Autophagy is a bulk degradation process conserved from yeast to mammals. To examine the roles of autophagy in cellular metabolism, we generated autophagy-defective (atg) mutants in the X2180-1B strain background. We compared the growth of wild-type (WT) and atg cells in minimal (synthetic dextrose, SD) and rich (yeast extract/peptone/dextrose, YEPD) medium, and we found that mutations in the core autophagy machinery result in defects in the diauxic shift, the transition from fermentation to respiratory growth upon glucose depletion, specifically in SD. Furthermore, we confirmed that autophagy was induced prior to the diauxic shift, implying that it plays a role in this process. In YEPD, atg mutants grew normally, so we assumed that the insufficiency of certain nutrients in SD was responsible for the defects. We ultimately identified iron, which is a necessary cofactor for respiratory activity, as the nutrient required for the diauxic shift in atg mutants. Indeed, atg mutants exhibited defects in respiration, which was rescued by supplementation with iron. Based on these data, we hypothesized that autophagy is involved in iron recycling during the diauxic shift. smf3Δfet5Δ or smf3Δfet1ΔΔ cells, which are unable to export iron from the vacuole, also exhibit defects in the diauxic shift, so iron released from the vacuole is important for the shift in SD medium. Finally, we observed that smf3Δfet5Δ cells accumulated nearly twice as much vacuolar iron as smf3Δfet5Δatg2Δ cells, suggesting that autophagy is involved in iron recycling by the vacuolar transport and degradation of iron-containing cargos.

Autophagy is an intracellular self-degradation process conserved from yeast to mammals. A predominant form of autophagy (macroautophagy, hereafter referred to as autophagy) degrades cytoplasmic components, including proteins, organelles, and even some invasive bacteria. When autophagy is induced, cytoplasmic components are sequestered by de novo formation of double membrane-bound structures, called autophagosomes, in the cytosol. These structures are targeted to the vacuoles, and their outer membranes fuse with the vacuole membrane. Ultimately, inner membrane-bound structures, termed autophagic bodies, are delivered to the vacuoles and degraded.

In Saccharomyces cerevisiae, the molecular machinery for autophagy has been studied intensively for the past 2 decades (1). By contrast, the roles of autophagy on cellular metabolism remain relatively unexplored. In studies of the autophagic machinery, nitrogen starvation is frequently used to trigger autophagy to study the associated membrane dynamics, mainly because this condition induces autophagy most strongly. Moreover, most experiments conducted by our group and others have used cells grown in yeast extract/peptone/dextrose (YEPD) medium, which is nutrient-rich. However, in their natural habitat, yeast cells survive large variations in nutrient availability, suggesting that autophagy has unrevealed functions that allow cells to cope with such nutritional fluctuations. Accordingly, an exhaustive study of autophagy under various nutrient conditions should uncover novel insights.

When glucose is available, yeast cells preferentially generate ATP by fermenting it to ethanol, irrespective of the presence of oxygen. Upon glucose depletion, cells induce mitochondrial respiration and begin to consume ethanol for energy production (2, 3). This physiological change, referred to as the diauxic shift, is a complex process temporally organized by numerous cellular events (4). This transition from glycolytic to respiratory metabolism has attracted research interest in part because it is analogous to cancer metabolism, which is associated with enhanced glycolytic activity and impaired oxidative phosphorylation (5). Furthermore, because budding yeast is a suitable model organism for comprehensive studies, several studies have characterized the yeast diauxic shift at the system level. DeRisi et al. (6) found, using microarrays, that expression of more than 1,700 genes changes during the shift. More recently, using advanced proteomics techniques, Murphy et al. (7) investigated the abundance of more than 4,500 proteins during the diauxic shift and showed that levels of more than 2,000 proteins are altered. Their results suggest that during the diauxic shift the glyoxylate/TCA cycle, fatty acid oxidation, stress responses, glycogen metabolism, oxidative phosphorylation, and proteolysis are up-regulated, whereas glycolytic flux and ribosomal biogenesis are down-regulated.

In this study we sought to elucidate novel roles of autophagy by analyzing this process in cells growing in minimal
Iron recycling via autophagy

(synthetic dextrose, SD) medium (8). For that purpose, we generated autophagy (atg)-defective mutants in the prototrophic strain X2180-1B, and we compared the growth of WT and atg mutants in YEPD and SD. Prototrophic atg mutants exhibited defects in the diauxic shift specifically in SD. We subsequently found that bulk autophagy is induced during cell growth in SD and acts to produce substances required for acquisition of respiratory activity during the diauxic shift.

