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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

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 F₀-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 (-NH₃-aa) for nitrogen starvation, or YNB medium without
126 amino acid (+NH₃-aa) for amino acid starvation. For ammonium starvation experiments, (-
127 NH₃ + 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) (-NH₃+aa). Autophagy induction with rapamycin was achieved with
130 rapamycin 200nM.

131 To obtain rho⁰ 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% β -mercaptoethanol) followed by
143 trichloroacetic acid precipitation [7]. Protein extracts were subsequently washed with acetone,
144 dried and suspended in 100 μ L of 5% (w/v) SDS. Finally, 100 μ L of Laemmli buffer (20%
145 glycerol, 2% SDS, 2% β -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 4×10^6 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 α (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 2×10^8 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-dT₂₅. 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 rho⁰ mutant and analyzed autophagy in response to nitrogen starvation. Rho⁰ 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 rho⁰ proved unable to accomplish autophagy even in response to ammonium starvation (Fig. 3B). Importantly, autophagy was still observed in the rho⁰ 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 H₂O₂ facilitated autophagy in response to nitrogen starvation and to a lesser extent, to amino acid starvation. H₂O₂ alone did not significantly activate autophagy (Fig. 3C). H₂O₂ 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 H₂O₂ 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* rho⁰ 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* rho⁰
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

394 **References**

- 395 1 Yang, Z. and Klionsky, D. J. (2010) Eaten alive: a history of macroautophagy. *Nature cell*
396 biology. **12**, 814-822
- 397 2 Tsukada, M. and Ohsumi, Y. (1993) Isolation and characterization of autophagy-defective
398 mutants of *Saccharomyces cerevisiae*. *FEBS letters*. **333**, 169-174
- 399 3 Weidberg, H., Shvets, E. and Elazar, Z. (2011) Biogenesis and cargo selectivity of
400 autophagosomes. *Annual review of biochemistry*. **80**, 125-156
- 401 4 Mari, M., Tooze, S. A. and Reggiori, F. (2011) The puzzling origin of the autophagosomal
402 membrane. *F1000 biology reports*. **3**, 25
- 403 5 Dupont, N., Chauhan, S., Arko-Mensah, J., Castillo, E. F., Masedunskas, A., Weigert, R.,
404 Robenek, H., Proikas-Cezanne, T. and Deretic, V. (2014) Neutral lipid stores and lipase PNPLA5
405 contribute to autophagosome biogenesis. *Current biology : CB*. **24**, 609-620
- 406 6 Li, D., Song, J. Z., Li, H., Shan, M. H., Liang, Y., Zhu, J. and Xie, Z. (2015) Storage lipid
407 synthesis is necessary for autophagy induced by nitrogen starvation. *FEBS letters*. **589**, 269-276
- 408 7 Regnacq, M., Voisin, P., Sere, Y. Y., Wan, B., Soeroso, V. M. S., Bernard, M., Camougrand, N.,
409 Bernard, F. X., Barrault, C. and Berges, T. (2016) Increased fatty acid synthesis inhibits nitrogen
410 starvation-induced autophagy in lipid droplet-deficient yeast. *Biochemical and biophysical*
411 research communications. **477**, 33-39
- 412 8 Shpilka, T., Welter, E., Borovsky, N., Amar, N., Mari, M., Reggiori, F. and Elazar, Z. (2015)
413 Lipid droplets and their component triglycerides and steryl esters regulate autophagosome
414 biogenesis. *The EMBO journal*. **34**, 2117-2131
- 415 9 Velazquez, A. P., Tatsuta, T., Ghillebert, R., Drescher, I. and Graef, M. (2016) Lipid droplet-
416 mediated ER homeostasis regulates autophagy and cell survival during starvation. *The Journal of*
417 cell biology. **212**, 621-631
- 418 10 Meyers, A., Weiskittel, T. M. and Dalhaimer, P. (2017) Lipid Droplets: Formation to Breakdown.
419 *Lipids*. **52**, 465-475

