Manganese co-localizes with calcium and phosphorus in Chlamydomonas acidocalcisomes and is mobilized in Mn-deficient conditions

**Munkhtsetseg Tsednee a,1, Madeli Castruita a,1, Patrice A. Salomé a,b,1, Ajay Sharma, c Brianne Elizabeth Lewis d, Stefan R. Schmollinger a,b, Daniela Strenkert a,b, Kristen Holbrook a,c, Marisa S. Otegui f, Kaustav Khatua g, Sayani Das g, Ankona Datta g, Si Chen b, Cristina Ramon i, Martina Ralle i, Peter K. Weber i, Timothy L. Stemmler d, Jennifer Pett-Ridge i, Brian M. Hoffman c and Sabeeha S. Merchant a,b,k**

From the a Department of Chemistry and Biochemistry, and b Institute for Genomics and Proteomics, University of California, Los Angeles, CA 90095; c Department of Chemistry, Northwestern University, Evanston, IL 60208; d Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI 48201; e Present address: Amgen, 1 Amgen Center Dr, Thousand Oaks, CA 91320; f Departments of Botany and Genetics, University of Wisconsin-Madison, WI 53706; g Department of Chemical Sciences, Tata Institute of Fundamental Research, Mumbai, 400005, India; h Advanced Photon Source, Argonne National Laboratory, Lemont, IL 60439; i Physical and Life Sciences Directorate, Lawrence Livermore National Laboratory, Livermore, CA 94550; j Department of Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR 97239; k Present address: Departments of Plant and Microbial Biology, Molecular and Cell Biology, UC Berkeley, Berkeley, CA 94720

1: equal contribution

**Running title:** Robust Chlamydomonas acidocalcisome-mediated Mn homeostasis

* to whom correspondence should be addressed: Sabeeha S Merchant: 176 Stanley Hall QB3, UC Berkeley, Berkeley, CA 94720; sabeeha@chem.ucla.edu

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**ABSTRACT**

Exposing cells to excess metal concentrations well beyond the cellular quota is a powerful tool for understanding the molecular mechanisms of metal homeostasis. Such improved understanding may enable bioengineering of organisms with improved nutrition and bioremediation capacity. We report here that Chlamydomonas reinhardtii can accumulate manganese (Mn) in proportion to extracellular supply, up to 30-fold greater than its typical quota and with remarkable tolerance. As visualized by X-ray fluorescence microscopy and nanoscale secondary ion MS, Mn largely co-localizes with phosphorus (P) and calcium (Ca), consistent with the Mn-accumulating site being an acidic vacuole, known as the acidocalcisome. Vacuolar Mn stores are accessible reserves that can be mobilized in Mn-deficient conditions to support algal growth. We noted that Mn accumulation depends on cellular polyphosphate (polyP) content, indicated by 1) a consistent failure of C. reinhardtii vtc1 mutant strains, which are deficient in polyphosphate synthesis, to accumulate Mn and 2) a drastic reduction of the Mn storage capacity in P-deficient cells. Rather surprisingly, X-ray absorption spectroscopy, EPR, and electron nuclear double resonance revealed that only little Mn²⁺ is stably complexed with polyP, indicating that polyP is not the final Mn ligand. We propose that polyPs are a critical component of Mn accumulation in Chlamydomonas by driving Mn relocation from the cytosol to acidocalcisomes. Within these structures, polyP may, in turn, escort vacuolar Mn to a number of storage ligands, including phosphate and phytate, and other, yet unidentified, compounds.
INTRODUCTION
In algae and land plants, manganese (Mn) is the cofactor for at least 30 enzymes: phosphoenolpyruvate carboxylase and oxalate oxidase among others (1–3). By far, the two major Mn sinks in the green lineage are the various Mn-containing superoxide dismutases (MnSOD) distributed in the cytosol, mitochondria and plastids (4–6), and photosystem II in chloroplasts. MnSOD protects against superoxide (generated in the bioenergetic membranes of photosynthesis and respiration among other metabolic reactions), while PSII houses the water oxidation complex in photosynthesis (7). Mn is therefore a key nutrient for plants and algae, with a quota typically much higher than in animals and respiring bacteria.

Although Iron (Fe) and Mn are two of the most abundant transition metals in the Earth’s crust, the rise in oceanic oxygen from oxygenic photosynthesis caused their massive precipitation as oxide complexes: their availability dropped by orders of magnitude (2 for Mn, and 4 for Fe), placing tremendous pressure on early unicellular organisms to meet their transition metal quotas for enzymes that had incorporated Fe- or Mn-cofactors (8) and presumably providing the driving force for the evolution of metal-selective transporters and intracellular metal sequestration mechanisms.

Mn and Fe deficiency are symptomatically similar in plants, as they both decrease the efficiency of photosynthesis and are both accompanied by leaf chlorosis (9), although the underlying causes are different. In the case of Fe, chlorosis results from inhibition of chlorophyll biosynthesis (10), now attributed to the di-iron cyclase in the pathway (11), and also from programmed reorganization of the photosynthetic apparatus for photoprotection and iron sparing (12–15). Mn deficiency likely destabilizes the PSII complex, perhaps due to the release of the extrinsic luminal proteins (5). Mn deficiency also increases intracellular redox stress because of decreased SOD activity, which manifests as necrotic regions on leaves (3, 16) or replacement of Mn in enzymes with Fe (17).

On the other end of the nutrient assimilation spectrum, excess Fe or Mn can be detrimental for growth, although for different reasons. High cellular Fe levels generate reactive oxygen species via the Fenton reaction, whose toxicity is exacerbated under high photon flux densities (18). For Mn, there is the danger of mismetalation and potential inactivation of the substituted metalloenzyme (17). Two notable exceptions are the budding yeast and the bacterium Deniococcus radiodurans. In both organisms, high concentrations of cellular Mn help protect proteins against oxidative stress damage due to the high superoxide dismutation activity displayed by Mn-phosphate low molecular weight metabolites (19–21).