Results

Autophagy is required for the yeast diauxic shift in minimal defined medium

To study autophagy in cells growing under nutrient-poor conditions, we examined autophagy during cell growth in SD medium. To rule out unexpected metabolic perturbations caused by auxotrophy, a characteristic generally exploited for genetic manipulation, we used the prototrophic strain X2180-1B and its derivatives throughout this study. As a representative autophagy-defective (atg) mutant, we used a deletion strain of ATG2, one of the core ATG genes required for both non-selective and selective autophagy. First, we compared the growth curves of WT and atg2Δ cells in nutrient-rich YEPD and nutrient-poor SD (8, 9). In aerobic batch culture, both WT and atg2Δ cells exhibited biphasic growth curves in YEPD; cells halt growth upon glucose depletion and then restart growing using ethanol after a lag phase referred to as the diauxic shift (Fig. 1A). By contrast, in SD only WT cells exhibited the biphasic growth curve, whereas atg2Δ cells showed a growth defect following the diauxic shift (Fig. 1B).

We next examined cell growth of other atg mutants in SD medium. atg1Δ cells also exhibited growth defects following the diauxic shift (Fig. 1C), as did core atg mutants such as atg5Δ, atg6Δ, atg9Δ, and atg14Δ (data not shown), suggesting that all functional units of Atg proteins are required for the diauxic shift. By contrast, cells disrupted at the locus of ATG19 or ATG32, which encode receptor proteins of the cytoplasm-to-vacuole targeting (Cvt) pathway and mitophagy, respectively, exhibited normal growth following the diauxic shift. Likewise, deletion of ATG11, which encodes the canonical adaptor of selective autophagy, did not result in any growth defects (Fig. 1C). These observations indicate that these forms of selective autophagy are not involved in the diauxic shift. pep4Δ prb1Δ and atg15Δ cells, which lack vacuolar proteases and a putative lipase required for the disintegration of autophagic bodies (Fig. 1D), respectively, also failed to exhibit the diauxic shift. To validate this observation, we generated the atg2Δ mutation in an auxotrophic strain, SEY6210, and monitored growth in SD supplemented with essential amino acids. This mutant also exhibited defects in the diauxic shift (Fig. 1E), suggesting that the defect does not depend on strain background. Taken together, these observations suggest that consequent degradation of cytoplasmic components, rather than sequestration per se, is necessary for the diauxic shift in cells growing in SD.

Non-selective autophagy is induced during the diauxic shift in SD

Next, we examined autophagic flux during growth in SD using the GFP-Atg8 processing assay (Fig. 2A). In this assay, N-terminally GFP-tagged Atg8 is delivered to the vacuole upon autophagy induction. There, GFP is cleaved from Atg8, and the appearance of free GFP can be followed as a semi-quantitative reporter of autophagy. The GFP moiety released began to appear at cell arrest, suggesting autophagy is induced prior to glucose exhaustion (Fig. 2A, at sampling point #4), and the levels increased during the diauxic shift (from the sampling point #5 to #8). However, in atg2Δ cells, no free GFP was detectable. Measurements of glucose concentration in the medium revealed that WT and atg2Δ cells consumed glucose at similar rates and depleted glucose at almost the same time (Fig. 2A, lower graph/dotted lines). In addition, we confirmed that atg2Δ cells produced ethanol at rates similar to those in WT cells (Fig. 2B), supporting that atg2Δ cells have normal glycolytic activity.

Furthermore, we examined the autophagy flux in other atg mutants 0 and 5 h after glucose depletion. In atg15Δ and pep4Δprb1Δ cells (Fig. 2C), autophagy flux was barely detected as expected. In addition, in atg32Δ and atg19Δ cells, autophagy flux was similar to WT (Fig. 2C), suggesting that mitophagy and the CVT pathway are not associated with the defective growth phenotype observed upon diauxic shift.

Next, we used transmission electron microscopy (TEM) to observe cell morphology during the diauxic shift. For this purpose, we used the pep4Δ mutant to prevent degradation of autophagic bodies in the vacuoles (10). We collected the pep4Δ cells 4–5 h after glucose depletion. The collected cells were subjected to rapid freezing and the freeze substitution method and were observed by TEM. Autophagic bodies were clearly present in SD-grown cells but not in YPD-grown cells (Fig. 2D). The autophagic bodies induced in SD contained cytoplasmic components such as electron-dense ribosomes and occasionally also lipid droplets, mitochondria, and other structures. These data suggest that, in SD, autophagy is induced prior to glucose exhaustion and plays an essential role in the diauxic shift.

