# Ral GTPase and the Exocyst Regulate Autophagy in a Tissue-Specific Manner

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**Transaction Report:**

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

Thank you for your patience while your study has been under peer-review for EMBO reports. We have now received the three enclosed reports on it. As you will see, although referee 3 is very positive, referees 1 and 2 are less so, and raise various concerns regarding the conclusiveness of the findings.

There are two important underlying concerns: that the evidence of the lack of a role for Ral and the exocyst in fat body autophagy is not sufficiently conclusive, and that the study does not advance our understanding of how Ral and the exocyst facilitate autophagy during developmentally regulated cell death. Upon further discussion with the referees, it became clear that sufficient support for publication would not be obtained unless both issues were addressed. As this is a tall order of uncertain outcome, we feel it is beyond the scope of a revision and have decided to return your manuscript to you at this stage.

Given the potential interest of the findings, however, we would be open to considering the resubmission of a related study if you were to develop the study along the lines indicated above. Please note that if you were to send a new manuscript, this would be treated as a new submission rather than a revision and would be editorially assessed afresh, especially with respect to novelty at the time of resubmission. If no novelty concerns arise and the referee concerns have been appropriately addressed, we would aim to engage the same three referees in the assessment of this resubmission.

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I am very sorry to disappoint you on this occasion, and nevertheless hope that the referee comments are helpful in your continued work in this area.

REFEREE REPORTS

Referee #1:

The paper by Tracy and Baehrecke explores the role of RalGTPase in the regulation of autophagy. Previous work in mammalian cells using knockdown or knockout strategies (PMID: 21241894 and 23473774) has suggested that RalB and its regulator RalGDS are involved in the regulation of autophagy that is induced in response to starvation or to invading pathogens and also may affect autophagy involved in stress responses in cardiomyocytes (PMID 23473774).

The current manuscript explores the function of the single Drosophila Ral gene in autophagy. Using Ral knockdown, expression of dominant negative Ral or a weak hypomorphic allele of Ral the authors test the role of Drosophila Ral in autophagy. The authors show a requirement for Ral and the exocyst complex in autophagy-mediated cell death in salivary gland. Nevertheless the authors show that these reductions of Ral activity fail to cause any effect on starvation-induced cell death in fat bodies. The proposed cell and context-specific roles of Ral and the exocyst complex in autophagy are interesting and important observations, but there are some issues that should be addressed before publication.

An important weakness of the data is that only a viable weak hypomorphic allele of Ral was used to test its effect on autophagy in fatbodies. Stronger, lethal alleles are available (e.g. PMID: 18552769) but are not tested here. Given the very high expression levels of Ral reported for fatbodies (Flybase) that should be tested before negating any effect of Ral on autophagy in fatbodies. The authors indicate that lethality of such strong alleles precludes their use in larvae, but the clonal analysis in mosaic tissues should still be possible as it was in ovaries (e.g. PMID: 18552769).

Remnant salivary tissue in late pupae is identified histologically, which seems straightforward and clear in some cases (e.g. Fig. 1 A or G), but much less clear in other cases. For example, the criterion by which salivary gland tissue is recognized in panels 1E, 3A or 6E is not obvious to me (maybe before compression of the PDF file that was more obvious). It is also not clear what the criterion is to distinguish between gland fragments and cell fragments in some of the quantifications of phenotypes. As these are key experiments in the current paper, those criteria should be made clear here and not just by referring to previous papers.

Another concern is that only a single assay was used to assess autophagy in fatbodies, the accumulation of mCherry-ATG8 positive punctae. While those results are very clear and convincing, the impact of the paper surely would be much greater if more than one assay would be used to assess autophagy as has been widely agreed to as a standard (PMID: 22966490). Especially an assay that measures autophagic flux would be important to complement the included data to buttress the claim that loss of Ral function has no effect on starvation induced autophagy.

other minor issues:

• On page 4 the authors appear to say that the Bodemann paper (PMID: 21241894) claimed a near universal requirement for Ral function in autophagy ("model makes several predictions, including that Ral functions as a broad regulator of autophagy in most if not all cell contexts." This seems to exaggerate the original papers claims and should be rephrased. The current observation that some but not all autophagy induction in Drosophila requires Ral seems interesting enough without such reinterpretation of the previous paper.
• Ral[35d] is claimed to be a weak hypomorphic allele of Ral, but this is not supported by any experimental data or reference.
• Considerable work has been done to implicate Ral in Notch and Jak/Stat signaling in Drosophila (e.g. PMID: 18552769 and PMID: 21350007) and it seems worthwhile to at least mention possible roles of these pathways, but I recognize that the character limit constitutes a serious issue in that
context
• at last, the paper may benefit from a little rewriting to untangle word monsters such as "an additive more intact salivary gland tissue fragment phenotype" (page 6) and to clarify phrases like "ral35d/wild type background " (page 6; presumably referring to ral35d heterozygous flies).

Referee #2:

Tracy et al study the regulation of autophagy in different tissues and under different growing conditions. The authors utilize salivary gland degradation in drosophila to study autophagic cell death during development and fatbodies to examine starvation induced autophagy. From their data the authors suggest Ral GTPase is an upstream regulator of autophagy through the exosyst complex in autophagic cell death yet not in starvation induced autophagy.

