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Storage lipid synthesis is necessary for autophagy induced by nitrogen starvation

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ABSTRACT

Nitrogen starvation is a universal stimulus of autophagy. At present, little is known about the relationship between carbon metabolism and autophagy under nitrogen starvation. Here, we show that yeast cells continue to consume glucose and downregulate fermentation under nitrogen starvation. Storage lipid production is increased, with concurrent proliferation of lipid droplets. Furthermore, we provide evidence that triacylglycerol synthesis is crucial for autophagosome biogenesis. It is involved in a step downstream of PAS (phagophore assembly site) scaffold assembly, and upstream of the recruitment of Atg1, Atg14, Atg5 and Atg8. Finally, we demonstrate that lipid droplets transiently interact with Atg8-containing membranes. Our study reveals a novel connection linking neutral lipid metabolism, lipid droplets and autophagy.

1. Introduction

Autophagy is a major intracellular degradation and quality control mechanism in eukaryotes [1–4]. During this process, cytosolic materials including proteins and organelles are sequestrated into double membrane autophagosomes. Cargos are eventually degraded upon fusion of autophagosomes with endosomal/lysosomal compartments. The molecular machinery of autophagosome biogenesis is mainly composed of the Atg proteins [5–7]. In the budding yeast, *Saccharomyces cerevisiae*, most Atg proteins localize to the PAS (phagophore assembly site, also known as the pre-autophagosomal structure), and are hypothesized to carry out their functions at this site. One universal physiological stimulus of autophagy is nutrient limitation. Research in the past years has revealed an elegant pathway connecting availability of nitrogen sources, the TOR complex and autophagy [8–10]. In contrast, relatively little is known about the status of carbon metabolism under nitrogen starvation conditions and what role, if any, it has on autophagy.

In general, the metabolism of most carbohydrates merges into the glycolysis pathway. Under aerobic conditions, pyruvate produced through glycolysis is further converted to acetyl-CoA, which can be ultimately metabolized to CO_2. Alternatively, pyruvate can be fermented to lactate or ethanol to regenerate essential cofactors in glycolysis. Complete oxidation of acetyl-CoA to CO_2 is the preferred mode of energy metabolism for most cells under aerobic conditions. Some prominent exceptions exit, however. One is the Warburg effect commonly seen in tumor cells. The other is the Crabtree effect found in the budding yeast. In both cases, even under aerobic conditions, glycolysis and fermentation are highly active, whereas the citric acid cycle and oxidative phosphorylation are downregulated [11,12]. Besides being consumed for energy production, excess acetyl-CoA can be incorporated into neutral lipids, such as triacylglycerol (TAG), and stored in lipid droplets (LDs) for later use [13,14].

2. Materials and methods

2.1. Biochemical assays for glucose and TAG

Glucose concentration in liquid medium was quantified by an assay kit (E1010, Applygen Technologies, Beijing) using the glucose oxidase (GOD) method. To measure cellular TAG content, yeast

Abbreviations: ALP, alkaline phosphatase; Atg, autophagy-related; Cvt, cytoplasm-to-vacuole targeting; LD, lipid droplet; PAS, phagophore assembly site; PE, phosphatidylethanolamine; TAG, triacylglycerol; SE, steryl ester

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cells were collected by centrifugation. After a freeze–thaw cycle, cells were treated with yeast lyticase for 30 min. The amount of TAG was then quantified by an assay kit (E1013, Applygen Technologies, Beijing) using the glycerol phosphate oxidase (GPO) method.

2.2. Calculation of glucose consumption rate

During mid-logarithmic phase, assuming that (1) yeast culture growth follows $OD_{600}(t) = be^{at}$, where a and b are constants, and that (2) the rate of glucose consumption follows $-dG(t)/dt = gOD_{600}(t)$, where $G(t)$ is the remaining glucose concentration and g is a constant, then the value of a can be deduced from the growth curve, and the value of g can be obtained as $-a\Delta G/\Delta OD_{600}$. For starved culture, considering that the total amount of cellular protein remains constant, an equivalent rate of glucose consumption is computed as $-\Delta G/OD_{600}(0)\Delta t$.

2.3. Fluorescence microscopy

Glass bottom 35 mm petridishes were coated with 1 mg/ml Concanavalin A to immobilize yeast cells. For each sample, a *Z*-stack of 15 sections was collected, with 0.5 μm stepping. A max-intensity projection was then created from 10 consecutive sections covering the entire depth of the cells. For staining of lipid droplets, cells were incubated with 0.05 μg/ml BODIPY 493/503 in SD-N for 10 min at room temperature. Cells were subsequently washed 5 times with SD-N before imaging. For labeling of vacuoles with FM4-64, mid-log yeast cells were stained with 64 μM FM4-64 in YPD for 2 min. Cells were washed and resuspended in YPD to resume growth for 1 h before shifting to starvation conditions.