- 420 11 Sandager, L., Gustavsson, M. H., Stahl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M.,
421 Ronne, H. and Stymne, S. (2002) Storage lipid synthesis is non-essential in yeast. *The Journal of*
422 *biological chemistry*. **277**, 6478-6482
- 423 12 Garbarino, J., Padamsee, M., Wilcox, L., Oelkers, P. M., D'Ambrosio, D., Ruggles, K. V.,
424 Ramsey, N., Jabado, O., Turkish, A. and Sturley, S. L. (2009) Sterol and diacylglycerol
425 acyltransferase deficiency triggers fatty acid-mediated cell death. *The Journal of biological*
426 *chemistry*. **284**, 30994-31005
- 427 13 Petschnigg, J., Wolinski, H., Kolb, D., Zellnig, G., Kurat, C. F., Natter, K. and Kohlwein, S. D.
428 (2009) Good fat, essential cellular requirements for triacylglycerol synthesis to maintain
429 membrane homeostasis in yeast. *The Journal of biological chemistry*. **284**, 30981-30993
- 430 14 Rockenfeller, P., Ring, J., Muschett, V., Beranek, A., Buettner, S., Carmona-Gutierrez, D.,
431 Eisenberg, T., Khoury, C., Rechberger, G., Kohlwein, S. D., Kroemer, G. and Madeo, F. (2010)
432 Fatty acids trigger mitochondrion-dependent necrosis. *Cell cycle*. **9**, 2836-2842
- 433 15 Ecker, N., Mor, A., Journo, D. and Abeliovich, H. (2010) Induction of autophagic flux by amino
434 acid deprivation is distinct from nitrogen starvation-induced macroautophagy. *Autophagy*. **6**, 879-
435 890
- 436 16 Gueldener, U., Heinisch, J., Koehler, G. J., Voss, D. and Hegemann, J. H. (2002) A second set of
437 loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic acids*
438 *research*. **30**, e23
- 439 17 Janke, C., Magiera, M. M., Rathfelder, N., Taxis, C., Reber, S., Maekawa, H., Moreno-Borchart,
440 A., Doenges, G., Schwob, E., Schiebel, E. and Knop, M. (2004) A versatile toolbox for PCR-
441 based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution
442 cassettes. *Yeast*. **21**, 947-962
- 443 18 Sesaki, H., Dunn, C. D., Iijima, M., Shepard, K. A., Yaffe, M. P., Machamer, C. E. and Jensen, R.
444 E. (2006) Ups1p, a conserved intermembrane space protein, regulates mitochondrial shape and
445 alternative topogenesis of Mgm1p. *The Journal of cell biology*. **173**, 651-658

- 446 19 Noda, T., Matsuura, A., Wada, Y. and Ohsumi, Y. (1995) Novel system for monitoring
447 autophagy in the yeast *Saccharomyces cerevisiae*. *Biochemical and biophysical research*
448 *communications*. **210**, 126-132
- 449 20 Stephan, J. S., Yeh, Y. Y., Ramachandran, V., Deminoff, S. J. and Herman, P. K. (2009) The Tor
450 and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to
451 control autophagy. *Proceedings of the National Academy of Sciences of the United States of*
452 *America*. **106**, 17049-17054
- 453 21 Shintani, T. and Klionsky, D. J. (2004) Cargo proteins facilitate the formation of transport
454 vesicles in the cytoplasm to vacuole targeting pathway. *The Journal of biological chemistry*. **279**,
455 29889-29894
- 456 22 Li, J., Liu, J., Wang, X., Zhao, L., Chen, Q. and Zhao, W. (2009) A waterbath method for
457 preparation of RNA from *Saccharomyces cerevisiae*. *Analytical biochemistry*. **384**, 189-190
- 458 23 Cardenas, M. E. and Heitman, J. (1995) FKBP12-rapamycin target TOR2 is a vacuolar protein
459 with an associated phosphatidylinositol-4 kinase activity. *The EMBO journal*. **14**, 5892-5907
- 460 24 Stracka, D., Jozefczuk, S., Rudroff, F., Sauer, U. and Hall, M. N. (2014) Nitrogen source
461 activates TOR (target of rapamycin) complex 1 via glutamine and independently of Gtr/Rag
462 proteins. *The Journal of biological chemistry*. **289**, 25010-25020
- 463 25 Vlahakis, A., Lopez Muniozguren, N. and Powers, T. (2016) Calcium channel regulator Mid1
464 links TORC2-mediated changes in mitochondrial respiration to autophagy. *The Journal of cell*
465 *biology*. **215**, 779-788
- 466 26 Yuan, W., Guo, S., Gao, J., Zhong, M., Yan, G., Wu, W., Chao, Y. and Jiang, Y. (2017) General
467 Control Nonderepressible 2 (GCN2) Kinase Inhibits Target of Rapamycin Complex 1 in
468 Response to Amino Acid Starvation in *Saccharomyces cerevisiae*. *The Journal of biological*
469 *chemistry*. **292**, 2660-2669
- 470 27 Cheong, H., Yorimitsu, T., Reggiori, F., Legakis, J. E., Wang, C. W. and Klionsky, D. J. (2005)
471 Atg17 regulates the magnitude of the autophagic response. *Molecular biology of the cell*. **16**,
472 3438-3453

