THE EMBO JOURNAL

Manuscript EMBO-2010-75353

Mitochondria regulate autophagy by conserved signaling pathways

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Review timeline:

Submission date:	12 July 2010
Editorial Decision:	13 August 2010
Revision received:	24 November 2010
Editorial Decision:	21 December 2010
Revision received:	28 February 2011
Accepted:	16 March 2011

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 13 August 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, all three reviewers find your results suggesting a regulatory link between mitochondria and autophagy upon amino acid starvation potentially interesting. However, especially referees 1 and 2 also raise a number of substantive points that would need to be adequately addressed before publication may eventually be warranted. In this respect, while I appreciate that a full mechanistic determination of this link may be somewhat beyond the scope of the current submission, the key issue would however be to strengthen the study in a way so as to exclude the possibility of indirect secondary effects underlying the current observations. Of particular importance in this respect will be the use of more specific loss-of-function approaches to validate the conclusion on mitochondrial/respiratory chain function, for which referee 1 offers a number of constructive suggestions.

Should you be able to satisfactorily address these main concerns, we should be happy to consider a revised version of the manuscript further for publication. I would thus like to invite you to prepare such a revision on the basis of the three referee reports, keeping in mind that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various major and minor points raised at this stage. When preparing your revision, please also bear in mind that your letter of response will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Please also briefly specify the individual author contributions, either in the acknowledgements section or in an adjacent separate section, as we are attempting to adopt this as a common policy now. Should you need any additional clarifications regarding the requirements of this revision, I shall be happy to discuss this further once you had a

chance to consider the comments in detail.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Graef and Nunnari analysed the impact of mitochondrial dysfunction on the autophagic response in Saccharomyces cerevisiae. They find that mitochondrial respiratory deficiency compromises induction of autophagy genes (regulated by PKA) and autophagic flux (regulated by TORC1 and PKA). The control of autophagy by mitochondria in yeast is a novel and interesting finding. Nevertheless, essential experiments are missing to support authors conclusions and the proposed molecular mechanism of a "cross-talk between TORC1, PKA and mitochondria" should be deeper analysed.
1) Attention must be paid to the models used for mitochondrial dysfunction.
a. The authors used rho0 yeast as the genetic model for mitochondrial dysfunction, synonymous of depolarized mitochondria, unable to produce mitochondrial ATP. Rho0 cells lack all the components encoded by mitochondrial DNA. Using strains depleted for specific subunits of the respiratory complexes or ATPase would allow to understand the relevance of mitochondrial depolarization versus lack of ATP produced by mitochondria. To avoid mtDNA instability due to the deletions, the authors should replace the coding sequence of the specific subunits with a non-respiratory genetic marker, as done, for instance, by Rak et al., 2007, JBC, for Atp6p gene deletion.
b. Moreover, the authors used antimycin A that inhibits complex III. Antimycin A blocked ATG8 induction but oligomycin did not, so the authors concluded that ATG8 induction requires maintenance of mitochondrial membrane potential (page 8, last paragraph). This should be measured. It is possible that wt yeast revert ATPase in order to maintain mitochondrial membrane potential if the proton gradient across the respiratory complexes is inhibited. Moreover, to address whether ATG8 induction depends on the mitochondrial membrane potential, it would be better to use an uncoupler like CCCP. Finally, antimycin A is a known ROS producer, raising the possibility that ROS block ATG8 induction. Therefore, control experiments with antioxidants should be performed here
2) In figs 1B, 2B the authors examined the localization of GFP signal after induction of autophagy. They should add the untreated controls to the figure. Moreover, a fluorescent protein resident in the vacuole should be used as a vacuolar marker, since vacuolar morphology of wt and rho0 cells are apparently different in fig 1B.
3) In page 7, the authors claim that Atg8 recruitment to PAS is not impaired by mitochondrial dysfunction, because they observed GFP-Atg8 in punctuate structures in aminoacid starved rho0 cells. This should be better addressed by analysing for instance Atg8-PE formation.
4) Based in the results presented in fig. 2, the authors concluded that the autophagic flux, as opposite to ATG8 induction, "depends on the ability to generate ATP by respiration even when glycolysis can occur". This should be better sustained by measuring total and mitochondrial ATP produced under the different conditions presented in fig. 2.
5) According to the authors, the results presented in fig. 3, are explained by the fact that inhibition of TORC1 is only partial upon aminoacid starvation, while rapamycin or N- starvation lead to a more complete inhibition. TORC1 activity should be measured under the different conditions, as done for instances by Neklesa and Davis 2009, PLOS Genetics. Moreover, authors affirm in page 10, 1st paragraph, that mitochondrial function regulates autophagic flux in a TORC1-dependent fashion. This is not supported by the presented data: activity of TORC1 should be compared betwen wt, rho0, cells treated with antimycin A and oligomycin.
6) In fig. 3, the authors should add the western-blot with the GFP reporter only to better measure ATG8 induction.

7) The effect of PKA on the ATG8 induction is not clear from the results presented in fig. 3B. Both hyperactivation and inhibition of PKA impaired ATG8 induction. The authors suggested that a certain level of PKA activity is required for full ATG8 induction. This point should however be better addressed, for instance, by titrating the inhibitor PP1.
8) In fig 3c, the authors show that in rho0 cells activity of the PKA is increased. Antimycin A and oligomycin treated cells should also be included here.
9) In fig. 4b, together with localization of Atg1, the authors should evaluate the phosphorylation levels of the protein.
10) The western-blot of fig 4c should be quantified.
11) The model presented in fig 5 should be described in the legend.