2.4. Culturing of yeast cells

Unless otherwise noted, yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). SD-N medium was used for nitrogen starvation (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose). For glucose starvation experiments, yeast cells were grown instead in SD + CA medium (0.67% yeast nitrogen base, 0.5% casamino acids, 20 mg/l uracil, 50 mg/l tryptophan, and 2% glucose), and shifted to SC-D medium for starvation (0.67% yeast nitrogen base, 0.5% casamino acids, 20 mg/l uracil, 50 mg/l tryptophan).

2.5. Construction of strains and plasmids

Plasmids for the tagging of Atg proteins are described elsewhere (Autophagy, manuscript submitted). The construction of additional plasmids is summarized in Table S1. Gene knockout and tagging were performed using the standard PCR based methods [15,16]. The sequences of the primers are listed in Table S2. Strains used in this study are listed in Table S3.

2.6. Other methods

The Ape1 maturation assay, the GFP-Atg8 processing assay, and the Pho8Δ60 assay were performed as described previously [17–20].

3. Results

3.1. Starved yeast cells continue glucose uptake and increase storage lipid production

To investigate the relationship between autophagy and carbon metabolism, we first examined the basic status of carbon metabolism. During mid-logarithmic phase, yeast cells are highly active at glycolysis. Glucose in the medium was quickly consumed, at a rate of 6 ± 1 mM/OD_{600}/h (Fig. 1A) (See Section 2 for calculation formula). Complete elimination of nitrogen sources in the medium results in inhibition of TOR activity and cell growth [9,21,22]. Accordingly, we found that the optical density of the liquid culture ceased logarithmic increase (Fig. 1A). Glucose consumed under growing conditions are primarily channeled into the fermentation pathway by the consecutive actions of pyruvate decarboxylases (PDC) and alcohol dehydrogenases (ADH) (Fig. S1) [23–26]. Upon shifting to nitrogen starvation medium, we observed a moderate drop in the expression of Pdc1, the major form of PDC (Fig. 1B). The major specie of ADH, Adh1 [27], was substantially downregulated (Fig. 1B), suggesting that ethanol fermentation is inhibited. In contrast, the uptake of glucose continued at a rate of 3 ± 1 mM/OD_{600}/h (Fig. 1A). Despite the increase of optical density under starvation, we found that the total amount of protein in the entire culture remained constant (Fig. 1A, the gray lines in right panel). Based on these results, we hypothesized that some of the glucose consumed under nitrogen starvation is channeled into certain storage species. Nitrogen starvation treatment resembles stationary phase in that both are nutrient limiting conditions. It is known that yeast cells entering stationary phase accumulate TAG in LDs [28,29]. We therefore checked whether the amounts of TAG and LDs increase under nitrogen starvation. Logarithmically growing yeast cells contained very little TAG and LDs (Fig. 1C and D). In contrast, the amount of TAG in starved cells increased significantly, which is accompanied by rapid proliferation of LDs (Fig. 1C and D). The paces of TAG production, LD proliferation, and autophagic turnover correlated well with each other (Fig. 1D and S2). Taken together, these data indicate that cells under nitrogen starvation continue glucose uptake, reduce fermentation and increase storage lipid production.

We further examined the impact of autophagy in the remodeling of carbon metabolism in *atg1Δ* cells. The downregulation of Pdc1, but not that of Adh1, was blocked in *atg1Δ* cells (Fig. 1B). In general, the impact of autophagy on the final outcomes, including glucose consumption, growth, and TAG production was less than 25% (Fig. 1A and C). These data suggest that the role of autophagy in the metabolic remodeling under nitrogen starvation is limited.

3.2. Storage lipid synthesis is required for autophagy induced by nitrogen starvation

We then investigated whether storage lipid synthesis has any role in autophagy. The production of TAG from diacylglycerol is mainly catalyzed by Dga1 and Lro1 [14,30–32]. Some very minor activity comes from Are2 and Are1 [31,32], which are mainly involved in sterol ester (SE) synthesis [33,34].

