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TOR and MAP kinase pathways synergistically regulate autophagy in response to nutrient depletion in fission yeast

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ABSTRACT

General autophagy is an evolutionarily conserved process in eukaryotes, by which intracellular materials are transported into and degraded inside lysosomes or vacuoles, with the main goal of recycling those materials during periods of starvation. The molecular bases of autophagy have been widely described in *Saccharomyces cerevisiae*, and the specific roles of Atg proteins in the process were first characterized in this model system. Important contributions have been made in *Schizosaccharomyces pombe* highlighting the evolutionary similarity and, at the same time, diversity of Atg components in autophagy. However, little is known regarding signals, pathways and role of autophagy in this distant yeast. Here, we undertake a global approach to investigate the signals, the pathways and the consequences of autophagy activation. We demonstrate that not only nitrogen but several nutritional deprivations including lack of carbon, sulfur, phosphorus or leucine sources, trigger autophagy, and that the TORC1, TORC2 and MAP kinase Sty1 pathways control the onset of autophagy. Furthermore, we identify an unexpected phenotype of autophagy-defective mutants, namely their inability to survive in the absence of leucine when biosynthesis of this amino acid is impaired.

Abbreviations: ATG: autophagy-related; cAMP: cyclic adenosine monophosphate; cDNA: complementary deoxyribonucleic acid; GFP: green fluorescence protein; Gluc: glucose; Leu: leucine; MAP: mitogen-activated protein; MM: minimal medium; PI: propidium iodine; PKA: protein kinase A; RNA: ribonucleic acid; RT-qPCR: real time quantitative polymerase chain reaction; *S. cerevisiae*: *Saccharomyces cerevisiae*; *S. pombe*: *Schizosaccharomyces pombe*; TCA: trichloroacetic acid; TOR: target of rapamycin; TORC1: target of rapamycin complex 1; TORC2: target of rapamycin complex 2; YE5S: yeast extract 5 amino acid supplemented.

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Introduction

Macroautophagy, hereafter referred to as autophagy, has an important role in differentiation, and it is an evolutionarily conserved process that degrades cellular organelles as well as proteins. It requires rearrangement of membranes to create vesicles that sequester cytosolic lumen containing the organelles/proteins to be degraded: the autophagosome. Its outer membrane will fuse to lysosomes/vacuoles (in yeast and plants), to create the autophagic bodies, to be released and degraded by the proteases of these compartments (for recent reviews, see [1–3]).

Using *Saccharomyces cerevisiae* (*S. cerevisiae*) as a model system, a set of genes, termed *ATG*, were discovered that participate in autophagy. All the yeast mutants lacking Atg proteins lose viability during nitrogen starvation, with 80% viability loss after 5 days of nutrient depletion, and they cannot sporulate. In *S. cerevisiae*, both the PKA (protein kinase A) and the TOR (Target of Rapamycin) pathways are involved in the regulation of autophagy during conditions of nutrient scarcity (for reviews, see [4–7]).


Regarding the biological roles of autophagy, defects in this process are clearly linked to aging and to diseases (for reviews, see [8,9]). This degradative pathway causes turnover of organelles or proteins, and this autophagic degradation may breakdown proteins into amino acids, to use them again in subsequent protein synthesis pathways when environmental supplies are limiting.

Schizosaccharomyces pombe (*S. pombe*) has been used in the last years as a new model system to study autophagy. This process was first studied in the fission yeast through the characterization of vacuolar functions, with the identification and characterization of vacuolar proteases [10–16]. With the isolation of the first *atg* mutants [17], it was early discovered that autophagy is required during nitrogen starvation to supply enough nitrogen intracellularly, explaining the mating defects of these mutants. In general, during nutritional starvation cells exit the normal growth cycle to undertake alternative paths such as sexual differentiation in fission yeast. In particular, nitrogen depletion causes a G₁ arrest and triggers sexual differentiation, and in parallel, protein degradation

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 Supplemental data for this article can be accessed [here](#).

linked to autophagy occurs as well [13]. It was important to demonstrate that, even though autophagy promotes the entry into the sexual differentiation fate, *atg* mutants are not sterile in the presence of nitrogen [17]. Therefore, the major role of autophagy is to provide a nitrogen supply in the absence of external sources. Furthermore, the group of Takegawa demonstrated that the mating defects of some *atg* mutants are not complete, proposing that fission yeast is capable of storing a large enough intracellular nitrogen pool to allow partial sporulation under nitrogen-limiting conditions even in the absence of autophagy, which provides the majority of the nitrogen source for protein synthesis during sporulation [18].

Due to all the above, most *atg* mutants of fission yeast have been identified through screenings of deletion mutant collections searching for defects in mating [19]. Data from the Du lab has shed light on the conservation but at the same time diversity of the autophagy components, demonstrating the usefulness of fission yeast as a unique model for studying autophagy. In fact, the characterization of the molecular bases by which the Atg proteins rule general and/or selective autophagy are currently being unraveled by the Du laboratory.

For instance, they have identified a selective autophagy pathway for the import of soluble cargos into the vacuole which, surprisingly, does not require core Atg proteins for function, contrary to the system described in budding yeast, confirming the unexpected versatility of eukaryotic autophagy mechanisms [20]. Regarding the engulfment of bulky organelles instead of soluble proteins, specific Atg proteins such as Atg20, Atg24 and Atg24b/SPB1711.11 have been shown to participate in the nitrogen starvation-induced autophagy of endoplasmic reticulum and mitochondria [21]. The Du laboratory has also provided mechanistic details on the biochemical principles of the onset of autophagy [22].

Even though most Atg components ruling autophagy in *S. pombe* have been identified and the participation of autophagy in mating has been largely demonstrated, little is known about the pathways driving the onset of autophagy in this model organism, nor on the role of this degradation system in other physiological processes. Here, we have performed a global study on the signals, the pathways and the consequences of activated autophagy in fission yeast. Using a modified version of the GFP (green fluorescent protein)-Atg8 reporter of autophagy, we show that not only nitrogen depletion but also glucose, sulfate, phosphate or leucine deprivation can trigger autophagy to different extents. We also reveal the unexpected participation of the MAP (mitogen-activated protein) kinase Sty1-driven stress pathway in autophagy regulation, in concert with the well characterized target of rapamycin (TOR) cascades. We also demonstrate that mutants auxotrophic for leucine are extremely dependent on autophagy to survive in front of leucine deprivation, suggesting that the intracellular pools of this amino acid are very limiting.

Results

General autophagy can be triggered not only by nitrogen depletion but also by phosphate, sulfate or glucose starvation

In most organisms, from yeast to human, autophagy has been monitored using the GFP-tagged Atg8 protein as reporter, which allows to locate autophagosomes by microscopy and to quantify autophagy by western blot [18,23]. The reporter designed in Takegawa's laboratory to study autophagy in fission yeast presents some limitations: the GFP-Atg8 fusion protein is expressed from an episomal plasmid, with a *ura4* selection marker under the control of the *nmt* (no-message in thiamine) promoter. Therefore, it can only be used in MM (minimal media) cultures of strains auxotrophic for uracil under non-repressive conditions, so that the plasmid copy number and the concentration of the reporter (which can change depending on the number of duplications after thiamine withdrawal) vary depending on strains and experimental conditions. We designed a new plasmid to express the fusion protein GFP-Atg8 under the control of the constitutive *sty1* promoter from a chimeric gene integrated at the *leu1-32* locus. Immunoblot analysis showed the formation of a free GFP band, due to the autophagic delivery of GFP-Atg8 into vacuoles and the subsequent proteolysis that releases the protease-resistant GFP moiety, few hours after nitrogen withdrawal (Figure 1, without [w/o] N).

It has been proposed that the conditions that induce autophagy in fission yeast are not the same as those in budding yeast. In *S. cerevisiae*, the induction of autophagy has been described in response to a variety of nutrient starvation conditions, such as the depletion of nitrogen, carbon, sulfur, phosphorus and zinc [10,24,25]. However, in *S. pombe*, autophagy is mainly related with nitrogen starvation, and no evidence of induction has been detected upon glucose depletion or through rapamycin treatment [18]. We decided to test, using our new integrative reporter, whether other nutrient deprivation conditions could trigger autophagy in fission yeast. As shown in Figure 1A, we also detected GFP-Atg8 cleavage after depriving the sulfur or phosphorus sources, although with slower kinetics and less intensity than in the absence of nitrogen. We then tested whether glucose depletion can trigger autophagy. We have previously demonstrated that glucose concentrations below 1%, which do not allow maximal optical densities of cultures due to glucose exhaustion, promote longer lifespan probably due to higher respiratory rates [26]. As shown in Figure 1B, when cells were grown in standard YE, with 3% glucose, a weak but reproducible GFP-Atg8 cleavage could be observed at the stationary phase; this cleavage was exacerbated (1% glucose) or occurred earlier (0.08% glucose) in cultures grown in respiratory-prone conditions. In fact, processing of the reporter started so early in 0.08% glucose that free GFP was rather undetectable at long times (day 2 in Figure 1B). Similar results were obtained in cells grown in minimal medium (MM) with different concentrations of the carbon source (Fig. S1A).

