

1 **TORC1 Inhibition Induces Lipid Droplet Replenishment in Yeast**

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3 **Running Title:** Rapamycin and Lipid Droplet Replenishment

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17 Word count for the Materials and Methods section: 1,263

18 Word count for the Introduction, Results, and Discussion sections: 3,674

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20 **ABSTRACT**

21 Lipid droplets (LDs) are intracellular structures that regulate neutral lipid homeostasis. In  
22 mammals, LD synthesis is inhibited by rapamycin, a known inhibitor of the mTORC1  
23 pathway. In *S. cerevisiae*, LD dynamics is modulated by the growth phase, however the  
24 regulatory pathways involved are unknown. Therefore, we decided to study the role of the  
25 TORC1 pathway on LD metabolism in *S. cerevisiae*. Interestingly, rapamycin treatment  
26 resulted in a fast LDs replenishment and growth inhibition. The discovery that osmotic  
27 stress (1M sorbitol) also induced LD synthesis but not growth inhibition, suggested that the  
28 induction of LDs in yeast is not a secondary response to reduced growth. Induction of LDs  
29 by rapamycin was due to increased triacylglycerol but not sterol esters synthesis. Induction  
30 was dependent on the TOR downstream effectors, the PP2A-related phosphatase Sit4p and  
31 the regulatory protein Tap42p. The TORC1-controlled transcriptional activators *GLN3*,  
32 *GATI*, *RTG1* and *RTG3* but not *MSN2* and *MSN4* were required for full induction of LDs  
33 by rapamycin. Furthermore, we show that deletion of *GLN3* and *GATI* transcription  
34 factors, which are activated in response to nitrogen availability, led to abnormal LD  
35 dynamics. These results reveal that the TORC1 pathway is involved in neutral lipid  
36 homeostasis in yeast.

37

38 **INTRODUCTION**

39 Lipid droplets (LDs) are intracellular structures formed by a core of neutral lipids, mainly  
40 triacylglycerols (TAG) and sterol esters (SE), which are delimited by a phospholipid  
41 monolayer embedded with proteins primarily related to lipid metabolism (1, 2, 3). At first,  
42 LDs were believed to be mere neutral lipid deposits, but later it became clear that LDs play  
43 other important roles in cellular physiology. The main function of LDs is to maintain

44 cellular lipid homeostasis (4, 5) and it was shown that defects in the mobilization of neutral  
45 lipids from LDs are related to type 2 diabetes, inflammation, neurodegenerative disorders  
46 and cancer (6, 7, 8). In addition to their importance in health, LDs are also studied in  
47 oleaginous yeast for their exploitation as cellular oil factories for biofuel production (9).

48 Although the metabolic steps of LD biogenesis are well known, the signals that govern  
49 LD dynamics are not clear yet. Because the signaling pathways are well-conserved between  
50 yeast and mammals, *S. cerevisiae* has been a model for studying LD dynamics. In *S.*  
51 *cerevisiae*, LDs follow a particular dynamic tightly linked to the growth phase and to the  
52 nutritional status of the cell (10, 11). When quiescent yeast cells encounter a rich medium  
53 containing glucose, cells must exit G0 in order to start duplication and cell growth (12).  
54 This start demands a high amount of sterols and fatty acids leading to a strong mobilization  
55 of the neutral lipids stored in LDs. As a result, LDs are diminished in number and in size  
56 (10). After this strong lipolytic phase, yeast cells shift to a lipogenic phase to replenish the  
57 levels of LDs reaching its maximum at early stationary phase (10, 11).

58 The regulatory mechanisms that orchestrate the correct expression and activity of  
59 lipolytic/lipogenic enzymes are not completely understood. However, some clues were  
60 given by the discovery that nitrogen limitation induces LD accumulation in  
61 microorganisms, such as the oleaginous yeast *Yarrowia lipolytica*, microalgae and bacteria  
62 (3, 9, 13, 14). Thus, it is tempting to speculate that the TOR pathway, a well-established  
63 nitrogen sensing and discriminating pathway (15, 16), coordinates lipid metabolism with  
64 growth.

65 While in mammals there is only a single TOR kinase that participates as part of both  
66 mammalian TOR complexes 1 and 2 (mTORC1 and mTORC2) (17), the yeast *S. cerevisiae*  
67 owns two different TOR kinases, Tor1p and Tor2p. Both isoforms can interchangeably

68 form the TOR complex 1 (TORC1) but only Tor2p can participate in the TOR complex 2  
69 (TORC2) (18, 19). Aside from this difference, the activities of TORC1, which regulates  
70 energetic metabolism and growth, and TORC2, which regulates cytoskeleton organization,  
71 are well conserved among organisms (20). Particularly, only TORC1 is inhibited by  
72 rapamycin (19). In both yeast and mammals, mTORC1 is activated when nutrients are  
73 available promoting protein synthesis and growth. During nutrient starvation or rapamycin  
74 treatment, TORC1/mTORC1 is inhibited (17, 21). In yeast, TORC1 regulates the  
75 localization of several transcriptional factors coordinating transcriptional programs through  
76 two major distinct effectors: Sch9p, involved in ribosome biogenesis and translational  
77 regulation and Tap42p-PP2A, involved in metabolic regulation (15, 20, 22, 23). The PP2A  
78 branch was the first to be discovered. When TORC1 is activated, Tap42p is phosphorylated  
79 and forms heterodimers with PP2A (Pph21p and Pph22p) and a PP2A-like protein  
80 phosphatase (Sit4p) preventing the activity of downstream transcription factors. Upon  
81 TORC1 inhibition, (rapamycin treatment or nitrogen starvation), Tap42-PP2A/PP2A-like  
82 interaction is lost, the transcription factors Gln3p and Gat1p are dephosphorylated and  
83 transiently localized to the nucleus (20, 23, 24, 25). TORC1 also regulates other outputs  
84 through the Tap42-PP2A branch such as: the retrograde pathway that coordinates  
85 mitochondrial function to changes in transcription, through Rtg1p and Rtg3p transcription  
86 factors among others; and the environmental stress response, that coordinates a general  
87 transcriptional response to different stresses through the transcription factors Msn2p and  
88 Msn4p; among other outputs (20, 26, 27).

89 In mammals, there are evidences that the mTORC1 must be active to allow the induction  
90 of lipid biosynthesis genes by growth factors (28). It is also known that LD formation  
91 caused by leptin treatment is mTORC1-dependent (29). Besides its lipogenic role, the

92 activation of mTORC1 also results in suppression of lipolysis in adipocytes (30). Although  
93 reports on the regulation of mammalian LD formation are increasing, the regulation of  
94 yeast lipid metabolism by TORC1 has not been studied.

95 In this work, we explored the role of the TORC1 pathway in the metabolism of LDs in *S.*  
96 *cerevisiae* focusing on its Tap42p-Sit4p branch. Contrary to what has been shown in  
97 mammals, we discovered that treatment of cells with rapamycin, a specific TORC1  
98 inhibitor, promotes the shift from lipolytic to lipogenic phase by activating TAG but not SE  
99 synthesis. One of the proposed mechanisms is that the inhibition of the TOR pathway by  
100 rapamycin de-represses the nitrogen catabolite repression (NCR) pathway, since deletion of  
101 Gln3p and/or Gat1p blocks rapamycin effect on LD dynamics.

102

### 103 MATERIALS AND METHODS

104 **Yeast strains.** *Saccharomyces cerevisiae* strain BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0*  
105 *ura3Δ0*), the derived open reading frame knock out collection (MATa deletion library) and  
106 strains expressing proteins tagged with the TAP epitope (TAP collection) were obtained  
107 from Open Biosystems. BY4742 (MATa *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), *dga1Δlro1Δ*  
108 (MATa *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dga1::KanMX lro1::KanMX*) and *are1Δare2Δ*  
109 (MATa *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1::KanMX are2::KanMX*) were kindly provided  
110 by Sepp D. Kohlwein (University of Graz, Austria). Mutants *tor1-1* and *tor2-1* derived  
111 from the JK9-3da (MATa *leu2-3,112 ura3-52 rme1 trp1 his4 GAL+ HMLa*) strain. Mutants  
112 *gln3Δgat1Δ* (MATa *leu2 ura3 rme1 trp1 his3Δ GAL+ HMLa gln3::KanMX gat1::HISMX*)  
113 and *tap42-11* (MATa *leu2-3,112 ura3-52 rme1 GAL+ HMLa tap42::KanMX/*  
114 *YCplac111::tap42-11ts*) strains derived from the TB50a (MATa *leu2 ura3 rme1 his3Δ*

115 *GAL+* HMLa) strain were kindly provided by Dr. M. N. Hall (University of Basel, Basel,  
116 Switzerland).