In wild type cells, nitrogen starvation induced non-selective autophagy, as reflected by the increased alkaline phosphatase (ALP) activity in the Pho8Δ60 assay (Fig. 2A) [19,35]. In contrast, the increase of ALP activity was significantly reduced in *dga1Δ lro1Δ* cells, and completely eliminated in *dga1Δ lro1Δ are1Δ are2Δ* cells (Fig. 2A). A minor effect was observed in *are1Δ are2Δ* cells (Fig. 2A). As a control, we verified that the reduction in ALP activity in both double mutants was recovered by ectopic expression of the deleted genes (Fig. 2A). In addition, the inability to activate Pho8Δ60 was not caused by defective vacuole proteolysis, as none of the mutations eliminated the activity of endogenous Pho8 (Fig. S3A), a resident vacuolar enzyme [36]. We further confirmed the autophagy defects by the GFP-Atg8 processing assay, which reflects the turnover of autophagosomal membrane [17,18]. In wild type cells, a significant portion of GFP-Atg8 was processed

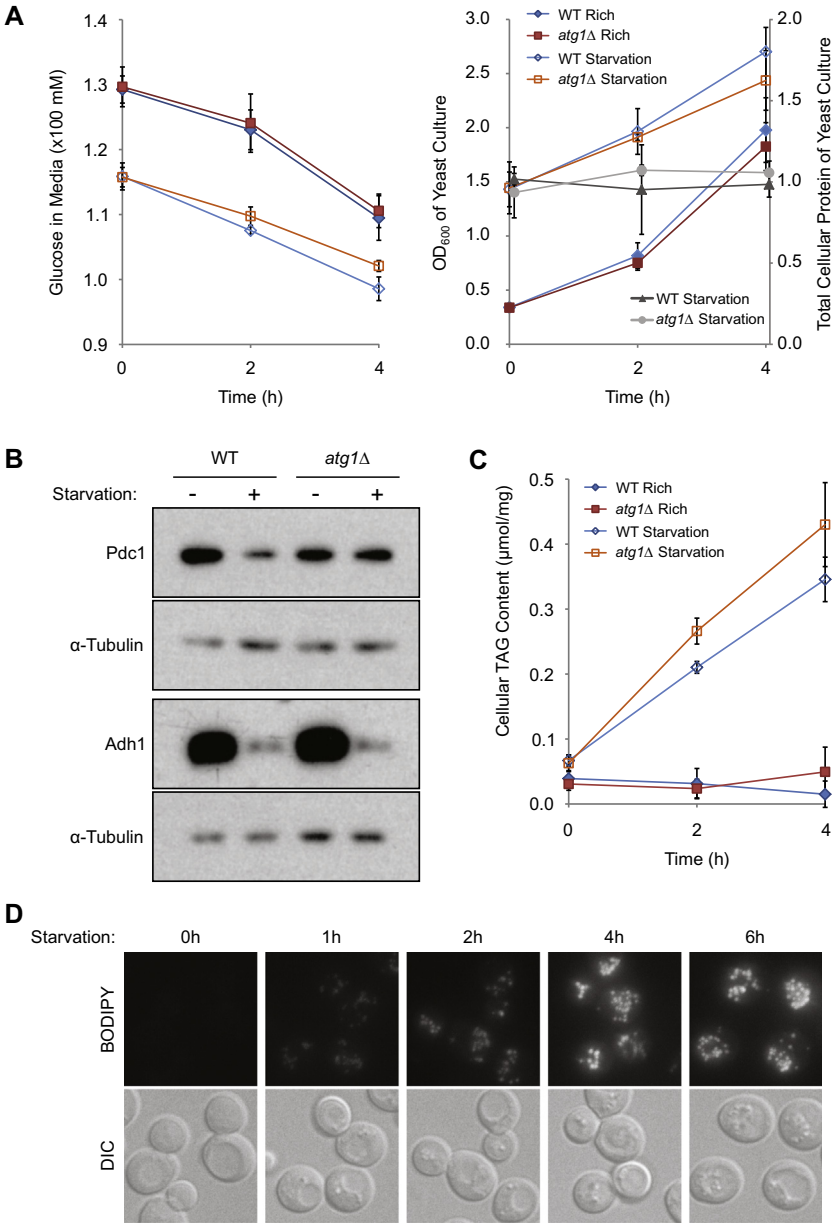

Fig. 1. Under nitrogen starvation, yeast cells continue to consume glucose, downregulate fermentation enzymes and increase storage lipid synthesis. (A) Glucose consumption and growth of yeast cells in rich medium and nitrogen starvation medium. Concentration of glucose in the media, optical density (OD_{600}) and total cellular protein of the culture were measured for wild type and *atg1Δ* yeast cells. For samples in rich medium, time zero is when OD_{600} reached approximately 0.35. For starved samples, time zero is when starvation began. Note that in rich medium, slightly more glucose was added to shift all data points up. For total cellular protein, the average of each repeat was normalized as 1. Error bar, standard deviation from three independent experiments. (B) Expression of key enzymes involved in fermentation. The indicated enzymes were chromosomally tagged with 3xHA at the carboxyl termini. Protein samples were collected from wild type and *atg1Δ* yeast cells at mid-log phase or after 4 h of starvation, and analyzed by immunoblotting. The experiment was repeated three times and representative images are shown. α-tubulin was used as a loading control. (C) Cellular TAG content of yeast cells in rich medium and in nitrogen starvation medium. Cellular