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ESCRT machinery mediates micro-autophagy of the endoplasmic reticulum in yeast.

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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.)

1st Editorial Decision 26th June 2019

Thank you for submitting your manuscript entitled "ESCRT machinery mediates selective microautophagy of endoplasmic reticulum" [EMBOJ-2019-102586] to The EMBO Journal. Your study has been sent to three reviewers for evaluation, whose reports are enclosed below.

As you can see, the referees find your work potentially interesting. However, they also raise several key points that need to be addressed before they can support publication in The EMBO Journal. In particular, the reviewers request you to i) better characterize the role of Pho8 in ER microautophagy (referee #1; points 1-4 from referee #3), ii) discuss in detail the role of ESCRT proteins in micro- and macro-ERphagy and lysosomal membrane scission (referee #1), iii) provide images of macro- and micro-autophagy-derived ER and investigate the underlying morphological differences (referee #2), iv) examine viability of micro- and macro-ERphagy-deficient cells (referee #2), and v) improve the EM analyses of the ESCRT mutants (referee #3).

Given the overall interest of your study, I would like to invite you to revise the manuscript as requested in the referees' reports. I would like to point it out that addressing the above points and all minor issues, as well as a strong support from the reviewers, will be essential for publication here.

REFEREE REPORTS

Referee #1:

In the manuscript "ESCRT machinery mediates selective micro-autophagy of endoplasmic reticulum" Schuck and colleagues proposed that micro-autophagy process is responsible for ER-phagy in yeast and it works in a parallel respect to macro-autophagy. Micro-autophagy can be

trigged by over-expression of ER resident transmembrane proteins and Nem1-Spo7 and ESCRT proteins are essential for the process. Interestingly, the classical autophagy genes and the known ER-phagy receptors seem not to be required. Even if micro-ER-autophagy is probably more oriented to the elimination of ER whorls after protein overexpression or after chemical stimuli, while macro-ER-phagy is fundamental during starvation, they are both required for cell homeostasis. Moreover, a common point between the two processes are the ESCRT proteins which seem to play a key role in both pathways.
The manuscript is well written, and data presented in a logic and comprehensive manner. The idea of the existence of two types of ER-phagy (micro and macro - ER - phagy) depending on the stimuli that the cell is receiving is also interesting and well fit with the complexity of ER biology. The manuscript is quite mature; however, the authors should take into consideration few points:
- Activation of micro-ER-phagy after ER resident protein over-expression is interesting and should be further investigated. More examples will help to understand if all ER resident proteins over-expression/accumulation potentially can trigger micro-ER-phagy or there is a specific subset like luminal, intramembrane or transmembrane proteins...
- The reporter Yop1-Pho8Δ60 is functional. To have a more complete picture, would be appropriate to add some different readouts for examples some biochemistry, protein degradation assay, ...
- The ESCRT proteins role in micro-ER-phagy, macro-ER-phagy and lysosomal membrane scission is the most interesting point and should be elaborate and discussed more in details. Apparently, these proteins coordinate the all autophagy process in the yeast. Would be nice to further investigate if there is a division of the ESCRT proteins in specific subgroups dedicated to each function (micro-, macro-autophagy, lysosomal membrane scission ..). Moreover, some hints on the molecular mechanisms of ESCRT protein activation in the different scenarios will add a huge value to the manuscript.

Referee #2:

Cells remodel their internal complement of organelles in response to the external environment. The authors examined ER degradation by macro-autophagy and micro-autophagy. Firstly, electron microscopic observation and fluorescence microscopic observation clearly indicated that the vacuolar ER whorl, which is induced by tunicamycin treatment, DTT treatment, or GFP-Pho8 expression, is formed by micro-autophagic pathway. Next, using the formation of ER whorl by GFP-Pho8 expression as an index, the authors performed visual screening of the yeast knockout collection. As a result, the authors identified that the Nem1-Spo7 complex and ESCRT machinery are essential for the formation of the ER whorls and are thereby key components for micro-ER-phagy. Furthermore, the authors also tried to determine the contribution of micro- and macro-ER-phagy under nitrogen starvation or tunicamycin treatment using a modified Pho8 derivative which localizes to the ER. From this analysis, the authors concluded that core autophagy machinery and Nem1-Spo7 drives macro- and micro-ER-phagy, respectively, and that the ESCRT machinery contributes to both pathways. Finally, it was shown that ESCRTs mediates vacuolar membrane scission at the final step of micro-ER-phagy.

Micro-autophagy and its mechanism are not well understood. In addition, the contributions that macro-autophagy and micro-autophagy make to cellular degradation have not been clearly defined. This manuscript addresses these unresolved issues by examining ER-phagy, and is an excellent study for its description of not only micro-autophagy but also ER-phagy. The experiments are well presented and the descriptions are also very clear. For those reasons, I recommend this manuscript for publication in the EMBO journal. However, I would make some minor suggestions, as described below. The authors should consider these points.

1. Fig 3A, 3B, and S3: The authors showed one image for each strain. The authors should indicate the fraction of cells in terms of GFP-Pho8 structure as in Fig 5B.

