagocytose cell debris. Honestly, I do not believe
that this is the case here. Some of the Atg proteins are indeed required for phagocytosis
during LC3-associated phagocytosis (LAP) (see: PMID: 30787029), but LAP is independent
form Atg1

and if LAP happened in the cells, surrounding the wound then GFP-Atg8a positive
phagosomes would appear in those cells. However, it is clearly not the case here.

We are a little confused. We agree that phagocytosis can indeed be independent of Atg1.
But is this a counterargument to the idea that phagocytosis in the observed situation
nevertheless is involved in removing debris (as we have also reported previously, see Fig.
3b and Video 7 in PMID: 27713427)?

But perhaps more importantly, the cells surrounding the wound do in fact contain GFP-Atg8a
positive phagosomes.

Reviewer #2 (Significance (Required)):

I highly recommend this manuscript to be uploaded to a relevant journal and I believe the
findings presented here will be interesting for biologists specialized in regeneration and
readers from the autophagy fields alike.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

**Summary:**

The larval epidermis of Drosophila is a prime model for studying wound healing by
combining live imaging with cellular, genetic and molecular analysis of the processes
involved. Autophagy is known to be activated and necessary for efficient wound healing in
animal models through secretion of cytokines and clearance of bacteria. This manuscript
implicates autophagy in cellular syncytium formation during wound healing. Live imaging
demonstrates autophagy activation in cells surrounding the wound. Inhibition of autophagy
by RNAi against atg1 or atg5, required for autophagy initiation and autophagosome
formation had no effect on the rate of constriction and closing of the wound site. However,
elegant live imaging demonstrates that autophagy is required cell autonomously for cell
fusion, a normal process during wound healing in flies. Autophagy can also be instructive for
cell fusion. Strong induction of autophagy by TORC1 inhibition, TSC1/2 overexpression or Atg1 overexpression induce cell fusion that is genetically dependent on atg5, a gene acting downstream of atg1 in autophagosome formation. As Chloroquine treatment, a chemical inhibiting autophagosome fusion to the lysosome and lysosomal breakdown showed no effect, the authors suggest that later steps of autophagy are not involved. Live imaging with a selection of cellular fluorescently tagged markers of apical, lateral and basolateral membrane domains, combined with electron microscopy show clearly that lateral membrane are disrupted and removed within the epithelium. During this process, membranous large vesicles "drift" away from the plasma membrane. If these vesicles relate to autophagy is not addressed. In addition to the effect on cell fusion, strong autophagy induction also leads to autophagy within the nucleus, chromatin condensation and distortion of the nuclear membrane. The manuscript is well written and easy to follow. Figure panels and data are clearly presented. All experiments are well described throughout and skillfully executed with appropriate controls and statistical analysis. It remains unknown what induces autophagy in response to wounding. It also remains unclear whether autophagy deconstructs or engulfs parts of the plasma membrane, or if parts of the autophagy machinery has additional roles in plasma membrane fusion.

**Major comments:**

- Are the key conclusions convincing?
  - Conclusions are generally balanced and convincing.
  - I have seldom seen a paper so well written, presented and balanced by first pass. Hence my experimental suggestions are few.

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?
  - Claims are well founded.

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary to evaluate the paper as it is, and do not ask authors to open new lines of experimentation.

  - The inhibition of autophagy is performed using knockdown of two genes acting in autophagy initiation (atg1, a part of the ULK1 kinase complex) and atg5, required for autophagosome formation. Later acting genes in the autophagy process such as autophagosome closure, fusion with the lysosome or degradation were not analyzed. In the abstract, the authors state "Proper functioning of TORC1 is needed to prevent autophagy from destroying the larval epidermis which depends on membrane isolation and phagophore expansion, but not fusion of autophagosomes to lysosomes". As far as I can see, the last statement on fusion derives from experiments with Chloroquine. Although frequently used for qualitative experiments, CQ is not suited for conclusive experiments. Without genetic experiments targeting genes for autophagosome-lysosome fusion such as snap29, stx17, vamp7 this statement is in my mind not well supported.