TAG content (expressed as TAG/total protein) was measured for wild type and *atg1Δ* yeast cells. Data are presented as in panel A. (D) BODIPY staining of LDs. At time zero, mid-log phase wild type yeast cells were shifted to nitrogen starvation medium. At the indicated time points, cells were stained with BODIPY and imaged by fluorescent microscopy. The experiment was repeated three times and representative images are shown. For the BODIPY channel, a maximum intensity projection of a *Z*-stack is presented. DIC: differential interference contrast. Note that our imaging parameters were optimized for starved cells, which resulted in LDs in growing cells being almost invisible.

into free GFP after 4 h of starvation (Fig. 2B). In contrast, only a tiny amount of free GFP was detected in *dga1Δ lro1Δ* cells (Fig. 2B). The amount of free GFP in *dga1Δ lro1Δ are1Δ are2Δ* cells was essentially undetectable (Fig. 2B).

We also examined the role of storage lipid synthesis in the cytoplasm-to-vacuole targeting (Cvt) pathway and in autophagy induced by glucose starvation. The Cvt pathway is a selective form of autophagy [2,6,7,20]. Under nutrient rich conditions, the amounts of mature Ape1 in *dga1Δ lro1Δ*, *are1Δ are2Δ*, and *dga1Δ lro1Δ are1Δ are2Δ* cells were comparable to that in wild type cells (Fig. S4A), suggesting that storage lipid synthesis is dispensable for the Cvt pathway. In both Pho8Δ60 assay and GFP-Atg8 processing assay, the magnitude of autophagy induction under glucose starvation was lower than that under nitrogen starvation (Fig. S4B and C). Nevertheless, significant reductions of autophagy activity were observed when the pathways of storage lipid production were

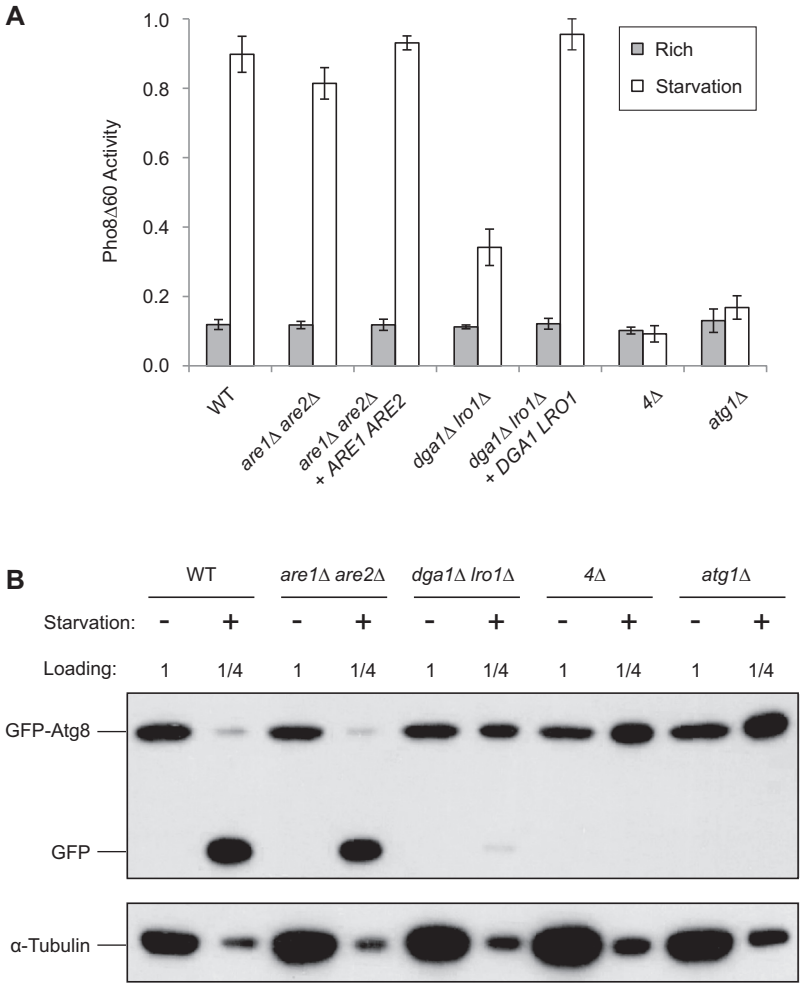

Fig. 2. Storage lipid synthesis is required for nitrogen starvation induced autophagy. Yeast cells carrying the indicated genotypes were grown to mid-log phase and then shifted to nitrogen starvation medium for 4 h. The magnitudes of autophagy response were measured by the Pho8Δ60 assay (A) and the GFP-Atg8 processing assay (B). *4Δ*: *are1Δ are2Δ dga1Δ lro1Δ*. (A) Error bar, standard deviation from three independent experiments. (B) Representative images from three independent repeats are shown. α-tubulin was used as a loading control.

