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Increased fatty acid synthesis inhibits nitrogen starvation-induced autophagy in lipid droplet-deficient yeast[1]

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[1] *Abbreviations:* ER, endoplasmic reticulum; GFP, green fluorescent protein; HA, hemagglutinin; TOR, target of rapamycin; YNB, yeast nitrogen base; YPD, yeast extract/peptone/dextrose.

Abstract

Macroautophagy is a degradative pathway whereby cells encapsulate and degrade cytoplasmic material within endogenously-built membranes. Previous studies have suggested that autophagosome membranes originate from lipid droplets. However, it was recently shown that rapamycin could induce autophagy in cells lacking these organelles. Here we show that lipid droplet-deprived cells are unable to perform autophagy in response to nitrogen-starvation because of an accelerated lipid synthesis that is not observed with rapamycin. Using cerulenin, a potent inhibitor of fatty acid synthase, and exogenous addition of palmitic acid we could restore nitrogen-starvation induced autophagy in the absence of lipid droplets.

Keywords: autophagy; fatty acid; fatty acid synthase; lipid droplets; rapamycin; *Saccharomyces cerevisiae*

1. Introduction

Macroautophagy, hereafter referred to as "autophagy", is a degradative pathway widely conserved in eukaryotes from yeast to mammals [1]. It is induced by various physiological stresses, such as starvation, and allows degradation of cytoplasmic materials and organelles to create novel building blocks to adapt a changing environment [2]. Defects in this process have been identified in the pathology of several diseases including cancer, some neurodegenerative diseases and myopathies [3-6]. Autophagy begins with the expansion of a cup-shaped membrane structure called the isolation membrane, or phagophore, which encapsulates the cytoplasmic materials or organelles that are to be degraded, while expanding. The phagophore eventually closes to form a double membrane structure called the autophagosome [7]. Its content is then delivered to the vacuole for degradation.

To date, 38 autophagy-related genes have been identified in the yeast *Saccharomyces cerevisiae* [8]. Many of them are shared by the Cytoplasmic to Vacuole (CVT) pathway that constitutively addresses proteins to the vacuole [9] and others are required for selective autophagy processes. A possible contribution of lipid droplets to autophagy has been reported [10-12]. Lipid droplet is a conserved structure found in most eukaryotic cells. It consists in a core of neutral lipids, mainly triacylglycerol and steryl-esters, which are surrounded by a phospholipid monolayer. In *S. cerevisiae*, lipid droplets biogenesis is strictly controlled by the enzymes necessary for neutral lipid synthesis [13]. These comprise the two diacylglycerol acyl transferases encoded by *DGA1* and *LRO1* which are responsible for TAG

biosynthesis [14] whereas steryl-esters are synthesized by the action of the *ARE1* and *ARE2* gene products [15, 16]. Thus, yeast mutants deleted for these 4 genes do not produce detectable neutral lipids and are completely devoid of lipid droplets [13]. Two studies in yeast have shown that such mutants are incapable of autophagy in response to nitrogen starvation [10, 12]. In mammals too, lipid droplets have been implicated in the regulation of autophagy [11]. From these studies it was suggested that lipid droplet contribution to autophagy would consist in providing fatty acids and lipids necessary for membrane autophagosome biogenesis [17]. Recently, this view was challenged by the observation that lipid droplet-deficient yeast is still capable to induce autophagy in response to rapamycin [18]. Moreover the authors showed that in the absence of lipid droplets, nitrogen starvation-induced autophagy defects could be corrected by inhibiting fatty acid biosynthesis and altering phospholipid composition. This observation was somewhat unexpected since it is known that inhibition of fatty acid synthesis in a wild type strain strongly impairs autophagy [10]. In the present paper, we come to a similar conclusion as Velazquez *et al* (2016) through a different experimental approach. Firstly, we establish that nitrogen starvation, but not rapamycin treatment, triggers an increase in lipid biosynthesis. Then we show that nitrogen starvation-induced autophagy can be restored in lipid droplet-deficient yeast by a controlled supply of fatty acid. Finally, we correlate the defects in autophagy with electron microscopic evidence of tangled ER membranes and we show that our strategy of a controlled fatty acid supply to restore autophagy also leads to the disappearance of these aberrant membrane tangles.