Iron ion supplementation restores post-diauxic shift growth of the atg2Δ mutant

Because atg2Δ cells exhibited normal biphasic growth curves in YEPD, we hypothesized that a shortage of a particular nutrient in minimal media is responsible for the defect in the diauxic shift in SD-grown atg2Δ cells. Therefore, we attempted to identify nutrients that restored the post-diauxic shift growth of atg2Δ cells in SD. Supplementation with a mixture of 20 amino acids (1 mM each) did not recover the post-diauxic shift growth of atg2Δ cells (Fig. 3A, +aa). Next, we examined the effect of various components of SD, including inorganic salts (monopotassium phosphate, magnesium sulfate, sodium chloride, and calcium chloride), vitamins, and trace elements on growth of atg2Δ cells. Supplementation with inorganic salts or vitamins did not affect SD-grown atg2Δ cells. We then tested four divergent ions of trace elements (copper, iron, manganese, and zinc), and we found that supplementation with Fe2+ at concentrations 10-fold higher than in normal SD restored the post-di-
auxic shift growth of atg2Δ cells (Fig. 3A). Fe³⁺ ion also restored growth, indicating that iron was effective regardless of its valence. As YEPD contains much more iron than SD (1650 μg of Fe/liter and 68.9 μg of Fe/liter, respectively), we hypothesized that the concentration of iron ion of SD is insufficient as cells undergo the metabolic changes associated with the diauxic shift.

To clarify the effect of iron on cell growth, we prepared modified SD with iron concentrations 0.5- and 10-fold higher than the standard concentration in SD (0.74 μM Fe³⁺), referred to as 0.5× Fe SD (0.37 μM Fe³⁺) and 10× Fe SD (7.4 μM Fe³⁺), respectively. We then compared the growth of WT and atg2Δ cells in SD, 0.5× Fe SD, 10× Fe SD, and Fe-free SD (Fig. 3B). In Fe-free SD, neither WT nor atg2Δ cells grew after the diauxic shift, indicating that iron is essential for this process. In 0.5× Fe SD, WT cells grew after the diauxic shift, whereas atg2Δ cells did not, indicating that this concentration of iron was insufficient to support the diauxic shift in the mutant (Fig. 3B, green solid and dotted lines, respectively). In 10× Fe SD (Fig. 3B, red line), both WT and atg2Δ cells exhibited biphasic growth curves. The post-diauxic growth of WT cells was also enhanced in 10× Fe SD, indicating that the concentration of iron ion in standard SD was insufficient for maximal post-diauxic shift growth even in WT cells. Under iron-limited growth conditions, atg2Δ cells exhibited a more pronounced decrease in post-diauxic shift growth than WT cells. These observations suggest that autophagy is involved in iron homeostasis under iron-poor conditions.

To test whether atg mutants have a defect in iron uptake, we used inductively coupled plasma-mass spectrometry (ICP-MS) to measure iron content in WT and atg2Δ cells 5 h following
Iron recycling via autophagy

Figure 2. Induction of bulk autophagy in SD. A, GFP-Atg8 cleavage assay of cells growing in SD. Sampling points are indicated on the growth curves (lower). Blue and red solid lines represent growth curves of WT and atg2Δ cells expressing GFP-Atg8, respectively. Glucose concentrations in media were determined simultaneously (WT, blue dotted line, and atg2Δ, red dotted line). Western blotting analysis of cell lysates from WT and atg2Δ cells expressing GFP-Atg8 at each sampling point (#1–8) (upper). Blots were probed with anti-GFP or anti-Pgk (loading control) mouse monoclonal antibodies. B, ethanol concentration in media during growth. Growth curves of WT (blue solid line) and atg2Δ cells (red solid line) and ethanol concentration in media of WT (blue dotted line) and atg2Δ cells (red dotted line). C, GFP-Atg8 cleavage assay of atg15Δ, atg17Δ, atg32Δ, and pep4Δprb1Δ cells. Cells were collected at t = 0 or 5 h after glucose depletion. Western blotting analysis of cell lysates from WT and atg15Δ, atg17Δ, atg32Δ and pep4Δprb1Δ cells. D, autophagic bodies accumulate in the vacuole during the diauxic shift. Electron microscopy of SD-grown (left) and YPD-grown (right) pep4Δ cells during the diauxic shift. Arrows point to autophagic bodies in the vacuole (scale bar, 500 nm).

glucose depletion. Iron contents (in nanograms/mg dry weight) were similar in WT and atg2Δ cells before the onset of respiratory growth when cells were grown in SD (Fig. 4A). We also monitored expression of Fet3, which is a component of the high affinity iron transporter complex on the plasma membrane, during growth (11). However, we observed defects in neither the protein levels nor cellular localization of Fet3 (Fig. 4B). We also examined the transcriptional levels of the iron regulon genes, FIT2 and FIT3, that are involved in iron uptake and are known to be up-regulated during the diauxic shift (12). The results indicate that induction of FIT2 and FIT3 was observed to a comparable degree upon the diauxic shift, suggesting that the iron regulon is induced independently of the core autophagy machinery (Fig. 4C). These data indicate that iron uptake in atg2Δ cells is not defective.