2. In Fig 4: If possible, the authors should include images which show macro-autophagy derived ER and micro-autophagy derived ER, and investigate the morphological differences (as shown in the model diagram in Fig 7). Blocking the degradation of autophagic bodies (deletion of ATG15 or double deletion of PEP4 and PRB1) may allow the authors to obtain those images.

3. It is unclear how much ER-phagy contributes to cellular homeostasis because Atg39 or Atg40 deficient cells are viable for several days (Mochida et al., Nature 2015). If micro- and macro-ER-phagy are able to compensate for each other, inhibition of both pathways could shorten life span duration. The authors should examine cell viability in macro- and micro-ER-phagy deficient cells.

4. It would be nice to show that DTT/tunicamycin induced whorls could be stained with a dye for the ER. Some dyes like R18 (Hirata et al., Plos one 2017) are known to stain the ER membrane.

5. In lines 60-62 of the manuscript, the authors described endolysosomal membrane consumption and replenishment by micro- and macro-autophagy. However, according to my understanding, the impairment of macroautophagy does not shrink the size of the vacuole. Macroautophagy is not only the process to supply lipids to the vacuolar membrane. Lipid transfer protein, such as Vps13, and other factors could contribute to maintain vacuolar lipid supplementation.

Referee #3:

Jasmin Schaefer, Sebastian Schuck and co-workers report that the 'ESCRT machinery mediates selective micro-autophagy of endoplasmic reticulum' in budding yeast.

First, the authors use fluorescence microscopy, freeze fracture and transmission electron microscopy and correlative light and electron microscopy to morphologically describe different stages of ER micro-autophagy. The show at least three different stages: (1) stacked ER is piling up on vacuoles (2) the stacked ER bends into a 'circular whorl' and invaginates the limiting membrane of the vacuole (3) the whorl is engulfed and taken up into the vacuole. These processes are independent of the autophagy machinery.

To identify the molecular machinery that executes these processes, a genetic screen was conducted, which returned 119 genes required for ER micro-autophagy. Among the hits were several major vacuolar trafficking machinery and the role of the ESCRT machinery was analyzed in more detail. The experiments indicate ESCRT proteins localize transiently to the site of ER-whorl engulfment, that they function directly in ER micro-autophagy and scission the vacuolar membrane to complete the uptake of ER whorls into the vacuole. This is a very interesting and important study. Also there is no doubt that ESCRT play a role in ER micro-autophagy, but certain points require clarification.

Major points:

(1) A better characterization of GFP-Pho8 as an inducer and as a reporter of ER-microautophagy is required. Pho8 is normally transported to the vacuole via the AP3 pathway, but apparently upon overexpression, GFP-Pho8 is stuck in the ER and then becomes a cargo for ER microautophagy. How much is GFP-Pho8 over-expressed relative to endogenous Pho8? What happens to the GFP(L221K)-Pho8 control? Does it reach the vacuole via the AP3 pathway? It would be helpful to show the data and compare GFP(L221K)-Pho8 and GFP-Pho8 localization and protein expression levels in cell lysates.

(2) The signal for GFP-Pho8 remains restricted exclusively to whorls, even inside vacuoles. Since the whorls will (most likely) be surrounded by vacuolar membrane, they might indeed be protected from efficient degradation. The whorls also appear to be morphologically intact and 'undigested'. This is different for autophagic bodies and MVB cargo, which diffuse immediately inside vacuoles and are degraded. Is GFP-Pho8 proteolytically processed inside vacuoles and if so how much?

(3) I wonder if biochemical assays, e.g. protease protection assays could be used on purified vacuoles with whorls to perhaps characterize the final scission step. That is, asses the sensitivity of GFP-Pho8 to exogenous proteases.

(4) Along the same lines, for Yop1-Pho8delN60 to function, this reporter needs to be exposed to vacuolar hydrolases, yet whorls appear to be protected (see above) to large extend. Is that the reason why Pho8 background activity in pep4 mutants was subtracted? What is the activity compared to endogenous Pho8 activity?

(5) It would be helpful to better describe the results of the screen. I quickly looked at the results and found in addition to ESCRTs, many other important vacuolar protein sorting complexes, including subunits od HOPS/CORVET, Retromer, Mon1-Czz1, and some SNARE-complex components. I believe that it would be worthwhile comparing these phenotypes to the phenotypes of the ESCRT mutant yeast cells. Also, it would be interesting to classify the phenotypes e.g. are some mutants unable to form ER stacks, and others perhaps unable to bend the stacks. The data is there and much insight will be gained from such an analysis.

(6) Since many different trafficking machineries - including ESCRTs - contribute to ER-microautophagy, can the author fully exclude that ESCRT function has an indirect effect on 'ER-micro-autophagy' by restricting/slowing down the trafficking of other factors required for certain steps in ER micro-autophagy. This should be pointed out better in the discussion.

(7) The authors show that an ESCRT-III subunit transiently localizes to the site of whorl engulfment, possibly at the time when scission takes place. From that result and from serial section TEM analysis (see below), the authors conclude, that ESCRTs function in a late step, subsequent to membrane bending and whorl formation and perhaps at the right time to execute scission. I would like to see a quantification of these events. I understand that ESCRT recruitment can be very transient, but a 'ballpark' number might be useful here: is it less than 10% of all events or more than 50% of all events?