- 473 28 Kabeya, Y., Kamada, Y., Baba, M., Takikawa, H., Sasaki, M. and Ohsumi, Y. (2005) Atg17
474 functions in cooperation with Atg1 and Atg13 in yeast autophagy. *Molecular biology of the cell*.
475 **16**, 2544-2553
- 476 29 Dong, J., Qiu, H., Garcia-Barrio, M., Anderson, J. and Hinnebusch, A. G. (2000) Uncharged
477 tRNA activates GCN2 by displacing the protein kinase moiety from a bipartite tRNA-binding
478 domain. *Molecular cell*. **6**, 269-279
- 479 30 Hinnebusch, A. G. (2005) Translational regulation of GCN4 and the general amino acid control
480 of yeast. *Annual review of microbiology*. **59**, 407-450
- 481 31 Bernard, A., Jin, M., Xu, Z. and Klionsky, D. J. (2015) A large-scale analysis of autophagy-
482 related gene expression identifies new regulators of autophagy. *Autophagy*. **11**, 2114-2122
- 483 32 Castilho, B. A., Shanmugam, R., Silva, R. C., Ramesh, R., Himme, B. M. and Sattlegger, E.
484 (2014) Keeping the eIF2 alpha kinase Gcn2 in check. *Biochimica et biophysica acta*. **1843**, 1948-
485 1968
- 486 33 Graef, M. and Nunnari, J. (2011) Mitochondria regulate autophagy by conserved signalling
487 pathways. *The EMBO journal*. **30**, 2101-2114
- 488 34 Dimmer, K. S., Fritz, S., Fuchs, F., Messerschmitt, M., Weinbach, N., Neupert, W. and
489 Westermann, B. (2002) Genetic basis of mitochondrial function and morphology in
490 *Saccharomyces cerevisiae*. *Molecular biology of the cell*. **13**, 847-853
- 491 35 Knupp, J., Martinez-Montanes, F., Van Den Bergh, F., Cottier, S., Schneider, R., Beard, D. and
492 Chang, A. (2017) Sphingolipid accumulation causes mitochondrial dysregulation and cell death.
493 *Cell death and differentiation*. **24**, 2044-2053
- 494 36 Kissova, I., Deffieu, M., Samokhvalov, V., Velours, G., Bessoule, J. J., Manon, S. and
495 Camougrand, N. (2006) Lipid oxidation and autophagy in yeast. *Free radical biology & medicine*.
496 **41**, 1655-1661
- 497 37 Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L. and Elazar, Z. (2007) Reactive
498 oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *The*
499 *EMBO journal*. **26**, 1749-1760

500 38 Sere, Y. Y., Regnacq, M., Colas, J. and Berges, T. (2010) A *Saccharomyces cerevisiae* strain
501 unable to store neutral lipids is tolerant to oxidative stress induced by alpha-synuclein. *Free*
502 *radical biology & medicine*. **49**, 1755-1764

503 39 Schuller, H. J. (2003) Transcriptional control of nonfermentative metabolism in the yeast
504 *Saccharomyces cerevisiae*. *Current genetics*. **43**, 139-160

