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Pex3-anchored Atg36 tags peroxisomes for degradation in *Saccharomyces cerevisiae*

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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 18 August 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. While their reports are explicit and recommend revision, I would just highlight the most critical concerns here:

As you will see all 3 referees pointed out a number of technical issues that they have clearly listed. In addition, Ref.#2 and #3 recommend extending and detailing the discussion section a bit further, together with fixing typos and missing or unformatted references.

Particularly, they suggest specific experiments to strengthen your message:

- Ref.#3 strongly recommends investigating the interactions of ATG36/ATG8 (point 12) and in agreement with Ref.#1, looking into ATG36/ATG11-phosphorylation status, in order to give some mechanistic insights to possible regulation of ATG36 (Ref.#1 point 11 and Ref.#2 point 14).
- GFP fluorescence signal is a concern, a monomeric GFP should be used, to ascertain that peroxisome dimerization is not an artefact of GFP dimerization (Ref.#2 point 1, Ref.#3 points 4, 8-9, 11) and GFP expression should be enhanced, to solve the detection issues (Ref.#2 point 1)
- Ref.#2 recommends using cell line FM4-64 to ascertain peroxisome degradation (point 2) and the nature of the signal should be clarified (Ref.#1 point 8 and Ref.#2 point 2).
- Non specific autophagy needs to be addressed as recommended by Ref.#2 (point 3)

Given the referees' overall positive recommendations, and providing that you are able and willing to address all referees' comments, we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal within 3 months (see below). I should add that it is EMBO

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Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The work by Motley et al reveals a new piece of selective autophagy apparatus controlling removal of peroxisomes in Saccharomyces cerevisiae. Peroxisomes carry out important metabolic and immune functions, which stipulates their proper maintenance: generation, segregation and degradation. Macropexophagy (or selective degradation of peroxisomes via autophagy, hereafter pexophagy) has been thoroughly investigated in methylotrophic yeasts Pichia pastoris and Hansenula polymorpha (Dunn et al., 2005; FarrÈ et al., 2008; Manjithaya et al., 2010), and marginally in plant pathogenic fungus Colletotrichum orbiculare (Asakura et al., 2009) and mammals (Hara-Kuge et al., 2008; Kim et al 2008). In P. pastoris, Atg30 has been shown to be the principal receptor which via interaction with peroxin Pex14 and Atg11 delivers peroxisomes for autophagic degradation (FarrÈ et al., 2008).
Here, a new autophagy receptor in S. cerevisiae, termed Atg36, is identified which specifically binds Pex3 and Atg11 thus linking peroxisomes to PAS. Atg36 is shown to be crucial for peroxisome degradation after switching from replete to starvation growth conditions. Atg36 overexpression accelerates pexophagy dependent solely on Pex3 whereas Pex14 is dispensable. Authors also show that if targeted to mitochondria Atg36 could mediate selective removal of the organelle, i.e. participate in mitophagy. This emphasizes the conservation and likely interconnection of selective autophagy pathways functioning in organelle quality control.

In general, the work is done at high level, results are well interpreted and discussed, and findings are integrated into the context.
Points to be addressed:

1. Page 3, 15th line, term 'PMP' is used for the first time and therefore should be defined.
2. Page 4, 15th line; xenophagy is another important type of selective autophagy worth mentioning. Also, it would be recommended to refer to the original publications at the end of the sentence.
3. Page 8, 2nd line from the bottom, reference to Knoblach et al (2010) should be appended to the end of the corresponding sentence, and the paper must be added to the list of references.
4. Page 9, 13th line, 'protease-resistant breakdown product' could be indicated here as GFP, since the latter is then used throughout the text.
5. Page 15, 10th line, 'pex3 cells' should be 'pex3 cells'

6. If the statement of different frequency of peroxisomes is made (e.g. Fig. 1A,C), numbers of peroxisomes on immunofluorescence images should be quantified and presented.
7. Expression of endogenous and ectopically overexpressed Atg36 before and after galactose stimulation at indicated time points needs to be defined and presented (Fig. 3) for the comparison.
8. Fig. 4B shows, that in absence of Pex3, Atg36-GFP is not only cytosolic but also forms certain distinct puncta (central cell). What is the nature of that puncta?
9. The input of Pex13-GFP used for co-IP experiment on Fig. 4D is much lower than of Pex3-GFP (or the proteins are not well separated) and therefore not suitable for the specificity control.
10. Sup 3C could be added as one of the main text figures. It provides a proof that pexophagy defect of pex3-177 cells stems from the inability to bind Atg36.
11. It would be interesting to test if Atg36 upregulation on oleate is caused transcriptionally or posttranslationally (Fig. 6). If at the level of transcription, would Slt2p MAPK be involved (Manjithaya et al., 2010)?
12. Could Atg36-Atg11 interaction (Fig. 7A) be enhanced in atg1 cells consistent with experiments on Fig. 7B?
13. Either 'BF' (defined in figure legends) or 'bright field' should be used exclusively on all the figures (Fig. 4C).
14. Page 14, 4th line, 'ie' should be 'i.e.'.
15. Page 17, 6th line from the bottom, a reference should be added to the stated information.
16. Page 26 12th line; page 28, 9th line, references not recognized by Endnote should be corrected.
17. Vacuole staining could be used more frequently to facilitate discrimination between vacuolar and cytosolic signals (e.g. Fig. 2A,B)
18. In Fig. 5 legend, it is not clear what '(-ve control)' means.
19. What is the frequency of Atg11 positive (or proximal) peroxisomes in pex3-177 and pex3-177atg1 cells?
20. Bellu et al reported that removal and degradation of Pex3 is important for pexophagy in H. polymorpha (Bellu et al., 2002). What is Pex3 stability in cells grown on glucose, oleate and starvation medium?