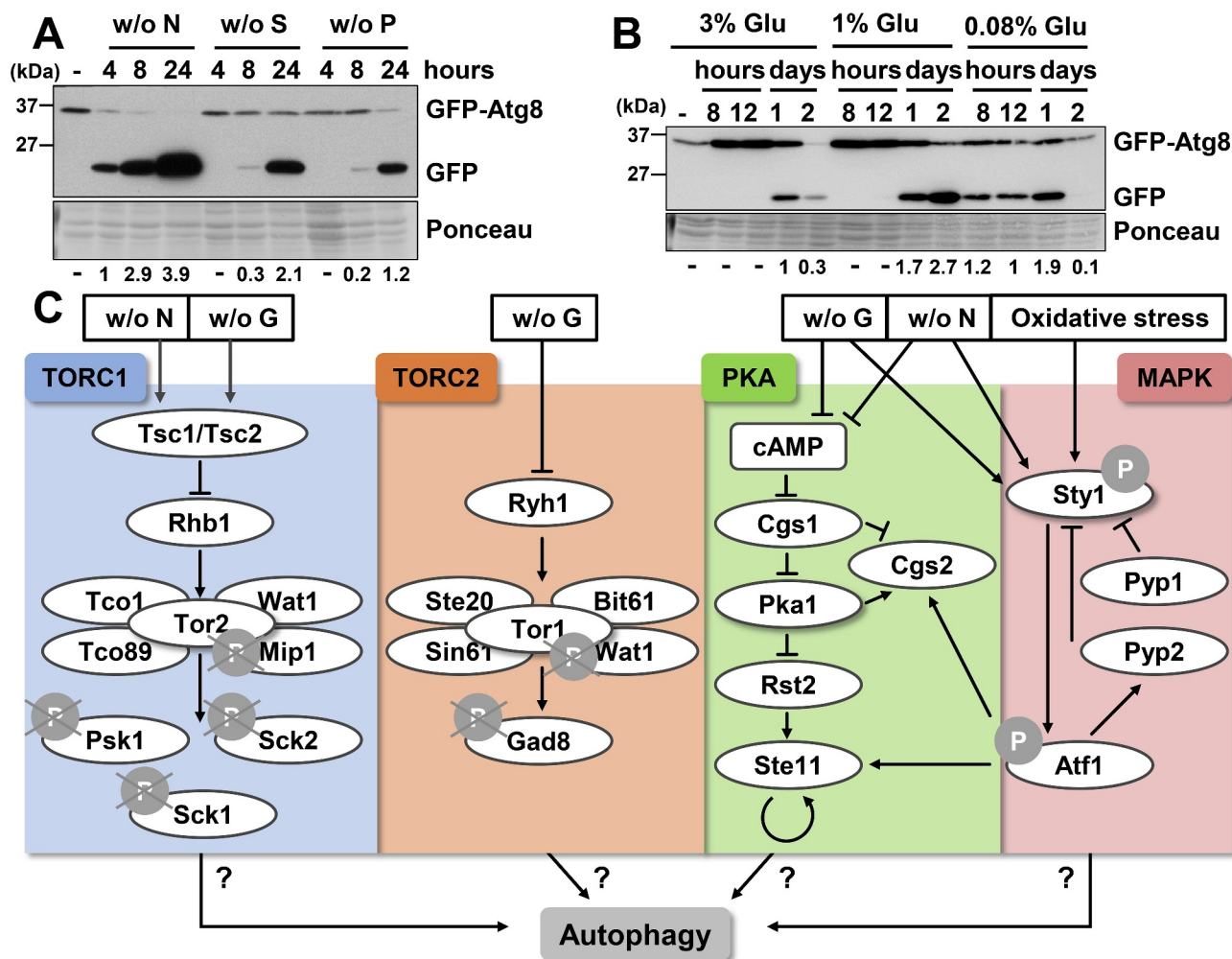


Figure 1. Autophagy is triggered not only by nitrogen starvation, but also by depletion of sulfur, phosphorus or carbon sources. (A) GFP-Atg8 cleavage is caused by nitrogen, sulfur or phosphorus starvation. MM cultures from the wild-type strain CC10, expressing GFP-Atg8 under the control of the constitutive *sty1* promoter from an integrative plasmid, were collected at the logarithmic phase (-) and 4–8–24 h after switching cells to media without nitrogen (w/o N), sulfur (w/o S) or phosphorus (w/o P) sources. Native extracts were analyzed by western blot using anti-GFP antibody. Ponceau staining of the membrane after immuno-detection is shown as a loading control. Numbers below the panels represent the amount of cleaved GFP (quantified as indicated in Materials and Methods) relative to cleaved GFP after 4 h without nitrogen (with an assigned value of 1). (B) GFP-Atg8 cleavage is also induced by glucose exhaustion. The wild-type strain CC10 was grown in standard YE55 (3% glucose) and modified YE55 (1% glucose). Cells were collected at the logarithmic phase and at the indicated times after reaching an OD_{600} of 0.5. For glucose-starvation in YE55 0.08% of glucose, cells were switched at the logarithmic phase from standard YE55 (3%) to YE55 0.08%. Samples were treated and analyzed as in (A). Numbers below the panels represent the amount of cleaved GFP relative to the amount after 1 day in standard YE55 (3%) (with an assigned value of 1). (C) Scheme of the different cellular pathways studied in this work, related to sensing and responding to nitrogen and glucose deprivation in fission yeast. See text for details.

We discarded that autophagy induction upon depletion of the sulfur, phosphorus or carbon sources was caused by simultaneous nitrogen depletion in the media: further addition of a nitrogen supply 5 h after starvation did not cause any significant decrease in GFP-Atg8 cleavage (Fig. S1B). We conclude that not only nitrogen starvation but also depletion of sulfur or phosphorus sources and, importantly, low glucose conditions known to contribute to longer lifespan [26] (Fig. S1C) can trigger the onset of autophagy in fission yeast with different kinetics and to different extents.

Role of the TORC1 complex in autophagy

Once established that any type of nutritional starvation can trigger autophagy in *S. pombe*, we tested the participation of several nutrient-sensing cascades in the regulation of

autophagy: the TORC1 (target of rapamycin complex 1), TORC2, PKA and MAP kinase pathways (Figure 1C). These regulatory routes, which respond to nitrogen and/or glucose deprivation, are involved in G_1 arrest, sexual development, amino acid uptake, cell growth, meiosis, lifespan and stress response (Fig. S1D).

Budding and fission yeast have two TOR kinases whereas higher eukaryotes generally have only one [27,28]. In fission yeast, TORC1 kinase complex is composed of the essential catalytic subunit Tor2 and Mip1, and the non-essential Tco89, Wat1 and Tco1 [29–31] (Figure 1C). Tuberous sclerosis complex, the heterodimer Tsc1-Tsc2, acts as an upstream negative regulator of TORC1 during starvation [32–34] (Figure 1C). Tor2 is essential during physiological conditions in *S. pombe*, and its inactivation by nitrogen starvation induces cell cycle arrest in the G_1 phase, initiation

of alternative cellular programs such as sexual development and induction of the nitrogen starvation gene expression program [29,30,35,36]. When TORC1 is active under nutrient-rich conditions, it phosphorylates members of the AGC (protein kinase A/protein kinase G/protein kinase C) kinase family: Psk1, Sck1 and Sck2. Psk1 is an RPS6KB1/S6K1 homolog, which has as a substrate Rps6 (ribosomal protein S6; for a review see [4]) (Figure 1C). Tsc1-Tsc2 senses nitrogen depletion, and causes dephosphorylation/inactivation within minutes of TORC1 complex, Psk1 and Rps6, leading to translation inhibition, among other effects [37,38].

To test whether autophagy is one of the alternative programs activated through the TORC1 pathway upon nutrient depletion, we studied the phosphorylation state of Psk1 and Rps6 in response to not only to nitrogen, but also to glucose, sulfur or phosphorus deprivation, all capable of inducing autophagy. As shown in Figure 2A and previously reported [37,38], not only nitrogen depletion but also glucose starvation triggered Psk1 and Rps6 dephosphorylation. In addition, a moderate dephosphorylation of Psk1 was observed 30 min after removing the sulfur or phosphorus sources, which was exacerbated after 8 h of nutrient depletion (Figure 2A).

In order to test the role of TORC1 pathway in autophagy, we first searched for mutants in which the inactivation of downstream kinases, i.e. Psk1 and Rps6, was altered in response to nutrient deprivation. As previously reported by the group of Tamanoi [37,39], cells carrying the *tor2^{LI310P}* allele were largely blind to nitrogen depletion and displayed very slow dephosphorylation of the Psk1 and Rps6 kinases; very similar behavior was seen in cells lacking the upstream sensors Tsc1 and Tsc2 (Figure 2B, upper panels). The TORC1 component Tco89 is dispensable for Psk1 and Rps6 dephosphorylation upon nitrogen starvation, and as expected cells lacking this subunit had slightly but reproducibly lower levels of phosphorylated Rps6 during nitrogen-rich growth (Figure 2B, third panel). As expected, the lack of Sck2 did not have any impact on Rps6 phosphorylation (Figure 2B, bottom panel).

We used these mutants to test the role of the TORC1 pathway in autophagy. As shown in Figure 2C and Fig. S2, *tsc1Δ* and *tsc1Δ tsc2Δ* mutants showed lower levels of GFP-Atg8 cleavage after nitrogen depletion (red circles in Figure 2C), while the *tco89Δ* and *sck2Δ* mutants displayed a slight but significant faster induction of GFP release (green circles in Figure 2C). These results indicate that TORC1 cascade plays a role in regulating autophagy as previously suggested [40].

The direct autophagy substrate of TORC1 in budding yeast is Atg13, which is hyper-phosphorylated under nutrient-rich conditions and is dephosphorylated during starvation facilitating the dephosphorylation of downstream Atg1 (for a review, see [41]). The electrophoretic mobility shift upon phosphorylation of Atg13 or Atg1 has proven to be a good indicator of autophagy in *S. pombe* [17]. As shown in Figure 2D, Atg1 was dephosphorylated 1 h after nitrogen depletion in wild-type cells, while this mobility shift was not observed in mutants defective in TORC1 inhibition, *tor2^{LI310P}* and *tsc1Δ* (Figure 2D, upper panel). However, Atg1 dephosphorylation did not seem to discriminate a moderate gain of TORC1 kinase activity: *tco89Δ* and *sck2Δ* displayed Atg1

dephosphorylation kinetics similar to those of the wild type strain (Figure 2D, bottom panel). On the basis of these evidences we propose that during nutrient depletion Tsc1-Tsc2 inhibits the TORC1 pathway, triggering the dephosphorylation of Sck2 that would stop Atg1 phosphorylation, inducing autophagy (Figure 2E).

The TORC2 pathway also modulates autophagy in fission yeast

In fission yeast TORC2 complex responds to low glucose conditions, but it is also related to processes that are triggered during nitrogen starvation. Thus, it is required for G₁ arrest, sexual development and for resistance to osmotic and oxidative stresses [42–46]. The TORC2 complex is composed of the kinase Tor1 and the regulatory subunits Ste20, Sin1, Wat1 (also present in TORC1) and Bit61 [30,31,46] (Figure 1C). Tor1 recruits and phosphorylates the fission yeast AGC kinase Gad8 [47] to regulate cell proliferation, the switch to bipolar cell growth, cell fusion during mating, and the meiotic program [42]. As previously shown [48], Gad8 was dephosphorylated in a TORC2 dependent manner during glucose starvation, but not after nitrogen depletion, which instead triggered a slower mobility shift in gels (Figure 3A).

To test a possible cross-talk between the TORC1 and the TORC2 pathways, we tested Psk1 and Rps6 dephosphorylation, located downstream of TORC1, after nitrogen depletion in TORC2 mutants (Figure 3B). In untreated conditions, *tor1Δ* and *gad8Δ* showed significantly lower Psk1 or Rps6 phosphorylation levels compared to wild type cells, whereas there were no differences in the concentration of total Psk1 (Psk1 in Figure 3B). Dephosphorylation of Psk1 and Rps6 upon nitrogen depletion in *tor1Δ* and *gad8Δ* seemed as fast as that of a wild-type strain. These results suggest a connection between both cascades under nutrient-rich conditions. To test whether this cross-talk also affects autophagy induction upon nitrogen starvation, we monitored GFP-Atg8 cleavage in the mutants *wat1Δ*, *gad8Δ*, *tor1Δ* and *tor1Δ gad8Δ* after nitrogen depletion. As shown in Figure 3C all mutants exhibited higher induction of autophagy than wild-type cells 2 h after nitrogen depletion. We conclude that mutants of the TORC2 cascade display enhanced levels of autophagy upon nutrient deprivation, and we propose that this probably occurs by modulation of starting TORC1 activity, which was partially inhibited in *gad8Δ* and *tor1Δ* mutants even prior to nutrient depletion (Figure 3B). Furthermore, no cumulative effect was observed in the double deletion of *tor1* and *tco89*: *tor1Δ tco89Δ* mutant exhibited an increase in autophagy similar to the single deletes *tor1Δ* or *tco89Δ*, supporting the theory that both TORC1 and TORC2 complexes cooperate modulating autophagy (Fig. S3A). As shown above for the partially active *tco89Δ* and *sck2Δ* mutants (Figure 2D), dephosphorylation of Atg1-HA was not significantly different from wild-type cells (Figure 3D).

