Inhibition of Target of Rapamycin Signaling and Stress Activate Autophagy in Chlamydomonas reinhardtii*

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Autophagy is a catabolic membrane-trafficking process whereby cells recycle cytosolic proteins and organelles under stress conditions or during development. This degradative process is mediated by autophagy-related (ATG) proteins that have been described in yeast, animals, and more recently in plants. In this study, we report the molecular characterization of autophagy in the unicellular green alga Chlamydomonas reinhardtii. We demonstrate that the ATG8 protein from Chlamydomonas (CrATG8) is functionally conserved and may be used as a molecular autophagy marker. Like yeast ATG8, CrATG8 is cleaved at the carboxyl-terminal conserved glycine and is associated with membranes in Chlamydomonas. Cell aging or different stresses such as nutrient limitation, oxidative stress, or the accumulation of misfolded proteins in the endoplasmic reticulum caused an increase in CrATG8 abundance as well as the detection of modified forms of this protein, both landmarks of autophagy activation. Furthermore, rapamycin-mediated inhibition of the Target of Rapamycin signaling pathway, a major regulator of autophagy in eukaryotes, results in identical effects on CrATG8 and a relocalization of this protein in Chlamydomonas cells similar to the one observed upon nutrient limitation. Thus, our findings indicate that Chlamydomonas cells may respond to stress conditions by inducing autophagy via Target of Rapamycin signaling modulation.

Protein turnover is essential for the adaptation of cells to variable environmental conditions. Similar to other eukaryotes, plants have developed two distinct mechanisms to regulate protein degradation, a selective ubiquitin/26S proteasome pathway (Vierstra, 2009) and macroautophagy (hereafter referred to as autophagy), a nonselective membrane-trafficking process (Bassham, 2009). During autophagy, a large number of cytosolic components, including entire organelles, organelle fragments, and protein complexes, are enclosed in bulk within a double-membrane structure known as the autophagosome and delivered to the vacuole/lysosome for degradation to recycle needed nutrients or degrade toxic components (Xie and Klionsky, 2007; Nakatogawa et al., 2009). The autophagosomes appear to arise from isolation membranes usually observed in close proximity to the vacuole called the preautophagosomal structure (PAS). These membranes expand and fuse to encircle portions of the cytoplasm, generating an autophagosome that is targeted to the vacuole. The outer membrane of the autophagosome then fuses with the vacuole membrane, and the remaining vesicle, known as the autophagic body, is finally released to the vacuole for its degradation (Xie and Klionsky, 2007).

The evolutionary conservation of autophagy among eukaryotes indicates that structural and regulatory components of this cellular process must be also conserved. Accordingly, a significant number of autophagy-related (ATG) genes that participate in autophagy regulation and autophagosome formation have been identified, initially through genetic approaches in yeast and subsequently in higher eukaryotes, including mammals, insects, protozoa, and plants (Bassham et al., 2006; Bassham, 2007; Meijer et al., 2007). In yeast, two protein conjugation systems composed of the ubiquitin-like proteins ATG8 and ATG12 and the three enzymes ATG3, ATG7, and ATG10 play an essential role in autophagosome formation and seem to be conserved through evolution (Geng and Klionsky, 2008). ATG8 becomes modified with the lipid molecule phosphatidylethanolamine (PE) by the consecutive action of the ATG7 and ATG3 enzymes in a process mechanistically similar to ubiquitination (Ichimura et al., 2000). Prior to this modification, ATG8 must be cleaved by the Cys protease ATG4 to expose a C-terminal Gly residue that is conjugated to PE (Kirisako et al., 2000; Kim et al., 2001). ATG12 becomes covalently attached to the ATG5 protein in a conjugation reaction that is catalyzed by ATG7 and ATG10 (Mizushima et al., 1998). ATG8-PE and ATG12-ATG5 conjugates localize to autophagy-related membranes and are required for the initiation and expansion of autophagosomal membrane and hemifusion of this membrane with the vacuolar membrane (Hanada et al., 2007; Nakatogawa et al., 2007, 2009;
Fujita et al., 2008; Geng and Klionsky, 2008; Xie et al., 2008).

Our understanding of the autophagy process has substantially increased with the development of specific markers for autophagy. In plants, two markers for autophagosomes have been described, the monodansydacavine dye and GFP-ATG8 fusion protein (Yoshimoto et al., 2004; Contiento et al., 2005; Thompson et al., 2005). As in other species, binding of ATG8 to autophagosomes has been used to monitor autophagy in plants. In contrast to yeast, where a single ATG8 gene is present, plants appear to contain a small gene family with several ATG8 isoforms, suggesting that autophagy is more complex in these photosynthetic organisms. For example, Arabidopsis (Arabidopsis thaliana) and maize (Zea mays) encode nine and five ATG8 genes, respectively (Doelling et al., 2002; Hanaoka et al., 2002; Ketelaar et al., 2004; Chung et al., 2009). However, despite the high complexity of the ATG8-conjugating system in plants, important findings have been recently reported on the molecular characterization of autophagy using ATG8 as an autophagy marker in these organisms. The use of specific markers for autophagy in plants has revealed that this process is active at a basal level under normal growth and is induced upon nitrogen- or carbon-limiting conditions as well as in response to oxidative stress (Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005, 2007; Chung et al., 2009). Reverse genetic approaches have also been applied to a number of Arabidopsis ATG genes using T-DNA insertion mutants or RNA interference in order to investigate the physiological roles of autophagy in plants. The initial characterization of autophagy-deficient plants demonstrated that the ATG system is not essential under nutrient-rich conditions. However, a detailed analysis of these mutants indicated that autophagy is required for the proper response of the plant to nutrient limitation or pathogen infection. Plants lacking the ATG4, ATG5, ATG7, ATG9, or ATG10 gene display premature leaf senescence and are hypersensitive to nitrogen or carbon limitation (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004; Thompson et al., 2005; Phillips et al., 2008). Arabidopsis plants with reduced levels of ATG18, which is required for autophagosome formation, are more sensitive to methyl viologen treatment and accumulate high levels of oxidized proteins, demonstrating that autophagic processes participate in the response of the plant to oxidative stress (Xiong et al., 2005, 2007). Plants deficient in the autophagy genes ATG6/Beclin1, ATG3, ATG7, and ATG9 exhibit unrestricted hypersensitive response lesions in response to pathogen infection (Liu et al., 2005; Hofius et al., 2009). These findings implicate autophagy as a prosurvival mechanism to restrict programmed cell death associated with the pathogen-induced hypersensitive response in plants. Arabidopsis ATG6 has also been shown to mediate pollen germination in a manner independent of autophagy (Fujiki et al., 2007).