Referee #2 (Remarks to the Author):

Manuscript: EMBOJ-2011-78820
Pex3-anchored Atg36 tags peroxisomes for degradation in <i>Saccharomyces cerevisiae</i>.
A.M. Motley et al.

This manuscript describes the isolation of a novel autophagy factor, designated Atg36, required to specifically degrade peroxisomes by pexophagy in baker's yeast. Remarkably, this factor is only conserved in species closely related to <i>S. cerevisiae</i> and is not a homolog of the pexophagy-receptor previously identified in the methylotrophic yeast species <i>Pichia pastoris</i> and <i>Hansenula polymorpha</i>. ScAtg36 is a peroxisomal protein that binds to the peroxisomal membrane protein Pex3 and links peroxisomes to be degraded via Atg11 to the autophagy machinery. Furthermore, relocation of Atg36 to mitochondria using mitochondrially targeted Pex3 can trigger autophagic degradation of these organelles (via mitophagy). This implies that the Atg36/Pex3 complex functions as a degradation tag.

The subject matter of this manuscript is highly suitable for EMBO Journal.
However, I have a number of reservations that preclude publication in its current form.

My comments:

1. Concerning all fluorescent pictures:
In many fluorescent pictures, the signal of the fluorescent protein is either very poor or not visible in all cells. Furthermore, the phenotypes as described in the text are not always properly visible in the indicated Figures. The authors note that plasmid-based expression of fusion constructs results in highly variable fluorescence levels (page 12, line 4-6). This expression problem is probably the reason why the expression/localization results with fluorescent proteins is so variable in cells in even a single experiment. I would like to urge the authors to integrate all constructs expressing fluorescent fusion genes to circumvent such problems.
Similarly, the authors comment at least in two places in their manuscript that peroxisomes tend to

cluster upon production of a GFP fusion protein (page 9, line 3; page 11, section 2, line 6). The authors still utilize GFP to localize membrane proteins, while they should use monomeric GFP for such studies. It is now well established that dimerization of GFP can cause organelle clustering.

2. Figure 1 C & 1 D (also page 10): the use of <i>pep4-3</i> and <i>prb1-1122</i> cells:
It is expected that in these mutant cells, used as controls, peroxisomes are taken up by the vacuole but not degraded. Consequently, the organelles are retained inside the vacuole. Unfortunately, in none of the pictures of Fig. 1C can we actually see the vacuoles. I would urge the authors to utilize FM4-64 to localize these structures. In Fig 1D, FM4-64 has been used. Surprisingly, in WT cells the vacuoles are round, as expected, but in <i>atg36</i> cells, red FM4-64 spots appear at the vacuolar surface. Can the authors comment on these spots?

3. Fig. 2, the assays are intended to demonstrate the presence of the Cvt pathway and of non-selective autophagy: The Cvt assay (Fig. 2A) is performed by fluorescence microscopy showing uptake of Ape1-GFP in the vacuole. Surprisingly, in WT cells almost no fluorescence is observed, while in <i>atg36</i> cells a massive fluorescence is visible. Does this mean that the Cvt pathway is highly stimulated in <i>atg36</i> cells ?
The assay to determine whether non-specific autophagy occurs (Fig. 2B) utilizes GFP-Atg8 as a marker without inducing autophagy using nitrogen limitation. This is not correct. The authors do not seem to realize that in a mutant that can still perform certain types of autophagy (either selective or non-selective) GFP-Atg8 uptake in the vacuole will not be disturbed. To determine whether in <i>atg36</i> cells non-selective autophagy is disturbed, another more specific assay is required (e.g. uptake of a cytosolic form of alkaline phosphatase into the vacuolar lumen via nitrogen starvation-induced autophagy).

4. Page 13.
The authors indicate that Atg36 binding to peroxisomes is saturable. Would overproduced non-peroxisomal (cytosolic) Atg36 not also bind to Atg11? If this would be the case, overexpression of <i>ATG36</i> should block pexophagy by competition. Is such a phenotype observed ?

5. Suppl. Fig. 3C. From these pictures it would appear that Inp1-GFP and Atg36-GFP are recruited to peroxisomes less efficiently by Pex3-177 and Pex3-1, respectively, than by WT Pex3. Is this correct ? Why was the reduction in binding between Atg36 and the Pex3-177 mutant not demonstrated biochemically as in Fig. 4A ?

6. The authors mention the isolation of three mutant <i>PEX3</i> alleles that function normally in peroxisome formation and inheritance, but no longer allow pexophagy. Remarkably, the information regarding the mutations in these alleles is only present in the Materials & Methods section. Similar to their previous manscript (Munck et al. 2009) the identified mutant <i>PEX3</i> alleles carry multiple mutations, while the mutations are apparently not distinguishing a hot-spot that could represent the potential interaction site of Pex3 with Atg36. Minimally, the authors should include a short section in their Results section where they mention the identified mutations and draw the proper conclusions. Ideally, the authors should identify the mutated residues that cause the observed phenotype.

