Oxidative Stress Contributes to Autophagy Induction in Response to Endoplasmic Reticulum Stress in *Chlamydomonas reinhardtii*[1[W]]

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The accumulation of unfolded/misfolded proteins in the endoplasmic reticulum (ER) results in the activation of stress responses, such as the unfolded protein response or the catabolic process of autophagy to ultimately recover cellular homeostasis. ER stress also promotes the production of reactive oxygen species, which play an important role in autophagy regulation. However, it remains unknown whether reactive oxygen species are involved in ER stress-induced autophagy. In this study, we provide evidence connecting redox imbalance caused by ER stress and autophagy activation in the model unicellular green alga *Chlamydomonas reinhardtii*. Treatment of *C. reinhardtii* cells with the ER stressors tunicamycin or dithiothreitol resulted in up-regulation of the expression of genes encoding ER resident endoplasmic reticulum oxidoreductin1 oxidoreductase and protein disulfide isomerases. ER stress also triggered autophagy in *C. reinhardtii* based on the protein abundance, lipidation, cellular distribution, and mRNA levels of the autophagy marker ATG8. Moreover, increases in the oxidation of the glutathione pool and the expression of oxidative stress-related genes were detected in tunicamycin-treated cells. Our results revealed that the antioxidant glutathione partially suppressed ER stress-induced autophagy and decreased the toxicity of tunicamycin, suggesting that oxidative stress participates in the control of autophagy in response to ER stress in *C. reinhardtii* In close agreement, we also found that autophagy activation by tunicamycin was more pronounced in the *C. reinhardtii sor1* mutant, which shows increased expression of oxidative stress-related genes.

All living organisms have evolved sophisticated mechanisms to efficiently respond and adapt their growth and metabolism to different types of stress. A well-documented example of such stress-induced responses is the process of autophagy or self-degradation, which is structurally and functionally conserved in all eukaryotes. During autophagy (also known as macroautophagy), cytoplasmic components, including proteins, membranes, and even organelles, are nonselectively enclosed within a double-membrane vesicle known as autophagosome and delivered to the vacuole/lysosome for degradation of toxic or damaged components and recycling of needed nutrients (Xie and Klionsky, 2007; Nakatogawa et al., 2009; Li and Vierstra, 2012; Liu and Bassham, 2012).

Autophagy is mediated by a set of proteins coded by *ATG* (autophagy-related) genes that are widely conserved from yeast (*Saccharomyces cerevisiae*) to humans. Homologs of *ATG* genes have been reported in plant and algal genomes, indicating that autophagy is also conserved in photosynthetic organisms (Thompson and Vierstra, 2005; Bassham et al., 2006; Diaz-Troya et al., 2008b; Avin-Wittenberg et al., 2012). Some ATG proteins play a structural role in autophagy and are essential for the formation of the autophagosome. For instance, the ATG8 protein anchors to the autophagosome membrane through its covalent binding to phosphatidylethanolamine, and the ATG8-phosphatidylethanolamine conjugate is essential for the formation and completion of the autophagosome (Ichimura et al., 2000). *ATG* genes are conserved in *Chlamydomonas reinhardtii* (algae; Diaz-Troya et al., 2008b; Pérez-Pérez et al., 2010), and autophagy has been investigated in this model system by monitoring the abundance, lipidation state, and cellular distribution of the ATG8 protein under several growth and stress conditions. As reported for other systems, nitrogen or carbon depletion triggers autophagy in *C. reinhardtii* (Pérez-Pérez et al., 2010). Moreover, entry of *C. reinhardtii* cells into stationary growth phase activates autophagy in a reversible manner, because the process is quickly down-regulated when cells return to exponential growth (Pérez-Pérez et al., 2010). Autophagy is also induced in *C. reinhardtii* cells treated with hydrogen peroxide or

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methyl viologen (MV), indicating that oxidative stress triggers this process in algae (Pérez-Pérez et al., 2010, 2012a). Reactive oxygen species (ROS) are potent inducers of autophagy in *C. reinhardtii* and plants (Liu and Bassham, 2012; Pérez-Pérez et al., 2012b). Indeed, a link between photo-oxidative damage, ROS accumulation, and autophagy activation has been shown in *C. reinhardtii* cells with a decreased carotenoid content caused by either the mutation of phytoene synthase or the inhibition of phytoene desaturase by the herbicide norflurazon (Pérez-Pérez et al., 2012a). Moreover, ROS generated in the chloroplast of carotenoid-deficient cells or the chloroplast of wild-type cells subjected to high light stress activate autophagy (Pérez-Pérez et al., 2012a).

The accumulation of unfolded/misfolded proteins in the endoplasmic reticulum (ER) is known to trigger autophagy in yeast (Bernales et al., 2006; Yorimitsu et al., 2006) and mammals (Ogata et al., 2006). More recently, induction of autophagy by ER stress has also been reported in land plants and algae (Pérez-Pérez et al., 2010; Liu et al., 2012), indicating that the signaling pathways controlling autophagy activation in response to this intracellular stress might be conserved in photosynthetic organisms. In *C. reinhardtii*, tunicamycin, which induces ER stress by inhibition of N-linked glycosylation, strongly increases the abundance and lipidation of ATG8 and modifies its cellular distribution (Pérez-Pérez et al., 2010). The accumulation of misfolded proteins in the ER is a potent stress signal that induces the expression of chaperones and other proteins required for the reestablishment of cell homeostasis, a signaling process known as the unfolded protein response (UPR; Walter and Ron, 2011). ER stress is perceived in the cell by key signaling proteins, such as the highly conserved inositol-requiring enzyme1 kinase, which transduces stress signals to the nucleus by promoting the splicing of basic leucine zipper (bZIP)-like transcription factors (Walter and Ron, 2011). Eukaryotic cells use a quality control mechanism that recognizes aberrantly folded proteins in the ER for their degradation through the proteasome, a process that is known as ER-associated degradation (Walter and Ron, 2011). Prolonged ER stress also triggers autophagy to remove unfolded proteins and counterbalance ER expansion caused by UPR (Bernales et al., 2006).

Unlike other cellular compartments, the ER provides, through ER-resident oxidoreductases, an oxidative environment that facilitates the oxidation of cysteines and thereby, the formation of disulfide bonds (Tu and Weissman, 2004). In yeasts and mammals, it has been shown that endoplasmic reticulum oxidoreductin1 (ERO1) is a major source of ROS in the ER and the cell (Haynes et al., 2004; Tu and Weissman, 2004). Oxidative protein folding in the ER occurs, in part, through the formation of disulfide bonds by protein disulfide isomerases (PDIs). To introduce disulfides into client proteins, PDIs must be maintained in an oxidized state, and ERO1 is mainly responsible for keeping PDI oxidized and active in the ER (Frand and Kaiser, 1999). ERO1 uses molecular oxygen as the final electron acceptor and hence, forms one molecule of hydrogen peroxide for every disulfide that it introduces (Tu and Weissman, 2004). Several studies have established an association between ER stress and ROS generation (Malhotra and Kaufman, 2007; Rutkowski and Kaufman, 2007; Ozgur et al., 2014), and the molecular mechanisms by which ROS are produced during UPR have been thoroughly reviewed (Santos et al., 2009).