We also measured the cellular contents of iron, zinc, copper, and manganese ions at the time of glucose depletion in 10× Fe SD. Cellular iron content increased almost 2-fold following iron supplementation, whereas cellular zinc, copper, and manganese were not altered (Fig. 4A), indicating that 10× iron supplementation did not affect the cellular content of other metals that we tested.

We next asked whether a chelator of iron could recapitulate the growth defect in WT cells. To this end, we treated cells with a range of concentrations of bathophenanthroline (BP), an iron-chelating reagent. Following BP treatment, even WT cells growing in YEPD failed to undergo the diauxic shift, although fermentative growth was unaffected (Fig. 5A), suggesting that iron is an essential element for acquisition of respiratory activity during the diauxic shift. We also examined whether iron chelation induces autophagy using the GFP-Atg8 cleavage assay. WT and atg2Δ cells expressing GFP-Atg8 were treated with either 50 μM BP or 0.2 μM/ml rapamycin for 4 h, and GFP-Atg8 and free GFP were analyzed by Western blotting analysis. BP did not induce autophagy, whereas rapamycin induced autophagy in WT cells (Fig. 5B).

Finally, we sought to determine the time point at which elevated iron was required for growth. Iron supplementation just prior to the diauxic shift fully restored growth (red line in Fig. 5C), whereas iron supplementation 12 h after glucose exhaustion (the time of initiation of post-diauxic shift growth) did not (purple line in Fig. 5C). These results suggest that iron is required for the onset of the diauxic shift.

Iron supplementation improves the respiratory activity of atg2Δ cells

The respiratory system requires a large amount of iron in catalytic co-factors, Fe/S clusters, and heme (13). Therefore, we determined the oxygen consumption rates (VO₂) in WT and atg2Δ cells during growth. WT and atg2Δ cells exhibited similar low respiratory activity just prior to glucose depletion. Sub-
sequently, the VO$_2$ of WT cells increased linearly, whereas atg2Δ cell VO$_2$ also increased, but to a significantly reduced extent (Fig. 6A). We also examined levels of C-terminal GFP-tagged Sdh2 and endogenous Cox4, an iron-sulfur protein subunit of succinate dehydrogenase and a subunit of cytochrome c oxidase in WT and atg2Δ cells in SD (Fig. 6B). Protein levels of Sdh2-GFP and Cox4 increased to a similar degree in both WT and atg2Δ cells, suggesting that autophagy disruption does not affect the expression or stability of components of the electron transport chain.

Next measured the respiratory activity of cells during the diauxic shift in the presence or absence of a 10× iron supplement. Irrespective of autophagic activity, we found that iron supplementation enhances respiratory activity in both WT and atg2Δ cells. This suggests that the iron concentration of standard SD medium is not sufficient for maximal respiratory activity, even for WT cells (Fig. 6C). The acquisition of respiratory activity therefore appears to depend on the availability of iron in the medium, which in standard SD medium is unable to meet the requirements of mitochondria as metabolism is adjusted toward the generation of energy by respiration.

**Induction of autophagy during the diauxic shift increases vacuolar iron flux**

Among its many roles in the cell, the vacuole serves as an iron reservoir, maintaining a pool of this element for subsequent use. Although the role of export proteins in mobilizing vacuolar iron to the cytosol has been demonstrated, we hypothesized that autophagy may play a role in the delivery of iron-containing cellular components to the vacuole to supply the pool of iron within this organelle. As we established that the acquisition of respiratory activity is linked to the amount of iron present in the medium, we therefore investigated whether a link between autophagy and vacuolar iron flux can be demonstrated during the diauxic shift.

Under iron-poor conditions, CCC1, which encodes a transporter that moves iron from the cytosol into the vacuole, is transcriptionally repressed, and the vacuolar iron pool is extremely low (14, 15). Yeast has two redundant exporters on the vacuole membrane, Smf3 and Fet5/Fth1 (16, 17), so we deleted smf3Δ and either fet5Δ or fth1Δ. Single deletion of either smf3Δ or fet5Δ did not cause a defect in the post-diauxic shift growth (data not shown), whereas double disruption of
**Iron recycling via autophagy**

**Figure 5. Iron is essential during the diauxic shift.** 
A. Growth curves of WT and atg2Δ cells growing in YEPD containing 10 or 50 μM BP, an iron-chelating reagent. B. GFP-Atg8 cleavage assay of cells treated with BP or rapamycin. WT and atg2Δ cells expressing GFP-Atg8 were grown in YEPD to A600 = 1.0, and treated with 50 μM BP or 0.2 μg/ml rapamycin for 4 h. Cell lysates were subjected to Western blotting analysis using anti-GFP and anti-Pgk (loading control) antibodies. C. Growth curves of atg2Δ cells supplemented with 10× Fe (Fe³⁺, 7.4 μM) at t = 0 h (just prior to the diauxic shift) and t = 12 h (onset of post-diauxic shift growth) are indicated by red and purple lines, respectively. Gray triangles and squares represent growth curves of atg2Δ cells grown in 10× Fe and standard medium, respectively.

