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Review

Regulation of autophagy in yeast *Saccharomyces cerevisiae*

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ABSTRACT

Autophagy is a conserved catabolic process that initially involves the bulk or the selective engulfment of cytosolic components into double-membrane vesicles and successively the transport of the sequestered cargo material into the lysosome/vacuole for degradation. This pathway allows counteracting internal and external stresses, including changes in the nutrient availability, that alter the cell metabolic equilibrium. Consequently, the regulation of autophagy is crucial for maintaining important cellular functions under various conditions and ultimately it is essential for survival. Yeast *Saccharomyces cerevisiae* has been successfully employed as a model system to study autophagy. For instance, it has allowed the isolation of the factors specifically involved in autophagy, the Atg proteins, and the characterization of some of their molecular roles. In addition, this organism also possesses all the principal signaling cascades that modulate the cell metabolism in response to nutrient availability in higher eukaryotes, including the TOR and the PKA pathways. Therefore, yeast is an ideal system to study the regulation of autophagy by these signaling pathways. Here, we review the current state of our knowledge about the molecular events leading to the induction or inhibition of autophagy in yeast with special emphasis on the regulation of the function of Atg proteins.

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1. Introduction

Unicellular organisms such as the budding yeast *Saccharomyces cerevisiae* are subjected to continuous and extreme changes in the surrounding conditions in nature. In order to guarantee survival, cells must be able to sense the external alterations and generate effective responses that allow the maintenance of the intracellular homeostasis. One of the most common stresses that cells have to face is the quality and the quantity of nutrients available in the environment. When nutrients are limited or exhausted, sensor proteins detect those changes and trigger signal-transduction pathways to produce an integrated series of responses that allow cells to shut off certain metabolic pathways but also to mobilize new energy sources in order to extend survival during starvation. In these situations, maintaining the steady-state involves orchestrated changes in the cellular machinery ranging from modification in the localization and/or activity of proteins to severe rearrangement of internal components, such as membranes and organelles, passing through global changes in genomic expression, transcription and translation [1].

Induction of autophagy is one of the general responses that is triggered in eukaryotic cells to overcome nutritional limitations [2,3]. This pathway involves the delivery of cytoplasmic components into the mammalian lysosome or the plant and yeast vacuole for degradation to generate an internal pool of molecules ready to be recycled. Nitrogen starvation is the stimulus that leads to the most rapid and ample autophagy response in yeast, but other nutritional deprivations such as removal of the carbon source, auxotrophic amino acids or nucleic acids also induce autophagy, but in a lower extent [3]. Together with its function as an adaptation to starvation, autophagy is important for other cellular and organismal functions as well. For example, in yeast under growing conditions, it fulfills the selective transport of the precursor vacuolar hydrolases aminopeptidase I (prApe1) and α-mannosidase (prAms1) from the cytosol to the vacuole in a process called the *c*ytosol to *v*acuole *t*argeting (Cvt) pathway [4–6]. In addition, autophagy is required for the specific clearance of certain organelles such as damaged mitochondria or superfluous peroxisomes [7,8]. These two types of selective autophagy also take place in mammalian cells, where autophagy carries out numerous other essential cellular functions [9,10]. For instance, this pathway participates in cellular differentiation and development, lifespan extension, type II programmed cell death, antigen presentation, tumor suppression and protection against pathogens such as viruses and bacteria [11–13]. Autophagy has also been related to the pathophysiology of several diseases including tumors, neurodegeneration and cardiomyopathies [14].

Mechanistically, the induction of autophagy involves the *de novo* synthesis of cytosolic double-membrane vesicles called autophagosomes that sequester portions of the cytoplasm during their formation (Fig. 1) [15]. Once completed, the autophagosomes dock and fuse with

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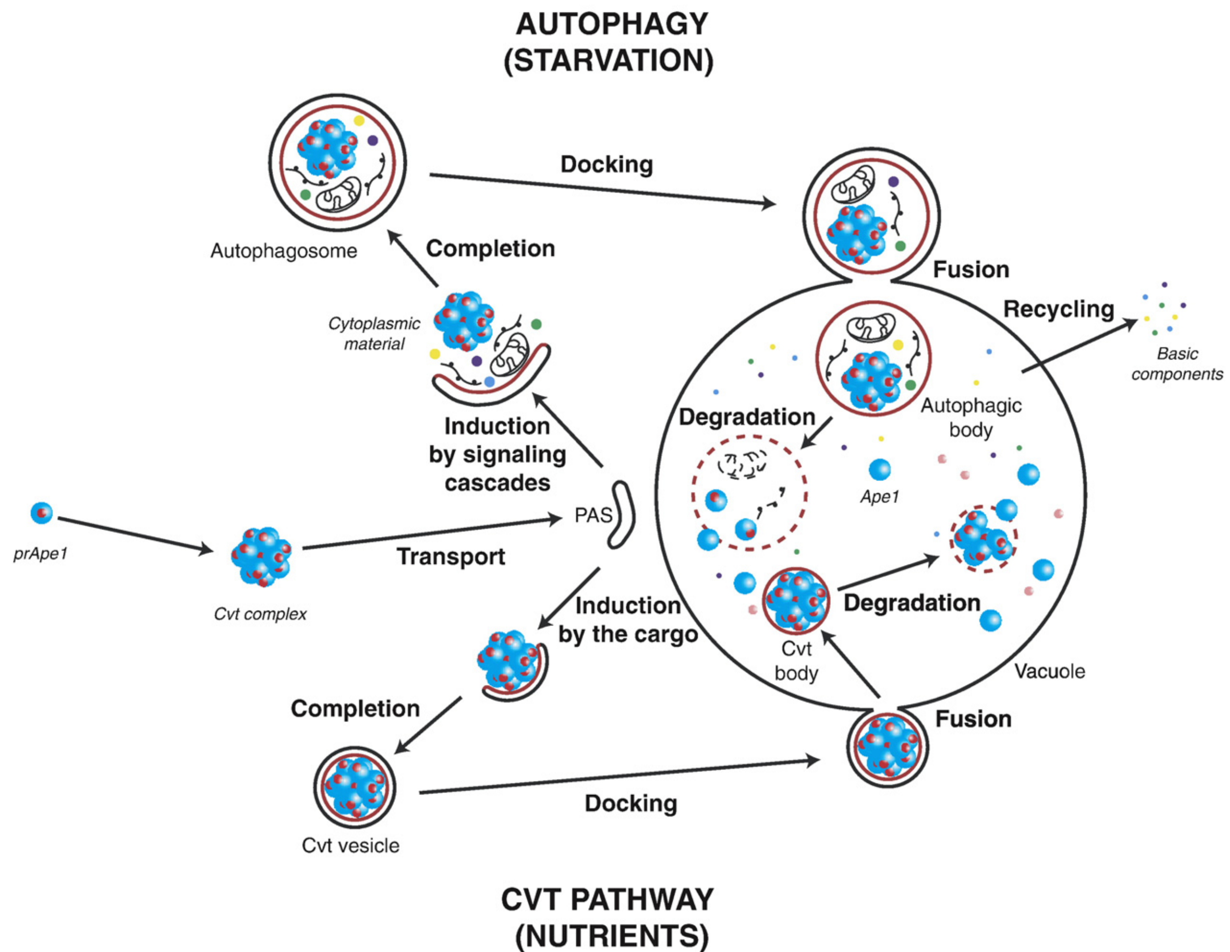

