



# Amino acid starvation inhibits autophagy in lipid droplet-deficient cells through mitochondrial dysfunction

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1 **Amino acid starvation inhibits autophagy in lipid droplet-deficient cells through**  
2 **mitochondrial dysfunction.**

3

4 Pierre Voisin, Marianne Bernard, Thierry Bergès and Matthieu Régnacq \*

5

6 From Laboratoire Signalisation & Transports Ioniques Membranaires (STIM) EA7349

7 Université de Poitiers, Bâtiment B31, 3 rue Jacques Fort, TSA51106, 86073 Poitiers Cedex

8 9, France

9

10 \* To whom correspondence should be addressed; [matthieu.regnacq@univ-poitiers.fr](mailto:matthieu.regnacq@univ-poitiers.fr)

11 Tel. (33)5 49 45 38 64

12

13 **Running title**

14 Autophagy requires functional lipid droplets and mitochondria

15

16

17 **Abbreviations**

18 *ATG*, autophagy-related gene; eIF2, eukaryotic initiation factor; ER, endoplasmic reticulum;

19 GAAC, general amino acid control; GCN, general control nonderepressible; GSH, glutathione

20 reduced form; GSSG, glutathione oxidised form; PtdCho, phosphatidylcholine; PtdIns,

21 phosphatidylinositol; ROS, reactive oxygen species; STE, sterylester; TAG, triacylglycerol;

22 TORC, target of Rapamycin complex.

23

24 **Keywords**

25 Autophagy; lipid droplets; *Saccharomyces cerevisiae*; mitochondria; catabolite repression.

## 26 **Abstract**

27 Lipid droplets are ubiquitous organelles in eukaryotes that act as storage sites for neutral  
28 lipids. Under normal growth conditions they are not required in the yeast *Saccharomyces*  
29 *cerevisiae*. However, recent works have shown that lipid droplets are required for autophagy  
30 to proceed in response to nitrogen starvation and that they play an essential role in  
31 maintaining ER homeostasis. Autophagy is a major catabolic pathway that helps degradation  
32 and recycling of potentially harmful proteins and organelles. It can be pharmacologically  
33 induced by rapamycin even in the absence of lipid droplets. Here, we show that amino acid  
34 starvation is responsible for autophagy failure in lipid droplet-deficient yeast. It not only fails  
35 to induce autophagy but also inhibits rapamycin-induced autophagy. The general amino acid  
36 control pathway is not involved in this paradoxical effect of amino acid shortage. We  
37 correlate the autophagy failure with mitochondria aggregation and we show that amino acid  
38 starvation-induced autophagy is restored in lipid droplet-deficient yeast by increasing  
39 mitochondrial biomass physiologically (respiration) or genetically (*REG1* deletion). Our  
40 results establish a new functional link between lipid droplets, ER and mitochondria during  
41 nitrogen starvation-induced autophagy.

## 42    **Introduction**

43    Macroautophagy (hereafter referred to as autophagy) is a major cellular response to maintain  
44    homeostasis [1]. It is induced in response to nutritional stress and results in bulk turnover of  
45    cytoplasmic content which ultimately allows the cell to adapt to a changing environment [2].  
46    Research in the yeast *Saccharomyces cerevisiae* has significantly contributed to identifying  
47    genes responsible for autophagy, the so-called *ATG* genes (autophagy-related). Autophagy  
48    begins with the formation of a cup-shaped membrane structure called the isolation membrane,  
49    or phagophore, which expands and encapsulates the cytoplasmic macromolecules or  
50    organelles that are to be degraded [3]. The phagophore eventually closes to form a double  
51    membrane structure called the autophagosome [3]. Its content is then delivered to the  
52    lysosome - or vacuole in yeast - for degradation. Several cellular structures have been  
53    proposed as contributing to autophagosome biogenesis by providing lipids required for  
54    membrane expansion. These include the ER (endoplasmic reticulum), the Golgi apparatus, ER  
55    exit sites, endosomes, mitochondria and plasma membrane [4]. Recently, lipid droplets have  
56    also been shown to participate in autophagosome biogenesis in HeLa cells [5]. Although a  
57    direct contribution of lipid droplets to autophagosome membrane expansion in yeast is still  
58    controversial, several studies have pointed to a functional link between lipid droplets and  
59    autophagy [6-9].

60        Lipid droplets are cellular organelles filled with neutral lipids (*i.e.* TAG  
61    (triacylglycerols) and STE (steryl esters)) and delimited by a monolayer of phospholipids with  
62    associated proteins [10]. A yeast mutant has been generated that is unable to synthesize TAG  
63    and STE, resulting in the complete lack of lipid droplets [11] (Supplemental Figure 1). This  
64    mutant is viable, although it is highly sensitive to excess unsaturated fatty acids which cause  
65    mitochondrial dysfunction and necrotic cell death [12-14]. In addition, this mutant is unable to  
66    achieve autophagy in response to nitrogen starvation and the autophagy blockade in starved

cells is accompanied by the formation of membrane tangles initially observed in electron microscopy [7, 8]. The autophagy failure in lipid droplet-deficient cells was originally interpreted as a possible requirement for fatty acids generated by TAG or STE hydrolysis in autophagosome membrane biogenesis [6, 8]. This view was, however, challenged by two observations. Firstly, rapamycin, a potent inhibitor of TORC1 (Target of Rapamycin complex 1), was very efficient at inducing autophagy in the lipid droplet-deficient mutant, a result indicating that autophagy is not strictly dependent on lipid droplets [7, 9]. Secondly, nitrogen starvation - but not rapamycin - caused an increase in fatty acid biosynthesis and an alteration of phospholipid composition that proved detrimental to autophagy [7, 9]. In fact, the partial inhibition of fatty acid synthesis by cerulenin, or experimental elevation of the PtdCho (phosphatidylcholine)/PtdIns (phosphatidylinositol) ratio, restored autophagy in response to nitrogen starvation and also eliminated the membrane tangles [7, 9]. Thus, the inability of the lipid droplet-deficient mutant to buffer free fatty acid into lipid droplets or its inadequate phospholipid composition appeared more likely to be responsible for the autophagy defect. Restoration of autophagy by cerulenin or by PtdCho/PtdIns clamp was, however, only partial when compared to the intense autophagy triggered by rapamycin, suggesting that the mechanism of impairment had not been fully addressed. To gain access to this mechanism, a better definition of the origin of the problem appeared to be required. One question that arose was whether the absence of autophagy upon nitrogen starvation was a simple failure to activate the process or if it reflected an inhibitory mechanism capable of blocking the autophagic response to other kinds of stress (*i.e.* rapamycin). Another aspect of the situation that had not been examined is the fact that nitrogen starvation is a composite stress in a medium lacking both ammonium ion and amino acids. Previous studies in lipid droplet-proficient yeast have shown that ammonium ion deficiency and amino acid deficiency induce autophagy through different mechanisms and have different requirements for the General

92 Amino Acid Control (GAAC) pathway [15]. To obtain a better definition of the situation  
93 encountered in lipid droplet-deficient yeast, we sought to examine separately the effects of  
94 ammonium ion or amino acid deficiencies on autophagy.

95 Here, we show that ammonium ion deficiency induces autophagy in lipid droplet-  
96 deficient yeast (RS4Δ strain). In contrast, amino acid deficiency not only fails to activate  
97 autophagy but also inhibits the responses to ammonium deficiency and to rapamycin. Amino  
98 acid starvation also alters mitochondrial morphology. We report that this phenotype is  
99 dependent upon the carbon source present in the medium since it is observed in glucose-  
100 grown cells, but not in respiratory medium. Finally, we show that this phenotype can also be  
101 suppressed by deletion of the *REG1* gene which is necessary for carbon catabolite repression  
102 in glucose-grown cells.