117 **Reagents.** Rapamycin (Sigma-Aldrich St. Louis, MO) was prepared at 1 mg/ml in  
118 dimethylsulfoxide (DMSO). BODIPY® 493/503 (referred to here as BODIPY) was  
119 purchased from Invitrogen, prepared as a 10 mM stock solution in DMSO and kept at  
120  $-80^{\circ}\text{C}$ . All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

121 **Growth conditions and rapamycin treatment.** Yeast cells were grown to stationary  
122 phase for 48 hours in liquid rich medium (YPD; 1% yeast extract, 2% peptone and 2%  
123 glucose) or in SD medium (2% glucose, 0.67% yeast nitrogen base, and the required  
124 supplements) by shaking at  $30^{\circ}\text{C}$ . At this time, the cells achieve a maximum of LD content.  
125 The cells were then seeded in fresh YPD medium at a density of 0.25  $\text{OD}_{600\text{nm}}$  and grown  
126 by shaking at  $30^{\circ}\text{C}$ . After six hours, when the cultures reached the mid-log phase, the  
127 cultures were divided and either rapamycin at a final concentration of 100 ng/mL or vehicle  
128 alone was added. The cultures were further incubated for 12 hours with shaking at  $30^{\circ}\text{C}$ .  
129 Aliquots were taken for optical density ( $\text{OD}_{600\text{nm}}$ ) or LD index readings at the indicated  
130 time points.

131 **Lipid droplet quantification by liquid fluorescence recovery (LFR) assay.** Lipid  
132 droplets were quantified by incubating formaldehyde-fixed cells with a solution containing  
133 the fluorescent dye BODIPY 493/503 quenched with potassium iodide. In this assay  
134 BODIPY passively enters the cells, staining the LDs, as described (11). Briefly, aliquots (5  
135  $\text{OD}_{600\text{nm}}$ ) were withdrawn from the yeast cultures, harvested and fixed with formaldehyde  
136 (final concentration of 3.7% v/v) for 15 min at room temperature. Fixed cells were  
137 collected by centrifugation (3000 x g, 5 min) and washed once with distilled water. Pellets  
138 were resuspended in water and kept at  $4^{\circ}\text{C}$  until use. Readings were performed by adding

139 fixed cells to a reading medium containing the neutral lipid probe BODIPY 493/503  
140 (Invitrogen) (5  $\mu$ M) and the fluorescence quencher potassium iodine (KI) (0.5 M).  
141 Fluorescence was read at 495/510 nm in a Spectramax M5 plate reader (Molecular  
142 Devices) sequentially with absorbance ( $Abs_{600nm}$ ). The LD index, which reflects the cell  
143 neutral lipid content is expressed as the ratio of fluorescence intensity to  $Abs_{600nm}$  of the  
144 cells. To obtain the relative LD index (rLD index) results were normalized to the LD index  
145 of stationary phase cells.

146 **Confocal fluorescence microscopy.** LDs were visualized by microscopy. Yeast cells  
147 were fixed with formaldehyde, as described above, and resuspended in water. 1  $\mu$ l of 10  
148 mM BODIPY 493/503 and 3  $\mu$ l of vector mounting medium for fluorescence  
149 (VECTASHIELD®) were added to 2  $\mu$ l of fixed cells. Images were captured using an  
150 Olympus IX 81 microscope with a 100x oil immersion objective.

151 **Lipid analysis.** Yeast cells were harvested by centrifugation at 3000 x g for 5 min at  
152 room temperature and washed once with cold distilled water. Lipids were extracted based  
153 on a modified protocol described by Bligh and Dyer using chloroform/methanol/water as  
154 solvents (31) and final extract was dried under a stream of nitrogen and stored at 20°C.  
155 Lipids were resuspended in chloroform and applied to silica plates to perform thin layer  
156 chromatography (TLC) employing triolein and cholesteryl oleate as standards (Sigma-  
157 Aldrich St. Louis, MO). Neutral lipids were separated in an ascending manner by using a  
158 two-step separation system: light petroleum/diethyl ether/acetic acid (35:15:1 v/v) as a  
159 solvent system developed to 2/3 of the height of the plate, followed by light  
160 petroleum/diethyl ether (49:1 v/v) solvent system until 1 cm of the top (32). Lipids were  
161 revealed with iodine vapor and spots were quantified by densitometry using Image Master  
162 TotalLab 1.11 (Amershan Pharmacia Biotech, England).

163 For the enzymatic determination of triacylglycerol content, cells were centrifuged and  
164 resuspended in 300  $\mu$ l of extraction buffer (50 mM Tris-HCl, 0.3% Triton X-100, pH 7.5)  
165 and lysed with glass beads, by vortexing for 5 cycles of 30 seconds each. Lysed cells were  
166 separated, and the glass beads were washed with 300  $\mu$ l of extraction buffer. The total  
167 lysate was centrifuged at 3000 rpm for 10 min. Neutral lipids were extracted from 200  $\mu$ l of  
168 the supernatant as described by Bligh & Dyer (33). Triacylglycerols were measured as  
169 previously described (11) using the triacylglycerol reagent kit (Doles, Brazil) according to  
170 the manufacturer's instructions against glycerol standards. Intracellular TAG was  
171 normalized by the protein concentration.

172 **Preparation of protein homogenates and western blot.** Protein homogenates were  
173 prepared as previously described (34). Briefly cells were centrifuged and the pellet was  
174 resuspended and incubated on ice for 10 min 0.2 M NaOH and 0.2% of 2-mercaptoethanol.  
175 After addition of 5% trichloroacetic acid, they were further incubated for 10 min on ice.  
176 Total protein was collected by centrifugation, resuspended in Laemlli sample buffer and  
177 immediately heated for 5 min at 80 °C. 10  $\mu$ l aliquots, corresponding to approximately 0.16  
178 OD cells, were separated in 6% SDS-acrylamide gel using the Mini-Protean II (BioRad)  
179 and electro-transferred to Immobilon-P for 30 min at 18 V in 25 mM Tris, 192 mM glycine  
180 and 10% methanol, using a trans-blot semi-dry cell (BioRad). After transfer, membranes  
181 were treated with 5% non-fat dry milk solution in TBS-T buffer for 1 h at room temperature  
182 and then incubated overnight at 4°C with 1:10.000 dilution of anti-TAP antibody (Open  
183 Biosystems). Blots were detected using the chemiluminescence ECL Plus kit (GE  
184 Healthcare). For total protein quantification after anti-TAP western blots, membranes were  
185 incubated for 2 min in staining solution (0.1% Coomassie brilliant blue R in 50% methanol,



186 7% acetic acid) and then for 10 min in destaining solution (50% methanol, 7% acetic acid).

187 Membranes were washed with distilled water and air dried.

188 **qRT-PCR.** RNA was isolated as previously described (35). The cDNA was synthesized  
189 from extracted RNA using the High Capacity cDNA Reverse Transcription Kit (Applied  
190 Biosystems) as indicated by the manufacturer and further used as a template for Real Time  
191 PCR using SYBR-Green PCR Master Mix (Applied Biosystems, CA, USA).  
192 Amplifications were performed in a Step One Plus or 7500 Real-Time PCR System  
193 (Applied Biosystems, CA, USA). PCR amplification was carried out under the following  
194 conditions: 95°C for 10 min, 40 cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 90 s.  
195 The threshold cycle (Ct) for each gene of interest was calculated using Step One Software  
196 v2.2 or 7500 Software v2.0.5. Normalization for each gene was performed against the  
197 mock sample (treated with vehicle alone) and gene expression levels were based on the 2-  
198  $\Delta\Delta C_t$  method (36)(37) by calculating the relative copy number (RCN) using *ACT1* gene as  
199 an internal control. The primers used were:

200 CIT2F: 5'GCATTTGGTATTCTTGCTCAA3'

201 CIT2R: 5'TGCTTTCAATGTTTTTGACCA3',

202 ACT1F: 5'TTCCCAGGTATTGCCGAAA3'

203 ACT1R: 5'TTGTGGTGAACGATAGATGGA3'

204 **Statistical analysis.** The statistical significance of the results was evaluated using  
205 student *t*-tests. Analysis for differences in drug treatment and control during time courses  
206 were performed by two-way ANOVA with the repeated measures test and the Bonferroni  
207 post-test using the GraphPrism 5.0 software.