Referee #2 (Remarks to the Author):

The authors of this work investigate the possible role of mitochondria in regulating yeast autophagy. They show that the mitochondrial respiratory chain play a crucial when autophagy is induced by amino acid starvation but not nitrogen deprivation. In addition, they show that protein kinases Atg1, TORC1 and PKA regulate autophagic flux, whereas autophagy gene induction depends solely on PKA. In that regard, the authors find that mitochondrial respiratory deficiency block autophagic flux, and possibly autophagy gene induction by impairing the Atg1 recruitment to the PAS.

Major criticisms

1) All the experiments are well planned, executed and controlled. The basic observations are also very interesting and the manuscript is well written. My major concern, however, is that while a dysfunction of mitochondrial respiration affects autophagy triggered by amino acid starvation, no direct molecular link between the signalling cascades regulating autophagy and mitochondria function has been identified. Therefore, the conclusion that mitochondria regulate autophagy is not directly demonstrated. What observed could be caused by one or more secondary effects.

2) TORC1 directly phosphorylates Atg13. As an important control and to further sustain the conclusion that there is not a dominant regulatory role of TORC1 in autophagy induced by amino acid depravation, the authors have to analyze the phosphorylation status of Atg13 in some of their experiments, mainly those shown in figure 3. This is particularly important because the phosphorylation status of Atg13 dictates the association between this protein and Atg1. Therefore, the authors have also to analyze the association between Atg1 and Atg13 in rho cells starved for amino acids. Atg13 is required to recruit Atg1 to the PAS [Suzuki et al, (2007), Genes Cells, 12:209-18]; absence of interaction between Atg13 and Atg1 could thus explain the observed phenotype (Figure 4B). Does Atg13 localize to the PAS in rho cells starved for amino acids?

Minor criticisms

1) A very recent publication [Ecker et al (2010), Autophagy, 6, Epub ahead of print] has shown that there are differences in the regulation of autophagy induced by amino acid and nitrogen starvation. They authors have to integrate/discuss these findings in their manuscript.

2) Page 3 line5. Autophagosome do not fuse with lysosome. In high eukaryotes, they first fuse with endosomes to become amphisomes and then amphisomes fuse with lysosomes.

3) Figures 1B, 2B and 4B. The authors assume that the GFP-Atg8-positive dot is the PAS in either rho cells or antimycin A-treated cells. How they are sure about this conclusion? Showing the presence of a second PAS marker such as another Atg protein or Ape1 could sustain their conclusion. My concern is due to the fact that using the same evaluation standards, one would conclude that GFP-Atg8 localizes to the PAS in atg7 cells as well based on what depicted in Figure 1B and 2B (lower panel). It has been shown, however, that in absence of Atg7, Atg8 is not lipidated and as a result is not recruited to the PAS. The authors have to explain this discrepancy.

4) Figure 1. It will be interesting to show that mutant affecting other mitochondrial function (lipid synthesis, maintenance of the mitochondrial DNA...) have not an impairment in autophagy when this pathway is induced by amino acid starvation.

5) Figure 2A. It would be nice if the authors analyze the effects of Antimycin A and Oligomycin on autophagy induced by nitrogen deprivation. The absence of effects will further support their conclusion of a different way of regulating this pathway.

6) It is important to indicate somewhere for how long the cells were treated with Antimycin A and Oligomycin.Where those drugs added at the beginning of the starvation period?

7) Figure 4A. Why does the deletion of ATG11 block amino acid-induced autophagy to the same extend than the rho mutation? An explanation has to be provided

Referee #3 (Remarks to the Author):

The manuscript by Graef & Nunnari describes an important study on the role of mitochondrial respiration in starvation-induced autophagy in yeast cells. Autophagy is the adaptive catabolic pathway responsible for cell survival during starvation and stress. While important for the pathogenesis of major human diseases, such as neurodegeneration and cancer, the mechanisms of autophagy appear to be evolutionarily highly conserved. Thus, elucidation of its mechanisms in the relatively simple organism, yeast, promises a great impact on our understanding of autophagy in human biology.

The relationship between mitochondria and autophagy is of extremely importance, even though understudied: 1) defective mitochondria are eliminated via a selective form of autophagy, and 2) mitochondrial membranes can be the source for the autophagosomal envelopes. Graef & Nunnari have now extended the notion of this intimate relationship in that they demonstrate the dependence of autophagy in yeast on the intact mitochondrial respiration. Using elegant genetic and biochemical tools, the authors show that respiration-deficient mitochondria inhibit both the autophagic flux and the induction of the key autophagy genes. Further, the authors propose a mechanistic link between the respiratory block and the autophagy inhibition in yeast.

The experiments for this study have been performed accurately and with technical excellence. The structure of the manuscript is also very good.

Comments:

1) Fig. 4C: "...Atg1 was present at similar steady levels in cells as monitored by Western (blot) analysis..." The authors seem to have performed this experiment to control for Atg1 levels in the preceding experiment (Fig. 4B), in which GFP-Atg8 and Atg1-mCherry co-localization in the perivacuolar puncta (phagophore assembly site, PAS) is assessed as a measure for the PKA activity and autophagy induction. However, this is an independent experiment using a different fusion protein (Atg1-GFP vs. Atg1-mCherry). It appears that extraction of the Atg1 protein from the same cells that had been imaged would have been a more relevant control. Also, despite the authors' claim regarding the similar levels of Atg1, there are some differences observed in Atg1 levels in the samples shown in the Fig. 4C, which should have also been quantified as previously done in the Western blot-based analyses.

