Analysis of autophagy activated during changes in carbon source availability in yeast cells

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Autophagy is a conserved intracellular degradation system in eukaryotes. Recent studies have revealed that autophagy can be induced not only by nitrogen starvation but also by many other stimuli. However, questions persist regarding the specific conditions. In experimental studies, abrupt nutrient changes are often used to induce autophagy. In this study, we investigated autophagy induction in batch culture on low-glucose medium, in which growth of yeast (Saccharomyces cerevisiae) is clearly reflected exclusively by carbon source state. In this medium, cells pass sequentially through three stages: glucose-utilizing, ethanol-utilizing, and ethanol-depleted phases. Using GFP cleavage assay by immunoblotting methods, fluorescence microscopy, and transmission electron microscopy ultrastructural analysis, we found that bulk autophagy and endoplasmic reticulum-phagy are induced starting at the ethanol-utilizing phase, and bulk autophagy is activated to a greater extent in the ethanol-depleted phase. Furthermore, we found that mitophagy is induced by ethanol depletion. Microautophagy occurred after glucose depletion and involved incorporation of cytosolic components and lipid droplets into the vacuolar lumen. Moreover, we observed that autophagy-deficient cells grow more slowly in the ethanol-utilizing phase and exhibit a delay in growth resumption when they are shifted to fresh medium from the ethanol-depleted phase. Our findings suggest that distinct types of autophagy are induced in yeast cells undergoing gradual changes in carbon source availability.

Autophagy is a conserved eukaryotic intracellular degradation system by which cytoplasmic components are delivered to the vacuolar (lysosomal) lumen and degraded by the vacuolar hydrolytic enzymes. During autophagy, the autophagosome, a key double-membrane structure, encloses various cellular components, including cytosolic proteins and organelles. Subsequently, the outer membrane of the autophagosome fuses with the vacuolar membrane, leading to release of the inner spherical structure, called the autophagic body, into the vacuolar lumen (1). Finally, the autophagic body disintegrates, and its contents are degraded by a variety of hydrolases. Although it was initially thought that autophagy exclusively entailed bulk engulfment of the cytoplasm, many types of autophagy that selectively degrade targets have been discovered (2). In addition, it is widely recognized that autophagy is involved in a variety of physiological processes.

The types of autophagy described above are categorized as macroautophagy. Two other types of autophagy have been described: microautophagy and chaperone-mediated autophagy (3). In microautophagy, substrates are directly enveloped by the vacuolar membrane and delivered to the vacuolar lumen (4). In chaperone-mediated autophagy, which has been characterized in higher eukaryotes, substrates for degradation directly translocate across the lysosomal membrane. Of these three types of autophagy, macroautophagy has been studied most extensively; accordingly, hereafter we will mostly refer to macroautophagy as autophagy.

Over the past 3 decades, a number of ATG genes, which play important roles on autophagy, have been identified, and the functions and structures of their protein products have been studied in the yeast Saccharomyces cerevisiae (1, 5). Fifteen Atg proteins, Atg1–10, Atg12–14, Atg16, and Atg18, play essential roles in autophagy, and these proteins are referred to as the core machinery for membrane formation. In starvation-induced autophagy, three Atg proteins, Atg17, Atg29, and Atg31, are additionally required. Most of the components of the core machinery are assembled at the pre-autophagosomal structure (PAS) in close proximity to the vacuole (6). In starvation-induced autophagy, Atg17 is required for PAS organization, suggesting that it acts as a scaffold protein (7). In contrast, in some types of selective autophagy, in which specific targets are sequestered by the autophagosome, Atg11 acts as a scaffold protein (8, 9). Selective autophagy requires receptors that are localized on targets and become landmarks for degradation: Atg19 and Atg34 for Ape1 (Cvt pathway) (10, 11); Atg32 for mitochondria (mitophagy) (12, 13); Atg36 for peroxisomes (pexophagy) (14, 15); and Atg39 and Atg40 for endoplasmic...
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Results

In synthetic medium containing low glucose, yeast growth is reflected exclusively by carbon source availability

The yeast *S. cerevisiae* exhibits diauxie when cultured in glucose-containing medium. Although many reports have described diauxic growth in *S. cerevisiae*, there is little detailed information about the relationships among cell density, glucose, and ethanol concentration in batch culture. To investigate this issue, we cultured prototrophic WT cells (X2180-1B strain) in standard SD medium containing 0.5% casamino acids (SDCA medium) and measured those parameters in detail (Fig. 1A).

