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Activation of Autophagy by Metals in *Chlamydomonas reinhardtii*

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Autophagy is an intracellular self-degradation pathway by which eukaryotic cells recycle their own material in response to specific stress conditions. Exposure to high concentrations of metals causes cell damage, although the effect of metal stress on autophagy has not been explored in photosynthetic organisms. In this study, we investigated the effect of metal excess on autophagy in the model unicellular green alga *Chlamydomonas reinhardtii*. We show in cells treated with nickel an upregulation of ATG8 that is independent of CRR1, a global regulator of copper signaling in *Chlamydomonas*. A similar effect on ATG8 was observed with copper and cobalt but not with cadmium or mercury ions. Transcriptome sequencing data revealed an increase in the abundance of the protein degradation machinery, including that responsible for autophagy, and a substantial overlap of that increased abundance with the hydrogen peroxide response in cells treated with nickel ions. Thus, our results indicate that metal stress triggers autophagy in *Chlamydomonas* and suggest that excess nickel may cause oxidative damage, which in turn activates degradative pathways, including autophagy, to clear impaired components and recover cellular homeostasis.

Eukaryotic cells are able to degrade and recycle their own material when they are exposed to nutrient starvation or other adverse conditions through a catabolic pathway known as macroautophagy or autophagy. This process is characterized by the formation of double-membrane vesicles termed autophagosomes that engulf and deliver cytosolic components to the vacuole/lysosome for degradation (1–4). The primary function of autophagy is to recycle cytoplasmic material as well as to clear damaged organelles or toxic cellular components generated during stress in order to maintain cellular homeostasis. In higher eukaryotes, autophagy has also been implicated in cell differentiation, development and cell death, and several human pathologies, such as cancer and neurodegenerative diseases (5, 6).

Autophagy is mediated by highly conserved autophagy-related (*ATG*) genes, which have been described in organisms ranging from yeasts to mammals. Some ATG proteins are required for the formation of the autophagosome and constitute the core autophagy machinery (4, 7, 8). This group of proteins includes the ATG8 and ATG12 ubiquitin-like systems required for vesicle expansion. The ATG8 protein has been widely used to monitor autophagy in many systems (9) because, unlike other ATG proteins, this protein firmly binds to the autophagosome membrane through a covalent bond to phosphatidylethanolamine (PE). Most of the core ATG proteins are conserved in land plants (10–12) and in evolutionarily distant algae, including freshwater species, such as the model green alga *Chlamydomonas reinhardtii* (herein referred to as *Chlamydomonas*) (13) and marine species (14). Our current knowledge about autophagy in algae is still limited compared to our knowledge about autophagy in other eukaryotes, but recent studies, mainly performed in *Chlamydomonas*, have shown that this degradative process is elicited under various stress conditions. Deprivation of nutrients (nitrogen, carbon, or sulfur) or progression into stationary growth phase activates autophagy (15–19). Oxidative stress, photooxidative damage generated by carotenoid deficiency, high light stress, cold stress, or the accumulation of unfolded proteins in the endoplasmic reticulum (ER) also triggers autophagy in *Chlamydomonas* (19–22). Moreover, a loss of chloroplast integrity due to depletion of the chloroplastic ClpP protease has been shown to activate autophagy in this alga (23). Recent studies have also linked this catabolic process with the degradation of lipid droplets in the green alga *Auxenochlorella protothecoides* (24) or with the propagation of DNA viruses in the marine alga *Emiliania huxleyi* (25). In land plants, the availability of mutant lines defective in key *ATG* genes has contributed to the identification of cellular processes mediated by autophagy. Functional studies have revealed that autophagy is required for the proper response of plant cells to abiotic stresses, senescence, and pathogen infection (for recent reviews, see references 1, 2, and 26).

The signaling mechanisms that regulate autophagy in plants and algae are still poorly understood, but mounting evidence indicates that reactive oxygen species (ROS) generated during specific stresses may be involved in the activation of autophagy. Supporting this hypothesis, it has been shown that treatment of *Arabidopsis* plants or *Chlamydomonas* cells with the ROS inducer hydrogen peroxide (H_2O_2) or methyl viologen results in severe oxidative stress and leads to autophagy induction (19, 21, 27). Moreover, carotenoid depletion in *Chlamydomonas* causes ROS accumulation, which in turn triggers autophagy (21). Metal toxicity has been associated with ROS production and oxidative stress signaling in plants and algae (28–32), but it remains unknown whether the cellular response to metals in these organisms may include the activation of autophagy. *Chlamydomonas* has been

Received 3 May 2015 **Accepted** 5 July 2015

Accepted manuscript posted online 10 July 2015

Citation Pérez-Martín M, Blaby-Haas CE, Pérez-Pérez ME, Andrés-Garrido A, Blaby IK, Merchant SS, Crespo JL. 2015. Activation of autophagy by metals in *Chlamydomonas reinhardtii*. Eukaryot Cell 14:964–973. doi:10.1128/EC.00081-15.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00081-15.

doi:10.1128/EC.00081-15

widely used to investigate metal metabolism and the cellular response to metal excess and metal-limiting conditions (28, 33, 34), and the development of genome-wide technologies has increased our current understanding about metal signaling in this alga. In this study, we show that high concentrations of nickel, cobalt, or copper trigger autophagy in *Chlamydomonas*.

MATERIALS AND METHODS

Strains and growth conditions. *Chlamydomonas reinhardtii cw15* 4B+, a cell wall-deficient strain, was obtained from the laboratory of Jean-David Rochaix. The *crr1-1* mutant strain (strain CC-3959) has been previously described (35). *Chlamydomonas* cells were grown under continuous illumination at 25°C in Tris-acetate phosphate (TAP) medium as described previously (36). All treatments were performed in liquid cultures in exponential growth phase (10^6 cells/ml).