To prevent experiencing either metabolic extreme, all living organisms have devised elaborate systems for the uptake, transport, storage and remobilization of micronutrients. Mn is typically transported as Mn2+ species, often by members of the same transporter families that transport Fe2+. These transporters include members of the NRAMP (Natural Resistance-Associated Macrophage Protein), MTP (Metal Tolerance Protein) and VIT1/CCC1 (Vacuolar Iron Transporter in Arabidopsis) families (16, 22–27). Primary Mn2+ uptake takes place at the plasma membrane by NRAMPs, and is subsequently routed to the endoplasmic reticulum, Golgi, mitochondria (in algae, fungi and plants) and chloroplasts (in algae and plants) to fulfill the metalation needs of each compartment. In addition, cells can store Mn2+ in vacuoles for later use under conditions of nutrient limitation, or to minimize the toxic effects of over-accumulation when placed in an environment with an over-abundance. In this regard, the vacuole acts as a temporary cellular sponge for excess Mn2+.

Because Mn2+ and Fe2+ can be transported by the same transporters, a greater understanding of Mn metabolism is relevant not only for appreciating Mn biochemistry but also for a systems-level view of Fe homeostasis that includes potential cross-talk and interactions with other metals. For instance, high Mn availability can induce Fe-deficiency symptoms (28, 29) by competing for the same transporters (22, 30–32). The Arabidopsis high-affinity Mn2+ transporter NRAMP1 can also function as a low-affinity Fe2+ transporter under Fe-replete conditions (33). Yet, despite its critical function in photosynthesis, Mn metabolism is under-investigated. In the unicellular alga Chlamydomonas reinhardtii (hereafter referred to as Chlamydomonas), Mn deficiency strongly induces the expression of Cre NRAMP1 and can also be associated with...
secondary Fe deficiency (5). The effects of high Mn supply in Chlamydomonas are unknown.

High concentrations of Mn in the soil typically rise after heavy rains and are exacerbated in acid soils. The analysis of various yeast mutants pointed to a role for phosphate in Mn uptake, perhaps as a co-transported counterion on phosphate transporters (21, 34–36). Polyphosphate chains are also critical for magnesium (Mg$^{2+}$) uptake, suggested to act as a cation filter that attracts Mg$^{2+}$ to the vacuole (Klompmaker et al., 2017). It is becoming more appreciated that lysosome-type organelles (of which the vacuole is one), called acidocalcisomes (because of their low pH and high calcium content), are key sites for metal storage (38–41).

We tested the metal content of Chlamydomonas as a function of Mn$^{2+}$-EDTA supply in the medium. Surprisingly, we found that cells accumulate Mn$^{2+}$ in linear proportion to supply many times over their demands for metalating all Mn sites in proteins (also referred to as quota). Spectroscopic analysis (XANES) indicates that intracellular Mn is mostly present as Mn$^{2+}$ species, as expected. Based on various imaging methods, including nanoSIMS, scanning X-ray fluorescence microscopy (XFM), energy-dispersive X-ray spectroscopy (EDX), and fluorescence imaging, we conclude that Mn$^{2+}$ is concentrated in the acidocalcisome. Mutants blocked in polyphosphate (polyP) synthesis do not accumulate Mn$^{2+}$. Yet, spectroscopic studies (EXAFS and ENDOR) do not support Mn$^{2+}$ association with polyP. Therefore, we conclude that polyP functions as an escorting and temporary ligand within acidocalcisomes rather than an end-point sequestration agent as suggested for Mg ions (37).

RESULTS

Chlamydomonas cells have a linear capacity for Mn accumulation

Manganese (Mn) deficiency in Chlamydomonas is associated with slow growth and decreased chlorophyll content due to compromised photosynthetic electron transfer (5). To determine the behavior of Chlamydomonas cells under conditions of Mn excess, we exposed cultures to Mn concentrations ranging from 6 µM to 1000 µM and monitored growth, chlorophyll levels and photosynthetic parameters. All Mn concentrations were well-tolerated by Chlamydomonas, and even provided an apparent growth advantage at 50 µM and above (Figure 1A). Higher concentrations of cellular Mn may participate in quenching of reactive oxygen species harmful to cells as was shown in yeast (20, 42). All cultures were healthy, as demonstrated by robust chlorophyll accumulation and $F_v/F_m$ values, with no indication of cellular stress (Figure S1). Other transition metals like Fe, Cu, Zn and P remained constant over the range of Mn concentrations tested here, with the exception of a slight increase in intracellular Ca under very high Mn concentrations (Figure S2).

The observed tolerance to excess external Mn was accompanied by intracellular accumulation of the metal well beyond its normal quota (Figure 1B). Chlamydomonas cells indeed displayed a linear capacity for Mn uptake from the medium that was not saturated even at 1 mM Mn. This behavior is unique among the metals we have tested and suggests that a distinct control mechanism operates for Mn$^{2+}$ uptake and storage. Indeed, we have previously observed rapid saturation of Cu uptake that does not extend beyond 2-3 x10$^7$ atoms/cell (41, 43). The Fe quota is much higher and its accumulation continues even after the quota is reached, but nevertheless more slowly (13).

We conclude that Chlamydomonas cultures exhibit a linear capacity for Mn uptake to many times its normal quota for photosynthesis, and that this intracellular accumulation appears to be beneficial to cell growth.