eliminated (Fig. S4B and C), indicating that synthesis of storage lipid is essential for glucose starvation induced autophagy.

3.3. TAG synthesis is required for recruitment of select Atg proteins after assembly of PAS scaffold

To further characterize how storage lipid synthesis participates in autophagy, we first examined the subcellular localization of GFP-Atg8. Atg8 serves as reliable marker of autophagosomal membrane [6,7]. After 1 h of nitrogen starvation, wild type cells contained on average 1.6 GFP-Atg8 puncta per cell (Fig. 3A and B). In contrast, the average number of GFP-Atg8 puncta dropped to 0.5 in *dga1Δ lro1Δ* cells (Fig. 3A and B). The number of puncta in *dga1Δ lro1Δ are1Δ are2Δ* cells was higher than that in *dga1Δ lro1Δ* cells, but lower than that in wild type cells (Fig. 3A and B). In *are1Δ are2Δ* cells, the number of GFP-Atg8 puncta was comparable to that in wild type cells (Fig. 3A and B). These results indicate that Dga1 and Lro1 (mainly responsible for TAG synthesis) are required for the generation Atg8-containing autophagic structures, and that Are1 and Are2 do not act in exactly the same manner. Considering that Are2 and Are1 participate in the synthesis of both TAG and SE, yet only have minor contribution to autophagy, we focused on *dga1Δ lro1Δ* in the remaining part of our analysis to reduce the complexity of data interpretation. As a further verification, we observed few GFP-Atg8 puncta in the vacuoles of *dga1Δ lro1Δ pep4Δ* cells (Fig. S3B), indicating that these cells failed to accumulate autophagic bodies. In contrast, GFP-Atg8 puncta were readily detectable in the vacuoles of otherwise wild-type *pep4Δ* cells.

Next, we examined localization of protein complexes that act upstream of Atg8: the Atg12-Atg5-Atg16 complex involved in Atg8-PE conjugation, the autophagy-specific phosphatidylinositol 3-kinase complex (containing Atg14), and the Atg1 complex (containing PAS scaffold proteins Atg13, Atg17 and Atg31) [6–8]. In wild type cells, all proteins displayed perivacuolar punctate signal, indicating that they are properly recruited to the PAS (Fig. 4A and B). In contrast, the formation of Atg5, Atg14 and Atg1 puncta was significantly reduced in *dga1Δ lro1Δ* cells (Fig. 4A and B). The numbers of Atg13, Atg17 and Atg31 puncta were comparable between wild type cells and *dga1Δ lro1Δ* cells (Fig. 4A and B). As controls, we verified that the proper recruitment of Atg5, Atg14 and Atg1 was restored by ectopic expression of *DGA1* and *LRO1*. Taken