Generation of the SATG8 *Chlamydomonas* strain. To obtain a *Chlamydomonas* strain expressing nickel-induced ATG8, *cw15* cells were transformed by electroporation (37) with the pMPM1 plasmid harboring the cDNA of the *Chlamydomonas ATG8* gene under the control of the *CYC6* promoter (P_{CYC6}). To generate the pMPM1 plasmid, an 857-bp DNA fragment containing the promoter of the *CYC6* gene from *Chlamydomonas* (38) was amplified by PCR using primers 5′-CCGGCTCGAGG CCTTGGACAAGGCACTTCAGTAAC-3′ and 5′-CCGGCATATGGGA GTAGGTTGAGTTAGTTCTGTG-3′, digested with flanking XhoI and NdeI restriction enzymes, and cloned into the pSL18 plasmid (S. Lemaire and J. D. Rochaix, unpublished data) at the XhoI and NdeI restriction sites. Next, a synthetic cDNA of the *Chlamydomonas ATG8* gene with an N-terminal Strep tag (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) (39) was cloned into pSL18 containing the *CYC6* promoter at the SpeI restriction site.

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 (catalog no. HATF00010; Millipore). Anti-*Chlamydomonas* ATG8 (anti-CrATG8) (19) and secondary antibodies were diluted 1:2,500 and 1:10,000, respectively, in phosphate-buffered saline containing 0.1% (wt/vol) Tween 20 (catalog no. A4974; Applichem) and 5% (wt/vol) milk powder. A Luminata Crescendo immunoblotting detection system (catalog no. WBLUR0500; Millipore) was used to detect the proteins with horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibodies (catalog no. A6154; Sigma-Aldrich).

Fluorescence microscopy. *Chlamydomonas* cells were fixed and stained for immunofluorescence microscopy as previously described (19). Affinity-purified polyclonal anti-ATG8 was used as the primary antibody at a 1:500 dilution. For signal detection, a fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin antibody (1:500; catalog no. F4890; Sigma-Aldrich) was used. Preparations were photographed on a DM6000B microscope (Leica) with an ORCA-ER camera (Hamamatsu) and processed with Leica Application Suite advanced fluorescence software.

RNA isolation and quantification. *Chlamydomonas* total RNA was isolated from frozen cell pellets as previously described (40). First-strand cDNA was produced using 2 μg total RNA, an oligo(dT) primer, and 100 units of SuperScript II RNase H-reverse transcriptase (catalog no. 18064-014; Invitrogen) in a 50-μl reaction mixture. Quantitative real-time reverse transcription-PCR was performed on an iCycler apparatus (Bio-Rad). The PCR mixtures, in a final volume of 20 μl, contained 10 μl of FastStart Universal SYBR green master mix (catalog no. 04913850001; Roche), 1 μl of cDNA dilution, 250 nM each primer, and distilled water. All reactions were performed in triplicate with 2 to 4 biological replicates. *CBLP* was used as a constitutively expressed gene (41). The primer pairs used for quantitative PCR (qPCR) were 5′-CTTCTCGCCCATGACCA C-3′ and 5′-CCCACCAGGTTGTTCTTCAG-3′ for *CBLP*, 5′-TCCCCG ATATCGACAAGAAG-3′ and 5′-TGCGGATGACGTACACAAAT-3′ for *ATG8*, 5′-CGCAGTTCGAAAAGGGTGCA-3′ and 5′-ATGACTGGAAT TCGGTCTGG-3′ for Strep-*ATG8*, 5′-GCTTCAAGGTGGAGAGCAT C-3′ and 5′-TAGTACTTCCAGGCGGCATC-3′ for *CYC6*, 5′-GCGGTC GCCAATAACCAAT-3′ and 5′-AAGGGCTGTCCCGAAAGC-3′ for *GPXH/GPX5* (42), 5′-CAGAGGTGAAAGGCGGATAC-3′ and 5′-GTGT TGCAATGGACTTCAGC-3′ for *GSTS1* (43), 5′-TGTCAACCTGCTCAT CAACC-3′ and 5′-CTGCTGCTGCTACTGCTGTC-3′ for *ERO1*, and 5′-GGTGTGGCTGGTTGAGTTCT-3′ and 5′-CTCTTTGGCGTCCTCACA GT-3′ for *PDI6*.

Transcriptome analysis. To identify transcripts whose abundance was affected by the addition of Ni^{2+} independently of CRR1, we reanalyzed transcriptome sequencing (RNA-Seq) data previously collected to identify CRR1-dependent Ni^{2+}-induced transcripts (C. E. Blaby-Haas et al., unpublished data). Our analysis included all transcripts from strains CC-5071 (*crr1-2*:*CRR1* mt^+ [referred to as the CRR1 strain]) and CC-5073 (*crr1-2*:*CRR1*-Δ*Cys* mt^+ [referred to as the CRR1-ΔCys strain]) whose abundance was considered to be significantly differentially abundant by the Cuffdiff algorithm (44) (*q* value, <0.05) between growth in the absence and growth in the presence of 50 μM $NiCl_2$ and whose abundance was greater than or equal to 10 fragments per kilobase per million (FPKM). From this data set we generated the final list of CRR1-independent Ni^{2+}-induced transcripts by identifying those transcripts not considered to be significantly differentially abundant by the Cuffdiff algorithm (*q* value, >0.05) between the CRR1-ΔCys and CRR1 strains in the presence of Ni^{2+}. For comparison of this list of transcripts to H_2O_2-responsive transcripts, the RNA-Seq reads presented previously (45) were realigned using the v5 genome assembly and v5.5 gene models as a reference. The set of transcripts whose abundance was considered to be significantly differentially abundant (as described above) between 1 h after addition of 1 mM H_2O_2 and immediately prior to addition was generated. For comparisons of transcripts with increased abundance in the presence of Ni^2 in the CRR1 strain and increased abundance in previously published RNA-Seq experiments, before determining the overlap we applied the cutoffs of ≥10 FPKM/reads per kilobase per million and a ≥2.0-fold change in expression between mock-treated cultures and cultures to which rose bengal was added (46), 0 h and either 12 h, 31 h, 43 h, or 48 h of ClpP1 depletion (23), 0 h and either 2 h or 8 h after rapamycin addition (23), 0 h and 6 h after transfer to dark anoxic conditions (47), conditions with 20 μM Fe supplementation and conditions with either 1 μM or 0.25 μM Fe supplementation (48), the presence and absence of Cu (49), and the presence and absence of Zn (50). The list of upregulated transcripts (that met these cutoffs) from each data set was then compared to the list of transcripts that increased in abundance (using the same cutoffs) in the CRR1 strain after Ni^{2+} addition. The *P* value for each overlap was calculated using R with the command sum(dhyper((*q*:*m*, *k*, 17301-*k*, *m*))), where *q* is the number of transcripts in the overlap, *m* is the number of transcripts that increased in abundance in the data set being compared, and *k* is the number of transcripts that increased in abundance following Ni^{2+} addition. Because most of the data sets that we performed these comparisons with were aligned to the v4 assembly of the *Chlamydomonas* genome, we converted locus identifiers in the Ni^{2+} and H_2O_2 data sets from v5 to v4 (which contains 17,301 loci, the total population in the equation) and the Cu deficiency data set from v3 to v4 using the correspondence table available at http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=PhytozomeV10.