2) Fig. S4: Data shown in this figure suggest that selective autophagy of mitochondria (mitophagy) is also inhibited under the conditions of the respiratory block. It is however unclear whether PKA also mediates inhibition of this type of autophagy during the respiratory block. It is thus requested that mitochondrial degradation is assessed in yeast strains with overactive and inhibited PKA, respectively, like the data shown in Fig. 3B.

3) An important type of selective autophagy in yeast is the Cvt pathway. The study would greatly gain in impact if it could show that the delivery and degradation of the substrate of this selective autophagy pathway (i.e. prApe1) also depends on the intact mitochondrial respiration and the PKA signaling.

4) On page 12 (second to last sentence) it is said that in various conditions Atg1 was present at similar steady state levels in cells as monitored by western analysis however the figure does not

clearly show this. Could authors provide a more convincing image and the confirmation by qRT PCR? Is induction of Atg1 and Atg13 ablated in rho0 cells under conditions of starvation? Again, Fig. 4C is not convincing whereas Atg13 and quantification of the effects are missing.

5) It would be interesting to assay if the ratio of phosphorilated/dephosphorilated Atg13 is affected in cells with mitochondrial respiratorydeficiency and if this it directly regulated by PKA?

6) Quantification of the results on Fig. 4B is missing. On the page 5 (second to last and last sentences) and throughout the text,whenever authors claim that the difference is significant p values must beprovided, and the statistics method should be included into materials and methods section.

7) It would be helpful for the reader if the order of figure appearance in the text corresponded to their numbering, i.e. Fig. S1A, Fig. S1B, Fig. 2A, Fig. 2B, ...)

1st Revision - authors' response 24 November 2010

Thank you for overseeing the review of our manuscript entitled ëMitochondria regulate autophagy by conserved signaling pathways (EMBOJ-2010-75353). We have considered the your comments and those made by the reviewers and we agree with your assessment that a full understanding of the regulatory link between mitochondria and autophagy is beyond the scope of the manuscript. We have, however, conducted a thorough analysis of respiratory complex specific loss of function mutants that fully supports our conclusions regarding the role of mitochondria in the regulation of autophagy and have addressed all other points raised by the reviewers in our revised manuscript. We have provided our detailed response in the document that follows. We would like to thank the reviewers for their efforts. The revised manuscript is improved.

We thank the reviewers for their constructive comments. Many of the reviewers were focused on further delving into aspects of the Atg1-Atg13 kinase complex regulation and PAS organization in our system. To address these comments, we extended our analysis by further cytological characterization as shown in revised figure 5B. Together with published data our findings in total strongly support a model in which respiratory deficiency results in increased PKA activity and the consequent impaired recruitment of the Atg1-Atg13 kinase complex to the PAS. As discussed in our response to reviewer 2, our preliminary data indicate that the regulation of the Atg1-Atg13 complex differs in some aspects under amino acid starvation versus nitrogen starvation and depends also on carbon source. Thus, we think that a complete and comprehensive analysis of Atg1 and Atg13 regulation is required to address the significance of these observed differences and is beyond the scope of our manuscript.

Response to Reviewer 1:

" 1) Attention must be paid to the models used for mitochondrial dysfunction.
a. The authors used rho0 yeast as the genetic model for mitochondrial dysfunction, synonymous of depolarized mitochondria, unable to produce mitochondrial ATP. Rho0 cells lack all the components encoded by mitochondrial DNA. Using strains depleted for specific subunits of the respiratory complexes or ATPase would allow to understand the relevance of mitochondrial depolarization versus lack of ATP produced by mitochondria. To avoid mtDNA instability due to the deletions, the authors should replace the coding sequence of the specific subunits with a non-respiratory genetic marker, as done, for instance, by Rak et al., 2007, JBC, for Atp6p gene deletion."

It is technically difficult to generate specific mtDNA alleles. We instead addressed the selective nature of the role of mitochondria in autophagy regulation by exploiting nuclear genes that encode proteins required for the translation of key mitochondrially encoded respiratory subunits of complex III and complex IV, which are noted parenthetically, namely CBS1 (Cob1) and MSS51 (Cox1),

PET111 (Cox2), and PET122 (Cox3), respectively. Similarly, we used a strain deleted for the nuclear gene ATP10, which encodes an essential assembly factor for the Fo sector of the ATP synthase. Importantly, mtDNA is stably maintained in strains harboring deletions for all of these genes. Data from an analysis of autophagy in these strains (revised Figure 2) are consistent with our observations from rhoo cells and wild type cells treated with chemical inhibitors and thus strengthen our conclusions.

"b. Moreover, the authors used antimycin A that inhibits complex III. Antimycin A blocked ATG8 induction but oligomycin did not, so the authors concluded that ATG8 induction requires maintenance of mitochondrial membrane potential (page 8, last paragraph). This should be measured. It is possible that wt yeast revert ATPase in order to maintain mitochondrial membrane potential if the proton gradient across the respiratory complexes is inhibited."

ATP synthase activity is indeed used to generate mitochondrial membrane potential in the absence of respiratory competence. However, it is well documented that respiratory deficient cells generate a significantly reduced mitochondrial membrane potential and that antimycin A at the concentrations we use significantly diminish membrane potential. We now cite the relevant literature to support a role for mitochondrial membrane potential as a signal for respiratory deficiency in mitochondria to regulate ATG8 induction.

