Potassium starvation induces autophagy in yeast

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ABSTRACT

Autophagy is a conserved process that recycles cellular contents to promote survival. Although nitrogen limitation is the canonical inducer of autophagy, recent studies have revealed several other nutrients important to this process. In this study, we used a quantitative, high-throughput assay to identify potassium starvation as a new and potent inducer of autophagy in the yeast \textit{Saccharomyces cerevisiae}. We found that potassium-dependent autophagy requires the core pathway kinases Atg1, Atg5, Vps34, as well as other components of the phosphatidylinositol 3-kinase complex. Transmission electron microscopy revealed abundant autophagosome formation in response to both stimuli. RNA sequencing indicated distinct transcriptional responses – nitrogen affects transport of ions such as copper while potassium targets the organization of other cellular components. Thus, nitrogen and potassium share the ability to influence molecular supply and demand but do so in different ways. Both inputs promote catabolism through bulk autophagy, but result in distinct mechanisms of cellular remodeling and synthesis.
INTRODUCTION

Many eukaryotic organisms experience nutrient starvation and their ability to adapt is important for survival. Adaptation to starvation is often characterized by alterations to signaling, transcription and metabolism (1-4). To support these changes, cellular components are recycled into useable building blocks by two distinct and complementary mechanisms. Whereas the ubiquitin proteasome system breaks down specific short-lived proteins into their constituent amino acids, autophagy targets a wider variety of cytoplasmic cargo for degradation (5).

Much of our understanding of proteostasis comes from genetic studies conducted in the budding yeast Saccharomyces cerevisiae. As first shown in yeast, and later in animals, autophagy can be induced by diverse nutritional and pharmacological signals that converge at the target of rapamycin complex 1 (TORC1) (6-10). In nutrient-rich environments, TORC1 remains active and inhibits autophagy via phosphorylation. Under pro-autophagy conditions, deactivation of TORC1 promotes assembly of autophagy-related (ATG) proteins (such as Atg1) and lipid chains. This step requires the phosphatidylinositol 3-kinase (PI 3-kinase) Vps34, in complex with Vps15 (regulatory kinase), Vps30 (adaptor) and either Atg14 or Vps38 (11-18). Activation of Vps34 leads to increased levels of phosphatidylinositol 3-phosphate, which enables downstream proteins such as Atg5 to assemble into a functional complex and catalyze the conjugation of the ubiquitin-like protein Atg8 (LC3, GABARAP and GATE-16 in animal cells) to phosphatidylethanolamine (19). This conjugate leads to membrane expansion around portions of the cytoplasm (20). This structure, known as the autophagosome, is a unique double-membrane vesicle that fuses with the vacuole (lysosome, in higher eukaryotes), resulting in the degradation and reuse of cytoplasmic contents (21-26).

In addition to removing cytoplasmic proteins, autophagy helps to replenish the cellular pool of biologically important metals, which are required in large abundance (calcium, potassium, sodium) or in trace amounts (iron, copper, zinc) to maintain physiological parameters such as cell volume, pH and protein synthesis (27-29). Under basal conditions, iron is recycled through the vacuolar transport and degradation of iron-containing cargo (30). Nitrogen limitation results in the release of calcium ions from lysosomes and the subsequent induction of autophagy through the activation of the transcription factor EB (31,32). In addition, the availability of certain ions influences basal as well as stress-induced autophagy. For example, zinc starvation promotes autophagic targeting of zinc-binding proteins in yeast (33). Therefore, autophagy and ion homeostasis share a complex reciprocal relationship that is only beginning to be appreciated.

Here we describe a new and important regulator of autophagy. Using a panel of independent and complementary methods, we show that short-term potassium starvation induces bulk autophagy. Using RNA sequencing, we demonstrate that nitrogen limitation and potassium starvation result in substantially different transcripational profiles. While nitrogen affects genes important to ion transport, potassium is less specific and regulates genes related to cellular organization. Taken together, our findings point to converging mechanisms for autophagic recycling of cellular materials in conjunction with distinct and complementary routes to transcriptional adaptation.
RESULTS

Potassium starvation promotes autophagy

Nitrogen limitation has long been the canonical inducer of autophagy. However, recent reports suggest other salts, amino acids and micronutrients are also important (33-36). Autophagy may indeed be upregulated to compensate for limited external availability of essential nutrients. We hypothesized that a quantitative and parallel analysis of growth medium components might reveal new pro-autophagy regulatory pathways.

To enable high-throughput comparison of autophagy responses across diverse nutritional conditions, we used Rosella, a fluorescent reporter of autophagy (Figure 1A) (37,38). Rosella is comprised of super-ecliptic pHluorin (green, pH sensitive) fused with DsRed.T3 (red, pH stable). Upon induction of autophagy, the reporter is transported to the lumen of the vacuole where the acidic pH lowers green fluorescence, while red fluorescence remains unaffected. Rosella response is presented as a ratio of red and green fluorescence. As shown in Figure 1B, cells transferred to nitrogen limitation conditions exhibited a large increase in Rosella response, as compared to control cells in standard medium.