Smf3Δfet5Δ or Smf3Δfet5Δfth1Δ did result in a defect (Fig. 7A). This suggests that Smf3 and Fet5/Fth1 have redundant functions in the diauxic shift and that iron exported from the vacuole is important for this process. Total cellular iron did not differ between WT and atg2Δ cells during the diauxic shift. These observations suggest that autophagy is involved in the recycling of iron ions via the vacuole. Therefore, to determine whether induction of autophagy during the diauxic shift contributes to vacuolar iron flux, we measured vacuolar iron content in Smf3Δfet5Δ and Smf3Δfet5Δatg2Δ cells during the shift. We isolated vacuoles from other organelles (such as mitochondria, which contain large quantities of Fe/S proteins and other iron-containing complexes) in cells at the diauxic shift (Fig. 7B, left), and we determined their iron contents. In Smf3Δfet5Δ cells, the iron contents of the vacuolar fraction were more than 2-fold higher than that of the triple mutant Smf3Δfet5Δatg2Δ (Fig. 7B, right), whereas levels of vacuolar phosphorus in both mutants did not significantly differ. Deficiency in vacuolar iron export resulted in vacuolar iron accumulation, whereas in the absence of autophagy, no such accumulation was observed. These data clearly indicate that induction of autophagy during the diauxic shift contributes to iron recycling via degradation in the vacuoles of iron-containing substrates, such as Fe/S clusters, heme proteins, and mitochondria.

It has been reported that autophagy plays a critical role in the maintenance of mitochondria, as the disruption of autophagy causes mitochondrial dysfunction in cells grown in rich nonfermentable medium as well as cells subjected to nitrogen star-
vation medium for long periods. Such dysfunction manifests as respiratory defects, increased generation of reactive oxygen species, and an elevated rate of petite cell formation (18, 19). In contrast, in this study the disruption of autophagy caused a post-diauxic growth defect that is rescued specifically by the addition of iron (Figs. 3A and 6C). In addition, mitochondrial protein levels were normal, even in atg2Δ cells (Fig. 6B). Together, these observations suggest that autophagy is necessary for intracellular iron homeostasis during the diauxic shift, leading to perturbations in mitochondrial maintenance.

Discussion

In this study, we found that core atg mutants are defective in the diauxic shift in nutrient-poor SD but not in nutrient-rich YEPD (Fig. 1). To acquire respiratory activity, mitochondria require a large amount of iron. During the shift, the iron regulon, including the iron uptake pathway, is positively controlled by Snf1/Snf4 kinase and Aft1, which are the yeast homologues of mammalian AMP-activated protein kinase and the transcription factor controlling the iron regulon, respectively (12). This suggests that yeast cells prepare to take up external iron during the shift for use in respiratory metabolism. The concentration of iron in standard SD was insufficient for maximal respiratory activity even in WT cells (Fig. 3B). Furthermore, even in YEPD, WT cells treated with a certain concentration of bathophenanthroline, an iron-chelating reagent, failed to undergo the diauxic shift, whereas growth during fermentation was normal (Fig. 5A). These observations suggest that iron is a critical nutrient during the diauxic shift and subsequent respiratory growth. We propose that bulk autophagy plays a key role in the recycling of iron during the diauxic shift.

Autophagy is induced by carbon starvation (10). However, we did not assume that induction of autophagy during the diauxic shift was directly triggered by glucose exhaustion; in
Iron recycling via autophagy

YEPD, autophagy was not induced during the diauxic shift (Fig. 2D), even though glucose was exhausted prior to that point, as it is in SD. Moreover, neither iron deprivation nor treatment with an iron-chelating reagent triggered autophagy (Fig. 5B). At present, the molecular mechanisms that trigger autophagy during the diauxic shift remain unknown. Previously, Piggott et al. (20) performed a genome-wide study aimed at identifying genes involved in optimal survival during 14 days of anaerobic fermentation in synthetic grape juice. They found that the majority of core atg genes are involved in optimal fitness during fermentation and that autophagy is induced on the 2nd day, during the early stages of fermentation (20). Therefore, they suggested that under harsh nutrient conditions, an alternative signal triggers autophagy despite the presence of sufficient nitrogen and amino acids (21, 22). It is likely that yeast cells have multiple systems for induction of autophagy in response to various external cues. Indeed, in the accompanying paper (45), we demonstrate that zinc starvation induces autophagy via TORC1 inactivation.

