Control of Autophagy in *Chlamydomonas* Is Mediated through Redox-Dependent Inactivation of the ATG4 Protease

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Autophagy is a major catabolic pathway by which eukaryotic cells deliver unnecessary or damaged cytoplasmic material to the vacuole for its degradation and recycling in order to maintain cellular homeostasis. Control of autophagy has been associated with the production of reactive oxygen species in several organisms, including plants and algae, but the precise regulatory molecular mechanisms remain unclear. Here, we show that the ATG4 protease, an essential protein for autophagosome biogenesis, plays a central role for the redox regulation of autophagy in the model green alga *Chlamydomonas reinhardtii*. Our results indicate that the activity of *C. reinhardtii* ATG4 is regulated by the formation of a single disulfide bond with a low redox potential that can be efficiently reduced by the NADPH/thioredoxin system. Moreover, we found that treatment of *C. reinhardtii* cells with norflurazon, an inhibitor of carotenoid biosynthesis that generates reactive oxygen species and triggers autophagy in this alga, promotes the oxidation and aggregation of ATG4. We propose that the activity of the ATG4 protease is finely regulated by the intracellular redox state, and it is inhibited under stress conditions to ensure lipidation of ATG8 and thus autophagy progression in *C. reinhardtii*.

Macroautophagy (hereafter termed autophagy) is a catabolic process by which eukaryotic cells degrade and recycle intracellular material, including proteins, membranes, ribosomes, and entire organelles, in response to stress or for proper differentiation and development. During autophagy, cytosolic components are engulfed in bulk within a double-membrane vesicle known as the autophagosome and delivered to the vacuole/lysosome for degradation to recycle needed nutrients or degrade toxic or damaged components (He and Klionsky, 2009; Mizushima et al., 2011; Liu and Bassham, 2012). Therefore, autophagy plays a fundamental role in cellular homeostasis and the response to stress. The degradation of intracellular components via autophagy can be selective or nonselective depending on the nature of cargo that is targeted to the vacuole, and several types of selective autophagy have been reported such as mitophagy, pexophagy, or chlorophagy (Floyd et al., 2012; Schreiber and Peter, 2014; Oku and Sakai, 2016; Young and Bartel, 2016).

Autophagy is highly conserved through evolution. The autophagy process is mediated by autophagy-related (*ATG*) genes, which were originally identified in yeasts (Tsukada and Ohsumi, 1993) and subsequently in other eukaryotes, including animals, plants, and algae (Bassham et al., 2006; Díaz-Troya et al., 2008b; Avin-Wittenberg et al., 2012; Shemi et al., 2015). Among these *ATG* genes, a subgroup of around 18 genes is required for autophagosome formation and constitutes the autophagy core machinery in most eukaryotes. Core ATG proteins include the ATG8 and ATG12 ubiquitin-like conjugation systems that catalyze the covalent binding of the ubiquitin-like protein ATG8 to the phospholipid phosphatidylethanolamine (PE), an essential event in the formation of the autophagosome (Mizushima et al., 2011; Feg et al., 2014). The conjugation of ATG8 to PE is catalyzed by the sequential action of ATG4 (a Cys protease), ATG7 (E1-activating enzyme), ATG3 (E2-activating enzyme), and the ATG12-ATG5-ATG16 complex (E3-like ligase). First, the ATG4 protease cleaves nascent ATG8 at a conserved Gly residue located at the C terminus of the protein. Processed ATG8 is then activated by the E1-like enzyme ATG7, transferred to the E2-like enzyme ATG3, and finally conjugated to PE at the exposed Gly in a reaction that requires the participation of the E3-like ligase ATG12-ATG5-ATG16 (Mizushima et al., 2011; Feng et al., 2014). ATG8 is also recycled from

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the autophagosome membrane by the delipidating activity of ATG4 that cleaves the amide bond between ATG8 and PE (Kirisako et al., 2000). Hence, ATG4 can act both as a conjugating and deconjugating enzyme, and the fine regulation of these two activities is essential for the normal function of autophagy (Nair et al., 2012; Nakatogawa et al., 2012; Yu et al., 2012).

The autophagy machinery is well conserved in the plant kingdom, although some differences have been reported between plants and algae. Higher plants such as Arabidopsis (Arabidopsis thaliana), maize (Zea mays), or rice (Oryza sativa) evolved either small or large families for many of the core ATG genes (Bassham et al., 2006; Chung et al., 2009; Avin-Wittenberg et al., 2012) likely allowing specialized functions. However, all predicted ATG genes appear to be in single copy in the genome of several algal species, including the model green alga Chlamydomonas reinhardtii (Diaz-Troya et al., 2008b; Avin-Wittenberg et al., 2012; Perez-Perez and Crespo, 2014; Shemi et al., 2015). The availability of plant mutant collections and established techniques for gene silencing has been fundamental to understand the role of autophagy in the plant response to stress. The analysis of Arabidopsis mutants defective in core autophagy genes such as ATG3, ATG4, ATG5, ATG7, or ATG10 revealed that autophagy plays a central role in the plant response to nutrient (nitrogen or carbon) starvation, different abiotic stresses, and pathogen infection (Li and Vierstra, 2012; Liu and Bassham, 2012; Avila-Ospina et al., 2014; Minina et al., 2014; Michaeli et al., 2016). Moreover, this catabolic process has been involved in the selective degradation of chloroplasts and peroxisomes in plants (Ishida et al., 2008; Farmer et al., 2013; Kim et al., 2013; Shibata et al., 2013). In algae, it has been reported that autophagy is activated in response to nutrient or carbon limitation, oxidative stress, metal toxicity, endoplasmic reticulum (ER) stress, or virus infection (Perez-Perez et al., 2010, 2012; Davey et al., 2014; Goodenough et al., 2014; Perez-Martin et al., 2014, 2015; Schatz et al., 2014).

Control of autophagy has been associated with the production of reactive oxygen species (ROS) in several systems. Upon starvation, human cells generate ROS, specifically H2O2, that seem to be essential for autophagosome biogenesis and autophagic degradation (Scherz-Shouval et al., 2007). In the model yeast Saccharomyces cerevisiae, autophagy has also been linked with the cellular redox state. Incubation of yeast cells with the ROS inducer H2O2 triggers autophagy, whereas activation of autophagy by the TOR inhibitor rapamycin results in a pronounced increase of the total glutathione (reduced glutathione [GSH] + oxidized glutathione [GSSG]) pool, a marker of redox unbalance (Perez-Perez et al., 2014). In Arabidopsis, it has been demonstrated that oxidative damage caused by the ROS generators methyl viologen and H2O2 triggers autophagy, and accordingly plants defective in autophagy show enhanced sensitivity to oxidative stress (Xiong et al., 2007). In the same line, a strong correlation between the activation of autophagy and the accumulation of ROS in carotenoid-depleted cells or in response to methyl viologen or H2O2 treatment has been shown in C. reinhardtii (Perez-Perez et al., 2010; Perez-Perez et al., 2012).

