**Running title:** ROS trigger autophagy upon ER stress

**Corresponding author:**

José L. Crespo

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Sevilla, 41092 Seville, Spain

e-mail: crespo@ibvf.csic.es

Tel: +34 954489574

Fax: +34 954460065

**Research Area:**

Signaling and Response

**Reactive oxygen species mediate autophagy induction in response to endoplasmic reticulum stress in Chlamydomonas**

Marta Pérez-Martín[1], María Esther Pérez-Pérez[2,3], Stéphane D. Lemaire[2,3] and José L. Crespo[1]*

[1]Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas (CSIC)-Universidad de Sevilla, 41092 Seville, Spain

[2]Centre National de la Recherche Scientifique, UMR8226, Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, Institut de Biologie Physico-Chimique, 75005 Paris, France

[3]Sorbonne Universités, UPMC Univ Paris 06, UMR8226, Laboratoire de Biologie Moléculaire et Cellulaire des Eucaryotes, Institut de Biologie Physico-Chimique, 75005 Paris, France

**Summary:**

Reactive oxygen species generated during endoplasmic reticulum stress contribute to autophagy activation in Chlamydomonas.

**Footnotes:**

This work was supported in part by an IEF EU Marie Curie Fellowship (PIEF-GA-2011-298652-REDOXDYNAMICS to MEPP), by LABEX DYNAMO ANR-11-LABX-0011 (to MEPP and SDL), by the Spanish Ministry of Economy and Competitiveness (grants BFU-2009-07368 and BFU2012-35913 to JLC), and by Junta de Andalucía (grant CVI-7336 to JLC).

**Corresponding author:**

José L. Crespo (crespo@ibvf.csic.es)

**ABSTRACT**

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 (ROS), which play an important role in autophagy regulation. However, it remains unknown whether ROS are involved in ER stress-induced autophagy. In the present study, we provide evidence connecting ROS generated during ER stress and autophagy activation in the model unicellular green alga *Chlamydomonas reinhardtii*. Treatment of Chlamydomonas cells with the ER stressors tunicamycin or dithiothreitol resulted in upregulation of the expression of genes encoding ER resident ERO1 oxidoreductase and protein disulfide isomerases. ER stress also triggered autophagy in Chlamydomonas based on the protein abundance, lipidation, cellular distribution and mRNA levels of the autophagy marker ATG8. Moreover, an increase in the level of ROS, in the oxidation of the glutathione pool and in 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, indicating that oxidative stress participates in the control of autophagy in response to ER stress in Chlamydomonas. In close agreement, we also found that autophagy activation by tunicamycin was more pronounced in the Chlamydomonas *sor1* mutant, which shows increased expression of oxidative stress-related genes.

## INTRODUCTION

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 non-selectively 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 yeasts 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., 2008; 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 (PE), and the ATG8-PE conjugate is essential for the formation and completion of the autophagosome (Ichimura et al., 2000). *ATG* genes are conserved in Chlamydomonas (Diaz-Troya et al., 2008; Perez-Perez 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 Chlamydomonas (Perez-Perez et al., 2010). Moreover, entry of Chlamydomonas cells into stationary growth phase activates autophagy in a reversible manner since the process is quickly downregulated when cells return to exponential growth (Perez-Perez et al., 2010). Autophagy is also induced in Chlamydomonas cells treated with $H_2O_2$ or methyl viologen (MV), indicating that oxidative stress triggers this process in algae (Perez-Perez et al., 2010; Perez-Perez et al., 2012a). Reactive oxygen species (ROS) are potent inducers of autophagy in Chlamydomonas and plants (Liu and Bassham, 2012; Perez-Perez et al., 2012b). Indeed, a link between photo-oxidative damage, ROS accumulation and autophagy activation has been shown in Chlamydomonas cells with a decreased carotenoid content caused by either the mutation of phytoene synthase or the inhibition of phytoene desaturase by the herbicide norflurazon (Perez-Perez et al., 2012a). Moreover, ROS generated in the chloroplast of carotenoid-deficient cells or in the chloroplast of wild-type cells subjected to high light stress activate autophagy (Perez-Perez et al., 2012a).

The accumulation of un/misfolded proteins in the endoplasmic reticulum (ER) is known to trigger autophagy in yeasts (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 (Perez-Perez 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 Chlamydomonas, tunicamycin, which induces ER stress by inhibition of *N*-linked glycosylation, strongly increases the abundance and lipidation of ATG8 and modifies its cellular distribution (Perez-Perez 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 IRE1 kinase, which transduces stress signals to the nucleus by promoting the splicing of bZIP-like transcription factors (Walter and Ron, 2011). Eukaryotic cells employ a quality control mechanism that recognizes aberrantly folded proteins in the ER for their degradation via the proteasome, a process that is known as ER-associated degradation (ERAD) (Walter and Ron, 2011). Prolonged ER stress also triggers autophagy to remove unfolded proteins and to 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 the ER oxidoreductin 1 (ERO1) is a major source of ROS in the ER and in 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 via protein disulfide isomerases (PDIs). To introduce disulfides into client proteins, PDI must be maintained in an oxidized state, and ERO1 is the main responsible for keeping PDI oxidized and active in the ER (Frand and Kaiser, 1999). ERO1 utilizes molecular oxygen as final electron acceptor and hence forms one molecule of $H_2O_2$ for every disulfide 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; Perez-Perez 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 the present study, we provide evidence connecting autophagy activation in ER-stressed cells with ROS generated from the UPR-regulated oxidative folding machinery in Chlamydomonas, and propose that oxidative stress contributes to the induction of autophagy by ER stress.

## RESULTS

### Tunicamycin triggers ER stress and autophagy in Chlamydomonas

In a previous study, we showed that tunicamycin triggers autophagy in Chlamydomonas (Perez-Perez 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). 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 and also with other stressors previously shown to induce autophagy such as $H_2O_2$, methyl viologen or norflurazon (Perez-Perez et al., 2010; Perez-Perez et al., 2012a) (SFig. 1). As expected, tunicamycin had no effect on the expression of *FKBP12*, an ER stress unrelated gene (SFig 2).

The cellular response to ER stress in Chlamydomonas is still poorly characterized and no markers have been established to investigate this process in this model alga. In Arabidopsis, it has been reported that expression of some UPR genes such as the ER-resident chaperone Calreticulin-2 (At1g09210) increases in response to tunicamycin (Martinez and Chrispeels, 2003). We identified a member of the calreticulin family in Chlamydomonas nuclear genome (SFig. 3), which we denoted as *CAL2*. To investigate whether the expression of *CAL2* is subject to UPR regulation in Chlamydomonas, transcription of this gene was determined by qPCR

analysis in cells treated with tunicamycin or with other stressors that should not trigger ER stress. *CAL2* mRNA levels were increased with tunicamycin (Fig. 1C) but not with $H_2O_2$, methyl viologen or norflurazon (SFig. 1A), demonstrating that this gene can be used to monitor ER stress in Chlamydomonas.

ERO1 has not been previously described in algae but based on its high evolutionary conservation we identified an *ERO1* homologue in the Chlamydomonas genome (SFig. 3). To investigate the participation of Chlamydomonas *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 yeasts, ERO1 and PDI expression 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 Chlamydomonas, although other PDI-like proteins appear to be conserved in the Chlamydomonas genome (Lemaire and Miginiac-Maslow, 2004; Filonova et al., 2013). Rb60 appears 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 upregulated (Fig. 1C). Searching for PDI proteins other than Rb60 in Chlamydomonas, we identified a PDI-like protein that we denoted as PDI6 containing an N-terminal DnaJ domain (SFig. 3). 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 Chlamydomonas.