In general, cellular iron trafficking must be tightly regulated because iron easily generates deleterious reactive oxygen species via the Fenton reaction (23). When iron is taken up into cells, it is immediately transported into mitochondria, which is the sole site of heme biosynthesis and is the major site of Fe/S cluster biosynthesis. Thus, mitochondria are not only the organelles with the greatest requirement for iron but also the center of the iron metabolism. In contrast, a subset of Fe/S cluster proteins is exported into the cytosol through Atm1, an iron transporter on the mitochondrial membrane, and inserted into apoenzymes via the cytosolic iron-sulfur protein assembly complex. Cytosolic Fe/S proteins are involved in several fundamental biological processes, including translation and several aspects of nuclear DNA metabolism such as DNA replication and repair (24–27).

In SD, iron is mainly present in the cytosol and mitochondria (15). Although bulk autophagy can in principle degrade non-selective substrates, we showed previously that Adh6 is the preferential target of bulk autophagy during nitrogen starvation (28). Therefore, we hypothesize that autophagy degrades unnecessary substrates to generate bioavailable iron for post-diauxic shift growth. The ribosome-associated factor Rli1, an essential cytosolic Fe/S protein, is a candidate substrate of bulk autophagy during the diauxic shift (25, 29). Ribosome biogenesis is strictly related to the growth rate of cells and is down-regulated during the diauxic shift (6, 7). Because respiratory growth after the diauxic shift is much slower than fermentative growth, the number of ribosomes required for growth is reduced. Although we have not identified preferential substrates of autophagy induced during the diauxic shift, it is likely that autophagy degrades iron proteins such as Rli1, which are required for rapid growth, to supply iron to mitochondria. In other words, autophagy serves as a balance on the demand for iron between respiratory activity and cellular growth under iron-poor conditions.

We showed that the vacuole plays a key role in iron recycling via proteolysis of iron proteins in the vacuole (Fig. 7). Vacuole is the major cellular site for iron storage. Under iron-replete conditions (>5 μM), excess cytosolic iron is imported into the vacuole via endocytosis or through Ccc1, a transporter on the vacuole membrane (30). In the vacuole, iron exists as Fe^{3+} due to the acidic intravacuolar pH, and it associates with (poly)-phosphate to form Fe^{3+}-P nanoparticles (31). However, as mentioned above, under iron-poor conditions, CCCI is down-regulated (14), so vacuolar iron is extremely scarce. Fet5/Fth1 and Smf3 were required for the diauxic shift in SD (Fig. 7A). In fact, FET5, FTH1, and SMF3 are also members of the iron regulon, which is up-regulated during the shift (32). These observations strongly suggest that under iron-poor conditions, iron recycling via the vacuole is required for the transition to respiratory metabolism and that bulk autophagy plays roles in such recycling by transporting iron proteins from the cytoplasm to the vacuole.

We previously showed that protein synthesis activity in atg mutants is lower than in WT cells during nitrogen starvation and that the products of bulk autophagy contribute to the free amino acid pool in these cells (33). Recent work showed that diverse products of autophagy, including not only amino acids but also fatty acids and sugars, are reutilized (33, 34). Here, we demonstrated that bulk autophagy is induced during the diauxic shift in SD and that bioavailable iron produced by autophagy is reused in the transition from glycolytic to oxidative energy production.

Experimental procedures

Yeast strains and culture conditions

Yeast strains used in this study are listed in Table 1. Strains were generated using one-step gene disruption or replacement methods, as described previously (35, 36). All deletion and epitope-tagged constructs in this study were validated by PCR. Cells were grown in synthetic defined (SD) or YEPD (1% yeast extract, 2% peptone, and 2% dextrose) medium. SD medium was prepared according to Ref. 46, with modifications as indicated in the text. For supplementation with amino acids, 1× Yeast Synthetic Drop-out Medium supplements without uracil (Sigma, Y1501) were used. Flask batch cultures were performed in polycarbonate Erlenmeyer culture flasks (Nalgene™) at 30 °C and 180 rpm.

Immunoblotting

Immunoblot analyses were performed as described previously (37, 38). Samples corresponding to 0.5 A_{600} units of cells were separated by SDS-PAGE followed by Western blotting. Antibodies against GFP (1:1,000, Roche Applied Science), Pdr1 (Invitrogen), Cox4 (Invitrogen), Pho8 (Invitrogen), Prb1 (39), Cox2 (a gift from Dr. Endo, Kyoto Sangyo University, Japan), and Pgk (Novex, Life Science) were used as primary antibodies.