ROS have been proposed to play an important role in the mechanisms of autophagy induction in response to various stress conditions in mammals, plants, and algae (Huang et al., 2011; Scherz-Shouval and Elazar, 2011; Szumiel, 2011; Li et al., 2012; Pérez-Pérez et al., 2012b). Although ER stress promotes ROS production and induces autophagy, it remains unknown whether ROS are involved in ER stress-induced autophagy. In this study, we provide evidence connecting autophagy activation in ER-stressed cells with redox imbalance generated from the UPR-regulated oxidative folding machinery in *C. reinhardtii* and propose that oxidative stress contributes to the induction of autophagy by ER stress.

RESULTS

Tunicamycin Triggers ER Stress and Autophagy in *C. reinhardtii*

In a previous study, we showed that tunicamycin triggers autophagy in *C. reinhardtii* (Pérez-Pérez et al., 2010). To further characterize the effect of tunicamycin on autophagy, we analyzed the expression of the *ATG8* gene by quantitative real-time PCR (qPCR). Tunicamycin treatment resulted in a progressive increase of *ATG8* transcript levels (Fig. 1A). In close agreement, an increase in ATG8 protein abundance and the appearance of modified ATG8 forms were observed in ER-stressed cells (Fig. 1B). No effect was observed on the amount of FK506 Binding Protein12 (FKBP12), an ER stress unrelated protein (Crespo et al., 2005). Transcription of *ATG3*, encoding an E2-like enzyme involved in ATG8 lipidation (Ichimura et al., 2000), was also analyzed in tunicamycin-treated cells to investigate the participation of other *ATG* genes in ER stress. qPCR analysis revealed that, similar to *ATG8*, *ATG3* expression was upregulated with tunicamycin as well as other stressors previously shown to induce autophagy, such as hydrogen peroxide, MV, or norflurazon (Pérez-Pérez et al., 2010, 2012a; Supplemental Fig. S1). As expected, tunicamycin had no effect on the expression of *FKBP12* (Supplemental Fig. S2).

The cellular response to ER stress in *C. reinhardtii* is still poorly characterized, and no markers have been established to investigate this process in this model alga. In Arabidopsis (*Arabidopsis thaliana*), it has been reported that expression of some UPR genes, such as the ER-resident chaperone Calreticulin2 (CAL2; At1g09210), increases in response to tunicamycin (Martínez and Chrispeels, 2003). We identified a member of the calreticulin

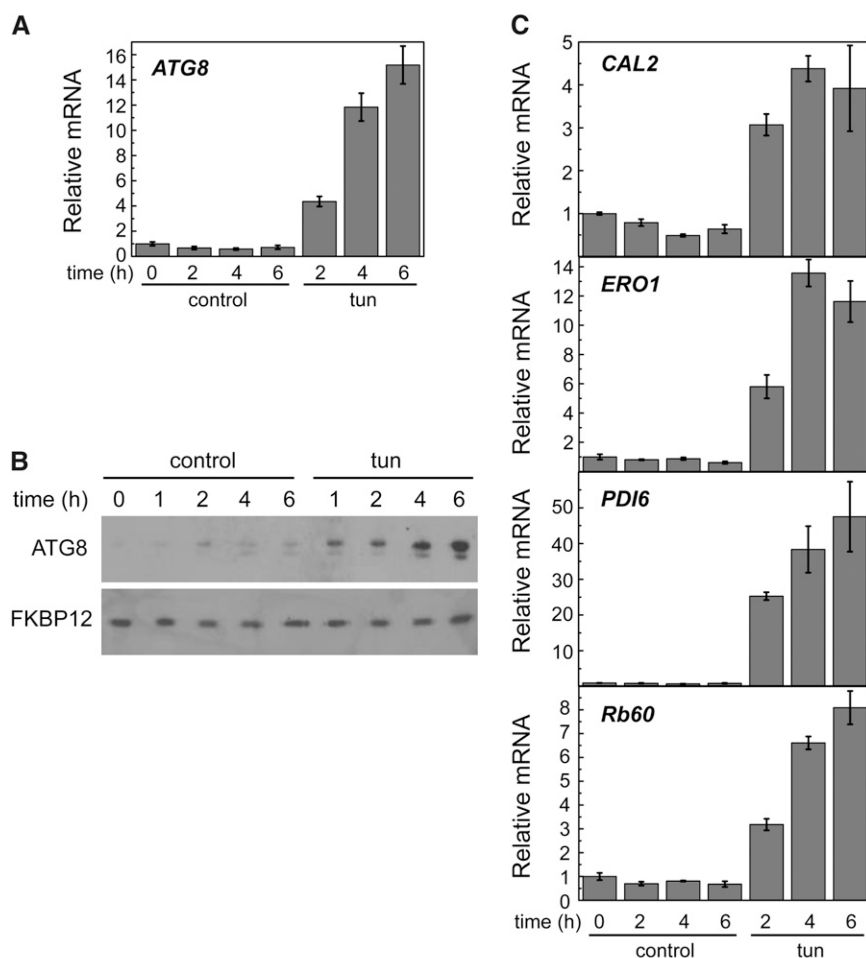

Figure 1. Tunicamycin triggers autophagy and ER stress in *C. reinhardtii* Log-phase cells grown in TAP medium were treated with 5 μg mL^{-1} tunicamycin, and samples were taken at the indicated times and processed for expression analysis of *ATG8* (A) and *CAL2*, *ERO1*, *PDI6*, and *Rb60* (C) by qPCR (A and C) or western blot (B). Thirty micrograms of total extracts were resolved by 15% SDS-PAGE followed by western blotting with anti-ATG8 and anti-FKBP12 antibodies. Values are means of three independent experiments. tun, Tunicamycin.

family in the *C. reinhardtii* nuclear genome (Supplemental Fig. S3), which we denoted as *CAL2*. To investigate whether the expression of *CAL2* is subject to UPR regulation in *C. reinhardtii*, transcription of this gene was determined by qPCR analysis in cells treated with tunicamycin or other stressors that should not trigger ER stress. *CAL2* mRNA levels were increased with tunicamycin (Fig. 1C) but not hydrogen peroxide, MV, or norflurazon (Supplemental Fig. S1A), showing that this gene can be used to monitor ER stress in *C. reinhardtii*.

ERO1 has not been previously described in algae, but based on its high evolutionary conservation, we identified an *ERO1* homolog in the *C. reinhardtii* genome (Supplemental Fig. S3). To investigate the participation of *C. reinhardtii ERO1* in ER stress, ERO1 expression was analyzed by qPCR in cells treated with tunicamycin. We found a strong induction of *ERO1* expression in ER-stressed cells (Fig. 1C). In yeast, ERO1 and PDI expressions are coordinately regulated and subjected to UPR regulation (Frand and Kaiser, 1998; Pollard et al., 1998). Rb60/PDI1A is the only canonical PDI that has been studied in *C. reinhardtii*, although other PDI-like proteins seem to be conserved in the *C. reinhardtii* genome (Lemaire and Miginiac-Maslow, 2004; Filonova et al., 2013). Rb60 seems to have a dual localization in the ER and the chloroplast (Levitan et al., 2005), but its role in ER stress has not been investigated. We analyzed the expression of *Rb60* in response to tunicamycin treatment and found that, similar to *ERO1*, this gene was strongly up-regulated (Fig. 1C). Searching for PDI proteins other than Rb60 in *C. reinhardtii*, we identified a PDI-like protein that we denoted PDI6 containing an N-terminal J domain (Supplemental Fig. S3). This domain structure is conserved in ER proteins involved in the recognition and binding of misfolded proteins that fail to achieve their correct conformation in the ER (Schroda, 2004). We investigated the participation of PDI6 in ER stress by analyzing the expression of this gene in tunicamycin-treated cells. *PDI6* mRNA level dramatically increased in ER-stressed cells (Fig. 1C), strongly suggesting that this gene is involved in the cellular response to this stress. Taken together, these results indicated that *CAL2*, *ERO1*, *Rb60*, and *PDI6* expression can be used to monitor ER stress in *C. reinhardtii*.