RESULTS

Nickel ions trigger autophagy in *Chlamydomonas*. The toxic effect of transition metals, such as nickel, copper, cobalt, or cadmium, in algae is well documented (29, 51–55), but the molecular

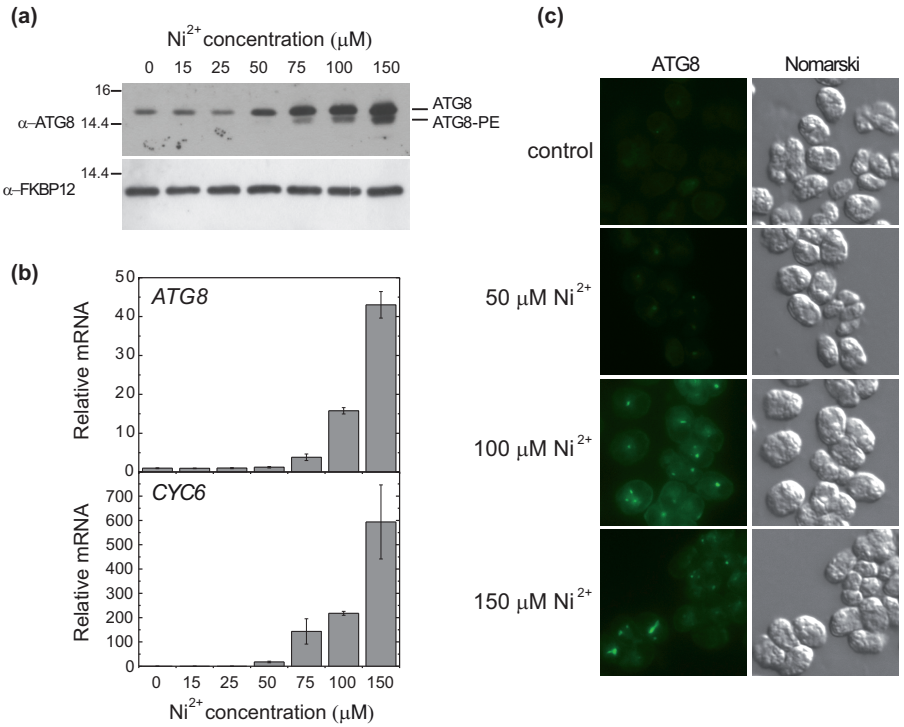

FIG 1 Exposure to high concentrations of nickel triggers autophagy in *Chlamydomonas*. (a) Immunoblot demonstrating the accumulation and lipidation of ATG8 in *cw15* cells grown to log phase (10^6 cells/ml) in TAP medium and treated with the indicated concentrations of Ni^{2+} for 8 h. Immunoblot analysis with an anti-FKBP12 antibody was used as a loading control. Molecular mass markers (in kilodaltons) are indicated on the left. (b) Analysis of *ATG8* and *CYC6* gene expression by qPCR in *cw15* cells treated as indicated in the legend to panel a. mRNA levels were compared and normalized to those for untreated cells (for which the level of mRNA expression was given a value of 1). The data are represented as the mean ± standard deviation from three independent experiments. (c) Immunolocalization of ATG8 in *cw15* cells grown to log phase in TAP medium and treated with 50, 100, or 150 μM Ni^{2+} for 8 h. Control refers to untreated cells.

processes that mediate the cellular response to these metals are still poorly understood. In this study, we investigated the effect of nickel and other transition metals on autophagy in the model alga *Chlamydomonas reinhardtii*. Toward this goal, *Chlamydomonas* cells were treated for 8 h with different concentrations of nickel (0, 15, 25, 50, 75, 100, 150 μM) and autophagy was monitored by analyzing the protein abundance and lipidation of ATG8. Our results revealed an increase in ATG8 abundance and the detection of higher levels of lipidated forms in cells treated with 100 and 150 μM Ni^{2+} than in cells treated with 75 μM Ni^{2+} (Fig. 1a). We also analyzed the transcript abundance of the *ATG8* gene by qPCR since it has been shown that enhanced transcription of this gene correlates with the activation of autophagy in *Chlamydomonas* (20). In close agreement with the ATG8 protein abundance, expression of *ATG8* was progressively upregulated when cells were treated with 75, 100, or 150 μM Ni^{2+} (Fig. 1b). The levels of mRNA for the *CYC6* gene, whose expression is tightly regulated by Ni^{2+} (53), were also determined as a positive control for Ni^{2+} treatment. As expected, expression of *CYC6* was strongly induced at Ni^{2+} concentrations above 25 μM (Fig. 1b). To further characterize a possible effect of nickel on autophagy, we analyzed the cellular distribution of ATG8 by immunofluorescence since in previous studies we showed that autophagy activation has a strong effect on the localization of ATG8 in *Chlamydomonas* (19). In consonance with the findings of immunoblotting and qPCR analysis, treatment of *Chlamydomonas* cells with 50 μM Ni^{2+} had only a moderate effect on ATG8 localization compared to that found in untreated cells, although a single spot could be observed in most cells (Fig. 1c). Higher concentrations of Ni^{2+} resulted in a pronounced increase in the ATG8 signal and the detection of several spots per cell (Fig. 1c). A similar punctate pattern has been observed for ATG8 in *Chlamydomonas* cells subjected to different autophagy-activating conditions (19–21). Taken together these results indicate that the presence of high concentrations of Ni^{2+} ions in the medium triggers autophagy in *Chlamydomonas*. To our knowledge, this is the first experimental evidence showing the activation of autophagy by high concentrations of metal in a photosynthetic organism.