Next, we used Rosella to test other nutrients important for proper cellular growth and homeostasis. As described in Table 1, synthetic complete medium with 2% dextrose (SCD) is composed of a nitrogen source (ammonium sulfate), sugar (dextrose), amino acids and nucleotides, as well as a base mixture (yeast nitrogen base or YNB) comprised of vitamins, trace elements and salts. YNB does not provide nitrogen, which is included separately as ammonium sulfate. Other investigators have shown that sugar starvation does not lead to completion of autophagy (39,40). Therefore, we turned our attention to other nutrients, and in particular, components of yeast nitrogen base. As shown in Figure 1B, cells displayed an increase in response that was similar in magnitude and substantially faster ($t_{1/2} \sim 1.25$ h) than that observed in nitrogen limitation media ($t_{1/2} \sim 4.00$ h). To identify the component(s) of YNB that individually contributed to the Rosella response (Figure 1B) we supplemented SCD(-YNB) medium with each individual vitamin, trace element or salt (Table 2 and Figure 1C), and monitored fluorescence over time. As shown in Figure 1D, Rosella response was significantly diminished upon addition of potassium phosphate (+KH$_2$PO$_4$) alone, while readdition of other salts had no effect (Figure 1D, inset). We note the comparatively small response to zinc sulfate. Depletion for Zn$^{2+}$ ions was reported to induce autophagy, but over longer timescales of $>16$ h (33). We conclude that, by this measure, potassium is the primary driver of autophagy observed in the absence of YNB.

To distinguish between the contributions of the constituent ions ($K^+$ and H$_2$PO$_4^-$), we measured autophagy in cells exposed to growth medium in which potassium phosphate (in the YNB) was replaced with ammonium phosphate [SCD(-potassium)]. As shown in Figure 1E, Rosella signal was elevated in these conditions, indicating that autophagy is specifically regulated by potassium cations. The response was recovered by the addition of 0.1-3 mM KCl, which has been used by others to replenish extracellular K$^+$ ions (41,42). In complete SCD medium, K$^+$ ions are present at a concentration of $\sim 7$ mM. As shown in the time profiles in Figure 1E, the response at 2 h was similar for both nitrogen and potassium but diverged substantially at later time points. The Rosella response at 2 h showed a half-maximal effective concentration of 1.2 mM KCl (Figure 1F), which was substantially lower than that required to activate the Hog1 kinase ($>50$ mM), which is crucial for cellular adaptation to external osmotic changes (43,44).

We observed substantial differences when comparing the response to SCD(-YNB) (Figure 1B) with SCD(-potassium) (Figure 1E). While KH$_2$PO$_4$ appears to have a predominant role in mediating the autophagic response in SCD(-YNB), we infer that the lack of additional components in SCD(-YNB) contributes in some way, perhaps in a synergistic or cooperative fashion, to the kinetics and magnitude of the differences when comparing these conditions. The differences are not due to specific anions,
given that autophagy is absent in SCD(-potassium) medium supplemented with KCl (Figure 1E) or SCD(-YNB) medium supplemented with KH$_2$PO$_4$ (Figure 1D, inset).

Potassium ions are highly abundant within yeast cells (~150-300 mM) and regulate ionic strength, turgor pressure, and enzyme function (28,45,46). In accordance with these requirements and as shown previously, cell growth is slowed by the lack of extracellular potassium (47). Addition of 0.3 mM KCl to SCD(-potassium) medium resulted in partial recovery of growth (Figure 1G). At 1.0 and 3.0 mM KCl, long term recovery of growth was near complete. The growth response at 20 h showed a half-maximal effective concentration of 0.9 mM KCl (Figure 1H). To ensure that cells remained viable, we monitored Propidium Iodide fluorescence (48) from individual cells in microplate wells exposed to the same growth media as in Figure 1G. As shown in Figure 1I, Propidium Iodide fluorescence remained low under potassium starvation conditions and was elevated upon nitrogen limitation. Thus, potassium is required for proper cell growth and autophagy. The autophagy response to potassium starvation is approximately 30% of that seen in response to nitrogen limitation. In contrast to nitrogen however, potassium is not required to maintain cell viability, at least in the short term.

**Potassium-dependent autophagy is mediated by autophagosomes**

Our findings presented in Figure 1 indicate that the potassium-dependent autophagy response is lower than that for nitrogen. The magnitude of autophagy is regulated by many factors that control the number or size of autophagosomes (49,50). To further compare potassium starvation and nitrogen limitation, we examined the accumulation of autophagosomes in individual cells using transmission electron microscopy (TEM). For these experiments, we used cells lacking the vacuolar protease Pep4, which is required for breakdown of autophagosomes within the vacuole (6). We first grew the cells for 6 h in SCD(-nitrogen) or SCD(-potassium) medium, harvested by centrifugation and prepared for TEM imaging (fixed, dehydrated, embedded, cut and stained). As expected, nitrogen limitation resulted in the formation of multiple autophagosomes in ~95% of the cells. On average, we observed 6.9 ± 0.9 autophagosomes per cell with a cross-sectional area of 0.102 ± 0.042 µm$^2$ (Figures 2A and 2B). In potassium-starved samples, autophagosomes were evident in only ~10% of the cells, far fewer than that observed in nitrogen-limited cells. However, when present, the number (6.5 ± 1.0 per cell) and size (0.089 ± 0.037 µm$^2$) of autophagosomes was similar under both conditions. The presence of autophagosomes is a hallmark of macroautophagy, as opposed to other types of autophagy and in particular microautophagy. In both conditions, we observed accumulation of glycogen granules (G) and lipid droplets (L) in the cytoplasm (Figure 2A and 2C). These features are characteristic of bulk autophagy (6). While most of the autophagosomes observed in our TEM images were already engulfed by the vacuole, we also observed some that were fused to the vacuolar membrane (Figure 2C) prior to engulfment. From these results, we conclude that potassium starvation leads to autophagosome formation, but does so in only a subset of cells.