(8) If it was indeed the case that ESCRTs only function directly during scission, I would expect that the majority of GFP-Pho8 is no longer detected in stacks, but rather in whorls that get stuck during the last step of the engulfment process. Yet this is not the case. The total number of 'flat' ER-stacks in WT cells and in ESCRT mutants appears to be roughly the same, however the localization of the stacks to the surface of the vacuoles is reduced by 50% in ESCRT mutants. Hence it seems that ESCRTs are also involved in the localization of ER stacks to the surface of the vacuole and perhaps even in bending the stacks? Along these lines, wouldn't it also be expected that more whorls jam up on the vacuole in ESCRT mutants? Perhaps following a 'pulse chase' of GFP-Pho8 (induction and shutoff) would be useful to address these points.

(9) Serial section EM is technically demanding. Unfortunately, the images for the ESCRT mutant are not convincing. In particular, it is difficult to see if there is indeed membrane connecting the whorl to the limiting membrane of the vacuole in the ESCRT mutant. The membrane preservation / contrast is simply not sufficient. The authors have used very good CLEM data in Figure1 and beautiful TEM in Figure2, hence I wonder if tomography of the CLEM images would not provide better images (including quantification) to make this important point. In my view, the authors need stronger data here to substantiate their point. This point needs to be particularly clarified, since their earlier reports showed that ER-microautophagy does not require ESCRT function (Schuck et al. 2014).

Minor points:
(1) What is the fraction of cells with vacuolar whorls? In line 115 the authors state that 'most GFP-Pho8 rings were inside the vacuole' and in line 128 'most whorls (probably referring to HMG2-GFP induced whorls) were in the cytosol'. Please provide quantification and highlight and discuss that the nature of the over-expressed membrane protein influences whorl uptake processes (could it have to do with the role of HMG2 in regulating ergosterol synthesis?)

(2) Line 204: The authors state that Pho8 activity was 'almost normal' in ESCRT mutants. Yet the Pho8 activity was at least 25%-40% reduced in ESCRT mutants.

(3) Localize MVB cargo with GFP-Pho in WT cells and in ESCRT mutants.

(4) Could ESCRTs also scission ER membrane to release stacks/whorls? They have been implicated in scission of peroxisomes from the ER. Perhaps something to discuss...

(5) Line 139 - please put arrowheads to the vacuolar membrane in Figure 2B

(6) I could not open the supplementary movies

We have now uploaded our revised manuscript "ESCRT machinery mediates selective microautophagy of endoplasmic reticulum". First, we would like to thank all three reviewers for their thoughtful and constructive comments. We have taken up almost all of their suggestions for additional experiments and, while these experiments have not changed the main message of our study, they have strengthened the manuscript in quite a number of places. In particular, we have:

1. Tested another three ER transmembrane proteins for their capacity to induce micro-ER-phagy (reviewer 1, point 1 and your first point). We show that only a subset of ER proteins appears to be able to induce micro-ER-phagy (new Figure EV1G).

2. Determined that Yop1Pho8Δ60 is non-functional (reviewer 1, point 2). However, Pho8Δ60-tagged Yop1 still localizes correctly and does not disturb ER structure, so that its use as a reporter for micro-ER-phagy is justified (new Figures EV4B, C).

3. Added stress-induced cleavage of Sec63-GFP as a complementary biochemical assay for micro-ER-phagy (reviewer 1, point 3), which confirmed the roles of the Nem1-Spo7 complex and ESCRTs (new Figures 3F, G).

4. Extended the discussion of the role of ESCRTs in autophagy (reviewer 1, point 4 and your second point), which we hope will stimulate further investigation (see revised discussion).

5. Quantified the frequency of vacuolar whorls in mutants lacking core autophagy genes (*Δatg* mutants) as well as in *Δnem1* and ESCRT mutants (reviewer 2, point 1). We show that *Δatg* and *Δnem1* mutants can still form whorls (although the ones in *Δnem1* mutants are morphologically aberrant) whereas ESCRT mutants cannot (new Figures EV3B, E and F).

6. Added electron micrographs of ER taken up into the lysosome by macro- and micro-ER-phagy (reviewer 2, point 2 and your third point), illustrating the profound morphological differences between the two (new Figure 4A).

7. Performed growth assays of macro-ER-phagy and ESCRT mutants (reviewer 2, point 3 and your fourth point), which show that macro-ER-phagy receptors and ESCRTs cooperate to counteract ER stress (new Figures 4F, G).

8. Extended the characterization of GFP-Pho8 (reviewer 3, points 1-4 and your first point) by showing that monomeric GFP-Pho8 cannot induce whorls, determining GFP-Pho8 levels relative to endogenous Pho8 and indicating phosphatase activity of Yop1-Pho8Δ60 compared to that of endogenous Pho8 (new Figures EV1D, E and additions to the text).

9. Quantified the fraction of whorls that show association with Snf7 and the duration of this association (reviewer 3, point 7). This proved to be a very informative analysis we had not considered before the reviewer prompted us to do it (new Figure EV5G).