Nickel and tunicamycin-induced ER stress activate autophagy through different mechanisms. The finding that high concentrations of nickel activate autophagy prompted us to compare this effect with the effects of other stresses that also upregulate this catalytic process in *Chlamydomonas*, such as ER stress (19, 20). To achieve this aim, we have generated a *Chlamydomonas* strain (termed SATG8) that expresses a Strep-tagged form of ATG8 under the control of the *CYC6* promoter (P_{CYC6}), in addition to the endogenous ATG8 protein. The *CYC6* promoter has been widely used as an inducible gene expression system in *Chlamydomonas* (56–59) because it is repressed by copper and induced by nickel (53, 60). Therefore, in the absence of nickel, SATG8 cells express only endogenous ATG8, whereas addition of this metal to the medium resulted in the detection of both endogenous and P_{CYC6}-driven ATG8 in an Ni^{2+} concentration-dependent manner (Fig. 2a). Expression of P_{CYC6}-driven *ATG8* in SATG8 cells was confirmed by qPCR (see Fig. S1 in the supplemental material). The two different ATG8 proteins could be easily and unambigu-

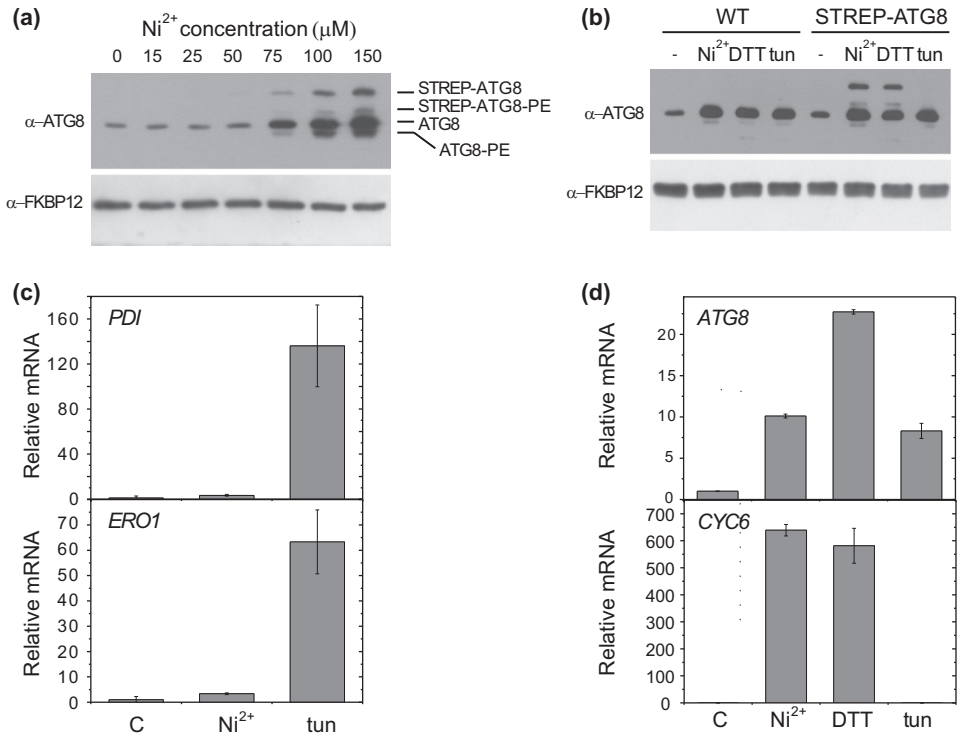

FIG 2 Nickel- and tunicamycin-induced ER stress induces autophagy through different mechanisms. (a) Immunoblot showing the accumulation and lipidation of endogenous and Strep-tagged ATG8 in SATG8 cells grown to log phase in TAP medium and treated with the indicated concentrations of Ni^{2+} for 8 h. Immunoblot analysis with an anti-FKBP12 antibody was used as a loading control. (b) Immunoblot analysis of endogenous and Strep-tagged ATG8 in *cw15* and SATG8 cells grown to log phase in TAP and treated with 100 μM Ni^{2+}, 2.5 mM DTT, or 5 μg/ml tunicamycin (tun) for 8 h. (c) Analysis by qPCR of *ERO1* and *PDI6* gene expression in *cw15* cells treated with 100 μM Ni^{2+} or 5 μg/ml tunicamycin for 8 h. C, untreated control cells. mRNA levels were compared and normalized to those for control cells (for which the level of mRNA expression was given a value of 1). The data are represented as the mean ± standard deviation from three independent experiments. (d) Analysis by qPCR of *ATG8* and *CYC6* gene expression in *cw15* cells treated as indicated in the legend to panel b.

ously detected by immunoblotting with an anti-ATG8 antibody due to the different sizes of the tagged and native proteins (Fig. 2a). *cw15* and SATG8 cells were treated with Ni^{2+} or tunicamycin, an inducer of ER stress and autophagy in *Chlamydomonas* (19, 20), and the abundance of the ATG8 protein was analyzed by immunoblotting. As expected, both treatments increased the abundance of ATG8 in *cw15* cells (Fig. 2b). In SATG8 cells, a similar effect on endogenous ATG8 was observed with Ni^{2+} and tunicamycin treatment, yet only Ni^{2+} treatment and not tunicamycin treatment induced the expression of P_{CYC6}-driven ATG8 (Fig. 2b). These results indicate that despite the ability of Ni^{2+} and tunicamycin to activate autophagy in *Chlamydomonas*, these two stressors likely operate through different mechanisms. Accordingly, we observed that Ni^{2+} had no effect on the abundance of mRNAs for *ERO1* and *PDI6* (Fig. 2c), which are upregulated in ER-stressed cells (20), suggesting that this metal does not induce autophagy via ER stress activation.