Whereas Rosella reports on the transport of cytoplasmic cargo to the vacuole, other methods directly monitor components of the autophagy machinery such as autophagosomes and vacuolar proteins. To confirm the pro-autophagy effect of potassium starvation, we monitored the spatial localization of the ubiquitin-like protein Atg8. Upon induction of autophagy, Atg8 conjugates with phosphatidylethanolamine to form autophagosomes, which deliver cytoplasmic contents to the vacuole (19,51). During this process, Atg8 assembles into autophagosomal clusters, which are observed as bright puncta within the cell (52). As shown in Figure 2D, we observed a substantial increase in GFP-Atg8 clusters under potassium starvation. This response was greater than that for nitrogen-limited cells, as is evident from the frequency distribution plots. These results indicate that autophagy is initiated in a majority of the cells,
but the process is completed in only a subset of those cells.

Potassium-dependent autophagy requires core ATG kinases and the PI 3-kinase complex

After being internalized by the vacuole, the autophagosomal cargo is released for degradation and recycling. This process can be monitored by cleavage of GFP-Atg8 (53). As shown in Figure 3A, GFP was observed after 3 h of potassium starvation and the response increased further at 6 h, indicating a sustained effect. Because GFP-Atg8 processing is typically more variable than the Rosella response, we validated our findings using the Pho8Δ60 enzymatic assay (54). The alkaline phosphatase Pho8 is translocated to the vacuole using the secretory pathway. While the truncated form is maintained in the cytosol (Figure 3B), it is transported to the vacuole under nitrogen limited conditions and subsequently activated via proteolytic processing. The increase in enzyme activity is a quantitative indicator of autophagy, and is measured as the conversion of a substrate to a fluorescent product. As shown in Figure 3B and in agreement with our findings from the other assays, potassium starvation resulted in an increase in activity that was approximately 38% of that observed with nitrogen limitation.

In addition to the bulk cytoplasm, organelles such as mitochondria, peroxisomes and ribosomes are also recognized as specific cargo for autophagic degradation. These selective forms of autophagy employ many of the core pathway components, as well as cargo-specific receptors (55-57). Previously, it was shown that pharmacological alterations to potassium ion homeostasis can lead to the production of reactive oxygen species, mitochondrial damage, and autophagic degradation of mitochondria (mitophagy) (58). Given these findings, we sought to determine the effects of our potassium starvation conditions on mitophagy. To that end, we monitored two GFP-tagged proteins, the mitochondrial outer membrane protein Om45 and the matrix protein Idh1 (59-61). As shown in Figure 3C, whereas nitrogen limitation resulted in the release of GFP from these proteins, potassium starvation had a negligible effect. Thus, bulk autophagy is regulated by nitrogen or potassium whereas mitophagy is dependent on nitrogen alone.

Vps34 is the sole phosphatidylinositol 3-kinase (PI3-kinase) in yeast and is essential for autophagy. Phosphatidylinositol 3-phosphate, generated by Vps34, is localized to autophagosomal membranes and is required for recruitment of other autophagy-related (ATG) proteins. Complex I is comprised of Vps34, a regulatory kinase Vps15, Vps30 (Beclin-1 in animal cells), and Atg14. Complex II contains Vps38 (UVRAG) in place of Atg14 (11-13). Whereas complex I mediates autophagy in nutrient-limiting conditions, complex II is essential for proper sorting of vacuolar enzymes under nutrient rich conditions. To test the role of the effector complexes in potassium-dependent autophagy, we analyzed individual gene deletions using the GFP-Atg8 immunoblotting assay. As anticipated, we observed no processing of GFP-Atg8 in cells lacking Vps34 and Vps15 (Figure 3D). Potassium-dependent autophagy required the canonical components Atg1 and Atg5, in a manner similar to the nitrogen response (Figure 3D). Moreover, autophagy was completely abrogated in cells lacking Atg14 (complex I) or the common adapter Vps30, and reduced by deletion of Vps38 (complex II) (Figure 3E). This is consistent with recent work suggesting reciprocal regulation of potassium signaling and TORC1 activity, which inhibits autophagy by phosphorylating Atg13, a component of the Atg1 kinase complex (41,62). Thus, both potassium- and nitrogen-dependent autophagy are mediated by Atg1, Atg5 and the PI 3-kinase complex.

Potassium starvation and nitrogen limitation exhibit distinct transcriptional profiles

Our data indicate that potassium and nitrogen regulate a common autophagy response to supply raw materials for cellular biosynthesis. To determine how this information is interpreted, we first considered phosphorylation-mediated activation of the mitogen-activated
protein kinase (MAPK) Kss1. In addition to its effects on autophagy, nitrogen limitation promotes cellular remodeling events such as filamentous growth (63), as well as the induction of genes involved in these processes, such as FUS1 (64-67). Notably, filamentation requires elements of the MAPK pathway (68) and Kss1 activation is important for the transcription of filamentation genes under nitrogen limitation conditions (69). Preliminary evidence from others suggested that potassium starvation also induces FUS1 transcription (42). However, the effect of nitrogen limitation or potassium starvation on Kss1 activation has not been investigated and more generally, the regulatory mechanisms shared by the autophagy and filamentous growth pathways are not fully understood (70, 71). To address this question, we used phos-tag gel electrophoresis to determine if either stimulus led to phosphorylation of Kss1. As expected, Kss1 was activated upon nitrogen limitation, although the effect was substantially less than that reported previously for addition of mating pheromone (Figure S1) (72). In contrast, Kss1 was unaffected by potassium starvation.