Fig. 1. The molecular mechanism of autophagy and the Cvt pathway. Under starvation conditions, signaling pathways trigger the assembly of the PAS and the subsequent expansion of what is believed to be an initial small cisterna. Fusion between the two extremities of this growing compartment leads to the formation of the autophagosome. During this event, cytoplasmic components including organelles and the prApe1 oligomer, are sequestered into this double-membrane vesicle. Complete autophagosomes dock and fuse with the vacuole releasing the internal autophagic body in the interior of this organelle. Vacuolar hydrolases successively degrade the autophagic bodies and their content. The resulting basic components (e.g. amino acids, sugars…) are recycled back into the cytoplasm for reuse. The biosynthetic Cvt pathway operates under growing conditions. The movement of the prApe1 oligomer in proximity of the vacuole surface induces the assembly of the PAS and that of the Cvt vesicle. Similarly to autophagosomes, Cvt vesicles dock and fuse with the vacuole liberating the Cvt bodies. Vacuolar hydrolases then break down the limiting membrane of this vesicle and protease process prApe1 into the active mature form (Ape1).

the lysosomes/vacuoles releasing their internal vesicles into the lumen of these organelles. These single-membrane vesicles, referred to as autophagic bodies and their content are then degraded by resident hydrolases. Finally, the degradation products resulting from this catabolic process are finally transported back into the cytoplasm and reused by the cell. Although the source of the membranes required for autophagosome formation is still unknown, the current model is that in yeast these large vesicles emerge at a single specialized perivacuolar location called the *p*re-*a*utophagosomal *s*tructure or the *p*hagophore *a*ssembly *s*ite (PAS) [16,17]. Among the 31 *au*tophagy-related (Atg) proteins that have been identified to date and that are specifically involved in different aspects of autophagy, most of them transitionally associate with the PAS and mediate the formation of the double-membrane vesicles [18]. Although almost all the *ATG* genes were initially isolated in yeast, orthologues of most of them have now been identified throughout the eukaryotic kingdom, revealing that both the molecular machinery and the mechanism of autophagy are highly conserved [19].

In this review, we will discuss our current knowledge about signal transduction pathways that regulate autophagy in yeast *S. cerevisiae* and how they potentially modulate the Atg machinery. Because the general molecular mechanism of autophagy has been extensively covered, we will exclusively focus on those molecular events involving Atg proteins that may have a key role during the induction of bulk autophagy or selective types of autophagy.

2. Regulation of the Atg machinery during starvation

In *S. cerevisiae*, the Cvt pathway operates under normal nutrient-rich growth conditions while nutrient depletion triggers autophagy as in all other eukaryotes (Fig. 1). In addition to the cargo, the Cvt pathway and bulk autophagy also differ in the size and formation rate of their respective vesicles (see also below). While biogenesis and transport to the vacuole of a small Cvt vesicle (140–160 nm in diameter) takes approximately 90–120 min, a large autophagosome (300–900 nm) can be formed and delivered into the vacuole in about 10–15 min [5,15–17]. In other words, when autophagy is induced, the specificity for the cargo is lost and more membranes need to be mobilized to sustain autophagy. Remarkably, most of the components required for the double-membrane vesicle formation are shared by the Cvt pathway and autophagy. A few Atg proteins, however, are specifically required for either one of these pathways, which may explain in part the differences between these processes [20–23]. Consequently, certain components of the Atg machinery that are already active during vegetative growth conditions must partially or completely adjust their function(s) when nutrients are limiting. How

are nutritional signals transmitted to the Atg machinery? Current data indicate that induction of autophagy entails changes in both the activity and the cellular level of determined Atg proteins.

2.1. The posttranslational regulation of the Atg machinery during starvation

The molecular mechanisms connecting the nutrient-sensitive signaling pathways to the Atg machinery are far from being understood, but most of the signal transductions seem to converge in the regulation of the activity of Atg1, a serine/threonine protein kinase essential for both the Cvt pathway and autophagy [24,25].