The cells grew logarithmically while utilizing glucose and producing ethanol. When glucose was exhausted, cell proliferation was temporarily arrested, and at the same time, ethanol concentration reached a maximum. After a while, the cells started their growth, causing the ethanol concentration to gradually decrease. Ethanol-utilizing growth did not follow a logarithmic pattern, as reported previously (18, 28). To our surprise, ethanol was not depleted even when ethanol-utilizing growth was almost arrested. This result suggests that growth arrest is not caused by depletion of ethanol in SDCA medium, but instead by other factors.

There are at least two approaches to making yeast growth directly reflect carbon source availability: increasing the concentrations of other nutrients or decreasing glucose concentration. As shown in Fig. 1A, the OD_{600} reached 8–9 by the time that glucose was depleted in SDCA medium. We considered that the effects of high cell density would interfere with yeast growth in the ethanol-utilizing phase even if we increased the levels of other nutrients in SDCA medium. To avoid those effects, we lowered the glucose concentration. When we cultured WT cells in synthetic medium containing 0.2% glucose and 0.5% casamino acids (SD_{0.2}CA medium), the cells temporarily stopped their growth at an OD_{600} of ~1.2 (Fig. 1B). After the growth arrest, the cells restarted their growth by using ethanol. In contrast to the growth pattern in SDCA medium, ethanol-utilizing growth was logarithmic in SD_{0.2}CA medium (Fig. 1D). During growth, the oxygen consumption rate was higher in SD_{0.2}CA medium than in SDCA medium in the absence or presence of FCCP, an uncoupling reagent, suggesting that respiratory potential is higher in SD_{0.2}CA medium than in SDCA medium (Fig. 1C). Importantly, arrest of ethanol-utilizing growth in SD_{0.2}CA medium occurred at the time when ethanol was exhausted. Furthermore, we confirmed that the yeast did not show significant growth on SCA medium, which did not contain glucose, in our experimental duration (Fig. S1A). Because OD_{600} exceeds 10 in SDCA medium, we considered that nitrogen sources are enough for yeast growth in SD_{0.2}CA medium. As expected, ammonium was not depleted during cultivation, and extra ammonium did not improve the yeast growth (Fig. S1, B and C). Therefore, we concluded that SD_{0.2}CA is a suitable model medium in which yeast growth is reflected exclusively by carbon source availability. In SD_{0.2}CA medium, three stages of yeast growth became obvious: glucose-utilizing, ethanol-utilizing, and ethanol-depleted (stationary) phases (Fig. 1E). Hereafter, we primarily used SD_{0.2}CA medium to analyze the effects of carbon source availability.
Autophagy is induced after glucose depletion

We examined induction of autophagy during growth in low-glucose medium using GFP-Atg8–expressing cells. When autophagy is induced, GFP-Atg8 is delivered to the vacuolar lumen via the autophagic pathway; the GFP moiety is released from GFP-Atg8 by vacuolar proteases, and free GFP is detected by immunoblot analysis (29). Endogenous expression of GFP-Atg8 did not alter the growth features observed in WT cells cultured in low-glucose medium (data not shown). We found that cleavage of GFP-Atg8 occurred in the ethanol-utilizing and ethanol-depleted phases but not in the glucose-utilizing phase (Fig. 2A). The cleavage depended strongly on ATG2 (Fig. 2A). In addition, we confirmed that GFP-Atg8 was delivered to the vacuolar lumen in WT but not atg2Δ cells (Fig. 2B). In a deletion mutant of ATG15, which encodes a putative lipase responsible for disintegration of autophagic bodies (30), we observed autophagic bodies moving around randomly (Movie S1). These autophagic bodies were not observed when ATG2 was simultaneously deleted (Movie S1). These results indicate that autophagy is induced after glucose depletion, i.e. in the ethanol-utilizing and ethanol-depleted phases. Moreover, our findings suggest that autophagy is activated to a greater extent in the ethanol-depleted phase than the ethanol-utilizing phase (Fig. 2A).