2. Materials and methods

2.1. Reagents, antibodies, and plasmids

Rapamycin (Santa-Cruz) and cerulenin (SIGMA) were solubilized in DMSO and added at a final concentration of 200 nM (0.2 µg/ml) and 40 µM (10 µg/ml) respectively unless otherwise stated. The vacuole labeling dye, FM4-64 (Santa Cruz) was dissolved in DMSO and used at 20 µM. Palmitic acid was dissolved in DMSO and used at a final concentration of 200 µM.

Mouse antibodies directed against GFP were obtained from Roche; mouse anti-Pgk1 antibodies and mouse anti HA antibodies were purchased from Invitrogen and SIGMA respectively. HRP conjugated anti-mouse secondary antibodies were from SIGMA.

For GFP–Atg8 processing, the plasmid pRS416 expressing GFP–Atg8 was used [19]. Plasmid pPHY2427 encoding 3xHA-Atg13 under the control of the CUP1 promoter in pRS426 was kindly provided by Pr. Hermann [20]

2.2. Yeast strains, media and growth conditions

All yeast strains described in this study are derived from BY (Euroscarf). BY4742 was used as the wild-type strain. RS4Δ (BY4742; *MATα are1::kanMX4 are2::kanMX4 lro1::TRP1 dga1::Lox-HIS3-Lox ura3 leu2 lys2*) was kindly provided by Roger Schneiter. Yeast strains were cultured in minimal YNB medium (yeast nitrogen base with ammonium sulfate and 2% glucose) supplemented with the appropriate amino acids and bases, or complete YPD medium (1% yeast extract, 1% peptone, 2% dextrose). For nitrogen starvation, unless otherwise stated, overnight cultures in YNB medium were inoculated in YPD medium and propagated for at least two

generations; cells were harvested by centrifugation, washed with distilled water and autophagy was induced by inoculation in YNB medium without ammonium sulfate and amino acid. Autophagy induction with rapamycin was achieved by adding rapamycin into YPD medium.

2.3. Immunoblotting analysis

Approximately 10^8 cells were harvested, and lysed by alkaline whole cell extraction (0.2 M NaOH; 0.3% β-mercaptoethanol) followed by TCA precipitation. Protein extracts were subsequently washed with acetone, dried and suspended in 100 µl of 5% (w/v) SDS. Finally, 100 µl of Laemmli buffer (20% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.04% Bromophenol Blue, 0.0625 M Tris-HCl pH 6.8) was added. Samples were incubated 10 min at 65°C prior to loading on a 12% polyacrylamide gel. Routinely, lysates from the equivalent of $4x10^6$ of cells were subjected to immunoblot analysis. After western blotting onto nitrocellulose and blocking the membrane with 5% non-fat milk, 0.1% Tween 20 in Tris-buffered saline, the blotted proteins were probed with mouse anti-GFP antibodies (1/2000) or with mouse anti-Pgk1 antibodies (1/10000) or mouse anti HA antibodies (1/2000). HRP conjugated anti-mouse secondary antibody (SIGMA) was used at 1/1000. Detection was performed with ECL prime from Amersham.

2.4. Fluorescence microscopy

Microscopy was performed on a FV1000 Olympus confocal microscope using a 60 X water immersion lens (NA 1.20) coupled to a 2.3X numerical zoom (0.12 µm per pixel). The excitation wavelengths for GFP or FM4-64 were set to 488 nm and 543 nm respectively. Image acquisition and conversion were performed separately for

green (520 nm) and red (603 nm) channels and processed with the Olympus Fluoview version 4.1 software. Transmission images were acquired with differential interference contrast optics.

2.5. Pulse labeling, extraction and analysis of lipids

An overnight culture in YPD medium at 27°C was propagated to OD~1 ($2x10^7$ cells/ml). The culture was divided into three fractions. The first fraction was immediately used for lipid labeling (T0 sample). Cells from the other two fractions were washed with distilled water and resuspended with rapamycin-containing YPD (R sample) or nitrogen free medium (SD-N sample) and incubated for 2 hours, before lipid labeling. Lipid labeling was performed in triplicate for 15 min at 27°C, after addition of 2 µCi/ml [2-^{14}C]-acetic acid, sodium salt (1994 MBq/mmol ; PerkinElmer, Courtaboeuf, France). Acetate incorporation was stopped by addition of 20 mM NaN_3. Cells were pelletted and stored at -20°C.

For total lipid extraction, the cell pellet was resuspended with 1 ml of 10 mM Trs HCl pH7.5, 40 mM β-mercaptoethanol and incubated for 5 min at room temperature. The cells were then centrifuged and resuspended with 600 µl of lysis buffer (20 mM Tris HCl pH7.5, 150 µg of lyticase 250 kU) and incubated for 30 min at 27°C.