As mentioned above, autophagy is triggered among other factors by a reduction in the availability of nutrients. This starvation signal is transmitted to the autophagic machinery by important regulatory factors, including the Ser/Thr kinases Target of Rapamycin (TOR), ATG1, and SNF1 and the phosphatidylinositol 3-kinase ATG6/Beclin1 (Diaz-Troya et al., 2008b; Bassham, 2009; Ceccolero and Reggiori, 2009). TOR has been identified as a negative regulator of autophagy in yeast, mammals, and fruit flies (Diaz-Troya et al., 2008b). The pharmacological inhibition of TOR by rapamycin leads to autophagy activation through a mechanism that requires the activation of the ATG1 kinase (Kamada et al., 2000). It has been recently demonstrated in mammals and fruit flies that a rapamycin-sensitive TOR signaling complex termed TORC1 directly phosphorylates and inhibits the ATG1 kinase and its regulatory protein ATG13 (Chang and Neufeld, 2009; Hosokawa et al., 2009; Jung et al., 2009). These regulatory proteins are conserved in plants, although except for ATG6 (Liu et al., 2005), there is no direct evidence for regulation of autophagy by these signaling pathways.

The unicellular green alga Chlamydomonas reinhardtii has been used as a model for the study of important cellular and metabolic processes in photosynthetic organisms (Harris, 2001). More recently, Chlamydomonas has also been proposed as a useful system for the characterization of the TOR signaling pathway in photosynthetic eukaryotes based on the finding that, unlike plants, Chlamydomonas cell growth is sensitive to rapamycin (Crespo et al., 2005; Diaz-Troya et al., 2008a). Treatment of Chlamydomonas cells with rapamycin results in a pronounced increase of vacuole size that resembles autophagy-like processes (Crespo et al., 2005). However, a role of TOR in autophagy regulation could not be demonstrated due to the absence of an autophagy marker in Chlamydomonas. Actually, no studies have been reported on any autophagy-related protein in green algae, despite the high conservation of ATG genes in Chlamydomonas (Diaz-Troya et al., 2008b).

This study reports the molecular and cellular characterization of autophagy in the green alga Chlamydomonas. We demonstrate that the ATG8 protein from Chlamydomonas (CrATG8) may be used as a specific autophagy marker. Nutrient limitation and cell aging trigger an autophagic response that can be traced as an increase at the level of CrATG8, the detection of modified forms of CrATG8, and a change in the cellular localization of this protein. Furthermore, we demonstrate that autophagy is inhibited by a rapamycin-sensitive TOR cascade in Chlamydomonas.

RESULTS

Identification of Chlamydomonas ATG8 cDNA

A recent survey of the Chlamydomonas genome has revealed the conservation of a single ATG8 gene in this
The putative CrATG8 gene spans about 2 kb of genomic DNA and consists of five exons and four introns. The full-length ATG8 cDNA was cloned by PCR from a *Chlamydomonas* cDNA library (see “Materials and Methods”). The CrATG8 gene encodes 134 amino acids with a predicted molecular mass of 15 kD. An evolutionary study of this protein evidenced a high sequence identity to plant ATG8s that ranges from 47% to 74% (data not shown). The critical Gly-116 residue of yeast ATG8 that is processed by the ATG4 protease is conserved in CrATG8 and corresponds to Gly-120 (Fig. 1A). However, a C-terminal extension of 14 residues that is not usually conserved among ATG8 proteins from other organisms was found after Gly-120 (Fig. 1A). To characterize CrATG8, we generated a polyclonal antibody against recombinant CrATG8 (see “Materials and Methods”) that reveals a protein of about 15 kD from *Chlamydomonas* soluble extracts (Fig. 1B). The anti-CrATG8 antibody cross-reacted with other putative ATG8 proteins from Arabidopsis and weakly recognized yeast ATG8 in total extracts (Fig. 1B).

**Figure 1.** A, Amino acid sequence alignment of the C-terminal segments of the ATG8 protein from *Chlamydomonas* and other organisms. Identical residues are shaded. The arrowhead indicates the processing site recognized by the ATG4 protease that exposes the C-terminal Gly for lipidation. The conserved Gly is shaded and in boldface, and Gly-120, present only in Chlamydomonas, is shown in boldface. The number of amino acids for each protein is indicated at the end of the sequence. Species abbreviations are as follows: Cr, *Chlamydomonas reinhardtii*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; At, *Arabidopsis thaliana*; Zm, *Zea mays*; Hs, *Homo sapiens*. The sequence from CrATG8 was taken from the *Chlamydomonas* database (http://www.chlamy.org/chlamydb.html). The rest of the sequences were obtained from the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov). B, Cross-reactivity of anti-CrATG8 antibody with ATG8 proteins from Arabidopsis and yeast. Total extracts from *Chlamydomonas reinhardtii*, Arabidopsis, or *S. cerevisiae* (wild-type [WT] and atg8 mutant strains) were prepared and resolved by 15% SDS-PAGE. ATG8 proteins were detected by immunoblot analysis with anti-CrATG8. Molecular mass markers (kD) are indicated on the left.