Cytosolic components, ER, and mitochondria are degraded via autophagy

Next, we sought to determine which substrates are degraded during growth in low-glucose medium. We performed the cleavage assay using WT cells expressing Pkg1-GFP (a cytosolic protein), Sec63-GFP (an ER membrane protein), Om45-GFP (a mitochondrial outer membrane protein), Pex11-GFP (a peroxisome membrane protein), or Osw5-GFP (a lipid droplet (LD) protein). Cells were collected from each culture at the indicated
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Our findings suggest that autophagy is activated to a greater extent in the ethanol-depleted phase in SD0.2CA medium (Fig. 2A). Consistent with this, the degradation rates of cytosolic components and the ER were higher in the ethanol-depleted phase than in the ethanol-utilizing phase (Fig. 3, B and C). The mitochondria were targets of autophagy during the ethanol-depleted phase (Fig. 3D). However, mitochondrial degradation was not observed during prolonged cultivation in SDCA medium (Fig. 5), suggesting that the mitochondrial degradation is induced not by long-term cultivation, but rather by ethanol depletion. It has been reported so far that mitophagy can be induced by prolonged cultivation on a nonfermentable carbon source or by shifting from a nonfermentable carbon source to nitrogen starvation medium containing glucose (12, 13, 31). In addition to those conditions, we propose that mitophagy is also induced by ethanol depletion after ethanol-utilizing growth.

Presence of amino acids may sustain autophagy activity during ethanol-depleted condition

The prototrophic WT yeast cells can synthesize the amino acids required for growth by themselves. However, as described above, we used medium containing casamino acids, acid hydrolysate of casein, to support the yeast growth. To evaluate effects of the presence of amino acids, we used low-glucose medium without casamino acids (SD0.2 medium). Growth feature in SD0.2 medium was similar to that in SD0.2CA medium; yeast growth is correlated with concentration of glucose and ethanol (Fig. 6A). However, there were two different aspects between those two conditions. In SD0.2 medium, the yeast growth was slower (Figs. 1B and 6A), and biomass was accumulated less in the ethanol-depleted phase (Fig. 6B), indicating that amino acids are used in the prototrophic yeast.

Using SD0.2 medium, in which ammonium was not depleted during cultivation (Fig. 6C), we repeated GFP cleavage assay of GFP-Atg8, Pgk1-GFP, Sec63-GFP, and Om45-GFP (Fig. 6, D–G). Regarding time points when autophagy is induced, results similar to SD0.2CA medium were obtained; bulk autophagy and ER-phagy were induced after glucose depletion, and mitophagy was induced in ethanol-depleted phase. We also

time points (Fig. 3A), and cell lysates were subjected to immunoblot analysis using anti-GFP antibodies (Fig. 3, B–F). The cytosol started to be degraded in the ethanol-utilizing phase, and its rate of degradation increased in the ethanol-depleted phase (Fig. 3B). A similar pattern was observed in the ER degradation, although the degradation rate was very low (Fig. 3C). On the contrary, the mitochondria were degraded only in the ethanol-depleted phase (Fig. 3D). In contrast to these organelles, degradation of peroxisomes and LDs was not detected during the experiment period (total cultivation time, 100 h) (Fig. 3, E and F). Rather, the amount of Pex11-GFP increased during the ethanol-utilizing and the ethanol-dephased phases, and Osw5-GFP increased during the ethanol-utilizing phase. We ascertained that Sec63-GFP and Om45-GFP were delivered and Osw5-GFP increased during the ethanol-utilizing and the ethanol-depleted phases, suggesting that these cellular components are degraded by the autophagic pathway. Degradation of the cytosol was highly dependent on ATG17, but not ATG11 (Fig. 4A). A similar pattern was observed for degradation of the ER (Fig. 4B). These results suggest that cytosolic components and the ER are degraded in an Atg17-dependent manner under our experimental condition. Importantly, double-deletion of ATG39 and ATG40 diminished ER degradation (Fig. 4B), suggesting that ER-phagy receptors are required for ER degradation under these conditions. In contrast, degradation of the mitochondria was highly dependent on ATG11 but only partially dependent on ATG17 (Fig. 4C). In addition, mitochondrial degradation was not detected in cells lacking ATG32 (Fig. 4C). These results indicate that mitophagy occurs in the ethanol-depleted phase. In summary, bulk autophagy and ER-phagy were induced after glucose depletion, whereas mitophagy was induced in the ethanol-depleted phase. The possible roles of Atg17 and Atg11 are discussed below (see “Discussion”).
ascertained that the degradation was dependent on ATG2. However, we noticed that some different points were observed in the ethanol-depleted phase between SD0.2CA medium and SD0.2 medium. In SD0.2CA medium, the longer the ethanol-depleted phase was, the greater the amount of free GFP accumulated (Fig. 3, B–D). In contrast, in SD0.2 medium, such accumulation was not observed (Fig. 6, E–G). In particular, ER degradation was reduced in the ethanol-depleted phase (Fig. 6F). It should be noted that the GFP cleavage assay only gives an indication of the amount of free GFP moieties in vacuoles at a certain time, which is determined by both influx from autophagy and degradation by vacuolar hydrolytic enzymes. Therefore, it cannot be reliably used as a quantitative measure to analyze or discuss data obtained under different conditions. Nevertheless, the results described above raised a possibility that the presence of amino acids may sustain autophagy activity during the ethanol-depleted phase.