103

## 104 **Materials and methods**

### 105 *Strains, oligonucleotides, plasmids and growth conditions*

106 All yeast strains described in this study are derived from BY (Euroscarf). BY4742 was used  
107 as the wild type strain (Y10000). RS4Δ (BY4742 derivative; *MATa are1 are2 lro1 dgal ura3*  
108 *trp1 leu2 lys2 his3 met15*) was kindly provided by Dr Schneiter. Deletions of *REG1* and  
109 *GCN2* were performed by PCR-based targeted homologous recombination, replacing the  
110 entire ORF with the *URA3* cassette [16]. PCR-mediated GFP tagging of *SEC63* was  
111 performed as described with pYM28 [17]. Plasmid pSu9-GFP expresses GFP fused to the  
112 presequence of subunit 9 of the F<sub>0</sub>-ATPases of *Neurospora crassa* from the *ADHI* promoter.  
113 This plasmid is based on pRS315 and was kindly provided by Dr Sesaki [18]. For GFP-Atg8  
114 processing, the plasmid pRS416 expressing GFP-Atg8 that was used [19] was kindly provided

115 by Dr Camougrand. Plasmid pPHY2427, expressing 3xHA-Atg13 under the control of the  
116 *CUP1* promoter in pRS426, was kindly provided by Pr Hermann [20].

117 Yeast strains were cultured in minimal YNB medium (yeast nitrogen base with  
118 ammonium sulfate and 2% glucose) supplemented with the appropriate Dropout mix  
119 (Formedium), or complete YPD medium (1% yeast extract, 1% peptone, 2% dextrose).  
120 Growth in respiratory medium was performed in YPLactate medium (1% yeast extract, 1%  
121 peptone, 2% (w/v) lactic acid pH5.3). For starvation experiments, unless otherwise stated,  
122 cultures in selective medium were inoculated in YPD medium and maintained in exponential  
123 growth phase for at least five generations; cells were harvested by centrifugation, washed with  
124 distilled water and autophagy was induced by inoculation in YNB medium without  
125 ammonium sulfate and amino acid (-NH<sub>3</sub>-aa) for nitrogen starvation, or YNB medium without  
126 amino acid (+NH<sub>3</sub>-aa) for amino acid starvation. For ammonium starvation experiments, (-  
127 NH<sub>3</sub> + aa), yeast cells were inoculated in YNB without ammonium sulfate but supplemented  
128 with histidine (10mg/L), tryptophane (10mg/L), leucine (50mg/L), methionine (10mg/L) and  
129 lysine (15mg/L) (-NH<sub>3</sub>+aa). Autophagy induction with rapamycin was achieved with  
130 rapamycin 200nM.

131 To obtain rho<sup>0</sup> mutants, a culture of RS4Δ grown to saturation on YPD medium  
132 containing ethidium bromide (10μg/mL) was diluted and re-inoculated at low density in the  
133 same medium. The resulting respiratory deficiency was confirmed by a complete lack of  
134 growth on YPLactate medium which is an obligatory respiratory medium.

135

136

137

138 *GFP-Atg8 cleavage assay and other western blot analyses*

139 The GFP-Atg8 cleavage assay takes advantage of the fact that GFP-Atg8, covalently attached  
140 to the autophagosome membrane, follows the autophagy flux until cleavage in the vacuole,  
141 where GFP resists further proteolysis [21]. Approximately  $10^8$  cells were harvested, and lysed  
142 by alkaline whole cell extraction (0.2M NaOH; 0.3%  $\beta$ -mercaptoethanol) followed by  
143 trichloroacetic acid precipitation [7]. Protein extracts were subsequently washed with acetone,  
144 dried and suspended in 100 $\mu$ L of 5% (w/v) SDS. Finally, 100 $\mu$ L of Laemmli buffer (20%  
145 glycerol, 2% SDS, 2%  $\beta$ -mercaptoethanol, 0.04% Bromophenol Blue, 0.0625M Tris-HCl  
146 pH6.8) were added. Samples were incubated for 10min at 65°C prior to loading for  
147 electrophoresis on a 12% polyacrylamide gel. Routinely, lysate aliquots corresponding to  
148  $4 \times 10^6$  cells were analyzed. After western blotting onto nitrocellulose and blocking the  
149 membrane with 5% non-fat milk, 0.1% Tween 20 in Tris-buffered saline, the blotted proteins  
150 were probed with mouse anti-GFP antibody (1/2000, Roche Applied Science, Meylan),  
151 followed by HRP conjugated anti-mouse IgG secondary antibody (1/10000, Jackson-  
152 Interchim). Detection was performed with ECL prime from GE Healthcare. Other western  
153 blot analyses were performed on similar extracts, using the appropriate acrylamide gel  
154 concentrations (8% or 12%) and the appropriate primary and secondary antibodies. These  
155 included mouse monoclonal anti-Pgk1 antibodies (1/10000, Invitrogen), mouse monoclonal  
156 anti-HA antibodies (1/2000, Santa Cruz), rabbit anti-phospho eIF2 $\alpha$  (1/1500, Thermofisher  
157 Scientific), and HRP conjugated anti-rabbit or -mouse IgG secondary antibody (1/10000,  
158 Jackson-Interchim).

159

160 *RNA extraction, reverse transcription (RT) and real-Time PCR*

161 Nucleic acids were extracted from  $2 \times 10^8$  cells as already described [22], then RNA was  
162 selectively precipitated in 3M LiCl final (25min on ice, then 16,000xg for 30min at 4°C),



163 washed with 70% cold ethanol, air-dried and resuspended in nuclease-free water. RNA was  
164 quantified (OD 260 nm) and adjusted to 0.25µg/µL. For cDNA synthesis, 1µg of total RNA  
165 was incubated for 2h at 37°C with Moloney Murine Leukemia Virus Reverse Transcriptase  
166 (M-MLV RT; Promega) and 0.1ng oligo-dT<sub>25</sub>. The RT reaction was terminated on ice by 1/5  
167 dilution in double-distilled water. For each sample, a control reaction was run without reverse  
168 transcriptase to estimate the amount of contaminating DNA. Real-time qPCR made use of a  
169 LightCycler® apparatus and SYBR Green mix (Roche Applied Science, Meylan, France),  
170 with the primers listed in Table 1. Each primer pair gave a single PCR product by melting  
171 curve analysis and agarose gel electrophoresis. The PCR steps were: enzyme activation at  
172 95°C for 10min followed by 40 cycles (for *ACT1*) or 45 cycles (for *PCL5*, *HIS4* and *ATG1*)  
173 comprising 10s at 95°C, 10s at 60°C (*ACT1*) or 65°C (*HIS4*) or 66°C (*PCL5* and *ATG1*), and  
174 10s (*ACT1*) or 20s (*PCL5*, *HIS4* and *ATG1*) at 72°C. PCR reactions on non reverse-  
175 transcribed RNA samples indicated that genomic DNA contaminants were always less than  
176 1/100. *ACT1* was used as the reference gene and the relative changes in target genes mRNA  
177 levels were calculated from ΔCt values.

#### 178 *Fluorescence microscopy*

179 Microscopy was performed on a FV1000 Olympus confocal microscope using a 100X oil  
180 immersion lens (NA 1.40) coupled to a 2.0X numerical zoom (0.08µm per pixel). The  
181 excitation wavelengths for GFP or FM4-64 were set to 488nm and 543nm respectively. Image  
182 acquisition and conversion were performed separately for green (520nm) and red (603nm)  
183 channels and processed with the Olympus Fluoview version 4.1 software. Transmission  
184 images were acquired with differential interference contrast optics.