505 40 Kojima, R., Endo, T. and Tamura, Y. (2016) A phospholipid transfer function of ER-
506 mitochondria encounter structure revealed in vitro. *Scientific reports*. **6**, 30777

507 41 Kornmann, B., Currie, E., Collins, S. R., Schuldiner, M., Nunnari, J., Weissman, J. S. and Walter,
508 P. (2009) An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science*.
509 **325**, 477-481

510 42 Hariri, H., Speer, N., Bowerman, J., Rogers, S., Fu, G., Reetz, E., Datta, S., Feathers, J. R.,
511 Ugrankar, R., Nicastro, D. and Henne, W. M. (2019) Mdm1 maintains endoplasmic reticulum
512 homeostasis by spatially regulating lipid droplet biogenesis. *The Journal of cell biology*. **218**,
513 1319-1334

514

515

516 TABLE 1 Oligonucleotides used in this study

517

Name :	Sequence :
GCN2KO forward	TGATTTTTTTTTCAATAATTTTCCGTTCCCCTAACACATACTATGTAT 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 Δ 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 Δ 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 Δ 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 Δ 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 Δ cells expressing Atg13-HA under the *CUP1* 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 Δ cells. The vertical line delineates different exposures of the same blot. **B)**
544 RS4 Δ cells were subjected to ammonium and/or amino acid starvation and examined on

western blot for eIF2 α phosphorylation. All treatments yielded the same level of eIF2 α phosphorylation. PGK: phosphoglycerate kinase 1 immunodetection as loading standard. The vertical line delineates different exposures of the same blot. C) RS4 Δ 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 Δ *gcn2* Δ 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 Δ or Y10000 cells expressing mitochondria-targeted Su9-GFP [18] were grown to exponential phase on complete medium (YPD) and shifted to either nitrogen-free (-NH₃-aa), or ammonium-free (-NH₃) 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 μ m.

B) RS4 Δ rho⁰ 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 Δ cells. C) RS4 Δ cells expressing GFP-Atg8 were starved for either nitrogen (-NH₃-aa), or amino acid (-aa), or cultured in complete medium YPD for 4h in the presence of the indicated concentration of H₂O₂. Western blot analysis of GFP-Atg8 cleavage indicates that H₂O₂ (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 T₀, 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 (-NH₃) or amino acid (-aa), or
583 containing 200nM rapamycin (R) for 6 hours. Cells were examined by confocal microscopy.

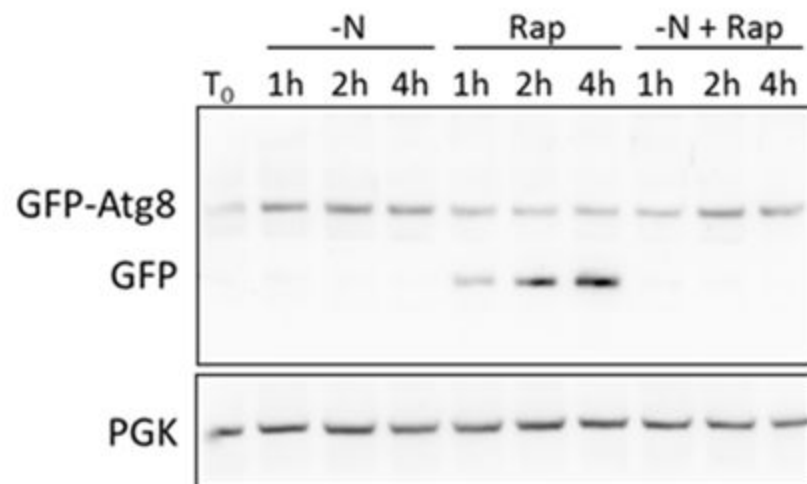
584 Left panel contains representative images of the cytosolic, vacuolar or uniform GFP
585 fluorescence in the cells. Quantification of GFP localization in each experimental group is
586 presented in the right panel, based on > 200 cells for each group. Scale bar: 2 μm. **C)** RS4Δ