In order to determine whether Tor1 and Gad8 work upstream of Tsc1 or converge synergistically with Tsc1-Rhb1 on the TORC1 kinase complex, we analyzed the genetic interactions between *tsc1Δ* or *tor2^{LI310P}* (mutants of TORC1 blinded to nutrient depletion; Figure 2B and C and Fig. S2)

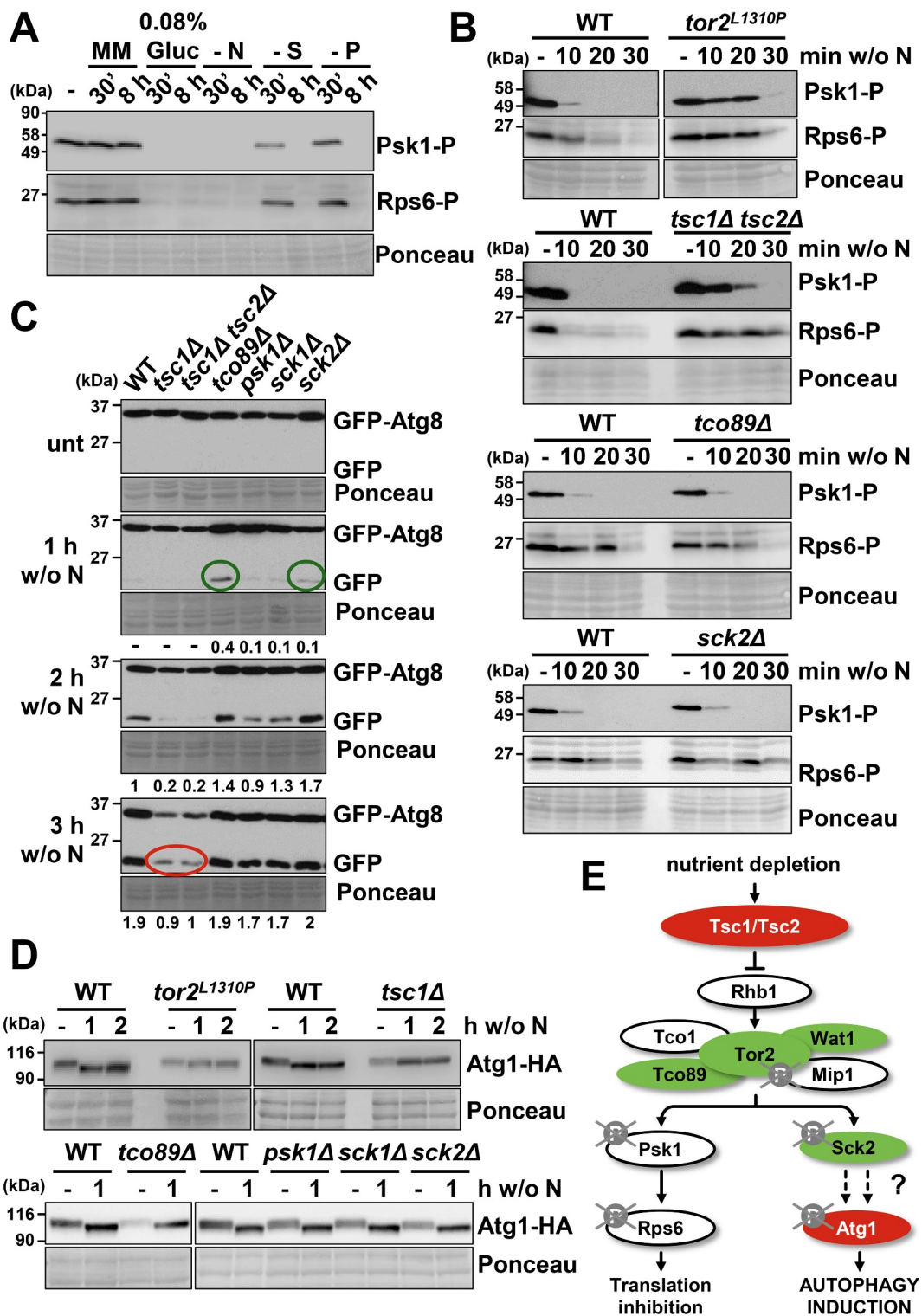


Figure 2. TORC1 negatively regulates autophagy. (A) TORC1 kinase activity and phosphorylation of its downstream consecutive targets, Psk1 and Rps6, are inhibited upon nutrient deprivation. MM cultures of strain CC10 (expressing GFP-Atg8) were collected at the logarithmic phase (-) and 30 min or 8 h after switching to modified MM with low glucose (0.08% Gluc), or without nitrogen (- N), sulfur (- S), and phosphorus (- P) sources. TCA extracts were analyzed by western blot with anti-phospho-Psk1 (anti-RPS6KB/p70) and anti-phospho-Rps6 (anti-AKT) antibodies. (B) Effect of TORC1 pathway mutants on the inhibition of Psk1 and Rps6 phosphorylation after nitrogen depletion. MM cultures of strains CC10 (WT), JUP1350 (*tor2^{L1310P}*), CC31 (*tsc1Δ tsc2Δ*), CC18 (*tco89Δ*) and CC21 (*sck2Δ*) were shifted to nitrogen depleted media for 10–30 min and analyzed as in (A). (C) Effect of TORC1 pathway mutants on autophagy activation upon nitrogen depletion. MM cultures of strains CC10 (WT), CC14 (*tsc1Δ*), CC31 (*tsc1Δ tsc2Δ*), CC18 (*tco89Δ*), CC66 (*psk1Δ*), CC89 (*sck1Δ*) and CC21 (*sck2Δ*), all expressing GFP-Atg8, were shifted or not at the logarithmic phase (unt) to nitrogen-free medium for 1–3 h, and were processed and analyzed as in Figure 1A. Accelerated GFP-Atg8 cleavage is indicated with green circles, while deficient cleavage is labeled with a red circle. Numbers below the panels represent the amount of cleaved GFP relative to the amount after 2 h without nitrogen in WT (with an assigned value of 1). (D) Atg1 dephosphorylation after nitrogen depletion is defective in *tor2^{L1310P}* and *tsc1Δ* mutants. MM cultures of strains CC99 (WT), CC115 (*tor2^{L1310P}*), CC101 (*tsc1Δ*), RB15 (*tco89Δ*), RB5 (*psk1Δ*), RB13 (*sck1Δ*), and RB6 (*sck2Δ*), all expressing Atg1-HA, were shifted or not to nitrogen-free medium, and processed and analyzed as in (A), using anti-HA antibody. (E) Scheme depicting the nutrient depletion-dependent antagonistic regulation by TORC1 of growth-related functions and of autophagy. Deletion or point mutants used in this study are represented in red (low autophagy) and green (high autophagy). See text for details.

and *gad8Δ* (Gad8 is downstream of TORC2, but *gad8Δ* displays decreased levels of TORC1 kinase activity during nutrient-rich conditions). Thus, the double mutant *gad8Δ tor2^{L1310P}* displayed a combination of phenotypes upon nitrogen depletion regarding phosphorylation of Psk1 and Rsp6, with lower starting levels and sustained phosphorylation upon nitrogen depletion (Figure 3E). A similar intermediate phenotype was observed in the double mutant *tsc1Δ gad8Δ* (Fig. S3B). Regarding GFP-Atg8 cleavage, *gad8Δ tor2^{L1310P}* displayed an intermediate behavior compared with the single deletes (Figure 3F); the same occurred with strain *tsc1Δ gad8Δ* (Fig. S3C). We also discarded an effect of TORC1 over Gad8 phosphorylation, since the electrophoretic mobility of Gad8-HA in response to starvation in *tsc1Δ* was identical to that of a wild-type strain (Fig. S3D). Thereby, we propose that TORC2 positively regulates TORC1 activity even in basal conditions, and that the lack of Tor1 or Gad8 mimics a mild nutrient deprivation decreasing growth and promoting autophagy (Figure 3G).

While mutations in the Pka1 pathway do not affect autophagy induction, cells lacking the MAP kinase Sty1 are severely defective in the onset of autophagy

Once the involvement of TORC1 and TORC2 in autophagy have been established, we compared the autophagy levels in mutants from other nutrients and stress response pathways: those ruled by Pka1 and by the MAP kinase Sty1 (Figure 1C). In *S. cerevisiae*, high levels of cAMP (cyclic adenosine monophosphate) or constitutive activation of PKA prevents autophagy in response to rapamycin or nutrient-depletion, indicating that PKA negatively regulates autophagy [49–51]. On the contrary, the fission yeast mutants *pka1Δ* and *cgs1Δ*, which display opposite global effects on the activity of the cascade [52], displayed kinetics of GFP-Atg8 cleavage after nitrogen depletion undistinguishable from those of wild type background (Figure 4A), indicating that the PKA cascade does not participate in autophagy regulation in *S. pombe*.

In *S. cerevisiae*, MAP kinases control specific autophagy programs in response to starvation; thus, Hog1 is required for mitochondrial autophagy (mitophagy) after nitrogen depletion [53]. The fission yeast Sty1 cascade is activated by environmental stresses, but also by nutrient deprivation (Figure 1C). We tested the role of the MAP kinase Sty1 and of its main downstream transcription factor, Atf1, in autophagy activation. We found that *sty1Δ* cells exhibited a dramatic reduction in autophagy during nitrogen starvation whereas *atf1Δ* presents a wild type pattern (Figure 4A). Therefore, the MAP kinase cascade induces autophagy through Sty1 in an Atf1-independent manner.

We have shown above that the TORC1, TORC2 and Sty1 pathways regulate the onset of autophagy in response to lack of nitrogen (Figure 4A); the effect of mutants of these cascades could be observed 1–3 h after nitrogen withdrawal, while all of them ended up displaying maximal GFP-Atg8 cleavage 24 h after nitrogen depletion (data not shown). We have shown above that not only nitrogen depletion but also the lack of glucose, sulfur or phosphorus sources could drive autophagy activation (Figure 1A,B). As shown in Figure 4B,

the three cascades shown to affect autophagy in response to nitrogen depletion also differentially responded to the other three types of stimuli, with cells lacking Sty1 displaying the more dramatic defects.