185 Vacuoles were visualised by overnight staining with FM4-64 (1µM) in complete medium  
186 prior to the shift in starvation medium.

187

## 188 **Results and discussion**

### 189 *Amino acid starvation inhibits autophagy in lipid droplet-deficient cells*

190 Previous reports have shown that rapamycin efficiently induced autophagy in RS4Δ cells,  
191 whereas nitrogen starvation failed to do so [7, 9]. Here, both stimuli were combined in our  
192 experiments and autophagy completion was assessed by the GFP-Atg8 cleavage assay [21].  
193 We observed that rapamycin-induced autophagy was completely inhibited by nitrogen  
194 starvation (Fig. 1A compare “Rap” and “-N+Rap”). Because nitrogen starvation is a  
195 combination of deficiencies in amino acids and in ammonium ion, both conditions were  
196 analyzed separately. In this experimental paradigm, autophagy could be observed when  
197 ammonium deficiency was imposed in the presence of amino acids (Fig. 1B), whereas amino  
198 acid starvation in the presence of ammonium ion failed to activate autophagy (Fig. 1B). As  
199 above, the combination of both deficiencies did not induce autophagy (Fig. 1B). Amino acid  
200 starvation also proved sufficient to block rapamycin-induced autophagy (Fig. 1C). The  
201 addition of increasing doses of the auxotrophic amino acids progressively restored rapamycin-  
202 induced autophagy, with a threshold observed around 1% of the standard amino acid supply  
203 (Fig. 1C). Similarly, ammonium starvation-induced autophagy was restored by amino acids in  
204 a dose-dependent manner, with a threshold around 10% of the standard amino acid supply  
205 (Fig. 1D). Together our data indicate that, in lipid droplet-deficient yeast, amino acid  
206 deficiency not only failed to induce autophagy but also blocked the effects of two signals that  
207 induce autophagy through direct inhibition of TORC1. In fact, rapamycin inhibits TORC1  
208 after forming a complex with FKBP12 (FK506 binding protein) [23] and ammonium ion  
209 deficiency causes a drop in glutamine concentration, an amino acid acutely required for the  
210 activity of the TORC1 complex [24]. In contrast, a deficiency in auxotrophic amino acids

211 inhibits TORC1 more indirectly, through the activation of TORC2, which activates Gcn2,  
212 which in turn inhibits TORC1 [25, 26].

213

#### 214 *Starvation sensing is functional in lipid droplet-deficient cells*

215 TORC1 represses autophagy through phosphorylation of the Atg13 protein [20]. When  
216 TORC1 is inhibited, Atg13 adopts a partially dephosphorylated form that initiates autophagy  
217 by recruiting Atg1 [27, 28]. We therefore examined whether amino acid starvation altered the  
218 efficiency of Atg13 dephosphorylation in lipid droplet-deficient cells. This hypothesis was not  
219 supported by our western blot analysis because ammonium starvation, amino acid starvation  
220 and rapamycin had similar effects on Atg13 dephosphorylation (Fig. 2A). This result  
221 suggested that the autophagy blockade caused by amino acid starvation did not take place at  
222 the initiation step. Because TORC1 inhibition by amino acid deficiency requires the activation  
223 of Gcn2 [15], the result also suggested that this indirect pathway was functional in RS4Δ  
224 cells. Gcn2 is a component of the GAAC pathway that plays a central role in the adaptation  
225 of yeast and animal cells to a reduction in amino acid supply [29]. In this pathway, Gcn2  
226 senses amino acid depletion and phosphorylates the translation regulator eIF2α  
227 (eukaryotic initiation factor), resulting in an accumulation of the transcription factor Gcn4 that  
228 in turn activates the expression of several genes required for amino acid synthesis [30]. In  
229 addition, Gcn4 increases *ATG1* expression [31] and is specifically required for autophagy in  
230 response to amino acid starvation [15]. Therefore, we examined whether the functionality of  
231 the GAAC pathway was altered in lipid droplet-deficient cells. Our experiments indicated that  
232 eIF2α phosphorylation was stimulated in all conditions of ammonium and/or amino acid  
233 starvation (Fig. 2B), thus confirming the functionality of Gcn2 in the GAAC pathway.  
234 Furthermore, transcriptional activation of three Gcn4-responsive genes, including *ATG1* [31],  
235 could be observed after ammonium and/or amino acid starvation, indicating that the GAAC

236 pathway was fully operational in lipid droplet-deficient cells (Fig. 2C). Together, our  
237 experiments indicated that Gcn2 fulfilled all its autophagy-related tasks in lipid droplet-  
238 deficient yeast (*i.e.* Atg13 dephosphorylation and induction of *ATG1*). Nevertheless,  
239 considering the prominent role of Gcn2 in amino acid depletion sensing and the diversity of  
240 effectors it may control [32], we felt compelled to examine whether the absence of lipid  
241 droplet might confer upon it the ability to inhibit autophagy downstream of Atg13 and Atg1.  
242 Such a paradoxical situation could be ruled out because *GCN2* deletion did not relieve the  
243 inhibitory effect of amino acid starvation on rapamycin-induced autophagy (Fig. 2D). This  
244 result further indicates that the mechanism of autophagy inhibition in amino acid-starved  
245 RS4Δ cells does not rely on Gcn2 to sense amino acid depletion. Having ruled out this central  
246 actor of cell adaptation to amino acid starvation, we explored other routes that could lead to  
247 autophagy inhibition.

248

#### 249 *Autophagy inhibition in lipid droplet-deficient cells is sensitive to the redox state of the cells*

250 The mitochondrial respiratory chain plays a role when autophagy is induced by amino acid  
251 starvation [33]. We sought to examine mitochondrial shape following autophagy induction in  
252 RS4Δ cells. Using plasmid pSu9-GFP as a reporter of mitochondrial shape, we observed that  
253 mitochondrial morphology in RS4Δ cells was differentially affected in response to amino acid  
254 deficiency and/or ammonium limitation. As shown in Fig.3A, 90% of the cells in the RS4Δ  
255 strain contained compact mitochondrial aggregates, clustered in one or two spots, when the  
256 cells were challenged by amino acid starvation for 2 hours. This shape is strikingly similar to  
257 the *mdm* (mitochondrial distribution and morphology) mutants, some of which are respiratory  
258 deficient [34]. The mitochondrial aggregates are clearly excluded from the vacuole ruling out  
259 the possibility that they result from mitophagy (Supplemental Figure 2). In response to  
260 ammonium starvation, RS4Δ displayed a less severe phenotype since aggregated

mitochondria were observed in only 43% of the cells. None of these starved conditions altered the typical tubular morphology of mitochondria in wild type cells (Fig. 3A). To substantiate the correlation between mitochondria integrity and autophagic ability in RS4Δ, we generated a RS4Δ rho<sup>0</sup> mutant and analyzed autophagy in response to nitrogen starvation. Rho<sup>0</sup> mutants lack all the components encoded by mitochondrial DNA and are thus unable to produce mitochondrial ATP. Interestingly, mitochondrial DNA ablation exacerbated the phenotype of RS4Δ since RS4Δ rho<sup>0</sup> proved unable to accomplish autophagy even in response to ammonium starvation (Fig. 3B). Importantly, autophagy was still observed in the rho<sup>0</sup> lipid droplet-proficient strain in response to ammonium starvation as reported before [33]. We concluded from these experiments that lipid droplet-deficient cells are more dependent on functional mitochondria than the wild type strain for autophagy in response to starvation.