In the course of these studies, we tested the response to dithiothreitol (DTT), another inducer of ER stress that is often used interchangeably with tunicamycin. Interestingly, we observed that DTT increased the abundance of endogenous and P_{CYC6}-driven ATG8 (Fig. 2b), indicating that DTT is able to activate the expression of the Ni^{2+}-responsive gene *CYC6* in *Chlamydomonas*. To confirm the effect of DTT on the *CYC6* promoter, we determined the transcript abundance of this gene by qPCR in *cw15* cells treated with Ni^{2+}, DTT, or tunicamycin. We found that, indeed, Ni^{2+} or DTT strongly increased the level of accumulation of *CYC6* transcripts, whereas no induction was observed with tunicamycin (Fig. 2d). The effect of Ni^{2+}, DTT, or tunicamycin in these cells was confirmed by the upregulation of the *ATG8* mRNA level (Fig. 2d). The finding that DTT was able to induce the expression of *CYC6* strongly suggests that this reducing agent acts on other cellular compartments, in addition to the ER, and may therefore cause damage in the cell other than ER stress.

Copper and cobalt but not cadmium or mercury activates autophagy in *Chlamydomonas*. In addition to nickel, the effects of other metals, including copper, cobalt, cadmium, and mercury, on autophagy were investigated. We analyzed the ATG8 abundance in *Chlamydomonas* cells treated with different concentrations of $CoCl_2$, $CuSO_4$, $CdCl_2$, or $HgCl_2$. Our results revealed that both Co^{2+} and Cu^{2+} induce the accumulation and lipidation of ATG8 to a level similar to the one observed in cells treated with Ni^{2+} (Fig. 3a and b). Immunofluorescence assays confirmed that the activation of ATG8 in cells treated with Co^{2+} or Cu^{2+} was like that in cells treated with Ni^{2+} (see Fig. S2 in the supplemental material). These results indicate that the presence of high concentrations of Co^{2+} and Cu^{2+} in the medium triggers autophagy in *Chlamydomonas*. In contrast to the effects of treatment with Co^{2+} and Cu^{2+}, treatment of *Chlamydomonas* cells with Cd^{2+} or Hg^{2+} had no significant effect on ATG8 protein abundance (Fig. 3c and d) or the cellular distribution (see Fig. S2 in the supplemental material). Consonant with these data, *ATG8* mRNA abundance

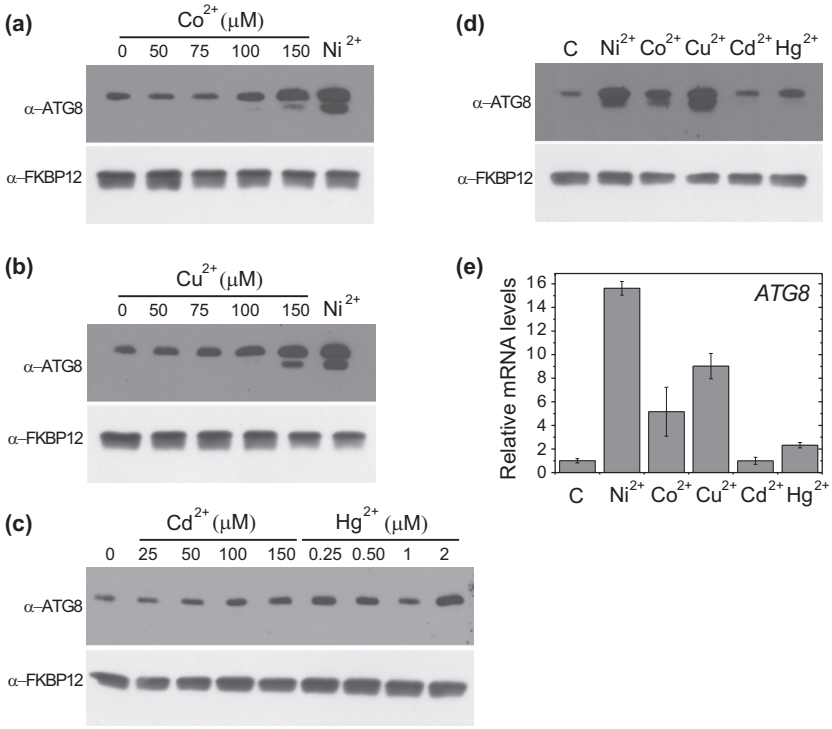

FIG 3 Cobalt and copper but not cadmium or mercury induces autophagy in *Chlamydomonas*. (a to c) Immunoblots showing the effect of Co^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+} on ATG8 abundance and lipidation. *cw15* cells grown to log phase in TAP medium were treated with the indicated concentrations of metals for 8 h. Immunoblot analysis with an anti-FKBP12 antibody was used as a loading control. (d) Immunoblot analysis of ATG8 from *cw15* cells treated with 100 μM Ni^{2+}, 150 μM Co^{2+}, 150 μM Cu^{2+}, 150 μM Cd^{2+}, or 2 μM Hg^{2+} for 8 h. (e) Analysis by qPCR of *ATG8* expression from cells treated as indicated in the legend to panel d. mRNA levels were compared and normalized to those for control cells (for which the level of mRNA expression was given a value of 1). The data are represented as the mean ± standard deviation from three independent experiments.

was upregulated by Co^{2+} or Cu^{2+} but not by Cd^{2+} or Hg^{2+} (Fig. 3e). Together these results indicate that the presence of high concentrations of Cd^{2+} or Hg^{2+} in the medium does not result in autophagy activation in *Chlamydomonas* like the presence of Ni^{2+}, Co^{2+}, or Cu^{2+} does.