Despite the extensive experimental evidence connecting autophagy and redox signaling, the precise molecular mechanisms by which ROS control this catabolic process are largely unknown. At present, ATG4 is the only ATG protein whose activity has been proposed to be redox regulated. The proteolytic activity of human ATG4A and ATG4B is reversibly inhibited by oxidation in a process that involves Cys-81, which may interfere with the activity of the nearby catalytic Cys-77 (Scherz-Shouval et al., 2007). The activity of the yeast ATG4 protease is also subject to redox regulation, although the underlying molecular mechanism differs from the one described in humans, because Cys-81 is not conserved in yeasts or other eukaryotes such as plants and algae (Perez-Perez et al., 2014). Indeed, we have recently demonstrated that yeast ATG4 is regulated by oxidoreduction of a single disulfide bond between Cys-338 and Cys-394. Mutation of any of these two cysteines resulted in loss of redox regulation of ATG4, and the mutant proteins displayed constitutive activation (Perez-Perez et al., 2014). Moreover, the small oxidoreductase thioredoxin (TrxB) was identified in this study as a regulator of ATG4 activity based on its ability to efficiently reduce the regulatory disulfide bond in this protease (Perez-Perez et al., 2014). In plants, it has been shown that the activity of Arabidopsis ATG4a and ATG4b is reversibly inhibited by oxidation (Woo et al., 2014), although the underlying molecular mechanism has not been reported.

In this study, we unraveled the molecular mechanism that mediates the redox regulation of C. reinhardtii ATG4. This regulation controls oxidative inactivation of ATG4 under conditions that promote autophagy in C. reinhardtii.

RESULTS

Identification of ATG4 in C. reinhardtii

We have previously reported that a single ATG4 gene is conserved in the nuclear genome of C. reinhardtii and that an ATG8-proteolytic activity can be detected in total extracts from C. reinhardtii (Perez-Perez et al., 2010). According to the most up-to-date annotated version of the C. reinhardtii genome at the Joint Genome Institute (gene model version 5.5 at the Phytozome 10.3 database), the C. reinhardtii ATG4 gene (Cre12.g510100.t1.1) codes for a protein of 640 amino acids that contains two peptidase domains highly conserved among ATG4 proteins (Sugawara et al., 2005; Fig. 1A). The predicted size of C. reinhardtii ATG4 (approximately 65 kD) is significantly higher compared to ATG4 proteins from other organisms, including algae, plants, yeasts, or mammals. Amino acid sequence comparison of ATG4 proteins revealed a low identity in this family of proteases, with Volvox carteri being the closest ortholog to C. reinhardtii ATG4 (Supplemental Fig. S1). Phylogenetic analysis of ATG4 proteins also showed that ATG4 homologs comprise three main branches: fungi, metazoans, and the green
lineage, which includes green algae in a separate clade from land plants (Supplemental Fig. S2).

To characterize the ATG4 protein from *C. reinhardtii*, an in vitro assay was established using recombinant purified ATG4 and the ATG8 protein from *C. reinhardtii* as substrate. The endoproteolytic activity of ATG4 can be monitored by SDS-PAGE based on the different mobility of processed and unprocessed ATG8, since *C. reinhardtii* ATG8 contains a 15-amino acid extension at the C terminus of the protein that is cleaved by ATG4 (Kirisako et al., 2000; Pérez-Pérez et al., 2010). Our results indicated that ATG4 efficiently processes the C terminus of ATG8 within 15 to 30 min using a low concentration of the protease in the assay (1 μM; Fig. 1, B–E). To confirm that ATG8 processing occurs at the highly conserved Gly residue that is recognized by ATG4 (Kirisako et al., 2000), we performed a proteolytic assay with a mutant version of ATG8 where Gly-120 has been replaced by Ala (ATG8G120A) or a truncated form lacking the last 15 amino acids (ATG8G120). As predicted, *C. reinhardtii* ATG4 cleaves ATG8 at the conserved Gly-120 (Fig. 1F).

**C. reinhardtii** ATG4 Activity Is Redox Regulated and Depends on a Single Disulfide Bond with a Very Low Redox Potential

It has been shown that the proteolytic activity of human and yeast ATG4 proteins is regulated by the reduction of a regulatory Cys adjacent to the catalytic Cys or the formation of a disulfide bond, respectively (Scherz-Shouval et al., 2007; Pérez-Pérez et al., 2014). To investigate whether the activity of *C. reinhardtii* ATG4 is subject to redox regulation, we performed an in-depth analysis of this protein under different redox conditions.

![figure 1](image-url)

**Figure 1.** *C. reinhardtii* ATG4 cleaves ATG8 at the conserved Gly Gly-120. A, Domain structure of the ATG4 protease from *C. reinhardtii*. Two peptidase domains comprising residues from positions 49-182 and 437-590, respectively, were identified using the Conserved Domains search tool at the NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). All cysteines present in *C. reinhardtii* ATG4 are indicated as gray balls, including the catalytic (red) and two putative regulatory (green) cysteines. B, *C. reinhardtii* ATG4 (1 μM) activity was monitored by following the cleavage of recombinant *C. reinhardtii* ATG8 (5 μM) from the unprocessed (ATG8) to the processed (pATG8) form (indicated by arrowheads) along time and subsequent SDS-PAGE and Coomassie Brilliant Blue staining. C, Quantification of ATG4 activity from B. The reference sample for quantification was the one at 60 min and it was considered as activity = 1 (in arbitrary units). D, Different concentrations of ATG4 were used to analyze C-terminal processing of ATG8 (5 μM) to pATG8 after 30 min. E, Quantification of ATG4 activity from D. The reference sample for quantification was the one with the maximum ATG4 concentration (9 μM was considered as activity = 1, in arbitrary units). F, *C. reinhardtii* wild-type ATG8 (ATG8WT) and the mutant forms ATG8G120A (Gly-120 is replaced by Ala) and ATG8G120 (a truncated protein at Gly-120 corresponding to a processed-like form and used as a control) were incubated in an assay mixture in the absence or presence of the ATG4 protease for 1 h.
First, ATG4 was incubated with increasing concentrations of reducing (dithiothreitol [DTT]) or oxidant (H$_2$O$_2$) agents and processing of ATG8 was analyzed. ATG4 activity was enhanced with increasing concentrations of DTT (Fig. 2, A and B), indicating that the protein is activated by reduction. By contrast, the activity of ATG4 prerduced with a low concentration of DTT (100 μM) was inhibited by H$_2$O$_2$ in a concentration-dependent manner (Fig. 2, C and D). Moreover, we found that both reduction and oxidation of C. reinhardtii ATG4 are fully reversible (Fig. 2E), strongly suggesting that the regulation of ATG4 activity is controlled by redox posttranslational modifications. ATG4 proteins are Cys proteases whose activity is strictly dependent on a catalytic Cys that can be irreversibly blocked by alkylating agents such as iodoacetamide (IAM). C. reinhardtii ATG4 previously reduced and activated with low DTT was irreversibly inactivated by IAM, demonstrating that this protein is a Cys protease (Fig. 2F). Next, to determine if C. reinhardtii ATG4 activity depends on the redox potential, we analyzed ATG8 processing in the presence of different ratios of reduced and oxidized DTT (DTT$_{red}$ and DTT$_{ox}$, respectively; Fig. 2G). It was found that ATG4 is activated at a DTT$_{red}$/DTT$_{ox}$ ratio between 10$^{-1}$ and 10$^{-2}$, suggesting that ATG4 activation requires a very low redox potential. In addition, we observed that C. reinhardtii ATG4 is activated by reduction with 1 mM DTT ($E_m$ = −330 mV) but not with GSH ($E_m$ = −240 mV) even at 5 mM (Fig. 2H), indicating again that a very low redox potential is required for ATG4 activation ($E_m$: midpoint redox potential at pH 7.0). Finally, we performed a complete DTT redox titration by varying the ambient redox potential ($E_h$ at pH 7.0) between −232 and −350 mV. ATG4 was incubated at each potential, and the range of ATG4 activation was determined with the ATG8 processing assay (Fig. 2I). Our data indicated that there is a strong correlation between ATG4 activation and the redox potential. No significant activity was detected when the $E_h$ was higher than −262 mV. The experimental data of this in-gel redox titration gave good fits to the Nernst equation for the reduction of a single 2-electron component, with a midpoint redox potential ($E_m$), at pH 7.0, of −278 mV (Fig. 2J). Thus, taken together, our data indicated that C. reinhardtii ATG4 activity is redox regulated and that the molecular mechanism of this redox regulation involves the oxi-doreduction of a single regulatory disulfide bond with a very low midpoint redox potential.