Previous studies have shown that nitrogen - or amino acid - starvation leads to a transient increase in mitochondrial respiration, even in glucose medium [35]. Moreover, the production of ROS (reactive oxygen species) along the respiratory chain appears to be required for optimal activation of autophagy [36, 37]. Having established that amino acid deficiency altered mitochondrial function in RS4Δ, we questioned whether the autophagic phenotype of RS4Δ resulted from failure to generate ROS. As illustrated in figure 3C, addition of 0.3-0.5mM H<sub>2</sub>O<sub>2</sub> facilitated autophagy in response to nitrogen starvation and to a lesser extent, to amino acid starvation. H<sub>2</sub>O<sub>2</sub> alone did not significantly activate autophagy (Fig. 3C). H<sub>2</sub>O<sub>2</sub> concentrations above 0.5mM had a toxic effect characterized by a reduced autophagy and the presence of multiple protein degradation bands. Additional evidence for a strong influence of the redox state on autophagy in RS4Δ cells could be obtained by showing that autophagy in response to ammonium ion deficiency was obliterated by the addition of the ROS scavenger N-acetylcysteine (Fig. 3D). The same concentrations of N-acetylcysteine did not abolish autophagy in lipid droplet-proficient cells (Fig. 3D). Together, the data are in

286 keeping with our previous report indicating that RS4Δ displays a high GSH (glutathione  
287 reduced form)/GSSG (glutathione oxidised form) ratio [38]. This increased ROS buffering  
288 capacity in RS4Δ cells might dampen the burst of ROS production that is required for  
289 autophagy [36]. The fact that the addition of H<sub>2</sub>O<sub>2</sub> facilitated autophagy but did not fully  
290 restore it suggests that ROS production was not the only contribution of mitochondria to the  
291 autophagic process. This prompted us to turn to experimental protocols that allow full  
292 restoration of mitochondrial function.

293

294 *Autophagy in lipid droplet-deficient cells is highly dependent on mitochondrial status*

295 We then examined whether autophagy could be restored in RS4Δ cells by culture conditions  
296 that favour mitochondria proliferation and activity. For this purpose, cells were grown in  
297 lactic acid media. In this fully respiratory medium, lipid droplet-deficient cells showed  
298 essentially the same growth-rate as wild type cells, although the growth rate of both strains  
299 was reduced by 50% as compared to fermentative conditions. Taking into account this growth  
300 delay, autophagy was monitored over 8 hours. Western blot analysis indicated the RS4Δ  
301 mutant was able to execute autophagy in response to the different combinations of ammonium  
302 and/or amino acid starvation (Fig. 4A, left panel). Comparison with the wild type strain (Fig.  
303 4A, right panel) revealed that the autophagy at 8 hours was nearly as intense in the RS4Δ  
304 mutant. A delayed autophagy could be observed in the mutant at 4 hours in the groups lacking  
305 amino acids (Fig. 4A, compare left and right panels). To obtain additional evidence, GFP-  
306 Atg8-expressing RS4Δ cells were examined by fluorescence microscopy after 6h of  
307 ammonium and/or amino acid starvation in respiratory growth conditions. In 70-100% of the  
308 cells, the GFP signal was either restricted to the vacuole (vacuolar) or encompassing the  
309 vacuole and the cytosol (uniform) (Fig. 4B). We interpreted these results as indicative of a  
310 completed autophagy or an autophagic flux in progress, respectively (Fig. 4B). For

comparison, effective autophagy in response to rapamycin in glucose medium resulted in 90% of the cells with GFP entirely or partially vacuolar, whereas autophagy blockade in glucose medium lacking both ammonium and amino acids yielded only 10% of that score (Fig. 4B). Taken together, our results indicated that autophagy was restored to near wild type levels in lactate-grown RS4Δ cells. Consistent with these results, the tubular morphology of the mitochondria was not affected by amino acid shortage in respiratory medium (Supplemental Figure 3). Because previous studies have indicated that RS4Δ cells generate ER membrane tangles during autophagy failure [7-9], we examined the effect of respiration on this process. To observe accurately the ER membrane, the ER-resident protein Sec63 was epitope-tagged with GFP (Supplemental Figure 4 and 5). Using Sec63-GFP, fluorescence membrane tangles could be observed in nearly all the cells upon nitrogen starvation in glucose medium (Fig. 4C, left panel), whereas they were extremely rare in respiratory medium (Fig. 4C, right panel). Therefore, respiration was also effective in resolving this other dysfunction of nitrogen-starved RS4Δ cells. In fermentable medium, mitochondria production is limited by the catabolic repression pathway, which is highly dependent on the phosphatase regulatory subunit Reg1 [39]. Deletion of the *REG1* gene in RS4Δ cells efficiently abolished the inhibitory effect of amino acid starvation on autophagy induced by ammonium starvation in glucose-grown cells (Fig. 4D). Moreover, amino acid starvation *per se* proved capable of inducing autophagy in this mutant (Fig. 4D). To further substantiate this conclusion, we analyzed GFP-Atg8 localisation in the RS4Δ-*reg1Δ* by fluorescence microscopy. As illustrated in Figure 4B, upon ammonium or amino acid starvation, GFP fluorescence reached the vacuole in more than 80% of the cells. Thus, *REG1* deletion restored the autophagic turnover of GFP-Atg8 in glucose medium. Because *REG1* deletion causes both an increase in mitochondria biomass and ATP production by respiration even in fermentative medium, we sought to separate the structural and metabolic contributions of mitochondria to autophagy

336 restoration. As illustrated in Fig. 4E, RS4Δ-*reg1Δ* rho<sup>0</sup> cells remained capable of activating  
337 autophagy in all conditions of ammonium and/or amino acid starvation. A slight decrease in  
338 GFP-Atg8 cleavage (compare Fig. 4D and E) may represent the loss of ROS contribution to  
339 autophagy restoration, although it also lies within the range of western blot variability.

340

341 In this report, we clarify the origin of the autophagy failure observed in lipid droplet-  
342 deficient cells by showing that it is singularly due to a toxic effect of amino acid starvation.  
343 Despite our best efforts, the amino acid starvation sensor that triggers this toxic effect has not  
344 been identified, although we were able to exonerate the GAAC pathway. We have been more  
345 successful at identifying the role of aggregated mitochondria in the mechanism of autophagy  
346 inhibition. This new information complements previous reports indicating that a rise in fatty  
347 acid synthesis was responsible for the autophagy blockade in lipid droplet-deficient cells  
348 challenged by nitrogen starvation [7, 9]. It could be anticipated that excess fatty acid was not  
349 the only cause of autophagy inhibition, because pharmacological correction of fatty acid  
350 synthesis merely achieved partial restoration of autophagy [7, 9]. In contrast, we show here  
351 that autophagy can be restored to near wild type level by increasing mitochondria activity and  
352 biomass (*i.e.* respiration or ablation of the *REG1* gene). Interestingly, although we observed  
353 that ROS production along the respiratory chain facilitated autophagy, the RS4Δ-*reg1Δ* rho<sup>0</sup>  
354 cells remained capable of activating autophagy upon amino acid starvation. Therefore, it  
355 would appear that mitochondria biomass and structural integrity are more meaningful than  
356 respiration for the restoration of autophagy. A noticeable correlate of autophagy inhibition in  
357 lipid droplet-deficient cells is an accumulation of tangled ER membranes whose mechanism  
358 remains to be elucidated [7, 9]. We would like to speculate that these tangled ER membranes  
359 may arise as a consequence of the mitochondrial aggregation described herein. They could, in  
360 fact, result from an interruption of phosphatidylserine transfer from the ER to the outer