Lipids were extracted using methanol-chloroform (2:1) [21], dried and counted.

2.6. Electron microscopy

Cells were collected after 2 hours of treatment and processed for electron microscopy as previously described [22] and observed on JEM1010 JEOL electron microscope.

3. Results and discussion

3.1. Lipid droplet-deficient yeast activates autophagy in response to rapamycin

The recent observation that autophagy induced by nitrogen starvation is impaired in lipid droplets deficient yeast led to the hypothesis that this structure contributes significantly to autophagy [10, 12]. The target of rapamycin (TOR) pathway is a central regulator of cell growth in response to nitrogen availability. Yeast possesses two TOR kinase complexes one of which, TORC1, controls cell growth in response to nutrient availability [23]. Consequently, inhibition of yeast TORC1 and nitrogen starvation induce a similar response [23], including autophagy induction [24]. We used the antifungal rapamycin, which specifically inhibits the TORC1 complex [23], to induce autophagy in a yeast strain deleted for all the four genes responsible for neutral lipid synthesis (strain RS4Δ). As a first step, autophagy completion was assessed by the GFP-cleavage assay [25] and fluorescence microscopy. Upon autophagy induction, autophagosomes containing a cytosolic GFP-Atg8 fusion protein are delivered to the vacuole for degradation. The GFP moiety of GFP-Atg8, being resistant to proteolysis, accumulates in the lumen of the vacuole. Thus, accumulation of free GFP, or increased fluorescence of GFP in the vacuole, is a good marker of autophagy completion.

As indicated by western blot analysis of GFP-Atg8 cleavage, strain RS4Δ that lacks lipid droplets is capable to induce autophagy in response to rapamycin (Fig. 1A). As reported before ([10, 12]), it is unable to do so in response to nitrogen starvation. Fluorescence microscopy confirmed that GFP-Atg8 cleavage in response to rapamycin took place in the vacuole (Fig. 1B) since the fluorescent signal accumulates in this compartment. We performed electron microscopy analysis of

RS4Δ to compare cell morphology in response to nitrogen starvation and rapamycin. Autophagic bodies were detected in vacuoles of RS4Δ treated with rapamycin for two hours (Fig 1C top panel) but not from cells that had been submitted for two hours to nitrogen starvation (Fig 1C bottom panel). We noted with interest the accumulation of tangled ER membranes in response to nitrogen starvation (Fig 1C bottom panel) as previously reported [10]. These membrane structures could correspond to collapsed cortical ER reported by Velazquez *et al* using fluorescence microscopy [18]. Remarkably, these membrane structures where not observed in rapamycin-treated cells suggesting that they may be functionally related to inhibition of autophagy under nitrogen starvation (Fig 1C top panel).

Atg13 is one of the main targets of the kinase TORC1 that controls autophagy induction. In growing cells, Atg13 is phosphorylated at multiple sites in a TOR dependent manner which prevents Atg13 association to Atg1 and autophagy induction [26]. It is rapidly dephosphorylated upon rapamycin treatment - or nitrogen starvation - which contributes to autophagy induction [27]. We wondered whether nitrogen starvation was efficient to induce Atg13 dephosphorylation in RS4Δ. As shown in Figure 1D, dephosphorylation of HA-tagged Atg13 could be observed in RS4Δ cells, both in response to rapamycin and to nitrogen starvation (Fig. 1D). Taken together these observations indicate that autophagy in RS4Δ cells is blocked selectively in response to nitrogen starvation and that the block takes place at a step located downstream of the early signalling cascade that initiates phagophore assembly. Further experiments were focused on trying to explain the differential effects of nitrogen starvation and rapamycin in RS4Δ cells.