  We had indeed ordered these strains from the Bloomington stock collection. However, they were dead on arrival and both our labs in Heidelberg and Cologne at the time had major problems with shipments from Bloomington and German customs. In the meantime, we were fortunately able to obtain these stocks and have done the experiments. None of the knockdowns suppress the phenotype, which confirms the chloroquine result. We have added these new findings

- Are the suggested experiments realistic for the authors? It would help if you could add an estimated cost and time investment for substantial experiments.
- Given the expertise of the authors, these experiments should be easy to perform within 3 months.

• Are the data and the methods presented in such a way that they can be reproduced?
  - The manuscript is well written and an excellent example of how methods and experiments should be presented. Methods, tools and experiments are all well described.

• Are the experiments adequately replicated and statistical analysis adequate?
  - Replicates and statistics are adequate and custom for the type of analysis performed.

**Minor comments:**

• Specific experimental issues that are easily addressable.
  Figure 3 h. The live imaging documents the striking disappearance of lateral cell membranes using SRC-GFP. In 3h, large vesicle formation and movement towards the cell interior is shown. How frequent is this?

This can only be seen clearly in experiments with time-controlled (Gal80ts) induction of autophagy where we can observe the process unfolding. We see these structures very frequently, but great variability in morphology and the structures are not always captured clearly in the plane of imaging. **We here provide further examples.**
**Figure D | Autophagy in unwounded epidermis.** a-c, Three additional examples showing apparent extrusions from lateral membranes after induction of autophagy (same experiment as Figure 3h). Time-lapse series of epidermal cells expressing Src-GFP and Atg1S. Transgene expression is induced at the end of the second larval instar, live imaging started 6 h later (t=0) and continued for an additional 6 hours. a-c, Src-GFP containing material appears to be taken out of and eventually detached from lateral cell membranes (arrows).

Is this believed to be the mechanism of lateral membrane removal?

We would of course like to believe that, but we have no proof, and would therefore only be able to speculate.

If so, is it dependent on the autophagy machinery. Are these vesicle positive for autophagy markers?

Some autophagy markers have indeed been reported to be associated with the plasma membrane (e.g. Atg9, Atg16), but a conclusive study, while highly desirable, in our view goes beyond the scope of this study.

Resolving this issue may lift the conclusions of the paper. Using 3xCherry-Atg8 together with SRC-GFP, this should be possible.
We carried out these experiments with two membrane markers, and despite the problem with the mCherry aggregates (see response to referees # 2) obtained nice results which we have now included. We did these not with artificially induced autophagy in the epidermis, but in the context of natural autophagy in the cells surrounding wounds.

Using CQ, the authors should be able to detect plasma membrane and junctional components in autophagosomes or autolysosomes (by confocal and electron microscopy) as degradation is inhibited. This should help to distinguish whether lateral membranes are engulfed and digested or if cells simply fuse, by using a part of the autophagy machinery.

We have many additional EM images on which we had extensive discussions with the Paolo Ronchi and Yannick Schwab at the EMBL (whom we embarrassingly forgot to acknowledge in our manuscript, which has now been corrected), and one of the authors of this paper (BM) is an expert in EM imaging of the larval epidermis. It was agreed that some structures could indeed be interpreted as autophagosomes with content resembling junctional material. We now include them in the paper.

The authors, state that strong autophagy activation also leads to syncytium formation of tracheal cells, salivary glands and gut EC cells. Representative images in a supplementary figure would be useful for future reference.

See response to other comments above (response to referees # 1). We have added some images in the revised manuscript.

• Are prior studies referenced appropriately?  
  -Yes. Key literature and findings are cited and discussed.

• Are the text and figures clear and accurate?  
  -Yes

• Do you have suggestions that would help the authors improve the presentation of their data and conclusions?  
  -See suggested experiments above.

Reviewer #3 (Significance (Required)):

• Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.  
  -The findings clearly document a role of autophagy in syncytium formation in the physiological process of wounding. This has parallels to muscle syncytium formation, but has to my knowledge not been demonstrated in any other cell type to be performed by autophagy. Moreover, the authors show that strong autophagy induction can lead to fusion of epithelial cells. This may have relevance for processes and diseases where polyploidy are observed.

• Place the work in the context of the existing literature (provide references, where appropriate).

• State what audience might be interested in and influenced by the reported findings.  
  -The data are very strong and the demonstration that autophagy controls syncytium
formation outside of muscle development is surprising and significant. It is of interest to the field of cell biology and development in general and the autophagy field in particular. It will also be of interest for the medical field that deals with multinuclear phenotypes, such as cancer.

• Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

-Development, cell signaling, autophagy, vesicle trafficking.

Table 1 | Fly stocks used in experiments

| Transgenes                  | Stock ID | Source                   | Publications using this construct                                      | Reference |
|-----------------------------|----------|--------------------------|-------------------------------------------------------------------------|-----------|
| UAS-GFP-Kuk (UAS-GFP-Kuk^{EY07696(w+)}) | Jörg Großhans | PMID: 16421189 | https://flybase.org/reports/FBal0161312 | 29        |
| UAS-Atg1^{RNAi} (UAS-Atg1^{RNAi}) | V # 16133 (GD7149) | PMID: 19363474 | PMID: 31995752 | PMID: 32032548 | PMID: 32915229 | https://flybase.org/reports/FBtp0034071.html |
| UAS-Atg5^{RNAi} (UAS-Atg5^{RNAi}) | V # 104461 (KK108904) | PMID: 31995752 | PMID: 32032548 | https://flybase.org/reports/FBtp0046851.html |
| UAS-Atg6^{RNAi} (UAS-Atg6^{RNAi}) | V # 110197 (KK102460) | PMID: 28581519 | PMID: 23599123 | PMID: 27542914 | PMID: 25644700 | Dissertation of Philipp Trachte, Abb. 23. https://refubium.fu-berlin.de/handle/fub188/27709 |
| UAS-Atg7^{RNAi} (UAS-Atg7^{RNAi}) | V # 45558 (GD11671) | PMID: 25882046 | PMID: 31995752 | PMID: 32032548 | PMID: 23599123 | https://flybase.org/reports/FBtp0025106.html |
| UAS-Atg12^{RNAi} (UAS-Atg12^{RNAi}) | V # 29791 (GD15230) | PMID: 25882046 | PMID: 17568747 | PMID: 31995752 | https://flybase.org/reports/FBtp0027770.html |
| UAS-TSC1,2 (UAS-TSC1, AUS-TSC2) | Iswar K. Hariharan | PMID: 15296714 | PMID: 11348592 | 64        |
| UAS-TSC1^{RNAi} (UAS-TSC1^{RNAi}) | V # 22252 (GD11836) | PMID: 23144631 | PMID: 29144896 | PMID: 29456138 | https://flybase.org/reports/FBtp0025266.html |
| Construct                              | BL #   | Maintainer               | PMID:                        | PMID:                      | BioBase Link                          |
|----------------------------------------|--------|--------------------------|------------------------------|---------------------------|---------------------------------------|
| UAS-Tor' (UAS-Tor'^RNAi)               | 33951  | Nobert Perrimon          | 25882046                     | 26395483                  | [FBtp0065159.html](https://flybase.org/reports/FBtp0065159.html) |
| UAS-TOR'^DN (UAS-TOR'^TED)             | 7013   | Thomas P. Neufeld        | 15296714                     | 29144896                  | [FBtp0016360.html](https://flybase.org/reports/FBtp0016360.html) |
| UAS-raptor' (UAS-raptor'^RNAi)         | 34814  | Nobert Perrimon          | 25882046                     | 31048465                  | [FBtp0081336.html](https://flybase.org/reports/FBtp0081336.html) |
| UAS-raptor'^2 (UAS-raptor'^RNAi)       | 41912  | Nobert Perrimon          | 32097403                     |                           | [FBtp0016360.html](https://flybase.org/reports/FBtp0016360.html) |
| UAS-rictor' (UAS-rictor'^RNAi)         | 36699  | Nobert Perrimon          | 25882046                     |                           | [FBtp0071419](https://flybase.org/reports/FBtp0071419) |
| UAS-Atg1S (UAS-Atg1'^RNAi)             |        | Thomas P. Neufeld        | 33253201                     |                           | [FBtp0016360.html](https://flybase.org/reports/FBtp0016360.html) |
| UAS-Atg1W, UAS-GFP (UAS-Atg1'^G10797') |        | Thomas P. Neufeld        | 33253201                     |                           | [FBtp0016360.html](https://flybase.org/reports/FBtp0016360.html) |
| UAS-S6K (UAS-S6K'^RNAi)                | 41895  | Nobert Perrimon          | 25284370                     |                           | [FBtp0071419](https://flybase.org/reports/FBtp0071419) |
| UAS-Sqa'^KD (UAS-Sqa'^T279A/CyO)       |        | Guang-Chao Chen          | 21169990                     |                           | [FBtp0071419](https://flybase.org/reports/FBtp0071419) |
| UAS-RhoA (UAS-RhoA'^RNAi)              | 12734  |                         | 23853710                     | 33789114                  | [FBtp0031970.html](https://flybase.org/reports/FBtp0031970.html) |
| UAS-Rok (UAS-Rok'^RNAi)                | 104675 |                         | 24995985                     | 33789114                  | [FBtp0046110.html](https://flybase.org/reports/FBtp0046110.html) |
| UAS-Rheb'^IV (UAS-Rheb'^IV')          | 9690   | Fuyuhiko Tamao           | 31909714                     | 28829944                  | [FBtp00141561.html](https://flybase.org/reports/FBtp00141561.html) |
Dear Parisa,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the original referee #3. As you can see from the comments below, the referee appreciates the introduced changes and supports publication here.