In addition to tunicamycin, we tested the effect of thapsigargin, an inhibitor of the ER Ca^{2+}-ATPase that triggers ER stress by depletion of luminal calcium stores (Urano et al., 2000), on autophagy and ER stress markers. Like tunicamycin treatment, thapsigargin led

to ER stress and autophagy activation in *C. reinhardtii* (Supplemental Fig. S4).

Tunicamycin Triggers Oxidative Stress

We explored the possible induction of oxidative stress caused by ER stress through different approaches. We analyzed the expression of *Glutathione Peroxidase Homologous* (*GPXH*) and *Glutathione-S-transferase1* (*GSTS1*) genes in ER-stressed cells. Both genes can be induced by several ROS, although *GPXH* is more significantly induced by singlet oxygen (Ledford et al., 2007; Fischer et al., 2007, 2012). Our results revealed a high induction of *GSTS1* and a moderate increase of *GPXH* expression in response to tunicamycin treatment (Fig. 2B), suggesting that ER stress may result in the activation of oxidative stress signaling in *C. reinhardtii* However, the intracellular pool of total glutathione, comprising reduced glutathione (GSH) and oxidized glutathione (GSSG), was determined in cells treated with tunicamycin. Our results indicated that ER stress raised the intracellular concentration of GSSG (Fig. 2B) and decreased the GSH-to-GSSG ratio (Fig. 2C), a hallmark of redox imbalance (Foyer and Noctor, 2011). Taken together, these results indicated that ER stress triggers oxidative stress in *C. reinhardtii*.

Dithiothreitol Causes ER Stress and Autophagy Activation

Disruption of disulfide bonds by dithiothreitol (DTT) results in accumulation of unfolded proteins in the ER and consequently, triggers ER stress. Massive aggregation of misfolded proteins caused by DTT has been shown to induce autophagy in various systems, including yeasts, mammals, and more recently, plants (Bernales et al., 2006; Yorimitsu et al., 2006; Liu et al., 2012). We investigated the effect of DTT on ER stress and autophagy in *C. reinhardtii* by different approaches. Autophagy was investigated in DTT-treated cells by examining *ATG8* expression. Our results showed that DTT induces *ATG8* expression (Fig. 3A), suggesting that DTT efficiently triggers autophagy in *C. reinhardtii* Activation of autophagy by DTT was confirmed by western-blot and immunofluorescence analyses of ATG8. The abundance of this protein progressively increased in response to DTT, which was shown by western blot (Fig. 3B). In agreement with this result, the cellular distribution of ATG8 drastically changed on DTT treatment, which was observed by immunofluorescence microscopy. In untreated cells, the ATG8 signal was very weak and localized in a single spot in some cells, whereas this signal was much stronger and detected as multiple spots in response to DTT or tunicamycin treatment (Fig. 3C).

ER stress was monitored in DTT-treated cells by qPCR analysis of *CAL2*, *ERO1*, *PDI6*, and *Rb60* genes. DTT treatment resulted in the up-regulation of these four genes (Fig. 3D). This result confirmed that *CAL2*, *ERO1*, *PDI6*, and *Rb60* genes might be used as ER stress markers in *C. reinhardtii* Given the tight correlation found between ER and oxidative stresses in tunicamycin-treated cells (Fig. 2), we also analyzed the expression of the oxidative stress-related genes *GSTS1* and *GPXH*. Interestingly, reductive stress induced by DTT may indirectly lead to oxidative stress in the cell, because transcription of both *GSTS1* and *GPXH* genes was enhanced (Fig. 3E).

Glutathione Partially Suppresses ER Stress-Induced Autophagy

To test whether redox imbalance contributes to autophagy activation during ER stress, autophagy and ER stress markers were analyzed in cells treated with DTT, which massively reduces disulfide bonds. DTT was used alone or combined with GSH, an abundant antioxidant in the cell that plays an important role in ROS scavenging and maintenance of cellular redox homeostasis (Foyer and Noctor, 2011). Our results show that *ATG8* induction caused by DTT is decreased by 50% in the presence of GSH (Fig. 4A), indicating

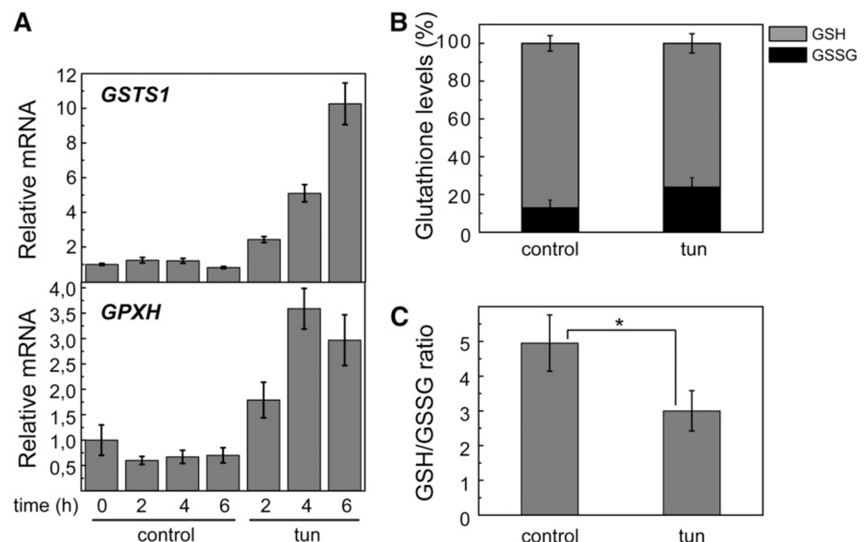

Figure 2. Tunicamycin triggers oxidative stress. A, Expression analysis of *GSTS1* and *GPXH* genes by qPCR in cells treated with 5 μg mL^{-1} tunicamycin at the indicated times. B, Intracellular pools of GSH and GSSG were determined in *C. reinhardtii* cells treated with 5 μg mL^{-1} tunicamycin or drug vehicle (control); a 100% glutathione pool corresponds to 837 $\pm$ 58 and 744 $\pm$ 64 pmol per 1 million cells for control and tunicamycin-treated cells, respectively. C, GSH-to-GSSG ratio in tunicamycin-treated cells compared with untreated cells. Values are means of three independent experiments. tun, Tunicamycin. *, Differences were significant at $P < 0.05$ according to Student's *t* test.