Induction of autophagy by nickel is independent of CRR1. The transcription factor CRR1 is required for the Ni^{2+}-responsive expression of *CYC6* and the Cu-responsive expression of many additional genes in *Chlamydomonas* (35, 53). Since our results indicated that nickel activates the expression of the *ATG8* gene in *Chlamydomonas*, we investigated whether CRR1 may participate in the upregulation of autophagy mediated by this metal. We analyzed ATG8 in wild-type and *crr1* mutant cells treated with Ni^{2+}, and a similar accumulation of this protein was detected in both strains (Fig. 4a). However, the increase in ATG8 abundance appeared to take place slightly earlier in the *crr1* mutant (Fig. 4b), possibly due to a higher sensitivity of this mutant to Ni^{2+}. In agreement with the immunoblotting data, we observed that *ATG8* transcript accumulation was upregulated by Ni^{2+} in *crr1* mutant cells (Fig. 4c). As previously reported (53), *CYC6* expression was abolished in the *crr1* mutant (Fig. 4c). Analysis of *ATG8* expression also revealed that the basal level of mRNA for this gene is higher in the *crr1* mutant cells than wild-type cells, even though no significant difference in ATG8 abundance in untreated cells was observed (Fig. 4a), suggesting that the posttranscriptional regulation of *ATG8* prevents the accumulation of this protein under conditions that do not require an active autophagy pathway. From these results, we concluded that there must be a CRR1-independent pathway that signals to autophagy in response to nickel toxicity.

RNA-Seq analysis of nickel-treated cells reveals an increase in the abundance of the protein degradation machinery and a substantial overlap with the H_2O_2 response. To understand the global effect of Ni^{2+} on autophagy, we undertook a genome-wide transcriptome analysis of *Chlamydomonas* cells treated with this metal. In these experiments, cells were treated with 50 μM $NiCl_2$ for 6 h with the objective of identifying the primary responses to a high concentration of Ni^{2+}. As mentioned above, Ni^{2+} induces the expression of several copper deficiency response genes in a CRR1-dependent manner (35, 53), although this transcription factor is dispensable for Ni^{2+}-induced autophagy. Therefore, we sought to identify those transcripts whose change in abundance is specific to Ni^{2+} addition versus those transcripts whose change in abundance is due to direct and indirect consequences of an Ni^{2+}-CRR1 interaction. To accomplish this goal, we determined the genome-wide response of *Chlamydomonas* to the addition of Ni^{2+} in the *crr1* mutant with either wild-type *CRR1* (strain CRR1) or a mutant version of *CRR1* (strain CRR1-ΔCys) added back. In the CRR1-ΔCys strain, the protein carries a deletion in the cysteine-rich domain near the C terminus of CRR1. This mutation abolishes the ability of Ni^{2+} to induce *CYC6* expression, but the strain CRR1-mediated Cu response is preserved (35).

We found 336 genes whose transcripts were upregulated in both the CRR1 and CRR1-ΔCys strains (overlap between A and B

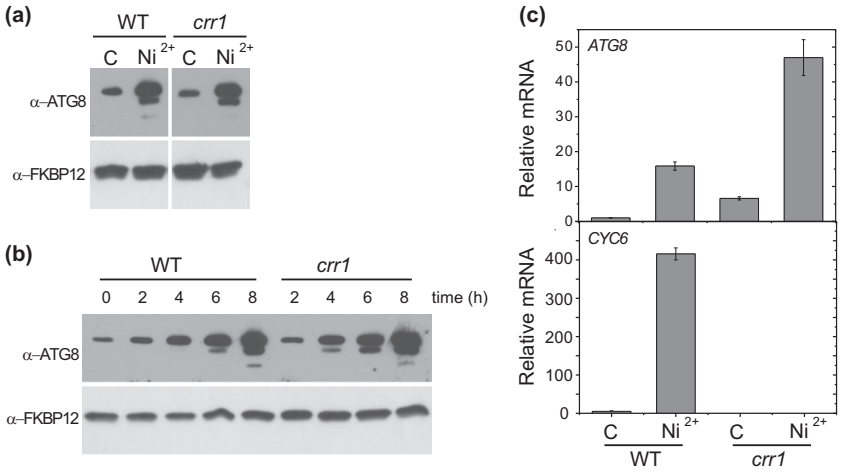

FIG 4 Activation of autophagy by nickel is independent of CRR1. (a) Immunoblots showing the accumulation and lipidation of ATG8 in *cw15* cells compared to those in *crr1* cells grown to log phase in TAP medium and treated with 100 μM Ni^{2+} for 8 h. WT, wild type; C, untreated control cells. Immunoblot analysis with an anti-FKBP12 antibody was used as loading control. (b and c) Analysis by qPCR of *ATG8* and *CYC6* gene expression in *cw15* and *crr1* cells treated as explained in the legend to panel a. mRNA levels were compared and normalized to those for control cells (for which the level of mRNA expression was given a value of 1). The data are represented as the mean ± standard deviation from three independent experiments.

in Fig. 5a and b). Of these genes, the mRNA abundance of 275 transcripts was not significantly different between the two strains (comparison C in Fig. 5a and b). We consider this core set of 275 genes to be the CRR1-independent Ni^{2+}-responsive transcriptome. Among this core set there is a substantial enrichment of the protein degradation machinery, including the autophagy genes *ATG3* and *ATG8*, genes for proteasome subunits and several classes of proteases, and genes involved in ubiquitin-dependent degradation of ER-associated protein degradation substrates, such as Cdc48, Ufd1, or Otu1 homologs (see Table S1 in the supplemental material). This subset of genes related to degradative pathways accounts for 25% of the upregulated transcripts (Fig. 5c; see also Table S1 in the supplemental material).