Mn co-localizes with Ca and P inside acidocalcisomes

In Arabidopsis, the major Mn storage site is the vacuole, from which it can be remobilized by NRAMP family members (16, 22, 44). We explored intracellular Mn distribution in Chlamydomonas cells grown in Mn-replete and excess conditions by multiple spectroscopic techniques: X-ray fluorescence microscopy (XFM), transmission electron microscopy coupled with energy-dispersive X-ray spectroscopy (TEM-EDX) and nanoscale secondary ion mass spectrometry (nanoSIMS). Each method offers particular advantages, and all three were largely congruent; we present the results from nanoSIMS below. XFM and TEM-EDX are shown in Figures S3 and S4.
Nanoscale secondary ion mass spectrometry (nanoSIMS) is a method that identifies elements, based on their mass, in surfaces of solid samples based on their mass with high spatial resolution. The method is compatible with visualizing populations of cells, which enables statistical analysis of regions of interest. In conjunction with the use of standards, quantitative information can be extracted from the data. Fixed cells grown in 6 µM or 1000 µM MnEDTA were analyzed with an O
analysis beam, and secondary ions were collected for carbon (12C+), phosphorus (31P+), calcium (40Ca+) and manganese (55Mn+). We report elemental abundances as uncorrected ion ratios, as reflected by the specific isotope species detected. As shown in Figure 2A, P, Ca and Mn showed clear co-localization in a field of cells grown in excess Mn. XFM observations of fixed whole cells painted a similar picture of intracellular metal distribution (Figure S3). Sites of P and Ca accumulation coincide with electron-dense particles seen by TEM-EDX (Figure S4) and are consistent with the defining elemental content of the acidocalcisome, a lysosome-related organelle. We exploited the sensitivity and dynamic range of nanoSIMS to quantify relative P, Ca and Mn levels by sub-dividing each cell into non-overlapping regions of interest (see Figure S5) and normalizing ion counts to 12C+ (41). We used the sites of high 40Ca+/12C+ and 31P+/12C+ concentration as proxy for acidocalcisomes (Figure 2B, 2C). The relative concentration of Ca and P did not change inside acidocalcisomes as a function of Mn supply (Figure S6). Other elements that differentiated VTC1 and vtc1-1 included P (Figure 3C) and Ca (Figure 3D): neither element content changed as a function of Mn supply in either genotype, but P levels in vtc1-1 were one third of those in VTC1, while Ca levels reached at most one tenth of those in VTC1, suggesting that Ca ions may associate directly with polyP. That vtc1-1 mutants have a lower P quota is expected, as vacuolar polyP

A mutant in the VTC complex does not accumulate manganese

Based on the yeast literature on manganese metabolism, and in view of its concentration within acidocalcisomes, we hypothesized that Mn ions were stored inside the organelle complexed with polyphosphate (polyP) (45, 46). The vacuolar transporter chaperone complex (VTC) catalyzes the synthesis of polyP from cytosolic inorganic phosphate and translocates the polymer across the vacuolar membrane in an ATP-dependent manner. The VTC complex is composed of five subunits in yeast, Vtc1-Vtc5 (35, 47). The Chlamydomonas genome encodes one orthologue for Vtc1 and Vtc4 each, and a mutant in VTC1, lacking detectable polyP, is available for phenotypic characterization (48). Growth of the vtc1-1 mutant was not adversely affected by excess Mn (Figure 3A), yet a role for polyP in Mn accumulation became evident when we determined the elemental profiles of vtc1-1 vs. VTC1 complemented strains cultured in medium with varied Mn content. The complemented strain accumulated Mn with the same linear capacity as did the wild-type laboratory strain CC-4532, with an 11-fold increase in Mn content between 6 µM and 600 µM (Figure 1B, Figure 3B). This capacity was greatly reduced in the vtc1-1 mutant: starting with only half of the Mn content normally seen in VTC1, the mutant only managed a two-fold rise in its Mn content, even when supplied with 600 µM MnEDTA (Figure 3B). Other elements that differentiated VTC1 and vtc1-1 included P (Figure 3C) and Ca (Figure 3D): neither element content changed as a function of Mn supply in either genotype, but P levels in vtc1-1 were one third of those in VTC1, while Mn levels reached at most one tenth of those in VTC1, suggesting that Ca ions may associate directly with polyP. That vtc1-1 mutants have a lower P quota is expected, as vacuolar polyP

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makes a substantial contribution to the total cellular P content in wild-type cells, equivalent to molar concentrations of P inside acidocalcisomes (49). The elemental profile of the Chlamydomonas vtc1-1 mutant is in line with the yeast vtc1 mutant, which has an even more pronounced decrease in internal P content (50).

If the lower Ca content of vtc1-1 were responsible for its inability to accumulate Mn, then growing wild-type cells with a fraction of the Ca normally supplied in our standard growth medium (340 µM) should phenocopy the vtc1-1 mutant phenotype. However, we did not discern any drop in Mn accumulation, even when the Ca supply was reduced to only 10% of normal levels, leading to a ~60% reduction in cellular Ca (Figure 5A, 5B). Cellular P contents only decreased slightly at the lowest Ca supply levels (Figure 5C). In contrast, withholding P from the growth medium, which resulted in a 40-fold drop in cellular P levels (Figure S8) did interfere with both Mn and Ca accumulation. Indeed, P-limited cells only contained one quarter of the Mn taken up by P-replete cells when exposed to excess Mn (Figure S8A); their Ca content was also very low and reminiscent of Ca levels in vtc1 mutants (Figure 3D, Figure S8C). By similarity with E. coli and yeast, we hypothesize that P limitation is accompanied by a severe drop in polyP that would prevent Mn (and Ca) accumulation, in agreement with our results with the vtc1-1 mutant (Figure 3). As shown recently for Mg ions (37), polyP therefore also plays a crucial role in Mn and Ca accumulation inside acidocalcisomes.