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 Chlamydomonas (SFig. 4).

**Tunicamycin triggers oxidative stress**

We explored the possible induction of oxidative stress caused by the accumulation of misfolded proteins in the ER through different approaches. First, we determined the level of ROS in tunicamycin-treated cells with $H_2$DCFDA, a widely used probe for detection of $H_2O_2$ in different systems (Rhee et al., 2010). A progressive accumulation of ROS was detected in tunicamycin-treated cells, reaching three times the level observed in untreated cells after 8 hours of treatment (Fig. 2A). Second, we analyzed in ER-stressed cells the expression of *GSTS1* and *GPXH* genes, which is regulated by ROS in Chlamydomonas (Ledford et al., 2007; Fischer et al., 2012). Our results revealed a high induction of *GSTS1* and a moderate increase of *GPXH* expression in response to tunicamycin treatment (Fig. 2B). Finally, the intracellular pool of total glutathione, comprising reduced (GSH) and oxidized (GSSG) glutathione, was determined in cells treated with tunicamycin. Our results revealed that ER stress raised the intracellular concentration of GSSG, decreasing

the GSH/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 Chlamydomonas.

**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 Chlamydomonas 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 Chlamydomonas. Activation of autophagy by DTT was confirmed by western blot and immunofluorescence analysis of ATG8. The abundance of this protein progressively increased in response to DTT as shown by western blot (Fig. 3B). In agreement with this result, the cellular distribution of ATG8 drastically changed upon DTT treatment as 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 upregulation of these four genes (Fig. 3D). This result confirmed that *CAL2*, *ERO1*, *PDI6* and *Rb60* genes might be used as ER stress markers in Chlamydomonas. 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 since transcription of both *GSTS1* and *GPXH* genes was enhanced (Fig. 3E).

**Glutathione partially suppresses ER stress-induced autophagy**

To test whether ROS contribute to autophagy activation during ER stress, autophagy and ER stress markers were analyzed in cells treated with DTT, which massively reduces disulfide bonds, in combination with reduced glutathione (GSH), an abundant antioxidant in the cell that plays an important role in ROS scavenging and in the 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 that autophagy might be partially suppressed by GSH. Interestingly, ER stress was also decreased by exogenous GSH in DTT treated cells, as revealed by the lower expression of PDI6 (Fig. 4A) and other ER stress-induced genes (SFig. 5A). Since GSH decreased both ER stress and autophagy, it was not possible to separate these two processes and therefore downregulation of autophagy might be due to the partial suppression of ER stress or to GSH-dependent ROS scavenging. Thus, further investigation of the

role of ROS in the control of ER stress-induced autophagy should rather be performed under experimental conditions where these processes can be independently tested. We found that GSH was also able to partially suppress tunicamycin-induced expression of *ATG8* although unlike DTT, tunicamycin did not decrease the expression of ER stress markers (Fig. 4B and SFig. 5B). In agreement with decreased *ATG8* transcription, the abundance of the ATG8 protein was also downregulated in cells treated with both tunicamycin and GSH compared to 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 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 (SFig. 6).

The effect of GSH on ER stress-induced autophagy was also examined by immunolocalization assays of ATG8 in Chlamydomonas cells. As previously shown (Perez-Perez 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.

**Regulation of ER stress-induced autophagy is independent of light and NADPH oxidases**

In a previous study we have shown that activation of autophagy upon photo-oxidative damage in Chlamydomonas is fully dependent of light (Perez-Perez et al., 2012a). However, the finding that tunicamycin or DTT trigger autophagy under light or dark conditions (SFig. 7A) excludes photosynthetically-generated ROS as regulators of this degradative process in ER-stressed cells. It has been shown that ROS generated by plant NADPH oxidases appear to be required for the activation of autophagy in response to nutrient depletion or salt stress (Liu et al., 2009) but not in response to ER stress (Liu et al., 2012). Our results indicate that, like in plants, NADPH oxidases are not involved in the control of ER stress-induced autophagy since the use of the NADPH oxidase inhibitor DPI did not prevent tunicamycin-induced autophagy (SFig. 7B). Thus, the regulation of autophagy in ER-stressed cells does not seem to be mediated by chloroplast- or NADPH oxidase-generated ROS in Chlamydomonas.

**Glutathione decreases the toxicity of tunicamycin in Chlamydomonas**

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 Chlamydomonas after 24 hours of treatment (Fig. 6A). Our results indicated that the presence of exogenous GSH in the medium decreased the toxicity of tunicamycin as 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 hours; Fig. 6A), supporting a pro-survival 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 homologue of defender against apoptotic cell death 1 that is conserved in many eukaryotes including Chlamydomonas (Kelleher and Gilmore, 1994; Gallois et al., 1997; Kelleher and Gilmore, 1997; Moharikar et al., 2007). The DAD1 protein is part of the oligosaccharyltransferase complex and appears 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 upregulated upon prolonged exposure (48 hours) 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 hours (SFig. 8), 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 Chlamydomonas we analyzed the activation of this degradative pathway by tunicamycin in the *sor1* mutant. This strain lacks the 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 cw15 and *sor1* cells with tunicamycin revealed a more pronounced accumulation of the ATG8 protein in the *sor1* mutant (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 to 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 demonstrated that ER stress induces autophagy in Chlamydomonas (Perez-Perez et al., 2010). In this study we show that the accumulation of misfolded proteins in the ER under tunicamycin or DTT treatment strongly increased the expression of the *ATG8* gene, 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 yeasts (Bernales et al., 2006; Yorimitsu et al., 2006), mammals (Ogata et al., 2006) and more recently in plants (Liu et al., 2012). Studies performed in yeasts 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 via autophagy during ER stress is conserved in plants since this type of membrane has been detected inside autophagic bodies in Arabidopsis plants treated with tunicamycin (Liu et al., 2012).

The present study also demonstrated that the accumulation of unfolded proteins in the ER triggers oxidative stress in Chlamydomonas. Formation of disulfide bonds in the ER and associated oxidative protein folding are linked to the generation of $H_2O_2$ by the activity of the ERO1 oxidoreductase that catalyzes the re-oxidation and activation of PDI (Tu and Weissman, 2004). Originally identified in yeasts (Frand and Kaiser, 1998; Pollard et al., 1998), ERO1 is widely conserved in all eukaryotes including plants (Aller and Meyer, 2012). In this study, we found that ERO1 is conserved in Chlamydomonas and its expression is highly induced by the ER-stressors tunicamycin and DTT (Figs. 1 and 3). ERO1 being an $H_2O_2$-generating

enzyme, its elevated expression may lead to increased ROS production under ER stress. Indeed, our results indicated that ROS accumulate in ER-stressed cells (Fig. 2A). Moreover, Chlamydomonas cells subjected to ER stress displayed elevated expression of oxidative stress-related genes and higher levels of the intracellular pool of GSSG (Fig. 2B, 2C and 3E). Thus, different lines of evidence indicate that ER stress triggers oxidative stress in Chlamydomonas. In yeasts, ROS such as $H_2O_2$ are generated during ER stress and overexpression of ERO1 causes a significant increase in ROS and GSSG pool, suggesting that upregulation 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 upon ER stress is widely conserved.