208

209 **RESULTS**210 **Inhibition of the TORC1 pathway by rapamycin induces LD accumulation in yeast.**

211 We investigated the effect of rapamycin, a TORC1 inhibitor, on LD dynamics in yeast by  
212 using a liquid fluorescence recovery (LFR) assay previously described by our group (11).  
213 Stationary cells were seeded at a low density in fresh rich medium and incubated for 6  
214 hours at 30°C before the addition of rapamycin. We have chosen the 6 hour time point to  
215 add rapamycin because this is the moment when the LD index reaches the lowest value  
216 under this experimental condition. We observed that rapamycin induced a faster increase in  
217 LD replenishment compared to non-treated yeast cells (Fig. 1A). Fluorescence microscopy  
218 showed that rapamycin treated cells stained more intensely with BODIPY than the non-  
219 treated yeast cells (Fig. 1C and D), reinforcing LFR assay results. We have also tested the  
220 effect of the addition of rapamycin just after the seeding in fresh rich medium and observed  
221 that in this case, rapamycin blocks the lipolytic phase and slightly induces LD accumulation  
222 (Fig.1B).

223 While mammalian cells present a unique TOR kinase named mTOR, the yeast *S.*  
224 *cerevisiae* presents two TOR isoforms, Tor1p and Tor2p (17, 18, 38). In yeast, both  
225 isoforms can participate with other proteins in the formation of the TOR complex 1  
226 (TORC1), but only Tor2p can participate in the TOR complex 2 (TORC2) (19). Despite  
227 this difference, like in mammalian cells, only TORC1 is affected by rapamycin treatment  
228 (19). Rapamycin acts forming a complex with FKBP12, which interacts with Tor1/2p  
229 kinases inhibiting TORC1 activity. The rapamycin-resistant mutants *tor1-1* and *tor2-1*, first  
230 reported by Heitman *et al* (18), present mutations that disrupt FKBP-rapamycin binding site  
231 in Tor1/2 avoiding TORC1 inhibition (39). The resistance to rapamycin conferred by these

232 alleles is dominant, as one TORC1-resistant complex is enough to guarantee TORC1  
233 function upon rapamycin treatment (18, 39).

234 In order to investigate whether the rapamycin effect is indeed caused by the specific  
235 inhibition of the TORC1, we studied the effect of rapamycin on the LD dynamics of *tor1-1*  
236 and *tor2-1* mutants. We observed that these mutations do not affect LD dynamics when  
237 compared to the isogenic WT strain under normal growth conditions, but both mutants fail  
238 to respond to rapamycin (Fig. 2A-C).

239 Previous studies show that *CIT2*, encoding the peroxisomal form of citrate synthase  
240 (40), is highly induced by rapamycin treatment (27, 41). In order to confirm that TORC1  
241 pathway is being activated under our experimental conditions, we determined the effect of  
242 rapamycin on the *CIT2* mRNA and protein levels by quantitative RT-PCR and immunoblot  
243 assays, respectively (Fig. 2D and E). We found that *CIT2* expression is highly induced by  
244 rapamycin treatment at both the mRNA and protein levels. Taken together, these results  
245 confirm that the TORC1 pathway is inhibited by rapamycin under the conditions tested and  
246 that the effects of rapamycin on the LDs are consequence of the inhibition of TORC1  
247 suggesting that TORC1 is involved in the regulation of LD homeostasis in yeast.

248 **Growth inhibition does not necessarily correlate to LD accumulation and vice-**  
249 **versa.** It has been described that in different microorganisms, as in microalgae and the  
250 yeast *Yarrowia lipolytica*, lipid accumulation could be a consequence of slower growth  
251 rates (9, 42, 43). To investigate if the lipogenic effect of rapamycin was related to inhibition  
252 of the growth we submitted cells to different types of stress, such as osmotic stress (1M  
253 sorbitol), saline stress (1M NaCl) and nitrogen-starvation (Fig. 3). Osmotic stress reduced  
254 growth but did not affect LD homeostasis after 2 hours of treatment. To confirm this effect  
255 we extended the incubation to 24 hours and no effect on LD dynamics was observed

256 compared to non-treated yeast cells (Fig. 3A). On the other hand, saline stress reduced  
257 growth and induced LD synthesis at short-time incubations (2 hours of treatment) (Fig. 3B).  
258 Nitrogen starvation did not reduced growth in the first 5 hours of treatment but induced the  
259 LD levels within this period (Fig. 3C). As a control for the nitrogen starvation experiment,  
260 we tested the effect of rapamycin in the same experimental conditions (SD medium). We  
261 observed that both induction of LD replenishment and inhibition of growth were similar as  
262 observed in complete medium (Fig. 3D). These results show that the LD accumulation  
263 observed in different stress conditions, including rapamycin treatment, are not necessarily  
264 related to the decrease in growth rate.

265 **LD replenishment induced by rapamycin depends on induction of TAG but not SE**  
266 **synthesis.** The core of LD is formed by TAG and SE. The LD index analyzed by the LFR  
267 assay might reflect changes in both neutral lipids, so we investigated whether the TAG  
268 and/or the SE levels were increased by rapamycin. Analysis of neutral lipids by TLC  
269 showed that only the TAG content was induced after 2 hours of rapamycin treatment (Fig.  
270 4A), which led to an increase in the TAG to SE ratio (Fig. 4B). Enzymatic determination of  
271 TAG content corroborated the TLC results showing a TAG content 40% higher in  
272 rapamycin-treated cells compared to non-treated cells ( $62.0 \pm 7.0$  nmol TAG x mg protein<sup>-1</sup>  
273 in rapamycin-treated cells in contrast to  $43.0 \pm 12$  nmol TAG x mg protein<sup>-1</sup> in control  
274 cells; n=3, p < 0.05). The specific effect of rapamycin on TAG synthesis was reinforced by  
275 the results showing that rapamycin does not affect LD in the strain deleted of the main  
276 DAG acyltransferases (*dgal1Δrol1Δ*), but it does on the strain deleted of the main sterol  
277 acyltransferases (*are1Δare2Δ*) (Fig. 4E-G).

278 It is believed that the LD levels reflects an equilibrium state between continuous  
279 synthesis and hydrolysis of neutral lipids (10). Thus, the rapamycin effect observed could  
280 be due to activation of neutral lipid synthesis and/or to inhibition of neutral lipid  
281 mobilization. Kurat and co-workers (34) have shown that the onset of the lipolytic phase  
282 depends on the activation of Tgl4p lipase by phosphorylation. Thus, we tested whether  
283 rapamycin decreased the phosphorylation level of Tgl4p. We observed that the ratio  
284 between phosphorylated and non-phosphorylated forms of Tgl4p was not changed by  
285 rapamycin treatment (Fig. 4C and D), although we observed a reduction in total Tgl4p level  
286 at two hours of rapamycin treatment. The latter effect could be related to general rapamycin  
287 induced protein degradation (44). This result suggests that rapamycin reduces the level of  
288 Tgl4p thus altering the balance between lipolysis and lipogenesis, favoring the latter.

289 **Involvement of the Tap42-PP2A branch of the TOR pathway on the LD induction**  
290 **by rapamycin.** It has become clear that in yeast the TORC1 monitors nutrients and stress  
291 signals, and then transduces these signals among two main signaling branches: the Sch9p  
292 branch, that regulates protein and ribosome synthesis genes; and the Tap42-PP2A branch,  
293 that participates in the regulation, mostly of metabolism genes during nitrogen starvation  
294 and rapamycin treatment (20, 22, 23). In the next experiments, we studied the participation  
295 of the PP2A-branch in the modulation of LD homeostasis by rapamycin. We chose to study  
296 this branch because we have shown that, just as rapamycin, nitrogen starvation enhances  
297 LD accumulation (Fig. 3C). Also, we have previously shown that the deletion of the PP2A-  
298 like Ser/Thr protein phosphatase Sit4 leads to lower levels of LDs in a growing culture via  
299 modulation of AMPK/Snf1p phosphorylation (11). This kinase is known to regulate lipid  
300 metabolism by controlling the activity of acetyl CoA carboxylase (ACCase) (45, 46). First,  
301 we tested the effect of rapamycin on LD levels in a *sit4Δ* strain. We observed that the

302 deletion of *SIT4* partially suppresses the induction of LD accumulation caused by  
303 rapamycin (Fig. 5A). The role of Sit4p on the response to rapamycin treatment is probably  
304 not related to its known role in regulating Snf1p (11) because rapamycin can induce LD  
305 levels in a *snf1Δ* strain (Fig. 5B). Furthermore, we could not detect any effect of rapamycin  
306 on the phosphorylation state of Thr210-Snf1p under this experimental condition (data not  
307 shown).

