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Conserved Atg8 recognition sites mediate Atg4 association with autophagosomal membranes and Atg8 deconjugation

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Transaction Report: This manuscript was transferred from *The EMBO Journal*, where it was originally reviewed. The following report contains those referee comments that were outstanding at the time of transfer.

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

1st Editorial Decision 03 August 2016

Thank you for the transfer of your research manuscript to EMBO reports. As I had indicated earlier, I would like to give you the opportunity to submit a modified and strengthened version of your work with the understanding that the referee concerns raised during the review process for EMBO Journal must be addressed and their suggestions taken on board - with the exception of the characterization of the role of Atg1 on Atg4 recruitment. This is beyond the scope of the current manuscript.

Importantly, in vitro evidence should be provided that the Atg4 LIR2 is indeed required to delipidate Atg8, as requested by both referees. Moreover, it should be tested at least either with Y2H or via pull-down assay if the other LIR domains are involved in Atg8 binding and if the LIR2 mutant is unable to directly bind Atg8 in vitro. All relevant controls have to be provided. Moreover, an additional assay to determine the effect on autophagy could be employed. A time-lapse analysis to verify the transient nature of the interaction of Atg4 with the PAS would certainly strengthen this claim.

Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #2:

Abreu et al. reported the identification of a new "conserved" LIR motif in Atg4 that is important for its association with autophagosome membrane and Atg8 deconjugation. This recruitment is crucial for deconjugation of Atg8-PE and then to autophagy efficiency, but not for post-translational C-terminal cleavage of Atg8. This study is novel and potentially important. The quality of presented data is high and supportive. However, several critical issues should be addressed before publication is considered.

1. Critical biochemical results are missing. It is important to confirm that LIR2 mutant loss its direct binding to Atg8 in vitro. More importantly, the authors should provide direct evidence that Atg4 LIR2 mutant is unable to deconjugate Atg8-PE in vitro. These biochemical evidences will strengthen the major conclusion raised in this study significantly.

2. The authors raised an interesting point that ATG4 localization at PAS is likely a transient event that takes place at a precise time point during autophagosome biogenesis. This point is novel but the authors failed to provide a direct evidence for this notion. A time-lapse experiment to catch this dynamic association under physiological conditions will help to establish this point, since it is not entirely clear whether the Atg8/ApeI positive dots in Atg1 deletion cells represent truly meaningful PAS.

3. The authors identified four LIRs in Atg4, likely according to their relative positions. If this is the case, it is confusing why 446-449 is named LIR3 but 424-427 is named LIR4. In Figure EV4B, the LIR sequence aligned crossing the species is LIR3 (446-449), but not LIR2 (102-105) as marked. This possible mislabel causes the big confusion about the predicted structure in Figure EV4C. Is this LIR LIR3 or LIR2? How about the rest of labeling? In Figure 3E and 3F, the authors should also include the LIR1, LIR3 and LIR4 mutants to test if these LIRs are involved in Atg8 binding. These LIR mutants should also be included in the rest of assays, especially the conserved LIR3.

Referee #3:

Atg4, a key autophagic enzyme responsible for the processing of Atg8 at its C-terminal to expose the glycine group that is later conjugated to PE on the membrane. Atg4 is also responsible for deconjugating Atg8 from the lipid. An interesting question in the field is how these two reactions are regulated in the context of autophagy. The present study deals with one aspect of this process, namely the mechanism by which Atg4 is recruited to the membrane to catalyze Atg8-PE cleavage. According to the proposed model, Atg8-PE recruits Atg4 by interacting with a LIR sequence identified in this study. Accordingly, the LIR-mediated interaction is specifically needed for deconjugating Atg8 from the lipid but not for the initial cleavage.

These are potentially interesting findings that may contribute to a better understanding of autophagy regulation. However, the data presented are limited and too preliminary to support the suggested model. Particularly, the notion that the identified Atg4 LIR (LIR3) is indeed specifically required for the delipidation process should be determined in vitro, using purified components. Moreover, the role of Atg1 on Atg4 recruitment to the PAS is only briefly mentioned and should be better characterized. The authors should also consider to add a schematic model.