7. Fig. 6A. The authors suggest concerning possible modification of Atg36 (page 16, second section, lines 10/11) that "the mobility of this fuzzy set of bands seems to change after switching to starvation medium". This can surely not be concluded from the gel in Fig. 6A, which clearly did not run straight (cf. the Pex11-GFP blot). The authors should better substantiate this claim.

8. When I read the Discussion section I was very disappointed. This is not a discussion, but rather a replay of data from the Results section. It is not understandable why the authors do not discuss in detail the significant differences between the role of Pex3 in pexophagy in <i>S. cerevisiae</i> and in methylotrophic yeasts. The authors should fully discuss the implications of their findings in relationship to all published data. This includes data on Pex3 and Pex14 in methylotrophic yeasts like <i>H. polymorpha</i> and <i>P. pastoris</i>, but also the role of Pex14 in pexophagy in human cells. All these other data seem to make baker's yeast the exception rather than the standard. This section should also discuss Yjl185c/Atg36 in more detail. Inspection of the SGD database shows that this protein interacts also with both Inp1 and Pex34. This would imply a role in peroxisome inheritance or proliferation. Although the authors mention these processes briefly in

their Results section, they fail to discuss these potential interactions at all.

9. The manuscript is hastily written and contains a large amount of typing errors.
This is especially annoying when the authors do not distinguish the protein from the gene or the mutant gene. E.g. in the manuscript "pex3" could stand for the protein Pex3, the gene <i>PEX3</i> or the mutant <i>pex3</i> . The authors should be much more consistent in their manuscript.

Small changes:

Abstract, page 2, lines 5/6 change to:
"We name this protein Atg36 as its absence blocks pexophagy"

Introduction, page 3, line 21 change to:
"Pex3-like protein has been implicated in this process in <i>Yarrowia lipolytica</i>."

Page 4, section 2, lines 11-16
Add "aggrephagy, the selective degradation of (ubiquitinated) aggregates in mammalian cells."

Page 5, line 8 change to:
"(PpAtg30) "

Page 5, section 2, line 2 change to:
"methylotrophic yeasts"

Page 6, lines 14/15 change to:
"Pex14 is not required for pexophagy in <i>S. cerevisiae</i>."

Page 8, line 6 change to:
"peroxisome numbers"

Page 8, second section, line 7 chage to:
"Knoblach et al (2010) showed"

Page 9, line 9 change to:
"labeling of the vacuole became apparent."

Page 13, second section, line 11. In the Pex3-Atg36 binding assay the text states that Atg36-GFP was used, while the Legend to Fig. 4A and the Materials & Methods section indicate this was Atg36. Please be consistent.

Page 15, second section, line 7 is not logical, replace by:
"that Pex11-GFP is degraded by autophagy-independent pathways (e.g. the ubiquitin/proteasome machinery) in the absence of Pex3."

Supplementary Figure Legends:
The line spacings in the Legends are not uniform, please adapt.

Referee #3 (Remarks to the Author):

1. Throughout the manuscript, genus and species names should be separate by a space.

2. On page 3, the authors refer to "PMPs" but I do not think they have defined this abbreviation.

3. On the top of page 4, the authors state that pexophagy is the autophagic breakdown of peroxisomes. They should include "selective" before "autophagic".

4. I think I must be missing something from Fig. 1A, but I am not understanding the Pex11-GFP fluorescence assay. Pex11 is a peroxisomal protein, and HcRed-PTS1 is targeted to the peroxisome. So how does colocalization of these two markers say anything about pexophagy? I understand that

vacuolar staining from GFP would indicate peroxisome degradation, but in that case I do not see what HcRed-PTS1 is adding. Is the HcRed-PTS1 only serving to verify that the labeled structures are peroxisomes? If so, I think this needs to be explained better in the description of the experiment.

5. On the bottom of page 9, the authors state that all of the mutants they examined were normal for pexophagy, but the data are only shown for pex14. Thus, they need to state "data not shown".

6. The plural of "punctum" is "puncta" and not "punctae".

7. In the legend to Fig. 2C the authors need to indicate the time point being examined.

8. In Fig. 3A, the authors examine N- and C-terminal tagged Atg36. It looks like the C-terminal GFP tagged construct is nonfunctional (a GFP signal is not seen in the vacuole at 5 h), but the authors do not refer to this part of the analysis. This becomes an issue with regard to Fig. 3B, where the authors use C-terminally tagged Atg36-mRFP. Is the C-terminal mRFP construct functional, but not the GFP construct? This needs to be discussed in the Results.

9. On page 14 the authors need to describe the split-GFP analysis better. For example, why was this done in atg8 null cells? What is the microscopy image supposed to show?

10. Fig. 5 and Fig. 6 need to be arranged in the portrait, not the landscape, orientation.

11. On page 17, the authors conclude that degradation of Atg36-GFP does not occur in the vacuole, presumably because it is still seen in the atg1 null strain. I do not think they can make this conclusion unless they examine an atg1 pep4 double null mutant. Otherwise, they can only conclude that degradation is not autophagy dependent.

12. The authors show that Atg36 interacts with Atg11. In the case of Atg19 (the receptor for the Cvt pathway) and Atg32 (the tag for mitophagy), these proteins interact with Atg11 and then subsequently bind Atg8. Thus, the authors should examine the interaction of Atg36 with Atg8.