mitochondrial membrane where it normally gets converted to phosphatidylethanolamine in the phospholipid synthesis pathway [40, 41]. This could explain why pharmacological correction of fatty acid synthesis, as performed in previous studies, only achieved partial restoration of autophagy while it completely resolved the ER tangles [7]. In contrast, rescuing mitochondria through respiration efficiently restored both autophagy and ER integrity. Thus, progress made in characterizing the autophagy failure in lipid droplet-deficient cells directs future studies towards a possible role of lipid droplets in the membrane contacts connecting mitochondria and ER. It can be hypothesized that the ER-mitochondria encounter structure (ERMES) [41] requires the presence of lipid droplets to function adequately when fatty acid synthesis is increased by amino acid starvation. In support of this hypothesis, a similar situation seems to arise at the nuclear ER-vacuolar junction (NVJ) where lipid droplets are recruited upon increased fatty acid concentration, so as to ensure neutral lipid storage, which in turn prevents excessive phospholipids synthesis and the formation of ER tangles [42]. Further analysis of the autophagy failure in lipid droplet-deficient yeast may thus provide a more general view of the mechanisms involved in organelle contacts and ER homeostasis.

376

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386

387 **Declarations of interest**

388 The authors declare no conflict of interest

389

390 **Author contribution**

391 MR, PV designed the research; MB, MR and PV performed the experiments; TB, MB, MR  
392 and PV analyzed data; MR and PV wrote the paper with comments from TB and MB.

393

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514

515

516 TABLE 1 Oligonucleotides used in this study

517

Name :	Sequence :
GCN2KO forward	TGATTTTTTTTTCAATAATTTCCGTTCCCCTAACACATACTATGTAT AACAGCTGAAGCTTCGTACGC
GCN2KO reverse	TATACTTTACCTTTAACTGATGCGTTATAGCGCCGCACAGATCTTTAAA GGCGCATAGGCCACTAGTGGATCTG
REG1KO forward	ATAAATCCTAAAGCAAGCATATTGACGAAGACGAGATAAGAAAAATC CAAAACAGCTGAAGCTTCGTACGC
REG1KO reverse	ACACTACCTGGATTTTTATTTTCTCTTCATGTTGACTTCAAAATTCTTTC TTGCATAGGCCACTAGTGGATCTG
S2-SEC63	ATATACGTCTAAGAGCTAAAAATGAAAACTATACTAATCACTTATATC TAATCGATGAATTCGAGCTCG
S3-SEC63	ATACTGATATCGATACGGATACAGAAGCTGAAGATGATGAATCACCA GAACGTACGCTGCAGGTCGAC
PCL5 forward	TCCACTGGCAGAAATCCGTATC
PCL5 reverse	GACACCTCTGGATTGGTGAATC
HIS4 forward	AGCTGAAGAACTGACTGAGGC
HIS4 reverse	TGGATTGGTCTGCTCAAAGCC
ATG1 forward	ATTCATTGGCAGACGAGGTTGC
ATG1 reverse	AATCTCGTTGATGCAATACCGAG
ACT1 forward	CCGGTGATGGTGTTACTC
ACT1 reverse	CAAATCTCTACCGGCCAAAT

518

519



520 **Figure legends**

521

522 **Figure 1. Amino acid deficiency inhibits autophagy in lipid droplet-deficient yeast.**

523 **A)** At time  $T_0$  RS4 $\Delta$  cells expressing GFP-Atg8 were transferred to nitrogen-free medium (-  
524 N: without amino acids and without ammonium sulfate) or treated with rapamycin (Rap) in  
525 either YPD or nitrogen-free medium. The GFP-Atg8 cleavage test indicated that nitrogen  
526 starvation failed to induce autophagy and also blocked the effect of rapamycin. **B)** RS4 $\Delta$  cells  
527 expressing GFP-Atg8 were subjected to ammonium starvation, amino acid starvation, or both.  
528 Ammonium starvation induced autophagy in the presence of amino acids, whereas amino acid  
529 starvation failed to induce autophagy and also blocked the effect of ammonium starvation. **C)**  
530 RS4 $\Delta$  cells expressing GFP-Atg8 were treated with rapamycin in medium containing  
531 ammonium ion and increasing doses of the auxotrophic amino acids (expressed as fractions of  
532 the standard supply). **D)** RS4 $\Delta$  cells expressing GFP-Atg8 were subjected to ammonium  
533 starvation in the presence of increasing doses of the auxotrophic amino acids (expressed as  
534 fractions of the standard supply).

535 PGK: phosphoglycerate kinase 1 immunodetection as loading standard.

536

537 **Figure 2. Amino acid deficiency sensing in lipid droplet-deficient yeast.**

538 **A)** RS4 $\Delta$  cells expressing Atg13-HA under the *CUPI* promoter were subjected to ammonium  
539 and/or amino acid starvation for 1h, or treated with rapamycin in YPD medium. The  
540 phosphorylated and dephosphorylated forms of Atg13-HA were separated on an 8%  
541 acrylamide gel and detected with anti-HA antibody. Compared to untreated cells ( $T_0$ ), all  
542 treatments caused Atg13-HA dephosphorylation, indicating the initiation of autophagy is  
543 functional in RS4 $\Delta$  cells. The vertical line delineates different exposures of the same blot. **B)**  
544 RS4 $\Delta$  cells were subjected to ammonium and/or amino acid starvation and examined on

western blot for eIF2 $\alpha$  phosphorylation. All treatments yielded the same level of eIF2 $\alpha$  phosphorylation. PGK: phosphoglycerate kinase 1 immunodetection as loading standard. The vertical line delineates different exposures of the same blot. C) RS4 $\Delta$  cells were subjected to the indicated treatments and the mRNA levels of three Gcn4 effector genes were quantified by real time RT-qPCR, using actin mRNA as normalizing standard. Error Bars indicate Mean  $\pm$  sd. D) A paradoxical inhibition of autophagy by the general amino acid control pathway could be ruled out, as amino acid deficiency completely inhibited autophagy to rapamycin in RS4 $\Delta$  *gcn2* $\Delta$  cells. GFP-Atg8 cleavage assay. PGK: phosphoglycerate kinase 1 immunodetection as loading standard.

554

**Figure 3. Mitochondrial status in lipid droplet-deficient cells.**

A) RS4 $\Delta$  or Y10000 cells expressing mitochondria-targeted Su9-GFP [18] were grown to exponential phase on complete medium (YPD) and shifted to either nitrogen-free (-NH<sub>3</sub>-aa), or ammonium-free (-NH<sub>3</sub>) or amino acid-free (-aa) media for 2h and mitochondria morphology was analyzed by confocal microscopy. Left panel contains representative images of tubular and aggregated mitochondria. Quantification of mitochondria aggregation is presented in the right panel, based on > 200 cells for each experimental group. Scale bar: 5 $\mu$ m.