The NADPH/NTR/Trx System Regulates C. reinhardtii ATG4 Activity

Trx are small oxidoreductases that are able to reduce disulfide bonds with very low redox potentials (Buchanan and Balmer, 2005). To reduce a disulfide bond in a target protein, Trx has to be reduced first by the NADPH-dependent thioredoxin reductase (NTR), which takes electrons from NADPH (Jacquot et al., 1994). Trx can also receive electrons from DTT, a classical electron donor widely used in biochemical assays to test the ability of Trx to reduce a target protein, since DTT is able to efficiently reduce Trx in vitro but reacts very slowly with the disulfide bond of the target protein. To test the ability of Trx to reduce and activate C. reinhardtii ATG4, TRXh1 from C. reinhardtii, the major cytosolic Trx (Lemaire and Miginiac-Maslow, 2004), was used in the ATG8 proteolytic assay. Our results indicated that TRXh1 efficiently activated the proteolytic activity of ATG4 in the presence of 10 μM DTT, whereas no ATG8 processing was detected in the presence of DTT only or when TRXh1 was replaced by a mutant form of this protein where the catalytic Cys is replaced by Ser (TRXh1$^{C39S}$; Fig. 3A). To further characterize the role of Trx in ATG4 reduction, we analyzed the time-course of ATG8 cleavage in the presence of TRXh1 and a very low concentration of DTT (10 μM). ATG4 was efficiently reduced and activated by TRXh1 but not with DTT alone or in the presence of TRXh1$^{C39S}$ (Fig. 3B). Nevertheless, the physiological electron donor to TRXh1 is NTR rather than DTT, and therefore to investigate the regulation of C. reinhardtii ATG4 by TRXh1 we reconstituted the complete NADPH/NTR/Trx system using NTRB from Arabidopsis as electron donor for TRXh1. We observed that in the presence of the complete system, ATG4 is able to efficiently process ATG8, whereas no cleavage occurred when the reducing system was not complete or the mutant TRXh1$^{C39S}$ was used (Fig. 3C). Therefore, our results strongly suggest a role of the NADPH/NTR/Trx system in the regulation of C. reinhardtii ATG4 by the oxi-doreduction of a regulatory disulfide bond.

Cys-400 Plays a Key Role in the Redox Control of C. reinhardtii ATG4

All our results indicate that a single disulfide bond may play a key role for the redox regulation of C. reinhardtii ATG4, as recently described for yeast ATG4 (Pérez-Pérez et al., 2014). Cys-338 and Cys-394 have been shown to establish a regulatory disulfide bond in yeast ATG4, and mutation of any of these residues results in constitutive activation of the protein (Pérez-Pérez et al., 2014). Cys-338 is widely conserved among ATG4 proteins, whereas Cys-394 seems to be restricted to some ascomycete species (Pérez-Pérez et al., 2014). Multiple sequence alignments revealed that the two regulatory cysteines identified in yeast ATG4 might be conserved in C. reinhardtii as Cys-400 and Cys-473, respectively (Supplemental Fig. S3). To test this hypothesis, Cys-400 and Cys-473 from C. reinhardtii ATG4 were mutated to Ser, and the corresponding recombinant proteins were produced and purified. We also identified Cys-84 as the catalytic Cys of C. reinhardtii ATG4. This Cys is the only one strictly conserved in all organisms (Supplemental Fig. S3). As expected, a mutant having this Cys replaced by Ser was constitutively inactive and was used as a negative control in these
Figure 2. The protease activity of *C. reinhardtii* ATG4 is redox regulated. A, Effect of DTT on ATG4 activity. ATG4 was incubated with ATG8 for 1 h in the presence of DTT concentrations ranging from 25 μM to 1 mM. Control (lanes 1 and 2): incubation with no addition. Lane 1 does not contain ATG4. B, Quantification of ATG4 activity from A. The highest DTT concentration sample was used as reference sample for quantification (activity = 1, in arbitrary units). C, Effect of H2O2 on the activity of prereduced ATG4. After pretreatment with 100 μM DTT for 30 min, ATG4 was treated with increasing H2O2 concentrations for 20 min and then incubated with ATG8 for 1 h. Control (lanes 1 and 2): incubation with no addition. Lane 1 does not contain ATG4. D, Quantification of ATG4 activity from C. The prereduced but not treated with H2O2 sample was used as reference for quantification (activity = 1, in arbitrary units). E, Inhibition of ATG4 activity by H2O2 is reversed by DTT. ATG4 protein was pretreated with 100 μM DTT for 30 min (lane 2); then, ATG4 was incubated with 500 μM H2O2 for 20 min (lane 3); finally, ATG4 was newly treated with 1 mM DTT for 30 min (lane 4). Control (lane 1): incubation with no addition. For all lanes, ATG8 was added to the reaction mixture for 1 h after each specific treatment to assess ATG4 activity. F, ATG4 is irreversibly inactivated by the alkylating agent IAM. ATG4 was pretreated with 100 μM DTT for 30 min (lane 2), then incubated with 20 mM IAM for 30 min (lane 3); next, ATG4 was again incubated with 50 mM DTT for 30 min (lane 4). Control (lane 1): incubation with no addition. For all lanes, ATG8 was added to the reaction mixture for 1 h after each specific treatment to study ATG4 activity. G, ATG4 activity is...
The ATG8 cleavage activity of all ATG4 variants was analyzed in the presence of a low concentration of DTT with or without TRXh1. As mentioned above, wild-type ATG4 was activated by reduction with TRXh1 but not by low DTT. As expected, the mutant lacking the catalytic Cys (ATG4C84S) was inactive in all tested conditions (Fig. 4). In contrast, the ATG4C400S mutant was readily active even in the absence of TRXh1 (Fig. 4), indicating that Cys-400 is required for the redox control of \textit{C. reinhardtii} ATG4. However, mutation of Cys-473 had no effect on the proteolytic activity of the protein, strongly suggesting that this Cys does not participate in the redox regulation of \textit{C. reinhardtii} ATG4 (Fig. 4). Nevertheless, the biochemical study performed on \textit{C. reinhardtii} ATG4 demonstrated that the activity of this protein is regulated dependent on the DTTred/DTTox ratio. ATG4 processing activity was monitored after incubation during 2 h in the presence of various DTTred/DTTox ratios (1/1, 1/10, 1/100, 10/1, 100/1 in mM). H, ATG4 is not activated by GSH. ATG4 activity was determined after incubation for 1 h in the presence of 0.5, 1, or 5 mM GSH (lanes 2, 3, and 4, respectively), 1 mM DTT (lane 5), or in the absence of reducing agent (lane 1). I, Redox titration of ATG4 activity. ATG4 activity was analyzed after incubation for 2 h at indicated $E_m$ poised by 20 mM DTT in various dithiol/disulfide ratios. ATG8 was added to the reaction mixture for 15 min after DTT treatment in all samples. The -∞ sample was used as reference for quantification. J, ATG4 activities monitored as in I were interpolated by nonlinear regression of the data using a Nernst equation for 2 electrons exchanged ($n = 2$) and one redox component. The average midpoint redox potential ($E_m$) of three independent experiments is reported in the figure as mean ± so.
through the formation of a disulfide bond efficiently reduced by thioredoxin, where Cys-400 plays an important role.