308 TORC1 signaling through the protein phosphatase Sit4 requires its regulatory protein  
309 Tap42p (23, 26). Tap42p is essential to cell viability so we tested the effect of rapamycin  
310 on a strain carrying the temperature-sensitive *tap42-11* allele, which is partially resistant to  
311 rapamycin at 25°C. We observed that this mutant was unresponsive to rapamycin and LD  
312 levels were not increased within 2 hours of treatment (Fig. 6). Sit4-Tap42 effectors include  
313 the GATA-family transcription factors Gln3p and Gat1p (15, 47), the heterodimeric  
314 transcriptional activators Rtg1p and Rtg3p (20, 41) and the Zn-finger transcription factors  
315 Msn2p and Msn4p (26, 48). These factors regulate the nitrogen catabolic response, the  
316 retrograde pathway and the general stress response, respectively. We next explored the  
317 involvement of these PP2A-branch effectors. We observed that LD induction by rapamycin  
318 was significantly diminished in the *gln3Δ*, *gat1Δ*, *rtg1Δ* and *rtg3Δ* strains, but not in the  
319 *msn2Δ* and *msn4Δ* mutant strains (Fig. 7). Failure to reduce the LD induction in *msn2Δ* and  
320 *msn4Δ* strains could be due to their overlapping function of Msn2p and Msn4p, however  
321 the double deleted strain *msn2Δmsn4Δ* still responded to rapamycin (data not shown) ruling  
322 out this possibility.

323 **Nitrogen starvation and lipid droplet synthesis in *S. cerevisiae*.** The transcription  
324 factors Gln3p and Gat1p regulate the expression of genes involved in nitrogen catabolism.

325 Upon nitrogen limitation, Gln3p/Gat1p are activated and promote the transcription of genes  
326 important for the utilization of alternative nitrogen sources. It is well known that  
327 Gln3p/Gat1p activation and nuclear translocation are dependent on TORC1 inhibition (16),  
328 (49, 50). We studied the LD profile of *gln3Δ*, *gat1Δ* and the double mutant *gln3Δgat1Δ*.  
329 Interestingly, we found that these strains had an aberrant LD profile, in which the lipolytic  
330 and lipogenic phases were not clearly identified (Fig. 8A and B).

331

## 332 **DISCUSSION**

333 **Rapamycin or nitrogen starvation induces LDs accumulation in *S. cerevisiae*.** We  
334 investigated the role of the TORC1 pathway in the regulation of LD dynamics in the yeast  
335 *S. cerevisiae*. Rapamycin, a TORC1 inhibitor that mimics several outputs of nitrogen  
336 starvation, was added to yeast cultures. As expected, addition of rapamycin (Fig. 1A and  
337 3D) and nitrogen starvation (Fig. 3C) readily promoted a shift to a lipogenic phase when  
338 cells are treated at the lipolytic phase. The rapid accumulation of LDs was accompanied by  
339 an increase in TAG but not SE levels (Fig. 4A and B). We could not observe differences in  
340 the phosphorylation state of the major TAG lipase in the first hour of rapamycin treatment  
341 (Fig. 4C and D), although Tgl4p was partially degraded at longer incubation times (2h).  
342 These results suggest that induction of LD synthesis could be due in part to induction of  
343 TAG synthesis and reduction of lipolysis.

344 Different metabolic pathways could be used by TORC1 to induce TAG synthesis, as *de*  
345 *novo* fatty acid synthesis by the glyceroneogenesis pathway or the use of free amino acid  
346 pool as a result of rapamycin induced-autophagy. From these we have discarded *de novo*  
347 synthesis of fatty acids by glyceroneogenesis since key enzymes in this pathway, such as

348 phosphoenolpyruvate carboxykinase, were not induced by rapamycin (data not shown). We  
349 also did not detect incorporation of labeled  $^{14}\text{C}$  from  $^{14}\text{C}$ -pyruvate into TAG after  
350 rapamycin treatment (data not shown). TORC1 inhibition was already demonstrated to  
351 promote autophagy (20), which might provide blocks for TAG synthesis. It has been shown  
352 that hepatocytes and cardiac miocytes accumulate LDs after short periods of starvation  
353 which is dependent on the autophagic protein Atg7 (51). Additional experiments are needed  
354 to study if in yeast the induction of TAG synthesis by rapamycin is indeed linked to  
355 autophagy.

356 **Growth impairment is not necessarily linked to LD accumulation.** A main concern  
357 about our results was that TORC1 is known for its role as a coordinator of cellular growth  
358 in response to nutrient availability. Since TORC1 inhibition leads to diminished growth  
359 rates and LD dynamics is profoundly affected by growth phase, we questioned if the  
360 replenishment of LDs induced by rapamycin was a non-specific effect of growth  
361 impairment. If this was true, we would expect that inhibition of growth by other stresses  
362 would affect LD dynamics. Our evidences showed that LDs accumulation was not always  
363 connected to growth inhibition. First, osmotic stress impaired growth without affecting LDs  
364 dynamics (Fig. 3A). Second, if reduced growth and LD synthesis were linked phenomena,  
365 the *sit4* $\Delta$  mutant would break this rule as it shows slow growth but lower levels of LDs  
366 (11).

367 **TORC1 is implicated in lipid homeostasis.** LDs dynamics are linked to progression of  
368 the cell cycle as evidenced by the activation of Tgl4p lipase by the cyclin-dependent kinase  
369 1, Cdk1/Cdc28 and the acute lipolytic activity observed in pre-logarithmic growth (10, 34,  
370 52). We can speculate that TORC1 is part of the cross-talk between regulators of cell cycle



371 and LD dynamics. Here we provide evidence that the TORC1 pathway is involved in LD  
372 homeostasis since deletion of downstream effectors such as the Ser-Thr phosphatase Sit4p  
373 (Fig. 5A) and the transcription factors Gln3p/Gat1p, led to abnormal LD dynamics (Fig. 8).

374 Besides TORC1, the AMP activated kinase (AMPK or Snf1p, in yeast cells) is also a  
375 hub that senses the energetic status of the cell and triggers the cellular response to a variety  
376 of stresses (53, 54). The existence of a crosstalk between TOR and Snf1p/AMPK pathways  
377 is still controversial in yeast. In mammals, AMPK and mTORC1 pathway are linked, since  
378 AMPK is able to inhibit mTORC1 through the phosphorylation of the upstream TOR1  
379 regulators, TSC2 and Raptor, when the cellular energy level is low (55, 56). In yeast, such  
380 regulation is not yet well established. Evidences suggest that TORC1 and Snf1p/AMPK  
381 converge in the regulation of fatty acid metabolism and other outputs, independently (57).  
382 A recent work provided strong evidences that Snf1p/AMPK would participate as a possible  
383 upstream regulator of TORC1 pathway in glucose limiting conditions (58).

384 In fact, previous studies demonstrated that Sit4p and Reg1p-Glc7 phosphatase, are  
385 negative regulators of Snf1p/AMPK activity (11, 59, 60). In *sit4Δ* and *reg1Δ* mutants,  
386 Snf1p/AMPK is hyper-activated which might inhibit acetyl-CoA carboxylase activity, a  
387 key enzyme in the synthesis of fatty acids (11). Preliminary, we hypothesized that the effect  
388 of rapamycin on LDs replenishment could be via Snf1p/AMPK. Thus, activation of TORC1  
389 pathway would have a negative effect on the accumulation of LD. We had to discard this  
390 hypothesis because the deletion of *SNF1* did not affect the cellular response to rapamycin  
391 (Fig. 5B).

392 By which mechanism did the inhibition of TORC1 induce the shift to the lipogenic  
393 phase? Summarizing our data, we believe that the modulation of LD homeostasis by

394 TORC1 requires the PP2A-like phosphatase, Sit4p, as well as the Gln3p and Gat1p  
395 transcriptional complex (Fig. 9). This assumption is based on the observation that strains  
396 deleted on the corresponding genes have aberrant LD dynamics during growth, where the  
397 transition between the lipolytic and lipogenic phase was not clearly observed (Fig. 8).  
398 Furthermore, *sit4Δ*, *gln3Δ* and *gat1Δ* mutants did not respond to rapamycin by inducing LD  
399 replenishment in the first 2 hours of treatment. Thus, rapamycin-induced NCR genes might  
400 be involved in the regulation of lipid homeostasis in batch cultures.