Additional comments:
- Autophagy should be determined throughout this study by additional assays such as GFP-Atg8 cleavage and vacuolar translocation. The signal to noise ratio of the PhoΔ60 assay used in this study should be improved.
- The data presented in figure 2 are lacking a positive WT control. Moreover, it would be more informative to include the atg1Δ data and present it as part of figure 1.
- It has been shown that deletion of Atg2, Atg18 also increase Atg8 localization to the PAS. It will be informative to determine whether in these strains too Atg4 follows Atg8.
- The data describe in figure 3 are mostly convincing. It would be important though to determine

whether IP shown in panel F can be detected in strains defective in Atg8 conjugation system (atg3Δ or atg7Δ).
- Atg8 lipidation shown in figure 4E should also be determined in cells expression Atg8ΔR.

1st Revision - authors' response 08 January 2017

We would like to thank the two reviewers for the very constructive comments and suggestions, which have helped to strength our story and improve the quality of the manuscript. In particular, three of the requested experiments has permitted to find 1) that the LIR4 motif, which is also conserved, participates to the interaction between Atg4 and Atg8, and 2) that LIR2 is not a LIR motif but a new sequence that permits Atg4 to recognize lipidated Atg8 (we have now renamed LIR2 as Atg8-PE Association Region, APEAR).

The considerable additional work has led us to acquire more information about the possible mechanism for Atg4-mediated deconjugation of Atg8-PE from autophagosomal membranes. Although not required for the normal progression of autophagy, the LIR4 motif of Atg4 strengths the biding between Atg4 and Atg8 in vivo and our data suggest that it might work cooperatively with the LIR2/APEAR domain. Overall, however, the major conclusions of our study remain unchanged: 1) Atg4 is recruited to the PAS and 2) its substrate (i.e. Atg8) is key determinant for Atg4 recruitment to this location. As mentioned above, a new conclusion reached with the new set of experiments is that Atg4 possesses a motif, the APEAR, which allows Atg4 to specifically recognize Atg8-PE on autophagosomal membranes. The title and the manuscript have been changed accordingly.

Reviewer #1

1. Critical biochemical results are missing. It is important to confirm that LIR2 mutant loss its direct binding to Atg8 in vitro. More importantly, the authors should provide direct evidence that Atg4 LIR2 mutant is unable to deconjugate Atg8-PE in vitro. These biochemical evidences will strengthen the major conclusion raised in this study significantly.

Because of the finding mentioned above, we have tested the in vitro binding between Atg8 and either Atg4, Atg4LIR2 or Atg4LIR4 (Figure 5E). This experiment has revealed two important things. First, LIR4 is a bona fide LIR motif and its relevance in Atg4 association to Atg8 is also underlined by the in vivo pulldown experiments (Figure 2B). Second, LIR2 is not a LIR motif and is not involved in Atg4 binding to non-lipidated Atg8. This observation is also confirmed by in vivo pull down experiment in cells where Atg8 cannot be conjugated to PE (Figure 5D). However, LIR2 domain is key for the binding of Atg8 to lapidated Atg8 (Figure 2B).
We have also performed the requested in vitro deconjugation assay (Figure EV7E) and the results mirror the ones obtained in vivo, i.e. the LIR2/APEAR motif plays a key role in Atg8-PE deconjugation.

2. The authors raised an interesting point that ATG4 localization at PAS is likely a transient event that takes place at a precise time point during autophagosome biogenesis. This point is novel but the authors failed to provide a direct evidence for this notion. A time-lapse experiment to catch this dynamic association under physiological conditions will help to establish this point, since it is not entirely clear whether the Atg8/ApeI positive dots in Atg1 deletion cells represent truly meaningful PAS.

We have performed live-cell imaging experiments where we have analyzed the dynamic association of Atg4-GFP to the PAS. As hypothesized, Atg4-GFP is always recruited after the formation of mCherry-Atg8-positive PAS and leaves these structures before what is a probable fusion of complete autophagosomes with the vacuole. These data are presented in Figure EV1 and Supplemental Video S1.

3. The authors identified four LIRs in Atg4, likely according to their relative positions. If this is the case, it is confusing why 446-449 is named LIR3 but 424-427 is named LIR4. In Figure EV4B, the LIR sequence aligned crossing the species is LIR3 (446-449), but not LIR2 (102-105) as marked. This possible mislabel causes the big confusion about the predicted structure in Figure EV4C. Is this LIR LIR3 or LIR2? How about the rest of labeling?

We apologize for the wrong information inserted in Figure EV4B. We have corrected it (still Figure EV4B). We have also verified very carefully the rest of the labeling and now they are all correct.