Another way to understand the consequences of autophagy is to measure global changes in gene transcription. To that end, we sequenced the transcriptome of cells exposed to nitrogen limitation or potassium starvation (in duplicate) prior to RNA extraction (Figure 4A). To compare the effects of the two stimuli, we carried out differential expression analysis, which revealed a dramatic impact on the transcriptome. Nitrogen limitation produced a highly dispersed response that included a large number of differentially expressed genes (DEGs) (log2(fold change) > 1 and adjusted p-value < 0.05) (Figure 4B). In comparison, potassium starvation resulted in fewer DEGs with substantially smaller fold-change values. Using this approach, we identified 107 DEGs common to the two conditions (Figure 4C). In addition to these shared targets, we found 68 and 626 unique DEGs for potassium and nitrogen, respectively. Principal component analysis (PCA) revealed that nitrogen limitation and potassium starvation lead to distinct transcriptional profiles (Figure 4D). To understand the functional processes targeted by these stimuli, we performed Gene Ontology (GO) enrichment analysis using the DEGs independently for each condition. Whereas potassium targets processes that organize and modify cellular components and organelles, nitrogen affects transport of copper and transition metal ions (Figure 4E). We conclude that potassium and nitrogen mediate their responses through distinct cellular mechanisms, as indicated by the appearance of differentially expressed genes (Figures 4B and 4C) and distinct GO annotations (Figure 4E).

**DISCUSSION**

Most pioneering studies of autophagy used nitrogen limitation as the stimulus, primarily due to its large and rapid response. Here we demonstrate, using a panel of reporter assays in individual cells and populations, that potassium starvation is a new and potent inducer of autophagy. The magnitude of the initial response is comparable to that observed with nitrogen limitation. Both stimuli transmit their effects via the canonical pathway components Atg1, Vps34, Vps15, Vps30, Atg14 and Atg5, which suggests a common route to cellular recycling. In contrast to these shared features, transcriptional responses to nitrogen limitation and potassium starvation are substantially different. Collectively, our data indicate that nitrogen and potassium share the ability to supply metabolic building blocks, but adopt distinct ways to reallocate these resources for future use.

Traditional methods to study autophagy require extensive sample preparation prior to readout and are not conducive to high-throughput analysis. In comparison, the Rosella plate reader assay provides a convenient, automated platform for quantitative measurements in diverse genetic backgrounds and growth conditions. Our comprehensive analysis showed that while nitrogen transmits the largest autophagy effects, potassium is also very effective, particularly in its induction of GFP-Atg8 clusters and the early Rosella response (<2 h). By integrating the Rosella reporter with other independent and well-established methods (electron microscopy, GFP-Atg8 immunoblotting and imaging, as well as the Pho8Δ60 alkaline phosphatase assay), we
were able to compare nearly 30 conditions and multiple time points, thereby accelerating the discovery process.

Our findings add to a growing list of novel regulators of autophagy such as amino acids, sulfate, glucose and, most recently, zinc ions (6,36). The potassium response is robust and mirrors that of nitrogen limitation particularly at early time points (<2 h). This is in contrast with zinc, which induces a response that is comparatively small and is evident only after prolonged treatment (>16 h) [Figure 1 and (33)]. Another class of inducers of autophagy operates through G protein coupled receptors (GPCRs). Prominent among these are the mammalian muscarinic cholinergic and β-adrenergic receptors, which bind to acetylcholine and epinephrine, respectively (73-78). Likewise, we have shown that the pheromone GPCR in yeast promotes vacuolar delivery of cytoplasmic contents (38,79). Whereas the response to nitrogen and potassium depends on both complex I and II of the yeast PI 3-kinase, mating pheromone requires complex II. Collectively, these studies demonstrate that multiple biochemically and physiologically distinct inputs converge on a common cellular process, one that is needed to support survival during changing growth conditions.

Although nitrogen and potassium induce a similar autophagy response, they are likely to serve distinct cellular needs. Nitrogen provides raw materials for synthesis of amino acids and nucleotides that enable the formation of proteins and nucleic acids. Potassium is accumulated within yeast and other eukaryotes to maintain important cellular parameters such as electroneutrality, pH, turgor pressure and cell volume. Although yeast is not well suited for direct (electrophysiological) analysis of potassium channel function, future studies could examine autophagy in mutants deficient in potassium transporter expression and activity. We observed that nitrogen targets genes that regulate transport of ions such as copper. In contrast, potassium impacts genes involved in a broader set of cellular processes, which overlap only partially with the nitrogen response. These data reveal that seemingly “redundant” input signals can nevertheless diverge and initiate distinct and complementary processes that prepare the cell for prolonged periods of nutrient deprivation.

In summary, our systematic and comprehensive studies highlight the power of high-throughput methods in hypothesis testing and discovery. Many important cellular pathways, including those mediated by the autophagy machinery, were first discovered in yeast and subsequently confirmed in more complex organisms. Given the conservation of pathways governing autophagy and ion homeostasis, we expect that potassium likewise regulates autophagy-related processes in humans. Such mechanisms may be important in understanding the functional consequences of hypokalemia, as occurs in chronic kidney disease, alcohol abuse and diabetic ketoacidosis.
EXPERIMENTAL PROCEDURES

Strains, plasmids and growth media. Yeast Saccharomyces cerevisiae strains used in this study were BY4741 (MATa leu2Δ met15Δ his3Δ ura3Δ), BY4741-derived gene deletion mutants obtained from the Yeast Knockout Collection (Invitrogen) or remade by homologous recombination of PCR-amplified drug resistance genes with flanking homology to the gene of interest (Table S1), and BY4741-derived GFP-gene fusions obtained from the Yeast GFP Clone Collection (Thermo Fisher Scientific) (80). PHO8ΔΔ0 (gift from Mara Duncan, University of Michigan) was stably integrated into the genome, replacing the native PHO8 gene. Plasmid pRS416-GFP-ATG8 (Addgene 49425) was a gift from Daniel Klionsky (81), and the GFP-ATG8 construct was subsequently introduced into the pRS415 vector (82). Rosella plasmid pAS1NB-DsRed.T3-SEP (2μ, ampR, LEU2+) was a gift from Mark Prescott and Rodney Devenish (37). The Kss1–9xMyc-tagged strain was generated by homologous recombination of a PCR-amplified 9xMyc cassette harboring a resistance gene to hygromycin B from plasmid pYM20 (pYM-9xMyc-hphNT1) at the C-terminus of the KSS1 open reading frame (ORF) (83,84).