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

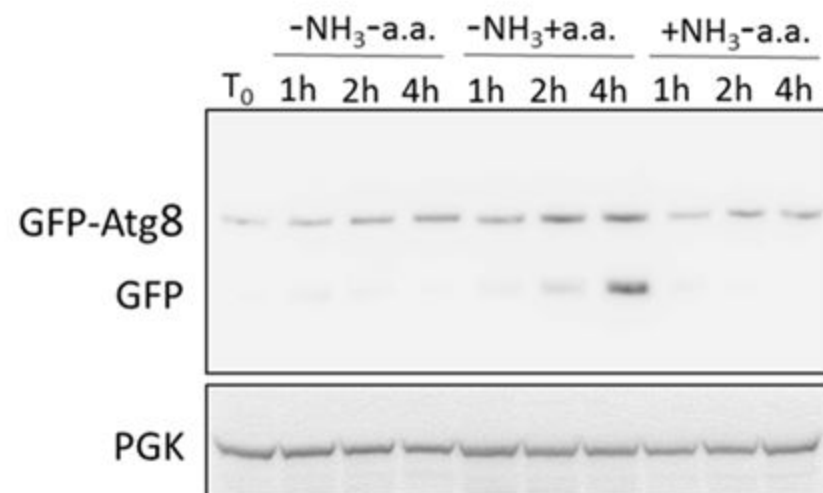
591 GFP-Atg8 were cultured in complete glucose medium, shifted at T₀ to the indicated starvation
592 media and analyzed by GFP-Atg8 cleavage assay. **E)** RS4Δ-*reg1Δ* rho⁰ cells expressing
593 GFP-Atg8 were treated as described in D.