13. In Fig. 8, the BF images are too dark and need to be modified.

14. As the authors note in the Discussion, phosphorylation of PpAtg30 is needed for interaction with PpAtg11. They suggest that a similar regulation may be occurring for the Atg36-Atg11 interaction. I think they need to investigate this further in the present manuscript. In particular, the authors use cells grown for 24 hours in glucose for the affinity isolation experiment in Fig. 7A. However, based on Fig. 6A Atg36 is not extensively modified under those conditions (compare lane 1 to lane 4 and 5). First, the authors need to verify that the mobility shift corresponds to phosphorylation by treating the samples with phosphatase. Second, they should examine the affinity of Atg36 from oleate grown cells at 3 versus 22 hours for the ability to pull down Atg11. They do not need to map the phosphorylation sites, or verify that these sites are needed for the interaction, but they should do the minimal experiment suggested here to obtain a hint as to the potential mechanism regulating the Atg36-Atg11 interaction.

In conclusion, I think this paper will ultimately be acceptable for publication. However, as noted above, I think the authors should examine the interaction of Atg36 with Atg8. In addition, they should further examine the interaction of Atg36 with Atg11 relative to the proposed phosphorylation of Atg36. At present, it seems like the authors have only done half of this experiment. That is, Atg36 may be phosphorylated, but they do not actually test this. Also, Atg11 might bind Atg36 with higher affinity in starvation conditions, but they are looking at a form of Atg36 that is identical (based on the total lysate PAP blot in Fig. 7A) before and after starvation; they need to test the "phosphorylated" and "non-phosphorylated" forms of Atg36 for the interaction with Atg11. If these last experiments can be completed, this paper will be a nice addition to the autophagy field.

1st Revision - authors' response 30 November 2011

Point to point response

Referee # 1

1. Done

2. Done

3. Done

4. Done

5. Done

6. Differences in frequency of peroxisomes are analysed in Suppl. Fig. 1, with Suppl. Fig. 1A showing the raw data and Suppl. Fig. 1B the quantification.

7. See new Suppl. Fig. 3B and C, where Atg36-GFP expressed from endogenous locus is compared to expression level of galactose-induced Atg36 GFP-tagged versions. We conclude that 6 h growth on galactose induces expression 50 ñ 100-fold higher than that from the endogenous locus. We have replaced previous experiments by the experiments shown in new Figs. 3B and C. Fig. 3B shows that increased levels of Atg36 tagged or untagged induce pexophagy even under non-starvation conditions, and the affect of subsequent starvation is analysed. These experiments indicate that N-tagged Atg36 is constitutively active, and this is confirmed in Fig. 3C, where N-tagged Atg36 induces pexophagy under peroxisome proliferating conditions.

8) See new Fig. 4B. The new figure shows atg36 pex3 cells expressing GFP-Atg36 in a separate panel from atg36 pex3 cells expressing both mitochondrial Pex3 and GFP-Atg36. The cytoplasmic puncta seen in pex3 cells expressing Atg36 are very mobile and do not colocalise with Atg11 or the vacuolar membrane. The observation that Atg36 remains intact in pex3 cells (Fig. 6C) indicates that Atg36 does not enter the vacuole in the absence of Pex3. Since these puncta are not related to any autophagic structures, are seen only in the absence of peroxisomes, and only in overexpressing cells, we think that they maybe aggregates of GFP.

9) IP represents immuno precipitation. L represents lysate. To clarify this confusion, we now change it to TL for total lysate as for other co-immunoprecipitation figures.

10) We have moved a panel from Suppl. Fig. 3C to Fig. 5D, where we also show using biochemistry that Pex3-177 does not bind Atg36 (Fig. 5E). Furthermore, we now show there is less colocalisation of Atg11 with peroxisomes in pex3-177 cells (Fig. 7D).

11) We agree that this would be interesting and will be studying this in the future. Thus far we have noticed that the level of Atg36-PtA is induced to WT levels on oleate in slt2 cells (not shown).

12) We have now done these experiments in atg1 cells (Fig. 7A) and are better able to detect the interaction. We have also used phosphatase inhibitors during the co-immunoprecipitation.

13) Done

14) Done

15) Done

16) Done

17) Done, see new Figs. 1D and 2A. We now use the alkaline phosphatase assay as a measure of non-selective autophagy (Fig. 2C).

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18) Corrected

19) See Fig. 7D. We found less colocalisation of Atg11 with peroxisomes in pex3-177 cells compared to cells expressing WT PEX3: whereas 75% of Atg11 puncta are in close proximity to peroxisomes in cells containing the WT PEX3 gene, in pex3-177 cells this is reduced to 30%. Quantitation is indicated in the text.

20) In new Fig. 6E, Pex3 stability is investigated by following Pex3-GFP in pex3 and atg36 pex3 cells grown in glucose, oleate, and starvation medium. We also follow degradation of endogenous Pex3 in WT cells under the same conditions (Fig. 6D). We find that the time course of both Pex3 and Pex3-GFP degradation during starvation is comparable to that of Atg36-PtA and Pex11-GFP (Fig. 6A), with most degradation occurring for all three proteins between 3 ñ 6 h on starvation medium. The finding that Pex3 is degraded with the same kinetics as the peroxisomal marker Pex11 and Atg36 suggests that S. cerevisiae Pex3 is not removed from peroxisomes and degraded prior to pexophagy, which has been found to be the case for H. polymorpha Pex3 (Bellu et al., 2002). Furthermore, H. polymorpha Pex3 is degraded even in cells blocked in autophagy, whereas S. cerevisiae Pex3 remains intact in atg36 cells (Figs. 6C and E). This clearly indicates a mechanistic difference between pexophagy in H. polymorpha and S. cerevisiae (see discussion).