3.2. Nitrogen starvation stimulates lipid synthesis in lipid droplet deficient yeast

Since RS4Δ cells do not store neutral lipids, it has been hypothesized that a deficit in fatty acid availability could be responsible for the impairment of autophagy [10, 12]. It seemed difficult however, to imagine why this should differentially affect the responses to nitrogen starvation and to rapamycin. In addition, previous attempts to restore the autophagic flux by providing exogenous fatty acids were not successful [10]. This prompted us to compare lipid synthesis between nitrogen deprived and rapamycin treated mutant. For this purpose, we performed a ^{14}C-acetate pulse labelling of the RS4Δ strain. Once internalized by the cells, ^{14}C-acetate is converted into acetyl CoA and can be readily incorporated into sterols and other lipids (Fig. 2A). Cells were pulsed labelled for 15 minutes with ^{14}C-acetate either before autophagy induction, or two hours after addition of rapamycin, or shift to nitrogen depleted medium. The amount of ^{14}C-acetate incorporated into the total lipid fraction was about 3-fold higher in nitrogen-starved cells than in rapamycin-treated cells and control cells (Fig. 2B). Thus, RS4Δ is not deficient in lipid synthesis in response to nitrogen starvation. This result is in agreement with the observation that exogenous addition of fatty acid was unable to restore autophagy in response to nitrogen starvation in this strain [10]. Therefore, we questioned whether the increased lipid synthesis could account for the impairment of autophagy in response to nitrogen starvation.

3.3. Reversal of the autophagy phenotype of lipid droplet-deficient yeast by controlled fatty acid availability

A previous study showed that total inhibition of fatty acid synthesis inhibited autophagy in wild type cells [10]. More recently, inhibition of fatty acid synthesis in

lipid droplets-deficient yeast was shown to restore 50% of the autophagic response to nitrogen starvation [18] and the authors suggested that unbuffered fatty acid synthesis in the absence of lipid droplets might lead to inhibition of autophagy. To examine this more carefully, we analysed the dose-dependent effect of cerulenin on starvation-induced autophagy. As illustrated in Figure 3A, the effect of cerulenin on nitrogen starvation-driven autophagy was clearly biphasic, with moderate doses (2 or 4 μM) restoring autophagy and high doses (20 or 40 μM) being inhibitory. These results suggested that autophagy in response to nitrogen starvation could take place only in a narrow window of fatty acid availability. This biphasic effect of cerulenin was specific of the autophagic response to nitrogen starvation. Indeed, when autophagy was induced by rapamycin, only the inhibitory effect of cerulenin could be observed (Fig. 3B). In both situations that were analysed, rapamycin and nitrogen starvation, full inhibition of fatty acid synthesis in lipid droplets deficient mutant severely impaired autophagy, suggesting a fatty acid requirement for autophagy.

In another experimental approach, we clamped fatty acid availability by complete inhibition of endogenous synthesis with cerulenin (40μM) combined with minimal supplementation with exogenous palmitic acid (200μM). As illustrated in Fig. 4A, this protocol efficiently restored autophagy in response to nitrogen starvation, whereas the simple addition of palmitate did not, as already observed [10]. Electron microscopy further indicated the presence of autophagic bodies in vacuoles (Fig. 4B) and the absence of tangled ER membranes in the cells, strengthening the notion that these membrane structures may be functionally related to the impairment of autophagy. Thus, the data indicate that the increase in fatty acid synthesis following nitrogen starvation is responsible for the inhibition of autophagy in RS4Δ.

Altogether, the present study of autophagy in lipid droplet deficient yeast has yielded three major findings. Firstly, lipid droplets are not required for autophagy as it can be induced by rapamycin in RS4Δ cells. Secondly, failure of RS4Δ cells to induce autophagy in response to nitrogen starvation is due to an overshoot of lipid production induced by starvation in the absence of lipid droplets. Thirdly, the autophagy deficient phenotype of RS4Δ cells can be reversed by imposing a moderate fatty acid supply. It can be achieved either by partial inhibition of fatty acid synthase (low cerulenin) or by a complete inhibition of the enzyme (high cerulenin) combined with supplementation with palmitic acid.

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Figure legends

Fig. 1. Lipid droplets are not required for autophagy in response to rapamycin. A- Lipid droplet depleted cells (RS4Δ) expressing GFP-Atg8 from a multicopy plasmid were grown to exponential phase in YPD medium before rapamycin (R) was added for the indicated time, or before the cells were washed in distilled water and shifted to nitrogen-free medium (-N) for the indicated time. Cells were then lysed and subjected to SDS-PAGE and Western blot analysis using anti-GFP and anti-Pgk1 antibodies. B- Exponentially growing RS4Δ expressing GFP-Atg8 cells were incubated with rapamycin for five hours in the presence of FM4-64 to stain vacuoles. Cells were observed by confocal fluorescent microscopy. Scale bars: 5μm. C- Exponentially growing RS4Δ were incubated for 2 hours with rapamycin (top panels) or starved in YNB-N medium for 2 hours (bottom panels) before being processed for electron microscopy. The right panels are magnification of the box from the corresponding left panels. Symbols: ER - endoplasmic reticulum; M - mitochondria; N - nucleus; V - vacuole; * - autophagic body; # - tangled membrane. Scale bars: 1 μm (left panels) and 400 nm (right panels). D- Exponentially growing RS4Δ cells expressing HA-tagged Atg13 (YPD) were treated with rapamycin for one hour (R), or washed with distilled water and incubated in nitrogen-free medium (YNB-N) for one hour. Cells were lysed and subjected to SDS-PAGE and Western blot analysis using anti-HA antibodies.