10. Improved the description of the EM analysis of ESCRT mutants (reviewer 3, point 9 and your fifth point). Importantly, the reviewer's comments made us realize that we had not explained well how the analysis was done and why the images we provide are informative despite the fact that the vacuole membrane cannot be visualized clearly (see revised text).

11. Analyzed localization of GFP-Pho8 relative to an MVB cargo (reviewer 3, minor point 3) and found that they are spatially distinct (new Figure EV5C).

12. Tested our assumption that tagged Snf7 is functionally neutral in our system (our addition) and found that this is indeed the case (new Figures EV5E, F).

Please see below for a point-by-point response to all of the reviewers' comments. We hope that the revised manuscript is now fit for publication in The EMBO Journal. Finally, we would like to thank you for your efforts in guiding this manuscript through the review process.

With kind regards,
Jasmin and Sebastian

Point-by-point response

Reviewer 1

(...) The manuscript is well written, and data presented in a logic and comprehensive manner. The idea of the existence of two types of ER-phagy (micro and macro - ER - phagy) depending on the stimuli that the cell is receiving is also interesting and well fit with the complexity of ER biology. The manuscript is quite mature; however, the authors should take into consideration few points:
- Activation of micro-ER-phagy after ER resident protein over-expression is interesting and should be further investigated. More examples will help to understand if all ER resident proteins over-expression/accumulation potentially can trigger micro-ER-phagy or there is a specific subset like luminal, intramembrane or transmembrane proteins...

We tested another three GFP-tagged ER transmembrane proteins that have been reported to induce stacks (Hmg1-GFP, GFP-Cyb5 and P450M4-GFP). Of these, Hmg1-GFP and GFP-Cyb5 indeed produced ER stacks. However, none of the three proteins induced whorls, even at the highest expression levels we could reach through genomic integration of four copies of *GAL* promoter-driven expression cassettes. Hence, it appears that only a subset of transmembrane proteins can induce micro-ER-phagy in our system. We provide schematics of all transmembrane proteins used (new Figure EV1C), include the imaging data (lines 128 - 133, new Figure EV1G) and emphasize in the discussion that multimerization may be important for the ability of transmembrane proteins to induce ER whorls (lines 468 - 473).

- [Determine whether] The reporter Yop1-Pho8Δ60 is functional.

To test whether Yop1-Pho8Δ60 is functional we took advantage of the fact that *YOP1* deletion by itself does not affect ER morphology but severely damages ER structure in *Δsey1* cells (Hu et al., 2009, PMID 19665976). Interestingly, we found that Yop1-Pho8Δ60 is non-functional. Nevertheless, since we also found that Yop1-Pho8Δ60 still shows the expected localization to the peripheral ER and that tagging of *YOP1* with Pho8Δ60 did not result in growth or ER morphology defects, we believe that Yop1-Pho8Δ60 is a valid reporter for ER-phagy (results section lines 198 - 202, new Figures EV4B-C and discussion lines 416 - 422).

To have a more complete picture, would be appropriate to add some different readouts for examples some biochemistry, protein degradation assay, ...

We now include ER stress-induced cleavage of Sec63-GFP as additional biochemical assay. Two Sec63-GFP cleavage fragments generated upon tunicamycin treatment are convenient readouts for micro-ER-phagy because they result from Atg7-independent processing in the vacuole, as we have shown previously (Schuck et al., 2014). Even though the Sec63-GFP cleavage assay is not as quantitative as the Yop1-Pho8Δ60 assay, it confirms that Nem1, Spo7 and ESCRTs are important for micro-ER-phagy (lines 233 - 238, new Figures 3F-G).

- The ESCRT proteins role in micro-ER-phagy, macro-ER-phagy and lysosomal membrane scission is the most interesting point and should be elaborate and discussed more in details. Apparently, these proteins coordinate the all autophagy process in the yeast. Would be nice to further investigate if there is a division of the ESCRT proteins in specific subgroups dedicated to each function (micro-, macro-autophagy, lysosomal membrane scission ..). Moreover, some hints on the molecular mechanisms of ESCRT protein activation in the different scenarios will add a huge value to the manuscript.

To address these comments, we have extended the discussion and elaborate on how the ESCRT machinery is adapted for and can simultaneously mediate different functions. However, since these are open new questions, we can at this point only offer some initial thoughts that hopefully inform future investigations (lines 424 - 452).

Reviewer 2

(...) Micro-autophagy and its mechanism are not well understood. In addition, the contributions that macro-autophagy and micro-autophagy make to cellular degradation have not been clearly defined. This manuscript addresses these unresolved issues by examining ER-phagy, and is an excellent study for its description of not only micro-autophagy but also ER-phagy. The experiments are well presented and the descriptions are also very clear. For those reasons, I recommend this manuscript for publication in the EMBO journal. However, I would make some minor suggestions, as described below. The authors should consider these points.

1. Fig 3A, 3B, and S3: The authors showed one image for each strain. The authors should

indicate the fraction of cells in terms of GFP-Pho8 structure as in Fig 5B.