For a long time, some controversy has surrounded the requirement of the Atg1 kinase activity for the Cvt pathway and autophagy. In an initial investigation, a kinase-dead version of this protein, Atg1^{K54A}, was generated by mutating a critical lysine residue in the catalytic site to an alanine [25]. Western-blot analyses of the processing of prApe1, and enzymatic analyses of autophagy in Atg1^{K54A}-expressing cells lead to the demonstration that the kinase activity of Atg1 is essential for both these processes [25]. In a subsequent study using a chemical genetic approach to inhibit the Atg1 kinase activity, however, Abeliovich et al. reached a different conclusion and conferred an exclusive role in the Cvt vesicle biogenesis to the Atg1 kinase activity [26]. The strategy employed in their work was based on the expression of the analogue-sensitive point mutant allele *atg1*^{M102A} that is specifically inhibited by the ATP analogous compound 1-NA-PP1, which is a competitive inhibitor not able to interact with the ATP-binding site of wild-type protein kinases [26,27]. When cells harboring the *atg1*^{M102A} allele were incubated in the presence of 1-NA-PP1, maturation of prApe1 was completely blocked in nutrient-rich conditions, while autophagic traffic remained as efficient as in the wild type strain [26]. Because of the discrepancy between the results of these two studies, the Yoshinori Ohsumi laboratory has also explored the same chemical genetic approach. In their hands, the Atg1 kinase activity was also needed for normal autophagy progression, although some autophagic transport was still observed in the kinase mutant strain [28]. Interestingly, when the two characterized point mutations were combined in the *atg1*^{K54A,M102A} allele, autophagic activity was reduced to a level indistinguishable from that observed in the *atg1*Δ deletion strain [28]. Thus, the current view is that Atg1 kinase activity is required for double-membrane vesicle formation under all nutritional conditions and consequently its regulation is crucial. The main difficulty in trying to understand how the Atg1 kinase activity is modulated has been the lack of a known physiological substrate. The use of the myelin basic protein as an artificial substrate has led to the finding that the Atg1 kinase activity increases under starvation conditions, suggesting that this change is required for autophagy induction [25,28]. Like several other protein kinases, Atg1 is able to phosphorylate itself [24]. When assayed *in vitro*, this autophosphorylation activity decreases when cells are nutrient-deprived [24,28]. This result seems to be in contrast with the fact that Atg1 kinase activity increases under the same conditions, but it is very likely an experimental artefact [24]. Atg1 isolated from starved cells has probably already undergone autophosphorylation and thus when this self-modification is tested *in vitro* a decrease in this activity is measured because fewer modification sites are still available [24]. In agreement with this idea, transfer of radioactive phosphate onto non-phosphorylated recombinant Atg1 is more efficiently catalyzed by Atg1 isolated from starved cells than that obtained from growing cells [28]. It is interesting to note that while the phosphorylation status of Atg1 increases during starvation, a partial dephosphorylation of this protein takes place *in vivo* shortly after induction of autophagy [29]. This result indicates that Atg1 is probably posttranslationally regulated by additional kinase(s) that are active during normal growth (see below). Taken all together, these observations indicate that an increase in the Atg1 kinase activity and its phosphorylation status plays a key role in autophagy induction. It still remains largely unknown however, which are the mechanistic implications of an activation of Atg1 kinase activity. Interestingly, the expression of the kinase-death Atg1^{K54A} does not affect the recruitment of Atg proteins to the PAS but leads to an accumulation of these factors at this specialized site [30]. Therefore, the Atg1 catalytic activity could be involved in the disassembly of Atg machinery components from the PAS once the double-membrane vesicles are completed. Because the kinetics of vesicle formation are faster during autophagy, one possibility is that a more active turnover of Atg proteins is required at the PAS and this could be important during the membrane expansion events needed for the creation of the large autophagosomes. This hypothesis is supported by a morphological study revealing that a strain expressing the *atg1*^{K54A} kinase-dead allele generates fewer and smaller autophagosomes than wild type cells [30].

It remains unclear which factors stimulate the Atg1 kinase activity and if autophosphorylation has a positive allosteric effect on this activity change. It is known that the Atg1-binding partner Atg13 is able to modulate Atg1 enzymatic activity in cooperation with Atg17 [25,28,31]. Atg13 is a phosphoprotein essential for both the Cvt pathway and autophagy, whereas Atg17 is required for autophagosome biogenesis but not for the Cvt pathway [6,25,31]. Starvation induces partial dephosphorylation of Atg13, increasing its affinity and indirectly that of Atg17 to Atg1, resulting in the concomitant upregulation of the Atg1 kinase activity [25,28] (Figs. 2 and 3). Accordingly, expression of specific mutants that disrupt the interaction between Atg1, Atg13 and/or Atg17 result in a severe impairment of the autophagic response [25,28,30]. Moreover, the *atg17*Δ strain forms very small autophagosomes similar to those observed in cells expressing the *atg1*^{K54A} kinase-dead allele [28,32].

Yeast two-hybrid analyses and co-immunoprecipitation experiments have revealed that Atg1 interacts directly or indirectly via Atg13 or Atg17 with several proteins specifically required for either the Cvt pathway (Atg11, Vac8, Atg20 and Atg24) or autophagy (Atg29, Atg31) [25,28,32–37] (Fig. 2). This finding has led to hypothesize that the role of Atg1 in switching between the two processes does not exclusively rely on its kinase activity, but could also depend on its capacity to bind distinctive proteins under different nutritional conditions. Some or eventually all of these interactions could be dictated by the Atg1 phosphorylation status. Alternatively, the association or dissociation to Atg1 of some of these pathway-specific Atg components could modulate the Atg1 kinase activity and eventually other functions of the protein. The existence of an Atg1 complex containing all of these proteins is only speculative as it is based on interaction studies in pairwise combinations (Fig. 2). The lack of knowledge about the composition of the Atg1 complex *in vivo* makes it difficult to unveil the molecular role and regulation of Atg1. Consequently, it is also unknown if the organization of it dynamically changes during the process of double-membrane vesicle formation.

