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Characterization of a novel autophagy-specific gene, *ATG29* ☆

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Abstract

Autophagy is a process whereby cytoplasmic proteins and organelles are sequestered for bulk degradation in the vacuole/lysosome. At present, 16 *ATG* genes have been found that are essential for autophagosome formation in the yeast *Saccharomyces cerevisiae*. Most of these genes are also involved in the cytoplasm to vacuole transport pathway, which shares machinery with autophagy. Most Atg proteins are colocalized at the pre-autophagosomal structure (PAS), from which the autophagosome is thought to originate, but the precise mechanism of autophagy remains poorly understood. During a genetic screen aimed to obtain novel gene(s) required for autophagy, we identified a novel ORF, *ATG29*/*YPL166w*. *atg29Δ* cells were sensitive to starvation and induction of autophagy was severely retarded. However, the Cvt pathway operated normally. Therefore, *ATG29* is an *ATG* gene specifically required for autophagy. Additionally, an Atg29-GFP fusion protein was observed to localize to the PAS. From these results, we propose that Atg29 functions in autophagosome formation at the PAS in collaboration with other Atg proteins.

Keywords: Autophagy; ATG; Atg29; PAS; Cvt; Yeast

Proper cellular homeostasis requires that a strict balance be kept between protein synthesis and degradation. Within cells, autophagy is the major pathway by which long-lived proteins and cytoplasmic organelles are degraded [1,2]. During autophagy, cytoplasmic components are enwrapped into an autophagosome and delivered to the vacuole for degradation [3]. From genetic analyses using budding yeast, at least 16 autophagy-related genes (*ATG*) have been identified as essential for autophagosome formation [2,4]. Recently, it has been demonstrated that most Atg proteins are colocalized at the pre-autophagosomal structure (PAS) [5]. Therefore, the PAS is implicated to be the center of autophagosome formation. However, how these Atg proteins function cooperatively at the PAS to create the autophagosome still remains to be elucidated.

In *Saccharomyces cerevisiae*, autophagy overlaps with another pathway, the cytoplasm to vacuole targeting (Cvt) pathway. This pathway is responsible for the biosynthetic delivery of vacuolar hydrolase aminopeptidase I (API) from cytoplasm to vacuole [2,6]. The Cvt pathway takes place in cells grown in nutrient-rich conditions, while autophagy is induced by starvation [1]. Furthermore, most of the Atg proteins required for autophagy are also needed for the Cvt pathway [2,4]. Although the Cvt and autophagy pathways are mechanistically similar, the double-membrane vesicles produced by these pathways are of different sizes [7]. Cvt vesicles are approximately 140–160 nm in diameter and exclude cytosol, whereas autophagosomes are 300–900 nm in diameter and include cytosol and

☆ *Abbreviations:* PAS, pre-autophagosomal structure; *ATG*, autophagy-related genes; Cvt, cytoplasm to vacuole targeting; API, aminopeptidase I; GFP, green fluorescent protein; Vps, vacuolar protein-sorting; PE, phosphatidylethanolamine.

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occasionally organelles. To date, a set of proteins specifically required for the Cvt pathway has been identified, while *ATG17* is the only gene known to function specifically in autophagy [8]. Clearly, the identification of additional molecule(s) required specifically for this pathway is crucial for a more thorough understanding of this process.

In this study, we searched for mutants that showed defects in autophagy and identified a novel gene, *YPL166w*/*ATG29*.

Materials and methods

Yeast strains and media. Growth media, culture conditions, and genetic manipulations were all as previously described [9]. The yeast strains used in this study are listed in Table 1. A deletion construct to disrupt *ATG29* with kanMX was amplified by PCR from BY4741-based *atg29* deletion mutants [10]. The resultant cassette was transformed into SEY6210, BJ2169, and KVY55 strains to make TMK4, TMK368, or TMK17, respectively. TMK372 was made by replacing *ATG8* gene with the *LEU2* gene [11]. TMK105 was made by replacing *ATG17* gene with the *URA3* genes in the strain TMK4. STY1133 was created as described previously [12]. TMK73 and TMK190 were created according to the one-step gene replacement method described previously [13].

Plasmid construction. A 1.7 kb fragment including the entire *ATG29* was cloned from yeast genomic DNA into pRS316 [14] to yield YCp*ATG29*. Plasmid YCp*ATG29-GFP* was generated as follows. The DNA fragment containing *ATG29-GFP*, which was PCR-amplified from TMK190, was inserted into pRS314 [14]. The plasmid pRS316 CFP-*ATG8* used in this study has been described previously [5].