Cells were grown in rich medium containing yeast extract (10 g/l), peptone (20 g/l) and 2% (w/v) dextrose (YPD) or synthetic complete medium (SCD) containing 2% (w/v) dextrose, ammonium sulfate, nucleotides, amino acids and a base mixture of vitamins, trace elements and salts (yeast nitrogen base, YNB) (Table 1). Plasmid selection was maintained by antibiotic supplementation or exclusion of appropriate nutrients. For nutrient starvation analysis, SCD(-nitrogen) medium was prepared by excluding ammonium sulfate, the major source of nitrogen. SCD(-YNB) medium was prepared by omitting yeast nitrogen base. To study individual YNB ingredients, SCD(-YNB) medium was supplemented with each component as listed in Table 2. Cells were cultured overnight with shaking at 30°C. Saturated cultures were diluted with fresh medium to optical density at 600 nm (OD600) = 0.1 and cultured for 6 h, then further diluted to OD600 = 0.001 and cultured for 18 h to maintain OD600 < 1 prior to use. For potassium starvation, cells were transferred to SCD(-potassium) medium, which was prepared using a modified YNB containing ammonium sulfate instead of potassium sulfate (Translucent K+ free medium containing ~15 μM K+, ForMedium CYN7501). As a benchmark for autophagy, cells were transferred to nitrogen limitation medium [SCD(-nitrogen)] lacking ammonium sulfate.

Rosella microplate-reader assay. Cells were transformed with pAS1NB-DsRed.T3-SEP and cultured in SCD(-leucine) medium. To start the starvation time-course, 1 ml of cells at OD600 ~ 1 were transferred to SCD(-potassium) medium after centrifugation at 13,000g for 1 min, washing and resuspension. 200 μL of cells were then added to individual wells in black clear-bottom 96-well microplates (Greiner 655087 or Corning 3631). Nitrogen-limited cells served as a reference treatment for autophagy. Untreated ‘control’ cells in SCD(-leucine) medium were included in separate wells to enable measurement of basal response. Microplates were sealed to reduce evaporation (adhesive Corning 3631) and placed in a microplate reader (Molecular Devices SpectraMax i3x) for 8 h at 30°C. At each timepoint, samples were shaken and fluorescence was measured for super-ecliptic pHluorin (SEP) (488 nm excitation, 530 nm emission) and DsRed.T3 (543 nm excitation, 587 nm emission). Background signal was measured using clear, cell-free medium. All experiments were performed in duplicate with three technical replicates. For each timepoint, Rosella response was calculated as the ratio of background-corrected dsRed.T3 and SEP fluorescence. Starvation–induced response was normalized with basal response observed for cells maintained in SCD(-leucine) medium. Dose-response profiles were calculated for the 2 or 8 h time points using a variable slope (four parameters) nonlinear regression with least squares fit (GraphPad Prism).

Statistical analysis. Statistical analysis of autophagy data was performed in Graphpad Prism with one-way ANOVA followed by Dunnett’s test for multiple comparisons.
Adjusted p-values were calculated relative to the untreated control (SCD) for Rosella response (Figures 1B, 1D and 1E), GFP-Atg8 cleavage (Figures 3A), Pho8Δ60 activity (Figure 3B) and Om45-GFP/Idh1-GFP cleavage (Figure 3C).

**Growth and viability measurements.** For cell growth measurements, wild-type BY4741 cells were cultured as described above to OD<sub>600</sub> ~1, then diluted to OD<sub>600</sub> = 0.05 into either SCD, SCD(-potassium) or SCD(-nitrogen) medium. 200 µl cells were transferred to a 96-well microplate and OD<sub>600</sub> was monitored in a SpectraMax i3x microplate reader at 30 min intervals for 48 h.

Cell viability was measured using a Celigo S Imaging Cytometer (Nexcelom) as described previously (83). Briefly, 200 µl of cells at OD<sub>600</sub> ~ 0.05 were mixed with 20 µM Propidium Iodide (P3566, Molecular Probes) and plated in half-area, black, clear-bottom 96-well plates (Greiner CELLSTAR) by centrifugation at 500g for 5 min at 4°C. Cells were imaged at 30 min intervals for 8 h at room temperature using the “Target 1+Mask” settings. Green fluorescence from Propidium Iodide was measured with the inbuilt GFP channel, while brightfield images were acquired simultaneously and used as masks for cell segmentation using Celigo’s native algorithm. Debris and cell clumps were excluded from the analysis by gating analysis based on the mean intensity and aspect ratio of Propidium Iodide. Background correction was performed by subtracting residual intensity from cell-free regions on the microplate. For data presentation, the mean Propidium Iodide intensity was averaged across 10,000 cells for each condition.