To confirm that these signals and pathways modulate the onset of autophagy, we have also used cytosolic Pgk1-GFP as a reporter of bulk autophagy, as previously described in budding yeast and, very recently, in fission yeast [54,55]. Pgk1-GFP cleavage occurred in response to nitrogen (Figure 4C) or glucose, sulfur or phosphorus deprivations (Figure 4D), and this cleavage was accelerated or impaired in mutants of the TORC1, TORC2 and Sty1 cascades, mimicking the results obtained with GFP-Atg8.

We conclude that, to different extents, TORC1, TORC2 and MAP kinase pathways are involved in the onset of autophagy in response to different types of nutrient depletions; namely, cells unable to fully inhibit TORC1 in response to nutrient deprivation (*tor2^{L1310P}* or *tsc1Δ tsc2Δ*, among others) or lacking the MAP kinase Sty1 displayed impaired efficiency to induce autophagy (Figure 4E).

Sty1 does not control autophagy through the TORC1 pathway, but rather at the level of transcription of atg genes

We first confirmed that Sty1 was activated/phosphorylated not only by environmental stresses such as hydrogen peroxide but also by the signals triggering autophagy, such as nitrogen and glucose starvation (Figure 5A). To test if the role of Sty1 on autophagy is due to a cross-talk with the TORC1 pathway, we tested whether the lack of Sty1 affects the basal phosphorylation levels of Psk1 and Rps6, or impairs their dephosphorylation during nitrogen depletion. As shown in Figure 5B, *sty1Δ* and wild type cells displayed undistinguishable Psk1 and Rps6 phosphorylation patterns before and after nitrogen deprivation. Indeed, the absence of Sty1 did not exacerbate the defective dephosphorylation of Psk1 and Rps6 of *tsc1Δ* cells, indicating that the MAP kinase does not regulate the TORC1 pathway (Fig. S4A). Furthermore, Sty1 does not seem to have an effect on the TORC2 pathway, since Gad8 phosphorylation levels upon nutrient starvation were unaffected in *sty1Δ* cells (Fig. S4B). Sty1 and TORC1 seem to affect autophagy independently. Indeed, GFP-Atg8 cleavage upon nitrogen withdrawal was significantly slower in the double *tsc1Δ sty1Δ* mutant than in the single mutants at all the time points tested (Figure 5C), supporting the idea that Sty1 and TORC1 activate the onset of autophagy through independent molecular events.

tsc1Δ cells displayed defects in Atg1 dephosphorylation after depletion of nitrogen (Figure 2D), but the absence of Sty1 did not jeopardize Atg1 dephosphorylation/activation (Figure 5D, Fig. S4C). During autophagy, Atg13 is dephosphorylated and mediates Atg1 dephosphorylation, which then triggers by an unknown mechanism the phosphorylation of Atg2 (for a review, see [56]). We tested whether Sty1 could directly promote Atg2 phosphorylation in response to nitrogen starvation. We detected a mobility shift, indicative of phosphorylation, in both wild-type and *sty1Δ* cell extracts obtained after nitrogen depletion (Figure 5E), indicating that

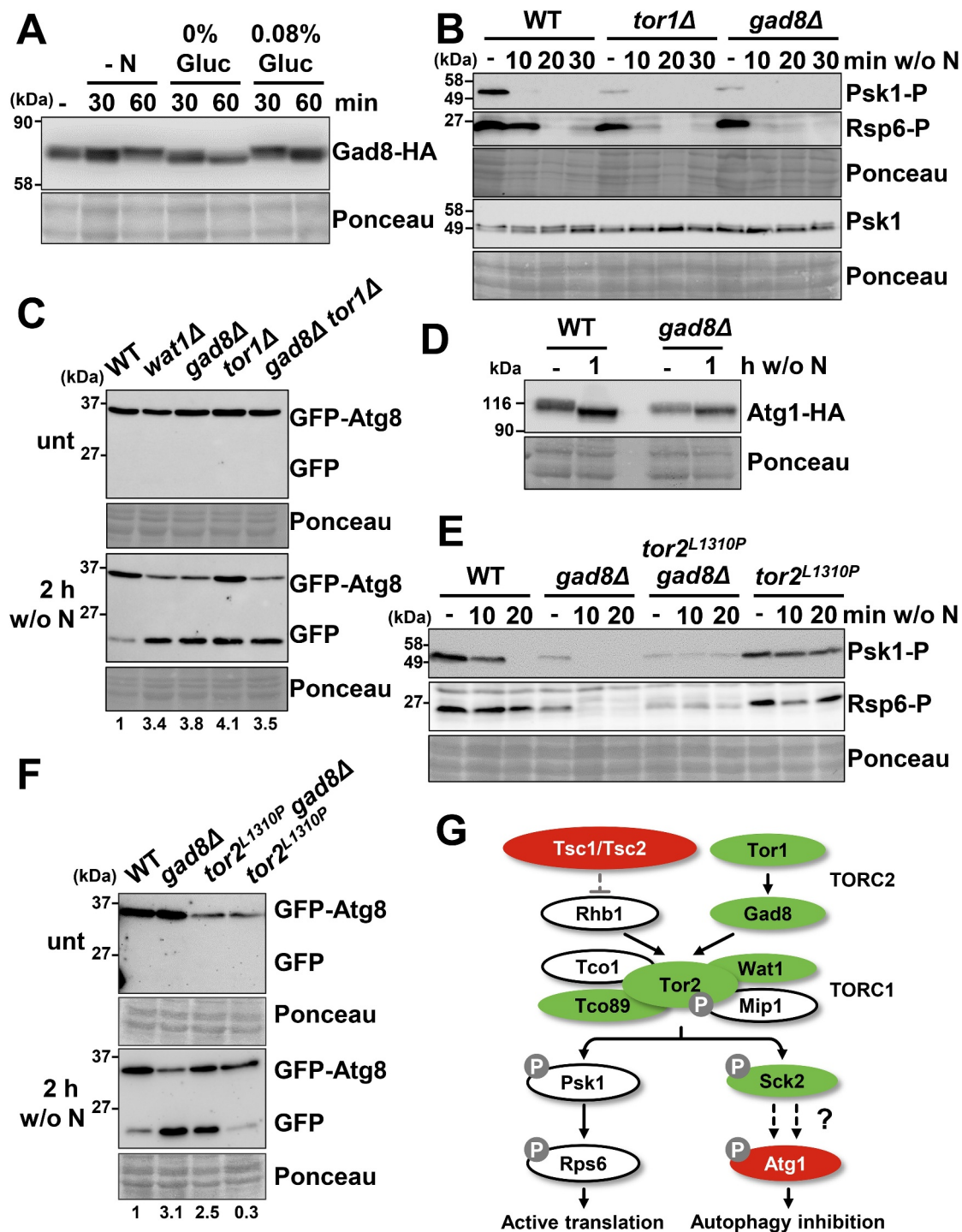


Figure 3. TORC2 interacts with TORC1 under basal condition and affects autophagy activation. (A) Inhibition of TORC2 kinase activity by glucose, but not nitrogen, depletion causes Gad8 dephosphorylation. MM cultures of strain AN0175 (WT), expressing Gad8-HA, were shifted or not to nitrogen-free or glucose-free medium, and processed and analyzed as in Figure 2A, using anti-HA antibody. (B) Effect of TORC2 pathway mutants on the inhibition of Psk1 and Rsp6 phosphorylation, downstream of TORC1, after nitrogen depletion. MM cultures of strains AN0179 (WT), RB17 (*tor1Δ*) and RB18 (*gad8Δ*), all expressing Psk1-Myc, were shifted or not to nitrogen depleted media for 10–30 min and analyzed as in Figure 2A. Anti-Myc was used as a loading control to demonstrate that Psk1 phosphorylation but not Psk1 levels are altered in the TORC2 mutants (Psk1). (C) Effect of TORC2 pathway mutants on autophagy activation upon nitrogen depletion. MM cultures of strains CC10 (WT), CC92 (*wat1Δ*), CC70 (*gad8Δ*), CC16 (*tor1Δ*) and RB4 (*gad8Δ tor1Δ*), all expressing GFP-Atg8, were shifted or not to nitrogen-free medium, and processed and analyzed as in Figure 2C. Numbers below the panels represent the amount of cleaved GFP relative to the amount after 2 h without nitrogen in WT (with an assigned value of 1). (D) Atg1 dephosphorylation after nitrogen depletion is unaffected in strain *gad8Δ*. MM cultures of strains CC99 (WT) and CC116 (*gad8Δ*), both expressing Atg1-HA, were shifted or not to nitrogen-free medium, and processed and analyzed as in Figure 2D. (E) Synthetic interaction between TORC1 and TORC2 mutants on TORC1 kinase activity. MM cultures of strains CC10 (WT), CC70 (*gad8Δ*), CC128 (*tor2^{L1310P} gad8Δ*) and JUP1350 (*tor2^{L1310P}*) were shifted or not to nitrogen-free medium, and processed and analyzed as in Figure 2A. (F) Synthetic interaction between TORC1 and TORC2 mutants on autophagy. MM cultures of strains CC10 (WT), CC70 (*gad8Δ*), RB32 (*tor2^{L1310P} gad8Δ*) and CC112 (*tor2^{L1310P}*), all expressing GFP-Atg8, were shifted or not to nitrogen-free medium, and processed and analyzed as in Figure 2C. Numbers below the panels represent the amount of cleaved GFP relative to the amount after 2 h without nitrogen in WT (with an assigned value of 1). (G) Scheme depicting the positive regulation of TORC1 kinase activity by the TORC2 components Tor1 and Gad8 under nutrient-rich conditions. Deletion or point mutants used in this study are represented in red (low autophagy) and green (high autophagy). See text for details.

Sty1 does not directly affect the phosphorylation state of Atg1 nor Atg2 (Figure 5E).

The Sty1 cascade is strongly activated by environmental stressors, and massive changes in the gene expression program occur to promote survival [57]. Hundreds of genes are upregulated in a Sty1-dependent manner, and many of the *atg* genes are triggered by some but not all the signals used [57]. We decided to test whether the induction of some *atg* genes could occur upon nitrogen starvation in a Sty1-dependent manner, to explain the role of the MAP kinase in autophagy activation. As shown in Figure 6A, many autophagy genes such as *atg1*, *atg4*, *atg6*, *atg9*, *atg22* and *atg13* were strongly induced during nitrogen starvation. Importantly, the expression pattern in wild type strain and *tsc1Δ* was similar for most genes, suggesting that TORC1 does not regulate autophagy at the transcriptional level. On the contrary, *sty1Δ* strain exhibited a significantly reduced upregulation of *atg* transcripts upon nitrogen depletion, except for *atg13* (Figure 6A), indicating that activated Sty1 contributes to the onset of autophagy indirectly by upregulating the expression of *atg* mRNAs (Figure 6B). It is worth noting that the upregulation of *atg* genes was Atf1-independent (Fig. S5), explaining why *atf1Δ* triggered autophagy upon nutrient deprivation similarly to wild-type cells (Figure 4A).

