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TORC1 Inhibition Induces Lipid Droplet Replenishment in Yeast

Running Title: Rapamycin and Lipid Droplet Replenishment

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ABSTRACT

Lipid droplets (LDs) are intracellular structures that regulate neutral lipid homeostasis. In mammals, LD synthesis is inhibited by rapamycin, a known inhibitor of the mTORC1 pathway. In *S. cerevisiae*, LD dynamics is modulated by the growth phase, however the regulatory pathways involved are unknown. Therefore, we decided to study the role of the TORC1 pathway on LD metabolism in *S. cerevisiae*. Interestingly, rapamycin treatment resulted in a fast LDs replenishment and growth inhibition. The discovery that osmotic stress (1M sorbitol) also induced LD synthesis but not growth inhibition, suggested that the induction of LDs in yeast is not a secondary response to reduced growth. Induction of LDs by rapamycin was due to increased triacylglycerol but not sterol esters synthesis. Induction was dependent on the TOR downstream effectors, the PP2A-related phosphatase Sit4p and the regulatory protein Tap42p. The TORC1-controlled transcriptional activators *GLN3, GAT1, RTG1* and *RTG3* but not *MSN2* and *MSN4* were required for full induction of LDs by rapamycin. Furthermore, we show that deletion of *GLN3* and *GAT1* transcription factors, which are activated in response to nitrogen availability, led to abnormal LD dynamics. These results reveal that the TORC1 pathway is involved in neutral lipid homeostasis in yeast.

INTRODUCTION

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Yeast strains. *Saccharomyces cerevisiae* strain BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), the derived open reading frame knock out collection (MATa deletion library) and strains expressing proteins tagged with the TAP epitope (TAP collection) were obtained from Open Biosystems. BY4742 (MATa *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), *dga1*Δ*lro1*Δ (MATa *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 dga1*::KanMX *lro1*::KanMX) and *are1*Δ*are2*Δ (MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 are1*::KanMX *are2*::KanMX) were kindly provided by Sepp D. Kohlwein (University of Graz, Austria). Mutants *tor1-1* and *tor2-1* derived from the JK9-3da (MATa *leu2-3*,112 *ura3-52 rme1 trp1 his4 GAL*+ HMLa) strain. Mutants *gln3*Δ*gat1*Δ (MATa *leu2 ura3 rme1 trp1 his3Δ GAL*+ HMLa *gln3*::KanMX *gat1*::HISMX) and *tap42-11* (MATa *leu2-3,112 ura3-52 rme1 GAL*+ HMLa *tap42*::KanMX/ YCplac111::*tap42-11*ts) 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°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°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 OD_{600nm} and grown
126 by shaking at 30°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°C.
129 Aliquots were taken for optical density (OD_{600nm}) 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 OD_{600nm}) 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°C until use. Readings were performed by adding

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

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

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

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

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

7% acetic acid) and then for 10 min in destaining solution (50% methanol, 7% acetic acid). Membranes were washed with distilled water and air dried.

qRT-PCR. RNA was isolated as previously described (35). The cDNA was synthesized from extracted RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as indicated by the manufacturer and further used as a template for Real Time PCR using SYBR-Green PCR Master Mix (Applied Biosystems, CA, USA). Amplifications were performed in a Step One Plus or 7500 Real-Time PCR System (Applied Biosystems, CA, USA). PCR amplification was carried out under the following 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. The threshold cycle (Ct) for each gene of interest was calculated using Step One Software v2.2 or 7500 Software v2.0.5. Normalization for each gene was performed against the mock sample (treated with vehicle alone) and gene expression levels were based on the 2-ΔΔCt method (36)(37) by calculating the relative copy number (RCN) using *ACT1* gene as an internal control. The primers used were:

CIT2F: 5'GCATTTGGTATTCTTGCTCAA3'

CIT2R: 5'TGCTTTCAATGTTTTTGACCA3',

ACT1F: 5′TTCCCAGGTATTGCCGAAA3′

ACT1R: 5′TTGTGGTGAACGATAGATGGA3′

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

RESULTS

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

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

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

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

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

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

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

LD replenishment induced by rapamycin depends on induction of TAG but not SE synthesis. The core of LD is formed by TAG and SE. The LD index analyzed by the LFR assay might reflect changes in both neutral lipids, so we investigated whether the TAG and/or the SE levels were increased by rapamycin. Analysis of neutral lipids by TLC showed that only the TAG content was induced after 2 hours of rapamycin treatment (Fig. 4A), which led to an increase in the TAG to SE ratio (Fig. 4B). Enzymatic determination of TAG content corroborated the TLC results showing a TAG content 40% higher in rapamycin-treated cells compared to non-treated cells (62.0 ± 7.0 nmol TAG x mg protein^{-1} in rapamycin-treated cells in contrast to 43.0 ± 12 nmol TAG x mg protein^{-1} in control cells; n=3, $p < 0.05$). The specific effect of rapamycin on TAG synthesis was reinforced by the results showing that rapamycin does not affect LD in the strain deleted of the main DAG acyltransferases (*dga1Δlro1Δ*), but it does on the strain deleted of the main sterol acyltransferases (*are1Δare2Δ*) (Fig. 4E-G).