Oligomerization of *C. reinhardtii* ATG4

In the course of the biochemical analysis of *C. reinhardtii* ATG4, we observed that this protein is able to oligomerize in the absence of 2-mercaptoethanol, an efficient reducer of disulfide bonds (Fig. 5A). Moreover, we found that the oligomerization of ATG4 is also redox regulated, since incubation of this protein with increasing DTT concentrations resulted in the monomerization of ATG4 (Fig. 5B), indicating that DTT prevents the aggregation of ATG4. However, treatment of ATG4 with H$_2$O$_2$ strongly stimulated the oligomerization of the protein in a reversible manner (Fig. 5, B and C). To further characterize the oligomerization of *C. reinhardtii* ATG4, we performed a DTT redox titration by incubating the protein with different concentrations of oxidized and reduced DTT. Our results revealed that there is a strong correlation between the oligomerization of *C. reinhardtii* ATG4 and the redox potential (Fig. 5D). Next, we investigated whether the oligomerization of *C. reinhardtii* ATG4 might be conserved in other ATG4 proteins. We recently demonstrated that the proteolytic activity of yeast ATG4 is also controlled by the redox potential (Pérez-Pérez et al., 2014), although the study of the aggregation of this protein was not addressed. A detailed analysis of yeast ATG4 revealed that, interestingly, this protein shows an oligomerization pattern similar to the one described in *C. reinhardtii* ATG4, including the redox control of this process (Supplemental Fig. S4). Thus, taken together, these findings indicate that the redox regulation of ATG4 proteins may also involve the formation of aggregates in vitro.

Stress Conditions Promote the Oxidation and Aggregation of ATG4 in *C. reinhardtii*

To study the regulation of ATG4 in *C. reinhardtii* cells, we used an antibody raised against the recombinant protein. This antibody was able to recognize low amounts of purified *C. reinhardtii* ATG4 (Fig. 6A). We also tested the ability of this antibody to detect ATG4 in total extracts from *C. reinhardtii* cells. A band with a size comparable to *C. reinhardtii* ATG4 was observed in total extracts, although additional bands of lower and higher sizes could also be detected (Supplemental Fig. S3). The bands of lower size may likely correspond to degradation products of ATG4, whereas those of higher size might be due to posttranslational modifications or aggregation of this protein in vivo as observed with purified ATG4. To explore this hypothesis, we analyzed if the *C. reinhardtii* ATG4 antibody is able to detect monomeric and oligomeric ATG4. Our results revealed that indeed the antibody reacted with both forms of purified ATG4 (Fig. 6A).

Next, we investigated whether the detection of oligomeric ATG4 in *C. reinhardtii* total extracts may be related with the function of this protein in vivo. To this aim, ATG4 was analyzed in *C. reinhardtii* cells subjected to different stress conditions that activate autophagy in this organism. First, ATG4 was examined in stationary phase cells, since autophagy is up-regulated when *C. reinhardtii* cells reach stationary growth (Pérez-Pérez et al., 2010). We found that the abundance of ATG4, in particular oligomeric forms, increased in stationary cells (Fig. 6B). As previously reported (Pérez-Pérez et al., 2010), lipidated ATG8 could also be detected in stationary cells (Fig. 6B). Second, we investigated the effect of tunicamycin on ATG4, since this drug triggers ER stress and autophagy in *C. reinhardtii* (Pérez-Pérez et al., 2010). Our results indicated that monomeric and oligomeric ATG4 forms increased in tunicamycin-treated cells (Fig. 6C). Third, autophagy was also induced in *C. reinhardtii* by treating cells with norflurazon, an inhibitor of the carotene biosynthesis pathway (Sandmann and Albrecht, 1990) that activates this degradative process in *C. reinhardtii* as a result of ROS production and photo-oxidative damage of the cell (Pérez-Pérez et al., 2012). ATG8 lipidation was investigated by treating *C. reinhardtii* cells with norflurazon. Our results revealed that indeed the antibody reacted with both forms of purified ATG4 (Fig. 6A).
detected in *C. reinhardtii* cells treated with norflurazon, as previously described (Pérez-Pérez et al., 2012; Fig. 6D). Monomeric and oligomeric forms of ATG4 were detected in cells with basal autophagy (Fig. 6D). However, activation of autophagy by norflurazon resulted in a pronounced detection of oligomeric ATG4 forms as well as an increase in the overall signal of ATG4 (Fig. 6D). Up-regulation of autophagy in stationary cells also increased the abundance of oligomeric ATG4 forms, although to a minor extent compared to cells treated with norflurazon for 48 h (Fig. 6D).

Finally, we also investigated the formation of ATG4 oligomers in the *C. reinhardtii* mutant *lts1-204*, which lacks phytoene synthase (McCarthy et al., 2004), one of the first enzymes in the carotenoid biosynthetic pathway. Carotenoid-less *lts1-204* cells grow in the dark on acetate-containing medium and are unable to survive in the light even under very low light conditions (McCarthy et al., 2004). We have previously shown that shifting *lts1-204* cells from dark to light results in a strong production of ROS and the activation of autophagy (Pérez-Pérez et al., 2012). Thus, we analyzed the effect of light on ATG4 abundance and oligomerization in the *lts1-204* mutant strain. In close agreement with the data obtained in norflurazon (NF)-treated cells, we found that both ATG4 abundance and oligomerization significantly increased in mutant cells exposed to light (Fig. 6E). As previously shown (Pérez-Pérez et al., 2012), ATG8 was also up-regulated in light-stressed *lts1-204* cells (Fig. 6E). Taken together, our results indicate that stress conditions that generate ROS and activate autophagy promote the oxidation and aggregation of ATG4 in *C. reinhardtii*.

**ATG4 Activity Depends on ATG4 Reduction and Monomerization**

We reasoned that the oxidation and aggregation of ATG4 observed in *C. reinhardtii* cells treated with NF might be due to the high production of ROS that takes place in these cells (Pérez-Pérez et al., 2012). To explore this hypothesis, we treated *C. reinhardtii* cells with NF in the presence or absence of the ROS scavenger N-acetyl Cys (NAC). We observed that NAC largely prevented the effect of NF on ATG4 (Fig. 7A). Remarkably, the presence of NAC also decreased the lipidation of ATG8 in cells treated with NF (Fig. 7A), again supporting the role of ROS in the activation of autophagy in these cells (Pérez-Pérez et al., 2012) through inhibition of ATG4.