B) RS4 $\Delta$  rho<sup>0</sup> cells expressing GFP-Atg8 were starved as indicated and processed through the GFP-Atg8 cleavage assay. The absence of a functional respiratory chain inhibits autophagy to ammonium starvation in RS4 $\Delta$  cells. C) RS4 $\Delta$  cells expressing GFP-Atg8 were starved for either nitrogen (-NH<sub>3</sub>-aa), or amino acid (-aa), or cultured in complete medium YPD for 4h in the presence of the indicated concentration of H<sub>2</sub>O<sub>2</sub>. Western blot analysis of GFP-Atg8 cleavage indicates that H<sub>2</sub>O<sub>2</sub> (0.3-0.5mM) partially restores autophagy in starvation media but does not induce it in complete medium. The vertical line delineates boundary in a composite

570 image generated from two different blots. **D)** N-acetylcysteine (NAC) treatment blocks GFP-  
571 Atg8 processing by autophagy in RS4Δ and but not in Y10000.  
572 PGK: phosphoglycerate kinase 1 immunodetection as loading standard.

573

574 **Figure 4. Respiration or release from catabolite repression restores autophagy in lipid**  
575 **droplet-deficient cells.**

576 **A)** RS4Δ (left panel) or Y10000 (right panel) expressing GFP-Atg8 were cultured in YP-  
577 Lactate medium. At time T<sub>0</sub>, cells were subjected to the indicated starvations in YNB lactic  
578 acid media and autophagy was monitored over 8h by GFP-Atg8 cleavage assay. Respiratory  
579 conditions restored autophagy to near wild type level in RS4Δ.

580 **B)** RS4Δ, Y10000 or RS4Δ-*reg1Δ* expressing GFP-Atg8 were cultured in complete glucose  
581 medium or lactate medium as indicated, in the presence of FM4-64 (1 μM), and shifted in the  
582 same medium lacking either nitrogen (-N) or ammonium (-NH<sub>3</sub>) or amino acid (-aa), or  
583 containing 200nM rapamycin (R) for 6 hours. Cells were examined by confocal microscopy.

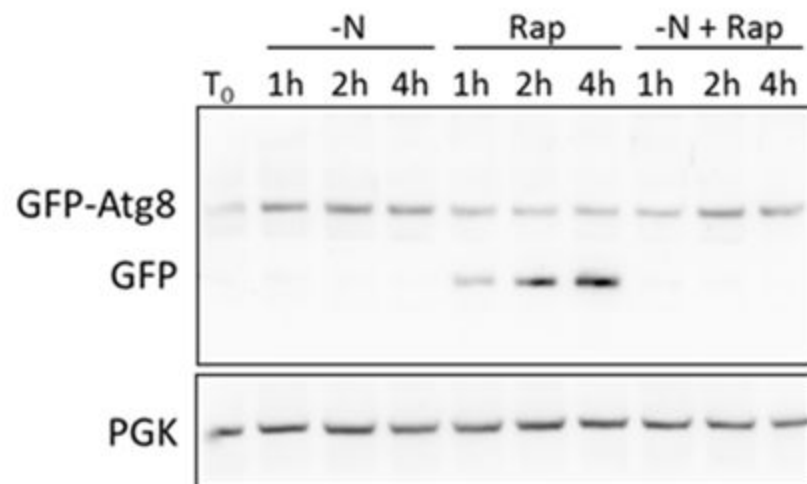
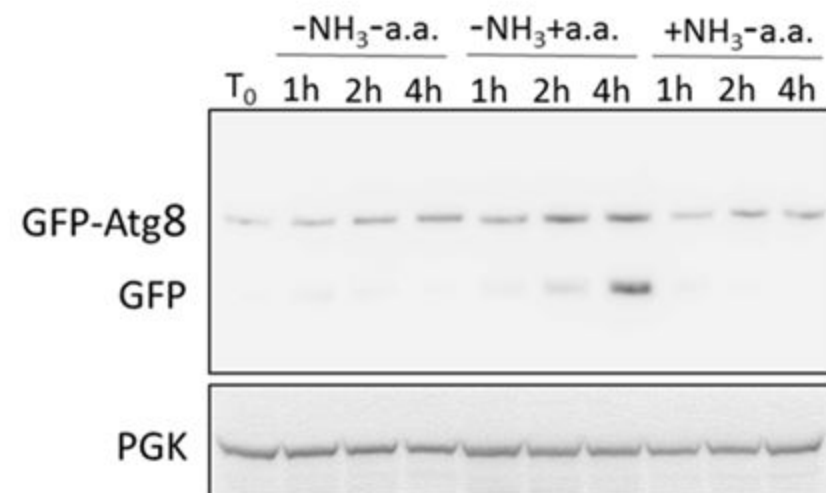
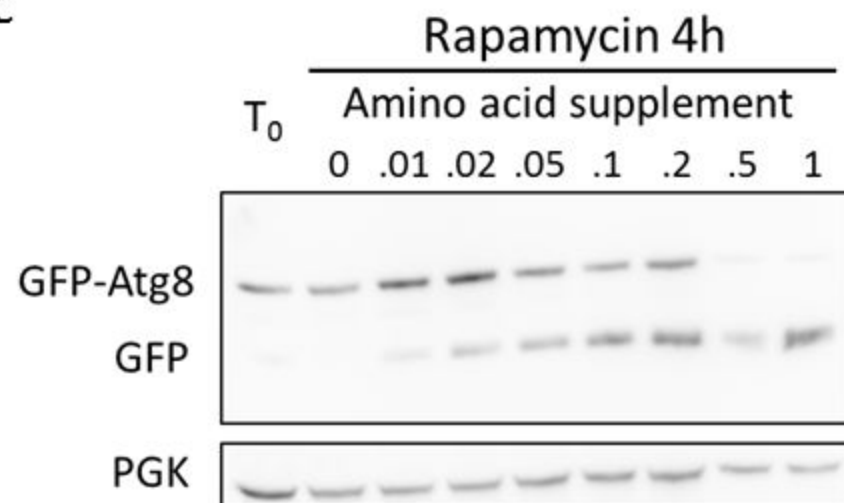
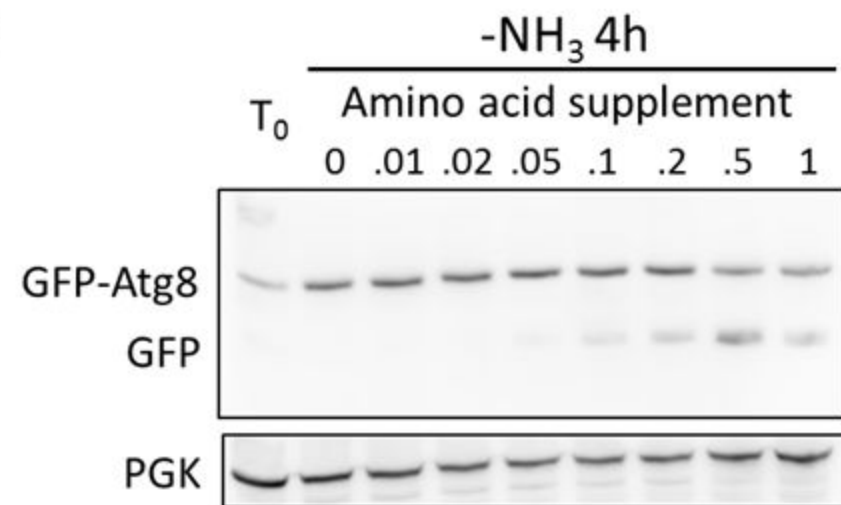
584 Left panel contains representative images of the cytosolic, vacuolar or uniform GFP  
585 fluorescence in the cells. Quantification of GFP localization in each experimental group is  
586 presented in the right panel, based on > 200 cells for each group. Scale bar: 2 μm. **C)** RS4Δ

587 cells containing an integrated copy of the ER resident membrane protein Sec63 tagged with  
588 GFP were grown in complete medium containing glucose or lactate as the sole carbon source  
589 as indicated. Cells were shifted in the same medium lacking nitrogen and the GFP signal was  
590 observed. Arrows: tangled membranes. Scale bar: 5 μm. **D)** RS4Δ-*reg1Δ* cells expressing