I am therefore very pleased to accept the manuscript for publication here. Before sending you the formal acceptance letter there are just some editorial points to resolve:

- You can only have 5 keywords
- Contributions should be re-labelled as Author Contributions
- Please also check the reference format
- The source data should be split into one file per figure
- Figure callouts are missing for Fig 5 C&D, Appendix Fig S4C, Appendix Fig S13C
- There's a callout to Appendix Fig S6F which doesn't exist.
- The legend to the movie should be provided as a word file and zipped with the respective movie file
- Table 1 should be moved to after the Reference section.
- We have changed Conflict of Interest to Disclosure & Competing Interests Statement.

- Also, as Maria Leptin has close ties to EMBO Press - you should state that in the Disclosure & Competing Interests Statement - see also https://www.embopress.org/page/journal/14602075/authorguide#conflictofinterest. Something like => "Maria Leptin is the former director of EMBO and a co-founder of Review Commons"

- Please upload the synopsis text and bullet points as a separate file (Cover art/synopsis image)
- Is the control panel in Fig 3F re-used in S5B? If so, please state this in the figure legend.
- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Take a look at the word file and the comments regarding the figure legends and respond to the issues.

That should be all - let me know if you have any further questions.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Link Not Available
Referee #3:

Original summary of the study

“The larval epidermis of Drosophila is a prime model for studying wound healing by combining live imaging with cellular, genetic and molecular analysis of the processes involved. Autophagy is known to be activated and necessary for efficient wound healing in animal models through the secretion of cytokines and clearance of bacteria. This manuscript implicates autophagy in cellular syncytium formation during wound healing. Live imaging demonstrates autophagy activation in cells surrounding the wound. Inhibition of autophagy by RNAi against atg1 or atg5, required for autophagy initiation and autophagosome formation had no effect on the rate of constriction and closing of the wound site. However, elegant live imaging demonstrates that autophagy is required cell-autonomously for cell fusion, a normal process during wound healing in flies. Autophagy can also be instructive for cell fusion. Strong induction of autophagy by TORC1 inhibition, TSC1/2 overexpression or Atg1 overexpression induce cell fusion that is genetically dependent on atg5, a gene acting downstream of atg1 in autophagosome formation. As Chloroquine treatment, a chemical inhibiting autophagosome fusion to the lysosome and lysosomal breakdown showed no effect, the authors suggest that later steps of autophagy are not involved. Live imaging with a selection of cellular fluorescently tagged markers of apical, lateral and basolateral membrane domains, combined with electron microscopy show clearly that lateral membrane are disrupted and removed within the epithelium. During this process, membranous large vesicles “drift” away from the plasma membrane. If these vesicles relate to autophagy is not addressed. In addition to the effect on cell fusion, strong autophagy induction also leads to autophagy within the nucleus, chromatin condensation and distortion of the nuclear membrane. The manuscript is well written and easy to follow. Figure panels and data are clearly presented. All experiments are well described throughout and skillfully executed with appropriate controls and statistical analysis. It remains unknown what induces autophagy in response to wounding. It also remains unclear by what mechanism autophagy deconstructs or engulfs parts of the plasma membrane, or if parts of the autophagy machinery has additional roles in plasma membrane fusion.”