**Microautophagy occurs in the ethanol-utilizing and ethanol-depleted phases**

As described above, it is considered that cleavage of GFP-Atg8 is not observed in autophagy-defective mutants. However,
we detected a significant amount of free GFP, even in atg2Δ cells expressing GFP-Atg8, in both the ethanol-utilizing and ethanol-depleted phases (Fig. 2A). Hence, we investigated how cleavage of GFP-Atg8 occurred in atg2Δ cells.

Initially, we sought to determine whether the GFP-Atg8 cleavage occurred in deletion mutants of the other core ATG genes in the ethanol-depleted phase (Fig. 7A). GFP-Atg8 cleavage was hardly detected in single deletion mutants of ATG3, ATG4, ATG5, ATG7, ATG10, ATG12, and ATG16, which encode components of the ATG conjugation system. In contrast, free GFP was clearly detected in single deletion mutants of ATG1, ATG2, ATG9, ATG13, ATG14, and ATG18. Because all components of the ATG conjugation system are required for the GFP-Atg8 cleavage, this result suggests that lipidation of Atg8 is essential for its delivery to the vacuolar lumen. Therefore, assuming that the amount of GFP-Atg8 that binds to the vacuolar membrane increases when GFP-Atg8 is lipidated, we speculated that lipidated GFP-Atg8 moieties are internalized into the vacuolar lumen via microautophagy, because it is reported that microautophagy occurs after the diauxic shift in YEPD medium (32). Consistent with this idea, we found that the free GFP bands detected in atg2Δ cells disappeared upon simultaneous deletion of VPS4 (Fig. 7B), which is involved in microautophagy after the diauxic shift (32). Furthermore, we demonstrated that cleavage of Vph1-GFP, a vacuolar membrane protein thought to be cleaved by microautophagy, occurred in the ethanol-utilizing and ethanol-depleted phases (Fig. 7D) and that this cleavage took place independently of the ATG genes (Fig. 7C). These results indicate that microautophagy occurs in the ethanol-utilizing and ethanol-depleted phases. On the basis of these findings, we propose that microautophagy degrades lipidated GFP-Atg8 without machineries for macroautophagy and that this is the reason that GFP-Atg8 was cleaved in atg2Δ cells. However, the possibility that lipidated GFP-Atg8 is transported into the vacuolar lumen via the multivesicular body pathway (33) cannot be excluded. As shown in Fig. 7A, we also found that cleavage of GFP-Atg8 was barely detectable in the deletion mutant of ATG6, which encodes a component of the phosphatidylinositol 3-kinase complex; however, the underlying reason remains to be elucidated.

Next, we performed electron-microscopic analysis to observe in detail WT cells in the glucose-utilizing and ethanol-depleted phases (Fig. 7E, upper panel). We observed spherical bodies, which we hereafter call microautophagic bodies, in the vacuolar lumen of cells in the ethanol-depleted phase; these bodies contained cytosolic components and LDs. We then confirmed, by BODIPY staining, that LDs were delivered to the
vacuolar lumen (Fig. 7F and Movie S2). In the ethanol-utilizing phase, it was often observed that the vacuolar membrane was directly enwrapping LD (Fig. S2). Microautophagic bodies containing the cytosol and/or LDs were also observed in the atg2Δ mutant (Fig. 7E, lower panel), consistent with the idea that microautophagy occurs without machineries for macroautophagy under our experimental condition. One discrepancy is that cleavage of Osw5-GFP was not detected despite incorporation of LDs into the vacuoles (Figs. 3F and 7, E and F). However, because disintegration of microautophagic bodies, whose membranes are derived from the vacuolar membrane, progresses very slowly, this cleavage may not be detectable.