**CrATG8 Functionally Replaces Yeast ATG8**

To investigate the possible role of CrATG8 in autophagy, we studied the functionality of this protein expressed in yeast mutant cells lacking endogenous ATG8. Yeast ATG8 is essential in both cytoplasm-to-vacuole targeting and autophagy pathways; therefore, *atg8* mutant cells fail to deliver proaminopeptidase I (pAPI) from the cytosol to the vacuole, where pAPI is processed to its mature form (mAPI; Kirisako et al., 1999; Huang et al., 2000). Therefore, processing of pAPI to mAPI in *atg8* mutant cells expressing CrATG8 was examined in order to study the functionality of CrATG8. Our results indicate that CrATG8 is able to functionally replace yeast ATG8, since mAPI was detected in *atg8* cells expressing CrATG8 (Fig. 2). To further demonstrate that the mAPI band detected in *atg8* cells expressing CrATG8 is due to the activity of this protein, processing of pAPI was also examined in cells transformed with a mutant form of CrATG8 where Gly-120 has been replaced by an Ala residue (CrG120A). A similar mutation in yeast ATG8 results in the complete loss of protein activity due to its inability for membrane attachment (Kirisako et al., 2000). As expected, no mAPI protein was detected in *atg8* cells expressing CrG120A (Fig. 2). To demonstrate that wild-type and mutant forms of CrATG8 were correctly expressed, we analyzed the transformant yeast extracts by western blot using the anti-CrATG8 antibody. Both CrATG8 and CrG120A were efficiently expressed, and due to the presence of the C-terminal extension in CrATG8, it was possible to distinguish the precursor (pCrATG8) and the mature (mCrATG8) forms of the protein (Fig. 2). Similar to what has been reported in yeast ATG8 (Kirisako et al., 2000), CrATG8 is predominantly processed when expressed in yeast cells, whereas the CrG120A mutant cannot be maturated. Overall, these results demonstrate that CrATG8 is cleaved at Gly-120 and presumably binds to membranes to functionally replace yeast ATG8 in delivering pAPI from the cytosol to the vacuole.

**In Vitro Processing of CrATG8 by Yeast ATG4 at Gly-120**

Yeast complementation assays of *atg8* mutant cells suggested that CrATG8 must be processed by yeast ATG4 at the conserved Gly-120 residue. To demonstrate that CrATG8 is recognized by yeast ATG4 (ScATG4), in vitro cleavage assays were performed using purified CrATG8 and ScATG4 proteins. The His6 tag was fused to the N terminus of these proteins, and recombinant CrATG8 and ScATG4 were purified from *Escherichia coli* cells by nickel affinity chromatography (see “Materials and Methods”). Processing of CrATG8 was monitored by the different mobilities in SDS-PAGE of the precursor and mature forms of the protein. Our results indicate that ScATG4 cleaves CrATG8, presumably at its C terminus (Fig. 3A). To investigate the participation of Gly-120 in CrATG8 processing, we studied the in vitro cleavage of the
G120A mutant. In agreement with the in vivo analysis of this mutant form of CrATG8 performed in yeast (Fig. 2), substitution of Gly-120 by Ala (G120A) completely abrogated processing of this protein by ScATG4 (Fig. 3B). Moreover, we investigated whether another Gly residue localized at the C terminus of CrATG8 at position 122, which is not conserved in other ATG8 proteins (Fig. 1A), may be recognized by ScATG4. Cleavage assays revealed that the G122A mutant was processed by ScATG4 whereas the double mutant G120/122A was not, indicating that substitution of Gly-122 by Ala has no effect on CrATG8 cleavage by ScATG4 and confirming that Gly-120 is the ScATG4 target (Fig. 3B).

**CrATG8 Is Processed at the C Terminus by an ATG4 Protease Activity in Chlamydomonas**

Our results demonstrated that CrATG8 is functional in a yeast system (Fig. 2). To investigate the functionality of CrATG8 in *Chlamydomonas*, we designed an ATG4 proteolytic assay in *Chlamydomonas* total extracts by taking advantage of the different sizes of the endogenous CrATG8 protein and recombinant His-tagged CrATG8. Total extracts were obtained from *Chlamydomonas* cells grown in acetate medium and then mixed with a fixed amount of different forms of recombinant CrATG8. Incubation of wild-type CrATG8 with *Chlamydomonas* extract resulted in the fast processing of the recombinant protein. A band with the same size as a truncated form of CrATG8 lacking the last 14 residues (6H-G120) was readily visible, indicating that CrATG8 was processed at Gly-120 (Fig. 4A). Mutation of Gly-120 to Ala abolished CrATG8 cleavage, while the substitution of Gly-122 to Ala had no effect in CrATG8 processing (Fig. 4A). As expected, the G120/122A double mutant could not be processed (Fig. 4A). These results indicated that *Chlamydomonas* total extracts have a proteolytic ATG4 activity that recognizes and processes the C terminus of CrATG8 at Gly-120. ATG4 proteases contain a catalytic Cys involved in substrate hydrolysis and therefore are sensitive to iodoacetamide (Kirisako et al., 2000; Kim et al., 2001). To investigate if the CrATG8 processing detected in *Chlamydomonas* extracts is mediated by a Cys protease, proteolytic assays were performed in the presence of iodoacetamide. CrATG8 cleavage was fully abolished by iodoacetamide, while the Ser protease inhibitor phenylmethylsulfonyl fluoride (PMSF) had no effect (Fig. 4A), indicating that the CrATG8 proteolytic activity in *Chlamydomonas* extracts is mediated by a Cys protease.

Having shown that *Chlamydomonas* cells have ATG4 activity, we next investigated if the CrATG8 band of 15 kD detected in total extracts corresponds to the mature form of the protein. To this aim, purified unprocessed CrATG8 and a C-terminal truncated form of the protein (G120), together with *Chlamydomonas* total extracts and extracts of yeast cells expressing wild-type or the G120A mutant of CrATG8, were resolved by SDS-PAGE and analyzed by western blot. Our results indicate that the band detected in *Chlamydomonas* extracts with the anti-CrATG8 antibody corresponds to the C-terminal mature form of CrATG8 (Fig. 4B). Moreover, unprocessed CrATG8 was not detected under these and other experimental conditions (Fig. 4B; data not shown), suggesting that newly synthesized CrATG8 is immediately cleaved by an ATG4 protease to expose the Gly residue, as described previously in yeast and plant cells (Kirisako et al., 2000; Kim et al., 2001; Yoshimoto et al., 2004).