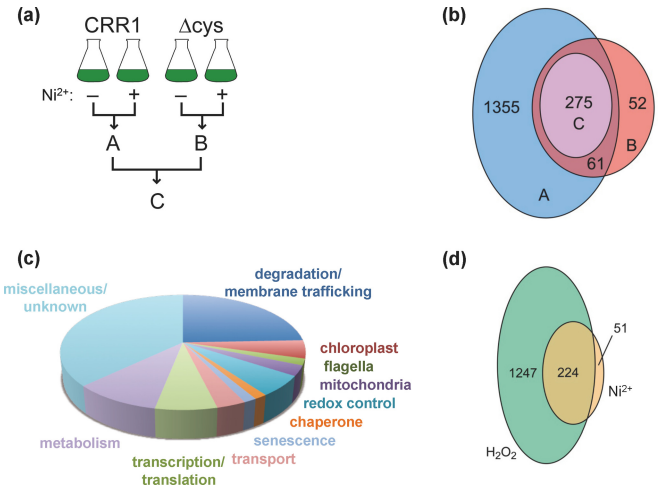

FIG 5 Transcriptomic analysis of 50 μM Ni^{2+}-treated cultures reveals a global impact on protein metabolism and overlap with the H_2O_2-induced stress response. (a) Schematic of the data sets used for the comparison shown in panel b. Ni refers to Ni^{2+} ions. (b) Venn diagram showing the overlap between the different sets of genes indicated in panel a. A, transcripts with a higher abundance in the presence of Ni^{2+} in the complemented (CRR1) strain; B, transcripts with a higher abundance in the presence of Ni^{2+} in the CRR1-ΔCys strain; C, transcripts in the overlap of A and B whose abundance in the presence of Ni^{2+} was not significantly different between the two strains. (c) Functional classification of the CRR1-independent Ni^{2+}-responsive transcriptome. The 275 transcripts with similar transcript abundances identified in the CRR1 and CRR1-ΔCys strains were clustered on the basis of their predicted function. (d) Venn diagram showing the overlap between transcripts with increased abundance in the presence of Ni^{2+} and H_2O_2.

Remarkably, we found a substantial enrichment of genes whose transcripts were also upregulated by the presence of exogenous H_2O_2 (45) (Fig. 5d): 80% of the 275 nickel-responsive genes were also upregulated by H_2O_2 (the overlap has a *P* value of 1.5e−227 on the basis of the hypergeometric distribution). Cellular damage caused by Ni^{2+} or H_2O_2 may lead to the activation of common cellular processes in *Chlamydomonas*, including autophagy. Indeed, the transcript abundance of some of the most highly H_2O_2-responsive genes is similarly affected by Ni^{2+} addition (see Fig. S3 in the supplemental material); the response of two sentinel oxidative stress-related genes, *GSTS1* and *GPXH* (43, 61, 62), was confirmed by qPCR (see Fig. S3 in the supplemental material). To further investigate a role of oxidative damage in Ni^{2+}-induced autophagy, ATG8 protein abundance and lipidation were examined in cells treated with Ni^{2+} in the presence of the antioxidant *N*-acetyl cysteine (NAC). The effect of Ni^{2+} on ATG8 was largely reduced in cells grown in NAC-containing medium (Fig. 6a). Moreover, the levels of mRNA for the *ATG8* and *GSTS1* genes remained low when cells were treated with Ni^{2+} in the presence of NAC (Fig. 6b). Taken together our results strongly suggest that treatment of *Chlamydomonas* cells with Ni^{2+} may cause oxidative damage, which in turn activates degradative pathways, including autophagy, as a defense mechanism to clear impaired components.

To further qualify the observed overlap between the Ni^{2+} and H_2O_2 data sets, we also compared those transcripts that increased in abundance in the CRR1 strain to those transcripts that increased in abundance following treatment of cells under conditions that are known to induce autophagy (ClpP1 depletion and rapamycin treatment [19, 23]) and conditions that have yet to be linked to autophagy induction in *Chlamydomonas*: singlet oxygen

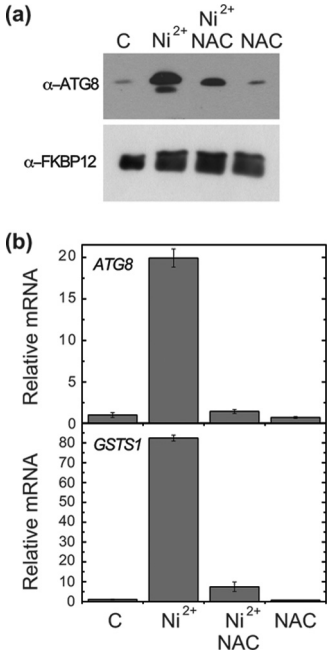

FIG 6 Effect of the antioxidant NAC on Ni^{2+}-induced autophagy. The protein abundance and lipidation of ATG8 (a) and the accumulation of *ATG8* and *GSTS1* gene transcripts (b) in *Chlamydomonas* cells treated with 100 μM Ni^{2+} for 8 h in the presence or absence of 10 mM NAC were determined. (a) Immunoblot analysis with an anti-FKBP12 antibody was used as loading control. (b) mRNA levels were determined by qPCR and normalized to those for control cells (for which the level of mRNA expression was given a value of 1). The data are represented as the mean ± standard deviation from three independent experiments.

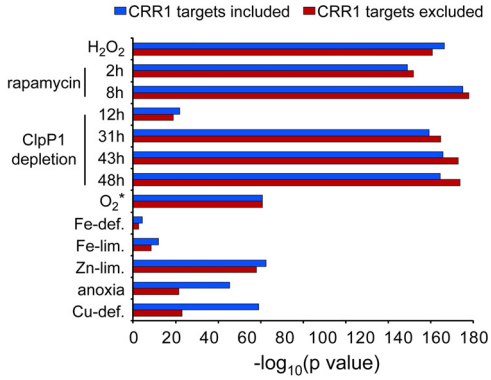

FIG 7 Overlap between the Ni^{2+} response in strain CRR1 and published stress transcriptomes. *P* values for enrichment of transcripts that were more abundant in the presence of Ni^{2+} than in the absence of Ni^{2+} in the CRR1 strain and in several published transcriptomes were calculated (using the hypergeometric distribution) after rose bengal (O_2*) addition, ClpP depletion, rapamycin addition, growth under anoxic conditions, Fe^{2+} limitation (Fe-lim.), Fe^{2+} deficiency (Fe-def.), Zn^{2+} limitation (Zn-lim.), and Cu^{2+} deficiency (Cu-def.). The analysis was performed with and without including CRR1 target transcripts in the data sets.

(46), acclimation to dark anoxia (47), poor Fe nutrition (48), poor Cu nutrition (49), and poor Zn nutrition (50). In addition to the H_2O_2 data set, we found that the most significant overlaps with Ni^{2+} induction in the CRR1 strain were with 8 h of rapamycin addition and 48 h of ClpP1 depletion (Fig. 7). There was a moderate overlap with singlet oxygen and Zn limitation, whereas the two Fe nutrition data sets had relatively little overlap with the data set for the Ni^{2+} response. Given the previous observation that Ni^{2+}, anoxia, and Cu deficiency all lead to the induced expression of a core set of CRR1 targets, we found that subtraction of CRR1 targets (49) affected only the overlap between Ni^{2+} and either anoxia or Cu deficiency.