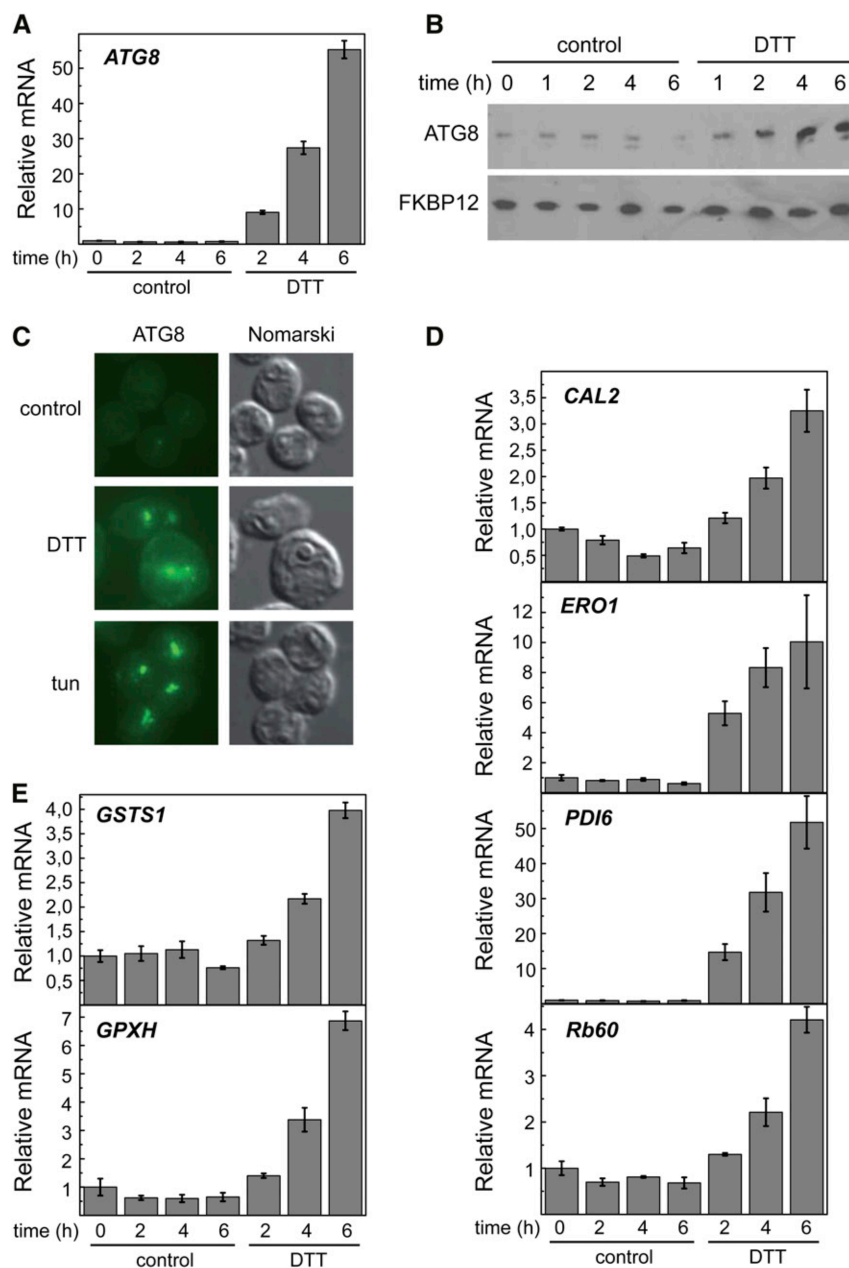

Figure 3. DTT triggers autophagy and ER stress in *C. reinhardtii*. Log-phase cells grown in TAP medium were treated with 2.5 mM DTT, and samples were taken at the indicated times for qPCR (A), western-blot (B), or immunofluorescence (C) analyses of ATG8. Immunofluorescence images correspond to 6 h of treatment. In addition to *ATG8*, expressions of the UPR-regulated genes *CAL2*, *ERO1*, *PDI6*, and *Rb60* (D) or the oxidative stress-related genes *GSTS1* and *GPXH* (E) were analyzed by qPCR. Values are means of three independent experiments.

that autophagy might be partially suppressed by GSH. Interestingly, ER stress was also decreased by exogenous GSH in DTT-treated cells, which was revealed by the lower expression of PDI6 (Fig. 4A) and other ER stress-induced genes (Supplemental Fig. S5A). Because GSH decreased both ER stress and autophagy, it was not possible to separate these two processes, and therefore, down-regulation of autophagy might be caused by the partial suppression of ER stress or might be caused by ROS scavenging or redox signaling mechanisms dependent on GSH. Thus, additional investigation of the role of oxidative stress in the control of ER stress-induced autophagy should be performed under experimental conditions, where ER stress and oxidative stress can be independently tested. We found that GSH was also able to partially suppress tunicamycin-induced expression of *ATG8*. However, unlike DTT, GSH did not significantly decrease the tunicamycin-induced expression of ER stress markers (Fig. 4B; Supplemental Fig. S5B). In agreement with decreased *ATG8* transcription, the abundance of the ATG8 protein was also down-regulated in cells treated with both tunicamycin and GSH compared with cells treated only with tunicamycin (Fig. 4C). Moreover, when the GSH concentration was increased from 5 to 10 mM, a stronger decrease of ATG8 protein level was observed (Fig. 4D). To analyze the specificity of the response to GSH, we also investigated whether ascorbate, an abundant metabolite in plant cells with antioxidant properties (Foyer and Noctor, 2011), is able to mitigate the increase of ATG8 protein level caused

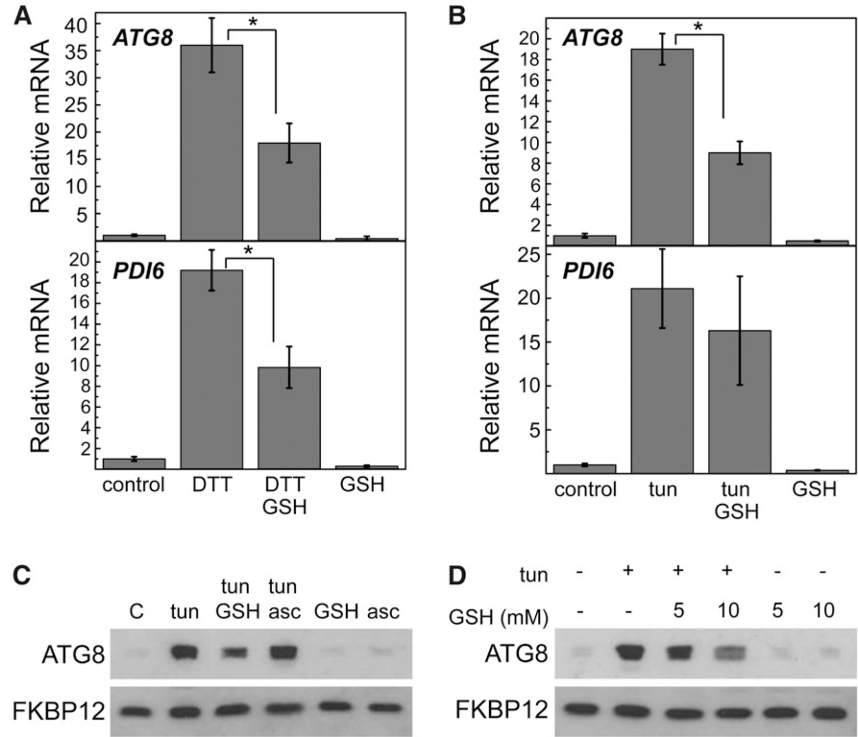

Figure 4. GSH partially prevents ER stress-induced autophagy. Log-phase cells grown in TAP medium were treated with 2.5 mM DTT (A) or 5 μg mL^{-1} tunicamycin (B) alone or combined with 10 mM GSH. After 8 h of treatment, cells were processed for expression analysis of *ATG8* and *PDI6* genes by qPCR. Values are means of three independent experiments. *, Differences were significant at $P < 0.05$ according to Student's *t* test between DTT and DTT-GSH or tunicamycin and tunicamycin-GSH. C and D, Western-blot analyses of ATG8 and FKBP12 proteins in log-phase cells treated with 5 μg mL^{-1} tunicamycin, 5 to 10 mM GSH, 2 mM ascorbate (asc), or combinations of these compounds. Thirty micrograms of total extracts were resolved by 15% SDS-PAGE followed by western blotting with anti-CrATG8 and anti-CrFKBP12 antibodies. tun, Tunicamycin.