An important consideration to keep in mind is that some of these interacting partners, but also other factors, could be crucial in the spatial regulation of Atg1 kinase activity, e.g. they might bring this protein in proximity of its substrate(s). Like the rest of the Atg proteins, Atg1 is recruited to the PAS and this targeting seems to be controlled by phosphorylation that is independent from the Atg1 autocatalytic activity [29]. Localization of Atg proteins at the PAS during vesicle biogenesis is a hierarchic process and the order of recruitment has been established [16,38,39]. Atg1, together with Atg13 and the specific autophagy proteins Atg17, Atg29 and Atg31 are among the first factors to be targeted to the PAS. Deletion of any of these genes blocks the recruitment of most of the Atg proteins to this site [39]. Moreover, Atg1, Atg13, Atg17 and Atg29 seem to be sufficient to assembly at the PAS in a multiple knockout strain lacking all the genes essential for double-membrane vesicle formation [40]. As a

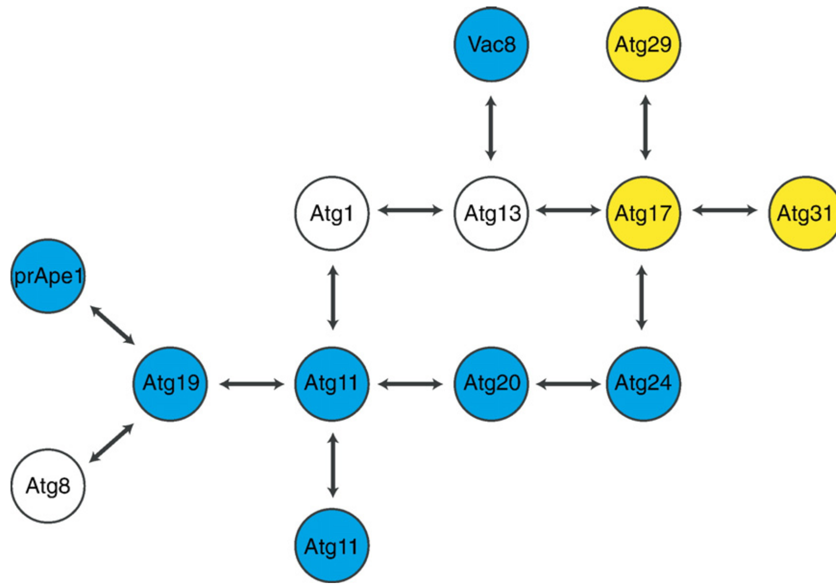

Fig. 2. Direct and indirect interactions between Atg1 and other components of the Atg machinery. Proteins essential for both the Cvt pathway and autophagy are represented with a white circle. Yellow and blue circles denote factors exclusively involved in the Cvt pathway and autophagy, respectively.

result, besides to its kinase function, Atg1 could also be crucial for the structural organization of the Atg machinery at the PAS.

2.2. Regulation of autophagy at the transcription level

In addition to posttranslational modifications that directly regulate the function of Atg components, an efficient autophagic response requires the upregulation of several *ATG* genes and perhaps that of other factors as well. Induction of autophagy under conditions in which protein synthesis is blocked with cycloheximide leads to the formation of abnormally small autophagosomes, indicating that protein synthesis is not necessary for induction of autophagy but crucial for the generation of normal sized autophagosomes [41]. Striking is the overproduction of Atg8, an ubiquitin-like protein involved in membrane fusion events during autophagosome formation that is bound to phosphatidylethanolamine by an ubiquitin-like conjugation system [42,43]. Therefore, it has been postulated that the higher levels of Atg8, which shows an approximately 8-fold induction [44,45], are necessary to mediate the increased number of membrane fusion events occurring during the formation of the large autophagosomes. This hypothesis is sustained by the finding that knockout strains lacking *ATG8* generate abnormally small autophagosomes [41,46]. An identical phenotype is also observed in cells expressing mutant versions of *ATG8* partially affecting its fusion properties [42]. Despite the relevance of Atg8 induction, the factors governing the transcription of this gene remain unknown.

The transcription of *ATG14* is also more than 20 times induced during autophagy [47]. Atg14 binds to Vps34, Atg6/Vps30 and Vps15 to form the class III phosphatidylinositol 3-kinase complex I, which is specifically involved in double-membrane vesicle formation [48,49]. The significance of the Atg14 upregulation remains to be investigated. Nevertheless, it is known that the *ATG14* gene is under the control of the nutrient-regulated transcription factors Gln3 (see below) and Gcn4 [47,50].

In addition to *ATG8* and *ATG14*, microarray analyses have revealed that *ATG1*, *ATG3*, *ATG4*, *ATG5*, *ATG7*, *ATG12* and *ATG13* are also upregulated under nutrient deprivation conditions, but to a lower extend than *ATG8* and *ATG14* [51]. The transcriptional factor Gcn4 has been shown to be involved in the induction of *ATG1* and *ATG13* during amino acids deprivation [50], but additional studies are required to acquire a more complete understanding about the transcriptional regulation of *ATG* genes.

3. Inhibition of autophagy by the *t*arget *o*f *r*apamycin (TOR) kinase

TOR belongs to a protein family of conserved serine/threonine protein kinases known as phospatidylinositol kinase-related kinases and is the main integrator of nutrient-derived signals in eukaryotes [52,53]. In the presence of preferred nitrogen sources, TOR is active and positively regulates numerous anabolic pathways while concomitantly represses processes required for the adaptation to nutrient availability, including autophagy. Starvation conditions lead to TOR inactivation, resulting in an inhibition of the metabolic pathways required for cell growth and a simultaneous initiation of a general response that allows cells to adapt to the new environmental situation [53].