**Roles of autophagy in the ethanol-utilizing and ethanol-depleted phases**

Because autophagy is induced after glucose depletion in low-glucose medium, we investigated the roles of the autophagy in growth. First, we cultured WT or atg2Δ cells in low-glucose medium and measured cell density, glucose, and ethanol concentration (Fig. 8A). In the ethanol-utilizing phase, growth and ethanol consumption rate were significantly slower in atg2Δ cells than in WT cells. Second, we measured the viabilities of WT and atg2Δ cells in the ethanol-depleted phase by phloxine B staining (Fig. 8B). The viabilities of these strains did not differ until 5 days after entry into the ethanol-depleted phase. Subsequently, we re-inoculated WT or atg2Δ cells in the ethanol-depleted phase into fresh SD0.2CA medium, and we then measured cell density of the cultures every 10 min using an automatically recording incubator (Fig. 8C). In WT cells, the length of lag phase barely changed until 3 days after entry into the ethanol-depleted phase, whereas in atg2Δ cells, the lag phase became longer as the ethanol-depleted phase became longer. This result suggests that autophagy in the ethanol-depleted phase is required to promote re-growth in fresh medium.

Above, we demonstrated that mitophagy was induced only in the ethanol-depleted phase. Finally, we investigated whether mitophagy was required for promotion of re-growth in fresh medium (Fig. 8D). The atg32Δ strain, a mitophagy-deficient strain, exhibited delayed re-growth compared with the WT strain when it was shifted to fresh medium on day 0 or day 4 of cultivation after entry into the ethanol-depleted phase. This observation suggests that mitochondrial quality and/or quantity control in the ethanol-depleted phase is important for subsequent re-growth. However, the ER-phagy–deficient atg39Δatg40Δ strain did not exhibit delayed re-growth. In light of the difference between atg2Δ and atg32Δ cells, this finding suggests that not only mitochondria but also other cellular components must be degraded for cells to undergo subsequent growth. Future work should seek to identify the autophagic targets that influence the ability to re-initiate growth.

**Discussion**

In natural environments, nutrient conditions change from moment to moment. Yeasts must adapt to these gradual
changes, and their growth is influenced by the availability of nutrients at that moment. Abrupt nutrient changes are often used to investigate cellular responses. In nature, however, nutrient changes are more likely to be gradual in most cases. Therefore, it is important to investigate cellular responses based on their growth along with nutrient changes.

Here, we found that carbon source availability is an important determinant of growth when yeast is cultured in SD0.2CA medium (Fig. 1B). Amino acids derived from the hydrolysate of casein contributed yeast biomass (Fig. 5B), but yeast growth was correlated with glucose and ethanol concentrations (Figs. 1B and 5A). We consider that casamino acids should be utilized for protein synthesis or others even in the prototrophic yeast used in this study, and that glucose and ethanol are important factors for growth state. Hence, we categorized the yeast growth in SD0.2CA or SD0.2 medium into three stages: the glucose-utilizing, ethanol-utilizing, and ethanol-depleted phases (Fig. 1E). We investigated autophagy in each phase and found...
that bulk autophagy and ER-phagy were induced starting in the ethanol-utilizing phase and that the level of bulk autophagic activity increased in the ethanol-depleted phase (Figs. 3–6). In contrast, mitophagy occurred only in the ethanol-depleted phase (Figs. 3–6). Induction of autophagy in the ethanol-utilizing phase is an interesting discovery because nutrient availability is expected to be sufficient for yeast growth at this stage. This result implies that autophagy is constitutively induced in the absence of glucose. Consistent with this, our group previously reported that autophagy is induced during the diauxic shift, i.e., after glucose depletion, in SD medium (18). In the ethanol-depleted phase, bulk autophagy is more strongly activated, and the mitochondria become targets of autophagy. Thus, we demonstrated that distinct types of autophagy are induced during gradual changes of the carbon source availability.