401 **TORC1 inhibition by rapamycin has different outcomes in yeast and mammalian**  
402 **cells.** Our results showing that TORC1 inhibition in yeast leads to LD synthesis do not  
403 agree with the role of the mTOR pathway in mammals, where it is well-established that the  
404 activation of the TORC1 pathway is essential to promote lipogenesis (28, 61).

405 One possible reason for the different outcomes of TORC1 activation/inhibition on LD  
406 homeostasis between yeast and mammals are the different roles of LDs in those organisms.  
407 Although LDs have common roles such as lipid storage and protection against lipotoxicity  
408 in both organisms (4, 62, 63), in mammals, LDs are involved in other biological processes  
409 such as immune response (64) and even viral replication (65). The role of LDs in those  
410 differential processes could explain the different regulatory role of TORC1 in LD  
411 homeostasis.

412 Another possible explanation for the different outcomes regarding LD homeostasis of  
413 activation/inactivation of TORC1 in yeast and mammals is the different strategies used by  
414 these organisms during nutrient limitation conditions. For example, contrary to what is  
415 expected for most mammalian cells, yeast cells consume glycogen when they are presented  
416 with proper growth conditions (e.g. fresh medium) which allows rapid growth, and

417 accumulate glycogen when the nutrients starts to become limited (66). As unicellular  
418 organisms, yeast cells cannot rely on other cells to offer them nutrients under nutrient  
419 starvation conditions. When nutrients starts to become limiting, they slow down division  
420 and start to accumulate nutrients to bear with the rough times ahead (12). If we apply this  
421 same reasoning to lipids, it does makes sense that yeast cells utilize lipids when they are  
422 presented proper growth conditions (e.g. fresh medium) and evidence shows that they use  
423 this lipids for rapid membrane synthesis (10, 52). When cells sense a stress or nutrient  
424 limitation conditions, they accumulate neutral lipids. In this scenario, it does make sense  
425 that a signaling pathway that coordinates growth with nutrient availability in yeast, such as  
426 the TORC1 pathway (20), would activate lipolysis under proper growth conditions to  
427 provide building blocks and energy for proliferation and would induce neutral lipids storage  
428 and growth arrest under nutrient limiting conditions. It will be interesting to understand  
429 evolutionarily how a well-conserved signaling cascade such as the TORC1 pathway  
430 diverged to completely opposite outputs to maintain lipid homeostasis in different  
431 organisms. Further work needs to be performed to address this question.

432

#### 433 **ACKNOWLEDGMENTS**

434 This work was supported by grants from Conselho Nacional de Ciência e Tecnologia  
435 (CNPq), Masters Degree and PhD degree Fellowship to JBM (CNPq), postdoctoral  
436 fellowship CAPES-PNPD Institucional to BBM, the Fundação de Amparo a Pesquisa do  
437 Rio de Janeiro (FAPERJ-Cientistas do Nosso Estado to MML), Iniciação Científica  
438 fellowship to GSM (CNPq). We gratefully acknowledge Suelene Francisca Bispo,  
439 Andressa Piedade Motta and Dr. Jens Rietdorf (CAPES/CDTS) for their technical  
440 assistance.

441

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- 620
- 621

622 **FIGURE LEGENDS**

623

624 **FIG 1** Rapamycin induces a rapid LD replenishment. Wild type (BY4741) yeast cells were  
625 pre-grown for 48 h to stationary phase and then inoculated into YPD fresh medium at low  
626 density (0.25 OD<sub>600nm</sub>). Rapamycin (100 ng/ml) (●) or vehicle alone (○) were added after 6  
627 h of growth (A) or at the time of seeding (B). The LD indexes were measured using the  
628 LFR assay and normalized to that obtained in pre-grown stationary cells (relative LD Index,  
629 rLD) (upper panel). Growth was determined by absorbance at 600 nm (lower panel). (C)  
630 Aliquots from the control culture (upper panel) or rapamycin-treated cells (lower panel)  
631 after two hours of rapamycin treatment, from experiment A, were analyzed by confocal  
632 fluorescence microscopy. Cells were stained with BODIPY<sup>®</sup> (green) and DAPI (blue)  
633 (scale bar = 2 μm). (D) Intensity of fluorescence was determined as described in Material  
634 and Methods section. Data are from three independent experiments ± standard deviations  
635 (\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 control vs rapamycin treatment).

636

637 **FIG 2** Rapamycin effect on LDs is due to the inhibition of the TORC1 pathway. (A) *tor1-*  
638 *1*, (B) *tor2-1* and (C) WT (JK9-3da) strains were treated with rapamycin (100 ng/ml) (●) or  
639 vehicle alone (○) as described in Fig. 1A. The LD indexes were measured and normalized  
640 to that obtained in pre-grown stationary cells (rLD Index) (upper panel) and growth was  
641 determined by absorbance at 600 nm (lower panel). (D) Rapamycin strongly induces *CIT2*  
642 expression. WT (BY4741) strain was treated with vehicle alone (white bars) or rapamycin  
643 (100 ng/ml) (gray bars) for 30 min up to 2 h and the relative copy number (RCN) of *CIT2*  
644 transcript was determined by real time PCR. The *ACT1* gene was used for normalization.  
645 Data are from three independent experiments respectively ± standard deviations (\*p<0.05

646 and  $p^{**}<0.01$ ). (E) Cit2p-TAP protein levels were analyzed by Western-Blot using the anti-  
647 TAP antibody (upper panel). Densitometry is from three independent experiments  
648 ( $***p<0.001$  between rapamycin and control).

649

650 **FIG 3** LD dynamics after saline stress, osmotic stress and nitrogen starvation. A and B  
651 Wild type (BY4741) yeast cells were inoculated into YPD fresh medium and after reaching  
652 exponential phase (5h) they were treated with none ( $\circ$ ) or 1M sorbitol ( $\bullet$ ) or 1M NaCl ( $\circ$ ).  
653 For nitrogen starvation (N-starvation) the wild type (BY4741) yeast cells were inoculated  
654 in complete SD (0.17% yeast nitrogen base, 0.5%  $\text{NH}_2\text{SO}_4$ , 2% glucose and supplements)  
655 and after reaching exponential phase the culture was filtered through a 0.22  $\mu\text{M}$  Millipore  
656 filter and re-seeded in complete SD ( $\circ$ ) or SD without  $\text{NH}_2\text{SO}_4$  ( $\bullet$ ). The effect of nitrogen  
657 starvation was compared to rapamycin treatment (100 ng/ml) ( $\bullet$ ) or addition of vehicle  
658 alone ( $\circ$ ) in cells grown in complete SD. The rLD index (upper panels) and growth (lower  
659 panels) was determined. Data are from three independent experiments  $\pm$  standard deviations  
660 ( $*p<0.05$ ,  $p^{**}<0.01$  and  $***p<0.001$ ).

661

662 **FIG 4** (A and B) Rapamycin-induced LD accumulation depends on TAG synthesis. WT  
663 (BY4741) cells were treated as in Fig. 1A with rapamycin (100 ng/ml) (gray bars) or  
664 vehicle alone (white bars). After 2 h of treatment cells were harvested and lipids were  
665 extracted and analyzed by TLC. (A) The content of sterol ester (SE) and triacylglycerol  
666 (TAG) after rapamycin treatment was estimated relative to samples treated with vehicle  
667 alone. (B) The ratio between TAG/SE was calculated in control yeast cells (white bars) and  
668 rapamycin treated cells (gray bars). Data are from three independent experiments  $\pm$

669 standard deviations. (\* $p < 0.05$ , ns = non-significant). (C and D) Phosphorylation of Tgl4p  
670 is not induced by rapamycin. (C) The Tgl4-TAP strain was grown as in Fig. 1A and treated  
671 with rapamycin (100 ng/ml) or vehicle alone after 6 h of seeding. Total protein was  
672 extracted and after separation of proteins in 6% SDS-PAGE, Tgl4p-TAP was revealed by  
673 Western Blotting using anti-TAP (1:10,000) antibody. (D) The ratio of phosphorylated  
674 Tgl4p to non-phosphorylated Tgl4p was quantified by densitometry. (E-G) Rapamycin-  
675 induced LD replenishment is dependent on TAG but not SE synthesis. (E) WT (BY4742)  
676 background, (F) *are1 $\Delta$ are2 $\Delta$* , a mutant strain deficient in SE synthesis, and (G)  
677 *dgal $\Delta$ tro1 $\Delta$* , a strain deficient in TAG synthesis, were treated with rapamycin ( $\circ$ ) or  
678 vehicle alone ( $\bullet$ ) as described in Fig. 1A. The rLD indexes (upper panel) and culture  
679 growth (lower panel) were recorded. Data are from three independent experiments  $\pm$   
680 standard deviations (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ).