Leucine starvation is a potent activator of autophagy in cells auxotrophic for leucine

Regarding the biological role of autophagy in fission yeast, it has only been reported that it contributes to maintain cell viability during very long periods, more than 20–25 days, of nitrogen starvation [17]. As shown in Figure 7A, wild-type cells deprived of nitrogen, sulfur or phosphorus sources displayed different degrees of viability 25 days after nutrient withdrawal, suggesting that the reservoirs for each essential nutrient are quantitatively different. Importantly, the *atg1Δ* and *atg2Δ* strains displayed decreased viability in all conditions, highlighting the role of this degradation pathway in providing nutrients from the digestion of non-essential molecular components.

It has been previously shown that the combination of *atg* deletions with auxotrophic mutations, especially those affecting leucine synthesis, exacerbates the viability defects upon nitrogen depletion [17]. We studied the viability of *atg1Δ leu1-32* during nitrogen starvation and after leucine depletion. Surprisingly, the effect of leucine withdrawal was even more dramatic than nitrogen depletion on *atg1Δ leu1-32* cells: only 12 h after leucine withdrawal the culture lost completely its colony-forming capacity (Figure 7B). The morphology of the starved *atg1Δ leu1-32* cells was undistinguishable from wild-type cells, suggesting that they are not dead but rather arrested (Figure 7C). Indeed, both wild-type *leu1-32* and *atg1Δ leu1-32* cells were refractory to propidium iodide internalization, as determined by flow cytometry, a classical reporter of viability (Figure 7D). We propose that autophagy is essential to escape from the leucine-induced arrest in cells that are unable to synthesize the amino acid [58]. We tested the role of autophagy on other amino acid deficiencies such as arginine or lysine in auxotrophic strains, to demonstrate that

deletion of *atg1* did not significantly affect cell viability in those cases (Fig. S6A).

Upon nitrogen depletion, cells auxotrophic and prototrophic for leucine followed very similar kinetics regarding TORC1 inactivation (Figure 7E) and autophagy induction (Figure 7F and Fig. S6B and C). On the contrary, leucine withdrawal induced Psk1 and Rps6 dephosphorylation (Figure 7G) and GFP-Atg8 cleavage (Figure 7F and Fig. S6C and D) only in wild-type *leu1-32* strain barely 2 h after amino acid starvation. We also checked the effect of leucine withdrawal in *leu1-32* TORC1 mutants: *tsc1Δ*, *tco89Δ* and *atg1Δ tco89Δ* (Figure 7H). Similar to *atg1Δ leu1-32*, 12 h after leucine depletion *tsc1Δ leu1-32* and *atg1Δ tco89Δ leu1-32* did not recover mitotic competence. However, *tco89Δ leu1-32* presented similar pattern that wild-type *leu1-32* strain: these mutant does not impair autophagy activation (see Figure 2C). Our experiments suggest that leucine depletion itself is a potent inducer of autophagy in cells auxotrophic for this amino acid, and that the requirement for leucine regulates and/or contributes to autophagy probably through TORC1 activity, revealing itself as an amino acid of great importance in cell survival.

Discussion

Even though the molecular components that govern autophagy upon nitrogen deprivation have been identified in fission yeast, little is known about the pathways that modulate its activation or the signals and biological roles linked to the onset of autophagy. Here, using an optimized reporter system based on the vacuolar cleavage of GFP-Atg8, we demonstrate that autophagy occurs not only in response to low nitrogen levels, but also in response to the depletion of a variety of nutrients, including carbon starvation, and that this activation is mediated through pathways related to cell growth, G₁ arrest, meiosis, lifespan and stress response. As in other eukaryotes, inactivation of the TORC1 pathway is an important contributor to the onset of autophagy, but unexpectedly we show here that activation of the stress-dependent MAP kinase pathway governed by Sty1 is crucial to promote full autophagy induction in response to nutrient depletion.

Thus, we have uncovered here new signals, all related to the depletion of essential nutrients, which are able to induce autophagy in fission yeast: not only nitrogen deprivation but also depletion of sulfur, phosphorus, glucose, or leucine sources were all capable of promoting GFP-Atg8 cleavage. Importantly, addition of nitrogen to cultures deprived of these other alternative nutrients did not prevent autophagy activation, dismissing that nitrogen is also becoming limiting in our experimental conditions. Thus, similar to nitrogen deprivation, depletion of sulfur or phosphorus sources induced autophagy (Figure 1A) and extended lifespan (Figure 7A). Supporting our results, in *S. cerevisiae* autophagy is induced during sulfur or phosphorus scarcity [10,24,25] and, in fission yeast, depletion of a sulfur supply extends chronological lifespan [59].

Regarding carbon depletion-mediated autophagy, previous studies suggested that the levels of glucose do not regulate autophagy in *S. pombe* [13,18], contrary to the classical

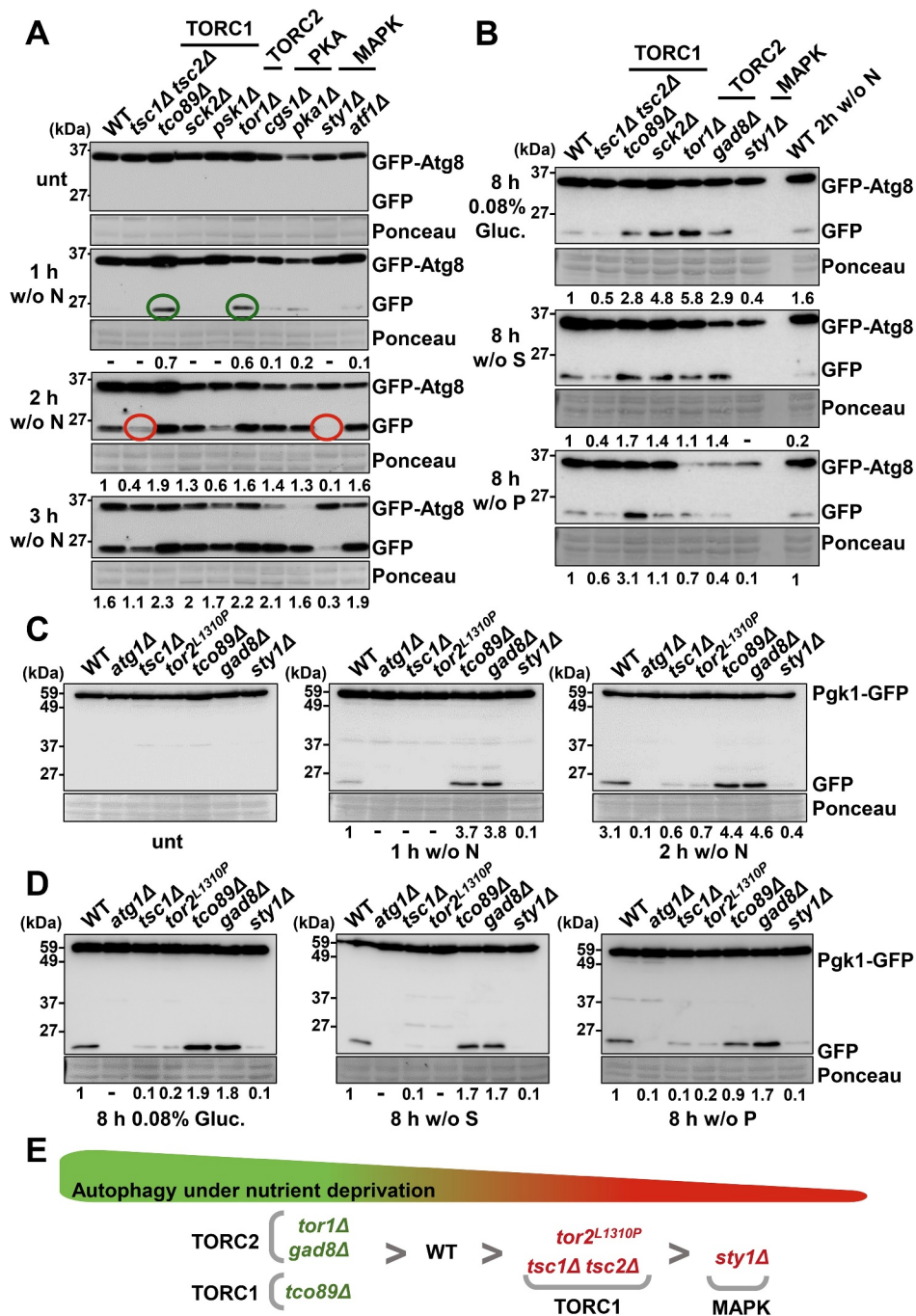


Figure 4. Comparative regulation of autophagy through the TORC1, TORC2 and MAP kinase pathways. (A) Effect of TORC1, TORC2, PKA and MAP kinase pathway mutants on autophagy activation upon nitrogen depletion. MM cultures of strains CC10 (WT), CC31 (*tsc1Δ tsc2Δ*), CC18 (*tco89Δ*), CC21 (*sck2Δ*), CC66 (*psk1Δ*), CC16 (*tor1Δ*), CC33 (*cgs1Δ*), CC22 (*pka1Δ*), CC13 (*sty1Δ*) and CC23 (*atf1Δ*), all expressing GFP-Atg8, were shifted or not at the logarithmic phase (unt) to nitrogen-free medium for 1–3 h, and were processed and analyzed as in Figure 1A. Accelerated GFP-Atg8 cleavage is indicated with green circles, while deficient cleavage is labeled with red circles. Numbers below the panels represent the amount of cleaved GFP relative to the amount after 2 h without nitrogen in WT (with an assigned value of 1). (B) Effect of TORC1, TORC2 and MAP kinase pathway mutants on autophagy activation upon depletion of the glucose, sulfur or phosphorus sources. MM cultures of strains CC10 (WT), CC31 (*tsc1Δ tsc2Δ*), CC18 (*tco89Δ*), CC21 (*sck2Δ*), CC16 (*tor1Δ*), CC70 (*gad8Δ*) and CC13 (*sty1Δ*), all expressing GFP-Atg8, were shifted at the logarithmic phase (unt) to modified MM with low glucose (0.08% Gluc), or without sulfur (w/o S) or phosphorus (w/o P) sources for 8 h, and were processed and analyzed as in Figure 1A. A sample of extracts from CC10 (WT) after 2 h of nitrogen-starvation was used as an autophagy control. Numbers below the panels represent the amount of cleaved GFP relative to the amount in WT after 8 h under indicated conditions (0.08% Gluc, without [w/o] S and without [w/o] P) (with assigned values of 1). (C) Effect of TORC1, TORC2 and MAP kinase pathway mutants on bulk autophagy activation upon nitrogen depletion. MM cultures of strains RB44 (WT), RB40 (*atg1Δ*), RB45 (*tsc1Δ*), RB41 (*tor2^{L1310P}*), RB48 (*tco89Δ*), RB39 (*gad8Δ*) and RB38 (*sty1Δ*), all expressing Pgk1-GFP, were shifted or not at the logarithmic phase (unt) to nitrogen-free medium for 1–2 h, and were processed and analyzed as in Figure 1A. Numbers below the panels represent the amount of cleaved GFP relative to the amount in WT after 1 h under nitrogen deprivation (with an assigned value of 1). (D) Effect of TORC1, TORC2 and MAP kinase pathway mutants on bulk autophagy activation upon depletion of the glucose, sulfur or phosphorus sources. MM cultures of strains RB44 (WT), RB40 (*atg1Δ*), RB45 (*tsc1Δ*), RB41 (*tor2^{L1310P}*), RB48 (*tco89Δ*), RB39 (*gad8Δ*) and RB38 (*sty1Δ*), all expressing Pgk1-GFP, were shifted at the logarithmic phase (unt) to modified MM with low glucose (0.08% Gluc), or without sulfur (w/o S) or phosphorus (w/o P) sources for 8 h, and were processed and analyzed as in Figure 1A. Numbers below the panels represent the amount of cleaved GFP relative to the amount in WT after 8 h under each condition (0.08% Gluc, without [w/o] S and without [w/o] P) (with assigned values of 1). (E) Scheme depicting the impact of mutations at the TORC1, TORC2 or MAP kinase pathways on autophagy induction. Accelerated nutrient starvation-dependent autophagy of some mutants is indicated in green, while deficient autophagy is indicated in red. See text for details.