"Moreover, to address whether ATG8 induction depends on the mitochondrial membrane potential, it would be better to use an uncoupler like CCCP."

We have now included a careful titration of CCCP during amino acid starvation (Supplemental Figure 5B). Consistently, this analysis indicates that decreasing membrane potential strongly impaired Atg8-induction. However, CCCP also uncouples the plasma and vacuolar membranes. Given that vacuolar acidification is required for autophagic turnover, we cannot exclude that the impairment in autophagic flux observed in the presence of CCCP is caused by non-mitochondrial changes.

"Finally, antimycin A is a known ROS producer, raising the possibility that ROS block ATG8 induction. Therefore, control experiments with antioxidants should be performed here"

To address this point, we performed starvation experiments with cells overexpressing superoxide dismutase 1 or 2 (Supplemental Figure 5A). Under these conditions, we did not observe any significant effects on autophagy induction in wild type or rho0 cells, or in cells treated with antimycin A or oligomycin. This suggests that ROS production does not play a major role in autophagy regulation under these conditions.

"2) In figs 1B, 2B the authors examined the localization of GFP signal after induction of autophagy. They should add the untreated controls to the figure. Moreover, a fluorescent protein resident in the vacuole should be used as a vacuolar marker, since vacuolar morphology of wt and rho0 cells are apparently different in fig 1B."

We have repeated our analysis and included the vital vacuolar dye FM4-64, which marked vacuoles in our cells. The results are presented in revised figure 1B and fully support our previous conclusions.

"3) In page 7, the authors claim that Atg8 recruitment to PAS is not impaired by mitochondrial dysfunction, because they observed GFP-Atg8 in punctuate structures in aminoacid starved rho0 cells. This should be better addressed by analysing for instance Atg8-PE formation."

We agree that the analysis of this regulatory step would add to the manuscript. Unfortunately, technical problems have hampered the analysis of Atg8-PE formation. Thus, instead, we have softened the significance of our observation that Atg8-GFP is punctate in rho0 cells in the manuscript. The revised text on p.7 now readsÖ. "GFP-Atg8 was observed in punctate structures in amino acid starved rho0 cells (Fig. 1B, Galactose), raising the possibility that Atg8 recruitment to the pre-autophagosomal structure (PAS) is not affected (Kirisako et al., 1999). " We have also carefully reworded the text on pages 13 and 14 so as to not overstate our results regarding Atg8-GFP.

"4) Based in the results presented in fig. 2, the authors concluded that the autophagic flux, as opposite to ATG8 induction, "depends on the ability to generate ATP by respiration even when glycolysis can occur". This should be better sustained by measuring total and mitochondrial ATP produced under the different conditions presented in fig. 2."

New data presented in Figure 2 indicate that impaired mitochondrial ATP production (oligomycin treated or atp10 cells) in the presence of a fermentable carbon source (galactose) did not impair autophagic flux significantly. Thus, ATP levels seem to play only a minor role under these conditions.

"5) According to the authors, the results presented in fig. 3, are explained by the fact that inhibition of TORC1 is only partial upon aminoacid starvation, while rapamycin or N- starvation lead to a more complete inhibition. TORC1 activity should be measured under the different conditions, as done for instances by Neklesa and Davis 2009, PLOS Genetics."

We analyzed TORC1 activity in vivo during amino acid or nitrogen starvation in the presence or absence of rapamycin by monitoring Npr1 phosphorylation (revised Figure 3B and Supplemental Figure 6B). Only small changes in the pattern of Npr1 phosphorylation were observed during amino acid starvation, indicating minor changes in TORC1 activity. In contrast, under nitrogen starvation or upon addition of rapamycin during amino acid starvation, we observed a significant accumulation of non-phosphorylated Npr1, indicating a strong inhibition of TORC1 activity under these conditions. These results support and strengthen our previous model of distinct roles for TORC1 in the regulation of autophagy induction during amino acid and nitrogen starvation.

"Moreover, authors affirm in page 10, 1st paragraph, that mitochondrial function regulates autophagic flux in a TORC1-dependent fashion. This is not supported by the presented data: activity of TORC1 should be compared betwen wt, rho0, cells treated with antimycin A and oligomycin."

Our phrasing was misleading as we did not intend to suggest that mitochondrial function regulated autophagic flux by modulating TORC1 activity. Rather, TORC1 contributes to the regulation of autophagic flux during amino acid starvation. We deleted this sentence from the manuscript.

"6) In fig. 3, the authors should add the western-blot with the GFP reporter only to better measure ATG8 induction."

These data are now included in revised Figures 3 and 4.

"7) The effect of PKA on the ATG8 induction is not clear from the results presented in fig. 3B. Both hyperactivation and inhibition of PKA impaired ATG8 induction. The authors suggested that a certain level of PKA activity is required for full ATG8 induction. This point should however be better addressed, for instance, by titrating the inhibitor PP1."

We tested a broad series of 1NM-PP1 inhibitor concentrations and all possible permutations of inhibitor sensitive mutations in the three genes (TPK1-3) encoding for the catalytic subunits of PKA to test whether intermediate activities or specific subunit combinations would be sufficient to support ATG8 induction in rhoo cells. Unfortunately, we did not identify conditions that allowed ATG8 induction in rhoo cells or that did not affect ATG8 induction in wild type cells.