681

682 **FIG 5** Rapamycin-induced LD replenishment is dependent on *SIT4*. *sit4 $\Delta$*  (A) or *snf1 $\Delta$*  (B)  
683 mutant strains were treated with (100 ng/ml) ( $\bullet$ ) or vehicle alone ( $\circ$ ) as in Fig. 1A. The  
684 relative LD index was normalized to the LD index obtained in WT (BY4741) cells pre-  
685 grown to stationary state (upper panels) and culture growth was determined (lower panels).  
686 Data are from three independent experiments  $\pm$  standard deviations (\* $p < 0.05$  and  
687 \*\*\* $p < 0.001$ ).

688

689 **FIG 6** Tap42p is important to mediate LD replenishment by rapamycin. (A) *sit4 $\Delta$*  and (B)  
690 the temperature sensitive *tap42-11* mutant strains were treated with (100 ng/ml) ( $\bullet$ ) or  
691 vehicle alone ( $\circ$ ) as in Fig. 1 for 2 h. The temperature sensitive *tap42-11* strain was grown



692 at 25 C, where it is partially resistant to rapamycin. The LD indexes were measured and  
693 normalized to that obtained in WT (BY4741 and TB50-a, respectively) pre-grown  
694 stationary cells using the LFR assay (upper panels) and culture growth was determined  
695 (lower panels). Data are from three independent experiments  $\pm$  standard deviations  
696 (\* $p$ <0.05 and \*\*\* $p$ <0.001).

697

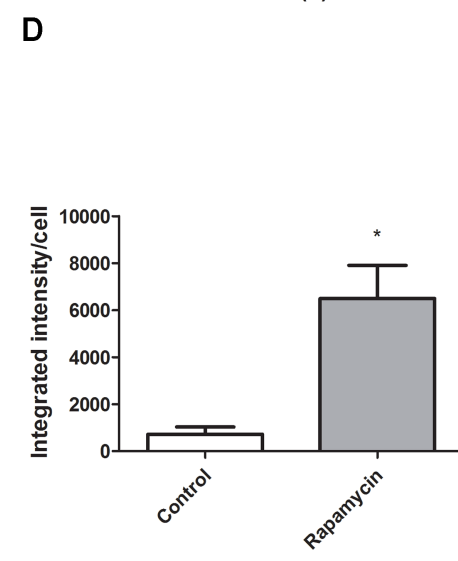
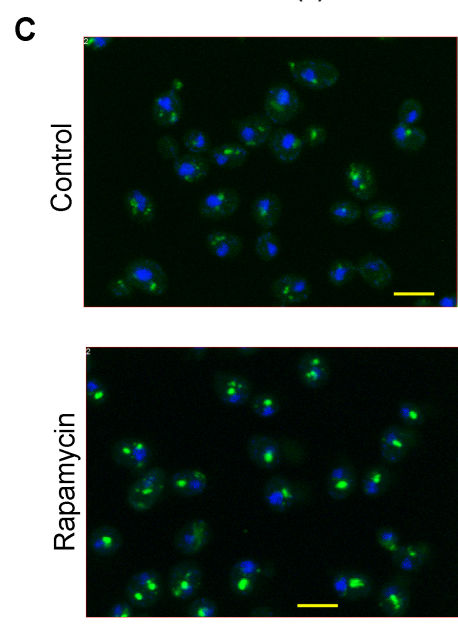
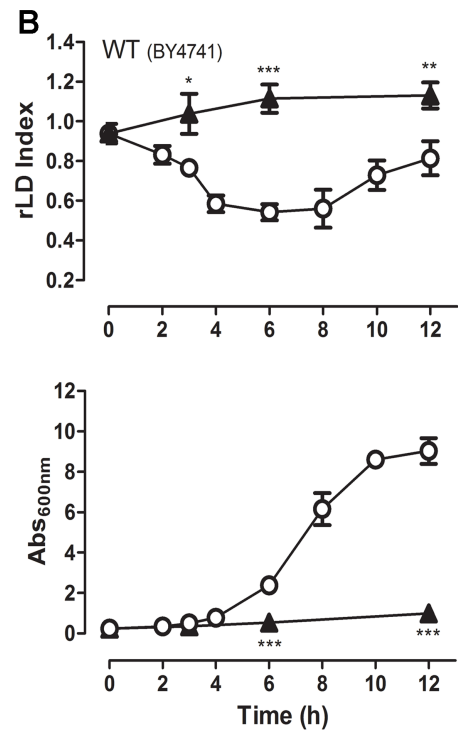
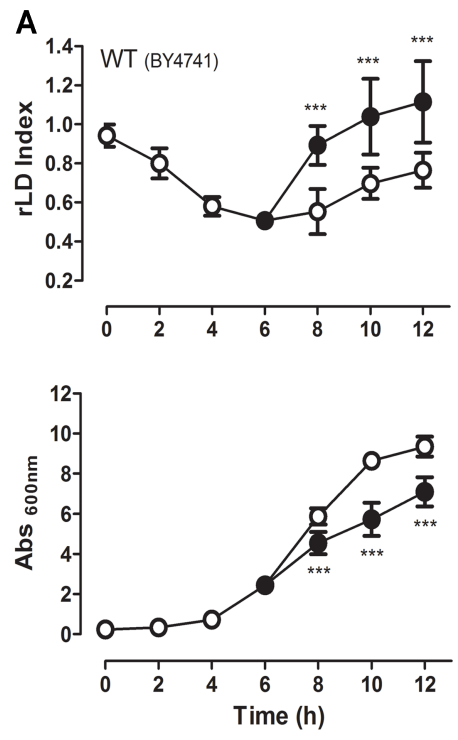
698 **FIG 7** TORC1-PP2A downstream effectors participate on LD replenishment induced by  
699 rapamycin. The indicated deleted strains were treated with rapamycin (100 ng/ml) or  
700 vehicle alone as shown in Fig. 1 and after 2 h of treatment. LD induction (A) as well as  
701 growth inhibition (B) caused by rapamycin treatment was calculated. Data are from three  
702 independent experiments  $\pm$  standard deviations (\* $p$ <0.05 and \*\* $p$ <0.01).