Two TOR homologue proteins, Tor1 and Tor2, have been identified in yeast *S. cerevisiae* and associate with several proteins to form two distinct functional complexes [54]. The TOR complex 1 (TORC1), which is inhibited by the immunosuppressor rapamycin, contains Tor1 or Tor2 plus Kog1, Tco89 and Lst8 [54–56]. TORC1 participates in controlling several aspects of cell growth and metabolism, such as the progression of the cell cycle, ribosome biogenesis, regulation of translational activity and amino acid uptake by permeases [53]. The rapamycin-insensitive TOR complex 2 (TORC2) is formed by Tor2, Avo1, Avo2, Avo3/Tsc11, Bit61 and Lst8, and it dictates the polarization of the actin cytoskeleton and the sphingolipid biosynthesis [53,54,56,57].

How the information about the extracellular nutrient levels is transmitted to TOR is far to be understood. There is evidence that TOR is able to sense the intracellular levels of glutamine, a main intermediate in the nitrogen metabolism that can serve as an indicator for the nutritional status of the cell [58]. The vacuolar-associated EGO (exit from rapamycin-induced growth arrest) protein complex could be involved in the activation of TOR in accordance with nutrient availability [59]. Importantly, not all downstream effectors directly or indirectly regulated by TOR are affected by the amount of glutamine. This observation has led to the hypothesis that TOR activity could also be responsive to other sensors/factors and consequently this kinase could be able to integrate and coordinate different signal transduction pathways depending on the nutritional situation [58].

The principal downstream effectors of TOR are the members of the type-2A protein phosphatase (PP2A) and the PP2A-like protein families, which in turn control a wide range of cellular events [60,61]. The PP2A catalytic subunits form heterotrimers together