"8) In fig 3c, the authors show that in rho0 cells activity of the PKA is increased. Antimycin A and oligomycin treated cells should also be included here."

These data are included in revised figure 4B and support our conclusions.

"9) In fig. 4b, together with localization of Atg1, the authors should evaluate the phosphorylation levels of the protein."

We conducted a more detailed cytological analysis of the PAS and our data are consistent with a model in which respiratory deficiency results in increased PKA activity that negatively affects the localization of Atg1 and Atg13 to the PAS. We think that a comprehensive analysis of Atg1

phosphorylation is beyond the scope of this manuscript.

"10) The western-blot of fig 4c should be quantified."

These data have been included in revised Figure 5C.

"11) The model presented in fig 5 should be described in the legend."

A description has been included (revised Figure 6).

Response to reviewer 2:

"1) All the experiments are well planned, executed and controlled. The basic observations are also very interesting and the manuscript is well written. My major concern, however, is that while a dysfunction of mitochondrial respiration affects autophagy triggered by amino acid starvation, no direct molecular link between the signalling cascades regulating autophagy and mitochondria function has been identified. Therefore, the conclusion that mitochondria regulate autophagy is not directly demonstrated. What observed could be caused by one or more secondary effects."

We agree that the question of how mitochondrial dysfunction is translated/transduced into changes in PKA (and possibly other) signaling pathways is outstanding. However, identification of the regulatory mitochondrial signaling mechanism(s) will require substantial work that is beyond the scope of the current manuscript.

"2) TORC1 directly phosphorylates Atg13. As an important control and to further sustain the conclusion that there is not a dominant regulatory role of TORC1 in autophagy induced by amino acid depravation, the authors have to analyze the phosphorylation status of Atg13 in some of their experiments, mainly those shown in figure 3."

To address this point (and reviewer 1's point #5), we analyzed the phosphorylation status of Npr1, a target of TORC1, during amino acid starvation or nitrogen starvation in the presence and absence of rapamycin (revised Figure 3B and Supplemental Figure 5B). These results fully support our conclusion that in contrast to nitrogen starvation, TORC1 plays a minor role in autophagy regulation during amino acid starvation.

"This is particularly important because the phosphorylation status of Atg13 dictates the association between this protein and Atg1. Therefore, the authors have also to analyze the association between Atg1 and Atg13 in rho cells starved for amino acids."

Preliminary data indicate that Atg1 and Atg13 association is not impaired during amino acid starvation under respiratory deficiency conditions. We also observe that mitochondrial dysfunction decreases the localization of both Atg1 and Atg13 to the PAS (see next comment). These data are consistent with our model in the context of published work showing that activation of PKA inhibits Atg1 and Atg13 PAS localization. Our Atg1-Atg13 interaction data, however, indicate that there are regulatory differences between amino acid starvation and nitrogen starvation in the presence of different carbon sources. Although it will likely be interesting and relevant, substantial additional work will be required to fully understand the basis for these differences and therefore, we have decided not to include them in this manuscript.

"Atg13 is required to recruit Atg1 to the PAS [Suzuki et al, (2007), Genes Cells, 12:209-18]; absence of interaction between Atg13 and Atg1 could thus explain the observed phenotype (Figure 4B). Does Atg13 localize to the PAS in rho cells starved for amino acids?"

We analyzed PAS recruitment of Atg13-mCherry in rhoo and wild type cells treated with either antimycin A or oligomycin. Atg13-mCherry localization to the PAS is strongly decreased in rhoo and wild type cells treated with antimycin A (revised Figure 5B). In contrast, both Atg1- and Atg13-mCherry localization to the PAS is unchanged in wild type cells the presence of oligomycin, consistent with our observation of wild type-like autophagy induction (revised Figures 2B and 5B). These differences correlate with PKA activity as we observed a relatively small increase in PKA activity in cells treated with oligomycin as compared to a larger increase in PKA activity in rhoo

cells and wild type cells treated with antimycin A (revised Figures 2B, 4B, 5B). Hence, our data are consistent with a model in which respiratory deficiency results in increased PKA activity negatively affecting PAS localization of Atg1 and Atg13.

"Minor criticisms

1) A very recent publication [Ecker et al (2010), Autophagy, 6, Epub ahead of print] has shown that there are differences in the regulation of autophagy induced by amino acid and nitrogen starvation. They authors have to integrate/discuss these findings in their manuscript."

We have referenced this recent work in our manuscript.

"2) Page 3 line5. Autophagosome do not fuse with lysosome. In high eukaryotes, they first fuse with endosomes to become amphisomes and then amphisomes fuse with lysosomes."

We corrected the introduction according to the reviewer's suggestion.

"3) Figures 1B, 2B and 4B. The authors assume that the GFP-Atg8-positive dot is the PAS in either rho cells or antimycin A-treated cells. How they are sure about this conclusion? Showing the presence of a second PAS marker such as another Atg protein or Ape1 could sustain their conclusion. My concern is due to the fact that using the same evaluation standards, one would conclude that GFP-Atg8 localizes to the PAS in atg7 cells as well based on what depicted in Figure 1B and 2B (lower panel). It has been shown, however, that in absence of Atg7, Atg8 is not lipidated and as a result is not recruited to the PAS. The authors have to explain this discrepancy."