by tunicamycin, but unlike GSH, no effect was observed on ATG8 abundance (Fig. 4C). We found, however, that ascorbate potently and rapidly increased the expression of *GSTS1*, indicating that this antioxidant is acting on the expression of some genes (Supplemental Fig. S6).

The effect of GSH on ER stress-induced autophagy was also examined by immunolocalization assays of ATG8 in *C. reinhardtii* cells. As previously shown (Pérez-Pérez et al., 2010), tunicamycin treatment strongly increased the ATG8 signal, which accumulated in a few intense spots (Fig. 5A). However, the presence of GSH in the culture medium significantly decreased the intensity of the ATG8 signal, although some spots were still clearly visible, indicating that autophagy was reduced but not fully abolished in these cells (Fig. 5A). Quantification of the immunofluorescence signal from individual cells confirmed the inhibitory effect of GSH on autophagy induced by ER stress (Fig. 5B). These results are in close agreement with qPCR and western-blot assays of ATG8 performed in tunicamycin-treated cells in the presence of exogenous GSH (Fig. 4) and strongly suggest that oxidative stress might be involved in the induction of autophagy in ER-stressed cells.

Glutathione Decreases the Toxicity of Tunicamycin in *C. reinhardtii*

Protein glycosylation in the ER is essential for cell growth, and thus, its inhibition by tunicamycin is toxic for the cell. Indeed, we found that tunicamycin reduced cell viability in *C. reinhardtii* after 24 h of treatment (Fig. 6A). Our results indicated that the presence of exogenous GSH in the medium decreased the toxicity of tunicamycin, which was revealed by Evans blue staining (Fig. 6A) or serial spot dilution assays (Fig. 6B). Interestingly, cells were still fully viable at the time of autophagy activation by tunicamycin (6–8 h; Fig. 6A), supporting a prosurvival role of this catabolic process in response to ER stress. In relation to the effect of tunicamycin on cell viability, we analyzed the expression of the *DAD1* gene, a homolog of defender against apoptotic cell death1 that is conserved in many eukaryotes, including *C. reinhardtii* (Kelleher and Gilmore, 1994, 1997; Gallois et al., 1997; Moharikar et al., 2007). The DAD1 protein is part of the oligosaccharyltransferase complex and seems to play an important role in N-linked glycosylation in the ER (Kelleher and Gilmore, 1994), a process specifically inhibited by tunicamycin. Our results revealed that *DAD1* expression was up-regulated on prolonged exposure (48 h) to tunicamycin (Fig. 6C), when cell viability is significantly compromised (Fig. 6A). No induction of *DAD1* was observed in cells treated with tunicamycin for 6 h (Supplemental Fig. S7), indicating that *DAD1* expression is associated to tunicamycin-induced loss of cell viability. In agreement with the protective role of GSH on cell growth in ER-stressed cells, the presence of this antioxidant decreased tunicamycin-mediated *DAD1* expression (Fig. 6C).

The *sor1* Mutant Displays Increased Autophagy in Response to ER Stress

To further investigate the redox regulation of ER stress-induced autophagy in *C. reinhardtii*, we analyzed the activation of this degradative pathway by tunicamycin in the *sor1* mutant. This strain lacks the

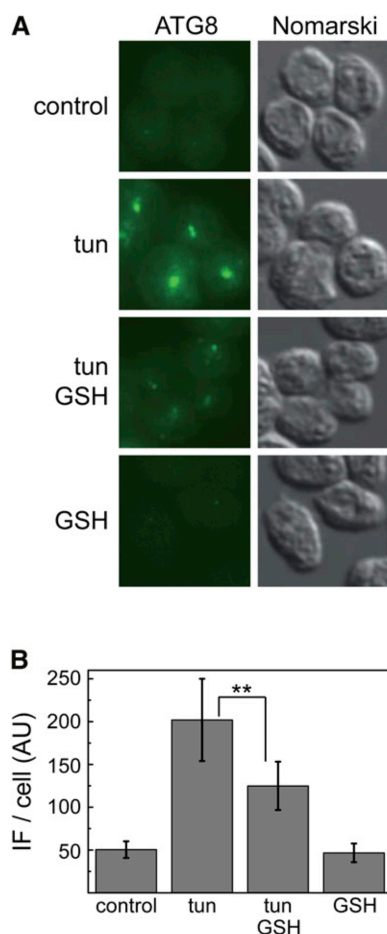

Figure 5. GSH decreases tunicamycin-induced cellular accumulation of ATG8 in *C. reinhardtii*. A, Immunolocalization of ATG8 in *C. reinhardtii* cells treated for 8 h with 5 μg mL^{-1} tunicamycin alone or combined with 10 mM GSH. Bar = 5 μm. B, Quantification of the immunofluorescence (IF) signal detected in individual cells from the experiment described in A. For each condition, a minimum of 100 individual cells was analyzed using ImageJ software. tun, Tunicamycin. **, Differences were significant at $P < 0.01$ according to Student's *t* test between tunicamycin and tunicamycin GSH.

Singlet Oxygen Resistant1 (SOR1) protein, a putative bZIP transcription factor that controls the expression of a large number of oxidative stress response and detoxification genes, including *GSTS1* (Fischer et al., 2012). Treatment of *sor1* cells with tunicamycin revealed a more pronounced accumulation of the ATG8 protein in the *sor1* mutant compared with wild-type cells (Fig. 7A), suggesting a higher induction of autophagy in this mutant in response to ER stress. Expression analysis of the *ATG8* gene also showed a higher response to tunicamycin in *sor1* compared with cw15 cells (Fig. 7B). As previously reported (Fischer et al., 2012), we found that *sor1* mutant cells show a constitutively higher expression of *GSTS1* (Fig. 7B). Interestingly, despite this elevated expression, tunicamycin further increased *GSTS1* mRNA abundance in the *sor1* mutant (Fig. 7B). Taken together, these results strongly suggested that autophagic activity triggered by ER stress is higher in cells with anomalous expression of oxidative stress-related genes.