Our previous report showed that iron levels are insufficient in SD medium after the diauxic shift and that growth after the diauxic shift is not logarithmic (18). Consistent with this, multiple studies have reported nonlogarithmic growth after the diauxic shift (28, 34, 35). Here, we showed that yeast growth was logarithmic in the ethanol-utilizing phase in low-glucose medium (Fig. 1D). In standard synthetic medium, certain nutrients are probably not present at sufficient levels for ethanol-utilizing growth. It is noteworthy that some essential nutrients in standard synthetic dextrose medium are insufficient to fully support yeast growth even during glucose-utilizing growth (36). These observations emphasize that the composition of growth media should be carefully considered when we perform long-term cultivation to investigate cellular responses to gradual environmental changes. In particular, the term “stationary
phase” is often used without precise definition. Importantly, under our experimental conditions, stationary phase was caused by ethanol depletion.

In this study, we revealed that three types of macroautophagy are induced after glucose depletion: bulk autophagy, ER-phagy, and mitophagy (Figs. 3, 4, and 6). However, the peroxisome and LDs were not degraded (Fig. 3, E and F). Rather, both organelles increased during the ethanol-utilizing phase. Because lipases for triacylglycerols and steryl esters are localized in LDs (37–40), fatty acids may be produced via lipolysis in the cytosol, converted to acyl-CoA, and assimilated in the peroxisome, which has β-oxidation activity in yeast, in the ethanol-depleted phase (41). The mitochondria should be needed to produce ATP, but the required amount of the mitochondria may be less in the ethanol-depleted phase than the ethanol-utilizing phase. Hence, the mitochondria may be degraded in the ethanol-depleted phase (Fig. 3D). There may be mechanisms so that organelle balance is appropriately adjusted according to the environment surrounding cells, and autophagy may contribute to the mechanisms.

Here, we further investigated the dependence of ATG17 or ATG11 on those types of autophagy (Fig. 4). It is now accepted that Atg11 functions as a scaffold protein to organize the PAS for selective autophagy. Indeed, ER-phagy induced by rapamycin treatment is partially dependent on ATG11 (16). Interestingly, under the ethanol-depleted condition, deletion of ATG11 did not influence ER degradation (Fig. 4B). One possible explanation for the weak dependence of ER-phagy on Atg11 in the ethanol-depleted phase is that a large fraction of Atg11 might be preferentially recruited to the mitochondria. It is noteworthy that ER-phagy receptors are still required for ER degradation under these conditions (Fig. 4B). In the case of mitophagy, Atg11 plays an essential role (12, 13). Consistent with this, the mitochondria were not degraded in atg11Δ cells in ethanol-depleted phase (Fig. 4C). Under our experimental conditions, the mitochondria should be well-developed at the starting point of the ethanol-depleted phase, because the cells had previously undergone the ethanol-utilizing phase. To degrade those mitochondria, many molecules of Atg11 might be needed. Detailed observation of the behavior of Atg11 and various organelles under the same conditions will reveal the roles of scaffold proteins, including Atg17. The observation that the mitochondrial degradation is partially dependent on ATG17 implies that Atg17 functions with Atg11 (Fig. 4C). If these two Atg proteins functioned independently, the mitochondria would be degraded in atg11Δ cells. Therefore, it is necessary to carefully examine the balance of various autophagy and scaffold protein(s).

Questions remain regarding the effects of autophagy on the intracellular environment. Here, we demonstrated that autophagy-deficient cells exhibited slower growth in the ethanol-utilizing phase (Fig. 8, A and B). Previously, our group reported that autophagy-defective mutants exhibit a growth defect after the diauxic shift and that autophagy during the diauxic shift in SD medium contributes to iron recycling (18). However, SD0.2CA medium contains enough iron to support respiratory growth (data not shown). Because the proteomic profile changes dynamically during the diauxic shift (35), autophagy induced after glucose depletion might degrade some glycolytic enzymes, which are abundant in glucose-containing medium, thereby causing the yeast to rapidly alter the intracellular proteome in preparation for the ethanol-utilizing phase. Alternatively, amino acids, which are important for ethanol-utilizing growth, may be released by autophagy. We also showed that autophagy in the ethanol-depleted phase was required to promote re-growth in fresh medium (Fig. 8, C and D). Interestingly, mitophagy was required for the rapid adaptation (Fig. 8D). Reactive oxygen species (ROS) accumulate in autophagy-deficient cells under nitrogen starvation (42, 43) and in mitophagy-deficient cells under starvation after respiratory growth (44). It is possible that ROS released from excess mitochondria accumulate in atg2Δ or atg32Δ cells in ethanol-depleted phase; if so, these ROS could damage cellular components in a way that inhibits rapid adaptation to fresh medium.