We find GFP-Atg8 puncta only occasionally in atg7 cells after 6h starvation. Indeed, quantification revealed that the vast majority of cells are deviod of Atg8 puncta, consistent with previously published work. We revised the Figure 1B with images of atg7 cells that are representative. We think that a detailed systematic analysis of the recruitment of Atg proteins to the PAS is beyond the scope of this manuscript. Thus, we phrased our conclusion concerning PAS formation in rhoo cells more carefully, but we think that it is likely that Atg8 puncta in wild type cells treated with antimycin represent PAS because short-term antimycin treatment affects Atg1 and Atg13 recruitment but does not change GFP-Atg8 puncta.

"4) Figure 1. It will be interesting to show that mutant affecting other mitochondrial function (lipid synthesis, maintenance of the mitochondrial DNA...) have not an impairment in autophagy when this pathway is induced by amino acid starvation."

This is an interesting suggestion and would support the specificity of the response. However, mutants that affect lipid synthesis, for example, display compromised respiration due to effects on respiratory chain complex assembly and thus would complicate any interpretation.

"5) Figure 2A. It would be nice if the authors analyze the effects of Antimycin A and Oligomycin on autophagy induced by nitrogen deprivation. The absence of effects will further support their conclusion of a different way of regulating this pathway."

These data are included in Supplemental Figure 2A and fully support our conclusions.

"6) It is important to indicate somewhere for how long the cells were treated with Antimycin A and Oligomycin.Where those drugs added at the beginning of the starvation period?"

Antimycin A and oligomycin were added at the initiation of starvation. The only exception is revised Figure 5B, where antimycin A was added 2.5 hrs after initiation of starvation (30 min before imaging). The figure legends accurately detail these conditions.

"7) Figure 4A. Why does the deletion of ATG11 block amino acid-induced autophagy to the same extend than the rho mutation? An explanation has to be provided."

We reproducibly observed impaired autophagic flux in the atg11 mutant under amino acid starvation, indicating a more general role for Atg11 under this condition. Further experimentation

beyond the scope of this manuscript will be required to characterize this role.

Response to reviewer 3:

"1) Fig. 4C: "...Atg1 was present at similar steady levels in cells as monitored by Western (blot) analysis..." The authors seem to have performed this experiment to control for Atg1 levels in the preceding experiment (Fig. 4B), in which GFP-Atg8 and Atg1-mCherry co-localization in the perivacuolar puncta (phagophore assembly site, PAS) is assessed as a measure for the PKA activity and autophagy induction. However, this is an independent experiment using a different fusion protein (Atg1-GFP vs. Atg1-mCherry). It appears that extraction of the Atg1 protein from the same cells that had been imaged would have been a more relevant control. Also, despite the authors' claim regarding the similar levels of Atg1, there are some differences observed in Atg1 levels in the samples shown in the Fig. 4C, which should have also been quantified as previously done in the Western blot-based analyses."

We quantitatively analyzed protein levels of Atg1- and Atg13-mCherry over the time course of the imaging experiment (revised Figure 5B and 5C). There are no differences in steady state levels of these proteins in wild type and rho0 cells.

"2) Fig. S4: Data shown in this figure suggest that selective autophagy of mitochondria (mitophagy) is also inhibited under the conditions of the respiratory block. It is however unclear whether PKA also mediates inhibition of this type of autophagy during the respiratory block. It is thus requested that mitochondrial degradation is assessed in yeast strains with overactive and inhibited PKA, respectively, like the data shown in Fig. 3B."

Unfortunately, expression of the constitutively active Ras2G19V mutant over prolonged (24h) time required for the requested analysis causes cells to die. As shown in revised Supplemental Figure 7 and previously by other laboratories, the general machinery (Atg8, Atg7, Atg1-13) is required for starvation-induced mitophagy. Taken together, this strongly suggests that increased PKA activity induced by mitochondrial respiratory deficiency suppresses the general autophagic machinery and thus mitophagy is inhibited.

"3) An important type of selective autophagy in yeast is the Cvt pathway. The study would greatly gain in impact if it could show that the delivery and degradation of the substrate of this selective autophagy pathway (i.e. prApe1) also depends on the intact mitochondrial respiration and the PKA signaling."

As shown by Budosvakya et al. 2004, increased PKA activity by expression of the constitutively active Ras2G19V mutant impairs the expression and processing of prApe1. Therefore, we think the addition of these data would not significantly add to the story.

"4) On page 12 (second‐to‐last sentence) it is said that in various conditions Atg1 was present at similar steady state levels in cells as monitored by western analysis however the figure does not clearly show this. Could authors provide a more convincing image and the confirmation by qRT‐PCR? Is induction of Atg1 and Atg13 ablated in rho0 cells under conditions of starvation? Again, Fig. 4C is not convincing whereas Atg13 and quantification of the effects are missing."

We analyzed PAS localization of Atg1- and Atg13-mCherry dependent on mitochondrial respiratory competence and provide careful quantifications of protein levels during the time course of our experiment (revised Figures 5B and C).

"5) It would be interesting to assay if the ratio of phosphorilated/dephosphorilated Atg13 is affected in cells with mitochondrial respiratory deficiency and if this it directly regulated by PKA?

The phosphorylation pattern of Atg13 that can be resolved by western blot analysis is dominated by the activity of TORC1. Our preliminary data indicate that the phosphorylation pattern of Atg13 is largely unchanged in rhoo cells as compared to wild type cells during amino acid starvation. As described above, a complete analysis of Atg1-Atg13 interaction/phosphorylation and PAS

organization under amino acid vs nitrogen starvation is required and beyond the scope of the manuscript.