To further investigate the formation of oligomeric ATG4, total extracts from stationary cells were incubated with DTT, and the aggregation of ATG4 was analyzed by western blot. Our results indicated that DTT stimulated the proteolytic activity of ATG4 (Fig. 7B, top panel). Next, we investigated whether aggregated ATG4 may represent an inactive state of the protein. To this aim, we assayed the ATG4 proteolytic activity present in total extracts from *C. reinhardtii* cells using recombinant 6His-ATG8 as substrate as previously described (Pérez-Pérez et al., 2010). Our results indicated that DTT stimulated the proteolytic activity of
ATG4 (Fig. 7B, bottom panel). Furthermore, we found that incubation with IAM resulted in ATG4 inactivation and had no effect on the conformation of the protein (Fig. 7, B and C), indicating that aggregated, oxidized ATG4 is inactive. This finding was also confirmed in vitro using purified ATG4 and ATG8 proteins. DTT efficiently reduced and activated recombinant ATG4, and a tight correlation between

Figure 6. Autophagy-activating conditions lead to the oligomerization and inactivation of ATG4 in C. reinhardtii cells. A, C. reinhardtii recombinant ATG4 was subjected to 12% SDS-PAGE in the presence (+) or absence (−) of β-mercaptoethanol (βME) in the loading buffer and/or treated with 20 mM DTT for 1 h and then stained with Coomassie Brilliant Blue (5 μM ATG4) or subjected to western-blot analysis with anti-ATG4 antibodies (0.25 μM ATG4). B, Western-blot analysis of ATG4 and ATG8 during the growth phase in C. reinhardtii. Cells were grown in TAP from 1 × 10⁶ cells/ml to 8 × 10⁶ cells/ml, and the ATG4 and ATG8 protein profiles were analyzed. C, Effect of ER stress on ATG4 and ATG8 in C. reinhardtii. Wild-type C. reinhardtii cells growing at exponential phase were treated with 5 μg/ml tunicamycin (tun) as previously described (Pérez-Pérez et al., 2010), and samples were taken at different times for its analysis. Untreated cells were used as control. D, Inhibition of the carotenoid biosynthetic pathway leads to ATG8 lipidation and ATG4 oligomerization in C. reinhardtii. Wild-type C. reinhardtii cells in exponential growth phase were treated with 20 mM NF during 48 h as previously described (Pérez-Pérez et al., 2012). Samples of nontreated cells were taken at the same time and used as control. D, Western-blot analysis of ATG4 and ATG8 proteins on dark-to-light transition in the lts1-204 mutant. C. reinhardtii lts1-204 cells growing exponentially in TAP under dark conditions were transferred to standard light illumination (25 μE) for 6 h. Cells maintained in dark conditions were used as control. Then 15 μg of total extracts were resolved by 12% (for ATG4) or 15% (for ATG8 and FKBP12) SDS-PAGE followed by western blotting with anti-ATG4, anti-ATG8, and anti-FKBP12 antibodies. The oligomeric (oATG4) and monomeric (mATG4) ATG4 forms are marked on the right. Molecular mass markers (kD) are indicated on the left. β-Mercaptoethanol-free buffer was used in B to E.
ATG4 reduction/monomerization and ATG8 processing was detected with increasing concentrations of DTT (Fig. 7, D and E).

Aggregated ATG4 Localizes to Punctate Structures in C. reinhardtii Cells

We analyzed the cellular localization of ATG4 by immunofluorescence microscopy in C. reinhardtii cells treated or not with NF. In control untreated cells, the ATG4 signal was weak and restricted to some spots localized to the apical end of the cell as revealed by basal body staining with an antiacetylated tubulin antibody (Díaz-Troya et al., 2008a; Fig. 8). Progression into stationary phase of untreated cells resulted in a moderate increase of ATG4 signal and the detection of intense spots in other parts of the cell in addition to the peri-basal body region (Fig. 8). Remarkably, NF treatment caused a pronounced increase of ATG4 signal and more intense dots were readily observed (Fig. 8). Based on these results and the finding that ATG4 aggregates in response to NF treatment, the punctate structures observed in treated cells may likely contain aggregated ATG4. To strengthen this hypothesis, we also investigated the localization of ATG4 in its1-204 mutant cells shifted from dark to light, since this stress strongly promotes ATG4 oligomerization (Fig. 6E). In agreement with the punctate pattern observed in wild-type cells treated with NF (Fig. 8), light stress in carotenoid-mutant

![Image](https://www.plantphysiol.org)
cells resulted in a sharp localization of ATG4 in a central region of the cell (Fig. 9). Altogether, these results indicate that ROS-generating stress leads to the detection of ATG4 as intense spots likely containing oligomeric forms of this protein.

**DISCUSSION**

Recent genomic analyses revealed that core autophagy genes are highly conserved among plants and algae (Díaz-Troya et al., 2008b; Avin-Wittenberg et al., 2012; Pérez-Pérez and Crespo, 2014; Shemi et al., 2015). However, our current knowledge about the molecular mechanisms that regulate most of the ATG proteins in the plant kingdom is limited compared to yeasts or mammals. In this study, we unraveled the molecular mechanism underlying the redox regulation of the ATG4 protease from the model green alga *C. reinhardtii*. A single ATG4 gene has been previously identified in *C. reinhardtii* (Díaz-Troya et al., 2008b) as well as in other green algae (Pérez-Pérez and Crespo, 2014; Shemi et al., 2015).
ATG4 antibodies. Scale bar = 8 μm.

Figure 9. Localization of ATG4 in C. reinhardtii carotenoid mutant cells exposed to light. Its-1-204 mutant cells growing exponentially in TAP medium in dark conditions were exposed to low light (20 μE m$^{-2}$ s$^{-1}$) for 6 h or maintained in dark conditions as control. Cells were collected and processed for immunofluorescence microscopy analysis with anti-ATG4 antibodies. Scale bar = 8 μm.

2015), whereas plants such as Arabidopsis, maize, or rice have two ATG4 genes (Yoshimoto et al., 2004; Chung et al., 2009; Avin-Wittenberg et al., 2012). Amino acid sequence comparison of ATG4 proteins from different organisms showed a low identity even in the green lineage (Supplemental Figs. S1 and S2). The ATG4 protein from C. reinhardtii is about 200 to 300 amino acids larger than its orthologs in yeasts, plants, or mammals, which might indicate that this protein has acquired additional domains in C. reinhardtii. In this regard, an unusually large (about 3,800 amino acids) ATG4 protein with ATG8-endoproteolytic activity has been reported in the parasitic protist Toxoplasma gondii (Kong-Hap et al., 2013). The low identity found among ATG4 proteases together with their variable size suggests that this key autophagy protein may have developed specific features and/or functions in evolutionarily distant eukaryotes.