DISCUSSION

In this study, we showed that exposure of *Chlamydomonas* cells to a high concentration of Ni^{2+} results in the upregulation of *ATG8* expression, an increased ATG8 protein abundance, and the detection of lipidated ATG8 forms (Fig. 1), all of which are landmarks of autophagy activation (9, 19). These results suggest that excess Ni^{2+} may trigger autophagy to mitigate the toxic effect of this metal in *Chlamydomonas*. Accordingly, it has been reported that similar concentrations of Ni^{2+} have a negative effect on *Chlamydomonas* cell growth (55). Immunofluorescence microscopy assays also revealed that the cellular localization of ATG8 drastically changed when cells were exposed to excess Ni^{2+}, which resulted in the detection of this protein as intense spots (Fig. 1). A similar ATG8 localization pattern has been reported in *Chlamydomonas* cells subjected to different stress conditions, including oxidative stress, photooxidative damage, or ER stress (19–21). Whether these ATG8-containing spots label autophagosome-like structures or vacuoles remains to be explored.

In addition to Ni^{2+}, we found that copper and cobalt also trigger autophagy in *Chlamydomonas*, although Ni^{2+} appeared to be more effective at similar metal concentrations (Fig. 3; see also Fig. S2 in the supplemental material). Activation of autophagy by Ni^{2+}, Cu^{2+}, or Co^{2+} has not been previously reported in photosynthetic organisms, although in mammalian cancer cells, copper complexes appear to induce oxidative stress, which in turn triggers autophagy (63, 64). Unlike the effect of Ni^{2+}, copper, or cobalt, no significant effect of Cd^{2+} or Hg^{2+} ions, even at high concentrations (150 μM and 2 μM, respectively), on ATG8 was observed (Fig. 3; see also Fig. S2 in the supplemental material). Similar amounts of these two metals have previously been shown to alter gene expression in *Chlamydomonas* (29, 65–67), although to our knowledge no effect on autophagy genes in algae or plants has been reported. However, cadmium toxicity has been linked to the activation of autophagy in human cells on the basis of the tumorigenic and cell death-inducing properties of this metal (68). The molecular mechanism by which Cd^{2+} activates autophagic programs in human cells is unclear, although it may involve ROS formation (69–71). Cadmium has also been shown to induce autophagy in sea urchin embryos, but the underlying signaling pathway remains to be identified (72). In photosynthetic systems, it has been reported that Cd^{2+} exposure appears to promote the formation of vacuole-like structures in the unicellular green alga *Micrasterias denticulata*, although no specific autophagy marker was analyzed in that study (73).

Our results demonstrate that Ni^{2+}-induced autophagy is independent of the transcription factor CRR1 in *Chlamydomonas* (Fig. 4). CRR1 is a key regulator of the acclimation of *Chlamydomonas* cells to copper deficiency (35, 74, 75) and is required for the Ni^{2+}-induced expression of genes within the Cu-responsive regulon, likely because Ni^{2+} interferes with Cu sensing by CRR1 (35, 53). The finding that Ni^{2+} activates autophagy in CRR1-deficient cells indicates that this transcription factor is fully dispensable for

Ni^{2+}-induced autophagy and that the misregulation of the CRR1 regulon by Ni^{2+} does not play a significant role in autophagy induction. Genome-wide transcript abundance analysis of *Chlamydomonas* cells with impaired CRR1 function (CRR1-ΔCys cells) revealed that there is a core set of 275 genes whose transcripts are similarly upregulated by Ni^{2+} in both CRR1 and CRR1-ΔCys cells (Fig. 5; see also Table S1 in the supplemental material). The autophagy genes *ATG3* and *ATG8* are included in this set of CRR1-independent Ni^{2+}-responsive genes, in close agreement with the finding that this transcription factor is dispensable for Ni^{2+}-induced autophagy.

The upregulation of genes coding for proteasome subunits, ubiquitin-related proteins, and several proteases in Ni^{2+}-treated cells supports the conclusion that metal stress must lead to massive damage in the cell that needs to be repaired to recover cell homeostasis. The considerable overlap between H_2O_2-responsive genes and the 275 Ni^{2+}-induced, CRR1-independent genes (Fig. 5; see also Table S1 in the supplemental material), together with the upregulation of two sentinel oxidative stress-regulated genes (see Fig. S3 in the supplemental material) and the decreased activation of autophagy observed in the presence of an antioxidant (Fig. 6), strongly suggests that this metal may lead to oxidative stress in *Chlamydomonas*, which in turn triggers autophagy. Accordingly, the ROS inducers H_2O_2 and methyl viologen have been shown to activate autophagy in *Chlamydomonas* (19, 21). A similar response may occur in cells exposed to Co^{2+} or Cu^{2+}, since high concentrations of these metals cause oxidative stress in plants and algae (30, 76–78).

A link between exposure to metals and oxidative stress has been shown in plants and algae (28–32, 76, 79), although we cannot rule out the possibility that the activation of autophagy by Ni^{2+}, Co^{2+}, or Cu^{2+} might be due to the effect of these metals on some metalloproteins by displacing cognate metals, such as Fe^{2+} or Mn^{2+}, from the active site and thus interfering with the activity of these enzymes, which may ultimately lead to an autophagy response. Moreover, this interpretation is compatible with the activation of autophagy by oxidative damage, since Ni^{2+} and other metals may indirectly cause oxidative stress by mismetallating and inactivating ROS-detoxifying enzymes (80).

ACKNOWLEDGMENTS

This work was supported by the Spanish Ministry of Economy and Competitiveness (grant BFU2012-35913 to J.L.C.), by the Junta de Andalucía (grant CVI->7336 to J.L.C.), and by National Institutes of Health (NIH) grants GM42143 and R24 GM092473 to S.S.M. I.K.B. and C.E.B.-H. were supported by training grants from the NIH (T32ES015457 and GM100753, respectively).

We thank Sorel Fitz-Gibbon for realignments of RNA-Seq reads.

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