6) Quantification of the results on Fig. 4B is missing. On the page 5 (second‐to‐last and last sentences) and throughout the text, whenever authors claim that the difference is significant p‐values must be provided, and the statistics method should be included into materials and methods section."

The quantification of Figure 4B was included in the text. In order to make these data more accessible to the reader we have moved it to the revised Figure 5B.

We have provided standard deviations throughout the manuscript in order to demonstrate statistical significance as described in the figure legends. While performed paired t tests on our data revealed significant p values, we think that they are somewhat redundant and do not strengthen our conclusions regarding significance.

"7) It would be helpful for the reader if the order of figure appearance in the text corresponded to their numbering, i.e. Fig. S1A, Fig. S1B,Fig. 2A, Fig. 2B, ...)"

We reorganized the supplemental figures to make them easier to follow. However, for Supplemental Figure 2 we preferred to present the data in a conceptual context rather than in a chronological order.

2nd Editorial Decision 21 December 2010

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. It has now been assessed once more by two of the original referees (apologies for the delay associated with this at this time of the year). Although both referees consider the manuscript considerably improved, they retain a number of substantive concerns, as you will see from the reports below. In particular the remaining issues detailed by referee 1, which pertain to measurements of membrane potential and ATP levels and which had already been raised in the original round of review, would in our opinion definitely still need to be addressed before publication may appear warranted. Adding these seemingly feasible experiments would probably also go a long way towards alleviating referee 2's remaining major concern regarding mechanistic understanding, so should you be able to comprehensively address the well-taken points of referee 1 I would be prepared to be less insistent on referee 2's remaining qualms (his/her second point is something they had not raised initially, only the third point - providing some non-shown data you already have - would be clearly important).

As mentioned in my original decision letter, we normally allow for only one round of major revision; however given that in this case both reviewers would in principle be supportive of publication, and that both recommend giving you an additional opportunity to deal with the addressable (yet substantial!) outstanding issues, I would therefore like to return the study to you once more for an exceptional extra round of revision here. Nevertheless this will have to be the final round of revision in this case, and please understand that if the next version does not address these issues in a satisfactory manner, we will see ourselves forced to reject the manuscript. Should you however be able to improve the study as requested by referee 1, then we should be happy to proceed further with publication of the manuscript.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In the revised version, Authors satisfactorily addressed most of the comments raised during the first reversion of the manuscript.

However, a big concern still remains and calls for additional experiments that were requested in the first revision, but unfortunately were not performed. The key question was to dissect the consequences of the functional manipulations performed here on ATP levels and mitochondrial membrane potential. The ultimate goal of these experiments was to differentiate the relative impact of the two on the induction of autophagy, a central question in the model proposed here.

In detail, authors should measure membrane potential upon AA poisoning as well as ATP levels in the different conditions used. Indeed, the conundrum of whether ATP levels (rather than mitochondrial membrane potential) are key to trigger ATG8 remains also in this revised manuscript. On page 9 of the revised, manuscript Authors state that "Together, these data indicate that in the presence of a non-fermentable carbon source autophagy induction depends on the cellular ability to generate ATP by respiration under amino acid starvation.". This would mean that if cells can not use glycolysis, they need mitochondria (that are the only source of ATP) to induce autophagy. "In contrast, during amino acid starvation in the presence of a fermentable carbon source, ATG8 induction and, to a lesser extent, autophagic flux require functional CIII and CIV respiratory chain complexes." If under these circumstances mitochondria reverted the ATPase to sustain their membrane potential, induction of autophagy could be explained by a decrease in total ATP levels. In the presence of a fermentable carbon source, however, the inhibition of F1F0 ATP-synthase has no consequence on ATG8 induction or on autophagic flux. Again, this lack of autophagy could be explained by the fact that, under this circumstance, ATP is sustained by glycolysis. In addition, authors did not measure mitochondrial and total ATP levels as previously requested (point 4). The combination of these requests was instrumental to clarify the relative role of membrane potential vs. ATP levels in the decisional process of autophagy induction. The data presented in revised Fig 2 are not what was asked for and are not conclusive (alternative mechanisms could play a role, see above). Thus, the same membrane potential and ATP measurement requested during the first round of revision are missing and are key to fully understand the model presented here. I believe that given the importance of this paper, the Authors should be given another round of revision to perform these rather easy experiments and to add them to the core of the paper.

Referee #2 (Remarks to the Author):

I think that this work is of high quality and the additional experiments and controls have made it better. I still, however, think that even if beyond the scope of this manuscript, a more direct connection between mitochondria and autophagy regulation is crucial. I agree that it will take time to unveil the mechanism underlying an eventual regulation of autophagy by mitochondria, but the authors could have at least shown that impairment of some mitochondrial functions is not affecting autophagy upon amino acid starvation. This would have supported the notion that specific functions such as the respiratory chain are linked to autophagy (= direct involvement of mitochondria in autophagy regulation) and excluded that a general malfunction of mitochondria affects of autophagy (= indirect effects due to the "sickness" of the cells).

Reading the text once more, I noticed that autophagy has been assessed by exclusively using one method, e.g. the GFP-Atg8 processing assay. To validate the use of this approach as a bona fide correct way to measure autophagy progression under the conditions analyzed by the authors, the use of an alternative method such as the Pho8delta60 assay or the counting of the autophagic bodies at least in one of the experiments (for example the one shown in figure 1a) is important.