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

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

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

TORC1signaling through the protein phosphatase Sit4 requires its regulatory protein Tap42p (23, 26). Tap42p is essential to cell viability so we tested the effect of rapamycin on a strain carrying the temperature-sensitive *tap42-11* allele, which is partially resistant to rapamycin at 25ºC. We observed that this mutant was unresponsive to rapamycin and LD levels were not increased within 2 hours of treatment (Fig. 6). Sit4-Tap42 effectors include the GATA-family transcription factors Gln3p and Gat1p (15, 47), the heterodimeric transcriptional activators Rtg1p and Rtg3p (20, 41) and the Zn-finger transcription factors Msn2p and Msn4p (26, 48). These factors regulate the nitrogen catabolic response, the retrograde pathway and the general stress response, respectively. We next explored the involvement of these PP2A-branch effectors. We observed that LD induction by rapamycin was significantly diminished in the *gln3Δ*, *gat1Δ*, *rtg1Δ* and *rtg3Δ* strains, but not in the *msn2*Δ and *msn4*Δ mutant strains (Fig. 7). Failure to reduce the LD induction in *msn2*Δ and *msn4*Δ strains could be due to their overlapping function of Msn2p and Msn4p, however the double deleted strain *msn2*Δ*msn4*Δ still responded to rapamycin (data not shown) ruling out this possibility.

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

Upon nitrogen limitation, Gln3p/Gat1p are activated and promote the transcription of genes important for the utilization of alternative nitrogen sources. It is well known that Gln3p/Gat1p activation and nuclear translocation are dependent on TORC1 inhibition (16), (49, 50). We studied the LD profile of *gln3*Δ*, gat1*Δ and the double mutant *gln3Δgat1Δ*. Interestingly, we found that these strains had an aberrant LD profile, in which the lipolytic and lipogenic phases were not clearly identified (Fig. 8A and B).

DISCUSSION

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

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

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

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

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

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

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

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

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

TORC1 requires the PP2A-like phosphatase, Sit4p, as well as the Gln3p and Gat1p transcriptional complex (Fig. 9). This assumption is based on the observation that strains deleted on the corresponding genes have aberrant LD dynamics during growth, where the transition between the lipolytic and lipogenic phase was not clearly observed (Fig. 8). Furthermore, *sit4*Δ, *gln3Δ* and *gat1Δ* mutants did not respond to rapamycin by inducing LD replenishment in the first 2 hours of treatment. Thus, rapamycin-induced NCR genes might be involved in the regulation of lipid homeostasis in batch cultures.

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

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

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

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

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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_{600nm}). 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® (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

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

FIG 3 LD dynamics after saline stress, osmotic stress and nitrogen starvation. A and B Wild type (BY4741) yeast cells were inoculated into YPD fresh medium and after reaching exponential phase (5h) they were treated with none (○) or 1M sorbitol (●) or 1M NaCl (○). For nitrogen starvation (N-starvation) the wild type (BY4741) yeast cells were inoculated in complete SD (0.17% yeast nitrogen base, 0.5% NH_2SO_4, 2% glucose and supplements) and after reaching exponential phase the culture was filtered through a 0.22 µM Millipore filter and re-seeded in complete SD (○) or SD without NH_2SO_4 (●). The effect of nitrogen starvation was compared to rapamycin treatment (100 ng/ml) (●) or addition of vehicle alone (○) in cells grown in complete SD. The rLD index (upper panels) and growth (lower panels) was determined. Data are from three independent experiments ± standard deviations (*p<0.05, p**<0.01 and ***p<0.001).

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

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) *are1Δare2Δ,* a mutant strain deficient in SE synthesis, and (G)
677 *dga1Δlro1Δ,* a strain deficient in TAG synthesis, were treated with rapamycin (○) or
678 vehicle alone (●) as described in Fig. 1A. The rLD indexes (upper panel) and culture
679 growth (lower panel) were recorded. Data are from three independent experiments ±
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*Δ (A) or *snf1*Δ (B)
683 mutant strains were treated with (100 ng/ml) (●) or vehicle alone (○) 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 ± 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*Δ and (B)
690 the temperature sensitive *tap42-11* mutant strains were treated with (100 ng/ml) (●) or
691 vehicle alone (○) 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 ± 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 ± standard deviations (*p<0.05 and **p<0.01).
703
704 **FIG 8** Disruption of Gln3p and Gat1p transcription factors affect LD dynamics. *gln3*Δ,
705 *gat1*Δ, *gln3*Δ*gat1*Δ and its isogenic strains were grown in YPD and the LD dynamics and
706 growth was followed. (A) *gln3*Δ (○) , *gat1*Δ (△) and the isogenic strain WT (BY4741)
707 (gray,dotted line) . (B) *gln3*Δ*gat1*Δ (○) and its isogenic strain WT (TB50-a) (gray, dotted
708 line). Data are from three independent experiments ± standard deviations (*p<0.05,
709 p**<0.01 and ***p<0.001).
710
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.