594 PGK: phosphoglycerate kinase 1 immunodetection as loading standard.

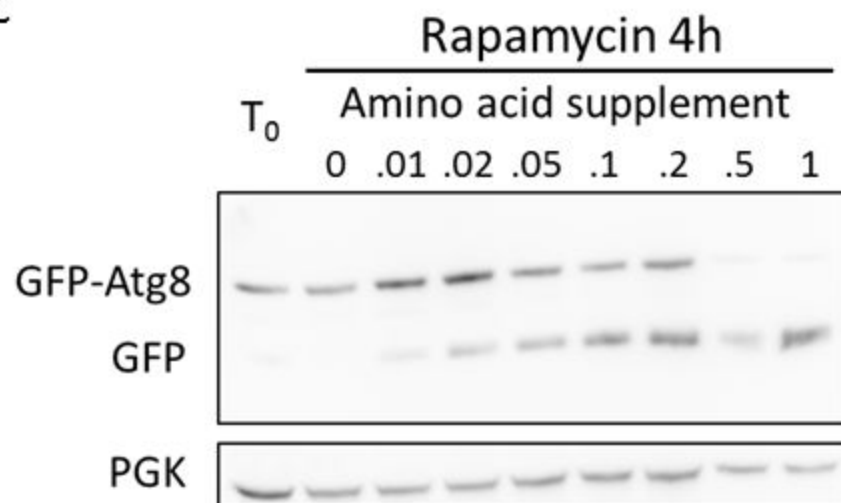
A



B



C



D

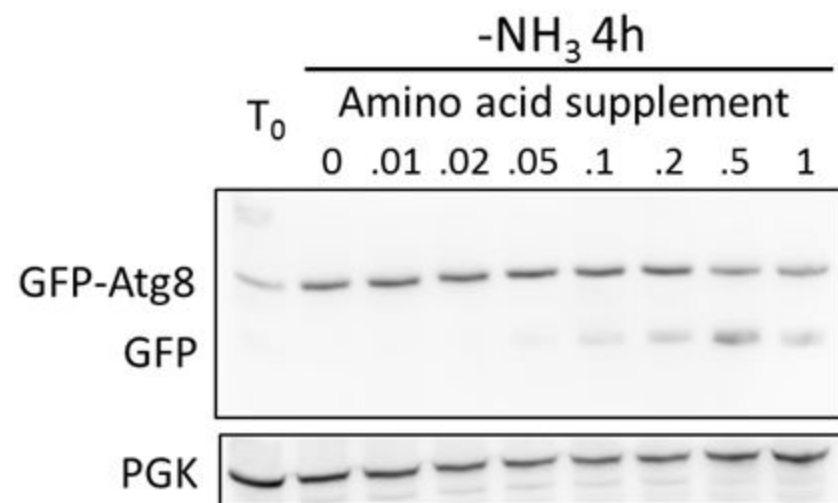
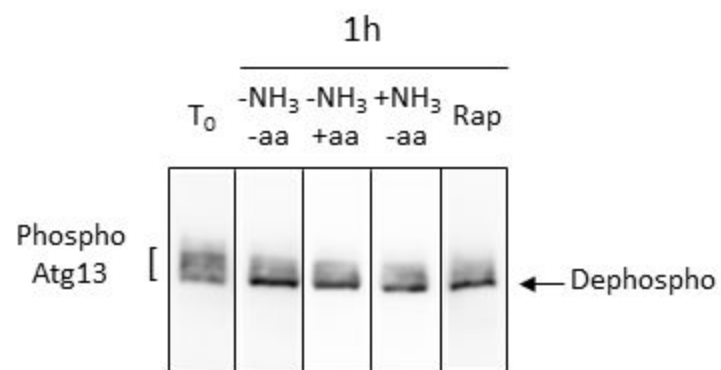
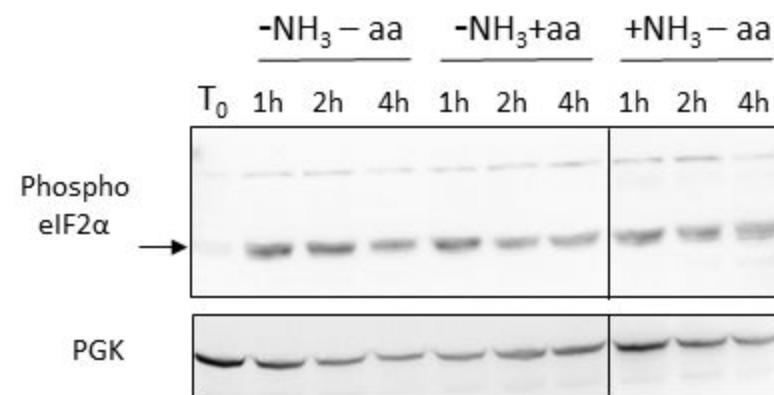