Our results demonstrated that the activity of C. reinhardtii ATG4 is subjected to redox regulation, since the protein is active in the reduced form and inactive in the oxidized form. Moreover, we found that this regulation is reversible, suggesting the involvement of redox posttranslational modifications. A similar redox regulation has been reported for yeast, human, and Arabidopsis ATG4 (Scherz-Shouval et al., 2007; Pérez-Pérez et al., 2014; Woo et al., 2014), indicating that the redox control of ATG4 activity is broadly conserved in eukaryotes despite the low identity observed among ATG4 proteins. The mechanism of action by which redox signals control ATG4 activity has been shown in only mammals and yeasts. In human ATG4A and ATG4B, this redox regulation involves Cys-81, which interferes with the activity of the adjacent catalytic Cys-77 (Scherz-Shouval et al., 2007). Regulatory Cys-81 from human ATG4s is not conserved in yeasts, and consequently the molecular mechanism that mediates the redox control of ATG4 in this unicellular model system is different. A detailed biochemical analysis demonstrated that Atg4 activity is controlled in yeasts through dithiol/disulfide exchange that involves the transfer of two electrons and the oxidation/reduction of a single disulfide bond (Pérez-Pérez et al., 2014). Our studies on the ATG4 protein from C. reinhardtii are in close agreement with these findings, since the redox characterization of this protein indicated that the activity of C. reinhardtii ATG4 is regulated by the reduction of a single disulfide bond with a very low redox potential. In the cell, these regulatory disulfides with very low redox potential are typically reduced by small oxidoreductases such as thioredoxins (Buchanan and Balmer, 2005). Indeed, it has been shown that yeast ATG4 can be efficiently reduced and activated by the cytoplasmic thioredoxin Trx1 (Pérez-Pérez et al., 2014). We found that C. reinhardtii ATG4 also can be readily reduced by the cytoplasmic thioredoxin TRXh1 from this alga, suggesting that control of ATG4 activity by thioredoxins might be evolutionarily conserved. In close agreement, thioredoxin and thioredoxin reductase have been identified as autophagosome-associated proteins in a quantitative proteomic study (Dengel et al., 2012). Moreover, we were able to reconstitute in vitro the whole ATG4 redox regulation process by the NADPH/NTR/thioredoxin system using NTRB from Arabidopsis as electron donor for C. reinhardtii TRXh1. The finding that NADPH can act as the electron donor of C. reinhardtii ATG4 suggests that this reducing molecule may link the energetic and redox state of the cell with the regulation of the autophagy process through the control of ATG4 activity.

Site-directed mutagenesis established that the regulatory disulfide bond in yeast Atg4 is formed between Cys-338 and Cys-394 (Pérez-Pérez et al., 2014). Cys-338 appears to be conserved in yeasts, plants, and mammals, whereas Cys-394 is restricted to some ascomycete species (Pérez-Pérez et al., 2014). Our results demonstrated that Cys-400 (the equivalent to Cys-338 in yeast Atg4) is required for the redox regulation of C. reinhardtii ATG4 activity and strengthen the hypothesis that the key role of this residue in the control of ATG4 is conserved through evolution. However, we found that Cys-473 (the counterpart to Cys-394 in yeast Atg4) does not participate in the redox regulation of ATG4 activity, since point mutation of this residue had no significant effect on the redox control of this protein. This observation is consistent with the poor conservation of Cys-394 from yeast ATG4 in other organisms (Pérez-Pérez et al., 2014). Nevertheless, the finding that C. reinhardtii ATG4 activity is regulated through the reduction of a single disulfide bond by thioredoxin and that one of the regulatory cysteines is conserved strongly suggests that the mechanism of ATG4 regulation is evolutionarily conserved in unicellular eukaryotes like yeasts and algae.
The formation of permanent or transient protein-protein complexes is essential for protein functionality and regulation. Some proteins such as the antioxidant enzyme peroxiredoxins undergo major redox-dependent conformational changes that are pivotal for its physiological function and regulation (Dietz, 2011). In this study, we report the formation of oligomers or aggregates of the *C. reinhardtii* ATG4 protein both in vitro and in vivo. Our results revealed that *C. reinhardtii* ATG4 aggregates upon oxidation, whereas the reduction of the protein prevents its aggregation, indicating that ATG4 oligomerization is tightly connected to the redox state of the protein. Remarkably, we found that yeast ATG4 also undergoes redox-dependent oligomerization, which may suggest this is a conserved feature among ATG4 proteins. We propose that *C. reinhardtii* ATG4 can be found in three different states that are modulated by the intracellular redox potential (Fig. 10). Under low redox potential, the thioredoxin system keeps ATG4 in a reduced and active form (State 1). The presence of ROS promotes the oxidation and consequent inactivation of ATG4 (State 2) that can be further oxidized as the redox potential increases, leading to the aggregation of the protein (State 3). Oxidation and aggregation of ATG4 can be reversed by reducing agents, which maintain the equilibrium for the proper redox regulation of ATG4 activity.

The proteolytic activity of ATG4 is essential for the conjugation of ATG8 to PE, a key step for autophagosome biogenesis, but this enzyme is also crucial during the final steps of autophagosome formation, since ATG4 can delipidate ATG8-PE to release it from its lipid anchor, a fundamental step for normal autophagic activity (Kirisako et al., 2000). Thus, ATG4 activity must be finely regulated to ensure autophagy progression. C-terminal maturation of ATG8 might be constitutive, since ATG4 cleaves newly synthesized ATG8 in yeasts (Kirisako et al., 2000). In close agreement, unprocessed ATG8 has not been detected in *C. reinhardtii* (Pérez-Pérez et al., 2010), suggesting that cleavage of ATG8 at the C terminus must be highly efficient. These findings suggest that regulation of ATG8 delipidation by ATG4 may play a central role in the control of the autophagy process. Mounting evidence indicates that ATG8 delipidation must indeed be tightly regulated. In human cells, it has been proposed that nutrient starvation generates ROS, which in turn inactivates ATG4 at the site of autophagosome formation to promote lipidation of ATG8 (Scherz-Shouval et al., 2007). According to this model, the accumulation of lipidated Atg8s detected in human cells under starvation might result from inhibition of Atg4 delipidating activity (Scherz-Shouval et al., 2007). In the same line, it has been shown that deconjugation of ATG8-PE is required for efficient autophagosome biogenesis in yeasts (Nair et al., 2012; Nakatogawa et al., 2012; Yu et al., 2012). Our results in *C. reinhardtii* strengthen the hypothesis that ATG4 is inactivated under stress conditions to prevent delipidation of ATG8 at the site of autophagosome formation.

![Diagram](https://via.placeholder.com/150)

**Figure 10.** Putative model for the redox regulation of ATG4 in *C. reinhardtii*. *C. reinhardtii* ATG4 can be found in at least three conformational states: an active and monomeric form (state 1), an inactive and monomeric form (state 2), and an inactive and oligomeric form (state 3). Taken together, our data strongly suggest that the presence of ATG4 in these three states depends on the intracellular redox potential. We propose a model in which two different oxidation levels result in the inactivation of the protease. A first level of oxidation (disulfide bond formation, state 2) results in the inactivation of ATG4, which can be either further oxidized to an oligomeric and inactive form (state 3) or newly reduced to a monomeric and active form (state 1). The presence of oxidants, ROS, or a positive redox potential may induce a high rate of state 2 and state 3 forms of ATG4. In contrast, the presence of reducing agents, the action of reducing proteins such as the Trx system, or a negative redox potential may lead to the reduction and activation of ATG4 (state 1). Thus, the activity of the ATG4 protease may be finely modulated depending on the redox conditions and the intracellular redox state in *C. reinhardtii* cells.
We found that ATG4 is oxidized and forms oligomers in *C. reinhardtii* cells treated with NF, an inhibitor of the carotenoid biosynthesis pathway that generates high levels of ROS and activates autophagy in this green alga (Pérez-Pérez et al., 2012). ATG4 aggregates were also observed in the carotenoid-less mutant *ls1-204* exposed to light, a stress condition that produces ROS and induces autophagy (Pérez-Pérez et al., 2012), or when wild-type cells reached stationary growth, a physiological condition that triggers autophagy in *C. reinhardtii* (Pérez-Pérez et al., 2010). Concomitant with ATG4 aggregation, we also detected an accumulation of lipitated ATG8 under all these stress conditions, strongly suggesting that aggregated ATG4 is inactive. Together, our results provide the molecular basis for the redox control of autophagy in a photosynthetic organism through the oxidation and inactivation of the ATG4 protease under stress conditions.