Chemiluminescence was induced using Femtoglow HRP Substrate (Michigan Diagnostics), and images were acquired on a LAS-4000 instrument and processed using the MultiGauge software (Fujifilm Life Sciences).

Measurement of oxygen consumption

Oxygen consumption was measured using a Fibox3 oxygen meter (PreSens Precision Sensing GmbH). Aliquots of cultures were transferred into a 1-ml cuvette equipped with sensor
spots. Oxygen concentration in the medium was recorded at sampling intervals of 1 s with stirring. Oxygen consumption rates (\(\%O_2/s\))/A were calculated based on measurements of oxygen consumption in the cuvette.

**Total RNA extraction, Northern blot, and quantitative RT-PCR**

Total yeast RNA was extracted by the hot phenol method, as described previously (40). For real-time RT-PCR, total RNAs were purified using the RNeasy mini kit (Qiagen). cDNA was prepared using SuperScript® VILO cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. RT-PCR experiments were performed based on the manufacturer’s instructions. RT-PCR experiments were performed based on the stepwise method of using specific primers. The reactions were used in the following steps: FIT2 (forward, TCTTGGGTTCTATCA; reverse, CACCGGAGCCAGGCGG); FIT3 (forward, CTCAACTCAGGTGCTTTAT; reverse, CTTTCCTGAGAAGGCAG); FIT4 (forward, ATTATGTTGTTGCTGTGTTGG; reverse, CATTCTTGGTGAAGGGAATGATGCAG). The PCR efficiency of the primer pairs was calculated by the dilution series method using cDNA as the template. Relative expression levels were determined by the 2\(\Delta\Delta C_t\) method.

**Determination of metal contents by inductively coupled plasma-mass spectrometry**

Cells were harvested and washed with deionized water three times. The cells were then freeze-dried, and 10 mg of dry cells or 400 \(\mu\)g of isolated vacuoles were placed in acid-washed volumetric flasks. They were then digested with concentrated trace metal-grade nitric acid at 110 °C in a heating dry bath for 5–6 h. For complete digestion, \(H_2O_2\) was added, and the sample was heated for 1 h at 120 °C. Mineralized samples were brought to 5 ml with deionized water and subsequently subjected to determination of metal concentration by ICP-MS (ELAN DRC-e; PerkinElmer Life Sciences). Concentrations of phosphorus, manganese, iron, copper, and zinc were determined by the external calibration method. For quantification of iron, a dynamic reaction cell mode was used if necessary.

**Preparation of yeast vacuoles**

Preparation of pure yeast vacuoles was carried out as described previously with minor modifications (42, 43). Briefly, 6 g of cells grown until 5 h after glucose depletion in SD were harvested, washed with 100 mM Tris-HCl (pH 9.5), 10 mM DTT, and suspended in 1.1 M sorbitol at a density of \(2 \times 10^8\) cells/ml. To this suspension, Zymolyase 100T (0.24 mg/ml) was added, and digestion proceeded at 30 °C for 120 min with gentle shaking. After digestion, cells were washed twice with 1.1 M sorbitol, and the spheroplast pellet was resuspended at 6 \(\times\) dilution in lysis buffer (10 mM MES-Tris (pH 6.9), 0.1 mM MgCl₂, 12% Ficoll), homogenized in a Dounce homogenizer, and centrifuged in a swinging bucket rotor at 4,500 \(\times\) g for 5 min. The spheroplast lysate was transferred to a centrifuge tube. Centrifugation was performed in a swing-out bucket rotor (CP70MX, Hitachi rotor type P40ST) at 50,000 \(\times\) g for 30 min at 4 °C. The fraction floating on top of the tube was resuspended in 5 ml of lysis buffer. The homogenized crude vacuoles were overlaid on a layer of 3 ml of 8% Ficoll, 10 mM MES-Tris (pH 6.9), 0.5 mM MgCl₂, and a second layer of 3 ml of the same buffer containing 4% Ficoll, and then centrifuged at 50,000 \(\times\) g for 45 min (CP70MX, Hitachi rotor type P40ST). Intact vacuoles, which were floating on top of the 4% Ficoll layer, were collected with a spatula. Protein concentrations were determined by the BCA method (Pierce).