In addition to *ERO1*, we identified other genes subjected to UPR regulation in Chlamydomonas. 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 dual localization in the ER and in 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 DnaJ domain that is conserved among ER co-chaperones (Schroda, 2004) and a C-terminal arginine-rich domain involved in ER stress quality control in mammals (Mizobuchi et al., 2007). The domain architecture of PDI6 appears to be well conserved in microalgae and less represented in plants, where the DnaJ domain-containing protein ATERDJ3A from *Arabidopsis thaliana* seems to be the closest

homologue (SFig. 3). No clear homologues 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 (Fig. 1, 3 and 4). Expression of the calreticulin *CAL2* gene was also found to be upregulated by the UPR in Chlamydomonas (Fig. 1, 3 and 4). Similar to ERO1, CAL2 is conserved in plants and other systems (SFig. 3), and microarray analysis of Arabidopsis seedlings treated with tunicamycin or DTT suggested that this gene is upregulated under ER stress conditions (Martinez 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 Chlamydomonas 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 (Perez-Perez et al., 2012b) and ER stress leads to ROS production in Chlamydomonas (Fig. 2), ER stress-derived ROS may likely contribute to autophagy regulation in response to this stress (Fig. 8). Indeed, the finding that the antioxidant GSH downregulates autophagy in ER-stressed cells (Figs. 4 and 5) strongly supports the implication of ROS in the control of ER stress-induced autophagy. 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). 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 Chlamydomonas since 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 upregulated when cell viability is compromised due to prolonged ER stress (Fig. 6C). *DAD1* is a putative anti-apoptotic gene widely conserved in eukaryotes including humans, plants, yeasts and algae (Nakashima et al., 1993; Kelleher and Gilmore, 1994; Gallois et al., 1997; Kelleher and Gilmore, 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 appears to be involved in preventing cell death and in regulating *N*-linked glycosylation. Our results are in agreement with these putative roles since expression of the Chlamydomonas *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 others the expression of *GSTS1* (Fischer et al., 2012), which is upregulated in ER-stressed cells (Fig. 2B). The high autophagic activity observed in *sor1* mutant cells treated

with tunicamycin might be related to the increased expression of *GSTS1* and other SOR1-controlled genes in this mutant, emphasizing again the link between ER stress-induced autophagy and redox signaling.

Overall, our results indicate that ER stress triggers ROS production and autophagy in Chlamydomonas. Under ER stress triggered by tunicamycin, GSH was found to decrease autophagy induction while UPR markers remained unaffected. This uncoupling of UPR and autophagy in the presence of a ROS scavenging molecule strongly suggests that, under ER stress, ROS production plays a prominent role in the induction of autophagy in Chlamydomonas (Fig. 8). Considering the high conservation of the mechanisms of ER stress and autophagy in eukaryotes, it is possible that ROS may also be involved in autophagy induction in other organisms. Further studies will be required to unravel how ROS control autophagy induction and to examine the interplay between ROS and other ER stress signaling pathways such as those involving the IRE1 kinase.

## MATERIALS AND METHODS

### Strains and growth conditions

*Chlamydomonas reinhardtii* cw15 4B+ and *sor1* mutant strains were obtained from the Chlamydomonas Culture Collection. Chlamydomonas 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 5 µg/ml tunicamycin (Calbiochem, 654380) from a 5 mg/ml stock in dimethylformamide, 2,5 mM DTT (Applichem, A2948), 1 mM $H_2O_2$ (Sigma, H1009), 1 µM methyl viologen (Sigma-Aldrich, 85617-7) or 20 µM norflurazon (Sigma-Aldrich, PS1044).

### Gene accession numbers

Chlamydomonas genes analyzed in this study were identified at the Phytozome website (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; *DAD1*, Cre02.g108400.t1.2.

### RNA isolation and quantification

Chlamydomonas total RNA was isolated from frozen cell pellets as previously described (Crespo et al., 2005). First-strand cDNA was produced using 2 µg total

RNA, oligo(dT) primer, and 100 units of SuperScript II RNase H- reverse transcriptase (Invitrogen, 18064-014) in a 50 µl reaction. Reverse transcriptase quantitative PCR 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 (Roche, 04913850001), 1 µL of cDNA dilution, 250 nM of each primer, and distilled water up to 20 µl. Conditions used for amplification in the thermocycler were: pre-incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing and elongation at 55-58°C depending on the gene analyzed for 60 s. All reactions were performed in triplicate with 2-4 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'-CCCACCAGGTTGTTCTTCAG-3' for *CBLP*; 5'-CGAGTTCAAGGTCGAGCAGT-3' and 5'-CCACCCACAGACATGGTGTA-3' for *ATG3*; 5'-TCCCCGATATCGACAAGAAG-3' and 5'-TGCGGATGACGTACACAAAT-3' for *ATG8*; 5'-ACCCTGACTACGTCCACGAC-3' and 5'-GTCCTCAGCGAACTTCTTGG-3' for *CAL2*; 5'-TGTCAACCTGCTCATCAACC-3' and 5'-CTGCTGCTGCTACTGCTGTC-3' for *ERO1*; 5'-GGTGTGGCTGGTTGAGTTCT-3' and 5'-CTCTTTGGCGTCCTCACAGT-3' for *PDI6*; 5'-CCAAGCGCTTTAAGAAGGTG-3' and 5'-GTAGGGAAGCCCTTGACCTC-3' for Rb60; 5'-AGGTTCTGGATGCGTTCCTA-3' and 5'-ACACAGTCAGGGCGAAGAAG-3' for *DAD1*; 5'-GCGGTCGCCAATAACCAAT-3' and 5'-AAGGGCTGTCCCGAAAGC-3' for *GPXH* (Fischer et al., 2009); 5'-CTGACCATCAGCCACGACT-3' and 5'-ACATCGAACACCAGGGTAGC-3' for *FKBP12*; 5'- CAGAGGTGAAAGGCGGATAC-3' and 5'-GTGTTGCAATGGACTTCAGC-3' for *GSTS1* (Fischer et al., 2012).

**Protein preparation and immunoblot analysis**

Chlamydomonas cells from liquid cultures were collected by centrifugation (4,000*g*, 5 min), washed once 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*, 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 (Bio-Rad, 162-0115). Anti-CrATG8 (Perez-Perez et al., 2010) and secondary antibodies were diluted 1:2,500 and 1:10,000, respectively, in phosphate-buffered saline containing 0.1% Tween 20 (Applichem, A4974) and 5% milk powder. The ECL-Plus immunoblotting detection system (GE Healthcare, RPN2132) was used to detect the proteins with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Sigma-Aldrich, A6154). Anti-FKBP12 antibody was diluted 1:3,000 and used as 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 (Bio-Rad, 500-0006).

**ROS determination**

The analysis of ROS was performed as previously described (Perez-Perez et al., 2012a). Total protein extracts from Chlamydomonas cells growing exponentially were obtained as described above. 200-400 µg of total protein were used for ROS quantification. Each measurement was performed on two equal aliquots, one of

them containing 100 mM ascorbate used as background signal. Samples were incubated for 15 min at 25°C. Then, 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) (Invitrogen, D-399) in dimethylsulfoxide (DMSO) was added to a final concentration of 25 µM and incubated for 30 min at 30°C. Fluorescence was measured using a Cary Eclipse fluorescence spectrophotometer (Varian) with excitation and emission wavelengths set at 485 and 525 nm, respectively. The ascorbate-insensitive fluorescence background was subtracted to each experimental sample. Six independent measurements were performed for each sample, and the obtained values were expressed as relative fluorescence units per microgram of protein. Each experiment was performed at least on three independent samples.