Referee # 2

1) We have enhanced the GFP signal in Figs. 1A, 3A, 5A, and Suppl. Fig. 4D. We have integrated GFP in Figs. 2A, 6B and C and PtA in Figs. 4D, 5E, 6A, and 7A for endogenous expression and for co-immunoprecipitations. We have expressed plasmid-encoded Atg36 from the GAL1 promoter to observe the activity of the protein by fluorescence, also because expression from the genomic locus is hard to detect (Suppl. Fig. 2). We have replaced the heterogeneous expression with Western blots in Figs. 3B and C, and now show separate fluorescent images in Fig. 4B for expression of Atg36 with or without mito-Pex3.
With regard to peroxisome clustering, we have repeated the experiments described in Fig. 1A and Suppl. 1E using monomeric GFP and find the results indistinguishable from those using the non-monomeric counterpart, i.e. Pex11-mGFP causes membrane clustering on oleate (Suppl. Fig. 1D). We find that integrated Atg36-monomeric GFP also causes peroxisomes to cluster (Suppl. Fig. 2), similar to our findings for the non-monomeric version.
We conclude that peroxisome clustering is not caused by GFP dimerisation of these fusions.

2) Fig. 1D now shows FM4-64 labelling for all the lines shown in Fig. 1C. The vacuoles in atg36 cells do not appear to be different to those in WT cells.

3) For Ape1-GFP see new Fig. 2A. The level of Ape1-GFP in the vacuole in WT and atg36 cells is comparable. We have performed the alkaline phosphatase assay as suggested (Fig. 2C) and this now replaces the GFP-Atg8 fluorescence of previous Fig. 2B.

4) No, we did not observe such a phenotype. On the contrary, we observe enhanced pexophagy upon overexpression of Atg36. Furthermore, overproduced Atg36 does not colocalise with Atg11 in cells lacking peroxisomes (not shown). This suggests that Atg36 needs to bind to peroxisomes to interact with Atg11.

5) The reviewer points out correctly that binding appears less. Both pex3 alleles were isolated in a microscopy-based screen and the specific phenotypes of these alleles are strong (see Fig. 5 and Suppl. Fig. 4). Analysis of the level of Pex3 protein shows that both mutant alleles are present at a somewhat reduced level (Suppl. Fig. 4E). However, the key point is that these alleles demonstrate differential phenotypes, with pex3-1 active in peroxisome formation and pexophagy, and pex3-177 active in peroxisome formation and segregation. Expression of Inp1-GFP results in loss of peroxisomes from the bud and gives the appearance of reduced binding. Similarly, pex3-1 cells have impaired peroxisome inheritance and so there are fewer Atg36-GFP puncta in pex3-1 mother cells. The reduction in binding between Atg36 and the Pex3-177 is now demonstrated biochemically (Fig. 5E).

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6) The lack of a hotspot for mutations in the pexophagy-deficient pex3 alleles may indicate the mutations affect the tertiary structure of the protein and this is now discussed. We are currently characterising the phenotype-causing mutations of the pex3 alleles and this will be part of a future publication.

7) Atg36-PtA migration after switching to starvation medium has been analysed in more detail in Suppl. Fig. 3D. (see also Fig. 7A for instance).

8) Please see new discussion, which includes discussion of interactions with Inp1 and Pex34 and differences between the role of Pex3 in S. cerevisiae and methylotropic yeasts.

9) Done

Small changes: all done and highlighted in red

Referee # 3

1) Done

2) Defined

3) Done

4) The HcRed-PTS1 in Fig. 1A is indeed serving only to verify that the Pex11-labeled structures are peroxisomes. We have included this control as Pex11 has previously been reported to translocate between ER and peroxisomes (Knoblach et al., 2010). We felt it necessary to validate that Pex11-GFP can be used as a marker to follow peroxisome degradation as it is used throughout the paper. We now explain this better in the Figure legend.

5) Done

6) Done

7) Done

8) The reviewerís comment led us to investigate the pexophagy activity of N- vs C- vs untagged Atg36 expression more fully by Western blotting, and this is now shown in Figs. 3B and C. What we found is that N-tagged Atg36 is constitutively active in that it induces pexophagy even under peroxisome proliferating conditions. In contrast, the untagged and C-tagged versions become fully active only when the cells are switched to starvation conditions.

9) Done (p14)

10) Done

11) Changed

12) Done, see Fig. 7.