Aksoy and colleagues reported that acidocalcisomes were missing in vtc1-1, which would easily explain the loss of Mn accumulation in the mutant. However, their conclusion relied on identifying acidocalcisomes solely as electron dense particles in TEM images (48). Such particles inside acidocalcisomes are likely polyP crystals (Ruiz et al., 2001; Azevedo and Saiardi, 2014; Hothorn et al., 2009). vtc mutants do not accumulate polyP in yeast (35) or Chlamydomonas (48), and we wondered whether the presented evidence for the lack of acidocalcisomes in the vtc1-1 mutant might be inconclusive. We therefore used the lysosensor dye DND189, which is used to visualize acidic organelles like lysosomes and acidocalcisomes. We observed many small acidic compartments in VTC1 and vtc1-1 cells grown under both standard conditions and Mn excess, which argues that the vtc1 mutant can generate a significant proton gradient across the acidocalcisome membrane (Figures 4A and 4B). Staining of polyP with DAPI was only positive in VTC1, as previously described (Figures 4C and 4D, Aksoy et al., 2014). The inability of vtc1-1 to accumulate Mn thus cannot be attributed trivially to a loss of acidocalcisomes in the mutant, and points to another process that is impaired.

To determine whether the acidocalcisome membrane was compromised in the absence of VTC1, we searched for the vacuolar membrane proteins H+-pyrophosphatase and V-ATPase in a proteomics dataset performed in another vtc1 allele (vtc1-2, with similar defects in P, Ca and Mn accumulation as vtc1-1, Figure S9A) and its associated wild-type strain CC-4533. We detected comparable spectral counts for the H+-pyrophosphatase and all subunits of the V-ATPase in vtc1-2 and its wild-type strain (Figure S9B), which is consistent with the presence of an organelle with a functional and energized boundary membrane (as noted from lysosensor DND189 staining, as mentioned above). These results also argue that although the yeast and Chlamydomonas vtc1 mutants are phenotypically close in terms of their elemental profile (Figure 3, Yu et al., 2012), they are not biochemically identical. Indeed, the yeast vtc1 mutant is distinct in that it causes a general drop in the levels of several V-ATPase subunits at the vacuolar membrane (47), and the locus is named for that phenotype (vacuolar transporter chaperone). Also not observed in the Chlamydomonas vtc1 mutant was a large change in the levels of the H+-pyrophosphatase, or in the predicted homologues to the yeast membrane H+-ATPase Pma1p (Figure S9C). We did however detect fewer peptides for the VTC4 subunit of the VTC complex in the mutant, as might be expected when a multi-subunit complex is missing a component. Although we cannot rule out more minute changes in the levels or localization of vacuolar membrane proteins in the Chlamydomonas vtc1 mutant, we conclude that the acidocalcisome boundary membrane is largely functional.

*Mobilization of stored Mn when challenged by Mn deficiency*

It is well-established that cells will prioritize particular metalloenzymes when faced
with limiting supply (43, 53). Although \( \text{vtc1} \) mutants only accumulate one third of the Mn normally seen in wild-type cells under standard growth conditions (Figure 3, Figure S9A), they show no signs of Mn deficiency, and Mn-containing proteins are just as abundant in the \( \text{vtc1} \) mutant as they are in the wild-type strain (Figure S9C). We therefore asked if the Mn pool that accumulates in wild-type cells was biologically accessible. Accordingly, we first grew \( \text{vtc1-1} \) and \( \text{VTC1} \) strains photo-autotrophically in 6 µM and 600 µM MnEDTA, washed cells with EDTA to remove trace metals from the cell surface before transferring them to fresh medium with no added Mn. We chose photo-autotrophic conditions to place a stronger pressure on the photosynthetic apparatus, the main Mn sink and with essential function in photosynthetic cells, to more easily discern a phenotype (54). Indeed, the growth potential of Chlamydomonas cells was determined by the genotype at the \( \text{VTC1} \) locus and by prior exposure to excess Mn. \( \text{VTC1} \) cultures grew well if the inoculum originated from a 600 µM MnEDTA pre-culture, but not from a 6 µM MnEDTA pre-culture (Figure 5A). The elemental profile for Mn across conditions confirmed Mn accumulation in \( \text{VTC1} \) cells grown in 600 µM MnEDTA at all times. This stored Mn was sufficient to sustain growth for at least one week when cells were transferred to growth medium with no added Mn, demonstrating that it was biologically accessible under Mn-limitation. The \( \text{vtc1-1} \) mutant presented an important control: in the absence of Mn accumulation typical for this mutant, cells could not survive unless provided with some MnEDTA in the growth medium, even when the pre-culture was exposed to 600 µM MnEDTA (Figure 5A). This control also argues against the trivial explanation of Mn carry-over from the pre-treatment condition. Actively-growing photosynthetic cells require 2-3 \( 10^7 \) atoms Mn per cell to perform adequately, and this held true in this experiment. Growth-arrested cells were well below this mark, with Mn levels closer to 0.5 – 0.7 \( 10^7 \) atoms Mn per cell (Figure 5B).

In summary, stored Mn from acidocalcisomes can be remobilized to sustain growth and photosynthesis when Chlamydomonas is challenged by Mn-limitation. **Determination of Mn oxidation and speciation within acidocalcisomes**

To validate the hypothesis that polyP was acting as the main Mn-ligand by defining the complexation and speciation of cellular Mn, we applied X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) spectroscopies to probe nearest-neighbor (XANES) and long-range (EXAFS) metal-ligand environments. We focused on the Mn K-edge, during which a metal 1s core electron is promoted to a vacant 3d orbital (in the pre-edge region) or to the continuum for all metal collected from cells grown in excess Mn (1000 µM MnEDTA). As shown in Figure 6A, all samples showed a limited pre-edge feature in the XANES, consistent with metal existing in an octahedral ligand symmetry (9, 55). The excitation edge for Mn in cells matched that from the MnSO\(_4\) standard in both inflection energy and overall shape, indicating that, as expected, accumulated Mn was predominantly present in the low-oxidation Mn\(^{2+}\) species and not in a high-oxidation state.