The manuscript is well written and the experimental questions and settings are clearly explained yet the data presented is not sufficient to support the conclusions drawn by the authors. First, the evidence for the regulation of autophagy by Ral is only correlative, as a direct regulation of the process was not examined. Second, the data regarding the involvement of autophagy in developmental cell death is too preliminary, utilization of additional autophagic markers and experimental procedures is necessary. Last, the effect of Ral manipulation in the various tissues is presented under starvation conditions only and it's comparison to control growing conditions is essential (Figures 3 and 4). In summary, the data presented are too preliminary to support the authors' model.

Referee #3:

This paper investigates the role of the Ral GTPase and the exocyst in autophagy in vivo. Drosophila is a useful model since different tissues undergo autophagy in response to different stimuli. The authors find that Ral and the exocyst affect autophagy during salivary gland programmed cell death but not starvation-induced autophagy in the fat body. The results are strong and demonstrate a clear difference between the tissues. This is an important finding as it has been proposed that Ral could function globally in autophagy. All of the comments below are minor and can be addressed with modifications to the text.

1. In the text referring to Figure 2, more detail about the difference between cell fragments and tissue fragments would benefit the reader.

2. Is there any indication that the Ral and exocyst mutants cause a developmental delay to salivary gland death, and thus an indirect effect on autophagy?

3. In the discussion the authors mention that there could be cell-type specificity for Ral regulation of autophagy. Have the authors looked at starved tissues other than the fat body?

4. In Figure 3, the % with salivary gland material is about 40% in ral mutants, although it was ~60% in Figure 1 and 2. Were experimental conditions different in Figure 3, or is this within the normal range of variability?

5. In Figure 4, the images seem to show an enhancement of puncta in the clones, although this is not apparent in the quantification. Perhaps these are not the best images to support the graphs, although they do support the conclusions that there is no block in autophagy. Likewise in Supplemental Figure 2, the sec3IR image shows increased puncta in the clones (although not in the graph). This may be due to bleed through from the GFP and should be explained.

6. Supplementary Figure 1. It would be helpful to explain the assay in more detail in the figure legend.
Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. I apologize for the delay in the editorial process, which was caused by a very high submission rate to our journal.

As you will see, both referees are very positive about the study and request only several changes to the text and the figures that I would like you to incorporate before we can proceed with the official acceptance of your manuscript. In particular, as referee 1 points out, the statement on Notch should be rephrased.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- We require an author's checklist and please also indicate the page numbers of the manuscript, where the relevant information can be found.

- The Expanded View format has replaced the Supplementary information. A maximum of 5 EV figures can be supplied for a manuscript. Please follow the nomenclature Figure EV1 and Table EV1 for the Supplement. The legends for these figures and tables are included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section.

- Every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text, summarizing in 2 sentences the study (max. 205 characters) as well as 2-4 one sentence bullet points that summarize the highlights. These should be complementary to the abstract, i.e., not repeat the same text. This will be accompanied by a thumbnail image and a Synopsis image (500 x 400 pixel) of your choice. Could you please provide the standfirst text, bullet points and a synopsis image?

Please contact me any time of you have any questions.

I look forward to seeing a revised form of your manuscript when it is ready.

We would like to thank the Reviewers and Editor for their thoughtful and constructive comments. Each reviewer and editor comment (bold) is followed by our response below.

Referee #1:
1) One change should be made, however. The authors added new data showing A) that activity of a single Notch reporter is reduced upon reduction of Ral activity by knockdown and B) that Notch knockdown reduces ATG8 punctae. These are intriguing data, but certainly not enough to conclude that Ral acts through Notch to regulate autophagy, as the authors do at the end of the result section ( "These data indicate that Ral regulates autophagy via Notch. " ) and also in the discussion. To support such a strong statement, additional epistasis experiments will be necessary. Alternatively, the authors could weaken that statement and phrase it as a possibility to be tested further.

We agree, thank the reviewer for this comment, and have changed the text as suggested.

Referee #2:
1. Figure 1 and elsewhere. The black dotted circle is hard to see in the histology panels. Perhaps another color would be easier to see.

We agree and have changed the color of the dotted circles to red in all of the appropriate figures.
2. Figure 3 is a little confusing since some mutant cells are positively marked and some are negatively marked. Clones could be marked with an asterisk to make it easier to understand the figure.

We agree and have marked the cell clones with a yellow asterisk in Figure 3.

3. In Figure 3I, the GFP puncta look larger in the clone. This does not change the conclusions but if this is reproducible, it could be mentioned/interpreted.

Although we agree with this comment about the image we had provided, this was not consistent across a large number of mutant clones that we analyzed. Therefore, we have changed the images in Figure 3I to more accurately reflect what we observed.

4. Was caspase activity examined in the exocyst mutants? This could be mentioned.

Caspase activity was not analyzed in exocyst mutants.

Editor:
1) We require an author's checklist and please also indicate the page numbers of the manuscript, where the relevant information can be found.

This has been completed.

2) The Expanded View format has replaced the Supplementary information. A maximum of 5 EV figures can be supplied for a manuscript. Please follow the nomenclature Figure EV1 and Table EV1 for the Supplement. The legends for these figures and tables are included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section.

We have corrected the expanded view as requested.

3) Every EMBO reports paper now includes a ‘Synopsis’ to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text, summarizing in 2 sentences the study (max. 205 characters) as well as 2-4 one sentence bullet points that summarize the highlights. These should be complementary to the abstract, i.e., not repeat the same text. This will be accompanied by a thumbnail image and a Synopsis image (500 x 400 pixel) of your choice. Could you please provide the standfirst text, bullet points and a synopsis image?

A synopsis and thumbnail image have been included with the revised manuscript.

2nd Editorial Decision
22 October 2015

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