13) Done

14) As suggested, the interaction between Atg11 and Atg36 has been examined, as well as the interaction between Atg8 and Atg36, and the phosphorylation state of Atg36 under pexophagy conditions (Figs. 7A and Suppl. Fig. 3D). Interaction of Atg36 with Atg11 is stimulated during starvation. In the new co-immunoprecipitation experiment, phosphatase inhibitors were added to preserve phosphorylation.
Phosphatase treatment of starved and oleate grown cells shows that Atg36 is phosphorylated. CIP treatment did clearly result in a faster migrating band of Atg36 in oleate grown cells. However, Atg36 from starved cells still displayed a fuzzy appearance and this smear seems to migrate between

the dephosphorylated oleate sample and untreated starved sample. This suggests that Atg36 is phosphorylated under the latter condition but contains either some CIP-resistant sites or distinct modifications as well as CIP-sensitive phosphorylation sites. Alhough these experiments do not completely clarify the modification status of Atg36 under various growth and experimental conditions, it nonetheless shows that Atg36 is differentially modified under these conditions and these modifications correlate with pexophagy and interaction with Atg11 and Atg8. As we have now indicated in the discussion it is only a correlation.

2nd Editorial Decision 22 December 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are enclosed. As you will see, all two referees express interest in your manuscript and are broadly in favor of publication, pending satisfactory minor revision.

Of particular concern, we would appreciate if you could provide a more causative effect of Atg36 phosphorylation by making phosphomimetic mutants (the phosphoproteome analysis, although recommended by the referee 1, will not be necessary).

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of the two reviewers. According to the nature of your revision I might send back your manuscript to the referees, so please make sure to reply to their concerns to the best of your capacities.

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

We generally allow up to three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

Yours sincerely,

Editor
The EMBO Journal

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors have improved the manuscript significantly by adding new data.
There are two main points the authors need to improve before the manuscript is accepted:
1. Possible phosphorylation of Atg36 has been only correlative, there are more opportunities for the authors to contribute to this topic by making phosphomimetic mutants and/or doing phosphoproteome analysis. It has been shown that serine phosphorylation next to the LIR domain increases affinity in binding to ATG8 orthologues. The discussion should be updated with different options on how phoshorylation can affect the pexophagy pathway.
2. References should be updated and more balanced. In the second paragraph about macroautophagy

the contribution of pioneering groups in the field have not been mentioned. In addition, referencing selective processes (labelled in red) several original and critical manuscripts have not been referenced.

Referee #3 (Remarks to the Author):

This paper has certainly improved, but there are still a few deficiencies.

1. Minor point: It seems a little strange to start the paper by referring to a figure in the supplement. In fact, the first six figure citations are all to Suppl. Fig. 1.

2. On page 10, the authors refer to the OM45-GFP assay and reference a paper by Okamoto et al. I checked that paper but could not find any reference to this assay.

3. Minor point: On page 12, the authors state that the N-tagged version of mRFP-Atg36 is not further activated by switching to starvation; however, the free GFP band looks more intense at the 1 and 2 hour time points (compare "N" lanes). Also, the abbreviations used in the figure ("W", "N", etc.) should be defined in the figure legend, not just the text.

4. A nice control for Fig. 4B would be a known mitochondrial marker or at least mitochondria-specific dye to verify the correct localization of the chimera.

5. The data in Fig. 5A and Suppl. Fig. 4D should be quantified.

6. I am confused by the statement on page 16, "Together with the overexpression experiments described in Fig. 3 it is clear that there is no direct correlation between the level of Atg36 expression and pexophagy." On page 12 in reference to Fig. 3, the authors conclude "Cells overexpressing any of the three versions of Atg36 induce pexophagy above endogenous level in WT cells...the N-tagged version induced pexophagy even under peroxisome proliferation conditions." That is, the overexpression of Atg36 appeared to drive pexophagy, but now, based on Fig. 6, they conclude that the level of Atg36 has no direct connection with pexophagy. Again, on page 20, the authors state, "...induction of mRFP-Atg36 causes pexophagy, even under conditions that normally stimulate peroxisome proliferation." These statements seem contradictory.

7. In regard to Fig. 6B, the authors state, "...Atg36-GFP disappears also from atg1 cells, but since no GFP cleavage product accumulates, this breakdown is not occurring in the vacuole." That is, the authors conclude that there is non-vacuolar degradation of Atg36-GFP, which explains the absence of the free GFP band. On the same page, in regard to Fig. 6C, the authors state, "...the protease-resistant GFP fragment appears during pexophagy in WT cells, it does not appear in pex3 or atg1 cells" and cite this as evidence that breakdown is occurring in the vacuole. They cannot first cite the lack of free GFP in atg1 as evidence for non-vacuolar degradation, and then cite the same lack of free GFP in atg1 as evidence for vacuolar degradation.

8. In Fig. 7A the authors need a negative control for something that Atg36 does not bind. They show that Atg8 and Atg11 do not bind the control construct, but as far as we know, anything will bind Atg36.

I think the paper minimally needs to go through one more round of revision to correct the errors (at least those in logic) that I point out above.

2nd Revision - authors' response 10 January 2012

Referee 1

1. We feel that to address this point, i.e., to make phosphomimetic mutants of serines and threonines around the LIR domain, requires us first to establish the LIR domain in Atg36. Using a pattern search comprising the following pattern [WY]XX[LIV] we found two potential LIRs. Both putative LIR motifs in Atg36 are surrounded by serines and threonines, and many mutants would have to be constructed and tested for their effect on Atg8 binding and pexophagy. We agree that there are many opportunities to contribute to the understanding of Atg36 phosphorylation and how this relates to function, and feel that a substantial study such as this is publishable as a standalone paper (see for example, Aoki et al., phosphorylation of serine 114 on Atg32 mediates mitophagy, MBC 2011 22, 3206-17).