**Glutathione determination**

Chlamydomonas cells were collected by centrifugation (5000*g*, 5 min), washed once 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 15000*g* for 20 min at 4°C. 500 µl of sample were 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 (reduced (GSH) plus oxidized (GSSG) glutathione) by the recycling assay initially described by Tietze (Tietze, 1969) and adapted from Queval and Noctor (Queval and Noctor, 2007). The method relies on the GR (Glutathione Reductase)-dependent reduction of 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB; Sigma, D8130). Oxidized glutathione was measured after treatment of neutralized sample with 10

mM 4-vinylpyridine (VPD, Sigma V320-4) for 30 min at 25°C. To remove excess VPD, the derivatized sample was centrifuged twice at 15000*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), 1 U/ml GR (Sigma, G3664), and DTNB reduction was measured at 412 nm. Different GSH (Sigma, G4251) concentrations ranging from 0 to 5 µM were used as standards.

**Viability assay**

Cell viability was estimated by determining the percentage of Chlamydomonas cells that excluded Evans blue dye (Sigma, E2129), which only stains nonviable cells. 450 µl of Chlamydomonas cells were incubated with 0,1 % Evans blue for 5 min, washed once 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**

Chlamydomonas cells were fixed and stained for immunofluorescence microscopy as previously described (Diaz-Troya et al., 2008; Perez-Perez et al., 2010). Affinity-purified polyclonal anti-ATG8 was used as primary antibody at 1:500 dilution. For signal detection, a fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:500; Sigma-Aldrich, F4890) 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.

**ACKNOWLEDGMENTS**

We thank Francisco J. Florencio for helpful discussions and advice.

**BIBLIOGRAPHY**


**Aller I, Meyer AJ** (2012) The oxidative protein folding machinery in plant cells. Protoplasma

**Avin-Wittenberg T, Honig A, Galili G** (2012) Variations on a theme: plant autophagy in comparison to yeast and mammals. Protoplasma **249:** 285-299

**Bassham DC, Laporte M, Marty F, Moriyasu Y, Ohsumi Y, Olsen LJ, Yoshimoto K** (2006) Autophagy in development and stress responses of plants. Autophagy **2:** 2-11

**Bernales S, McDonald KL, Walter P** (2006) Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS Biol **4:** e423

**Crespo JL, Diaz-Troya S, Florencio FJ** (2005) Inhibition of target of rapamycin signaling by rapamycin in the unicellular green alga Chlamydomonas reinhardtii. Plant Physiol **139:** 1736-1749

**Danon A, Mayfield SP** (1991) Light regulated translational activators: identification of chloroplast gene specific mRNA binding proteins. EMBO J **10:** 3993-4001

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

**Diaz-Troya S, Perez-Perez ME, Florencio FJ, Crespo JL** (2008) The role of TOR in autophagy regulation from yeast to plants and mammals. Autophagy **4:** 851-865

**Filonova A, Haemsch P, Gebauer C, Weisheit W, Wagner V** (2013) Protein Disulfide Isomerase 2 of Chlamydomonas reinhardtii Is Involved in Circadian Rhythm Regulation. Mol Plant

**Fischer BB, Dayer R, Schwarzenbach Y, Lemaire SD, Behra R, Liedtke A, Eggen RI** (2009) Function and regulation of the glutathione peroxidase homologous gene GPXH/GPX5 in Chlamydomonas reinhardtii. Plant Mol Biol **71:** 569-583

**Fischer BB, Ledford HK, Wakao S, Huang SG, Casero D, Pellegrini M, Merchant SS, Koller A, Eggen RI, Niyogi KK** (2012) SINGLET OXYGEN RESISTANT 1 links reactive electrophile signaling to singlet oxygen acclimation in Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A **109:** E1302-1311

**Foyer CH, Noctor G** (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol **155:** 2-18

**Frand AR, Kaiser CA** (1998) The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. Mol Cell **1:** 161-170

**Frand AR, Kaiser CA** (1999) Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. Mol Cell **4:** 469-477

**Gallois P, Makishima T, Hecht V, Despres B, Laudie M, Nishimoto T, Cooke R** (1997) An Arabidopsis thaliana cDNA complementing a hamster apoptosis suppressor mutant. Plant J **11:** 1325-1331

**Harris EH** (1989) The Chlamydomonas Sourcebook. Academic Press, San Diego

**Haynes CM, Titus EA, Cooper AA** (2004) Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. Mol Cell **15:** 767-776

**Huang J, Lam GY, Brumell JH** (2011) Autophagy signaling through reactive oxygen species. Antioxid Redox Signal **14:** 2215-2231

**Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, Ohsumi Y** (2000) A ubiquitin-like system mediates protein lipidation. Nature **408:** 488-492

**Kelleher DJ, Gilmore R** (1994) The Saccharomyces cerevisiae oligosaccharyltransferase is a protein complex composed of Wbp1p, Swp1p, and four additional polypeptides. J Biol Chem **269:** 12908-12917

**Kelleher DJ, Gilmore R** (1997) DAD1, the defender against apoptotic cell death, is a subunit of the mammalian oligosaccharyltransferase. Proc Natl Acad Sci U S A **94:** 4994-4999

**Ledford HK, Chin BL, Niyogi KK** (2007) Acclimation to singlet oxygen stress in Chlamydomonas reinhardtii. Eukaryot Cell **6:** 919-930

**Lemaire SD, Miginiac-Maslow M** (2004) The thioredoxin superfamily in Chlamydomonas reinhardtii. Photosynth Res **82:** 203-220

**Levitan A, Trebitsh T, Kiss V, Pereg Y, Dangoor I, Danon A** (2005) Dual targeting of the protein disulfide isomerase RB60 to the chloroplast and the endoplasmic reticulum. Proc Natl Acad Sci U S A **102:** 6225-6230

**Li F, Vierstra RD** (2012) Autophagy: a multifaceted intracellular system for bulk and selective recycling. Trends Plant Sci **17:** 526-537

**Li L, Ishdorj G, Gibson SB** (2012) Reactive oxygen species regulation of autophagy in cancer: implications for cancer treatment. Free Radic Biol Med **53:** 1399-1410

**Liu Y, Bassham DC** (2012) Autophagy: Pathways for Self-Eating in Plant Cells. Annu Rev Plant Biol **63:** 215-237

**Liu Y, Burgos JS, Deng Y, Srivastava R, Howell SH, Bassham DC** (2012) Degradation of the endoplasmic reticulum by autophagy during endoplasmic reticulum stress in Arabidopsis. Plant Cell **24:** 4635-4651

**Liu Y, Xiong Y, Bassham DC** (2009) Autophagy is required for tolerance of drought and salt stress in plants. Autophagy **5:** 954-963

**Malhotra JD, Kaufman RJ** (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal **9:** 2277-2293

**Martinez IM, Chrispeels MJ** (2003) Genomic analysis of the unfolded protein response in Arabidopsis shows its connection to important cellular processes. Plant Cell **15:** 561-576

**Mizobuchi N, Hoseki J, Kubota H, Toyokuni S, Nozaki J, Naitoh M, Koizumi A, Nagata K** (2007) ARMET is a soluble ER protein induced by the unfolded protein response via ERSE-II element. Cell Struct Funct **32:** 41-50

**Moharikar S, D'Souza JS, Rao BJ** (2007) A homologue of the defender against the apoptotic death gene (dad1 )in UV-exposed Chlamydomonas cells is downregulated with the onset of programmed cell death. J Biosci **32:** 261-270

**Nakashima T, Sekiguchi T, Kuraoka A, Fukushima K, Shibata Y, Komiyama S, Nishimoto T** (1993) Molecular cloning of a human cDNA encoding a novel

protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. Mol Cell Biol **13:** 6367-6374