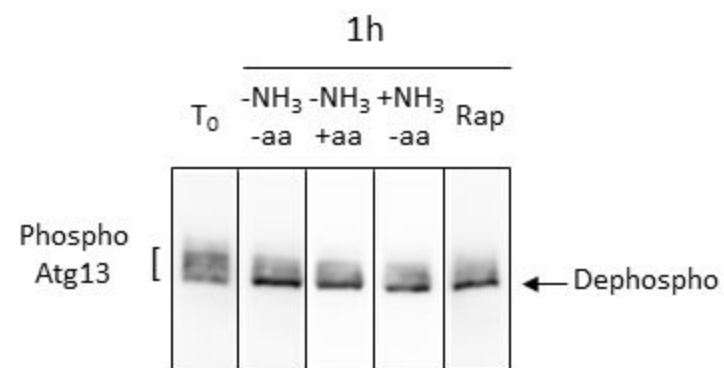
591 GFP-Atg8 were cultured in complete glucose medium, shifted at T<sub>0</sub> to the indicated starvation  
592 media and analyzed by GFP-Atg8 cleavage assay. **E)** RS4Δ-*reg1Δ* rho<sup>0</sup> cells expressing  
593 GFP-Atg8 were treated as described in D.

594 PGK: phosphoglycerate kinase 1 immunodetection as loading standard.

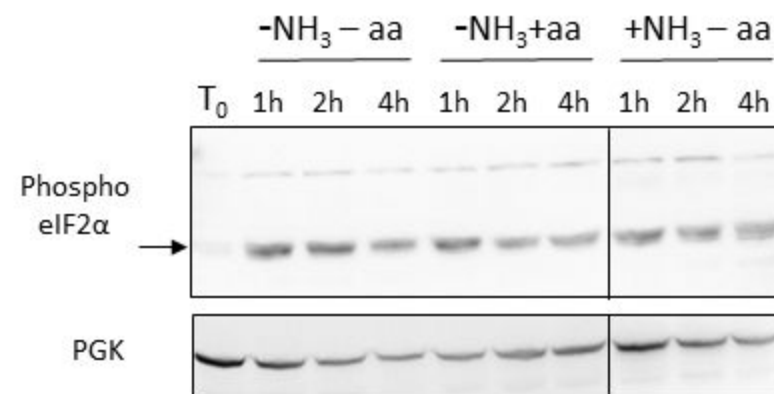


**A****B****C****D**

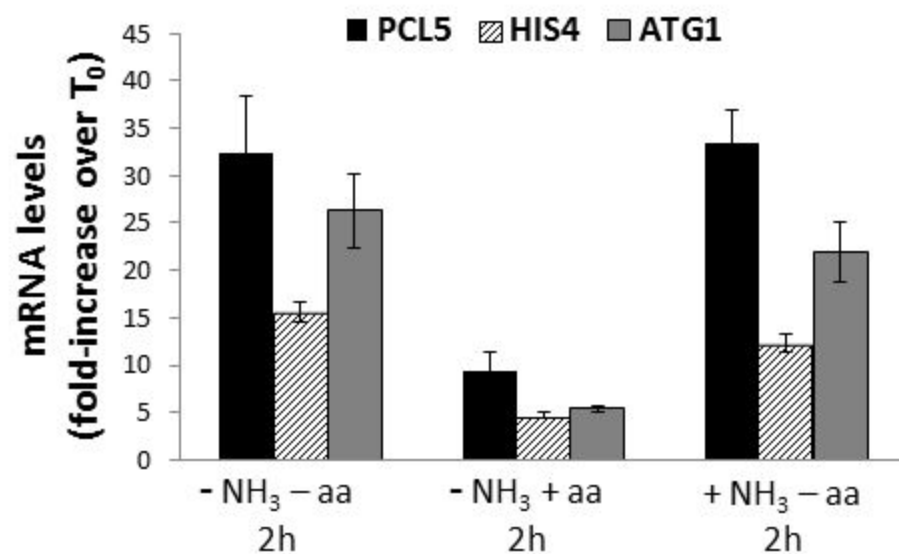
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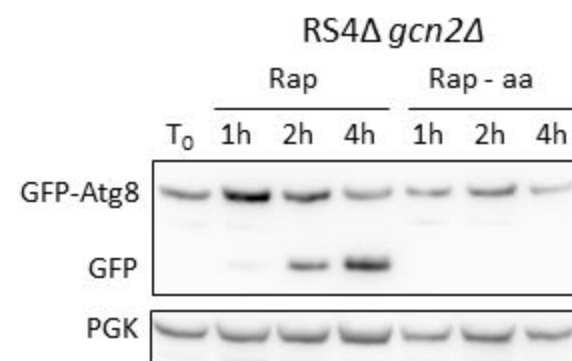
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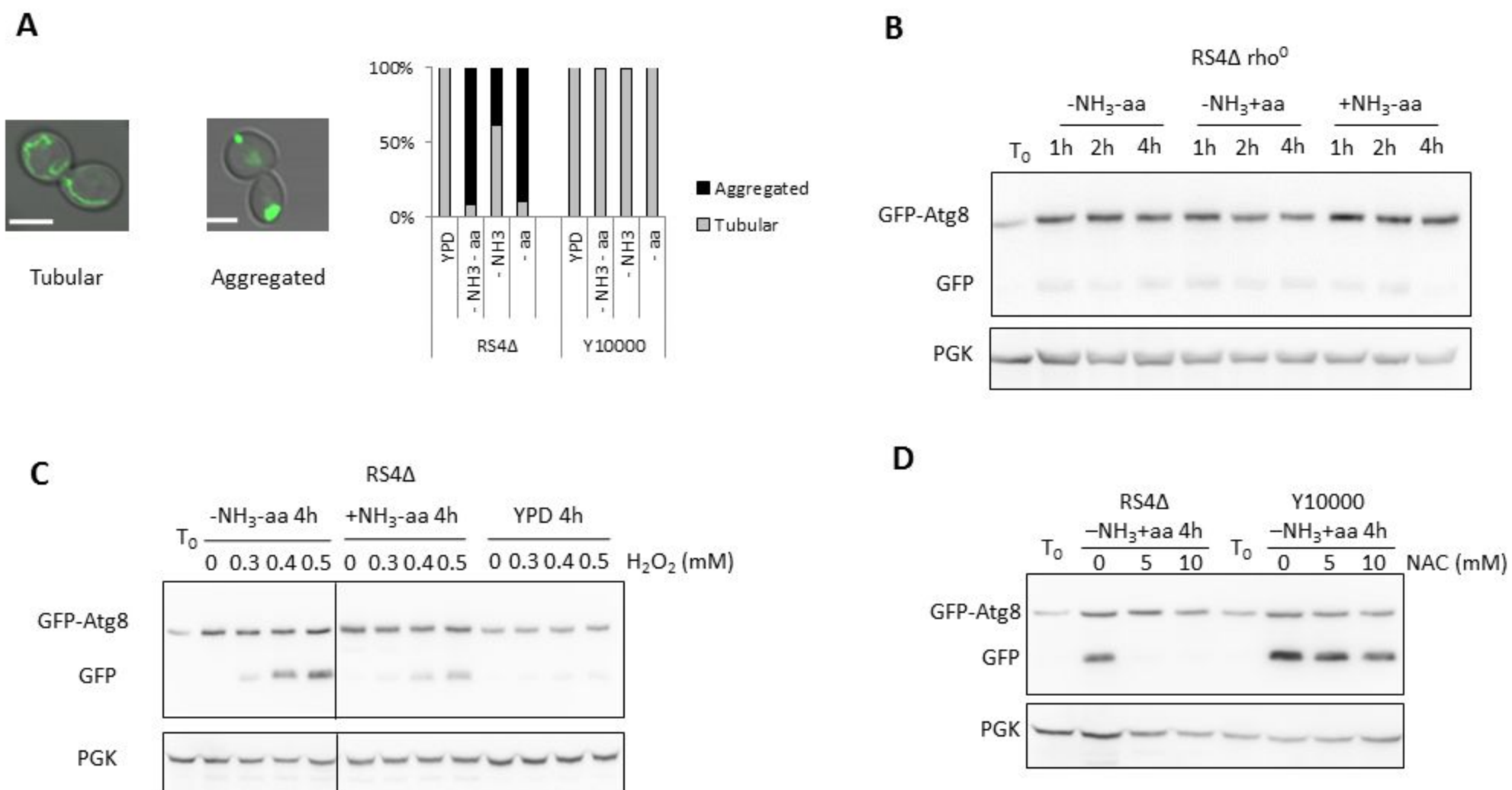