We have added quantification of the frequency of vacuolar whorls in representative mutants *(Δatg1*, *Δatg7*, *Δatg14*, *Δnem1*, *Δvps23*, *Δsnf7* and *Δvps4*). This more detailed analysis mostly confirmed our previous qualitative results but also showed that *Δnem1* cells can still form vacuolar whorls (new Figures EV3B, E). However, vacuolar whorls in *Δnem1* cells are morphologically aberrant (revised Figure EV3D and new Figure EV3F), consistent with the biochemical micro-ER-phagy defects in these cells presented in later figures.

2. In Fig 4: If possible, the authors should include images which show macro-autophagy derived ER and micro-autophagy derived ER, and investigate the morphological differences (as shown in the model diagram in Fig 7). Blocking the degradation of autophagic bodies (deletion of ATG15 or double deletion of PEP4 and PRB1) may allow the authors to obtain those images.

We added an electron micrograph of a DTT-treated cell that has in its vacuole both an ER whorl and macroautophagic bodies containing short ER fragments. These ER fragments are similar to those seen previously after nitrogen starvation, which also activates macro-ER-phagy (Hamasaki et al., 2005, PMID 15569245). These data highlight the morphological differences between macro- and micro-ER-phagy and set up the subsequent analysis of the genetic differences between these pathways (lines 245 - 255, new Figure 4A). On a technical note, use of *Δatg15* or *Δpep4 Δprb1* mutants was not necessary because DTT inhibits vacuolar proteolysis (Schuck et al., 2014), thus helping to preserve autophagic bodies.

3. It is unclear how much ER-phagy contributes to cellular homeostasis because Atg39 or Atg40 deficient cells are viable for several days (Mochida et al., Nature 2015). If micro- and macro-ER-phagy are able to compensate for each other, inhibition of both pathways could shorten life span duration. The authors should examine cell viability in macro- and micro-ER-phagy deficient cells.

We performed growth assays with WT, *Δatg39 Δatg40*, *Δsnf7* and *Δsnf7 Δatg39 Δatg40* cells. Combined deletion of *ATG39* and *ATG40* by itself did not affect growth under normal conditions or ER stress. However, it increased the tunicamycin sensitivity of *Δsnf7* mutants, indicating that macro-ER-phagy and ESCRTs indeed cooperate to promote ER stress resistance. A caveat is that ESCRTs have other functions besides mediating micro-ER-phagy. Therefore, a more incisive analysis will have to await the identification of ways to specifically disrupt micro-ER-phagy (lines 312- 321, new Figures 4F, G).

4. It would be nice to show that DTT/tunicamycin induced whorls could be stained with a dye for the ER. Some dyes like R18 (Hirata et al., Plos one 2017) are known to stain the ER membrane.

We appreciate this constructive suggestion. We tested R18 and confirmed that it labels the ER. However, it failed to reveal ER whorls. The exclusion of many ER markers from the whorls is striking and we will certainly investigate this phenomenon further in the future.

5. In lines 60-62 of the manuscript, the authors described endolysosomal membrane consumption and replenishment by micro- and macro-autophagy. However, according to my understanding, the impairment of macroautophagy does not shrink the size of the vacuole. Macroautophagy is not only the process to supply lipids to the vacuolar membrane. Lipid transfer protein, such as Vps13, and other factors could contribute to maintain vacuolar lipid supplementation.

The reviewer is completely right: vacuole size is normal in macroautophagy mutants (e.g. Chan & Marshall, 2014, PMID 24806931). We have deleted our previous problematic statement, which does not affect any other part of the manuscript.

Reviewer 3

(...) ***This is a very interesting and important study. Also there is no doubt that ESCRT play a role in ER micro-autophagy, but certain points require clarification***

Major points:

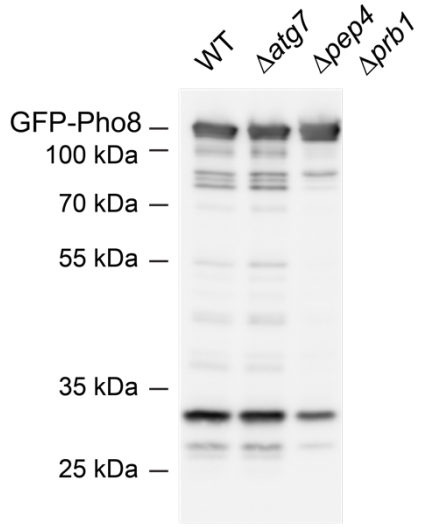
(1) A better characterization of GFP-Pho8 as an inducer and as a reporter of ER-microautophagy is required. Pho8 is normally transported to the vacuole via the AP3 pathway, but apparently upon overexpression, GFP-Pho8 is stuck in the ER and then becomes a cargo for ER microautophagy. How much is GFP-Pho8 over-expressed relative to endogenous Pho8? What happens to the GFP(L221K)-Pho8 control? Does it reach the vacuole via the AP3 pathway? It would be helpful to show the data and compare GFP(L221K)-Pho8 and GFP-Pho8 localization and protein expression levels in cell lysates.

We now show that *GPD* promoter-driven GFP-Pho8 and GFP(L221K)-Pho8 are expressed at similar levels, which exceed the levels of endogenous Pho8 by roughly two orders of magnitude. Nevertheless, while GFP-Pho8 is retained in the ER, GFP(L221K)-GFP is still transported to the vacuole membrane (lines 107 - 112, new Figures EV1D-E).