DISCUSSION

In a previous report, we showed that ER stress induces autophagy in *C. reinhardtii* (Pérez-Pérez et al., 2010). In this study, we show that ER stress mediated by tunicamycin or DTT treatment strongly increased the expression of the *ATG8* gene, which is in agreement with the pronounced accumulation of the ATG8 protein and its modified forms detected in ER-stressed cells (Figs. 1 and 3). Induction of autophagy by ER stress is evolutionarily conserved and has been reported in yeast (Bernales et al., 2006; Yorimitsu et al., 2006), mammals (Ogata et al., 2006), and more recently, plants (Liu et al., 2012). Studies performed in yeast strongly suggest that autophagy serves to counterbalance ER expansion that occurs as a consequence of UPR signaling (Bernales et al., 2006). Degradation of ER membranes by autophagy during ER stress is conserved in plants, because this type of membrane has been detected inside autophagic bodies in Arabidopsis plants treated with tunicamycin (Liu et al., 2012).

This study also showed that the accumulation of unfolded proteins in the ER triggers oxidative stress in *C. reinhardtii* Formation of disulfide bonds in the ER and associated oxidative protein folding are linked to the generation of hydrogen peroxide by the activity of the ERO1 oxidoreductase that catalyzes the reoxidation and activation of PDI (Tu and Weissman, 2004). Originally identified in yeast (Frand and Kaiser, 1998; Pollard et al., 1998), ERO1 is widely conserved in all eukaryotes, including plants (Aller and Meyer, 2013). In this study, we found that ERO1 is conserved in *C. reinhardtii* and that its expression is highly induced by the ER stressors tunicamycin and DTT (Figs. 1 and 3). ERO1 is a hydrogen peroxide-generating enzyme, and its high expression may lead to increased levels of hydrogen peroxide under ER stress. Indeed, our results indicated that *C. reinhardtii* cells subjected to ER stress displayed elevated expression of oxidative stress-related genes and higher levels of the intracellular pool of GSSG (Figs. 2, B and C and 3E). Thus, different lines of evidence indicate that ER stress triggers oxidative stress in *C. reinhardtii*. In yeast, ROS, such as hydrogen peroxide, are generated during ER stress, and overexpression of ERO1 causes a significant increase in ROS and GSSG pool, suggesting that up-regulation of the oxidative protein folding machinery by the UPR contributes to ROS accumulation (Haynes et al., 2004). In a recent report, it has also been shown that ER stress triggers ROS signaling in plants (Ozgur et al., 2014), indicating that ROS production on ER stress is widely conserved.

In addition to *ERO1*, we identified other genes subjected to UPR regulation in *C. reinhardtii*. The expression levels of two PDI genes, *Rb60* and *PDI6*, and the *CAL2* chaperone were increased in response to ER stress (Figs. 2 and 4). Rb60 is a canonical PDI with an atypical

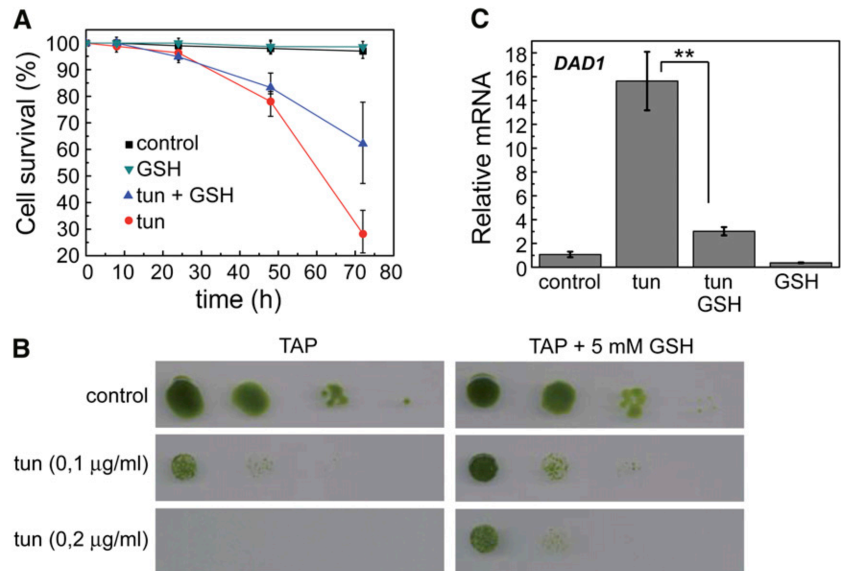

Figure 6. GSH reduces the toxicity of tunicamycin in *C. reinhardtii*. A, Log-phase cells grown in TAP medium were treated with tunicamycin (5 μg mL^{-1}), GSH (10 mM), or both compounds combined. Untreated cells were used as control. Cell viability was determined by Evans blue staining at the indicated times. Results are means of four independent experiments. B, Cells were subjected to 10-fold serial dilutions and spotted onto TAP plates containing the indicated concentrations of tunicamycin and GSH. Plates were grown at 25°C under continuous illumination for 5 d. C, Expression analysis of the *DAD1* gene by qPCR. Log-phase cells were treated with 5 μg mL^{-1} tunicamycin alone or combined with 10 mM GSH for 48 h and then processed for RNA isolation and analysis. Values are means of three independent experiments. tun, Tunicamycin. **, Differences were significant at $P < 0.01$ according to Student's t test between tunicamycin and tunicamycin-GSH.

dual localization in the ER and the chloroplast (Levitan et al., 2005), where it regulates psbA translation by light (Danon and Mayfield, 1991). The function of Rb60 in the ER is unclear, although based on its high identity to classical PDI and its localization, it likely participates in oxidative protein folding in the ER. *PDI6* encodes a PDI-like protein containing an N-terminal J domain that is conserved among ER cochaperones (Schroda, 2004) and a C-terminal Arg-rich domain involved in ER stress quality control in mammals (Mizobuchi et al., 2007). The domain architecture of PDI6 seems to be well conserved in microalgae and less represented in plants, where the J domain-containing protein ATERDJ3A from Arabidopsis seems to be the closest homolog (Supplemental Fig. S3). No clear homologs of this protein have been found in yeasts or humans. Our results revealed that *PDI6* mRNA levels are particularly sensitive to ER stress, and a massive increase in the expression of this gene was observed with tunicamycin or DTT (Figs. 1, 3, and 4). Expression of the calreticulin *CAL2* gene was also found to be up-regulated by the UPR in *C. reinhardtii* (Figs. 1, 3, and 4). Similar to ERO1, CAL2 is conserved in plants and other systems (Supplemental Fig. S3), and microarray analysis of Arabidopsis seedlings treated with tunicamycin or DTT suggested that this gene is up-regulated under ER stress conditions (Martínez and Chrispeels, 2003). Our results indicate that the abundance of *ERO1*, *Rb60*, *PDI6*, or *CAL2* mRNAs can be used as markers of the UPR in *C. reinhardtii* and strongly suggest that the mechanisms of oxidative protein folding described in other eukaryotes are conserved in green algae.