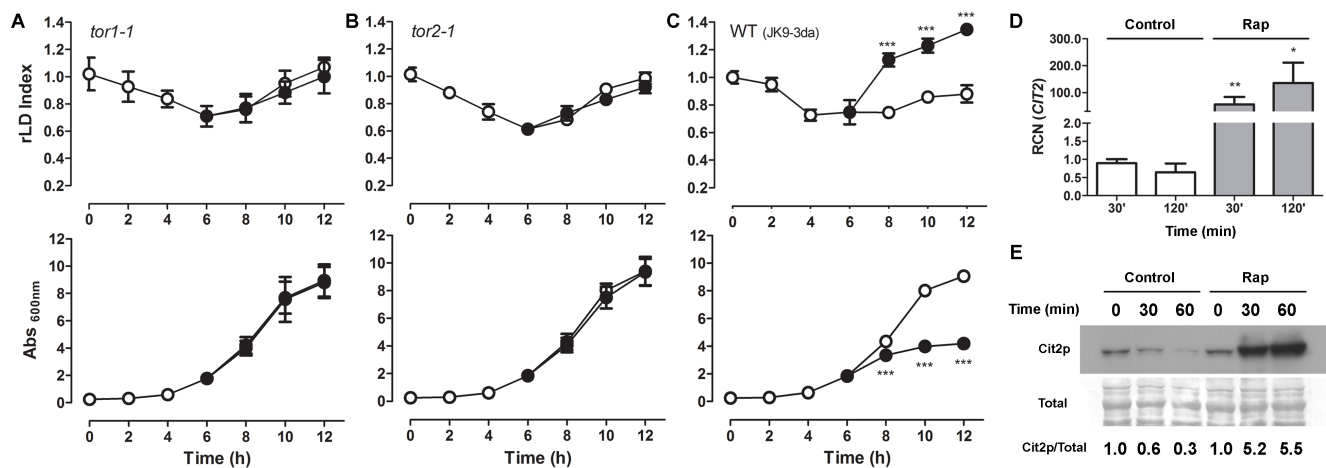
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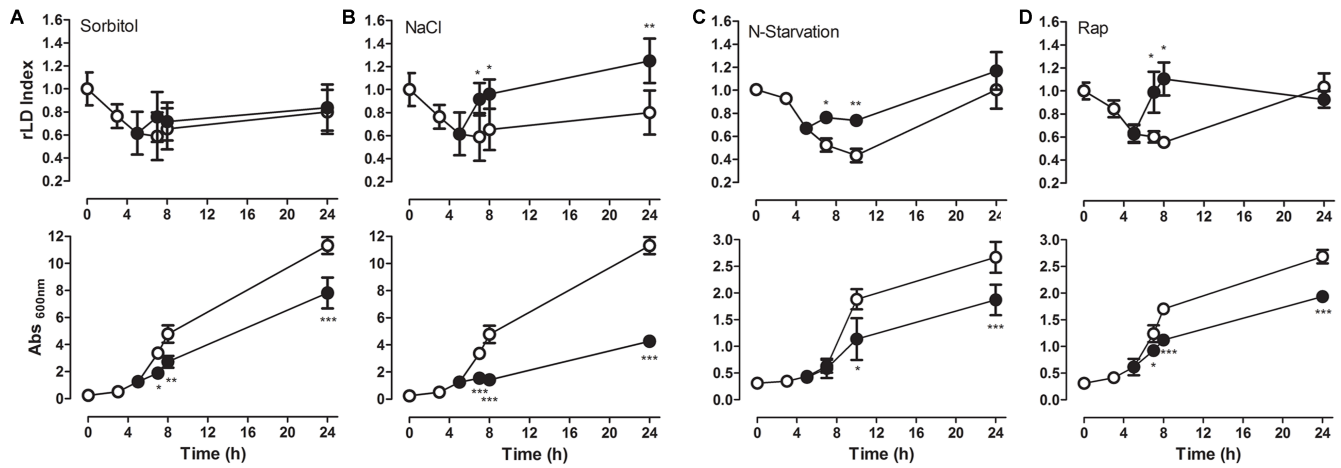
704 **FIG 8** Disruption of Gln3p and Gat1p transcription factors affect LD dynamics. *gln3* $\Delta$ ,  
705 *gat1* $\Delta$ , *gln3* $\Delta$ *gat1* $\Delta$  and its isogenic strains were grown in YPD and the LD dynamics and  
706 growth was followed. (A) *gln3* $\Delta$  ( $\circ$ ), *gat1* $\Delta$  ( $\Delta$ ) and the isogenic strain WT (BY4741)  
707 (gray,dotted line). (B) *gln3* $\Delta$ *gat1* $\Delta$  ( $\circ$ ) and its isogenic strain WT (TB50-a) (gray, dotted  
708 line). Data are from three independent experiments  $\pm$  standard deviations (\* $p$ <0.05,  
709  $p$ \*\*<0.01 and \*\*\* $p$ <0.001).

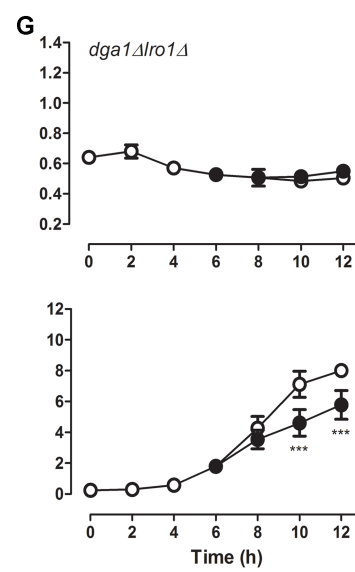
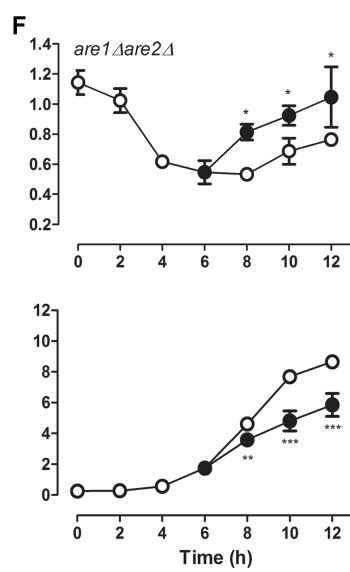
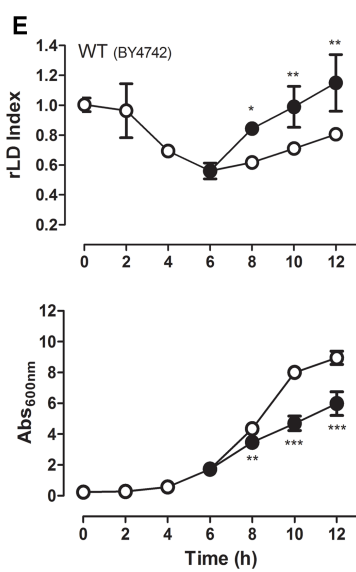
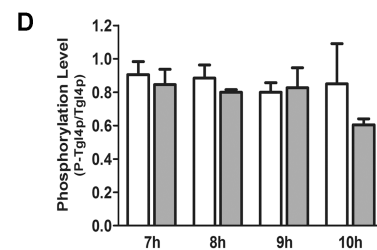
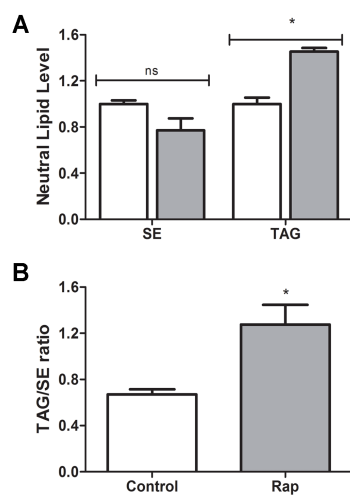
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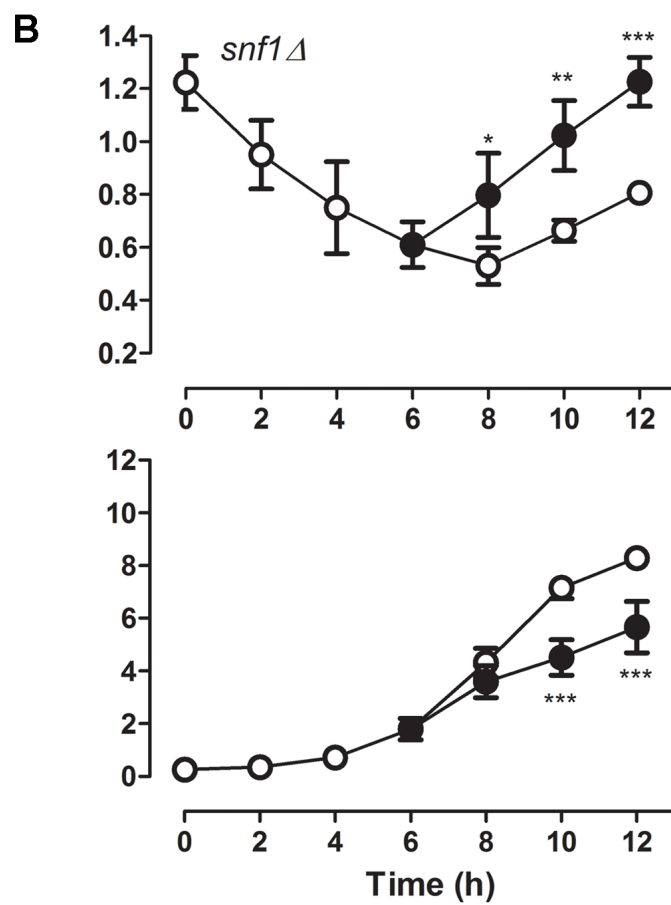
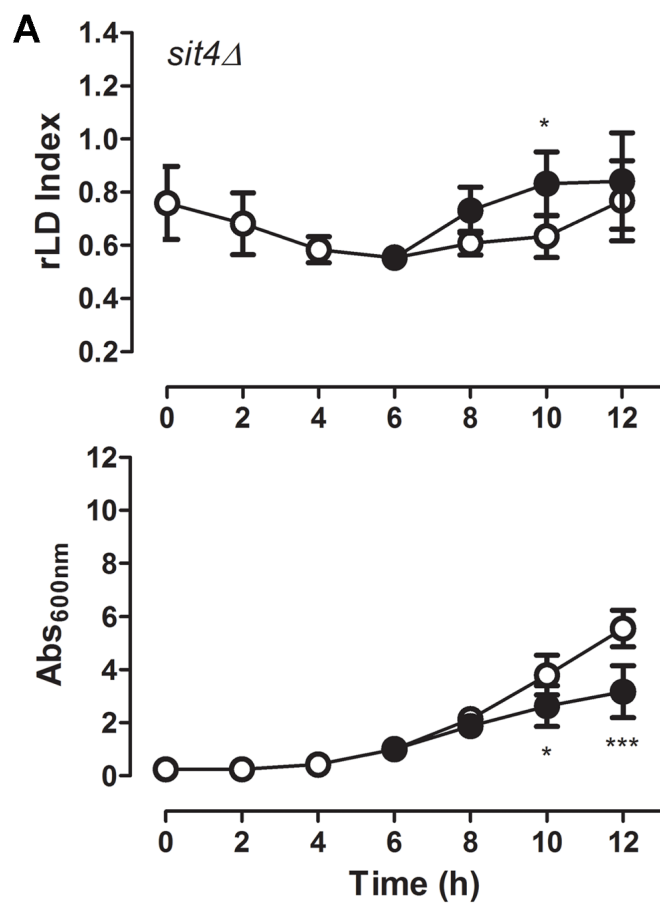
711 **FIG 9** Upon rapamycin treatment or nutrient starvation, TORC1 is inhibited relieving  
712 Tap42p-Sit4p to act on downstream effectors, such as Rtg1/3p and Gat1p/Gln3p.  
713 Gln3p/Gat1p are translocated to the nucleus and induce early TAG synthesis which reflects  
714 in increased LDs. Effect of rapamycin on LDs replenishment is also dependent on Rtg1/3p,  
715 but the role of these transcription factors is still to be investigated.