**MATERIALS AND METHODS**

**Strains, Media, and Growth Conditions** *Chlamydomonas reinhardtii* wild-type 4Ax (CC-4051) and the *ls1-204* mutant (CC4199) strains were obtained from the Chlamydomonas Culture Collection. *C. reinhardtii* cells were grown under continuous illumination at 25°C in Tris-acetate phosphate (TAP) medium as described (Harris, 1989). When required, cells in exponential growth phase (10^6 cells/mL) were treated with NF (Sigma-Aldrich; A16940; 10 μM) or 1 mM EDTA (Sigma-Aldrich; E6511) in 50 mM Trizma base, 138 mM NaCl, and 27 mM KCl, pH 8. When indicated, ATG4 was pre-incubated in the presence of reducing (DTT (Sigma-Aldrich; 43815) or GSH (Sigma-Aldrich; G2451)) or alkylating agent (20 mM iodoacetamide (IAM) or 50 mM N-ethylmaleimide) or alkylating agent (20 mM DTT) or alkylating agent (20 mM IAM) was added. The reaction mixture was incubated at 25°C for the indicated time and stopped by addition of 10% SDS-PAGE gels and stained with Coomassie Brilliant Blue (Sigma-Aldrich; 27816). For ATG4 activity quantification, gels were scanned with Odyssey (LI-COR, Biosciences) and the signals corresponding to unprocessed (ATG8) and processed (pATG8) ATG8 were quantified with the software Image Studio (LI-COR, Biosciences). The ATG4 activity was considered as the relation between pATG8/(ATG8+pATG8), and it was normalized between 0 and 1, with 0 corresponding to totally oxidized and inactive ATG4 and 1 corresponding to totally reduced and active ATG4. The ATG4 activities (in arbitrary units) were plotted using the software OriginPro 8 (OriginLab).

**Gene Cloning and Protein Purification**

The DNA coding sequence of the ATG4 gene from *C. reinhardtii* was synthesized and cloned into pBluescript SK (+) vector by GeneCust Europe (GeneCust Europe, 2016). The different ATG4 mutants (ATG4C84S, ATG4C473S, and ATG4C400S) were synthesized by GeneCust Europe and cloned into pBluescript SK (+) vector. The different ATG4 mutants were also cloned into the pET28a (+) (Novagen; 69864-3) plasmid at NdeI and XhoI sites for expression of the corresponding His-tagged protein. The different ATG4 mutants (ATG4C84S, ATG4C473S, and ATG4C400S) were synthesized by GeneCust Europe and cloned into pBluescript SK (+) vector. The different ATG4 mutants were also cloned into the pET28a (+) plasmid at NdeI and XhoI sites. All clones were verified by DNA sequencing and used to transform *Escherichia coli* BL21 (DE3) strain. Recombinant proteins were expressed by induction at exponential growth phase (OD_{600} ≈ 0.5) with 0.5 mM isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich; I6758) for 2.5 h at 37°C. The recombinant proteins ATG4C84S, ATG4C473S, and ATG4C400S were purified by affinity chromatography on a Hi-Select Nickel Affinity Gel (Sigma-Aldrich; P6611) following the manufacturer's instructions. Prior to purification, wild-type ATG4 and the Cys variants were first solubilized with 4 mM urea (Sigma-Aldrich; 51456) due to the presence of the recombinant proteins in inclusion bodies and subsequently purified from the soluble fraction by affinity chromatography as described above. The ATG8 protein from *C. reinhardtii* and the ATG4 protein from *Saccharomyces cerevisiae* were purified as previously described (Pérez-Pérez et al., 2010, 2014), respectively. The TRXh1WT and TRXh1C39S proteins from *C. reinhardtii* were purified as described in Goyer et al. (1999). The NTRB protein from Arabidopsis (*Arabi
dopsis thaliana*) was purified as described in Jacquot et al. (1994). All proteins used in this study are listed in Supplemental Table S1.

**In Vitro ATG4 Cleavage Assay**

A typical reaction mixture included 1 μM ATG4, 5 μM ATG8, 1 mM 1,4-DTT (Sigma-Aldrich; 48315), and 1 mM EDTA (Sigma-Aldrich; E6511) in 50 mM Tris base, 138 mM NaCl, and 27 mM KCl, pH 8. When indicated, ATG4 was pre-incubated in the presence of reducing [DTT (Sigma-Aldrich; 48315) or GSH (Sigma-Aldrich; G2451)], oxidizing [DTT (Sigma-Aldrich; D3511) or H_2O_2 (Sigma-Aldrich; H1009)], or alkylating [IAM (Sigma-Aldrich; I1149)] compounds alone or in combination at the indicated times and concentrations. For the analysis of TRX activity, 10 μM DTT and 5 μM TRXh1 or TRXh1C39S from *C. reinhardtii* were used at the indicated incubation time. The reaction mixtures were incubated at 25°C for the indicated time and stopped by addition of β-mercaptoethanol-free Laemmli sample buffer followed by 5 min of boiling. Proteins were resolved on 15% SDS-PAGE gels and stained with Coomassie Brilliant Blue (Sigma-Aldrich; 27816). For ATG4 activity quantification, gels were scanned with Odyssey (LI-COR, Biosciences) and the signals corresponding to unprocessed (ATG8) and processed (pATG8) ATG8 were quantified with the software Image Studio (LI-COR, Biosciences). The ATG4 activity was considered as the relation between pATG8/(ATG8+pATG8), and it was normalized between 0 and 1, with 0 corresponding to totally oxidized and inactive ATG4 and 1 corresponding to totally reduced and active ATG4. The ATG4 activities (in arbitrary units) were plotted using the software OriginPro 8 (OriginLab).

**Protein Preparation and Immunoblot Analysis**

*C. reinhardtii* cells from liquid cultures were collected by centrifugation (4,000g for 5 min), washed in 50 mM Tris-HCl (pH 7.5) buffer, and resuspended in a minimal volume of the same solution. Cells were lysed by two cycles of slow freezing to −80°C followed by thawing at room temperature. The soluble cell extract was separated from the insoluble fraction by centrifugation (15,000g for 20 min) in a microcentrifuge at 4°C. For immunoblot analyses, total protein extracts (15 μg) were subjected to 12% or 15% SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad; 162-0115). Anti-CrATG4 (obtained in collaboration with Agrisera Antibodies), anti-CrATG8 (Pérez-Pérez et al., 2010), anti-CrFKBP12 (Crespo et al., 2005), and secondary antibodies were diluted 1:50,000, 1:5,000, 1:5,000, and 1:10,000, respectively, in PBS containing 0.1% (v/v) Tween 20 (Applichem; A4974) and 5% (w/v) milk powder. The Luminata Crescendo Millipore immunoblotting detection system (Millipore; WBLURS) was used to detect the proteins with horseradish peroxidase-conjugated antirabbit secondary antibodies (Sigma-Aldrich; A6154). Proteins were quantified with the Coomassie dye binding method (Bio-Rad; 500-0000).