Fig. 2. RS4Δ display increased synthesis of lipids in response to nitrogen starvation. A - Simplified pathways connecting neutral and phospholipid and sterol metabolisms. Only the enzymes relevant to this study are indicated. For the sake of clarity, several steps have been omitted. B - Pulse labelling of RS4Δ with ^{14}C-acetate. Cultures were grown in complete medium to exponential phase and divided in three. One culture was immediately pulse labelled for 15 min with ^{14}C-acetate (T0). The remaining two cultures were treated with Rapamycin (R) or harvested, washed with distilled water and inoculated in nitrogen free medium (-N). Each of the two cultures was incubated for 2 hours and pulse labelled for 15 min with ^{14}C-acetate. Lipids were extracted as described in the Materials and methods section. Error bars represent the s.e.m. of three independent experiments. *** $P<0.005$ (Student's t-test).

Fig. 3. Partial inhibition of fatty acid synthase in RS4Δ restores autophagy in response to nitrogen starvation. A - The effect of cerulenin on nitrogen induced starvation is biphasic in RS4Δ. Overnight cultures of RS4Δ expressing GFP-Atg8 were grown overnight and inoculated for at least two generations in YPD medium (YPD). They were treated with 200nM rapamycin for three hours (R). Alternatively, cells were incubated with cerulenin at the indicated concentration for 30 min, before being harvested, washed with distilled water and inoculated in YNB-N to induce autophagy. Note that in YNB-N, cerulenin was added to maintain its original concentration. Cells were then processed for western blot analysis with anti GFP and anti Pgk1 antibodies as described in Figure 1. B - Fatty acid synthesis inhibition impairs rapamycin induced autophagy in RS4Δ. Overnight cultures of RS4Δ expressing GFP-Atg8 were grown overnight and inoculated for at least two generations in YPD medium (YPD). Cells were incubated with the indicated

concentration of cerulenin for 30 min, before rapamycin (200 nM) was added. Cells were further incubated for 3 hours, harvested and processed for western blot analysis with anti GFP and anti Pgk1 antibodies as described in Figure 1.

Fig. 4. Controlled supply with fatty acid restores autophagy in RS4Δ cells submitted to nitrogen starvation. RS4Δ cells expressing GFP-Atg8 were grown overnight in minimal medium supplemented (+ palmitate) or not (- palmitate) with 200 µM palmitate. They were re-inoculated in YPD medium with or without palmitic acid as indicated and grown for at least two generations; when indicated (+), cells were incubated with cerulenin (40 µM) for 30 minutes to inhibit fatty acid synthesis. Then, cells were harvested, washed with distilled water, and inoculated in YNB-N medium for 0 or 3 hours. When indicated, cerulenin and fatty acid were added at the same concentration as mentioned before. A - Cells were then processed for western blot analysis with anti GFP and anti Pgk1 antibodies as described in Figure 1. B- Cells were processed for electron microscopy. Symbols : N - nucleus; V - vacuole; * - autophagic body. Scale bars : 1 µm (left panel) and 200 nm (right panel).