2. Done, see p4.

Referee 2

1.We have included our validation of the pexophagy markers and assays used throughout the paper in Suppl. Fig 1, as well showing that replicative peroxisome multiplication and function is unaffected in atg36 cells. We felt it necessary to include this information prior to the first figure of the main paper, which shows the pexophagy defect of atg36 cells.

2. Corrected: OM45 assay is adaptation of that by Kanki et al. (2008) and Okamoto et al. (2009), both now referenced, see p 10.

3. Corrected in text (see p 12). The abbreviations were defined in figure legend.

4. Done, see Fig. 4B and legend.

5. Peroxisome distribution in glucose-grown PEX3, pex3-177 and pex3-1 cells is quantified in Suppl. Fig. 4B. Peroxisome numbers are hard to quantify in oleate grown cells because peroxisomes tend to cluster under this condition as explained on p 8. However, the Pex11-GFP signal in PEX3 and pex3-177 is shown in Fig. 5B by Western blots of oleate grown cells (t = 0) and starved cells (t=6 h and 22 h), and in Suppl. Fig. 4A by Western blot of all pex3 alleles identified in the screen after growth on oleate (-) and starvation (+). We have now quantified the Inp1-GFP puncta and the figures are shown in Suppl. Fig. 4 legend.

6. We have inserted some text (see p 16) to clarify the statement ëÖ there is no direct correlation between the level of Atg36 expression and pexophagyí

7. We are sorry for the confusion. We interpret the data as follows. Atg36-GFP is broken down during starvation by the following mechanisms: cotransport with peroxisomes into the vacuole in WT cells, or, if autophagy is blocked (e.g. in atg1 cells), Atg36 is degraded not via autophagy but via another process. The text has been altered to clarify this (see p 17).

8. The referee asks whether anything will bind Atg36, i.e., the referee asks for a negative control for the co-immunoprecipitation experiments using Atg36-ProtA as bait. We show in Fig. 4D that Pex13-GFP does not interact with Atg36-ProtA.
We show also under non-pexophagy conditions that very little Atg11 or Atg8 Co-IPs with Atg36 (the level is comparable to that of the negative control Mvp1-ProtA). This may reflect aspecific binding. However, when we shift to starvation medium, Atg8 and Atg11 binding to Atg36-ProtA is increased. This clearly shows that the increased binding under starvation conditions is specific. A similar experiment was performed by Kanki et al., Fig. 4B, Dev. Cell 17 (2009), 98-109.

Nevertheless, we have rerun the samples of Fig. 7A and analysed them for the presence of PGK1 and PEP12. Although both proteins are easily detectable in the total lysates, they are absent from the IP. We have not included this in the manuscript figure, but include it below for your inspection.

3rd Editorial Decision 16 January 2012

Thank you for submitting your revised manuscript to our editorial office. I have now extensively looked at your revisions and discussed with my colleagues your point-by-point response, especially in light of the requested experiments from referee #1 that you have not performed. I also corresponded with this reviewer to be sure that the experiments demanded where easy to perform.

We believe that your manuscript would be greatly improved if you could at least make mutations in the two LIR motifs you have found, test the outcome of phosphomimetics and show a functional effect on ATG36. As I said previously, we will not require a proteomic map of ATG36, nor will we need the identification of the responsible kinase, but rather a simple and doable experiment to test your hypothesis.

As you certainly know, EMBO policy normally stipulates that a single round of revision is allowed. However, for the sake of your manuscript, we are willing to accept yet another revision addressing the point I just mentioned. If you can't do this experiment, I am sorry to say that you will have to seek publication elsewhere at this stage.

I would appreciate if you could let me know at your earliest convenience whether you decide to perform the experiment or would like to withdraw your manuscript.

I am looking forward to hearing from you soon.

Yours sincerely,

Editor
The EMBO Journal

Additional correspondence 17 January 2012

We are disappointed with the decision but are willing to perform the suggested experiments for this manuscript. We confirm that we will try to identify the LIR domain and mutagenise serines and threonines in its vicinity, as well as analysing the effects of these mutations on pexophagy, and on whether these mutations affect Atg8 binding. This is more than just a simple and doable experiment, and will take considerable time.

We will get back to you in due course.

Additional correspondence 02 April 2012

For the revision of our manuscript, you asked us to make mutations in the putative LIR motifs of Atg36, and to construct and test phosphomimetic mutants around the motif important for Atg36 function.

There is one LIR motif in Atg36 that conforms to the LIR consensus in yeast (WxxL/I), but mutations in this motif have no consequence for Atg36 function, even deletion of this motif has no effect on pexophagy by Atg36. There are seven further motifs in Atg36 that resemble the Atg8/LC3 consensus (Y/FxxL/I/V), and we have tested all of these for function. One of these motifs indeed has a strong effect on Atg36 function, such that pexophagy is completely blocked in the mutant, as assessed by Pex11 pexophagy. This mutant version of Atg36 is still recruited to peroxisomes, but peroxisomes remain dispersed in the cytosol and are not degraded. We have replaced a serine and a threonine residue in the vicinity of the motif by alanine residues and are checking them for function.

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We are also currently making phosphomimetic mutants of these residues. To finish these experiments, we will need an additional 2-3 weeks and expect to resubmit before the end of this month.

We hope this is acceptable to you.