**CrATG8 Is Reversibly Modified during Stationary Growth Phase**

Previous studies in yeast, mammals, and plants demonstrated that the modified form of ATG8 generated upon autophagy activation migrates faster than unmodified ATG8 (Kabeya et al., 2000; Kirisako et al., 2000; Yoshimoto et al., 2004). We observed that besides the main 15-kD CrATG8 band detected by western-blot analysis in *Chlamydomonas* cell extracts, two less abundant bands of lower apparent molecular mass were visible (Figs. 1B and 4). To investigate whether these two bands may be generated as a result of CrATG8 modification, we performed western-blot analysis of *Chlamydomonas* cells under different growth conditions. First, we analyzed CrATG8 modification in exponential and stationary phase cells in acetate-rich or minimal medium. In addition to mature CrATG8, two bands became visible as cells approached the stationary phase in acetate-rich medium, whereas these bands were almost undetectable in exponential cells (Fig. 5A). Similar to what has been described in other systems, these forms likely repre-
sent lipidated CrATG8, although we cannot exclude the possibility that one of these bands may be the result of a different modification. A significant and progressive increase in CrATG8 abundance was also found in stationary phase cells compared with exponential cells, suggesting that the function of this protein is required when cells age. Western-blot analysis of the cytosolic protein FKBP12 and Coomassie Brilliant Blue staining of the gel were used as loading controls (Fig. 5A). Modified CrATG8 was also observed in minimal medium, although the faster migrating band was faintly detected (Fig. 5A). As in acetate-grown cells, the level of CrATG8 increased during the growth phase in minimal medium (Fig. 5A). Since the lower molecular mass variant of CrATG8 was concomitantly detected with an increase in the abundance of mature CrATG8, dilution series of total extracts obtained from log or stationary cells were analyzed by western blot in order to have similar amounts of the mature form from both conditions. Our results indicated that under uniform levels of mature CrATG8, modified forms are specifically detected in stationary cells (Fig. 5B).

To further characterize the process of CrATG8 modification associated with cell growth, CrATG8 was analyzed by western blot in Chlamydomonas cultures that have reached the stationary growth phase and then transferred to fresh medium to initiate a new cell growth cycle or maintained in the same medium. While modified CrATG8 was clearly detectable in cells that remained in the stationary growth phase, CrATG8 was delipidated within 6 h when stationary cells were transferred to new medium (Fig. 5C, middle and lower panels). Modified CrATG8 was again detected after 48 h of reinoculation (Fig. 5C, middle panel). None of the three bands detected correspond to unprocessed CrATG8, since this protein is immediately cleaved (Fig. 4). Taken together, these results indicate that CrATG8 modification can be efficiently reversed under optimal growth conditions.

Modification of CrATG8 under Different Stress Conditions

The participation of CrATG8 in the cellular response to different stresses such as nitrogen or carbon limitation and oxidative stress was examined by monitoring the modification state of this protein. To investigate whether nitrogen limitation may induce CrATG8 modification and autophagy activation in Chlamydomonas, exponentially growing cells were subjected to nitrogen depletion and CrATG8 modification was tested by western-blot analysis. Compared with untreated control cells, nitrogen limitation resulted in a
substantial increase in CrATG8 levels as well as the
detection of modified CrATG8 forms (Fig. 6A).
Carbon starvation induced by darkness has been
shown to induce autophagy in plants (Thompson et al., 2005; Phillips et al., 2008; Chung et al., 2009).
To test whether carbon-limiting conditions also trigger
autophagy in *Chlamydomonas*, cells grown under con-
tinuous light in minimal medium were shifted to
darkness or maintained under a short-day photope-
riod (8 h of light/16 of h dark) and the presence of
modified CrATG8 was examined by western blot.
Fixed-carbon limitation induced by darkness resulted
in an increase in CrATG8 abundance and in the
detection of modified CrATG8 forms (Fig. 6B). How-
ever, no effect was observed in cells grown under a
short-day photoperiod compared with cells main-
tained under continuous light (Fig. 6B).

Autophagy is involved in degrading oxidized pro-
teins during oxidative stress in plants (Xiong et al.,
2007). The possible participation of CrATG8 in oxida-
tive stress was analyzed by hydrogen peroxide (H$_2$O$_2$)
treatment, an established oxidative stress inducer, in
*Chlamydomonas* cells. Modified CrATG8 was readily
detected upon treatment with H$_2$O$_2$ (Fig. 6C). More-
over, as in nutrient-starved cells, the level of CrATG8
significantly increased under oxidative stress condi-
tions (Fig. 6C). Therefore, autophagy might contribute
to the degradation of oxidized proteins during oxida-
tive stress in *Chlamydomonas*.

In addition to nutrient or oxidative stress, we inves-
tigated the activation of CrATG8 in response to a
specific stress in the endoplasmic reticulum (ER) by
treating *Chlamydomonas* cells with tunicamycin, which
raises the level of unfolded polypeptides at this com-
partment by inhibiting protein glycosylation. Com-
pared with control, untreated cells, tunicamycin
strongly increased CrATG8 lipidation and abundance
(Fig. 6D), indicating that autophagy is involved in the
degradation of unfolded proteins in the ER.

**TOR Inhibits Autophagy in Chlamydomonas**

In a previous study, we showed that rapamycin-
mediated inhibition of TOR signaling caused in-
creased vacuolization (Crespo et al., 2005), a typical
morphological autophagy effect. To confirm that the
TOR pathway controls autophagy in *Chlamydomonas*,
we took advantage of the autophagy monitoring assay

Figure 4. ATG4 protease activity in cell-free ex-
tracts of *Chlamydomonas*. A, Ten nanograms of
the different recombinant ATG8 proteins (CrATG8, G120A, G122A, G120122A, or G120)
was incubated with 30 μg of cell-free extracts (SE)
of *Chlamydomonas* during 45 min on ice. When
required, cell-free extracts were previously incu-
bated with the corresponding protease inhibitor
for 45 min on ice. The reaction was stopped by
the addition of loading buffer and boiling at
100°C. Aliquots of the reaction mixtures were
analyzed by western blotting with the anti-
CrATG8 antibody. Exogenous, recombinant pro-
teins contained the His tag and were labeled as
6H to distinguish them from endogenous
CrATG8. The precursor (p) and mature (m) forms
of CrATG8 are marked with arrowheads. The top
panel corresponds to control (without protease
inhibitor) assays, while the middle and bottom
panels correspond to experiments carried out in
the presence of the protease inhibitor 3 mM
iodoacetamide or 5 mM PMSF, respectively. B,
CrATG8 is constitutively processed in *Chlamydo-
monas*. Western-blot analysis of the CrATG8 pro-
tein. Lane 1, His tag-free ATG8 purified protein;
lane 2, His tag-free G120 purified protein; lane 3,
total extract from *atg8* yeast cells expressing
CrATG8; lane 4, total extract from *atg8* yeast cells
expressing G120A; lane 5, cell-free extract from
*Chlamydomonas*. The precursor (p) and mature
(m) forms of CrATG8 are marked with arrow-
heads. A molecular mass marker (kD) is indicated
on the right.
using the anti-CrATG8 antibody and examined the presence of modified CrATG8 forms in rapamycin-treated cells. As shown in Figure 7, rapamycin treatment resulted in CrATG8 modification and an increase in the level of this protein. The rapamycin-induced effects were more pronounced in a *Chlamydomonas* mutant strain (Crespo et al., 2005) that exhibits high sensitivity to rapamycin (Fig. 7). As expected, no effect on CrATG8 was observed in the rapamycin-resistant mutant *rap2* strain (Fig. 7), which lacks the rapamycin primary target FKBP12 (Crespo et al., 2005). Thus, our results demonstrate that CrATG8 is under the control of a rapamycin-sensitive TOR signaling pathway in *Chlamydomonas*.