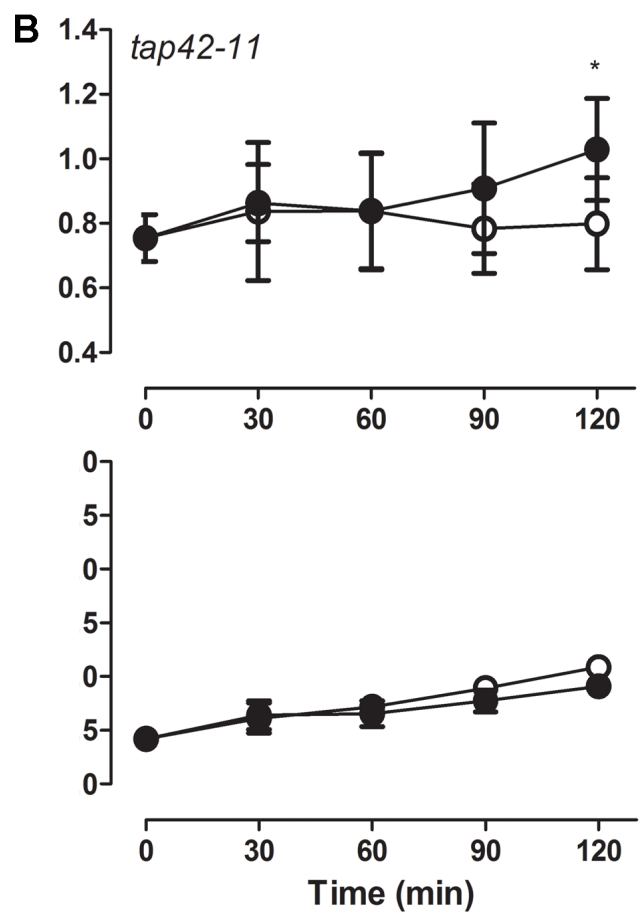
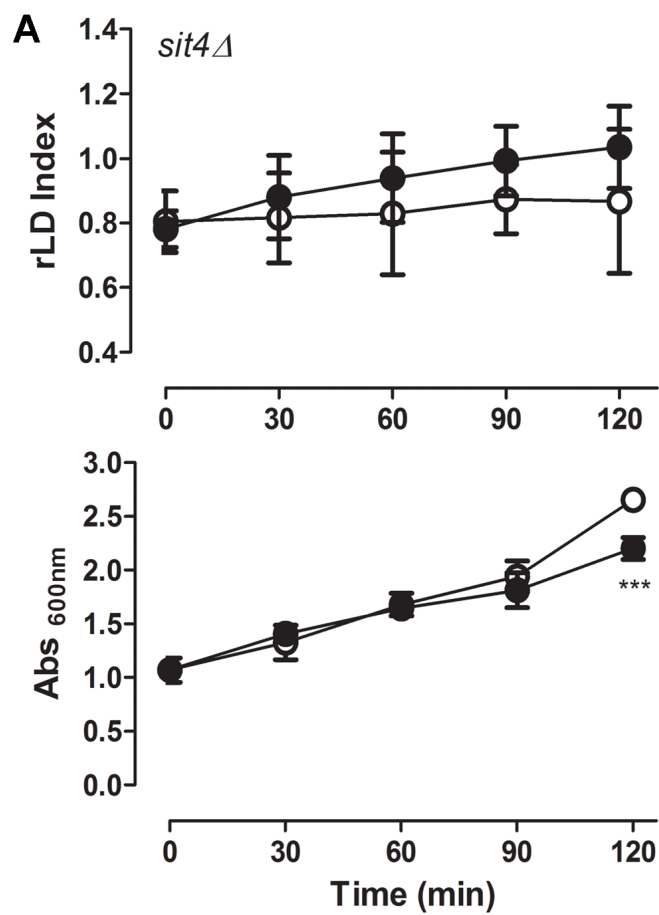




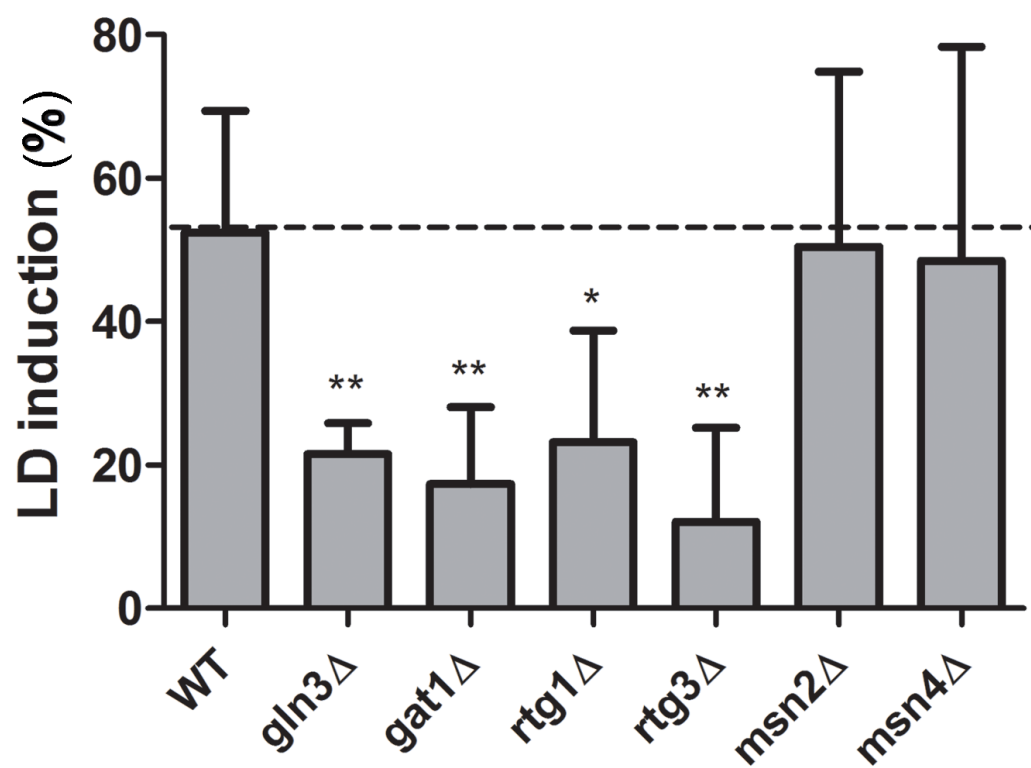








**A**



**B**