**Nakatogawa H, Suzuki K, Kamada Y, Ohsumi Y** (2009) Dynamics and diversity in autophagy mechanisms: lessons from yeast. Nat Rev Mol Cell Biol **10:** 458-467

**Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, Murakami T, Taniguchi M, Tanii I, Yoshinaga K, Shiosaka S, Hammarback JA, Urano F, Imaizumi K** (2006) Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol **26:** 9220-9231

**Ozgur R, Turkan I, Uzilday B, Sekmen AH** (2014) Endoplasmic reticulum stress triggers ROS signalling, changes the redox state, and regulates the antioxidant defence of Arabidopsis thaliana. J Exp Bot **65:** 1377-1390

**Perez-Perez ME, Couso I, Crespo JL** (2012a) Carotenoid deficiency triggers autophagy in the model green alga Chlamydomonas reinhardtii. Autophagy **8:** 376-388

**Perez-Perez ME, Florencio FJ, Crespo JL** (2010) Inhibition of target of rapamycin signaling and stress activate autophagy in Chlamydomonas reinhardtii. Plant Physiol **152:** 1874-1888

**Perez-Perez ME, Lemaire SD, Crespo JL** (2012b) Reactive oxygen species and autophagy in plants and algae. Plant Physiol **160:** 156-164

**Pollard MG, Travers KJ, Weissman JS** (1998) Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. Mol Cell **1:** 171-182

**Pootakham W, Gonzalez-Ballester D, Grossman AR** (2010) Identification and regulation of plasma membrane sulfate transporters in Chlamydomonas. Plant Physiol **153:** 1653-1668

**Queval G, Noctor G** (2007) A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: Application to redox profiling during Arabidopsis rosette development. Anal Biochem **363:** 58-69

**Rhee SG, Chang TS, Jeong W, Kang D** (2010) Methods for detection and measurement of hydrogen peroxide inside and outside of cells. Mol Cells **29:** 539-549

**Rutkowski DT, Kaufman RJ** (2007) That which does not kill me makes me stronger: adapting to chronic ER stress. Trends Biochem Sci **32:** 469-476

**Santos CX, Tanaka LY, Wosniak J, Laurindo FR** (2009) Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. Antioxid Redox Signal **11:** 2409-2427

**Scherz-Shouval R, Elazar Z** (2011) Regulation of autophagy by ROS: physiology and pathology. Trends Biochem Sci **36:** 30-38

**Schroda M** (2004) The Chlamydomonas genome reveals its secrets: chaperone genes and the potential roles of their gene products in the chloroplast. Photosynth Res **82:** 221-240

**Szumiel I** (2011) Autophagy, reactive oxygen species and the fate of mammalian cells. Free Radic Res **45:** 253-265

**Thompson AR, Vierstra RD** (2005) Autophagic recycling: lessons from yeast help define the process in plants. Curr Opin Plant Biol **8:** 165-173

**Tietze F** (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem **27:** 502-522

**Tu BP, Weissman JS** (2004) Oxidative protein folding in eukaryotes: mechanisms and consequences. J Cell Biol **164:** 341-346

**Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D** (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science **287:** 664-666

**Walter P, Ron D** (2011) The unfolded protein response: from stress pathway to homeostatic regulation. Science **334:** 1081-1086

**Xie Z, Klionsky DJ** (2007) Autophagosome formation: core machinery and adaptations. Nat Cell Biol **9:** 1102-1109

**Yorimitsu T, Nair U, Yang Z, Klionsky DJ** (2006) Endoplasmic reticulum stress triggers autophagy. J Biol Chem **281:** 30299-30304

**FIGURE LEGENDS**

**Figure 1.** Tunicamycin triggers autophagy and ER stress in Chlamydomonas. Log phase cells grown in TAP medium were treated with 5 μg/ml tunicamycin (tun) and samples were taken at the indicated times and processed for expression analysis of *ATG8* (A), *CAL2*, *ERO1*, *PDI6* and *Rb60* (C) by qPCR 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.

**Figure 2.** Tunicamycin triggers oxidative stress. (A), Log phase cells grown in TAP medium were treated with 5 μg/ml tunicamycin (tun) and samples were taken at different times for determination of ROS levels as described in Materials and Methods. (B), Expression analysis of *GSTS1* and *GPXH* genes by qPCR in cells treated with 5 μg/ml tunicamycin at the indicated times. (C), The ratio of intracellular pools of reduced (GSH) and oxidized (GSSG) glutathione was determined in cells treated with 5 μg/ml tunicamycin. Values are means of three independent experiments. "*", Differences were significant at $P<0.05$ according to Student's t-test.

**Figure 3.** DTT triggers autophagy and ER stress in Chlamydomonas. 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) analysis of ATG8. Immunofluorescence images correspond to 6 h of treatment. In addition to *ATG8*, expression of the UPR-regulated genes *CAL2*, *ERO1*, *PDI6* and

*Rb60* (D) or the oxidative stress-related genes *GSTS1* and *GPXH* genes (E) was analyzed by qPCR. Values are means of three independent experiments.

**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 tunicamycin (tun) (B) alone or in combination 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 tun and tun/GSH. (C, D), Western blot analysis of ATG8 and FKBP12 proteins in log phase cells treated with 5 µg/ml tunicamycin (tun), 5-10 mM GSH or 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.

**Figure 5.** GSH decreases tunicamycin-induced cellular accumulation of ATG8 in Chlamydomonas. (A), Immunolocalization of ATG8 in Chlamydomonas cells treated for 8 h with 5 µg/ml tunicamycin (tun) alone or in combination with 10 mM GSH. Scale bar, 5 µm. (B), Quantification of the immunofluorescence 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. "**" Differences were significant at $P<0.01$ according to Student's t-test between tun and tun/GSH.

**Figure 6.** GSH reduces the toxicity of tunicamycin in Chlamydomonas. (A) Log phase cells grown in TAP medium were treated with tunicamycin (5 μg/ml; tun), GSH (10 mM) or both compounds in combination. 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 (tun) and GSH. Plates were grown at 25°C under continuous illumination for 5 days. (C) Expression analysis of the *DAD1* gene by qPCR. Log phase cells were treated with 5 μg/ml tunicamycin (tun) alone or in combination with 10 mM GSH for 48 hours and then processed for RNA isolation and analysis. Values are means of three independent experiments. "**" Differences were significant at $P<0.01$ according to Student's t-test between tun and tun/GSH.

**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) 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) for 6 hours and subsequently subjected to RNA isolation and analysis. Values are means of three independent experiments.

**Figure 8.** Control of autophagy by ER stress and ER-derived ROS in Chlamydomonas. 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 via de oxidoreductase ERO1, which contributes to the upregulation 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.