**ATG8 Associates with Membranes in *Chlamydomonas***

ATG8 proteins from yeast, mammals, and plants have been shown to be membrane associated (Kirisako et al., 1999; Kabeya et al., 2000; Yoshimoto et al., 2004;...
Chung et al., 2009). To investigate whether CrATG8 is associated with membranes, we performed subcellular fractionation by ultracentrifugation. Total soluble extracts were obtained from exponentially growing Chlamydomonas cells, and the presence of CrATG8 in the different soluble and membrane-enriched fractions was analyzed by western blot. The first fractionation was performed at low speed (15,000 g), and CrATG8 was detected in the supernatant (S1) and pellet (P1) fractions (Fig. 8A), indicating that part of the protein is membrane bound. Interestingly, a faster migrating band likely corresponding to modified CrATG8 was observed in the membrane fraction but not in the soluble fraction. A second fractionation was achieved by ultracentrifugation of S1 to separate soluble (S2) and microsomal (P2) fractions. CrATG8 was again detected in both fractions and, as in the low-speed fractionation, putative modified CrATG8 was found in the membranous pellet (Fig. 8A). Thus, our results indicated that there might be at least two different pools of CrATG8 in exponentially growing Chlamydomonas cells, soluble and membrane-bound CrATG8. The presence of microsomes in the insoluble fraction after ultracentrifugation was confirmed by immuno-detection of a vacuolar membrane ATPase.

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**Figure 6.** Analysis of CrATG8 under different autophagy-activating conditions. A, CrATG8 modification upon nitrogen starvation. Chlamydomonas cells growing exponentially (1 \(\times\) 10^6 cells mL^-1) in Tris-acetate phosphate medium were washed with a nitrogen-free medium and grown under these conditions for 24 h. Cells grown in nitrogen-containing medium were used as controls. B, CrATG8 modification under light/dark transitions. Exponential cells grown under continuous light in minimal medium were maintained under similar conditions (L) or subjected to 8-h-light/16-h-dark cycles (L/D) or continuous darkness (D). Next, cultures were diluted to 4 \(\times\) 10^5 cells mL^-1 and maintained under the same conditions until a cell density of 1 \(\times\) 10^6 cells mL^-1. C, Analysis of CrATG8 modification under oxidative stress conditions. Chlamydomonas cells growing exponentially (1 \(\times\) 10^6 cells mL^-1) were treated with 1 or 2 mM H_2O_2 for 8 h. Untreated cells were used as controls. D, CrATG8 modification in Chlamydomonas cells subjected to ER stress. Chlamydomonas cells growing at exponential phase (1 \(\times\) 10^6 cells mL^-1) were treated with 5 \(\mu\)g mL^-1 tunicamycin (tun) for 8 h. Untreated cells at different times were used as controls. In all experiments, 30 \(\mu\)g of total extracts was resolved by 15% SDS-PAGE followed by western blotting with the anti-CrATG8 antibody. Western-blot analyses with anti-FKBP12 and Coomassie Brilliant Blue (CBB) staining were used as loading controls. CrATG8* corresponds to CrATG8 modifications.

**Figure 7.** TOR signaling inhibition induces autophagy in Chlamydomonas cells. Chlamydomonas cells from different strains (wild type [WT], rap2^+^ + M52V E54Q) growing exponentially in acetate-rich (Tris-acetate phosphate [TAP]) or minimal (Min) medium were treated with 500 nM rapamycin for 16 h. The CrATG8 protein profile was analyzed by western blot with the anti-CrATG8 antibody. Western blot analyses with anti-FKBP12 and Coomassie Brilliant Blue (CBB) staining were used as loading controls. C, Control cells; R, rapamycin-treated cells.
to CrFKBP12 (Crespo et al., 2005) showed that the microsome-containing fraction is not significantly contaminated with thylakoidal membranes or soluble cytoplasmic proteins (Fig. 8A).

To improve the detection of modified CrATG8 associated with membranes, a similar fractionation approach was performed in soluble extracts obtained from stationary cells, where these forms are more abundant (Fig. 5). High-speed centrifugation revealed that both mature and modified forms of CrATG8 are present in the pellet and soluble fractions (Fig. 8B). However, treatment of pellet membranes with detergent (1% deoxycholate) resulted in the complete solubilization of CrATG8, indicating that these forms are associated with the membrane (Fig. 8B). Anchoring of ATG8 to the membrane occurs through the covalent binding of the protein to a molecule of PE (Ichimura et al., 2000). Treatment of the ATG8-PE adduct with phospholipase D (PlpD) releases phosphatidic acid from the conjugate and consequently solubilizes membrane-bound ATG8 (Ichimura et al., 2000; Tanida et al., 2004; Fujioka et al., 2008; Chung et al., 2009). To investigate whether CrATG8 might be modified by lipidation, membrane-associated CrATG8 was treated with PlpD. Our results show that PlpD treatment caused the complete solubilization of CrATG8 from the membranous fraction (Fig. 8B), strongly suggesting that modified forms of CrATG8 are the phos-
pholipid conjugates of this protein. To further investigate the membrane association of CrATG8, we performed Suc gradient sedimentation assays. Unlike the CrFKBP12 soluble protein, CrATG8 cosedimented with protein membrane markers such as vacuolar ATPase, BiP, and FOX1 (Fig. 8C). Taken together, our results are consistent with the notion that CrATG8 associates with membranes through covalent binding to PE.