Oscillations in an EXAFS spectrum are caused by scattering of the excited electron from the absorbing atom interacting with neighboring scattering atoms, the deconvolution of which provides a means to determine the distance between Mn and the neighboring atom, and to estimate the chemical nature of this atom through simulation of a theoretical curve to the empirical data (56). Fourier transforms of EXAFS data provide a ~0.5 Å phase-shifted radial distribution function of the ligand environments around the absorbing metal (Figure 6B (right, top and bottom)) while simulations of the EXAFS data can be used to provide direct metrical details of the metal-ligand environment (Figure 6B (left, top and bottom)). The best fit simulation results suggested a nearest-neighbor ligand environment consisting of 5 to 6 oxygen and/or nitrogen ligands (based on error bars of the data) at a distance of 2.18 ± 0.02 Å, while long-range scattering could be equally explained by 2-4 carbon (C) ligands or a single phosphorus (P) ligand (around 4Å from Mn atoms), with weaker support for P scattering (Table 1, Figure 6). A double peak between 2-4k was poorly explained by a fit with either C or P. Hydrated Mn Mn-(H\(_2\)O)\(_6\) can exhibit such oscillations in the 2-4k range, especially at lower pH (9), which would be compatible with the acidic environment of the
acidocalcisome. A contribution by an imidazole-containing ligand, for example histidine, is another (non-exclusive) possibility, as Mn-histidine complexes only display a single peak in the 2-4 k range (9).

We next employed electron paramagnetic resonance (EPR), electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) spectroscopies to resolve the nature of the Mn ligands in our samples (McNaughton et al., 2010, Sharma et al., 2013). EPR spectra of intracellular Mn2+ qualitatively paralleled the elemental analysis (Figure 1B), showing an over 10-fold greater accumulation of Mn in wild-type cells grown in the presence of 600 µM MnEDTA (black line, Figure 7A) relative to cells grown with the standard 6 µM MnEDTA (red line). vtc1-1 cells exhibited reduced capacity to accumulate Mn (~4-fold) compared to CC-4532 and VTC1 complemented strain (Figure S10). We therefore used the Bodipy-based fluorescent probe M1, specific for Mn2+ (57) to assess Mn accumulation in vtc1-1 and the complemented strain. Figure S11 shows that the M1 probe both 1) supported reduced Mn accumulation in the mutant and 2) showed localization within a lysosensor-positive organelle. Note that the sensitivity of the M1 probe is insufficient to detect Mn within acidocalcisomes of cells grown under normal Mn nutrition conditions.

Mn2+ in both genotypes showed the narrow EPR signal (small zero-field splitting) with resolved six-line 55Mn hyperfine pattern centered at g-2 (~12 kG) of Mn2+ coordinated in the high-symmetry, octahedral geometry commonly observed in Mn-metabolite complexes (Figure S10). From the EXAFS results above, we hypothesized that Mn-metabolites could be P-containing compounds, for example inorganic phosphate (Pi), phytate (Phy) or polyphosphates (polyP), the last providing an elegant explanation for the loss of Mn accumulation in vtc1-1 mutants, defective in polyP biogenesis. We therefore applied ENDOR/ESEEM spectroscopies to distinguish among these candidate metabolites by comparing spectra derived from cells grown in 6 µM or 600 µM MnEDTA with spectra generated from standard solutions of Mn-Pi, Mn-Phy and Mn-polyP (shown in Figure 7B). The 1H signals represent protons from water molecules bound to Mn, their intensity decreasing as the H2O ligands of Mn2+(H2O)6 are replaced by metabolite ligands. For each Pi molecule binding to Mn, one water molecule is displaced, whereas in Mn-polyP complexes virtually all water molecules are displaced. The presence and abundance of P-based ligands can itself be inferred from the presence of their 31P signals: In short, the Mn2+ acts as an ‘indicator’ of the relative concentrations of competing ligands free within the cellular environment.

31P ENDOR spectra from Mn2+ in general show a doublet centered around the 31P larmor frequency and separated by a hyperfine coupling A_Pi ~4; the peaks at lower/higher frequency are labelled as ν−/ν+. Although in many spectra of Pi and polyP complexes the lower-frequency (ν−) peak is not well resolved, the single ν+ peak is sufficient to characterize the complex. Mn-Pi and Mn-polyP complexes show similar 31P couplings, but they exhibit different relative amplitudes of the 31P and 1H ENDOR responses, which together allow identification of the contribution from Pi/polyP (21). Phytate complexes exhibit two partially overlapping doublets with hyperfine coupling A_Phy ~4, 8 MHz. (Figure S12): again the ν+ peaks are sufficient to characterize the complex, and the relative amounts of Pi and phytate complexes can be deduced by decomposing an observed cellular 31P ENDOR spectrum into individual component contributions.