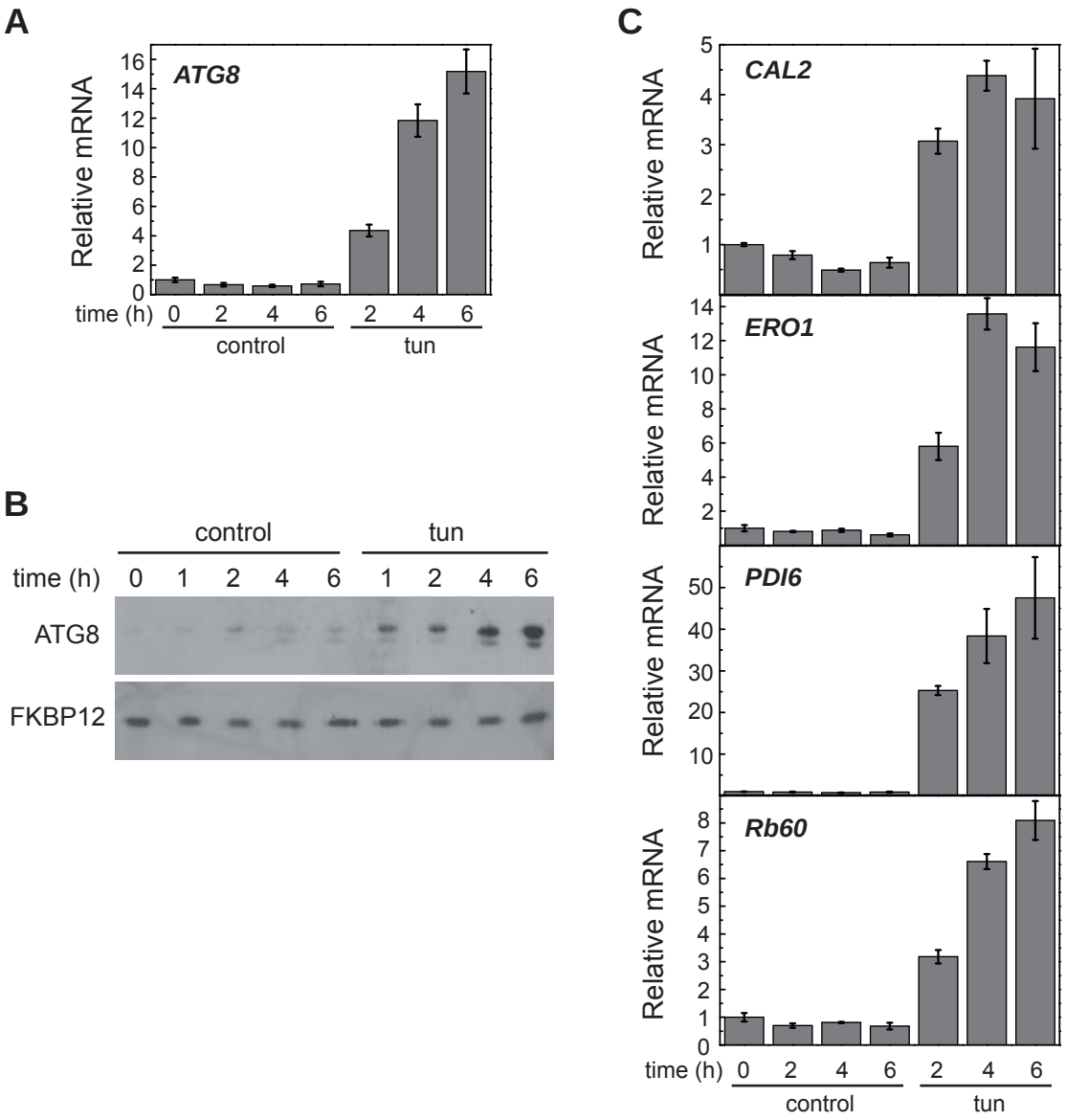


**Figure 1.** Tunicamycin triggers autophagy and ER stress in Chlamydomonas. Log phase cells grown in TAP medium were treated with 5 µg/ml tunicamycin (tun) and samples were taken at the indicated times and processed for expression analysis of *ATG8* (A), *CAL2*, *ERO1*, *PDI6* and *Rb60* (C) by qPCR 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.

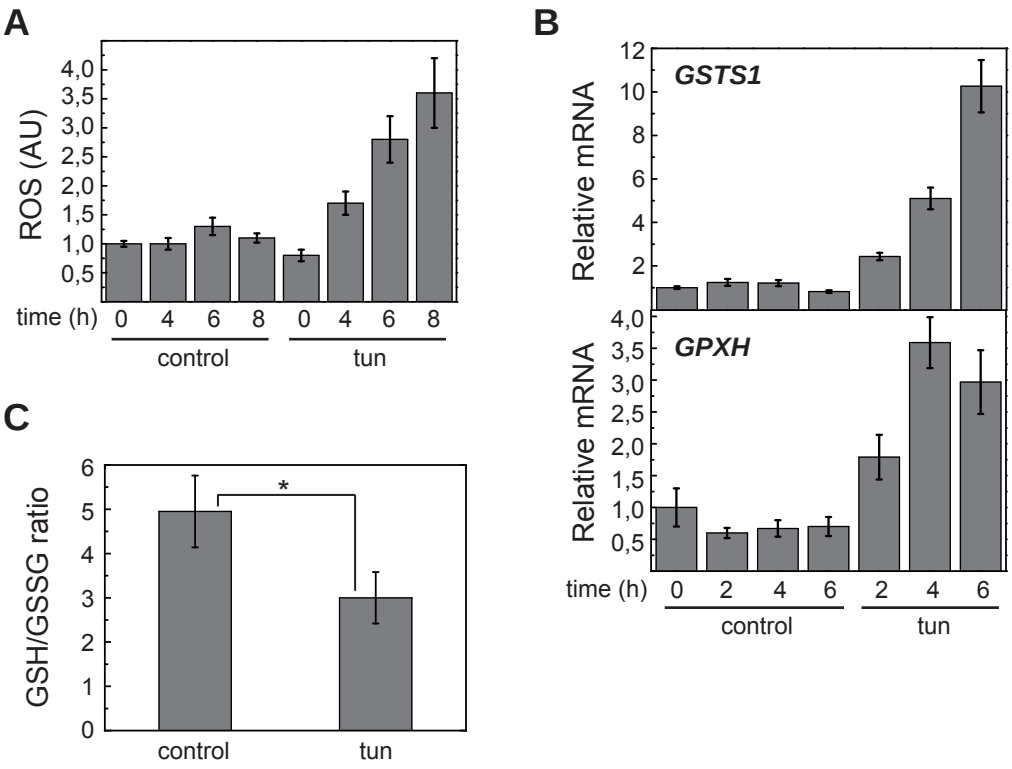


**Figure 2.** Tunicamycin triggers oxidative stress. (A), Log phase cells grown in TAP medium were treated with 5 µg/ml tunicamycin (tun) and samples were taken at different times for determination of ROS levels as described in Materials and Methods. (B), Expression analysis of *GSTS1* and *GPXH* genes by qPCR in cells treated with 5 µg/ml tunicamycin at the indicated times. (C), The ratio of intracellular pools of reduced (GSH) and oxidized (GSSG) glutathione was determined in cells treated with 5 µg/ml tunicamycin. Values are means of three independent experiments. "*", Differences were significant at $P<0.05$ according to Student's t-test.