Distinct CrATG8 Cellular Localization upon Autophagy Activation

Immunofluorescence (IF) microscopic studies and GFP-ATG8 fusion proteins have been utilized to monitor autophagy in several organisms. We used the anti-CrATG8 antibody to examine the subcellular localization of this protein by IF microscopy in *Chlamydomonas* cells under different conditions. In cells growing exponentially in acetate-rich medium, CrATG8 was localized to punctate structures and a single spot was detected in most of the cells (Fig. 9). Similarly, the signal of CrATG8 in cells grown in minimal medium was confined to discrete punctae, although the number of these spots was usually higher than in acetate-grown cells, likely due to a higher requirement of autophagy under these conditions (Supplemental Fig. S1). The distribution of ATG8 in the cell has been shown to be modified by the activation of autophagy (Kirisako et al., 1999; Kabeya et al., 2000; Kim et al., 2001). Thus, we investigated whether CrATG8 cellular localization may respond to autophagy induction. Exponentially growing cells were treated with rapamycin or tunicamycin or subjected to nitrogen limitation or oxidative stress to trigger autophagy, and CrATG8 localization was examined by IF microscopy. Treatment of acetate-grown cells with rapamycin resulted in a pronounced detection of CrATG8 in more punctate structures and an increase in cell size (Fig. 9). Rapamycin caused a similar effect on CrATG8 localization in minimal medium, although a diffuse CrATG8 signal could also be detected at the periphery and the anterior end of the cell (Supplemental Fig. S1). Accordingly, this region of the cell has been reported to become vacuolated upon rapamycin treatment under similar experimental conditions (Crespo et al., 2005). Nitrogen depletion also resulted in a strong modification of CrATG8 cellular localization, being detected as multiple small dots all over the cell with a predominant localization around the chloroplast (Fig. 9; Supplemental Fig. S1). Similar to nitrogen starvation, oxidative stress induced by H₂O₂ increased the overall intensity of the CrATG8 signal in the cell, and some large spots could also be detected (Fig. 9; Supplemental Fig. S1). The effect of tunicamycin on the cellular distribution of CrATG8 differed in some respects from other stresses. First, the signal was remarkably stronger, and second, CrATG8 accumulated at a specific region in the middle of the cell that might correspond to the ER, in agreement with the localized effect of tunicamycin at this compartment (Fig. 9; Supplemental Fig. S1). Taken together, these results indicate that the cellular distribution of CrATG8 strongly depends on the autophagic requirement of the cell.

**DISCUSSION**

The *Chlamydomonas* genome contains potential homologs to yeast and plant *ATG* genes, including those involved in the ATG8/ATG12-conjugating systems (Diaz-Troya et al., 2008b), which strongly suggests that *Chlamydomonas* might re-cycle intracellular components through autophagic processes. In agreement with this hypothesis, it has been recently shown that autophagosome-like structures are generated as a response to prolonged salt stress in the unicellular green alga *Micrasterias denticulata* (Affenzeller et al., 2009). However, the molecular features of autophagy have never been characterized in green algae. Here, we report the participation of the *Chlamydomonas* CrATG8 protein in autophagy and demonstrate that this protein may be used to monitor autophagic processes in this green alga.

Compared with the ATG8 proteins from other organisms, the C-terminal extension downstream of the conserved Gly120 of CrATG8 is particularly large (Fig. 1A). A comparative analysis of the C-terminal extension of ATG8 proteins from different organisms indicated that the sequence after the conserved Gly completely diverged (Kabeya et al., 2000). In Arabidopsis, two of the nine ATG8 homologs, AtATG8h and AtATG8i, do not possess additional amino acids downstream of the conserved Gly, while the ATG8 protein from other organisms such as rats or the symbiotic fungus *Laccaria bicolor* have large C-terminal extensions (Kabeya et al., 2000). The C-terminal extension does not seem to be required for the functionality of the protein, since all nine Arabidopsis ATG8s, including AtATG8h and AtATG8i, are active in plant autophagy (Yoshimoto et al., 2004). Unprocessed CrATG8 could not be detected in *Chlamydomonas* cells (Fig. 4), indicating that similar to what has been described in yeast or plants (Kirisako et al., 2000; Kim et al., 2001), newly synthesized CrATG8 is immediately processed. Whether the C-terminal extension of CrATG8 may play a role in the stability of the protein and/or its processing remains to be investigated.

Functional studies performed in yeast revealed that CrATG8 is able to restore pAPI processing in an atg8 mutant strain under normal growth conditions (Fig. 2), demonstrating the functionality of this protein in yeast ATG8-mediated processes. Yeast complementation assays have also been performed with Arabidopsis and *Trypanosoma cruzi* ATG8 homologs, and in contrast to CrATG8, these proteins were able to functionally replace yeast ATG8 under starvation conditions only (Ketelaar et al., 2004; Alvarez et al., 2008). Mutation of the conserved Gly residue to Ala in CrATG8 fully
abolished pAPI processing, suggesting that despite the presence of the large C-terminal extension, CrATG8 could be processed and lipidated in yeast cells (Fig. 2A). Indeed, in vitro cleavage assays demonstrated that CrATG8 is maturated at Gly-120 by yeast ATG4 (Fig. 3). Our in vivo and in vitro heterologous studies strongly suggested that CrATG8 performs a similar function to yeast ATG8 in *Chlamydomonas*. Several lines of evidence support this hypothesis. On the one hand, CrATG8 is processed in *Chlamydomonas* at the conserved Gly, as revealed by ATG4 proteolytic assays performed in cell-free extracts of *Chlamydomonas* (Fig. 4). In agreement with findings in plants and *T. cruzi*, where ATG4 proteases are constitutively expressed regardless of nutrient conditions (Yoshimoto et al., 2004; Alvarez et al., 2008), ATG4 activity was detected in *Chlamydomonas* cells under optimal and nutrient-limiting growth conditions (Fig. 4; data not shown). On the other hand, CrATG8 levels increased upon autophagy activation. The abundance of plant ATG8s is up-regulated at the protein and mRNA levels in response to autophagy induction by nutrient starvation (Yoshimoto et al., 2004; Contento et al., 2005; Thompson et al., 2005; Chung et al., 2009). Accordingly, a significant increase in CrATG8 protein abundance was detected in *Chlamydomonas* cells limited for nutrients (Fig. 6). Entry into stationary phase also resulted in the up-regulation of CrATG8 levels (Fig. 5), which links autophagy to cell aging in *Chlamydomonas*. Autophagy has been shown to play a role in plant senescence. The phenotypic analysis of Arabidopsis