Fluorescence microscopy. Intracellular localization was examined using a DeltaVision microscope system (Applied Precision, Issaque, WA) or an inverted fluorescence microscope (IX-71, Olympus) as described previously [5].

Other procedures. Isolation of mutants defective in autophagy was performed as described previously [15]. For measurement of autophagic activity, the ALP assay was performed as described previously [16]. Immunoblot analyses and in vitro protein kinase assays were performed as described previously [8,11].

Table 1
Yeast strains used in this study

Strain	Genotype	Source
BY4741	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	[10]
BYS7	BY4741 *atg7Δ::kanMX*	[10]
BYS16	BY4741 *atg16Δ::kanMX*	[10]
TMK181	BY4741 *atg29Δ::kanMX*	[10]
BJ2168	*MAT**a** ura3 leu2 trp1 ura3 pep4-3 prb1–1122 prc1–407*	Yeast Genetic Stock Center, Berkeley
TMK372	BJ2168 *atg8Δ::LEU2*	This study
TMK368	BJ2168 *atg29Δ::kanMX*	This study
SEY6210	*MATα ura3 leu2 his3 trp1 lys2 suc2*	[25]
KVY116	SEY6210 *atg13Δ::kanMX*	[5]
KVY117	SEY6210 *atg16Δ::LEU2*	[5]
JOY617	SEY6210 *atg17Δ::HIS3*	[26]
TMK4	SEY6210 *atg29Δ::kanMX*	This study
TMK105	SEY6210 *atg17Δ::URA3 atg29Δ::kanMX*	This study
KVY55	SEY6210 *pho8::pho8Δ60*	[11]
JOY687	KVY55 *atg7Δ::HIS3*	[27]
YYK382	KVY55 *atg17Δ::kanMX*	[21]
TMK17	KVY55 *atg29Δ::kanMX*	This study
STY1133	SEY6210 *RFP-APE1::LEU2*	This study
TMK190	SEY6210 *ATG29::ATG29-GFP-kanMX*	This study
TMK73	SEY6210 *ATG29::ATG29-YFP-kanMX*	This study

Results

Identification of ATG29/YPL166w

All previously discovered *atg* mutants, which are defective in autophagy, show a loss of viability under starvation conditions. Thus, sensitivity to starvation would seem to be a good indicator of autophagy defects [15]. To search for novel genes involved in autophagy, we performed a genome-wide screen using a collection of roughly 4500 yeast single-gene deletion mutants in the BY4741 background [10]. Each strain was first screened for the reduced viability under starvation conditions and over 250 strains were selected from this first screening. Strains that showed defects in the Cvt pathway were removed from the analysis. We next examined the autophagic activity of these candidates by measuring the accumulation of autophagic bodies. Of these strains, one of the most striking candidates was a disruptant of *YPL166w*. Here, we have named the ORF *YPL166w* as *ATG29*.

Atg29 is a previously unidentified protein of 213 amino acid residues with no predicted transmembrane domain, signal sequence, or other known motif. Coils 2.1 software [17] yielded a weak prediction of a coiled-coil in the region between amino acids 65 and 95 (Fig. 1). Atg29 has orthologues in other *Saccharomyces* species, but is not conserved in higher eukaryotes.

Vacuolar transport pathways are not impaired in the atg29Δ mutant

Autophagic degradation requires proper transport of vacuolar hydrolases. Thus we examined the processing of three kinds of vacuolar proteases, Pep4(PrA), PrB, and CPY, in *atg29Δ* to investigate whether the transport of these proteins was affected in the mutant. Immunoblot analyses of *atg29Δ* showed the presence of mature forms of these enzymes in similar amounts as wild-type cells, irrespective of growth conditions (data not shown). The fact that CPY processing was unaffected in *atg29Δ* indicates that *atg29Δ* is not a vacuolar protein-sorting (*vps*) mutant. Vacuolar acidification in *atg29Δ* was normal as judged by staining with quinacrine (data not shown). Furthermore, *atg29Δ* cells did not show sensitivity to 300 mM $CaCl_2$ (data not shown), indicating that *atg29Δ* cells have functional vacuoles.

ATG29 is essential for autophagy, but not for the Cvt pathway

atg29Δ cells displayed reduced viability under starvation conditions compared to wild-type cells (Fig. 2A). The autophagic activity of *atg29Δ* was assayed by two different approaches. First, we observed autophagic bodies by light