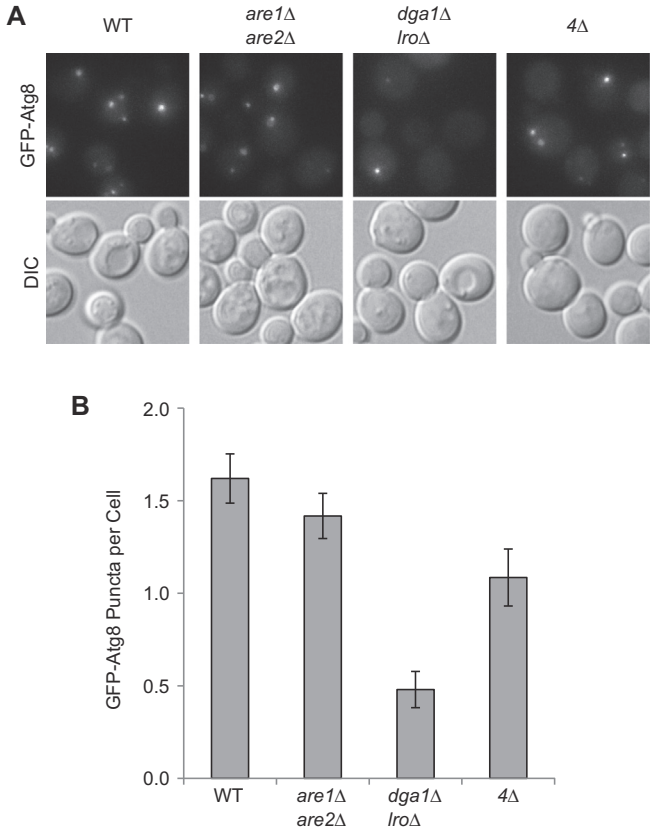

Fig. 3. TAG synthesis is involved in autophagosome biogenesis. Autophagosome biogenesis was assessed by fluorescence microscopy using GFP-Atg8 as a marker. Yeast cells expressing GFP-Atg8 were grown to mid-log phase and then shifted to nitrogen starvation medium for 1 h before observation. The experiment was repeated three times. *4Δ*: *are1Δ are2Δ dga1Δ lro1Δ*. (A) Representative images. For the GFP channel, a maximum intensity projection of a *Z*-stack is presented. DIC: differential interference contrast. (B) Quantification of the number of GFP-Atg8 puncta per cell. Error bar, standard error of the mean, $n \geqslant 75$.

together, these data indicate that TAG synthesis is not involved in the initial assembly of the PAS scaffold; instead, it is required for the efficient recruitment of downstream Atg proteins.

3.4. LDs may transiently interact with Atg8-PE containing membranes

Storage lipids including TAG and SE are stored and actively metabolized in LDs [13,14]. We therefore investigated the spatial relationship between LDs and the PAS. Under nitrogen starvation conditions, colocalization between the two entities (labeled by Dga1-GFP and Dudre-Atg8 [37], respectively) was observed in 9 ± 2% of the cells (Fig. 5A) (We did not use Lro1 for this experiment because it appeared to mainly reside in ER-like compartments (Fig. S5)). To see if the low rate of colocalization represents physiologically relevant transient interactions with autophagic compartments, we utilized an assay based on spatial restriction of Atg4 (Fig. S6A). Atg4 is a cytosolic protease responsible for the deconjugation of Atg8-PE. If the subcellular distribution of Atg4 is restricted by fusing Atg4 to an integral membrane protein, the success of the cleavage reaction will depend on the transient interaction of Atg4 containing compartments and Atg8-PE containing compartments. When Atg4 was restricted to peroxisomes, which have little known interaction with the PAS, the resulting autophagic flux was low (Fig. 5B and S6B) [38]. In contrast, when Atg4 was targeted to LDs (by fusing to Tgl3, a lipase residing on LDs that happens to express at a level comparable to that of Atg4), the resulting autophagic flux was significantly higher (Fig. 5B and S6B), confirming that LDs transiently interact with Atg8-PE containing compartments.

4. Discussion

In this study, we investigated the relationship between carbon metabolism and autophagy. We found that under nitrogen starvation conditions, yeast cells continued to consume glucose, yet downregulated fermentation. This was accompanied by increased TAG synthesis and proliferation of LDs. Inhibition of TAG synthesis resulted in severe defects in autophagosome biogenesis. Analysis of the subcellular localization of key Atg proteins suggests that the defects lie downstream of PAS scaffold assembly, and upstream of the recruitment of Atg1, Atg14, Atg5 and Atg8. We further demonstrate that LDs transiently interact with Atg8-PE containing membranes. Conversely, we found that the impact of autophagy on the remodeling of carbon metabolism to be minor.

Our data suggests that storage lipids and/or LDs are involved in an intermediate step of autophagosome biogenesis, albeit the precise mechanism is not clear. As TAG synthesis is closely linked with phospholipid synthesis [14,39], it is potentially plausible that interference with TAG synthesis might misdirect excess carbon into phospholipids, leading to pleotropic defects in the intracellular trafficking system. Although difficult to rule out completely, several pieces of evidence suggest that it might not be the main cause. First, the CPY pathway (a key route for the transport of zymogens from the Golgi to the vacuole) appeared operational in the quadruple knockout mutant [40]. This is consistent with our data demonstrating that the Cvt pathway was functional (Fig. S4A), and that endogenous Pho8 was active (Fig. S3A), both of which suggest that the trafficking within the endomembrane system remains active prior to starvation. Furthermore, we found that even under glucose starvation, in which there is no excess glucose, storage lipid synthesis was still required for efficient autophagy (Fig. S4B and C).