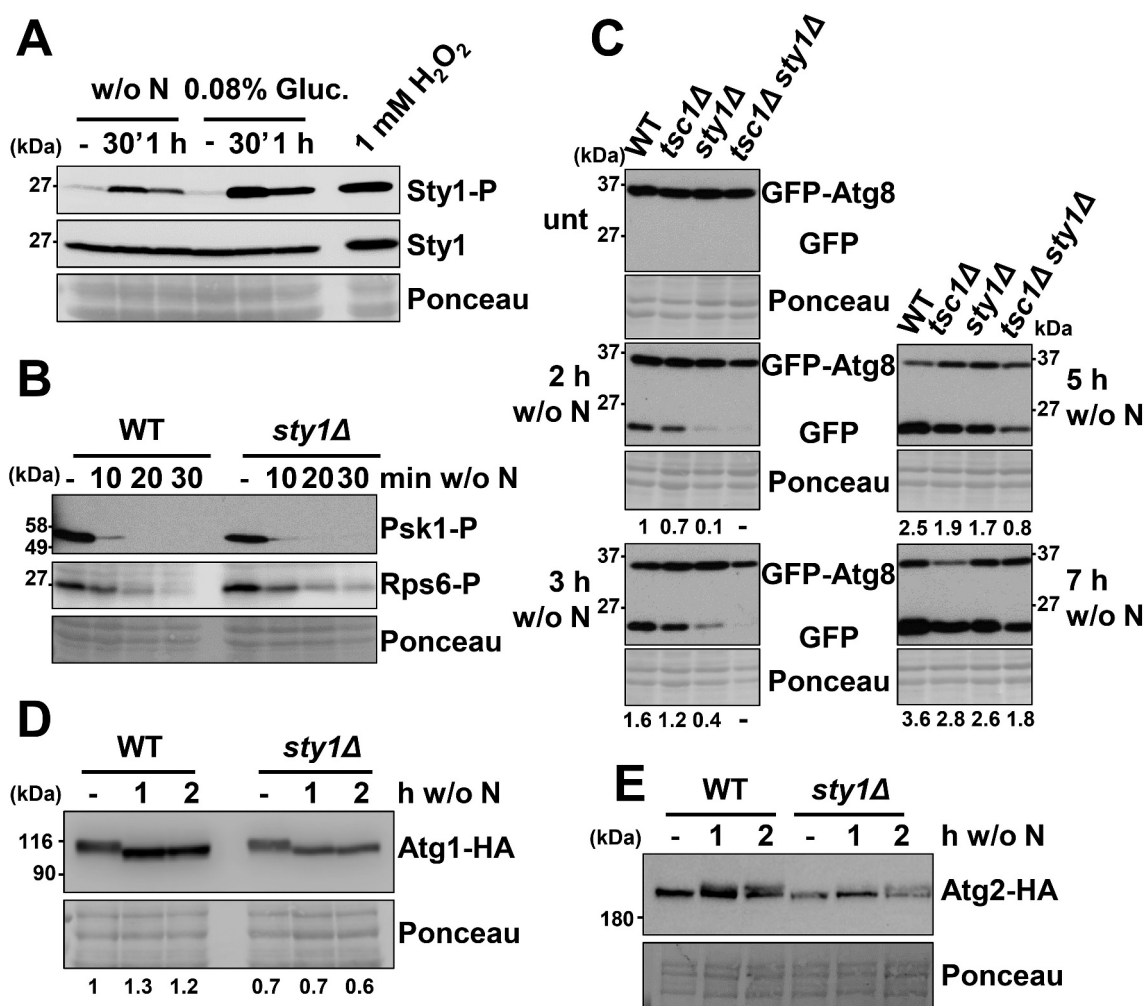


Figure 5. The MAP kinase Sty1 does not regulate TORC1-dependent growth promotion functions, nor affects phosphorylation levels of Atg1 and Atg2, but positively regulates autophagy. (A) Sty1 is phosphorylated upon nitrogen or glucose starvation. MM cultures of wild-type strain CC10 were grown and shifted or not from the logarithmic phase (-) to modified MM without nitrogen (w/o N) or with low glucose (0.08%). As a control, logarithmic cultures were treated 10 min with 1 mM H₂O₂. TCA extracts were analyzed by western blot using anti-phospho-MAPK/p38 (Sty1-P) and anti-Sty1 antibodies. (B) Lack of Sty1 does not impair dephosphorylation of Psk1 and Rsp6 after nitrogen depletion. MM cultures of strains CC10 (WT) and CC13 (*sty1Δ*) were shifted or not to nitrogen depleted media for 10–30 min and analyzed as in Figure 2A. (C) Synergistic role of TORC1 and MAP kinase pathways on autophagy activation. MM cultures of strains CC10 (WT), CC14 (*tsc1Δ*), CC13 (*sty1Δ*) and CC111 (*tsc1Δ sty1Δ*), all expressing GFP-Atg8, were shifted or not to nitrogen-free medium for 2–7 h, and processed and analyzed as in Figure 2C. Numbers below the panels represent the amount of cleaved GFP relative to the amount after 2 h without nitrogen in WT (with an assigned value of 1). (D) Lack of Sty1 does not impair dephosphorylation of Atg1 after nitrogen depletion. MM cultures of strains CC99 (WT) and CC110 (*sty1Δ*), both expressing Atg1-HA, were shifted or not to nitrogen depleted media for 1–2 h and analyzed as in Figure 2A, using anti-HA antibody. Numbers below the panels represent the amount of Atg1 relative to the amount in WT under physiological conditions (-) (with an assigned value of 1). (E) Lack of Sty1 does not impair phosphorylation of Atg2 after nitrogen depletion. MM cultures of strains CC122 (WT) and CC123 (*sty1Δ*), both expressing Atg2-HA, were shifted or not to nitrogen depleted media for 1–2 h and analyzed as in Figure 2A, using anti-HA antibody.

autophagy activation upon carbon starvation described in budding yeast [10]. We report here the early cleavage of our sensitive reporter GFP-Atg8 in stationary phase cultures grown in low glucose media, conditions linked to elongated lifespan (Figure 1B, Fig. S1A and C). We had previously demonstrated that the growth in low (1%) glucose media promotes respiratory metabolism and longer lifespan [26]. In addition, it has been also shown that *S. pombe* cells grown in media containing 0.08% glucose display hallmarks of respiratory metabolism, while fermentation is repressed [60]. We propose that autophagy mediated by glucose deprivation could be caused by an increase in respiratory metabolism, and that elimination of damaged mitochondria could be the biological goal linked to this activation.

We have here dissected the signaling cascades controlling autophagy activation in fission yeast. As expected, the TORC1 is crucial for the onset of autophagy, and mutants of the pathway controlling normal growth have the opposite impact on autophagy activation. Thus, cells lacking Tsc1 or expressing the signal blinded kinase *tor2^{L1310P}* could not trigger autophagy, and mutants decreasing TORC1 kinase activity (*tco89Δ*) had defects in cell growth (i.e. Psk1 phosphorylation) and were faster triggering GFP-Atg8 cleavage. We have also shown that not only TORC1 but also the TORC2 pathway had a significant impact on the onset of autophagy. As in other organisms, *S. pombe* has two types of TOR kinase complexes, TORC1 and TORC2, and interestingly the two have opposite roles in sexual differentiation, which is induced by nutrient