The revised manuscript satisfactorily addresses all of the concerns I raised for the initial submission.

The surprising effect on syncytium formation, both physiologically during wounding and artificially using genetic activation of autophagy is now convincingly shown. Additional use of membrane and lateral junction markers together EM analysis, lend further support to the idea that lateral plasma membrane is degraded through autophagy.

Suggestions for improvement:

- On page 4. The authors state that "knockdown of Atg6 (phagophore nucleation factor), Atg7 or Atg12 (phagophore elongation factors) also resulted in a decrease in autophagosomes, but the effect was much less pronounced (Fig 1A, B, D and Movie EV1"

And then follow on by saying: "This is consistent with previous findings that these components are accessory but not necessarily essential for the completion of autophagy1"

Although this can be said of Atg7, Atg12, it is not a general consensus in the field that this is the case for Atg6. Looking through the referenced paper, I guess the author may base this on the RNAi experiments in ref 21 (Xu, T, et al, 2015) however atg6 mutant analysis in ref 25 (Shravage, BV et al 2013) clearly show that atg6 is strictly required.

-I suggest avoiding the sweeping collective statement of the last sentence “This is consistent with previous findings that these components are accessory but not necessarily essential for the completion of autophagy1” by either removing it altogether or rephrasing appropriately.
Dear Parisa,

Thank you for submitting your revised manuscript. I have now looked at it and all looks good!

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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** Click here to be directed to your login page: https://emboj.msubmit.net
Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

The data shown in figures should satisfy the following conditions:

- The data 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 (e.g., cell line, species name).
- A description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, filters, 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 2 groups, 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-tailed?
  - 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.
  - What was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data. Please fill in these boxes. [Do not worry if you cannot see all your text once you press return]

B. Statistics and general methods

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

We do not have large numbers of experiments but whenever possible, carried out each experiment at least three times.

1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

This is not an animal study.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

Larvae that did not survive until post larval stages were excluded. This was pre-established. After live imaging, larvae were returned individually into food vials and monitored for survival.

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.

There was no pre-selection of larvae.

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.

The randomization was used. Larvae were selected on the basis of the genotypic interest and equivalent positions were always imaged: the dorsal larval epidermis in the abdominal segments 33–45 of L3 instar larvae.

4.b. For animal studies, include a statement about blinding even if no blinding was done.

No blinding was done.

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.

For statistical hypothesis testing, an independent and non-parametric Kruskal-Wallis tests were performed for the mean of spots in control and experimental conditions in the graphs in Figs 1J, 3B, and 5C. We assumed unequal sample size and unequal variances and calculations were performed using the SciPy library from Python 3.7 and GraphPad Prism version 8.1. For all other graphs (Appendix Figs S7D, S7G, and S12B) ordinary one-way ANOVA statistical tests were performed after we confirmed normality using the Shapiro-Wilk test (max α = 0.05). Values in this paper are presented as mean box plots. Box plot elements are: centre line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; points, outliers. P values are indicated as follows: *P < 0.04; **P < 0.003; ***P < 0.0002; ****P < 0.0001 and lack of an asterisk or ns means non-significant (P > 0.123).
**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), AntibodyRadar (see link list at top right).

7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

| Question | Answer |
|----------|--------|
| Is there an estimate of variation within each group of data? | Yes. The spread is plotted and described wherever necessary. |
| Is the variance similar between the groups that are being statistically compared? | Yes. The tests used do not assume equal variance (see above & Method section). |

**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.

9. For experiments involving the vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

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'. Please confirm you have followed these guidelines.

**E - Human Subjects**

11. Identify the committee(s) approving the study protocol.

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.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For phase I and II randomised 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.

17. For tumour marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.

**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 GEO, Proteomics data: PRIDE PRED000008 etc.) Please refer to our author guidelines for 'Data Deposition'.

- Data deposition in a public repository is mandatory for:
  - Proteomics data: PRIDE PXD000208 etc. Please refer to our author guidelines for 'Data Deposition'.
  - Proteomics and molecular interactions
  - Functional genomics data
  - Antibody-related information

19. Deposit 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 Ciro (see link list at top right) or Dryad (see link list at top right).

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).

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 formats (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 models 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.

**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.

| Question | Answer |
|----------|--------|
| Could your study fall under dual use research restrictions? | No |