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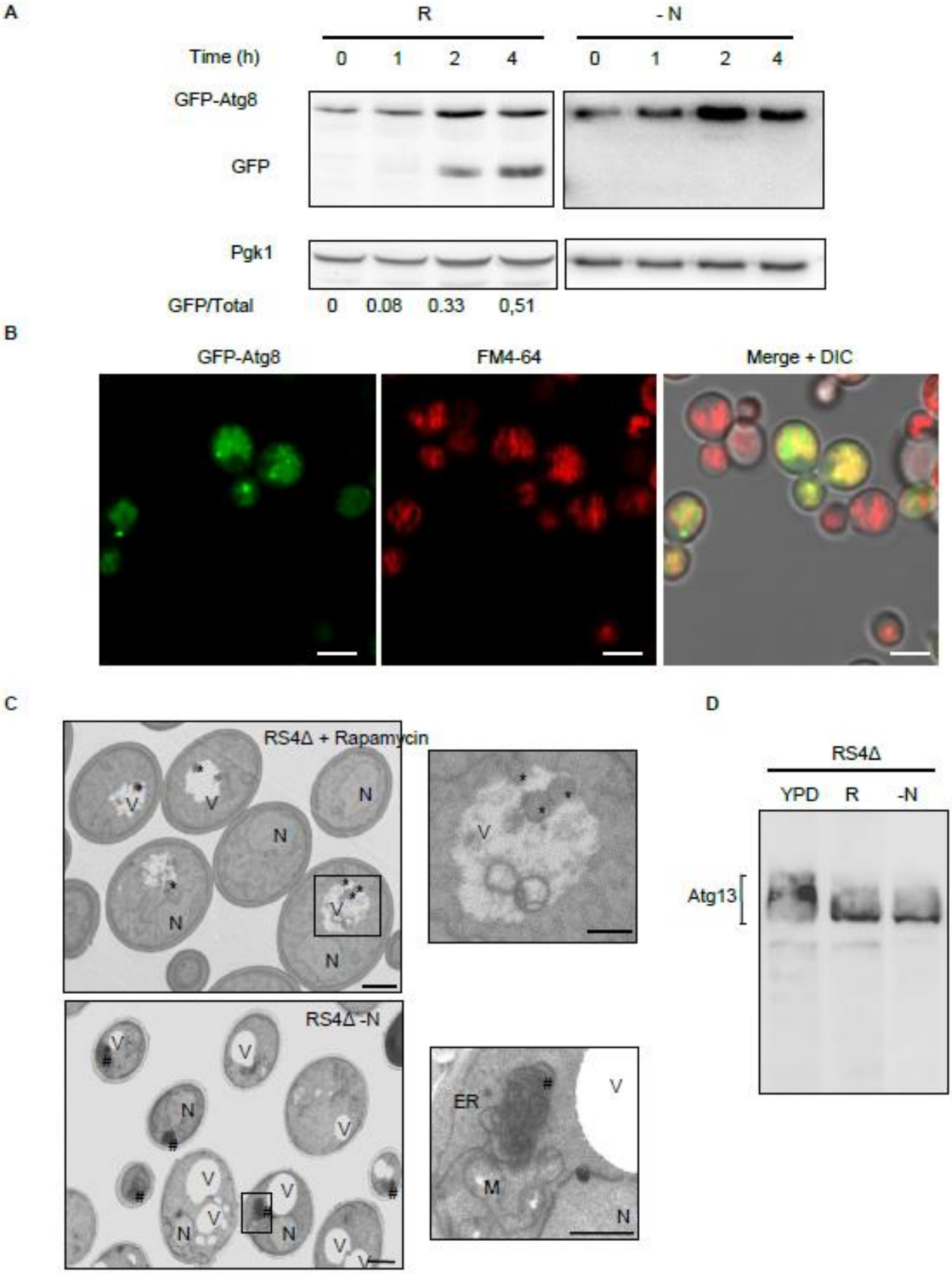
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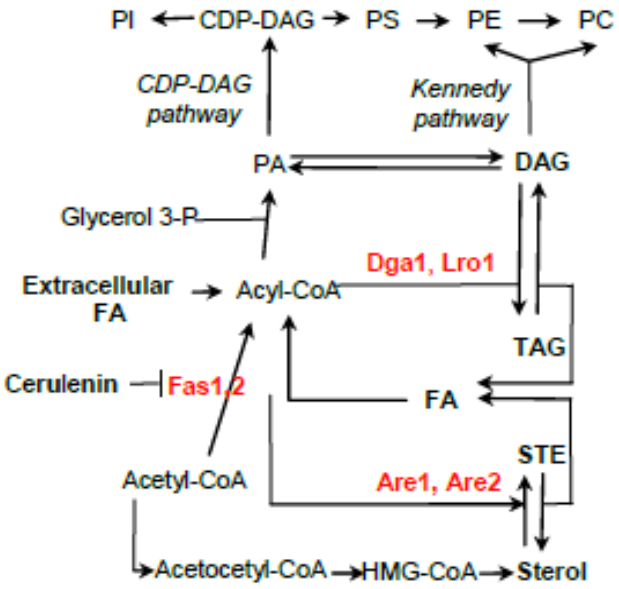