3rd Revision - authors' response 27 April 2012

We believe that your manuscript would be greatly improved if you could at least make mutations in the two LIR motifs you have found, test the outcome of phosphomimetics and show a functional effect on ATG36. As I said previously, we will not require a proteomic map of ATG36, nor will we need the identification of the responsible kinase, but rather a simple and doable experiment to test your hypothesis.

We proposed that Atg36 activates pexophagy by a two-step process, first accumulation on peroxisomes followed by an activation event under pexophagy-inducing conditions, which correlates with a shift in mobility of Atg36. Under these conditions, we also observed an increased interaction of Atg36 with Atg11 and Atg8. The experiments requested relate to the second event, and are based on the precedent of phosphorylation-dependent interaction of optineurin with LC3. We have done the experiments you requested to test whether phosphorylation of an AIM in Atg36 affects its activity. The mechanism of Atg36 action seems to be different from that of optineurin, however, in that we were unable to find an AIM that is required for Atg36 function, and so could not test the effect of phosphorylation of an AIM.

Below we describe our experiments and findings in detail, and discuss how it relates to the recent literature, which suggests the importance of AIMs may vary between receptors.

New data:
For this revision of the manuscript, you asked us to make mutations in the two putative LIR/AIM motifs of Atg36 we identified, and to construct and test phosphomimetic mutants around the motif important for Atg36 function.
Using the pattern search [WY]xx[LI] (Kirkin et la., 2009), we identified two putative Atg8-interacting motifs in Atg36. However, optineurin contains a phenylalanine at position 1 of the motif (Wild et al., 2011), and using Fxx[LIV] as a pattern search we identified six additional motifs. We substituted each motif to AxxA and assayed for pexophagy activity and localization to peroxisomes. All known S.cerevisiae AIMs contain a tryptophan (W) at position 1. Atg36 contains one such motif (W282/L285), but as shown in Suppl. Fig. 5, mutation of this motif had no consequence for function. Of the other seven putative motifs, mutation of only Y191/L194 impairs pexophagy. This mutant version of Atg36 is still recruited to peroxisomes, but peroxisomes remain dispersed in the cytosol and are not degraded (Suppl Fig 5). We do not think this motif is an AIM, however, since the tyrosine is not conserved and it does not contain the serine/ threonine residues or the negatively charged amino acids that characteristically flank position 1 of the motif. Nonetheless, as requested, we tested whether phoshomimetic mutations activated Atg36 by replacing the closest phosphorylatable residues (serine and threonine at -4 and + 6, respectively) with glutamate, but found no consequence for pexophagy. Similarly, replacing these residues by alanine did not affect pexophagy. Finally, mutation of the acidic residue at the x-2 position had no effect on pexophagy by Atg36. Only the YxxL to AxxA mutation blocks pexophagy. We tested the importance of the Y of Y191/L194 and found that Y191L (the residue present in most Atg36 orthologues) had normal pexophagy activity (Suppl Fig. 5). This confirms that the YxxL is not an AIM.
We did not investigate the effect of mutations around the remaining seven LIR-like motifs because we lack a functional read-out for an effect.
Our findings could be explained by redundancy between AIMs, ie that there are multiple motifs in Atg36 that are functionally redundant. A second interpretation is that there is no direct interaction between Atg36 and Atg8, as has recently been proposed to be the case for Atg30 (see below).
Of course we have tried to test whether Atg36 and Atg8 interact directly. However, we have been unable to show this interaction in vitro, or by yeast two hybrid (although we did find an interaction between Atg32 and Atg8 in yeast 2 hybrid). The in vivo interaction between Atg36 and Atg8 (Fig.

7) is the only interaction we see between these two proteins.
During the revision of this manuscript, a paper by Kondo-Okamoto et al studied the interaction of mitophagy receptor Atg32 with Atg8 and Atg11. They found that whereas disrupting the interaction between Atg32 and Atg11 had a strong effect on mitophagy (reducing it to 20% of WT levels), mutating the AIM in Atg32 had only a minor affect on mitophagy (reducing it to 88% of WT levels). Although mutating the AIM prevented the Atg8-Atg32 interaction by two hybrid, the two proteins still coimmunoprecipitated. Even mutating the Atg32 AIM binding interface of Atg8 IN ADDITION to mutating the AIM of Atg32 does not prevent coIP of the two proteins, and mitophagy remains at 60% of wt levels. Therefore, the interaction of Atg8 with the AIM of Atg32 is not essential for mitophagy but it does contribute. In contrast, interaction of the AIMs in the Cvt pathway proteins Atg19 and Atg34 is crucial for their interaction with Atg8 as well as Cvt pathway function. Similarly, in mammalian cells, interaction of LC3 with selective autophagy receptors is crucial for autophagic degradation of the cargos. Therefore, it seems that the importance of AIMs in autophagic receptor function varies.
Furthermore, a meeting report published in EMBO reports last month states that Subramaniís lab has identified a factor that links their autophagy receptor Atg30 to Atg8. Atg30 lacks an AIM and binds Atg8 via a novel protein (EMBO reports (2012) 13, 175 ñ 177). This is consistent with our data, and may also be relevant to that of Kondo-Okamoto et al for Atg32, who suggest that ëother protein-protein interfaces could contribute to the interaction between Atg32 and Atg8 in vivoí (Kondo-Okamoto et al, 2012).
We have included our new data in Suppl. Fig. 5, and show all changes in this third revision of the manuscript in red.
We sincerely hope you will now accept our manuscript for publication.

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