In addition to various lipid species, LDs also harbor a plethora of enzymes involved in lipid metabolism [13,14]. Therefore an alternative possibility is that LDs may serve as an intermediate reservoir and chemical factory that produces certain lipid species, which can then be utilized in autophagy. Transient contacts between LDs and autophagic membrane structures has been visualized by Dupont et al. in mammalian cells [41], which is in agreement with our microscopy data in yeast (Fig. 5A). Furthermore, our Atg4 spatial restriction assay provided an independent piece of evidence supporting the existence of interactions between LDs and autophagic membranes (Fig. 5B). Such interactions could facilitate the transport or exchange of lipid species between the two compartments. Consistent with this model, the same aforementioned study by Dupont et al. demonstrated that a neutral lipase, PNPLA5, was required for efficient autophagy [41]. In our system, elimination of Tgl3 and Tgl4, two major TAG lipases, was insufficient to cause substantial impairment in autophagy (Fig. S7A and B). As yeast has at least another four TAG lipases, we suspect that the activities of the remaining enzymes might be high enough to maintain normal autophagy. If TAG derived metabolites were indeed essential ingredients of the phagophore membrane, it would not be surprising that assembly of Atg proteins downstream of the scaffolds is affected in its absence. The functions of both phosphatidylinositol 3-kinase complex and the conjugation systems are clearly membrane dependent. Although Atg1 has often been portrayed as a close associate of the PAS scaffolds, recent publications revealed a physical and functional connection between Atg1 and Atg8 [42–44]. The