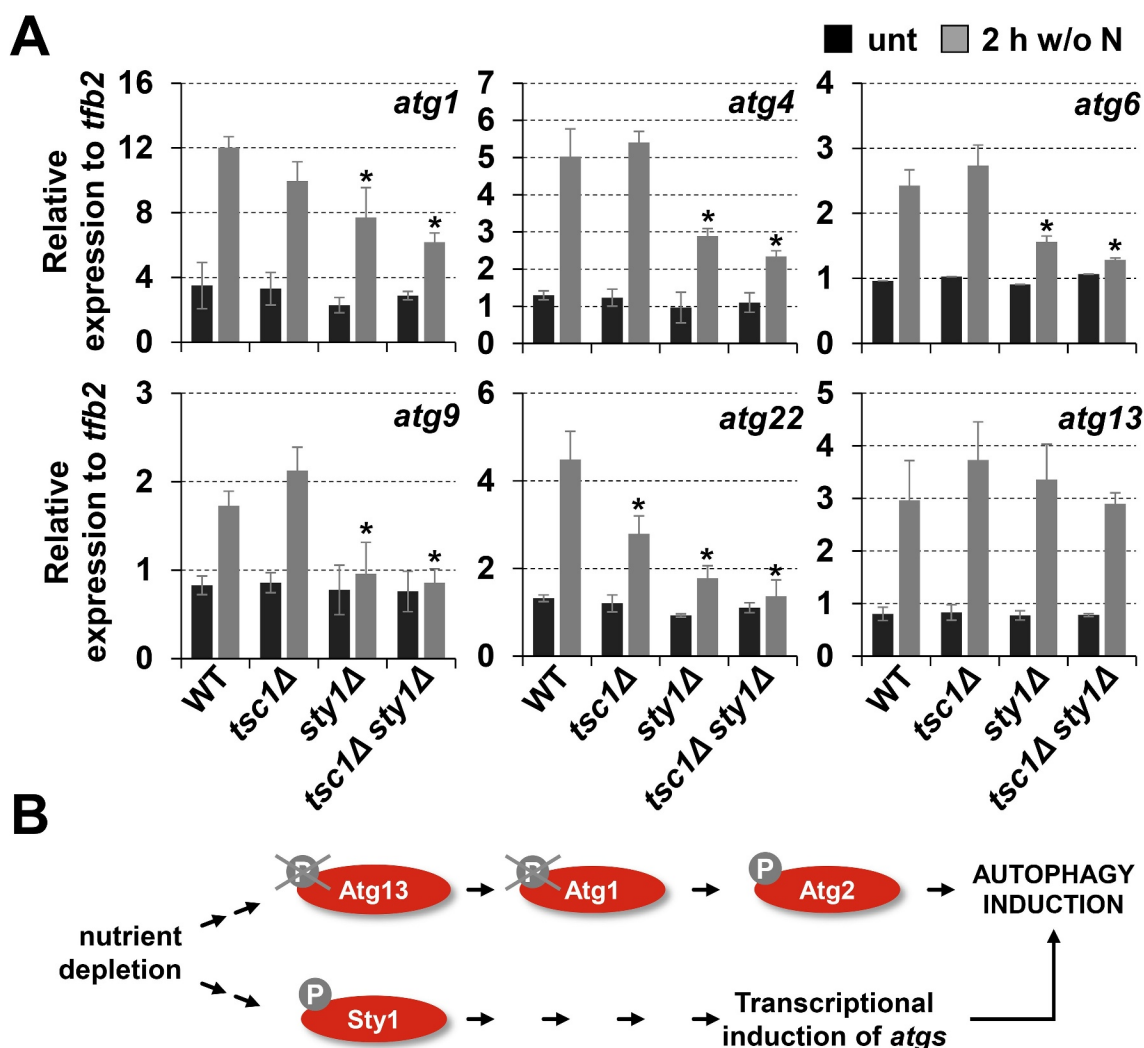


Figure 6. Sty1 plays a role in transcriptional upregulation of *atg* genes after nitrogen starvation. (A) Expression of some *atg* genes is dependent on Sty1. MM cultures of strains CC10 (WT), CC14 (*tsc1Δ*), CC13 (*sty1Δ*) and CC111 (*tsc1Δ sty1Δ*) were collected at the logarithmic phase (unt) and 2 h after switching to nitrogen-starved medium; total RNA was isolated and the transcripts specific for *atg1*, *atg4*, *atg6*, *atg9*, *atg22* and *atg13* were quantified by RT-qPCR. Amplification with *tfb2* primers was used as a normalization control. Each column represents the mean value and SD (standard deviation), calculated from four biological replicates. Asterisks represent significant differences, * $p < 0.01$ (Welch's t-test). (B) Scheme suggesting that Sty1 does not induce autophagy through a direct effect on Atg13-Atg1-Atg2 phosphorylation levels, but rather through the transcriptional upregulation of *atg* genes. See text for details.

starvation [61]. Nevertheless, we show here that both TORC1 and TORC2 kinases negatively regulate autophagy: mutants decreasing the kinase activity of the complexes (*tco89Δ* and *tor1Δ*) displayed an earlier induction of autophagy not only after nitrogen depletion but also upon low glucose, or upon depletion of sulfur or phosphorus source. Our data suggest that the direct regulator of cell growth promotion during nutrient-rich conditions and of autophagy activation during nutrient depletion is TORC1, but there is a positive crosstalk between both branches: the TORC2 mutants *gad8Δ* and *tor1Δ* displayed under nutrient-rich conditions impaired phosphorylation of substrates downstream of the TORC1 kinase (Psk1 and Rps6), and accelerated autophagy during nutrient depletion (Figure 3B and C). A similar crosstalk has been proposed in mammalian cells: TSC2 is directly phosphorylated and inhibited by the action of AKT, a homolog of Gad8 [33,62]. In fission yeast, our genetic interaction analysis suggests that

both branches impinge on the same substrates regarding autophagy: both *tor1Δ tco89Δ* and *tco89Δ* mutants showed similar accelerated autophagy upon lack of nutrients compared to wild-type cells (Fig. S3A). Furthermore, the double mutant *tor2^{L1310P} gad8Δ* displayed a mixed phenotype regarding basal phosphorylation of Psk1 and Rps6 and autophagy induction (Figure 3E), and *tsc1Δ gad8Δ* cells had an intermediate effect on GFP-Atg8 cleavage compared to the single deletes (Figure 3F). We propose that TORC2 kinase positively regulates TORC1 kinase independently of Tsc1-Tsc2 (Figure 3G).

Once established that carbon starvation induces autophagy, we investigated the participation of the PKA and the MAP kinase Sty1 pathways, since both unambiguously respond to glucose exhaustion. The fission yeast mutant *pka1Δ* and its antagonist, *cgs1Δ*, did not display significant defects in autophagy activation, even though the cAMP-dependent PKA

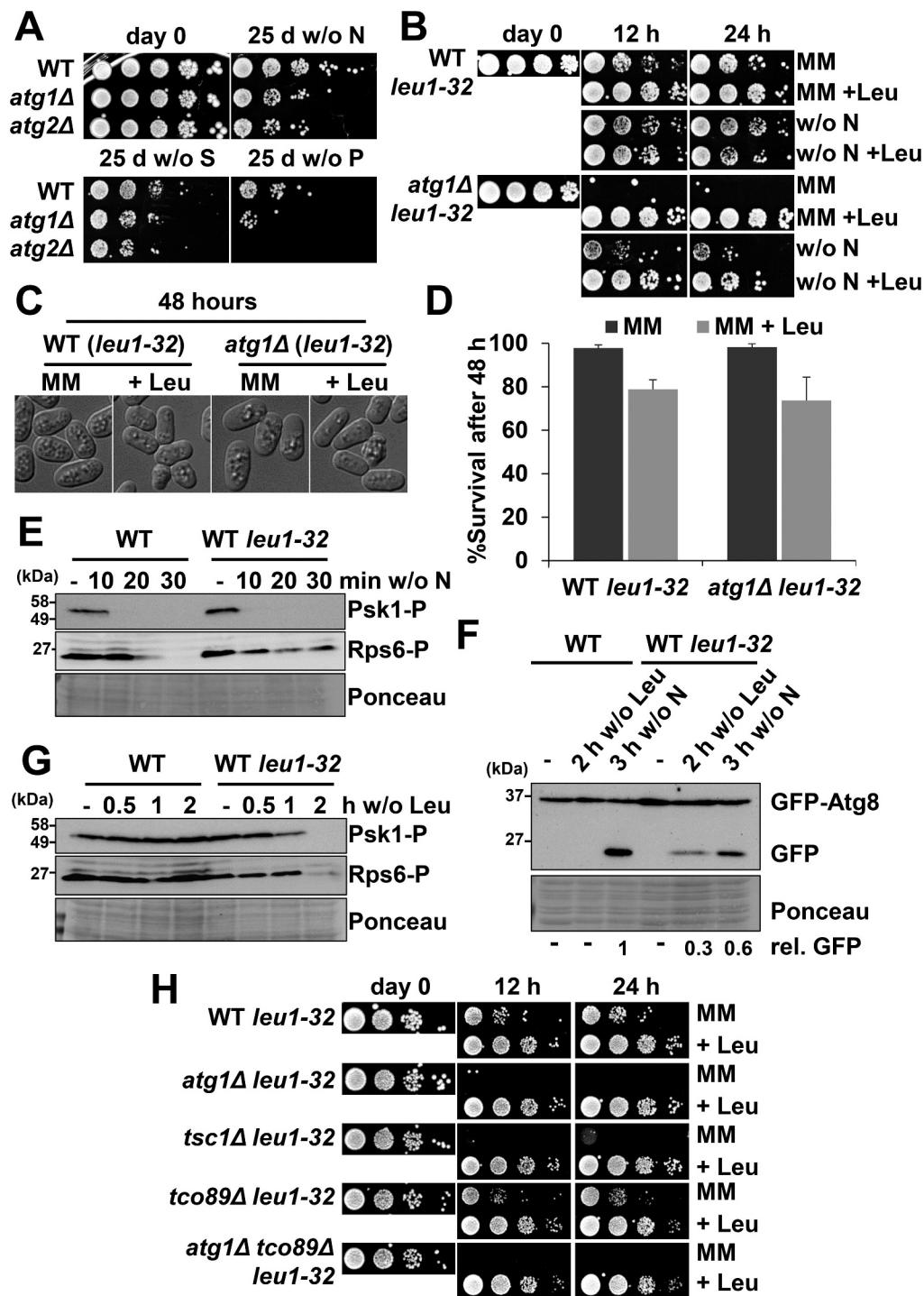


Figure 7. Biological role of autophagy in cells auxotrophic for leucine. (A) The negative effect on lifespan of *atg* mutants can only be observed 25 days after nutrient deprivation. MM cultures of strains CC10 (WT), CC11 (*atg1Δ*) and CC20 (*atg2Δ*) grown in MM until logarithmic phase (day 0) were switched to modified MM without nitrogen (w/o N), sulfur (w/o S) or phosphorus (w/o P) sources during 25 days. Serial dilutions of the cultures were plated onto YE55 plates. (B) The negative effect on lifespan of *atg* mutants auxotrophic for leucine can be observed 12 h after nitrogen or leucine deprivation. Strains HM123 (WT) and SK1 (*atg1Δ*), both carrying the *leu1-32* allele, were grown in leucine-supplemented MM until logarithmic phase (day 0) and switched to MM without leucine (MM), MM with leucine (MM +Leu), MM without nitrogen (w/o N) or MM without nitrogen with leucine (w/o N +Leu), for 12 or 24 h. Serial dilutions of the cultures were plated onto YE55 plates. (C, D) Microscopy images (C) and percent of survival (D) of HM123 (WT *leu1-32*) and SK1 (*atg1Δ leu1-32*) after 48 h in MM and leucine-supplemented MM (+ Leu). For survival measurements, cells were incubated with the permeability-dependent dye PI, and the percentage of non-fluorescent cells, indicative of viability, was determined by flow cytometry. (E) TORC1 kinase activity is unaffected in an autophagy-proficient *leu1-32* background. MM cultures of strains CC10 (WT) and CC88 (WT *leu1-32*) were shifted or not to nitrogen depleted media for 10–30 min and analyzed as in Figure 2A. (F) Leucine deprivation can trigger autophagy in *leu1-32* auxotrophic strains. MM cultures of strains CC10 (WT) and CC88 (WT *leu1-32*), both expressing GFP-Atg8, were shifted or not to leucine (2 h) or nitrogen (3 h) depleted MM and processed and analyzed as in Figure 2 C. Numbers below the panels represent the amount of cleaved GFP relative to the amount after 3 h without nitrogen in WT (with an assigned value of 1). (G) Leucine deprivation can inhibit TORC1 kinase activity in auxotrophic strains. Strains and conditions as in (F) were processed and analyzed as in Figure 2A. (H) The fast negative effect on lifespan of *atg* mutants auxotrophic for leucine is also displayed by TORC1 mutants with impaired autophagy activation. MM cultures of strains HM123 (WT) and *atg1Δ* (SK1), CC129 (*tsc1Δ*), CC134 (*tco89Δ*) and CC135 (*atg1Δ tco89Δ*), all carrying the *leu1-32* allele, were grown and processed as in (B).

cascade regulates autophagy from yeast to mammals [49,51,63,64]. Our genetic evidences are in accordance with previous reports indicating that autophagy is unaffected by cAMP levels [18]. We demonstrate here, however, that cells lacking Sty1 had striking defects in GFP-Atg8 cleavage, which are quantitatively more dramatic than those of TORC1 mutants (Figure 4C).