(2) The signal for GFP-Pho8 remains restricted exclusively to whorls, even inside vacuoles. Since the whorls will (most likely) be surrounded by vacuolar membrane, they might indeed be protected from efficient degradation. The whorls also appear to be morphologically intact and 'undigested'. This is different for autophagic bodies and MVB cargo, which diffuse immediately inside vacuoles and are degraded. Is GFP-Pho8 proteolytically processed inside vacuoles and if so how much?

GFP-Pho8 whorls in the vacuole indeed appear to be largely intact. It has been noted before that microautophagic bodies are likely to be more stable than macroautophagic bodies, presumably because they derive from the vacuole membrane (Iwama & Ohsumi, 2019, PMID 30755486). Furthermore, it is possible that the artificially large size of the GFP-Pho8 whorls interferes with lateral removal of glycosylated vacuolar transmembrane proteins from sites of microautophagy, which may be necessary to make the membrane of microautophagic bodies accessible to vacuolar hydrolases. We attempted to analyze proteolytic processing of GFP-Pho8 but find that only a small, difficult to quantify fraction

undergoes processing in the vacuole, as illustrated by the western blot below. Therefore, GFP-Pho8 cleavage is not a helpful assay. Instead, we have implemented ER stress-induced cleavage of the ER protein Sec63-GFP as an alternative biochemical assay for micro-ER-phagy (see response to reviewer 1 above), which confirms the involvement of Nem1, Spo7 and ESCRT proteins (lines 233 - 238, new Figures 3F-G). Furthermore, as we emphasize in the revised manuscript, we use GFP-Pho8 whorls as an informative substitute for whorls induced by ER stress but focus on the stress-induced whorls wherever possible (lines 133 - 136). For this reason, Sec63-GFP cleavage after ER stress treatment serves the purpose of a biochemical micro-ER-phagy assay better than GFP-Pho8 cleavage could.



Western blot of GFP from cells of the indicated genotype expressing GFP-Pho8. Note that to visualize GFP-containing cleavage fragments at all, the blot was exposed such that the chemiluminescence substrate became limiting for full-length GFP-Pho8, leading to an underestimation of its amount and making it difficult to accurately determine the extent of cleavage. Furthermore, comparison of the GFP-containing fragments in WT and Δatg7 cells versus Δpep4 Δprb1 cells shows that these fragments only partially arose from processing in the vacuole.

(3) I wonder if biochemical assays, e.g. protease protection assays could be used on purified vacuoles with whorls to perhaps characterize the final scission step. That is, assess the sensitivity of GFP-Pho8 to exogenous proteases.

This may be an informative approach in future analyses. However, we believe that it is outside the scope of this revision.

(4) Along the same lines, for Yop1-Pho8delN60 to function, this reporter needs to be exposed to vacuolar hydrolases, yet whorls appear to be protected (see above) to large extend. Is that the reason why Pho8 background activity in pep4 mutants was subtracted? What is the activity compared to endogenous Pho8 activity?

As the reviewer correctly suspects, Yop1-Pho8Δ60 activity is much lower than that of endogenous Pho8 under the same conditions (about 20-fold) and only just over 3-fold higher than the background activity in Δpep4 mutants, so that background subtraction is important. We have added this explanation and the specific phosphatase activities of Yop1-Pho8Δ60, cyto-Pho8Δ60 and endogenous Pho8 under the different conditions in the methods section (lines 844 - 857).

(5) It would be helpful to better describe the results of the screen. I quickly looked at the results and found in addition to ESCRTs, many other important vacuolar protein sorting

complexes, including subunits od HOPS/CORVET, Retromer, Mon1-Czz1, and some SNARE-complex components. I believe that it would be worthwhile comparing these phenotypes to the phenotypes of the ESCRT mutant yeast cells. Also, it would be interesting to classify the phenotypes e.g. are some mutants unable to form ER stacks, and others perhaps unable to bend the stacks. The data is there and much insight will be gained from such an analysis.

We agree and have expanded the summary of the hits in the results section (lines 174 - 180) and added descriptions of their phenotypes to Table EV1. Since the image analysis was done manually rather than computationally, we focused on defective whorl formation as the most obvious phenotype and currently do not have the morphological resolution to identify mutants with more subtle phenotypes, such as defective stack formation.

(6) Since many different trafficking machineries - including ESCRTs - contribute to ER-microautophagy, can the author fully exclude that ESCRT function has an indirect effect on 'ER-micro-autophagy' by restricting/slowing down the trafficking of other factors required for certain steps in ER micro-autophagy. This should be pointed out better in the discussion.

We provide evidence that the strong micro-ER-phagy defects in ESCRT mutants as measured by the biochemical Yop1-Pho8Δ60 assay are unlikely to result from general vacuole dysfunction (also see minor point #2 below). Nevertheless, it is difficult to exclude indirect effects and we now explicitly state this in the discussion (lines 445 - 447).

(7) The authors show that an ESCRT-III subunit transiently localizes to the site of whorl engulfment, possibly at the time when scission takes place. From that result and from serial section TEM analysis (see below), the authors conclude, that ESCRTs function in a late step, subsequent to membrane bending and whorl formation and perhaps at the right time to execute scission. I would like to see a quantification of these events. I understand that ESCRT recruitment can be very transient, but a 'ballpark' number might be useful here: is it less than 10% of all events or more than 50% of all events?