Figure 9. IF localization of CrATG8 in *Chlamydomonas* cells. Cells growing exponentially in acetate medium were subjected to different treatments as follows: cells were treated with rapamycin for 16 h (rap), shifted to nitrogen-free medium for 24 h (–N), treated with 0.2 mM H$_2$O$_2$ for 8 h to induce oxidative stress, or treated with 5 µg mL$^{-1}$ tunicamycin (tun) for 8 h to trigger ER stress. As a control, cells were maintained in exponential growth. Cells were collected and processed for IF microscopy analysis as described in “Materials and Methods.” The signal recognized by the affinity-purified anti-CrATG8 antibody is shown in green (CrATG8). The same acquisition time was used in all samples except for tunicamycin-treated cells, where it was reduced to 50% to avoid signal saturation. Bars = 2 µm.
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... and punctate structures representing autophagosomes or PAS (Yoshimoto et al., 2004; Thompson et al., 2005). This model is strengthened by the detection of most ATG8s in the pellet fraction in plant cells, an indication of ATG8 association with the autophagosomes (Yoshimoto et al., 2004). In exponentially growing Chlamydomonas cells, mature and modified forms of CrATG8 were also found in association with membrane fractions (Fig. 8A), which may imply the participation of CrATG8 in autophagy or other membrane-trafficking events under optimal growth conditions. Upon autophagy induction, CrATG8 becomes lipidated and binds to membrane, although mature, unmodified CrATG8 could also be detected with membrane fractions (Fig. 8B). The presence of unmodified CrATG8 in membranes is not exclusive to Chlamydomonas, since this phenomenon has also been reported in mammals (Tanida et al., 2004) and plants (Chung et al., 2009).

The TORC1 pathway perceives the nutritional status of the cell and transmits these signals to the autophagic machinery, acting as a negative regulator of autophagy (Noda and Ohsumi, 1998). The molecular mechanisms by which TORC1 inhibits autophagy were elucidated from studies performed in yeast (Noda and Ohsumi, 1998; Kamada et al., 2000; Kabeya et al., 2005), and significant advances in this regulatory pathway were recently reported in insects and mammals (Chang and Neufeld, 2009; Hosokawa et al., 2009; Jung et al., 2009). Components of TORC1, including the TOR kinase, are functionally conserved in plants (Menand et al., 2002; Anderson et al., 2005; Deprost et al., 2005; Mahfouz et al., 2006), although the participation of this signaling pathway in the control of autophagy has not been shown in photosynthetic eukaryotes. The data presented in this study demonstrate that TOR signaling inhibits autophagy in Chlamydomonas. Rapamycin-mediated inhibition of CrTOR resulted in increased CrATG8 modification and a relocalization of CrATG8 in Chlamydomonas cells similar to the one observed under nutrient-limiting conditions (Figs. 7 and 9). Thus, our results assign a prominent role to the TOR pathway in the control of autophagy in photosynthetic organisms. In yeast and animal cells, the ATG1 kinase and its regulatory proteins ATG11, ATG13, and ATG17 transmit the signal from TORC1 to the autophagosome-generating machinery. Whether these proteins may perform a similar function in plants or algae is unknown.

Autophagy has been recently involved in chloroplast degradation and Rubisco mobilization to the vacuole during plant senescence and stress (Ishida et al., 2008; Wada et al., 2009). Rubisco and other stroma-targeted proteins are mobilized from the chloroplast to the vacuole through an ATG-mediated autophagic process, which does not implicate entire chloroplast degradation. In Chlamydomonas, it has been shown that a substantial amount of chloroplast material, including the large subunit of Rubisco and ribosomes, is transferred to a class of vacuoles distinct...
from contractile vacuoles via protrusion of the outer membrane of the plastid envelope (Park et al., 1999). However, the participation of autophagy in this degenerative process has not been demonstrated. The availability of a specific autophagy marker in *Chlamydomonas* will certainly facilitate the investigation of the role of autophagy in this and other cellular events.

**MATERIALS AND METHODS**

**Strains and Growth Conditions**

*Chlamydomonas reinhardtii* wild-type strain 6C+ was obtained from the laboratory of J-D Rochais (University of Geneva). *cys2-8* and *cys2-8 + M52V E45Q* strains were characterized previously (Crespo et al., 2005). *Chlamydomonas* cells were grown under continuous illumination at 25°C in Tris-acetate phosphate or high-salt minimal medium as described (Harris, 1989). When required, 1.2% bacto agar (Difco) was added to the medium.

**DNA and Protein Sequence Alignments**

The amino acid *Chlamydomonas* ATG8 sequence was obtained from the *Chlamydomonas* database (www.chlamy.org/chlamydb.html). The ATG8 amino acid sequences from other organisms were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The multiple sequence alignment was performed with the ClustalW2 program (www.ebi.ac.uk/Tools/clustalw2).

**Cloning and Protein Purification**

The ATG8 coding region from *Chlamydomonas* (351 bp) was amplified by PCR from a *Chlamydomonas* cDNA library using the following primers: ATGFor (5'-CCCGCATAATGTTGGTCTCCAGGCCAGTCGCTGCTCCCGCACGAGTGTT3') and ATGRev (5'-CCCGCTGAGTCTACAACCGCAGCTCTCCACACACGCTGCTGCTCCCGCACGAGTGTT3'), designed to contain a Ndel or Xhol site (underlined), respectively. The resulting product was digested with Ndel and Xhol and inserted into pET28a (Novagen) for expression in *Escherichia coli*. Expression of the recombinant protein was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. The ATG8 His6-tagged protein was purified from the lysates by two consecutive affinity chromatographies on a nickel matrix from Novagen.