In *S. pombe*, the MAP kinase Sty1 is essential for survival under diverse environmental conditions such as oxidative stress, it is required for the initiation of the mating and meiosis program and is activated at the onset of stationary phase and during calorie restriction [26,65,66]. Our results reveal that Sty1 is activated after nitrogen or carbon depletion and is a main activator of bulk autophagy independently of its main effector Atf1 (Figure 4). The other two MAP kinase cascades in *S. pombe*, driven by Pek1 and Pmk1, have not yet been connected to autophagy, but it is worth to point out that cross-talks between the MAP kinase cell wall integrity and stress pathways have been described in plant pathogenic fungi [67,68], and a combination of both may affect the onset of autophagy. In *S. cerevisiae*, the stress MAP kinase Hog1 does not have a role in bulk autophagy, but it controls specific autophagy programs in response to starvation such as mitophagy [53]. In mammalian cells, the participation of the Sty1 ortholog MAPK/p38 is controversial, since it has a dual role in autophagy both as a positive and negative regulator [69].

We have shown here that the Sty1 kinase is not required for the phosphorylation of Atg1 or Atg2 (Figure 5D and E). We then tested whether the transcriptional autophagy program was regulated by the MAP kinase, since in budding yeast a transcriptional signature of *atg* genes has been reported in response to starvation (for a review, see [5]). Indeed, we show that several autophagy genes were induced upon nitrogen starvation, confirming a former genome-wide analysis [70], and this upregulation was, in most cases, strongly dependent on Sty1 but not on Atf1 (Figure 6 and Fig. S5). For some genes such as *atg1*, even the basal levels were decreased in a *sty1Δ* background (see Figure 6A), as well as the encoded protein Atg1 (see Figure 5D). Regarding nutrient-dependent autophagy activation, we propose that an alternative transcription factor downstream of Sty1 could trigger *atg* transcription. The zinc finger transcription factor Rsv2 was a likely candidate, since it is required for cell viability during stationary phase, its over-expression elongates lifespan and induces stress-related genes during sporulation [71–73], it is upregulated at the transcriptional level by environmental stresses [57], nitrogen starvation [70], and during stationary phase in a Sty1-dependent manner (our own unpublished data). However, GFP-Atg8 cleavage after nitrogen starvation was unaffected in *rsv2Δ* cells (our own unpublished data).

In budding yeast and in mammalian cells, autophagy is required to maintain viability under starvation conditions [74–76]. In *S. pombe*, the lack of viability of *atg* mutants is only observed after ≥ 25 days of growth in the absence of a nitrogen source, while few hours of nitrogen or leucine starvation impairs mitotic competence in auxotrophic autophagy mutants [17] (Figure 7B). We do not know why *atg* mutants depleted for nitrogen or leucine for only 12 h were

then unable to grow on plates, since the hallmarks of viability were unperturbed: the *atg* mutant cells displayed good microscopic morphological fitness, and propidium iodide staining, as an indicator of membrane integrity, did not differ from that of wild-type cells. Remarkably, we detected similar loss of colony-forming units upon nitrogen or leucine depletion in *tsc1Δ leu1-32* mutants (Figure 7H). As suggested by others [58], we believe that leucine or nitrogen depletion in cells auxotrophic for the amino acid causes severe cell arrest, and that the autophagy cascade is required for exiting the arrest and/or for the restoration of mitotic competence. Further studies will be required to elucidate the relationship between TORC1 and autophagy in leucine auxotrophic strains, and to decipher the interplay between growth and alternative gene programs regulated by the TORC1 pathway.

Materials and methods

Yeast strains and plasmids

Genotypes and origins of the strains used in this study are described in Table S1. Deleted and tagged strains including Atg1-HA, Atg2-HA and Pgk1-GFP were generated by homologous recombination using PCR fragments, amplified from plasmids derived from pFA6a derivatives [77] or by crossing deletion mutants from the Bioneer collection [78]. We PCR-amplified *atg8* from plasmid GFP-Atg8+ pREP41 [18], kindly provided by Kaoru Takegawa (Kyushu University, Japan), and cloned in plasmid p541' (pJK148 derivative [79], expressing GFP under the control of the constitutive *sty1* promoter [80]), yielding the integrative plasmid p750', allowing the integration of the *sty1':GFP-atg8::leu1* gene and the expression of GFP-Atg8. Strains with a chimeric gene *sty1':GFP-atg8::natMX6* were obtained by substituting the *leu1* locus of strain CC10 (wild-type, *sty1':GFP-atg8::leu1*) by homologous recombination using a PCR-amplified fragment obtained with a pair of primers flanking *leu1* using as a template a pFA-derivative plasmid containing nourseothricin resistance (*natMX6*). When required, leucine auxotrophy was recovered by crossing with *leu1-32* strains.

Experimental conditions

Cells were grown in rich medium YE5S (yeast extract 5 amino acid supplemented; Sunrise, 2011–500, with the standard glucose concentration (3%) or, when indicated, with only 1% or 0.08% glucose, or in synthetic minimal medium MM (Sunrise, 2005–250; 2% glucose) at 30°C as described previously [81]. Unless indicated in the figure legend, autophagy was induced by shifting logarithmic phase cells growing in MM (OD₆₀₀ of 0.5) to MM containing different concentrations of glucose (1% or 0.08%), or lacking the nitrogen source (NH₄Cl; Merck, 12,125–02-5), the phosphorus source (Na₂HPO₄; Sigma Aldrich, S-0876), or the sulfur source (Na₂SO₄; Sigma, S-5640). When indicated, MM was supplemented with leucine, lysine, or arginine. Culture media were pre-warmed to 30°C prior to media shift.

Stationary phase conditions and survival assays

To test lifespan in different glucose concentrations, wild type was grown at 30°C in YE5S or MM to the logarithmic phase (OD₆₀₀ of 0.5), and 1/10 serial dilutions (from 10 to 10⁵ cells) in 3 µl were spotted onto plates of rich media to measure viability. To test survival of auxotrophic strains in starved media, cells were grown at 30°C in supplemented MM media (with leucine, arginine or lysine, as required) to the logarithmic phase (OD₆₀₀ of 0.5). Then cells were washed twice and transferred to MM without amino acids and/or nitrogen. Again, colony forming units were determined by spotting 1/10 serial dilutions. Viability was measured using dye permeability of PI (propidium iodine) analyzed by flow cytometry, FACS Calibur (Becton Dickinson) at low flow, as described before [26]. Briefly, diluted cells at OD₆₀₀ of 0.1 were stained during 30 min at 30°C with 2 µg/mL of PI. PI was monitored in channel FL3 (detecting red fluorescence). Percent of survival was obtained comparing color-positive population with unstained cells.

Microscopy

Images were acquired using a Nikon Eclipse 90i microscope equipped with differential interference contrast optics. Images shown were taken by using PLAN APO VC 100 × 1.4 oil immersion objective, and an ORCA-II-ERG camera (Hamamatsu). Processing of all images was performed using Fiji (ImageJ, National Institutes of Health).

Protein extracts and immunoblot analysis

For immunoblot analysis of phosphorylated proteins and immunoblot analysis of GFP-Atg8 from Figures S3A and C, modified TCA (trichloroacetic acid) extracts from 5 ml cultures were prepared as previously described [82]. For most immunoblots of GFP-Atg8, 10 ml of cell cultures at an OD₆₀₀ of 0.5 were pelleted, and cells were resuspended in 200 µl of PBS with appropriate protease inhibitors. Cells were disrupted using a vortex (Vortex-Genie 2, Scientific Industries) with 0.5 mm glass beads. The membrane fraction was discarded by centrifugation and whole cell extracts were quantified by Bradford assay (Bio-Rad, 5,000,006), to load 30 µg of protein sample per lane. All samples were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and proteins were immuno-detected using the following antibodies: anti-GFP (Takara, 632,381), anti-HA (monoclonal homemade;12 C5A), anti-MYC (SIGMA, C3956), anti-RPS6KB/p70 (Cell Signaling Technology, 9206), anti-AKT (Cell Signaling Technology, 9611), anti-phosphorylated MAPK/p38 (Cell Signaling Technology, 9215) and anti-Sty1 [83]. Protein loading was determined by staining membranes with ATX Ponceau S (Sigma-Aldrich, 09276) in a dilution of Ponceau five-fold in 1% of acetic acid. Visualization, image processing and relative protein quantification were performed using ChemiDoc MP Imaging System (Bio-Rad, 17,001,402) and Fiji (ImageJ, National Institutes of Health).

Gene expression analysis

Total RNA (ribonucleic acid) extraction was performed from logarithmically growing cells and after nitrogen depletion. DNase I treatment of RNA, cDNA (complementary deoxyribonucleic acid) synthesis and RT-qPCR (real time quantitative polymerase chain reaction) were performed as previously described [84,85]. Briefly, reverse transcription was made using 1 µg of RNA using Reverse Transcription System (ThermoFisher, 4,374,967) following the manufacturer's instructions. Quantification of cDNA was analyzed by using real-time qPCR on LightCycler II using Light Cycler 480 SYBR Green I Master (Roche, 04887352001). Sequence and information of the oligonucleotides used are listed in Table S2.

Statistical analysis

Statistical analysis of gene expression was performed using R Studio software.

Data from four (main figures) or three (supplementary figures) biological replicates are represented as ± standard deviation (SD). We used unpaired Welch's t-test to calculate P-values and to determine significant differences between deletion strains and wild-type strain. P-values < 0.01 (main figures) or <0.05 (supplementary figures) were considered as significant.

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Data and code availability

All images included in the main and supplemental figures are available as Mendele dataset (doi: 10.17632/wpvb37w474.1).

Disclosure of potential conflicts of interest

The authors declare no competing interests.

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