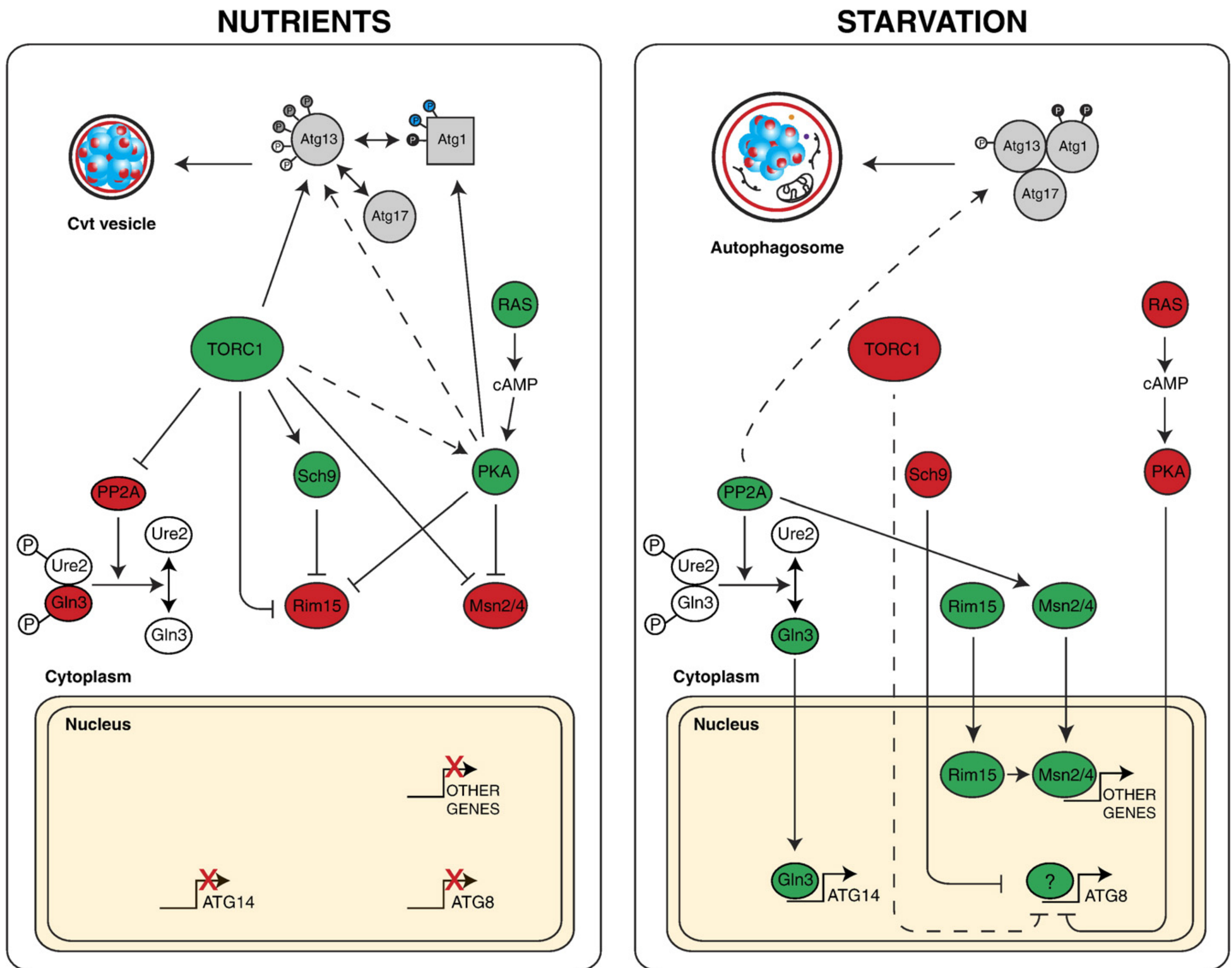

Fig. 3. Regulation of autophagy by the TOR and the Ras/cAMP-dependent PKA signaling pathways. (Left panel) Repression of autophagy in presence of nutrients. In the presence of nutrients, the TOR and the Ras/cAMP-dependent PKA signaling cascades are active and maintain Atg1 and Atg13 in a hyperphosphorylated status. This modification reduces the affinity between these two proteins and also decreases the Atg1 recruitment efficiency to the PAS leading to the formation of Cvt vesicles in stead of autophagosomes. TORC1 also negatively regulates the members of the PP2A phosphatase family and positively regulates the kinase Sch9. As a consequence, the transcription factors required for the adaptive response to starvation are retained in the cytoplasm through various mechanisms. PKA is also involved in retaining Msn2/4 and Rim15 in the cytoplasm. (Right panel) Induction of autophagy during starvation. Nutrient deprivation causes the inactivation of the TOR and the Ras/cAMP-dependent PKA signaling pathways. The PP2A phosphatases mediate the dissociation of Gln3 from its repressor Ure2. Translocation into the nucleus of Gln3 leads to the expression of multiple genes including *ATG14*. PP2A phosphatases could also be implicated in the dephosphorylation of Atg1 and Atg13, which promotes the association between these two proteins and Atg17, and their recruitment to the PAS. These changes are essential for the autophagosome formation. Another event following the inactivation of these two signaling cascades is the dephosphorylation of Rim15 and Msn2/4 and their successive translocation into the nucleus, where they induce the transcription of a different set of genes. The *ATG8* expression is also upregulated under those conditions. Transcription factors, protein kinases and phosphatases are represented in green when active and in red when inactive. Square and circle Atg1 symbolize low and high protein kinase activity, respectively. Phosphorylation by PKA is represented by blue phosphates (P). TOR phosphorylation is marked with grey P and those resulting from Atg1 autophosphorylation activity are in black. Continuous arrows denote established functional connections, whereas dashed ones indicate putative links.

with a scaffolding protein known as the A subunit and a member of the regulatory B subunit, which determine the substrate specificity and the subcellular location of the complex [60]. In addition, PP2A and PP2A-like proteins can also independently interact with Tap42, to which binding is stimulated by TORC1 by direct phosphorylation of Tap42 [62]. It is still an issue of debate if the regulatory subunit of Tap42 acts positively or negatively on this phosphatase family [63–67].

Importantly, abrogation of TOR function by using thermosensitive mutant alleles or after rapamycin treatment induces several phenotypes typical of starved cells, including autophagy even under nutrient rich conditions [51,68]. These data clearly demonstrate that an active TOR pathway negatively regulates autophagy through TORC1. A molecular change observed after TOR inhibition is the dephosphorylation of Atg13, which, as discussed above, triggers autophagy [25]. The enzymes involved in this conversion are unknown, even PP2A and PP2A-like phosphatases are the most obvious candidates, although this conversion probably occurs in a Tap42-independent way [25]. Alternatively, it is evident that the TOR signaling pathway maintains Atg1 and Atg13 in an inactive, hyperphosphorylated state. It remains unclear, however, if TORC1 directly phosphorylates these two Atg proteins or if a downstream kinase is responsible for this modification. It is interesting to note that Tor1 has recently been localized and part of it has been found concentrated in perivacuolar punctuated structures [69]. It is tempting to speculate that one of these could be the PAS. Alternatively, the active subpopulation of TORC1 on the vacuole surface [70] could act on the Atg machinery from there and eventually be also involved in the regulation of later stages of autophagy such as tethering and fusion.

Another main downstream effector of TOR, which is directly phosphorylated and activated by TORC1, is the protein kinase Sch9 [70]. Accordingly, Sch9 also participates in coordinating cell growth with nutrient availability, life span and stress resistance [71,72]. Importantly, the inhibition of Sch9 is essential to induce autophagy under particular circumstances, which strongly suggests that the TOR signaling cascade relies on this kinase to negatively regulate certain aspects of autophagy (see below) [73]. So far, however, there is no experimental evidence showing that Sch9 is directly or indirectly modulating the function of certain Atg proteins.

3.1. Transcriptional regulation of autophagic genes mediated by TOR

TOR acts as a negative regulator of the expression of genes required for the adaptation to starvation and other stress conditions by localizing the transcription factors involved in their transcription in the cytoplasm [53]. These transcription factors include Gcn4, the GATA-binding proteins Gln3 and Gat1, the partially redundant Zn^{2+}-finger Msn2 and Msn4, and the heterodimeric Rtg1–Rtg3 complex [64,74,75]. By direct or indirect phosphorylation of these transcription factors and/or of the respective cytoplasmic repressors, active TOR promotes the association between them preventing their transport into the nucleus [53]. When TOR is inhibited, dephosphorylation leads to the release of the transcription factors from their repressors and their subsequent translocation into the nucleus, when they trigger a global transcriptional adaptive response. For example, the transcription factor Gln3 and its specific repressor Ure2 are phosphorylated in the presence of preferential nitrogen sources and associated in the cytoplasm [64] (Fig. 3). Nitrogen starvation or rapamycin treatment stimulate PP2A-mediated dephosphorylation of both Gln3 and Ure2, and the consequent transport of Gln3 into the nucleus leads to the transcription of several genes including *ATG14* [47] (Fig. 3). Expression of *ATG8* is also negatively regulated by TOR [41], but as mentioned, the transcription factors modulating the transcription of this gene are unknown.

4. Regulation of autophagy by other signaling pathways

Diverse studies in yeast have demonstrated that additional signal transduction cascades such as the one involving the Ras/cAMP-dependent protein kinase A (PKA) cooperate together with TOR in the regulation of autophagy [29,73,76,77].

In the yeast *S. cerevisiae*, the Ras/cAMP-dependent PKA pathway regulates metabolism and cell growth in concert with the availability of carbon sources [78,79]. In yeast, there are two members of the Ras GTPase family, Ras1 and Ras2, which coexist in two different forms depending of the nucleotides they are binding: The inactive GDP-bound state becomes active when GDP is exchanged for GTP [80]. When cells are shifted from a medium containing a non-fermentable carbon source to one containing glucose, Ras proteins become activated and directly bind to adenylate cyclase stimulating the synthesis of cAMP [81]. In turn, cAMP activates PKA by promoting the dissociation of the catalytic subunits, Tpk1, Tpk2 and Tpk3, from the repressor Bcy1 [78]. Phosphorylation of different substrates by PKA then triggers the response that allows cells to rapidly adapt to the new conditions.