**GFP-Atg8 immunoblotting.** For the GFP-Atg8 processivity assay, cells were propagated in SCD(-leucine) medium prior to nitrogen limitation or potassium starvation (85). Cell extracts were obtained after 0, 3 and 6 h of starvation. Cells were lysed with TCA buffer (10 mM Tris-HCl pH 8.0, 10% (w/v) trichloroacetic acid (TCA), 25 mM NH<sub>4</sub> acetate, 1 mM Na<sub>2</sub>EDTA). Protein extracts were reconstituted in resuspension buffer (100 mM Tris-HCl, 3% (w/v) sodium dodecyl sulfate (SDS), pH 11.0), and protein concentration was determined using the Bio-Rad DC assay. Samples were normalized to 0.5-1 µg/µl with resuspension buffer and sample buffer (500 mM Tris-HCl, 20% (v/v) glycerol, 2% (w/v) SDS, 200 mM dithiothreitol, 0.01% (w/v) bromophenol blue, pH 8.5). Proteins were resolved on 10% SDS-PAGE gels, transferred to nitrocellulose membranes and detected by first incubating with blocking buffer (5% non-fat milk in TBST with 10 mM sodium azide) and, subsequently, immunoblotting with GFP antibodies (sc-999c, clone B-2, Santa Cruz Biotechnology) at 1:1,000 dilution in blocking buffer or glucose 6-phosphate dehydrogenase (G6PDH) antibodies (8866, Cell Signaling Technology, 1:1,000). Immuneactive species were detected with antibodies conjugated with horseradish peroxidase (715-035-150, Jackson ImmunoResearch) at 1:10,000 using ECL-plus reagent (Life Technologies). Protein bands were quantified on a BioRad Chemidoc Touch Imaging System using Image Lab software version 6.0.1 (Bio-Rad).

**GFP-Atg8 microscopy.** Clustering of GFP-Atg8 was measured using brightfield illumination on a Nikon Ti 2000 inverted microscope. Agar pads were prepared by dissolving 2% (w/v) agar in SCD(-leucine), SCD(-potassium) or SCD(-nitrogen) medium and spotting (200 µL) onto a clear glass slide. Another slide was quickly placed on top and the agarose was allowed to solidify. After removing the top slide, 5 µL of starved cells were mounted on the pad and sealed with a clean glass coverslip as described previously (86). GFP-Atg8 within single cells was imaged by excitation at 488 nm and emission at 500-550 nm. GFP-Atg8 spots were counted in 85-100 individual cells for each condition using FIJI image analysis software and graphs were plotted with Graphpad Prism (87).

**Om45-GFP and Idh1-GFP immunoblotting.** BY4741 cells expressing Om45-GFP or Idh1-GFP were sourced from the Yeast GFP Clone Collection (Thermo Fisher Scientific) and maintained OD<sub>600</sub> < 1 in YPD medium for ~24 h. To promote increased expression of the GFP-tagged proteins, cells were then diluted 100-fold.
with YPL medium (2% lactate instead of dextrose) and cultured for 12-16 h. Nitrogen limitation or potassium starvation was carried out for 6 h prior to protein extraction and immunoblotting analysis, as described above for GFP-Atg8.

**Pho8Δ60 enzymatic assay.** The Pho8Δ60 assay was performed as described previously (54). Cells were cultured for 24 h in SCD medium to OD$_{600}$ ~ 1 prior to transfer to SCD(-nitrogen) or SCD(-potassium) medium. After 0, 3 or 6 h of starvation, 5 ml cells were collected by centrifugation at 3000g for 5 min. Cell pellets were resuspended in 200 µl cold assay buffer (250 mM Tris-HCl pH 9.0, 10 mM MgSO$_4$ and 10 µM ZnSO$_4$), mixed with ~100 µl acid-washed glass beads (500 µm diameter) and lysed on an automatic vortex mixer (5 min at 4°C). The suspension was diluted with 50 µl assay buffer and cell debris were removed by centrifugation at 13,000g for 5 min. Protein concentration in the supernatant solution was determined with the BioRad DC protein assay. To measure enzyme activity, cell lysates were diluted 10-fold and incubated with substrate (5 mM α-naphthyl phosphate, Sigma-Aldrich N7255, dissolved in assay buffer) at 30°C for 20 min. The reaction was stopped by adding 1 ml of 2 M glycine-NaOH (pH 11.0) solution. Fluorescence was measured on a SpectraMax i3x microplate reader (345 nm excitation, 472 nm emission) and enzyme activity calculated as emission per unit protein (mg) in the reaction.

**Transmission electron microscopy.** BY4741 cells lacking the Pep4 vacuolar protease were maintained at OD$_{600}$ < 1 for ~24 h prior to nitrogen limitation or potassium starvation for 6 h. Cells were collected by centrifugation (1000g for 2 min) and samples were prepared for transmission electron microscopy according to standard methods (88,89). Cells were fixed with 2% (w/v) glutaraldehyde in 0.1 M PIPES buffer (pH 6.8 and containing 1 mM MgCl$_2$, 1 mM CaCl$_2$, and 0.1 M sorbitol) at room temperature for 1 h and stored at 4°C for several days. The cell wall material was permeabilized by mixing with 1% (w/v) sodium metaperiodate. Pellets were post-fixed with 1% osmium tetroxide/1.25% potassium ferrocyanide (w/v)/0.1 M PIPES buffer (pH 6.8) for 1 h and stained en bloc in 2% (v/v) aqueous uranyl acetate for 20 min. Cell pellets were dehydrated using a series of increasing ethanol concentrations, rinsed with 100% propylene oxide and embedded in Spurr’s epoxy resin (Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections (70-80 nm) were cut with a diamond knife, mounted on 200 mesh copper grids and stained with 4% (v/v) aqueous uranyl acetate for 12 min and with Reynold’s lead citrate for 8 min (90). Samples were observed using a JEOL JEM-1230 transmission electron microscope operating at 80kV (JEOL USA, Inc., Peabody, MA) and images were acquired with a Gatan Orius SC1000 CCD Digital Camera and Gatan Microscopy Suite 3.0 software (Gatan, Inc., Pleasanton, CA).