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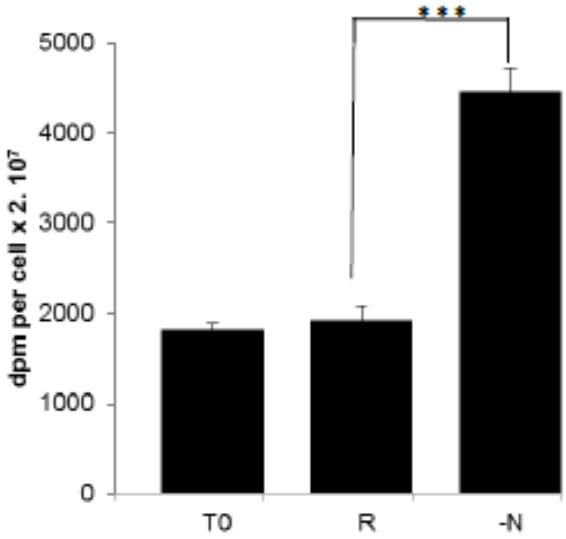
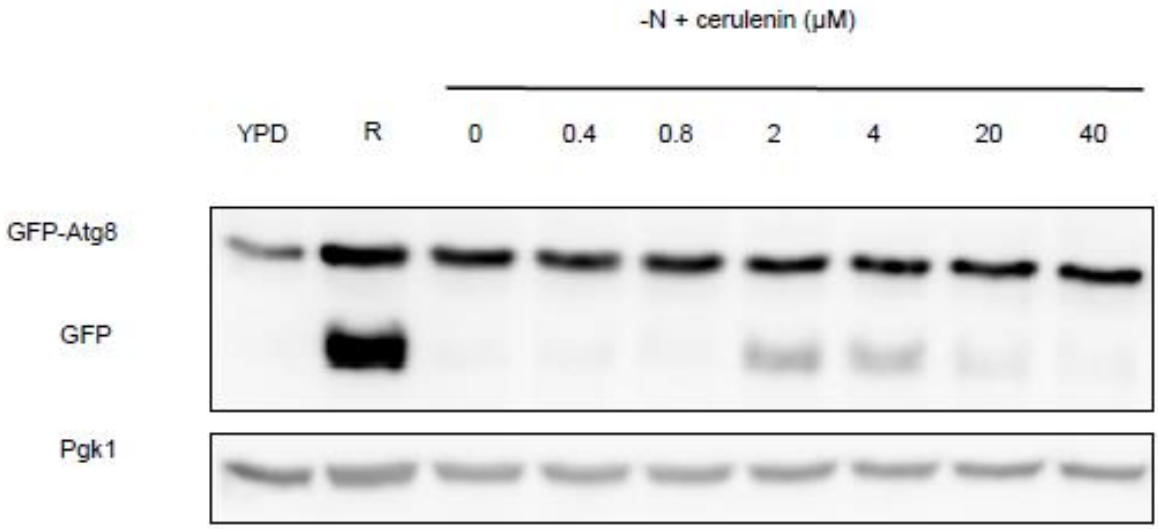
Are1, Are2
Acetocetyl-CoA
HMG-CoA
Sterol