Finally, in response to my second criticism, the authors have analyzed the phosphorylation status of Npr1 to determinate the activity of TORC1. While this analysis shows that TORC1 is active, the assessment of the Atg13 phosphorylation status would be a more direct way to determine the action of TORC1 on the autophagy machinery. The authors have indicated in their response to the criticism

5 of referee 3 that this analysis has been done and indeed the phosphorylation patter of Atg13 is not altered. I think that this result, which further supports the author's conclusions, has to be shown.

2nd Revision - authors' response 28 February 2011

Thank you for the opportunity to address the reviewers' comments on our revised manuscript entitled 'Mitochondria regulate autophagy by conserved signaling pathways' . As you will read in the detailed response that follows, we have performed the requested experiments measuring ATP and membrane potential under the relevant conditions and have addressed the additional points raised by the reviewers.

We look forward to hearing from you.

Response to Reviewer 1:

We thank the reviewer for supporting and encouraging comments.

We view the primary request of reviewer 1 as follows:

"In detail, authors should measure membrane potential upon AA poisoning as well as ATP levels in the different conditions used. Indeed, the conundrum of whether ATP levels (rather than mitochondrial membrane potential) are key to trigger ATG8 remains also in this revised manuscript."

In response to this request, we extended our analysis and measured ATP levels in total cells and mitochondria as well as mitochondrial membrane potential in vivo during amino acid starvation under all relevant conditions. The exception is that we were unable to measure mitochondrial membrane potential in rhoo cells. The lack of signal in rhoo cells is likely a consequence of failure to accumulate the dye as indicated by the lack of the potential insensitive staining of ER at high dye concentrations. As all other conditions worked, this technical problem did not hamper our analysis. We incorporated these new data into the manuscript in a new Figure 3. As described in the manuscript, we found reduced ATP levels in rho0 cells under growing conditions as compared to wild type. In addition, ATP levels in rho0 cells were essentially unchanged by starvation conditions. In contrast, we observed that ATP levels decreased significantly and to a similar degree in wild type cells and wild type cells treated with inhibitors of mitochondrial function. This reduced level of cellular and mitochondrial ATP observed under starvation conditions was similar to the level observed in rho0 cells, suggesting that there might be a cellular mechanism for maintaining ATP within a narrow range. Our previous data indicated that some form of ATP production, either mitochondrial respiration under non-fermentative conditions or glycolysis under fermentative conditions, is required for autophagy induction. In light of recent publications indicating a direct positive role for AMP kinase in mammalian autophagy regulation, our data point to the fact that autophagy induction is likely regulated by complex signals dependent on growth conditions. Importantly, our data also indicate that ATP levels are unlikely to play a pivitol role in the regulation of autophagy during amino acid starvation by mitochondria as described in our study. In contrast, our measurements of mitochondrial membrane potentials in vivo during the starvation period revealed clear differences dependent on mitochondrial respiration under non-fermentative and fermentative conditions. These data strongly correlate with autophagy induction responses revealed in our previous experiments under fermentative conditions (Fig. 2B). Hence, our data support a model in which mitochondrial membrane potential signals the functional state of mitochondria and regulates the cell's decision to induce autophagy during amino acid starvation.

Response to reviewer 2:
We thank the reviewer for the supporting remarks. Three points were raised that we will address below in the order in which they were raised in the comments.

1. We think that our data provides compelling evidence that respiratory activity, specifically mitochondrial membrane potential regulates autophagy induction during amino acid starvation. We agree with the reviewer that it would be interesting to determine whether other mitochondrial functions can also affect the regulation of autophagy, but we are not confident that such experiments would address the reviewer's concern that the regulation we observe is due to indirect effects as opposed to direct mitochondrial involvement. First, to our knowledge mutants that affect other central mitochondrial functions, such as mtDNA maintenance, lipid metabolism, protein import and quality control, also impair mitochondrial respiration and, therefore, we predict would also affect autophagy induction. Second, considering the central role of mitochondria in cellular metabolism, we would find the observation that additional mitochondrial functions affect autophagy induction interesting. Specifically, such a result might indicate a new regulatory link between a metabolic pathway and cell growth, division and autophagy regulation.

2. Our GFP-Atg8 based autophagy assay is corroborated by our cytological and biochemical analyses. Thus, we think it is accurate. At this stage of the study, we are focusing our efforts on determining the molecular basis for the role of mitochondria in the regulation of autophagy.

3. We have included the requested analysis of Atg13 phosphorylation during amino acid starvation in new Figure 4C.

Acceptance letter 15 March 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Before we will able to send you a formal letter of acceptance, there are just two minor things I need to ask you for: a 'conflict of interest' statement, and an 'author contribution' statement, both to be included at the end of the manuscript text. To expedite this, you may simply send them to us in the body of an email, from which we can easily copy them into the manuscript text file; alternatively you may send as a new text document including these two statements.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

Yours sincerely,

Editor
The EMBO Journal

Referee #1

(Remarks to the Author)
The authors exhaustively answered to the remaining concerns with the results presented in Fig. 3. According to the authors, we can conclude that mitochondrial membrane potential signals the functional state of mitochondria, modulating induction of autophagy (page 10 of the manuscript).

In conclusion, the manuscript is an important piece of work that will for sure stimulate further research on the emerging topic of the interconnection between mitochondria and autophagy.