TEM images were analyzed using FIJI image processing software (87). Autophagosome frequency (number per cell) was estimated by manual counting for 50 representative cells. To measure autophagosome size, outlines of individual autophagosomes were traced using the freehand selection tool. Next, the image was spatially calibrated using the ‘Set scale’ tool to convert pixels to micrometers. Area (in square micrometers) was measured using the ‘Measure’ tool within the region of interest (ROI) Manager. Lipid droplets and vesicles were excluded from the analysis based on their location (vacuole vs cytoplasm), brightness (lipid droplets were significantly brighter) and size (vesicles were 5-10 fold smaller) relative to autophagosomes.

**PhosphoMAPK analysis.** MAP kinase activation was measured using quantitative immunoblotting as previously described (83). Briefly, BY4741 Kss1-9xMyc cells were grown to OD$_{600}$ ~ 1 in SCD medium and transferred to SCD(-nitrogen) or SCD(-potassium) medium or treated with 3 µM α-factor pheromone (positive control). Aliquots were collected at the indicated time points, mixed with 5% (w/v) TCA, and collected by centrifugation at 4000g for 2 min. Cell pellets were resuspended in TCA buffer and protein extracts were normalized with resuspension buffer and sample buffer. Proteins were resolved at 150V for 1.5 h at 25°C with
10% SDS-PAGE gels containing 50 μM Phos-tag (Wako Chemicals) and 100 μM Zn(NO₃)₂. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore IPVH00010) in Phos-tag transfer buffer at 20V for 20 h at 4°C. Blots were probed with Myc antibodies (2276, Cell Signaling Technology, 1:1000) or glucose 6-phosphate dehydrogenase (8866, Cell Signaling Technology, 1:1000) antibodies and quantified as detailed above.

**RNA sequencing.** Cells were grown to OD₆₀₀ ~ 1 in SCD medium, then transferred to SCD(-potassium) or SCD(-nitrogen) media by centrifugation and resuspension, and shaken at 30°C for 1 h. For each condition, 8x10⁷ cells were collected by centrifugation at 11,000g for 30 s at 4°C. The supernatant was aspirated and cell pellets were frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the RNeasy Mini kit (Qiagen 74104) with on-column removal of DNA using the RNase-Free DNase Set (Qiagen 79254). The cDNA library was prepared with the KAPA mRNA HyperPrep Kit (Roche 08098115702, KAPA Code KK8580), barcoded with NEBNext Multiplex Oligos (Illumina E7710S), and sequenced with Illumina NextSeq 500 for 75 bp single-end reads.

Quality check of raw sequence data was performed using the FastQC algorithm (91). Genome indices for yeast were downloaded from Ensembl.org and sequence alignment was performed using the STAR algorithm (92). Transcripts were quantified with the SALMON algorithm (93). Data were filtered to remove genes with <10 counts across all conditions and analyzed with DESeq2 package in R, while controlling for batch effect (94,95). All reported log₂ fold-change values and adjusted p-values for genes were directly obtained from the DESeq2 model. Gene Ontology (GO) term enrichment analysis was performed with the GO Term Finder (version 0.86) from the Saccharomyces Genome Database (https://www.yeastgenome.org/).
DATA AVAILABILITY STATEMENT

All gene expression data reported in this manuscript have been deposited to the NCBI GEO database (accession number GSE151898). All other data described herein are presented within this manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
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FOOTNOTES

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ABBREVIATIONS

The abbreviations used are: PI, Phosphatidylinositol; ATG, Autophagy-related gene; SCD, Synthetic Complete medium with Dextrose; YNB, Yeast Nitrogen Base; TEM, Transmission Electron Microscopy; MAPK, Mitogen-Activated Protein Kinase; DEG, Differentially Expressed Gene.
**TABLE 1:** Components of Synthetic Complete medium with 2% Dextrose (SCD)

| Nutrient                          | Name                                      | Amount (g/l, unless specified otherwise) |
|----------------------------------|-------------------------------------------|-------------------------------------------|
| Sugar                            | Dextrose                                  | 20                                        |
| Nitrogen                         | Ammonium sulfate                          | 5                                         |
| Nucleotides + Amino acids        | CSM Dropout mixture (CSM-Ura)             | 0.69                                      |
|                                  | Uracil                                    | 20 mg                                     |
|                                  | Adenine                                   | 30 mg                                     |
| Vitamins, compounds supplying trace elements, and salts | Yeast Nitrogen Base (YNB) without amino acids and without ammonium sulfate | 1.7                                       |