We thank the reviewer for prompting us to do this informative analysis. We found that Snf7 was recruited to nearly 80% of the whorls captured by our movies. The duration of Snf7 recruitment often lasted only a few minutes. However, some recruitment events lasted for up to 60 minutes and some whorls were visited by Snf7 multiple times. This could indicate that Snf7 can get stuck on the (artificially large) GFP-Pho8 whorls and that recruitment events can be non-productive. The latter conclusion is consistent with work by Adell et al., 2017 (PMID 29019322) showing that ESCRT-III recruitment during multivesicular endosome formation can be non-productive (lines 365 - 369, new Figure EV5G).

(8) If it was indeed the case that ESCRTs only function directly during scission, I would expect that the majority of GFP-Pho8 is no longer detected in stacks, but rather in whorls that get stuck during the last step of the engulfment process. Yet this is not the case. The total number of 'flat' ER-stacks in WT cells and in ESCRT mutants appears to be roughly the same, however the localization of the stacks to the surface of the vacuoles is reduced by 50% in ESCRT mutants. Hence it seems that ESCRTs are also involved in the localization

of ER stacks to the surface of the vacuole and perhaps even in bending the stacks? Along these lines, wouldn't it also be expected that more whorls jam up on the vacuole in ESCRT mutants? Perhaps following a 'pulse chase' of GFP-Pho8 (induction and shutoff) would be useful to address these points.

At this point, the step during micro-ER-phagy for which we have evidence for a direct involvement of ESCRTs is vacuole membrane scission. Nonetheless, we agree that ESCRTs may have additional roles in micro-ER-phagy, including in association of stacks with the vacuole membrane, formation of uptake intermediates or even ER membrane scission, as suggested by the reviewer (see minor point #4 below). Experimentally resolving this complex mechanistic issue clearly is for future studies. For now, we have revised and expanded the discussion to explain these considerations more clearly (lines 447 - 452).

(9) Serial section EM is technically demanding. Unfortunately, the images for the ESCRT mutant are not convincing. In particular, it is difficult to see if there is indeed membrane connecting the whorl to the limiting membrane of the vacuole in the ESCRT mutant. The membrane preservation / contrast is simply not sufficient. The authors have used very good CLEM data in Figure1 and beautiful TEM in Figure2, hence I wonder if tomography of the CLEM images would not provide better images (including quantification) to make this important point. In my view, the authors need stronger data here to substantiate their point. This point needs to be particularly clarified, since their earlier reports showed that ER-microautophagy does not require ESCRT function (Schuck et al. 2014).

The reviewer's comment made us realize that we had provided a poor description of how we quantified uptake of whorls into the vacuole. The terms 'connected whorls' and 'disconnected whorls' apparently evoked the expectation to see the vacuole membrane, which is indeed rarely visualized clearly by conventional EM after aldehyde fixation. However, the electron micrographs provide very strong contrast between the light vacuole lumen, the dark cytosol and the even darker ER whorls. This enables quantification of whorl uptake from serial sections based on whether a whorl is entirely surrounded by vacuole lumen (in which case uptake is complete) or whether a whorl is still in direct contact with the cytosol (in which case uptake is incomplete). Importantly, this classification does not require that the vacuole membrane can be traced. We therefore believe that our approach is suitable to decide whether a whorl has completed uptake. We have rephrased the description of how we classified whorls (results section, lines 389 - 396) and expanded the description of the quantification procedure (methods section, lines 596 - 608). On a technical note, tomography of GFP-Pho8 whorls is impractical because they are 1.5 µm in diameter and covering them would require five or six serial tomograms.

Minor points:

(1) What is the fraction of cells with vacuolar whorls? In line 115 the authors state that 'most GFP-Pho8 rings were inside the vacuole' and in line 128 'most whorls (probably referring to HMG2-GFP induced whorls) were in the cytosol'. Please provide quantification and highlight and discuss that the nature of the over-expressed membrane protein influences whorl uptake processes (could it have to do with the role of HMG2 in regulating ergosterol synthesis?)

We quantified and found that about 80% of GFP-Pho8 whorls are vacuolar (consistent with Figure 5) and only about 20% of Hmg2-GFP whorls (lines 113 and 128). Furthermore, we have analyzed three additional ER transmembrane proteins for their capacity to induce ER whorls (see response to reviewer 1 above) and expanded the relevant paragraph in the discussion. There, we emphasize multimerization as an important factor for the capacity of ER transmembrane proteins to induce whorls and refer the reader to two papers that address this issue in depth (Koning et al., 1996, PMID 8744950 and Federovitch et al., 2008, PMID 18667535). The latter paper also shows that the enzymatic activity of Hmg2 has no role in the induction of ER stacks and whorls.

(2) Line 204: The authors state that Pho8 activity was 'almost normal' in ESCRT mutants. Yet the Pho8 activity was at least 25%-40% reduced in ESCRT mutants.