4. In Figure 3E and 3F, the authors should also include the LIR1, LIR3 and LIR4 mutants to test if these LIRs are involved in Atg8 binding. These LIR mutants should also be included in the rest of assays, especially the conserved LIR3.

We have examined all the LIR motif mutants in the experiments shown in Figure 3F (now Figure 2B), Figures 4C and 4D (now Figures 3C and 3D), Figure 4E (now Figure 3E) and Figures 5A-5C (still Figures 5A-5C).

Reviewer #2

1. Particularly, the notion that the identified Atg4 LIR (LIR2) is indeed specifically required for the delipidation process should be determined in vitro, using purified components. Moreover, the role of Atg1 on Atg4 recruitment to the PAS is only briefly mentioned and should be better characterized.

See rebuttal to point 1 of reviewer #1 for the in vitro experiments. In our manuscript, we have not mentioned a possible role of Atg1 or any other Atg protein that when knocked out, leads to a more pronounced Atg4 recruitment to the PAS.

2. The authors should also consider to add a schematic model.

We have taken this suggestion in account and added a putative mechanistic model as Figure EV8B.

3. Autophagy should be determined throughout this study by additional assays such as GFP-Atg8 cleavage and vacuolar translocation. The signal to noise ratio of the Pho 60 assay used in this study should be improved.

In addition to the Pho8 60 assay, another method that we originally employed to assess autophagy was the measurement of prApe1 maturation (Figures 3B, 3C, EV3A and EVB4; now Figures 2D, 2E, EV5A and EV5B). Nonetheless, we have now performed the vacuolar translocation for all the LIR mutants (Figure 3C and 3D) and the autophagic body counting (Figures 5A and 5B), which are two alternative approaches to measure autophagy progression. The conclusion has remained unchanged, i.e. the LIR2 motif plays an important role for autophagy in vivo.

4. The data presented in figure 2 are lacking a positive WT control. Moreover, it would be more informative to include the atg1 data and present it as part of figure 1.

We have followed the suggestion of this reviewer and integrated Figure 2 into Figure 1. Figure 2 has now become panel D of Figure 1. We have also added WT and atg1 controls.

5. It has been shown that deletion of Atg2, Atg18 also increase Atg8 localization to the PAS. It will be informative to determine whether in these strains too Atg4 follows Atg8.

This result was illustrated in Figure EV1 in the original version of the manuscript. It is now in Figure EV2 of the revised version of the manuscript.

6. The data describe in figure 3 are mostly convincing. It would be important though to determine whether IP shown in panel F can be detected in strains defective in Atg8 conjugation system (atg3 or atg7).

We have performed the requested experiment in atg3 cells and in accordance with the in vitro binding assay (Figure 5D), we did not observe a defect in the interaction between Atg4LIR2 and Atg8. This is a key result because when the pulldown experiment is done in a wild type background expressing Atg4LIR2, where almost the entire Atg8 population is conjugated to PE (Figure 3E), binding between Atg4 and Atg8 is severely reduced. The inability of Atg4 with a mutation in the LIR2/APEAR domain to recognize Atg8-PE, explain why this mutant protein is unable to deconjugate Atg8-PE in vivo and in vitro (Figures 3D, 3E and EV7E).

7. Atg8 lipidation shown in figure 4E should also be determined in cells expression Atg8 R.

The Atg8 lipidation has been assessed in cells expressing Atg8 R as requested and it is presented in Figures 3E. This experiment confirms that the strain expressing Atg4LIR2 display an in vivo defect in Atg8 deconjugation from PE.

2nd Editorial Decision 07 February 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. As you will see, both referees are positive about the study and recommend publication in EMBO reports.

REFEREE REPORTS

Referee #1: The authors addressed my concerns and the manuscript in its present form meets EMBOR scientific merit.

Referee #2: Acceptable

2nd Revision - authors' response 12 February 2017

Autbors made the requested editorial changes and resbumitted their manuscript.

3rd Editorial Decision 20 February 2017

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.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Prof. Dr. Fulvio Reggiori
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Tipycally, 100-150 cells were counted in the fluorescence microscopy experiments and 50 cell sections in the elctron microscopy examinations. Nonetheless, the cumulative presentation approach has been used to determine the convergence of the results at each time. If not converging, more cells/cell sections were conuted until reaching a convergence of the results.
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C- Reagents

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not applicable.

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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.	Not applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
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.	Not applicable.
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Not applicable.
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