For Mn-replete cells grown in 6 µM MnEDTA, by combining 31P, 1H absolute ENDOR response (Figure 7C) and comparing them with standards (Figure 7B), we discovered that Mn-Pi and Mn-Phy complexes accounted for ~50%, and ~21% respectively of the total cellular Mn2+ pool, with minimal contribution from polyP (Figure 7C, D, F; Figure S13): for every 10 cellular Mn2+ ions, 5 will have bound Pi, and another 2 will be in a complex with Phy. This result was surprising to us, as we had hypothesized a polyP-based ligand in light of the vtc1 mutant phenotype. ENDOR spectra from cells grown in 600 µM MnEDTA were likewise incompatible with a polyP ligand: in these cells the 31P peak decreased, while the 1H peak was higher, suggesting that the Mn was not predominantly associated with polyP, as predicted (Figure 7C, E, F). Indeed, the analysis suggested that on average only about one in ten Mn atoms was bound to Pi, while the fraction...
associated with Phy remained constant, with roughly two of ten Mn complexed to Phy for every 10 Mn (Figure 7E, F; Figure S13).

Cells grown under excess Mn conditions accumulate Mn to about 10-fold the levels of Mn$^{2+}$ found in cells grown in Mn-replete conditions (Figure 1B; Figure 7G). As a result, although the fraction of Mn-Pi decreases with increasing Mn accumulation (Figure 7D-F), ENDOR-derived speciation shows that the total cellular pool of Mn-Pi complexes in fact doubles between Mn supply levels of 6 µM and 600 µM. The fraction of Mn that binds Phy does not change when cells are grown under excess Mn, so the total Mn-Phy cellular pool sees a 10-fold increase from 6 to 600 µM Mn. Thus, using Mn$^{2+}$ as an indicator for its ligands and their concentrations, this means that as the cellular Mn$^{2+}$ increases, so does the ratio of ligand concentrations, [Phy]/[Pi].

Despite higher absolute contributions from Mn-Pi and Mn-Phy, even under excess Mn conditions little Mn is bound by polyP. In addition, a careful investigation using ENDOR/ESEEM revealed no $^{14}$N signals, which indicates that wild-type cells do not contain a significant population of Mn$^{2+}$ coordinated by nitrogenous ligands. A high percentage of Mn may therefore be in a complex with ligand(s) not visible by ENDOR spectroscopy (most probably carboxylato metabolites).

**Reversible sequestration of Mn by an ENDOR-silent ligand**

The ENDOR results described above did not support the initial hypothesis that intracellular Mn$^{2+}$ is bound to polyP. We wondered whether Mn$^{2+}$ might however be transiently bound to polyP, which could be visualized in a time-course experiment. To this end, we first grew cells in 6 µM MnEDTA and then shifted them to fresh medium containing 600 µM MnEDTA to induce accumulation. We collected aliquots daily and measured intracellular Mn by elemental analysis, and determined the fraction bound to Phy and Pi. Mn$^{2+}$ accumulation only took place when cells reached the end of their exponential growth phase and transitioned to stationary (Figure 8A). These results align well with the timing of polyP accumulation inside acidocacisomes in Chlamydomonas and other unicellular eukaryotes (58), again suggesting that polyP is an important contributor to Mn accumulation, even if we cannot detect substantial Mn-polyP metabolites. ENDOR spectroscopy of the same samples revealed constant Mn-Phy levels of about 20% and Mn-Pi levels around 60% before cells reached stationary phase. However, as cells began to accumulate 15 times the levels of Mn between 36 and 48 h after inoculation, Mn-Pi levels concomitantly decreased by 50%, with minimal contribution from Mn-polyP complexes (Figure 8B), further arguing against a role for polyP as the Mn ligand at any stage during Mn accumulation (Figure 8B).

In a reciprocal experiment, we transferred cells adapted to Mn excess into fresh medium with no added MnEDTA and collected samples every day. Cellular Mn content decreased drastically within 24 h, and stabilized within 2 days, as cells first mobilized their intracellular stores to sustain growth (Figure 8C). Mn-Pi levels mirrored the changes in intracellular Mn: the fraction of Mn-Pi complexes increased sharply within 2 days following the step down from 600 µM to 0 µM MnEDTA, but Mn-polyP complexes did not appear (Figure 8D).

These results demonstrate that cells 1) accumulate Mn at the end of the exponential growth phase, 2) do not rely on polyP as a final storage form of Mn-complexes, and 3) can quickly remobilize accumulated Mn during micronutrient scarcity, consistent with our earlier observation (Figure 5).

**Imidazole becomes a Manganese ligand in the absence of polyP**

What is the Mn$^{2+}$ speciation in the vtc1-1 mutant, which lacks polyP? ENDOR spectroscopy identified the Mn-P ligand complexes mainly as Mn-Phy (40%), with minimal contribution from Mn-Pi (Figure 9A, 9B). Surprisingly, in contrast to wild-type (CC-4533 and VTCl complemented strains), the vtc1-1 mutant had a significant population of Mn-$^{14}$N metabolites when grown under Mn-replete conditions. As depicted in Figure 9C, the Mn-imidazole complex shows a strong 3-pulse ESEEM time-wave modulation, which is caused by Mn-$^{14}$N electron nuclear hyperfine coupling to a bound imidazole. Under identical assay conditions (namely samples were set to have equal amounts of Mn during the assay), the vtc1-1 mutant generated time-wave traces similar to
those of the reference Mn-imidazole complex, but with a signal amplitude only 20% of that from Mn-imidazole.

These results therefore indicate that ~20% of the cellular Mn$^{2+}$ ions in vtc1-1 are bound to an imidazole-type ligand in vtc1-1, presumably histidine-based, which could arise from low molecular-weight metabolites and/or Mn-containing enzymes. In the green lineage, Mn-SODs and the oxygen-evolving complex are the main Mn-bound enzymes, but the EPR spectra show no contribution from either, and allow us to rule both out as the source of the imidazole-based ligand in vtc1 (59, 60). The two wild-type strains (CC-4532 and the VTC1 complemented strain) have no discernable Mn-imidazole signal (Figure 9C). Mn-imidazole metabolites may be present in wild-type strains but their signal could be masked by Mn-P metabolites.