**Table 1**

| Strain | Genotype | Source |
|--------|----------|--------|
| X2180-18 | MATa SUCl2 malgal2 CUP1 | Yeast Genetic Stock Center |
| STR210 | MAL3 ura3-112 his3-52 Δ200 rpl1Δ-h9262 suc2Δ-h9274 TAP2Δ-h9274 GAL | This study |
| MMY2    | MATa SUCl2 malgal2 CUP1 atg1Δ-h9274 kanMX6 | This study |
| MMY7    | MATa SUCl2 malgal2 CUP1 atg2Δ-h9274 kanMX6 | This study |
| MMY10   | MATa SUCl2 malgal2 CUP1 atg19Δ-h9274 kanMX6 | This study |
| MMY11   | MATa SUCl2 malgal2 CUP1 pep3Δ-h9274 kanMX6 | This study |
| MMY16   | MATa leu2-3,112 ura3-52 his3-Δ200 rpl1Δ-h9262 suc2Δ-h9274 TAP2Δ-h9274 GAL atg2Δ-h9274 kanMX6 | This study |
| MMY98   | MATa SUCl2 malgal2 CUP1 atg8Δ-GFP-ATG5-hphNT1 | This study |
| MMY104  | MATa SUCl2 malgal2 CUP1 atg8Δ-GFP-ATG5-hphNT1 atg2Δ-h9274 kanMX6 | This study |
| MMY128  | MATa SUCl2 malgal2 CUP1 SDH2-GFP-kanMX6 | This study |
| MMY158  | MATa SUCl2 malgal2 CUP1 SDH2-GFP-kanMX6 atg2Δ-h9274 hphNT1 | This study |
| MMY198  | MATa SUCl2 malgal2 CUP1 atg32Δ-h9274 kanMX6 | This study |
| MMY209  | MATa SUCl2 malgal2 CUP1 atg15Δ-h9274 kanMX6 | This study |
| MMY304  | MATa SUCl2 malgal2 CUP1 FET3-GFP-kanMX6 | This study |
| MMY306  | MATa SUCl2 malgal2 CUP1 FET3-GFP-kanMX6 atg2Δ-h9274 hphNT1 | This study |
| MMY490  | MATa SUCl2 malgal2 CUP1 fht1Δ-h9274 kanMX6 | This study |
| MMY492  | MATa SUCl2 malgal2 CUP1 fht5Δ-h9274 kanMX6 | This study |
| MMY494  | MATa SUCl2 malgal2 CUP1 fht5Δ-h9274 kanMX6 | This study |
| MMY502  | MATa SUCl2 malgal2 CUP1 snf3Δ-h9274 fht1Δ-h9274 hphNT1 | This study |
| MMY505  | MATa SUCl2 malgal2 CUP1 snf3Δ-h9274 fht5Δ-h9274 hphNT1 | This study |
| MMY521  | MATa SUCl2 malgal2 CUP1 atg8Δ-GFP-ATG5-hphNT1 atg19Δ-h9274 kanMX6 | This study |
| MMY602  | MATa SUCl2 malgal2 CUP1 snf3Δ-h9274 fht5Δ-h9274 hphNT1 atg2Δ-h9274 hphNT2 | This study |
| MMY679  | MATa SUCl2 malgal2 CUP1 atg8Δ-GFP-ATG5-hphNT1 pep4Δ-zeoNT3 prb1Δ-h9274 kanMX6 | This study |
| MMY711  | MATa SUCl2 malgal2 CUP1 atg8Δ-GFP-ATG5-hphNT1 pep4Δ-zeoNT3 prb1Δ-h9274 kanMX6 | This study |
| MMY731  | MATa SUCl2 malgal2 CUP1 atg8Δ-GFP-ATG5-hphNT1 pep4Δ-zeoNT3 prb1Δ-h9274 kanMX6 | This study |
| TMY971  | MATa SUCl2 malgal2 CUP1 pep4Δ-zeoNT3 prb1Δ-h9274 kanMX6 | This study |
| JOY67   | MATa leu2-3,112 ura3-52 his3-Δ200 rpl1Δ-h9262 suc2Δ-h9274 TAP2Δ-h9274 GAL atg7Δ-atg8 Δ9 lys2-801; GAL atg7Δ-atg8 Δ9 lys2-801; GAL atg7Δ-atg8 Δ9 lys2-801; | This study |

**Iron recycling via autophagy**

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Miscellaneous methods

Intracellular localization of proteins was examined using an inverted fluorescence microscope as described previously (38). Ultrastructural analysis of yeast cells was performed by Tokai-EMA (Japan). Measurements of cellular glucose concentration and ethanol were performed using a glucose assay kit (F-kit n-glucose, J.K. International) and an ethanol assay kit (F-kit ethanol, J.K. International). Statistical analysis was performed by the Student’s t test. p values less than 0.05 were considered significant.

Author contributions—T. H. and Y. O. designed experiments and wrote the manuscript; T. H., T. K., and M. M. performed the experiments.

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