Several experiments have lead to the conclusion that the Ras/PKA pathway negatively regulates autophagy. For example, autophagy is strongly inhibited when the dominant hyperactive allele RAS^{val19} or the PKA catalytic subunit Tpk1 are overexpressed in starved yeast [29,76,77]. Morphological analyses have revealed that there is a block at the level of autophagosome formation [77]. Conversely, the dominant negative allele RAS^{ala22} induces autophagy even under nutrient rich conditions [77].

A recent study designed to find evolutionary conserved functional sequences identified two potential PKA phosphorylation sites in Atg1. *In vitro* and *in vivo* experiments confirmed that PKA directly modifies Atg1 [29]. Surprisingly, point mutations of the PKA phosphorylation consensus sites have no effect on the kinase activity of Atg1 but alter the intracellular localization of this protein [29]. Fluorescence microscopy experiments have revealed that wild type Atg1 moves from a mostly cytoplasmic subcellular distribution in growing cells to a more predominant PAS localization after autophagy induction, however an Atg1 mutant lacking both PKA sites permanently associates with the PAS in all nutritional conditions. In agreement with this result, Atg1 is dispersed in the cytosol of cells expressing the constitutively active RAS^{val19} in all nutritional situations [29]. All together, these data demonstrate that the Ras/cAMP-dependent PKA signaling pathway inhibits autophagy through the negative control of the association of Atg1 to the PAS. On a note, the PKA phosphorylation consensus sequences are also present in Atg13 and Atg18 but it remains to be elucidated if they are functional. In addition, it is also unknown if the Ras/cAMP-dependent PKA signaling cascade regulates other steps of autophagosome biogenesis.

Crucially, depletion of the PKA kinase activity alone is not sufficient to induce autophagy in growing cells but requires the simultaneous inhibition of Sch9 [73]. This indicates that Sch9 and by extension its upstream regulator TOR are modulating supplementary pathways essential for triggering autophagy that are not under the direct control of the Ras/cAMP-dependent PKA signaling cascade. It is important to note that in absence of PKA and Sch9, the autophagy response is less robust than after rapamycin treatment [73], indicating that the TOR pathway modulates certain aspects of autophagy in a Sch9-independent way.

All together, these observations make evident that the signals generated by the TOR and PKA signaling cascades have to be integrated in order to accurately regulate autophagy. This idea is also strengthened by the data showing that the autophagy response is intensified by PKA inhibition when the TOR signaling is inactivated [73]. Consequently, it is very likely that these routes converge in the control of the activity and/or the levels of several proteins important for this degradative pathway. A clear example is Atg1. Another one is the transcription factors Msn2/4 and Rim15, the protein kinase involved in their activation [73,76,82,83]. Sch9 negatively regulates the nuclear import of Rim15, while PKA inhibits the kinase activity of Rim15 and the nuclear localization of Msn2/4 by direct phosphorylation [83–85] (Fig. 3). Finally, the transcription factors Gln3 and Sfp1 could also be another common control point.

Snf1 is the yeast homologue of the mammalian AMP-activated protein kinase and it is required to coordinate glycogen metabolism with the levels of glucose [86,87]. A genetic screen designed to identify downstream effectors of Snf1 has lead to the identification of Atg1 and Atg13 as putative candidate proteins to be regulated by this kinase [88]. Further characterization of this connection has revealed that Snf1 is a positive regulator of autophagy in contraposition to Pho85, a cyclin-dependent protein kinase repressing this pathway and required for glycogen anabolism [88]. Additionally, Snf1 might regulate autophagy at a transcriptional level because nuclear localization of the transcription factors Gln3 and Msn2 is also affected by this kinase [89,90]. It is in large part unclear how signals derived from the Snf1 kinase activity are integrated within the rest of the nutrient-sensing cascades, but what has been established is that TORC1 acts as a negative regulator of Snf1 [91].

5. The Cvt pathway and the induction of double-membrane vesicle biogenesis by the cargo

Although autophagy has been considered for a long time to be a nonselective degradation pathway, numerous types of autophagy involving the exclusive sequestration of a specific cargo have recently been described. The most well studied type of selective autophagy is the Cvt pathway (Fig. 1), which is morphologically analogous to bulk

autophagy and uses most of the components of the Atg machinery [4,6]. The Cvt pathway is devoted to the sorting of precursor prApe1 and prAms1 directly from the cytoplasm into the vacuolar lumen [92,93] (Fig. 1). Shortly after synthesis, prApe1 oligomerizes into homo-dodecamers that subsequently assemble into a large cytosolic structure, the Cvt complex. This is further recognized by a specific receptor, Atg19 that in addition also binds to prAms1, the other known cargo of the Cvt pathway [5,94–97]. The cargo-receptor complex is then transported to the PAS by a mechanism that depends on the actin cytoskeleton and Atg11 [98,99]. At the PAS, the Cvt complex is enwrapped into a Cvt vesicle that subsequently fuses with the vacuole and releases the inner Cvt body into the lumen of this organelle. There the limiting lipid bilayer is degraded and prApe1 and prAms1 are finally processed into their active forms [5] (Fig. 1). In addition to the two cargo proteins, Atg8 and Atg19 have also been identified as components of the Cvt vesicles and bodies, but upon arrival in the vacuole, they are rapidly degraded [96,100].