The His6-tagged Gly-to-Ala mutants of ATG8, denoted as G120A, G122A, and G120122A, whose Gly-120 was substituted by Ala, were both replaced by Ala, respectively, were obtained by PCR using the wild-type version of the ATG8 gene cloned into pET28a and reverse primers including the proper mutation as follows: G120A Rev (5'-TCACAGCGGTTGGTCTCCAGGCCAGTCGCTGCTCCCGCACGAGTGTT3') and G120122A Rev (5'-CCCGCTGAGTCTACAACCGCAGCTCTCCACACACGCTGCTGCTCCCGCACGAGTGTT3'), designed to contain a Ndel or Xhol site (underlined), respectively. The resulting product was digested with Ndel and Xhol and inserted into pET28a (Novagen) for expression in *Escherichia coli*. Expression of the recombinant protein was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37°C. The ATG8 His6-tagged protein was purified from the lysates by two consecutive affinity chromatographies on a nickel matrix from Novagen.

**Antibody Production and Immunoblot Analysis**

The anti-CrATG8 polyclonal antibody was produced by injecting the recombinant wild-type CrATG8 protein into a rabbit using standard immunization protocols. Polyclonal serum was affinity purified by coupling the recombinant protein CrATG8 (12 mg) to a solid resin (Aminolink Plus Immobilization kit; Thermo Scientific) according to the manufacturer’s instructions. For immunoblot analyses, total protein extracts that had been subjected to SDS-PAGE were transferred to nitrocellulose membranes (Bio-Rad). The anti-CrATG8 polyclonal antibody was used to detect the proteins with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. The anti-FOX1 antibody was obtained from Agrisera and diluted to 1:20,000, 1:10,000, 1:2,000, and 1:100, respectively. The ECL-Plus immunoblotting detection system (GE Healthcare) was used to detect the proteins with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. The anti-FKP12 antibody has been described previously (Crespo et al., 2005). The anti-pAPI antibody was obtained from the laboratory of D.J. Klionsky (University of Michigan). Both antibodies were used at 1:4,000 dilution. The anti-D1 and anti-vacuolar membrane, anti-BiP, and anti-FOX1 antibodies were purchased from Bio-Rad laboratories and diluted to 1:2,000, 1:1,000, 1:2,000, and 1:100, respectively.

**Subcellular Fractionation and Solubilization of CrATG8**

For fractionation assays, *Chlamydomonas* whole-cell extract was prepared from wild-type cells as described above. After freezing and thawing, samples were centrifuged at 5000g for 5 min to remove cell debris. The supernatant was centrifuged at 15,000g for 15 min to generate the soluble extract and the low-speed membrane fraction. The microsomal fraction was obtained by centrifugation at 105,000g for 1 h. The pellets were resuspended in a minimal volume of lysis buffer (50 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100) by vortexing them 10 times for 1 min each time with glass beads (Sigma-Aldrich). Crude extracts were cleared by centrifugation at 15,000g for 15 min at 4°C. Proteins were quantified with the Coomassie Brilliant Blue dye-binding method as described by the manufacturer (Bio-Rad).

**In Vitro ATG4 Cleavage Assays**

Recombinant wild-type CrATG8 and the different Gly-to-Ala mutant versions (5 µg) were incubated with different amounts of purified ScATG4 in 100 µl of Tris-buffered saline (TBS) containing 1 mM EDTA and 1 mM iodothreitol. The reaction mixtures were incubated at 30°C for different times and stopped by the addition of Laemmli sample buffer followed by 5 min of boiling. Proteins were resolved on a 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue. For the detection of ATG4 activity in *Chlamydomonas*, cell-free extracts were prepared as described above and incubated with 10 ng of the different recombinant ATG8 proteins in TBS buffer for 45 min. To determine which protease group *Chlamydomonas* ATG8 belongs to, proteolytic assays were carried out in the presence of the protease inhibitor iodoacetamide (3 µM) or PMSF (5 µM). The reactions were stopped by the addition of Laemmli sample buffer followed by 5 min of boiling. Proteins were resolved on a 15% SDS-PAGE gel and immunoblotted with the anti-CrATG8 antibody.
centrifuging the soluble extract in an 80-Ti rotor in a Beckman Coulter ultracentrifuge at 100,000g for 2 h at 4 °C. For CrATG8 solubilization, the membrane pellet fraction was resuspended in extraction buffer plus 1% deoxycholate and incubated on ice for 1 h. Samples were then centrifuged at 100,000g for 2 h to separate soluble from insoluble material. PpD treatment was performed by incubating the membrane fraction at 37 °C for 1 h with Streptomyces chromofuscus PpID (Sigma), and reactions were stopped by the addition of SDS-PAGE sample buffer. The different fractions were processed and subjected to western-blot analysis with the anti-CrATG8 antibody.Suc density gradient analysis was performed as described previously (Diaz-Troya et al., 2008a). Fluorescence Microscopy
Wild-type Chlamydomonas cells were fixed and stained for IF microscopy as described previously (Cole et al., 1998; Diaz-Troya et al., 2008a). The primary antibody used was rabbit polyclonal anti-CrATG8 (1:500). For signal detection, a fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:50; Sigma) was used. Preparations were photographed on a DM6000B microscope (Leica) with an ORCA-ER camera (Hamamatsu) and processed with the Leica Application Suite Advanced Fluorescence software package (Leica). Deconvolution analysis of images was performed with the same software. For the comparative analysis of the fluorescein isothiocyanate signal from different samples, the same acquisition time was fixed except for cyanomycin-treated cells, where it was reduced to 50% to avoid signal saturation.

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure S1. IF localization of CrATG8.

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LITERATURE CITED
Diaz-Troya S, Florencio FJ, Crespo JL (2008a) Target of rapamycin and LST8 proteins associate with membranes from the endoplasmic reticulum in the unicellular green alga Chlamydomonas reinhardtii. Eukaryot Cell 7: 212–222

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Meijer WH, van der Klei IJ, Veenhuis M, Kiel JA (2007) ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. Autophagy 3: 106–116


Xiong Y, Contento AL, Bassham DC (2005) AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in Arabidopsis thaliana. Plant J 42: 535–546