**In Vivo ATG4 Cleavage Assays**

A typical reaction mixture contained 50 μg total extract from *C. reinhardtii* and 10 ng of 6His-tagged ATG8 (denoted as 6H-ATG8). When indicated, reducing agent (25 μM DTT) or alkylating agent (20 μM IAM) was added. The reaction mixture was incubated at 25°C for the indicated time and stopped by addition of β-mercaptoethanol-free Laemmli sample buffer followed by 5 min of boiling. The samples were analyzed by western-blot analysis with anti-CrATG8 or anti-CrATG4 antibodies as described above.

**Immunofluorescence Microscopy**

*C. reinhardtii*-untreated (control 0 h and control 48 h) or NF-treated (NF 48 h) cells were fixed and stained for immunofluorescence microscopy as previously described (Crespo et al., 2005). The primary antibodies used were anti-CrATG4 (1:250) and anticycteoleylated Tubulin (Sigma-Aldrich; I7451; 1:500 dilution). For signal detection, fluorescein isothiocyanate-labeled goat anti-rabbit antibody (Sigma-Aldrich; F4890; 1:500 dilution) and Alexa Fluor 594 donkey anti-mouse (Jackson ImmunoResearch; 715-515-150; 1:5,000 dilution) were 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). For comparative analysis, the same acquisition time was fixed for the fluorescein isothiocyanate or Alexa Fluor 594 signals.

**Statistical Methods** All experiments were performed at least in triplicate and data are presented as mean ± SD. The statistical significance of data was verified by the Student's t test whenever required.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Table showing the identity (%) of ATG4 proteins from different species.

**Supplemental Figure S2.** Unrooted maximum likelihood phylogenetic tree of ATG4 proteins.

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Supplemental Figure S3. Multiple sequence alignment of ATG4 from diverse organisms.

Supplemental Figure S4. Redox control of yeast ATG4 oligomerization.

Supplemental Figure S5. Detection of ATG4 in total extracts from *C. reinhardtii*.

Supplemental Table S1. Proteins used in this study.

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We thank Christophe Marchand for sharing *C. reinhardtii* TRXh1 and TRXhIC395 proteins, Emmanuelme Isakidise-Bourguet for kindly providing Arabidopsis NTRB, Krishna Niyogi for making carotenoid mutant strains available to the research community, and Francisco J. Florencio for critical reading of the manuscript.

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LITERATURE CITED


**Figure S1.** Table showing the identity (%) of ATG4 proteins from different species (*Chlamydomonas reinhardtii*; *Volvox carteri*; *Coccomyxa subellipsioidea*; *Arabidopsis thaliana*; *Oryza sativa*; *Homo sapiens*; *Saccharomyces cerevisiae*; *Schizosaccharomyces pombe*). The identity was obtained by alignment of the two sequences using Pairwise Sequence Alignment EMBOSS Needle. The accession numbers are indicated in Figure S2.

**Figure S2.** Unrooted Maximum likelihood phylogenetic tree of ATG4 proteins. Sub-families are indicated by color-coded circles. Species abbreviations and the accession numbers are indicated. **Cr:** *Chlamydomonas reinhardtii* (Cre12.g510100.t1.1), **Vc:** *Volvox carteri* (Vocar.0016s0213.1), **Cs:** *Coccomyxa subellipsioidea* (EIE27123), **At:** *Arabidopsis thaliana* (AT2G44140.1 for A; AT3G59950.1 for B), **Al:** *Arabidopsis lyrata* (935334 for A; 486460 for B), **Zm:** *Zea mays* (GRMZM2G064212_T02 for A; GRMZM2G173682_T01 for B), **Sl:** *Solanum lycopersicum* (Solyc01g006230.2.1), **St:** *Solanum tuberosum* (PGSC0003DMT400081879), **Gm:** *Glycine max* (Glyma.18G248400.1 for A; Glyma.09G244800.1 for B), **Mt:** *Medicago truncatula* (Medtr7g081230.1), **Vv:** *Vitis vinifera* (GSVIVT01003913001), **Os:** *Oryza sativa* (LOC_Os03g27350.1 for A; LOC_Os04g58560.1 for B), **Sc:** *Saccharomyces cerevisiae* (KZV08400), **Sp:** *Schizosaccharomyces pombe* (CAC00556), **Ca:** *Candida albicans* (XP_713234), **Nc:** *Neurospora crassa* (EAA30202), **An:** *Aspergillus nidulans* (XP_661074), **Hs:** *Homo sapiens* (BAB83889 for A; BAB83890 for B), **Mm:** *Mus musculus* (NP_777364 for A; NP_777363 for B), **Zf:** *Zebrafish* (AAH95617), **Dm:** *Drosophila melanogaster* (NP_608563). ATG4 sequences were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) for algae and plants, and NCBI (https://www.ncbi.nlm.nih.gov/pubmed) for fungi and animals.

**Figure S3.** Multiple sequence alignment of ATG4 from diverse organisms. (A) Alignment of the region containing the catalytic Cys84. (B) Alignment of the C-terminus region containing putative regulatory Cys400 and Cys473. The proteins were aligned with the ClustalW program using default parameters. Residues boxed in red correspond to 70% identity. Residues boxed in blue correspond to 70% conservation with the PAM120 matrix. The accession numbers are indicated in Figure S2.
**Figure S4. Redox control of yeast ATG4 oligomerization.** (A) Yeast recombinant ATG4 (5 µM) was subjected to 12 % SDS-PAGE in the presence (+) or absence (-) of β-mercaptoethanol (βME) and stained with coomassie brilliant blue (CBB). mATG4, monomeric ATG4; oATG4, oligomeric ATG4. (B) ATG4 (5 µM) was preincubated with 100 µM DTT for 1 h (lane 2); then, ATG4 was treated with 500 µM H₂O₂ for 20 min (lane 3); and finally, ATG4 was incubated with 1 mM DTT for 30 min (lane 4). Control (lane 1): incubation with no addition. (C) Redox titration of ATG4 oligomerization/monomerization. ATG4 monomerization was analyzed after incubation for 2 h at indicated $E_h$ poised by 20 mM DTT in various dithiol/disulfide ratios. Arrowheads indicate oligomeric (oATG4) and monomeric (mATG4) ATG4 forms. β-mercaptoethanol-free buffer was used in (B and C).

**Figure S5. Detection of ATG4 in total extracts from Chlamydomonas.** Different amounts (2.5, 10, 20, 30 and 40 µg) of soluble extracts from stationary cells were resolved by 12 % SDS-PAGE followed by Western-blot analysis with the anti-ATG4 antibody. The oligomeric (oATG4) and monomeric (mATG4) ATG4 forms are marked on the right. Molecular mass markers (kDa) are indicated on the left. β-mercaptoethanol-free buffer was used.
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Figure Suppl 2

green lineage

higher plants

algae

fungi
Figure Suppl 4

A

β-mercaptoethanol
+ -

mATG4
oATG4

B

100 μM DTT
500 μM H2O2
1 mM DTT
- - + + +
- - + + +
- - - - +

C


oATG4
mATG4
Figure Suppl 5
Table S1. Proteins used in this study

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Cr: *Chlamydomonas reinhardtii*; Sc: *Saccharomyces cerevisiae*; At: *Arabidopsis thaliana*.