Given that autophagy is regulated by ROS (Pérez-Pérez et al., 2012b) and ER stress triggers oxidative stress responses in *C. reinhardtii* (Fig. 2), ER stress-derived ROS may likely contribute to autophagy regulation in response to this stress (Fig. 8). In close agreement with this hypothesis, we found that the antioxidant GSH down-regulates autophagy in ER-stressed cells (Figs. 4 and 5). Glutathione is the main free soluble thiol in the cell and acts as a redox buffer by maintaining the intracellular environment in a reduced state (Foyer and Noctor, 2011). Moreover, glutathione has also been shown to play a protective role from ER-generated oxidative stress. Prolonged UPR activation in a yeast strain that is genetically predisposed to sustained ER stress causes oxidative stress, ultimately leading to cell death (Haynes et al., 2004). In this strain, the presence of GSH in the culture medium relieved oxidative stress and prevented cell death without diminishing UPR activation, indicating that ROS accumulate during ER stress and are toxic for the cell (Haynes et al., 2004). The effect of GSH in alleviating oxidative stress and promoting growth of ER-stressed cells might be conserved in *C. reinhardtii*, because our results showed that GSH decreases the toxicity of tunicamycin (Fig. 6). The protective role of GSH was emphasized by the finding that this antioxidant decreased the expression of the *DAD1* gene, which is specifically up-regulated when cell viability is compromised because of prolonged ER stress (Fig. 6C). *DAD1* is a putative antiapoptotic gene widely conserved in eukaryotes, including humans, plants, yeasts, and algae (Nakashima et al., 1993; Kelleher and Gilmore, 1994, 1997; Gallois et al., 1997; Moharikar et al., 2007). The DAD1 protein is localized in the ER and forms part of the oligosaccharyltransferase complex, which catalyzes N-linked glycosylation (Kelleher and Gilmore, 1994). Although the precise function of this protein is not clear, DAD1 seems

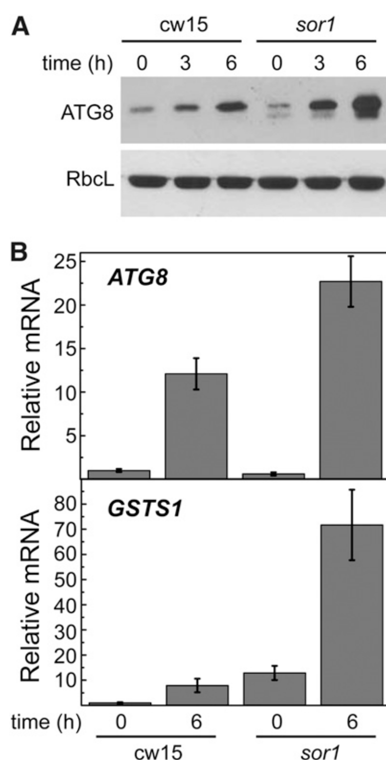

Figure 7. The *sor1* mutant displays increased autophagy in response to ER stress. A, Log-phase cw15 and *sor1* cells were treated with tunicamycin (5 μg mL^{-1}), and at the indicated times, samples were taken and processed for western-blot analysis. B, Expression analysis of *ATG8* and *GSTS1* genes by qPCR. Log-phase cw15 and *sor1* cells were treated with tunicamycin (5 μg mL^{-1}) for 6 h and subsequently subjected to RNA isolation and analysis. Values are means of three independent experiments. RbcL, Rubisco large subunit.

to be involved in preventing cell death and regulating N-linked glycosylation. Our results are in agreement with these putative roles, because expression of the *C. reinhardtii DAD1* gene is induced by tunicamycin treatment, which is toxic for the cell and specifically inhibits N-linked glycosylation.

The hypothesis that ER stress-induced autophagy and redox signaling are linked was strengthened by the finding that tunicamycin triggers autophagy more potently in *sor1* mutant cells (Fig. 7). The *sor1* strain was isolated in a screen for mutants with increased tolerance to singlet oxygen and shows a constitutively higher expression of a large number of oxidative stress response and detoxification genes (Fischer et al., 2012). Mapping of the *sor1* mutation identified a putative bZIP transcription factor denoted as SOR1 that controls, among other things, the expression of the glutathione *S*-transferase gene *GSTS1* (Fischer et al., 2012), which is up-regulated in ER-stressed cells (Fig. 2B). The high autophagic activity observed in *sor1* mutant cells treated with tunicamycin might be related to the enhanced expression of ROS-induced genes reported in this mutant, because our results indicated that ER stress-induced autophagy is linked to redox signaling in *C. reinhardtii* (Figs. 2, 4, and 5). Given the role of SOR1 in the control of glutathione-based ROS scavenging by regulating the expression of genes, such as *GSTS1* or *GPXH* (Fischer et al., 2012), and the regulatory function of this antioxidant in ER stress-induced autophagy (Figs. 4 and 5), it is possible that the up-regulation of autophagy observed in the *sor1* mutant is related to the high expression of these genes, which may result in reduced intracellular levels of glutathione in *sor1* cells.

Overall, our results indicate that ER stress triggers autophagy and oxidative stress in *C. reinhardtii*. Under ER stress triggered by tunicamycin, GSH was found to decrease autophagy induction, whereas UPR markers remained unaffected. This uncoupling of UPR and autophagy in the presence of an antioxidant participating in diverse ROS scavenging mechanisms suggests that ROS production may participate in the control of ER stress-induced autophagy in *C. reinhardtii* (Fig. 8). However, GSH effects on ER stress may involve other mechanisms not associated with ROS scavenging. For example, given the protective role that glutathione plays in the ER (Haynes et al., 2004), it may also be possible that glutathione specifically regulates autophagy by restoring the GSH-to-GSSG ratio in the ER, which is critical for maintaining the functionality and redox balance at this cellular compartment. Glutathione or redox imbalance may also play a signaling role during ER stress (e.g. through glutathionylation of specific target proteins; Zaffagnini et al., 2012). In this work, we have established the importance of the intracellular redox state and glutathione during ER stress-induced autophagy. Additional studies will be required to unravel how redox active molecules control autophagy induction and examine the interplay between these mechanisms and other ER stress signaling pathways.

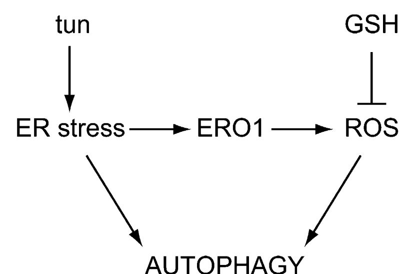

Figure 8. Control of autophagy by ER stress and ER-derived ROS in *C. reinhardtii*. Tunicamycin (tun) treatment results in toxic accumulation of unfolded proteins in the ER, leading to autophagy activation as a defensive mechanism. ER stress also increases ROS production in the ER by de oxidoreductase ERO1, which contributes to the up-regulation of autophagy. The antioxidant properties of GSH counterbalance ROS signaling to the autophagic machinery but do not abrogate ER stress, resulting in partial inactivation of autophagy.

MATERIALS AND METHODS

Strains and Growth Conditions

Chlamydomonas reinhardtii cell wall-deficient cw15 4B+ and *sor1* mutant 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 per milliliter) were treated with 5 μg mL^{-1} tunicamycin (654380; Calbiochem) from 5 mg mL^{-1} stock in dimethylformamide, 2.5 mM DTT (A2948; Applichem), 1 mM hydrogen peroxide (H1009; Sigma-Aldrich), 1 μM MV (85617-7; Sigma-Aldrich), or 20 μM norflurazon (PS1044; Sigma-Aldrich).

Gene Accession Numbers

C. reinhardtii genes analyzed in this study were identified at the Phytozome Web site (http://www.phytozome.net/cgi-bin/gbrowse/chlamy/) under the following accession numbers: *ATG3*, Cre02.g102350.t1.2; *ATG8*, Cre16.g689650.t1.2; *CAL2*, Cre01.g038400.t1.2; *ERO1*, Cre17.g723150.t1.3; *PDI6*, Cre12.g518200.t1.3; *Rb60*, Cre02.g088200.t1.2; *GPXH*, Cre10.g458450.t1.3; *GSTS1*, Cre16.g688550.t1.2; and *DAD1*, Cre02.g108400.t1.2.