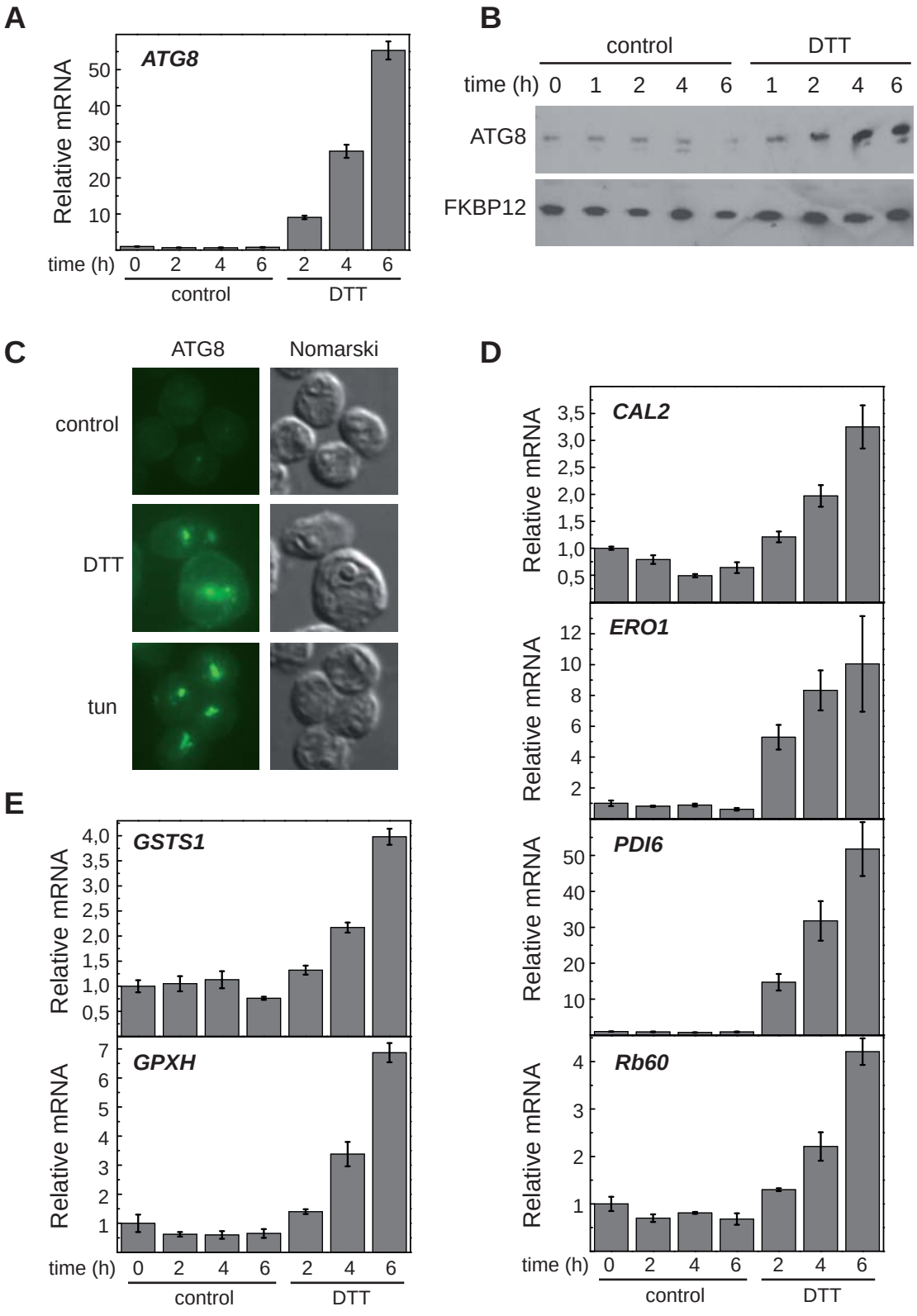


**Figure 3.** DTT triggers autophagy and ER stress in Chlamydomonas. Log phase cells grown in TAP medium were treated with 2.5 mM DTT and samples were taken at the indicated times for qPCR (A), western blot (B) or immunofluorescence (C) analysis of ATG8. Immunofluorescence images correspond to 6 h of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

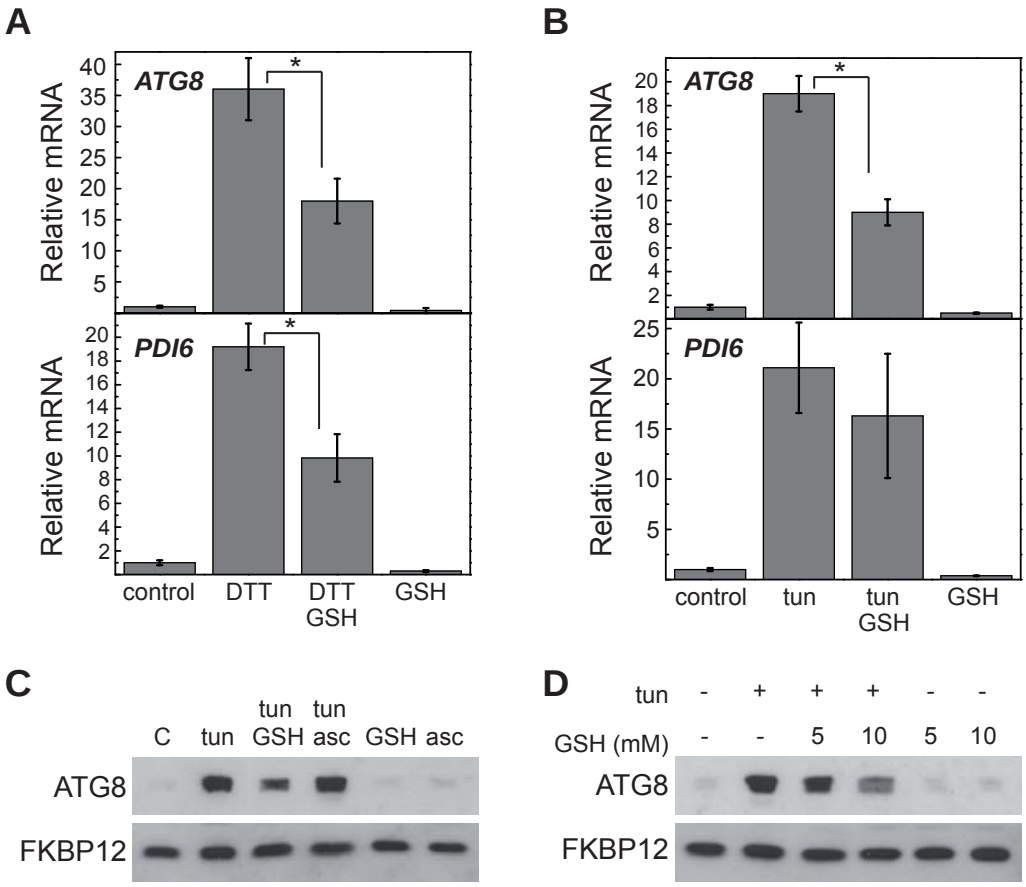


**Figure 4.** GSH partially prevents ER stress-induced autophagy. Log phase cells grown in TAP medium were treated with 2.5 mM DTT (A) or 5 µg/ml tunicamycin (tun) (B) alone or in combination with 10 mM GSH. After 8 h of treatment, cells were processed for expression analysis of *ATG8* and *PDI6* genes 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 tun and tun/GSH. (C, D), Western blot analysis of ATG8 and FKBP12 proteins in log phase cells treated with 5 µg/ml tunicamycin (tun), 5-10 mM GSH or 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.