To clarify this point, we rephrased the text, which now reads: "In contrast, endogenous Pho8 activities in *Δnem1* and ESCRT mutants were reduced by, on average, 30% at steady-state and 20% after tunicamycin treatment. Therefore, vacuole functions relevant for Yop1-Pho8Δ60 activation are largely intact in *Δnem1* and ESCRT mutants, and it appears unlikely that the mild general defects represented by reduced endogenous Pho8 activities are sufficient to explain the strong inhibition of micro-ER-phagy." (lines 226 - 231).

(3) Localize MVB cargo with GFP-Pho8 in WT cells and in ESCRT mutants.

We now show that vacuole-associated GFP-Pho8 in WT cells and *Δsnf7* mutants does not co-localize with the MVB cargo mCherry-CPS. In particular, the class E compartments in *Δsnf7* mutants are spatially distinct from GFP-Pho8 stacks (lines 343 - 345, new Figure EV5C).

(4) Could ESCRTs also scission ER membrane to release stacks/whorls? They have been implicated in scission of peroxisomes from the ER. Perhaps something to discuss...

We thank the reviewer for this good suggestion. We now point out the possibility that ESCRTs may mediate ER membrane scission during micro-ER-phagy (lines 450 - 452).

(5) Line 139 - please put arrowheads to the vacuolar membrane in Figure 2B

Done.

(6) I could not open the supplementary movies

We previously provided tif files containing series of z stacks, which could be opened with Image J but required the user to manually navigate the z-planes, individual channels and time points. We now provide avi files, which automatically play as movies when the user clicks on them. In these movies, all z-planes, the individual channels and the overlay for each time point are shown in a 5 x 4 matrix of tiles, which provides a much better overview.

2nd Editorial Decision 28th October 2019

As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, referee #1 requests you to tone down conclusions on the role of ESCRT proteins in ER-phagy, as well as to discuss the possibility that micro- and macro-ER phagy are present in mammalian cells. In addition to resolving these remaining points, there are a few editorial issues concerning text and figures that I need you to address before we can officially accept the manuscript.

REFEREE REPORTS

Referee #1:

In the revised manuscript, the authors addressed almost all the point raised by the referees. The manuscript now is significantly improved. The new data have been well incorporated in the previous manuscript and the discussion is more complete and gives a better overview of the biological meaning.
The molecular mechanisms have been discussed in more details even if the role of ESCRT machinery is still not completely unravelled. As the authors wrote, at this point they can give some hints but not a full description of the molecular pathways. This remain a point to address in the future. However, the paper is already quite elaborated so adding more details in this direction will probably make the manuscript quite heavy to read, especially for readers not directly from this field. In the text, the authors wrote that the mechanisms are still not clear so this could be sufficient. Maybe some statements on the role of ESCRT proteins could be smoothed a bit considering that some experiments are still missing.
The idea that ER-phagy could appear in two different forms: macro-ER-phagy or micro-ER-phagy is of interest and likely this idea will be tested in the mammalian system too. The authors should point this in the discussion. So far the micro-ER-phagy is a yeast biological process, but it could be extended to other systems too.

Overall, this review doesn't have major issues with the revised version of the manuscript. After some editing in the text the manuscript should be suitable for publication.

Referee #3:

The authors have done a great job and significantly improved the revised version of the manuscript. The new experiments in the revised version strengthen the original conclusions and support the overall hypothesis. The paper manuscript is well suited for publication in the EMBO Journal.

2nd Revision - authors' response 30th October 2019

Below, please find our response to the queries in your decision letter from October 28. We copied the relevant sections from your letter (in italics) and provide a point-by-point response. We hope that our responses satisfy the issues raised.

(...) As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, referee #1 requests you to tone down conclusions on the role of ESCRT proteins in ER-phagy, as well as to discuss the possibility that micro- and macro-ER phagy are present in mammalian cells.

Please see our response to referee #1 below.

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Overall, this review doesn't have major issues with the revised version of the manuscript. After some editing in the text the manuscript should be suitable for publication.

From these comments we understand that our revised discussion of ESCRTs is adequate. Unfortunately, we do not understand what exactly the referee means by saying that 'maybe some statements on the role of ESCRT proteins could be smoothed a bit (...)'. Since the referee indicates no specific point we should address, we suggest to leave the text as is. We have, however, taken up the suggestion to emphasize that it will be important in the future to test whether micro-ER-phagy is conserved in mammals and whether mammalian macro-ER-phagy and micro-ER-phagy can occur simultaneously (lines 489 - 497).

Referee #3:

The authors have done a great job and significantly improved the revised version of the manuscript. The new experiments in the revised version strengthen the original conclusions and support the overall hypothesis. The paper manuscript is well suited for publication in the EMBO Journal.

Accepted 11th November 2019

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Sebastian Schuck
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2019-102586

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Blindfolded analysis of time-lapses and electron micrographs was achieved by sample randomization during montage generation and phenotypic analysis, as described in methods section.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. Chi Square test for independence in Figure 5 and Fisher's exact test in Figure 6.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. Sampling was random and the tests used make no assumptions about data distribution.
Is there an estimate of variation within each group of data?	Yes, standard error of the mean.

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Is the variance similar between the groups that are being statistically compared?	NA

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.	NA

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E- Human Subjects

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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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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