Figure 2

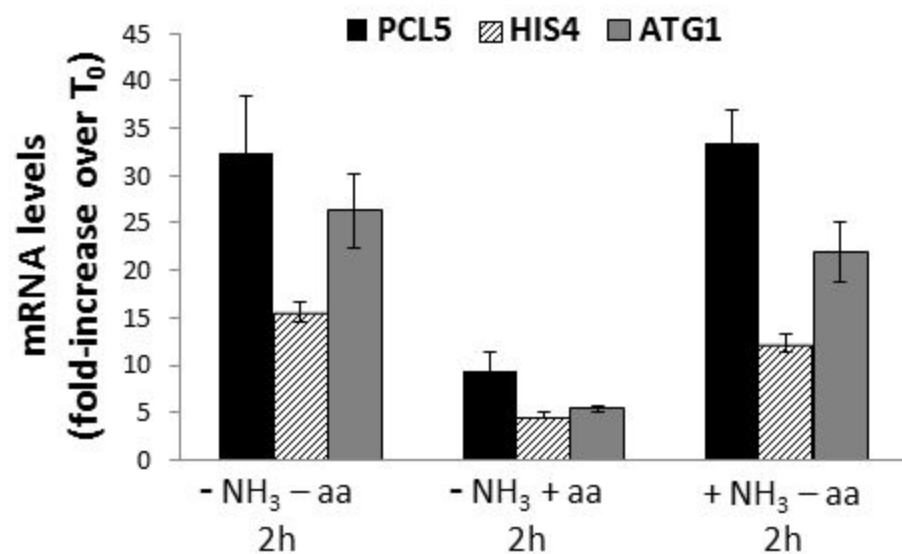
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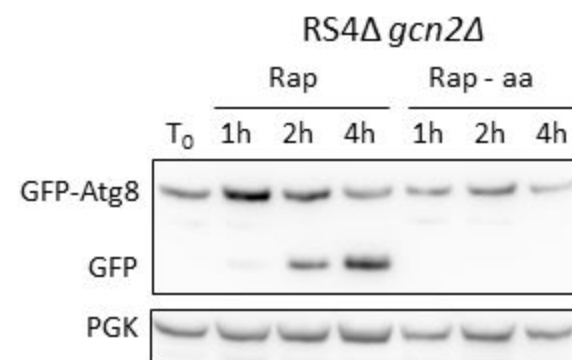
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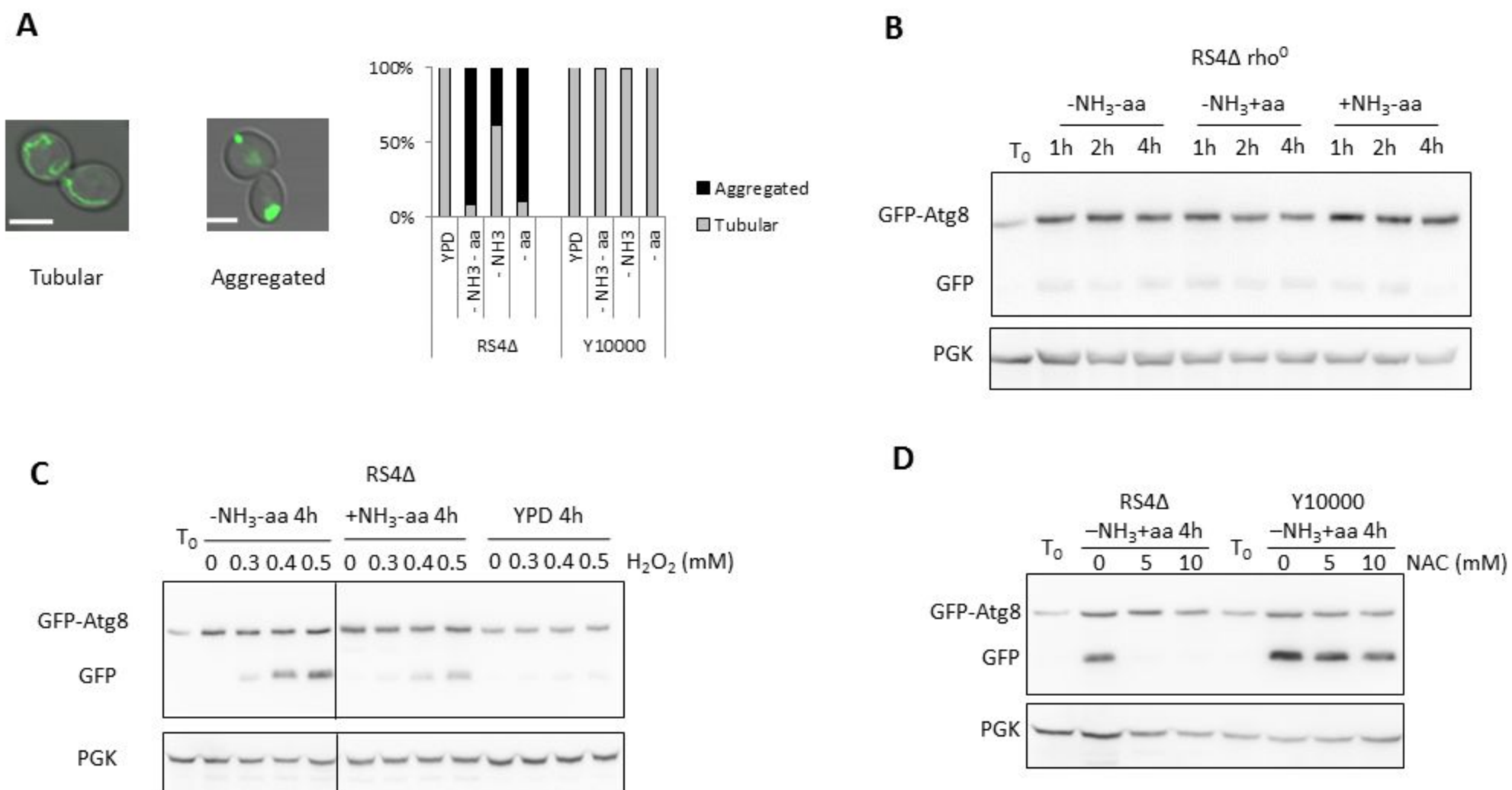