Interestingly, deletion of *APE1*, *ATG11* or *ATG19*, or the expression of certain alleles of *ACT1*, the gene encoding for actin, blocks the Cvt complex recruitment to the vacuole and organization of the PAS, resulting in the failure of Cvt vesicle formation [98–100]. These mutations, however, do not affect bulk autophagy [33,96,98,100], which makes it evident that in the Cvt pathway the cargo regulates the formation of the double-membrane vesicle rather than signaling cascades. Consequently, the cargo is not incorporated into a constitutively emerging vesicle. The fact that the Cvt complex dictates the PAS assembly could be the basis of the specificity of the Cvt pathway, e.g. the membrane expansion is induced in proximity of the cargo and possibly continues around it preventing the inclusion of cytosol into the vesicle [5]. Atg11 is most likely the molecule directly connecting the cargo to the Atg machinery because of its interaction with Atg1 [33] (Fig. 2). Similar to the regulation of Atg1 localization by PKA, Atg11 might activate the formation of the Cvt vesicle by recruiting Atg1 to the PAS. This model is supported by the experimental evidence that Atg11 mutants unable to bind Atg1 can recruit the Cvt complex to the proximity of the vacuolar surface but fail to organize the PAS [101].

The induction of double-membrane vesicle biogenesis by the cargo does not appear to be an exclusive peculiarity of the Cvt pathway. The selective degradation of peroxisomes by pexophagy is another Atg protein-dependent process that takes place when the metabolic functions of these organelles are no longer necessary [102]. Similar to the Cvt pathway, pexophagy requires Atg11 and the actin cytoskeleton [33,98]. Therefore it is imaginable that these two pathways induce the formation of double-membrane vesicles using analogous mechanisms. Another type of selective autophagy that requires Atg11 is mitophagy, a process mediating the clearance of excess and damaged mitochondria [7,103–105]. Finally, ER-phagy, another selective process that is responsible for the degradation of part of the endoplasmic reticulum (ER) during starvation or termination of an ER stress response, necessitates Atg19 and the actin microfilaments [106,107]. These requirements are appealing for an involvement of Atg11 as well, but the participation of this protein in ER-phagy remains to be elucidated.

A major difference between the Cvt pathway and the other selective types of autophagy is that the first is a biosynthetic route whereas the others are inducible processes triggered by changes in the nutritional conditions or by internal signals associated with dysfunctional situations. For example, sensing components of the cAMP-signaling pathway, such as the G-protein-coupled receptor Gpr1 and its interacting G-protein Gpa2, are important for the induction of pexophagy [108]. Consequently, in all these different situations, the various signaling cascades are probably cross-talking with the machinery responsible for the cargo-mediated induction of double-membrane vesicle biogenesis. Because Atg11 is able to indirectly bind various cargoes, the control of the signaling pathways could take place at the level of regulation of the interaction between this protein and the organelles that have to be eliminated. Moreover, the same cascades could integrate the need of additional membranes necessary to enwrap larger cargoes. This would explain why players essential for bulk autophagy such as Atg17, Atg29 and Atg31, have been shown to participate in pexophagy and mitophagy [7].

6. Pseudohyphal growth (PHG) in budding yeast

Most of the *S. cerevisiae* laboratory strains are genetically impaired for filamentous growth, but pseudohyphal differentiation and invasive growth are part of a general response to nitrogen starvation in wild type budding yeast [109]. Similar to autophagy, the PHG program is induced upon nutrient deprivation and allows the cell to extend its viability under nutrient stress conditions [110]. Consequently, it is not surprisingly that the nutrient-sensitive kinases involved in the regulation of autophagy such as TOR and PKA are also key elements in PHG modulation [111,112]. Experimental evidence, however, suggests that autophagy may precede and restrain PHG [110]. In absence of *ATG* genes, the PHG program is activated earlier, indicating that autophagy generates the basic constituents necessary for the cell at the beginning of starvation situation but successively, under prolonged deprivation, PHG is stimulated as an alternative way to obtain nutrients [110]. Thus, it appears that autophagy is only partially concomitant with PHG. How can the same signaling cascades modulate such different cellular responses? An interesting idea comes from data showing that a partial loss of TOR activity is required for induction of PHG, while a complete abolition of TOR activity blocks this process [111]. Therefore, a possible scenario could be that different levels of TOR activity, and maybe of additional nutrient-sensitive protein kinases, determine which of the downstream signaling branches are activated and eventually to which extent.

7. Conclusions

The two principal ways employed in the laboratory to induce autophagy in yeast have been complete nitrogen deprivation or addition of the Tor inhibitor rapamycin. However, yeast cells can also trigger autophagy during the transition from exponential growth to a stationary phase [88], a less dramatic environmental situation when nutrients are still available but they start to become limited. This event probably reflects more realistically what often occurs in nature. Is autophagy modulated in a similar way in our experimental setups as in nature? The answer is almost certainly no. Cells should be able to adjust the autophagy response to their specific metabolic requirements, which sometimes need the obtainment of more than one basic molecule but also the elimination of a specific organelle. In this review, we have mainly described how the different signaling pathways could regulate autophagy at the level of induction of autophagosome formation. Most of the research has focused on the Atg1–Atg13 complex, but the functions of other Atg proteins might be modulated as well. Gene expression is very likely one of the ways. Several transcription factors essential for normal autophagy have been identified, but others are still unknown as for example those involved in *ATG8* expression. It cannot be excluded that signaling cascades control other steps of the process of autophagy, such as for example the docking and/or fusion of autophagosomes with the vacuole. This event is probably taking place during the ER-phagy occurring at the termination of an ER stress, where ER-containing autophagosomes accumulate in the cytosol instead of immediately fusing with the vacuole [107]. Obviously, another important role played by the signaling pathways is dictating which organelle or structure has to be eliminated during a selective type of autophagy.

Further studies are definitively required to understand how autophagy is regulated and how signaling cascades accurately perform this. Because of the increasing list of physiological and pathological

situations that are linked to autophagy, it is not surprising that this process is governed by an intricate network of signaling cascades that cross-talk with each other to generate an integrated and specific response. Yeast remains a precious experimental tool to extricate ourselves in the certain aspects of this complex network and consequently it will certainly help us to improve our understanding of the molecular events that allows the accurate regulation of autophagy.

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