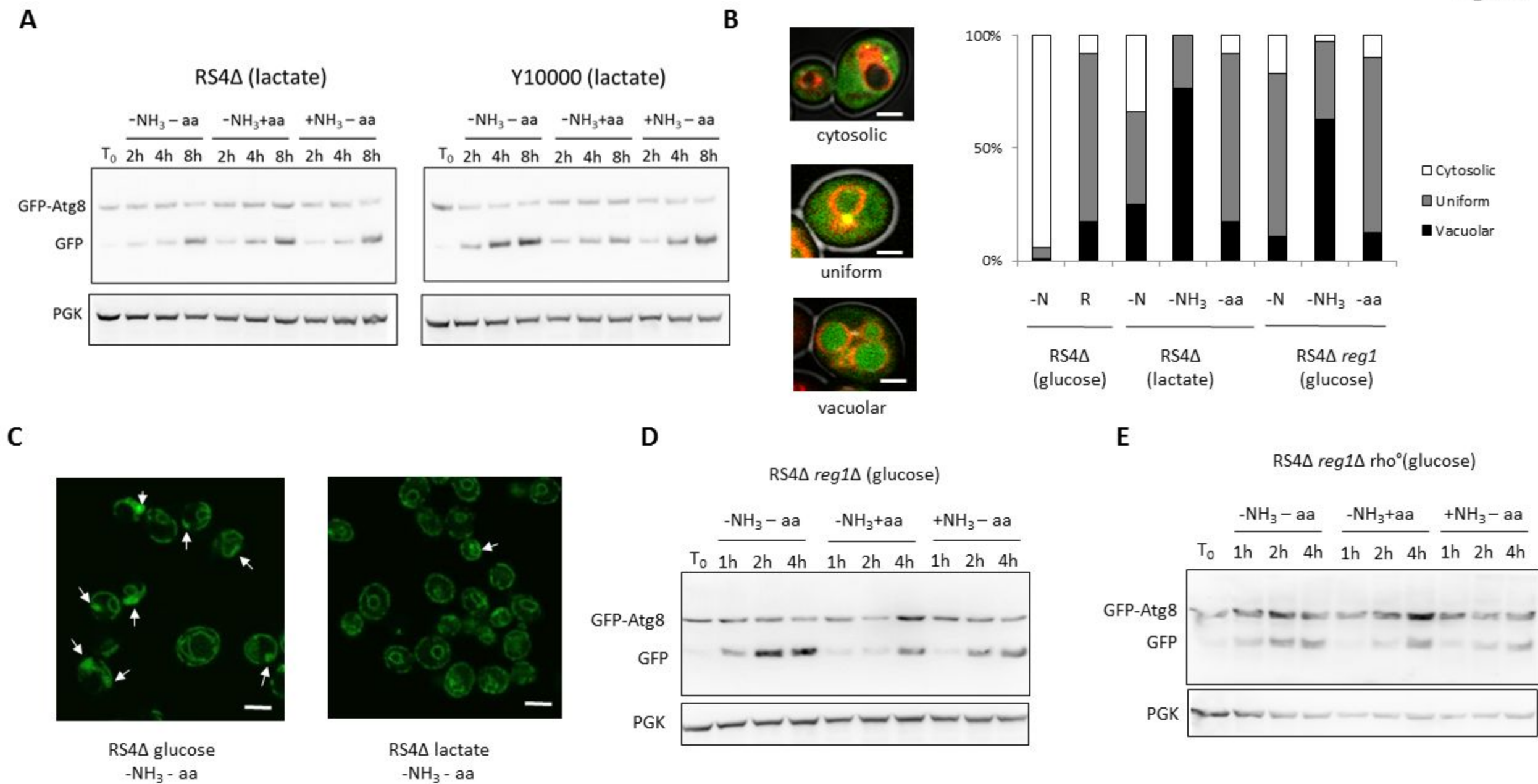
C



D









## **Supplemental Figure legends**

### **Supplemental Figure 1. RS4Δ strain does not contain lipid droplets.**

Y10000 (left panel) and RS4Δ (right panel) cells were incubated for 10 minutes with 1.5μg/mL Bodipy 493/503 and analyzed by confocal microscopy. Scale bar: 5μm.

### **Supplemental Figure 2. Mitochondria aggregates are formed in response to nitrogen starvation in RS4Δ.**

Y10000 (top panels) and RS4Δ (bottom panels) cells expressing Su9-GFP and stained with FM4-64 were grown in complete medium (left panels) or subjected to nitrogen starvation for 2h (right panels) and analyzed by confocal microscopy. Scale bar: 5μm.

### **Supplemental Figure 3. Mitochondrial status is normal in lipid droplet-deficient cells after amino acid starvation in respiratory medium.**

RS4Δ cells expressing Su9-GFP and stained with FM4-64 were grown in YP lactic medium (top panels) or subjected to amino acid starvation for 2h (bottom panels) and analyzed by confocal microscopy. Left panels: transmission image; middle panel: confocal image of a middle plane of the cells; right panel: confocal image of the top of the cells. Scale bar: 5μm.

### **Supplemental Figure 4. Sec63-GFP patches correlate with ER membrane tangles in RS4Δ.**

**A)** RS4Δ cells expressing Sec63-GFP were grown to exponential phase in YPD medium and analyzed by confocal microscopy. Scale bar: 5μm. **B)** Same experiment performed with cells subjected to nitrogen starvation for 2h. White arrows indicate several patches of Sec63-GFP fluorescence. Scale bar: 5μm. **C)** Same experiment as in **B** in the presence of cerulenin that

prevents accumulation of ER membrane tangles [7]. Only one patch of Sec63-GFP is observed (white arrow). Scale bar: 5 $\mu$ m. **D and E)** RS4 $\Delta$  cells were treated as in B and C respectively and processed for electron microscopy: as observed before with confocal microscopy, ER tangles were detected only in the absence of cerulenin. We interpreted these experiments as indicating that Sec63-GFP patches represent ER membrane tangles.

**Figure 5. Sec63-GFP patches are connected to the ER.**

Optically sliced images from top (1) to bottom (8) of RS4 $\Delta$  cells expressing Sec63-GFP and subjected to nitrogen starvation. Arrows point to connections between ER and Sec63-GFP patches. Bottom right: transmission image of the corresponding cells. Scale bar: 5 $\mu$ m.