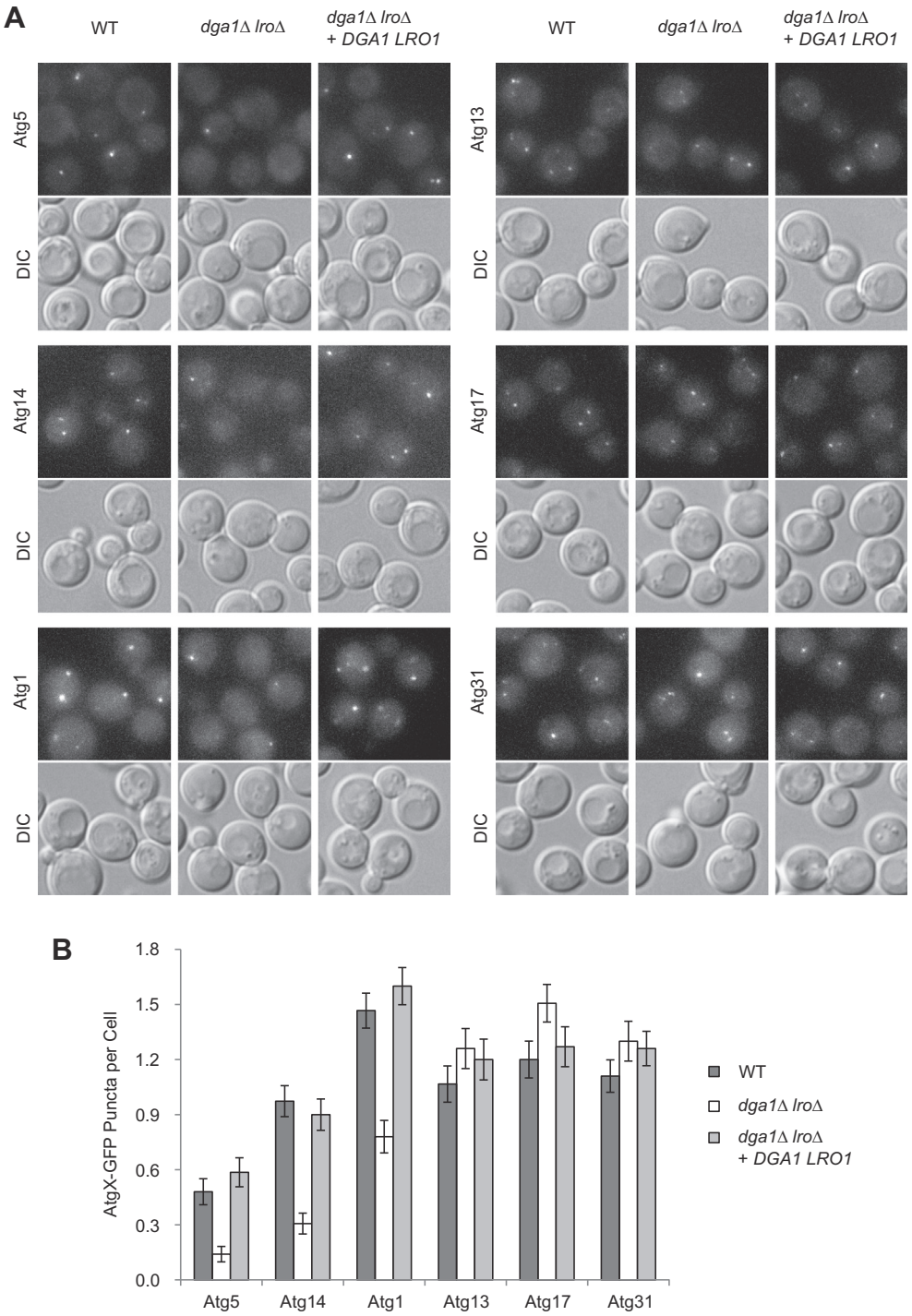

Fig. 4. TAG synthesis is necessary for the efficient targeting of Atg5, Atg14, and Atg1 to the PAS, but does not affect PAS scaffold assembly. Yeast cells expressing the indicated GFP constructs were grown to mid-log phase and then shifted to nitrogen starvation medium for 1 h before observation. The experiment was repeated three times. Images are presented as in Fig. 3.

dual personality of Atg1 might also explain why its PAS targeting was less dependent on TAG synthesis than other affected proteins (Fig. 4).

Finally, it is also possible that it is the formation of LDs, rather than TAG-related metabolism, that is important for autophagy. As the severity of LDs formation defects in these mutants correlates well with the degree of autophagy impairment (Fig. S8), it is not possible to draw a clear distinction between the role of lipid metabolism and the role of LDs from our current data. At present, we can only speculate what role of LDs might play beyond lipid metabolism. In the Cvt pathway, cargos constitute an important signal leading to the assembly of the PAS [18]. Given that LDs are also subject to autophagic degradation [45,46], a signaling role might be a tempting, if unorthodox, alternative.

In summary, our study highlights a novel connection between metabolism and autophagy. In response to depletion of nitrogen sources, yeast cells not only remodel their nitrogen metabolism, but also carbon metabolism. Importantly, the production of storage

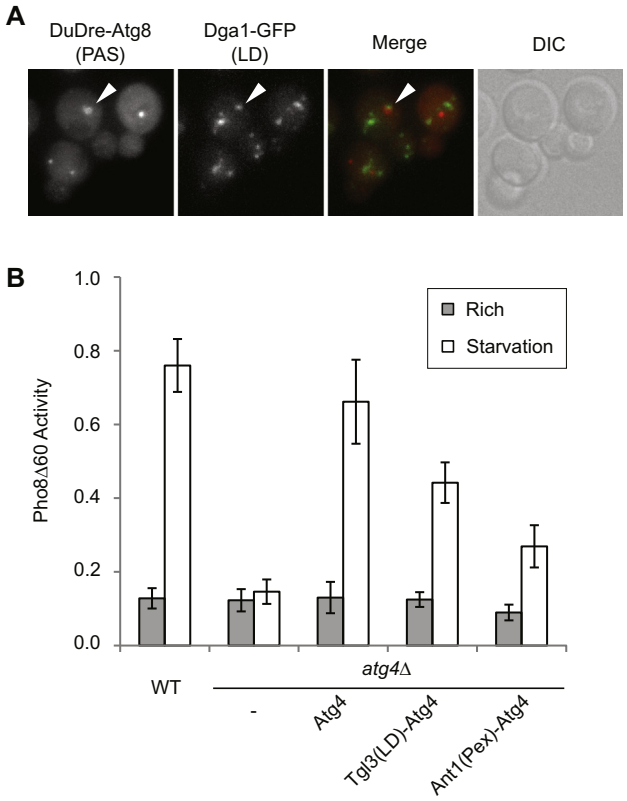

Fig. 5. LDs transiently interact with Atg8-containing membranes. (A) Occasional colocalization of LDs with the PAS. LDs and the PAS were labeled with Dga1-GFP and Dudre-Atg8, respectively. Cells were grown to mid-log phase and then shifted to nitrogen starvation medium for 1 h before observation. For fluorescent channels, maximum intensity projections of *Z*-stacks are presented. Arrow head, colocalized Dga1 and Atg8. Colocalization was present in 9 ± 2% of the cells (based on three independent repeats). (B) Atg4 restricted to LDs is partially functional. Atg4 was targeted to LDs or peroxisomes by fusing to Tgl3 or Ant1, respectively. The constructs were expressed in *atg4Δ* cells and the resulting autophagic flux was quantified by the Pho8Δ60 assay. Error bar, standard deviation from three independent experiments. See Fig. S6B for expression and localization of the Atg4 chimeras.

lipids is critical for the proper proceeding of autophagy, which in turn plays an irreplaceable role in the replenishment of nitrogen. Further research will be needed to test whether similar connections exist in tumor cells, which share certain characteristics of yeast carbon metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.11.050.

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