The type of microautophagy that involves invagination of the vacuolar membrane, recently named Type 2 microautophagy (4), occurs after the diauxic shift in YEPD medium or standard synthetic complete medium (32, 45). Our results are consistent with those findings: microautophagy occurred in the ethanol-utilizing and ethanol-depleted phases, i.e. after diauxic shift. The vacuole invagination process occurs under various conditions, and it results in incorporation of various organelles into the vacuolar lumen (46–49). Although we found that cytosol and LDs are delivered to the vacuolar lumen (Fig. 7, E and F), it is possible that microautophagy results in uptake of other cellular components. One discrepancy is that Pgo1-GFP was barely cleaved in atg2Δ cells, although cleavage of GFP-Atg8 and Vph1-GFP was clearly detected (Figs. 4A and 7, B and D). Moreover, Osh5-GFP was not cleaved in the WT cells despite the presence of LDs in the vacuolar lumen (Fig. 7F and Movie S2). In the ethanol-depleted phase, microautophagic bodies containing cytosol and LDs were detected in the vacuolar lumen of WT cells, implying that they were not being efficiently degraded (Fig. 7E, upper panel). A possible explanation about these discrepancies is that the disintegration speed of microautophagic bodies might differ depending on their contents, resulting in different rates of cleavage. As described above, it is possible that LDs are degraded in the cytosol. It will be an interesting issue to clarify the contribution of LD degradations between in the vacuole and in the cytosol.

Carbon metabolism is a fundamental biological process that is involved in the production of energy and basic cellular components. We should be aware that the metabolic pathways of various nutrients are intricately connected with one another. Here, we focused on carbon source availability, but these influences extend to metabolism of nucleic acids and amino acids, which are deeply involved in induction of autophagy. In the future, integrated metabolomic analyses will provide a better understanding of the physiological roles of autophagy.

**Experimental procedures**

**Yeast strains and growth conditions**

Yeast strains used in this study are listed in Table 1. Gene deletions and epitope tagging of genes at endogenous loci were
performed by PCR-based methods and validated by PCR (50, 51).

An appropriate carbon source was added to YNB medium (1 g/liter potassium dihydrogen phosphate, 0.5 g/liter magnesium sulfate heptahydrate, 0.1 g/liter sodium chloride, 0.1 g/liter calcium chloride dihydrate, 2 mg/liter myo-inositol, 0.4 mg/liter calcium (+)-pantothenate, 0.4 mg/liter nicotinic acid, 0.4 mg/liter pyridoxine hydrochloride, 0.4 mg/liter thiamine hydrochloride, 0.2 mg/liter p-aminobenzoic acid, 0.2 mg/ml riboflavin, 2 μg/liter (+)-biotin, 2 μg/liter folic acid, 0.5 mg/ml boric acid, 0.4 mg/liter manganese(II) sulfate pentahydrate, 0.4 mg/liter zinc sulfate heptahydrate, 0.2 mg/liter iron(III) chloride hexahydrate, 0.2 mg/liter disodium molybdate(VI) dihydrate, 0.1 mg/liter potassium iodide, 0.04 mg/liter copper(II) sulfate pentahydrate, 5 g/liter ammonium sulfate) containing 0.5% Bacto™ casamino acid (Difco), as follows: 2% (w/v) glucose (SDCA medium) or 0.2% (w/v) glucose (SDₐₐCA medium). To prepare SD₀.₂ medium, 0.2% (w/v) glucose was added to YNB medium.

Yeast cells were inoculated from YPD plates (1% Bacto™ yeast extract (Difco), 2% Bacto™ peptone (Difco), 2% glucose, 2% agar) into 2 ml of SDCA medium in a test tube and grown overnight (pre-culture) at 30°C and 187 rpm using a rotator (RT-550, TAITEC Corp.) until glucose was completely exhausted. These cells were inoculated into fresh medium and cultured under the indicated conditions. Unless otherwise mentioned, yeast cells were grown at 30°C and 180 rpm in a 125-ml Erlenmeyer baffled flask (Corning) on a BioShaker (BR-43FL, TAITEC Corp.).