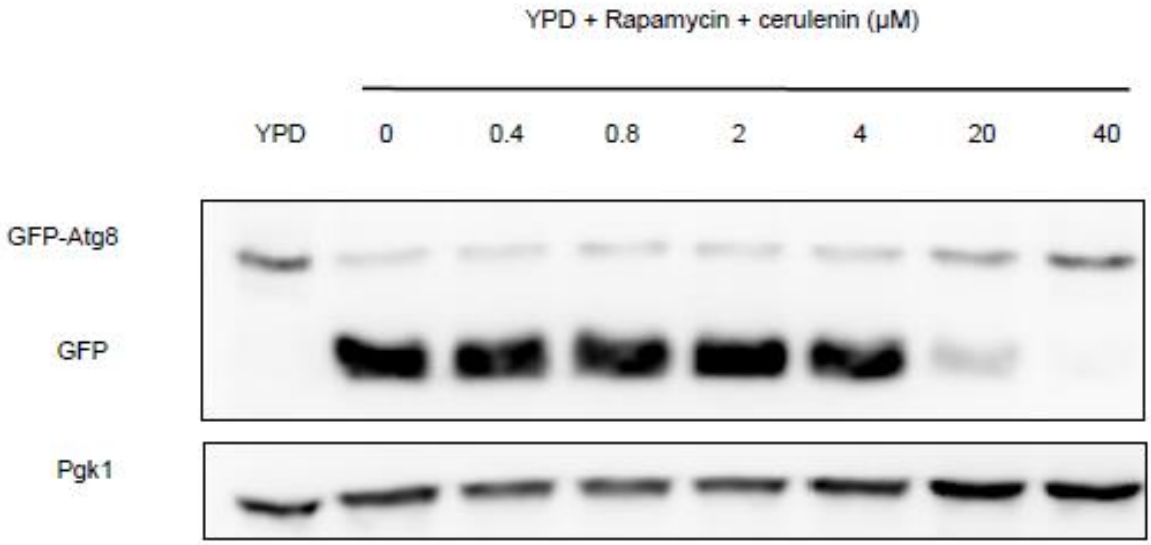
B


***
5000
4000
3000
2000
1000
0
dpm per cell x 2. 10⁷
T0
R
-N

A

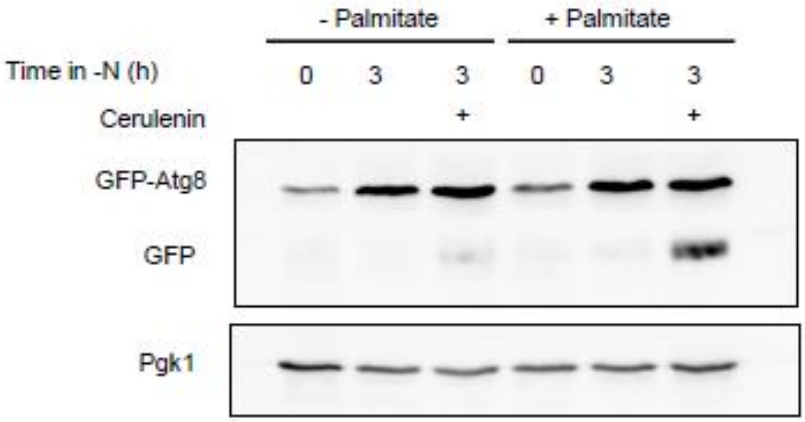

-N + cerulenin (μM)
YPD
R
0
0.4
0.8
2
4
20
40
GFP-Atg8
GFP
Pgk1

B

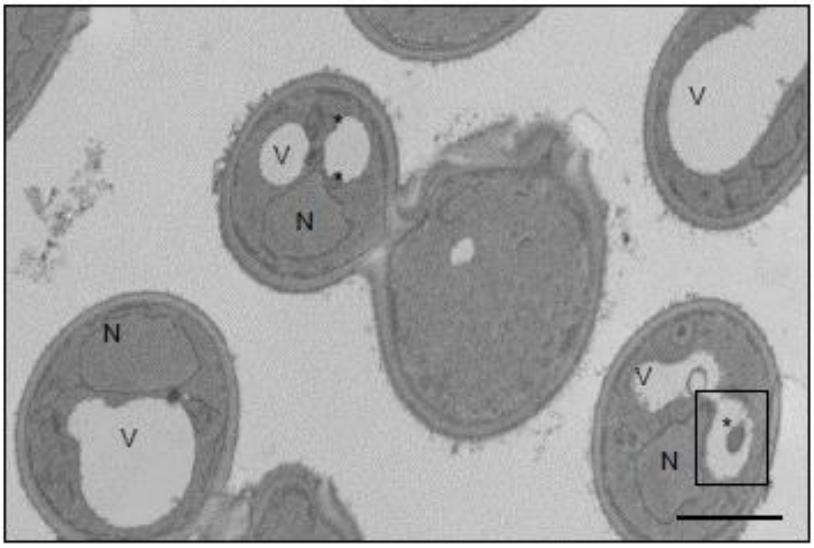

YPD + Rapamycin + cerulenin (μM)
YPD
0
0.4
0.8
2
4
20
40
GFP-Atg8
GFP
Pgk1

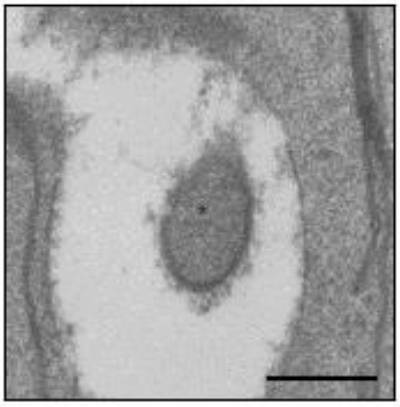
Figure

A


- Palmitate
+ Palmitate
Time in -N (h)
0
3
3
0
3
3
Cerulenin
+
+
GFP-Atg8
GFP
Pgk1

B


V
V
N
N
V
V
N



Conflict of interest

The authors declare no conflict of interest.