RNA Isolation and Quantification

C. reinhardtii total RNA was isolated from frozen cell pellets as previously described (Crespo et al., 2005). First strand complementary DNA was produced using 2 μg of total RNA, oligo(dT) primer, and 100 units of SuperScript II RNase H reverse transcription (18064-014; Invitrogen) in a 50-μL reaction. Reverse transcription qPCR was performed with a StepOne Real-Time PCR System (Applied Biosystems). PCR reactions, in a final volume of 20 μL, contained 10 μL of FastStart Universal SYBR Green Master (04913850001; Roche), 1 μL of complementary DNA dilution, 250 nM each primer, and distilled water up to 20 μL. Conditions used for amplification in the thermocycler were preincubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 55°C to 58°C (depending on the gene analyzed) for 60 s. All reactions were performed in triplicate with two of four biological replicates. The CBLP gene was used as a control constitutively expressed gene (Pootakham et al., 2010). The primer pairs used for qPCR were 5′-CTTCTCGCCCATGACCAC-3′ and 5′-CCCACCAG-GTTGTTCTTCAG-3′ for *CBLP*, 5′-CGAGTTCAAGGTCGAGCAGT-3′ and 5′-CCACCCACAGACATGGTGTA-3′ for *ATG3*, 5′-TCCCCGATATCGACA-AGAAG-3′ and 5′-TGCGGATGACGTACACAAAT-3′ for *ATG8*, 5′-ACCC-TGACTACGTCCACGAC-3′ and 5′-GTCCTCAGCGAACTTCTTGG-3′ for *CAL2*, 5′-TGTCAACCTGCTCATCAACC-3′ and 5′-CTGCTGCTGCTACTGCTGTC-3′ for *ERO1*, 5′-GGTGTGGCTGGTTGAGTTCT-3′ and 5′-CTCTTTGGCGTCCTCA-CAGT-3′ for *PDI6*, 5′-CCAAGCGCTTTAAGAAGGTG-3′ and 5′-GTAGG-GAAGCCCTTGACCTC-3′ for Rb60, 5′-AGGTTCTGGATGCGTTCCTA-3′ and 5′-ACACAGTCAGGGCGAAGAAG-3′ for *DAD1*, 5′-GCGGTCGCCAATAAC-CAAT-3′ and 5′-AAGGGCTGTCCCGAAAGC-3′ for *GPXH* (Fischer et al., 2009), 5′-CTGACCATCAGCCACGACT-3′ and 5′-ACATCGAACACCAGGGTAGC-3′ for *FKBP12*, and 5′-CAGAGGTGAAAGGCGGATAC-3′ and 5′-GTGTTGCA-ATGGACTTCAGC-3′ for *GSTS1* (Fischer et al., 2012).

Protein Preparation and Immunoblot Analysis

C. reinhardtii cells from liquid cultures were collected by centrifugation (4,000*g* for 5 min), washed one time 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 (15,000*g* for 15 min) in a microcentrifuge at 4°C. For immunoblot analyses, total protein extracts (30 μg) were subjected to 15% SDS-PAGE and then transferred to nitrocellulose membranes (162-0115; Bio-Rad). Anti-CrATG8 (Pérez-Pérez et al., 2010) and secondary antibodies were diluted 1:2,500 and 1:10,000, respectively, in PBS containing 0.1% (v/v) Tween 20 (A4974; Applichem) and 5% (w/v) milk powder. The ECL-Plus immunoblotting detection system (RPN2132; GE Healthcare) was used to detect the proteins with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (A6154; Sigma-Aldrich). Anti-FKBP12 antibody was diluted 1:3,000 and used as the loading control as described previously (Crespo et al., 2005). Proteins were quantified with the Coomassie Brilliant Blue dye-binding method as described by the manufacturer (500-0006; Bio-Rad).

Glutathione Determination

C. reinhardtii cells were collected by centrifugation (5,000*g* for 5 min), washed one time in 50 mM sodium phosphate (pH 7.5) buffer, resuspended in 0.2 N HCl, and lysed by two cycles of frozen-thawed at −80°C. Crude extracts were cleared by centrifugation at 15,000*g* for 20 min at 4°C, and 500 μL of sample was neutralized by adding 50 μL of 50 mM NaH_2PO_4 (pH 7.5) and 0.2 N NaOH to a final pH between 5 and 6. The neutralized sample was directly used for measuring total glutathione (GSH plus GSSG) by the recycling assay initially described by Tietze (1969) and adapted by Queval and Noctor (2007). The method relies on the glutathione reductase-dependent reduction of 5,5′-dithiobis(2-nitro-benzoic acid) (DTNB; D8130; Sigma). GSSG was measured after treatment of the neutralized sample with 10 mM 4-vinylpyridine (V320-4; Sigma) for 30 min at 25°C. To remove excess 4-vinylpyridine, the derivatized sample was centrifuged two times at 15,000*g* for 20 min at 4°C. To measure total glutathione or GSSG, sample was added to a mix containing 120 mM NaH_2PO_4 (pH 7.5), 300 μM DTNB, 500 μM NADPH, 1 mM EDTA (pH 8), and 1 units mL^{-1} glutathione reductase (G3664; Sigma), and DTNB reduction was measured at 412 nm. Different GSH (G4251; Sigma) concentrations ranging from 0 to 5 μM were used as standards.

Viability Assay

Cell viability was estimated by determining the percentage of *C. reinhardtii* cells that excluded Evans blue dye (E2129; Sigma), which only stains nonviable cells; 450 μL of *C. reinhardtii* cells was incubated with 0.1% (w/v) Evans blue for 5 min, washed one time with 500 μL of TAP medium, and resuspended in an equal volume of TAP medium. Cells were examined in a phase contrast microscope to visualize uptake of the dye.

Fluorescence Microscopy

C. reinhardtii cells were fixed and stained for immunofluorescence microscopy as previously described (Diaz-Troya et al., 2008a; Pérez-Pérez et al., 2010). Affinity-purified polyclonal anti-ATG8 was used as the primary antibody at 1:500 dilution. For signal detection, a fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:500; F4890; Sigma-Aldrich) 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). For the comparative analysis of the fluorescein isothiocyanate signal from different samples, the same acquisition time was fixed. Immunofluorescence signals in individual cells were quantified using the ImageJ software.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Analysis of autophagy induction by different stress conditions in *C. reinhardtii*.

Supplemental Figure S2. Expression analysis of the *FRKB12* gene by qPCR.

Supplemental Figure S3. Domain structure and amino acid sequence analysis of CAL2, ERO1, and PD16 proteins from *C. reinhardtii*.

Supplemental Figure S4. Effect of thapsigargin on autophagy and ER stress.

Supplemental Figure S5. Expression analysis of *CAL2*, *ERO1*, and *Rb60* genes by qPCR.

Supplemental Figure S6. Expression analysis of the *GSTS1* gene by qPCR in cells treated with ascorbate.

Supplemental Figure S7. *DAD1* expression is up-regulated upon prolonged ER stress.

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