**DISCUSSION**

To prevent mismetalation of enzymes that incorporate iron, zinc or manganese as cofactors, cells maintain a limited cytosolic pool of these transition metals by the concerted action of regulated uptake and sequestration from the cytosol (17). In the unicellular alga *Chlamydomonas reinhardtii* and all photosynthetic organisms, mitochondrial superoxidase dismutases and the chloroplast photosystem II complex represent the main Mn sinks in the cell. Although Mn sequestration by the plant vacuole, Golgi and endoplasmic reticulum have been established, how *Chlamydomonas* copes with and stores excess Mn is unclear. Our results, comprising observations from complementary approaches, implicate a lysosome-related organelle, the acidocalcisome, as the main sequestration site for cellular Mn.

NanoSIMS mass spectrometry (Figure 2), XFM (Figure S3), and EDX spectroscopy (Figure S4) demonstrated co-occurrence of Mn with Ca and P, both markers of acidocalcisomes (38, 48, 49, 61). A vtc1 mutant defective in the function of the VTC complex was unable to accumulate Mn to wild-type levels under Mn-replete conditions (6 µM MnEDTA) and failed to accumulate Mn under excess conditions (600 µM MnEDTA) (Figures 3 and 5). The VTC complex localizes to the vacuolar membrane and catalyzes the synthesis of polyP chains of variable length (from tens to hundreds of Pi residues) by pulling inorganic phosphate from the cytosol and translocating the elongating polyP chain into the vacuole in an ATP-dependent manner. The polyP inside acidocalcisomes is likely to accumulate in a low pH environment, surrounded by cations like Ca$^{2+}$, Mg$^{2+}$ and K$^+$. Unlike Mn$^{2+}$, which has no unpaired electrons and is hence invisible to paramagnetic resonance spectroscopies, the coordination state of Mn$^{2+}$ can be probed using EPR/ENDOR/ESEEM spectroscopies (19, 21). Hypotheses concerning divalent cation accumulation (37) can therefore be tested for and with Mn$^{2+}$, but not for Mg$^{2+}$. EPR spectra from Mn-accumulating cells categorically demonstrate that cellular Mn is not found complexed to EDTA, as the observed EPR signals display only the relatively narrow signal of a high-symmetry metabolite complex with well-defined six-line $^{55}$Mn hyperfine pattern, which is very distinct for Mn$^{2+}$.
from the broad and low-symmetry signal of Mn-EDTA, with a negligible $^{55}$Mn hyperfine pattern (62). EPR spectroscopy therefore argue against significant uptake of EDTA from the growth medium by Chlamydomonas cells, even in the presence of 1 mM EDTA.

Our results show that Mn$^{2+}$ accumulates in the acidocalcisomes and further reveal that Mn$^{2+}$ is found as mononuclear complexes with inorganic phosphate (Pi) and phytate (Phy), but not polyP. Further, our EPR measurements of Mn$^{2+}$ in the presence of polyP do not suggest that polyP can form antiferromagnetically coupled poly-nuclear polyP-Mn$^{2+}$ complexes (Figure S9), and the conclusion that virtually all the Mn is in the Mn$^{2+}$ state is supported by the parallelism of changes in total Mn and in EPR-detectable Mn$^{2+}$. Under replete conditions (6 µM MnEDTA), about 70% of total Mn$^{2+}$ was complexed to either Pi or Phy, but the contribution from polyP complexes was minimal (Figures 7 and 9; Figure S11 and S12). Under Mn excess conditions (600 µM MnEDTA), Mn-Pi levels doubled, while Mn-Phy levels increased 10 times; however, together, Mn-Pi and Mn-Phy only accounted for one third of the total Mn accumulated in these cells, with the remaining two thirds of the pool of Mn$^{2+}$ likely coordinated to carboxylato metabolites, which are ENDOR-silent. Nevertheless, polyP must be present in cells to permit translocation of Mn into acidocalcisomes, as Chlamydomonas vtc1 mutants cannot accumulate Mn, pointing to a transitory role for polyP in Mn homeostasis.

The speciation profile of Mn in vtc1 mutants was very distinct from wild-type cells: ENDOR features derived from $^{31}$P markedly decreased in the vtc1-1 mutant (Figure 9A, B), consistent with a much greater contribution from ENDOR-silent ligands. However, ESEEM spectroscopy shows that ~20% of Mn$^{2+}$ takes on an imidazole-based metabolite as a ligand in the absence of polyP, possibly acting to concentrate this divalent transition metal into acidocalcisomes. A similar population of Mn$^{2+}$-imidazole complexes was previously observed in Deinococcus radiodurans (19).

We were surprised by how effective Chlamydomonas cells were at accumulating Mn, which is in stark contrast to the rates of uptake for Cu (41) and Fe (13), which becomes restricted once the cell has the amount it needs to maintain metalloprotein quotas. Much of the work on high Mn tolerance comes from yeast: a loss of function in the MnSOD enzyme Sod1p causes elevated oxidative stress, but this can be rescued by high concentrations of exogenous Mn. Once inside the cell, cytosolic Mn forms complexes with inorganic phosphate and can then compensate for the loss of Sod1p due to its high intrinsic superoxide dismutase activity (20). Mn and P metabolism are largely co-dependent: Mn and P are thought to be co-transported by phosphate transporters, which is supported by the lower Mn content of phosphate transporter mutants (36, 63, 64). However, not all yeast mutants with altered P content agree with this model, unless their polyP pool is also taken into account. For example, pho80 mutants exhibit a constitutive P deficiency phenotype, leading to 1) increased P uptake, 2) high polyP levels and 3) much higher intracellular Mn content (34, 46). The Mn pool in pho80 mutants is presumed to be cytosolic, but we hypothesize a vacuolar localization, where polyP acts as a molecular magnet to sequester Mn away from the cytosol, thereby robbing pho80 cells from Mn-catalyzed detoxification protection. In agreement with our hypothesis, Chlamydomonas vtc1 mutants and yeast vtc mutants fail to accumulate Mn and have much lower polyP levels (35, 36, 48, 50), and may offer an alternative and more comprehensive explanation that ties Mn and P metabolism via polyP.