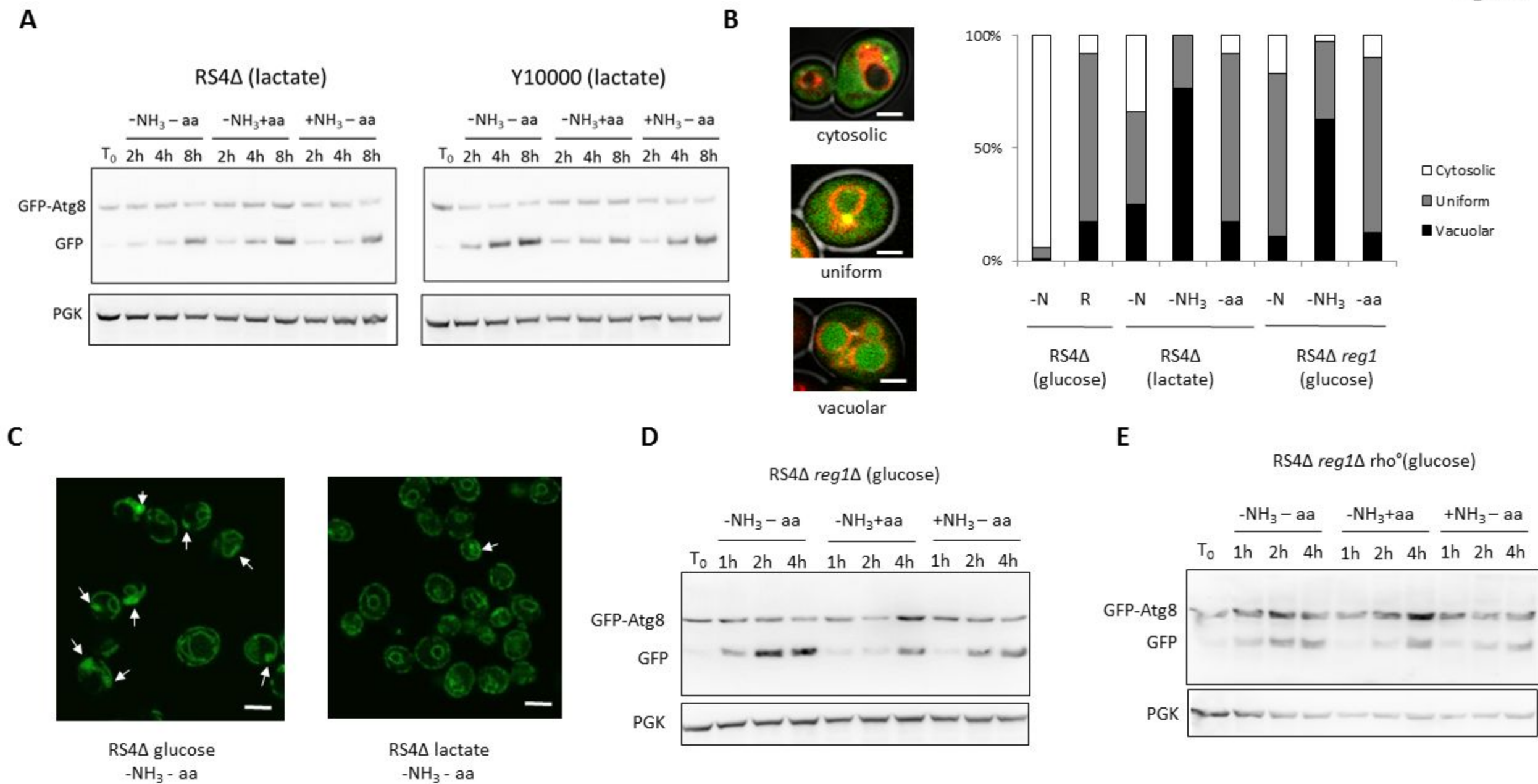
C



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 μ m. **D and E)** RS4 Δ 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 Δ 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 μ m.