**TABLE 2:** Components of Yeast Nitrogen Base (without amino acids and without ammonium sulfate)

| Nutrient                          | Name                                      | Amount (µg/l) |
|----------------------------------|-------------------------------------------|---------------|
| Vitamins                         | Biotin                                    | 2             |
|                                  | Calcium pantothenate                      | 400           |
|                                  | Folic acid                                | 2             |
|                                  | Inositol                                  | 2000          |
|                                  | Niacin                                    | 400           |
|                                  | p-Aminobenzoic acid                       | 200           |
|                                  | Pyridoxine hydrochloride                  | 400           |
|                                  | Riboflavin                                | 200           |
|                                  | Thiamine hydrochloride                    | 400           |
| Compounds supplying trace elements | Boric acid                                | 500           |
|                                  | Copper sulfate                            | 40            |
|                                  | Potassium iodide                          | 100           |
|                                  | Ferric chloride                           | 200           |
|                                  | Manganese sulfate                         | 400           |
|                                  | Sodium molybdate                          | 200           |
|                                  | Zinc sulfate                              | 400           |
| Salts                            | Potassium phosphate                       | 1000          |
|                                  | Magnesium sulfate                         | 500           |
|                                  | Sodium chloride                           | 100           |
|                                  | Calcium chloride                          | 100           |
Figure 1: Potassium starvation promotes autophagy. (A) Rosella is comprised of super-ecliptic pHluorin (green) and DsRed.T3 (red). Upon induction of autophagy, Rosella is transported to the vacuole where low pH results in attenuation of green fluorescence, whereas red fluorescence is unaffected. (B) Cells expressing Rosella were maintained in exponential growth (OD at 600 nm < 1) for 24 h prior to nitrogen limitation or starvation for Yeast Nitrogen Base (YNB). Fluorescence was measured at 30 min intervals for 8 h. Response is presented as the ratio of red and green fluorescence. Inset: Table showing adjusted p-values for data at 2 h. (C) Components of YNB and their abundance (in grams) in 1 liter of growth medium. (D) Cells were grown in SCD(-leucine) medium as described for (B), then transferred to SCD(-YNB) medium individually supplemented with each YNB component. Rosella response is shown as % relative to the response in SCD(-YNB) at 2 h. Inset: Time-course of response in SCD(-YNB).
medium after addition of the major individual salt components. Adjusted p-values were determined for KH$_2$PO$_4$ addition relative to SCD(-YNB) (E) Time-course of response in SCD(-potassium) medium upon addition of 0-3.0 mM KCl. Inset: Table showing adjusted p-values for data at 8 h. (F) Dose-dependence of Rosella response shown in (E), for the 2 h and 8 h timepoints. (G) Cellular growth reported by optical density at 600 nm (OD$_{600}$), under the same conditions as in (E). (H) Dose-dependence of cell growth data shown in (G), for the 20 h and 40 h timepoints. (I) Time-course of cell viability measured as fluorescence from Propidium Iodide. Rosella experiments were performed at least three times and data presented as ± standard deviation for four technical replicates. Growth and viability data are reported for three biological replicates. Data were analyzed in Microsoft Excel and GraphPad Prism. Adjusted p-values were determined with one-way ANOVA relative to untreated cells (SCD).
Figure 2: Potassium-dependent autophagy is mediated by autophagosomes. (A) Representative images of individual cell sections after 6 h of nitrogen limitation or potassium starvation. Vacuole (V),
autophagosomes (*), lipid droplet (L), glycogen (G). (B) Distribution of the abundance (per cell) and size (area in µm²) of autophagosomes (n = 50). (C) High magnification image (80,000x) showing an autophagosome (arrowhead) fused to the vacuolar membrane. (D) Representative single cell images and distributions of GFP-Atg8 spots formed in response to 2 h of nitrogen limitation or potassium starvation (n = 85 cells).
Figure 3: Potassium-dependent autophagy requires autophagy-related kinases Atg1 and Atg5, and the PI 3-kinase complex. (A) In pro-autophagy conditions, the GFP-Atg8 reporter is transported to the vacuole and enzymatically processed to release GFP. GFP-Atg8 processing was monitored in wild-type BY4741 cells after 0, 3 and 6 h of nitrogen limitation or potassium starvation. Protein bands were quantified using ImageLab and presented as ratio of GFP and GFP-Atg8 (% of maximum). (B) Induction of autophagy results in activation of the Pho8Δ60 enzyme, which converts a fluorogenic substrate (α-naphthyl phosphate) into a fluorescent reporter (α-naphthol). Shown are data for Pho8Δ60 activity in the same conditions as (A). (C) Processing of mitophagy reporters Om45-GFP and Idh1-GFP in wild-type BY4741 after 6 h of nitrogen limitation or potassium starvation. Data are presented as ratio of GFP and Om45-GFP/Idh1-GFP (% of maximum). (D) Processing of autophagy reporter GFP-Atg8 after 6 h of nitrogen limitation or potassium starvation in BY4741 cells lacking core components of the PI 3-kinase complex (Vps34 or Vps15), or the autophagy pathway (Atg1 or Atg5). (E) Processing of GFP-Atg8 after...
6 h of nitrogen limitation or potassium starvation in BY4741 cells lacking regulatory components of the PI 3-kinase complex (Vps30, Vps38 or Atg14). All experiments were repeated three times and data are ± standard deviation for three or four biological replicates. Adjusted p-values were determined by one-way ANOVA relative to untreated cells (SCD).
Figure 4: Potassium starvation and nitrogen limitation exhibit distinct transcriptional profiles. (A) Schematic representation of experimental approach to obtain RNA sequencing data for potassium starvation or nitrogen limitation. (B) Differential expression analysis reveals statistically significant changes in RNA abundance. Genes with adjusted p-value <0.05 and log₂(fold change) > 1 are highlighted in color. (C) Venn diagram showing overlap between differentially expressed genes (DEGs) for potassium starvation and nitrogen limitation. Numbers in parentheses represent genes with a known Gene Ontology (GO) annotation. (D) Principal component analysis of sequencing data showing the first two principal components (PC1 and PC2). Colors represent treatment conditions and shapes denote biological replicates. Axis labels describe the percentage of variance explained by each principal component. Together, principal components 1 and 2 explain 94% variance of the data. For all conditions, replicates are closely spaced, which indicates that variance arises primarily from the nutritional treatments. (E) GO process terms enriched amongst the DEGs for each condition. RNA abundance was quantified for two biological replicates for each condition.