**Growth curve analyses**

OD₅₀₀ of yeast cultures were obtained using a spectrometer (U-2900, Hitachi) at the indicated time points after cultures were diluted to appropriate concentrations. For automatic
growth curve analyses, pre-cultured cells were inoculated at a starting OD_{600} of 0.01 into 5 ml of the indicated medium in L-shaped tubes and then cultured at 30 °C at 60 rpm in an automatically recording incubator (TVS062CA, Advantec). OD_{600} of yeast cultures was recorded every 10 min. Growth curves were obtained by connecting the average values of the respective times (n = 3).

**Measurement of dry cell weight**

Yeast cells were collected by centrifugation (10,000 rpm, 1 min), washed with distilled water, and collected by centrifugation (10,000 rpm, 1 min). The supernatant was discarded; the resultant pellet was frozen by liquid nitrogen, and the frozen-pellet was freeze-dried overnight using a freeze dryer (VD-500F, TAIITEC Corp.). Weight of the dried yeast cells was measured, and the dry cell weight per 1-liter of culture was calculated.

**Quantification of glucose and ethanol concentration in cultures**

Medium or yeast cultures were centrifuged at 10,000 rpm for 1 min, and supernatants were collected. Supernatants were diluted to the appropriate concentrations. Glucose, ethanol, or ammonium concentrations in supernatants were measured using the F-kit D-glucose (JK International Inc.), F-kit ethanol (JK International), or F-kit ammonia (JK International), respectively.

**Measurement of oxygen consumption rate**

Respiration of yeast cells was measured noninvasively at room temperature using a Fibox3 oxygen meter (PreSens Precision Sensing GmbH) with continuous stirring. Cells were collected and resuspended in 3 ml of YNB medium containing 0.5% (w/v) casamino acids and 1% (v/v) ethanol in a 15-ml tube at appropriate density. Then, the tube was vortexed for 3 s, and the cell suspension was immediately transferred into a cuvette. Dissolved oxygen in the culture was measured every second, with stirring, by the Fibox3. The changes of dissolved oxygen (DO) in the solution per unit time and OD_{600} were calculated (ΔDO^{no treatment} (% O_{2}/OD_{600}/min)) using dissolved oxygen values from 61 to 120 s. The same procedures were performed in the presence of antimycin A (5 μM) or FCCP (10 μM). On the bases of these measurements, ΔDO^{antimycin A} and ΔDO^{FCCP} were calculated. Finally, the true values of dissolved oxygen change as ΔDO^{no treatment} − ΔDO^{antimycin A} (no treatment) or ΔDO^{FCCP} − ΔDO^{antimycin A} (FCCP) were calculated.

**Immunoblot analyses**

Immunoblot analyses were performed as described previously (20). Antibodies against GFP (Roche Applied Science), Pglk1 (Invitrogen), β-tubulin (Wako), and Ape1 (a laboratory stock) were used to detect relevant proteins. Chemiluminescence signals were detected using a CCD camera system (LAS4000, GE Healthcare, or Fusion FX, Vilber Lourmat). Quantification of each band intensity was performed using Fusion© software (Vilber Lourmat).

**Microscopic image acquisition**

Fluorescence microscopy was performed as described previously (20).

**Ultrastuctural analyses**

Ultraststructural analysis of yeast cells was performed by Tokai-EMA (Japan).

**BODIPY staining**

BODIPY 493/503 was dissolved in DMSO at a final concentration of 1 mg/ml (BODIPY stock solution). Five microliters of the BODIPY stock was added to 495 μl of yeast culture, and the mixture was incubated for 5 min at room temperature.

**Determination of cell viability**

Cell viability was determined by phloxine B staining (52). Phloxine B was added to yeast cultures at a final concentration of 2.5 μg/ml. Cells were observed using an inverted fluorescent microscope (IX81, Olympus) equipped with a cooled CCD camera (CoolSNAP HQ, Photometrics) and a x40 objective lens (Uplan Apo ×40, Olympus) at room temperature. A power supply (BH2-RFL-T3, Olympus) was used to excite phloxine B. To obtain phloxine B fluorescence, an excitation filter (BP535-555HQ, Olympus), a dichroic filter (DM565HQ, Olympus), and a fluorescence filter (BA570-625HQ, Olympus) were used. Images were acquired using the MetaMorph software (Molecular Devices).

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