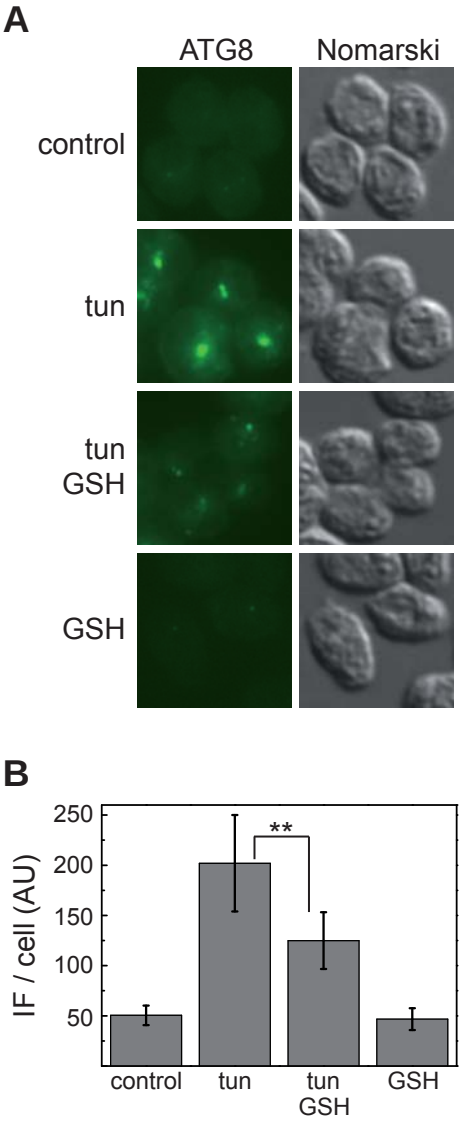


**Figure 5.** GSH decreases tunicamycin-induced cellular accumulation of ATG8 in Chlamydomonas. (A), Immunolocalization of ATG8 in Chlamydomonas cells treated for 8 h with 5 µg/ml tunicamycin (tun) alone or in combination with 10 mM GSH. Scale bar, 5 µm. (B), Quantification of the immunofluorescence 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. "**" Differences were significant at $P<0.01$ according to Student's t-test between tun and tun/GSH.

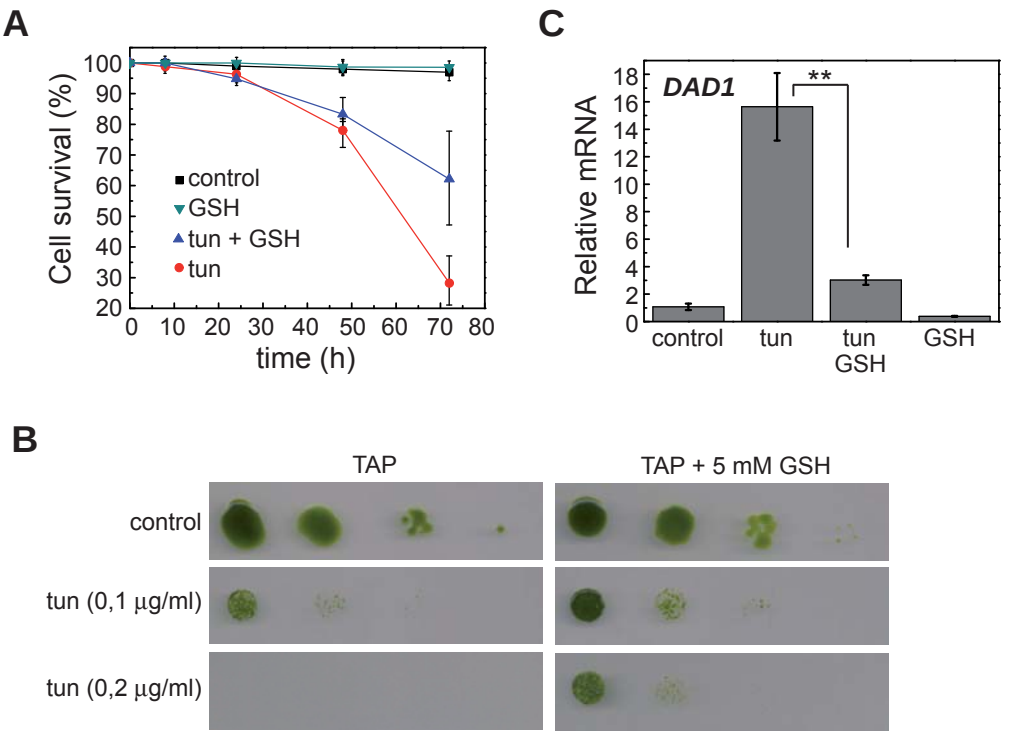


**Figure 6.** GSH reduces the toxicity of tunicamycin in Chlamydomonas. (A) Log phase cells grown in TAP medium were treated with tunicamycin (5 µg/ml; tun), GSH (10 mM) or both compounds in combination. Untreated cells were used as control. Cell viability was determined by Evans blue staining at the 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 (tun) and GSH. Plates were grown at 25°C under continuous illumination for 5 days. (C) Expression analysis of the *DAD1* gene by qPCR. Log phase cells were treated with 5 µg/ml tunicamycin (tun) alone or in combination with 10 mM GSH for 48 hours and then processed for RNA isolation and analysis. Values are means of three independent experiments. "**" Differences were significant at $P<0.01$ according to Student's t-test between tun and tun/GSH.

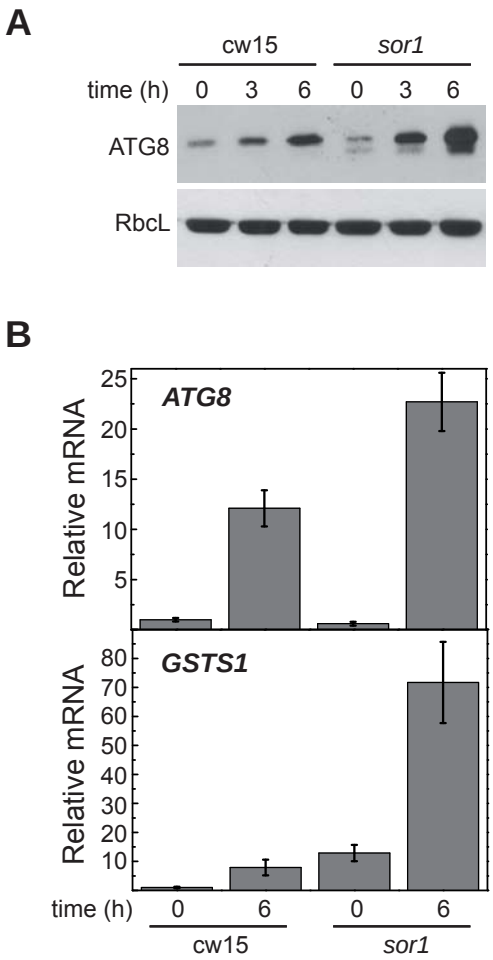


**Figure 7.** The sor1 mutant displays increased autophagy in response to ER stress. (A) Log phase *cw15* and *sor1* cells were treated with tunicamycin (5 µg/ml) and at the indicated times samples were taken and processed for western blot analysis. (B) Expression analysis of *ATG8* and *GSTS1* genes by qPCR. Log phase *cw15* and *sor1* cells were treated with tunicamycin (5 µg/ml) for 6 hours and subsequently subjected to RNA isolation and analysis. Values are means of three independent experiments.

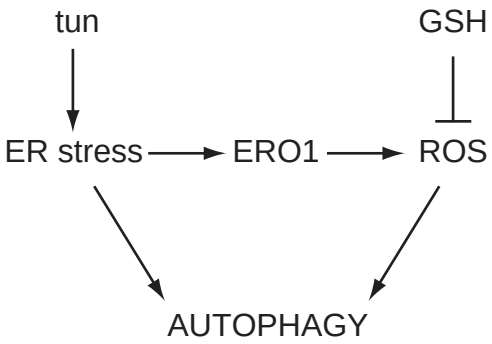


**Figure 8.** Control of autophagy by ER stress and ER-derived ROS in Chlamydomonas. Tunicamycin (tun) treatment results in toxic accumulation of unfolded proteins in the ER, leading to autophagy activation as a defensive mechanism. ER stress also increases ROS production in the ER via de oxidoreductase ERO1, which contributes to the upregulation 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.