The crystal structure of the catalytic domain of yeast Vtc4 exposes another factor potentially contributing to the high capacity of cells to accumulate Mn (52). The substrate-binding site is coordinated with Mn$^{2+}$ via six positively-charged and one negatively-charged amino acids and a tyrosine residue that are conserved between yeast and Chlamydomonas (Figure S14). Vtc4p is inactive without a metal cofactor, and Mn$^{2+}$ cations offered the strongest stimulation of polyP synthesis, followed by Zn$^{2+}$, Co$^{2+}$, Mg$^{2+}$ and Ni$^{2+}$ (52). The potential for accumulation may become obscured by the associated toxicity of the transition metal, as would be expected for Zn$^{2+}$, Co$^{2+}$ and Ni$^{2+}$. Only in the case of Mn$^{2+}$ (this study) and Mg$^{2+}$ (37) will the metals be taken up by cells and stored inside acidocalcisomes, all the more so that Mn$^{2+}$ and Mg$^{2+}$ will stimulate polyP synthesis in a feed-forward loop, as long as cytosolic inorganic P is available.
Previous attempts at estimating the Mn quota of healthy Chlamydomonas cells did not distinguish between Mn pools participating in photosynthesis or stored in acidocalcisomes. The pool of Mn sequestered inside the vacuole is fully bioavailable when cells are faced with Mn scarcity conditions (Figure 5). The Chlamydomonas vtc1 mutant cannot synthesize or store polyP in its acidocalcisomes, and therefore lacks one major cellular Mn sink, which grants us access to the photosynthetic Mn quota, which we estimate to be \( \approx 2 \times 10^7 \) Mn atoms per cell (instead of the previous estimate of \( 4-5 \times 10^7 \) Mn atoms per cell, Figure 3) (65). Likewise, we can estimate the polyP content of Chlamydomonas cells relative to total P content using the vtc1 mutant: about 60\% of all cellular P is engaged in polyP and other low molecular weight complexes.

In conclusion, we show here that Chlamydomonas cells can accumulate the transition metal Mn\(^{2+}\) to very high levels, complexed in part with inorganic phosphate and phytate metabolites. PolyP is critical for this process, but is not the final ligand for Mn, as little Mn-polyP complex is found in any of the variants studies. Instead, we propose that Chlamydomonas cells utilize polyP to concentrate Mn\(^{2+}\) cations into acidocalcisomes. At least in yeast, high cytosolic levels of polyP are toxic, and polyP cannot be translocated to the vacuole from the cytosol (66). The role of polyP in Mn\(^{2+}\) uptake is likely restricted to the acidocalcisomes, where we propose it plays an escorting role to a final, and currently unknown ligand(s). Our results may also cast the role of polyP in Mg\(^{2+}\) acquisition in a different light (37). The speciation state of Mn\(^{2+}\) will provide an excellent indicator for the yeast vacuolar environment in cells grown in the presence of high concentrations of EDTA. The ability to identify whether EDTA is an intracellular ligand will shed light on the sequestration of metals into acidocalcisomes/vacuoles in Chlamydomonas and yeast and will refine the role of polyP within this cellular context.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**

*Chlamydomonas reinhardtii* wild-type strains CC-4532, mutant strain vtc1-1 (CC-5321), and vtc1-1 strain complemented with the VTC1 locus (here referred to as VTC1, CC-5324) were cultured in Tris-acetate-phosphate (TAP) medium with revised trace elements (65). Cultures were incubated at 24 °C with constant agitation (180 rpm) in an Innova incubator (New Brunswick Scientific, Edison, NJ) in continuous light (\( \approx 100 \) μmol of photons per m\(^{-2}\) ⋅ s\(^{-1}\)) provided by cool white fluorescent bulbs (4100 K) and warm white fluorescent bulbs (3000 K) in the ratio of 2:1. High photon flux density (350 - 420 μmol of photons per m\(^{-2}\) ⋅ s\(^{-1}\)) was provided by a white fluorescent bulb (3800 K).

Where indicated, excess manganese was supplied as EDTA-chelated MnCl\(_2\). Manganese-deficient medium was prepared without Mn supplementation (5). For photoautotrophic growth, acetate was eliminated from TAP medium, the pH was adjusted to 7.4 with HCl, and the cultures were bubbled with air. Phosphorus-free medium (Tris-acetate, TA), was prepared by replacing potassium phosphate with 1.2 mM potassium chloride (67).

For all treatments, pre-cultures were grown in TAP medium until mid-log growth phase (3-5 × 10\(^6\) cells/ml), then used to inoculate test cultures at an initial cell density of 1× 10\(^6\) cells/ml. Cultures were collected for analysis at a cell density of 3 to 4 × 10\(^6\) cells/ml. For phosphorus deficiency, cells grown in TAP medium were collected by centrifugation (3,500 × g, 3 min), and washed twice with TA medium containing indicated amounts of manganese at cell density of 1× 10\(^5\) cells/ml (67).

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The vtc1-1 mutant and complemented strain (VTC1) were grown in photoautotrophic conditions for 2 rounds (one week each) with 6 μM Mn, with air bubbling. For pre-treatment, flasks were inoculated at 1×10\(^4\) cells/mL with 6 or 600 μM Mn and allowed to grow for 1 week with air bubbling. Cells were collected by centrifugation for 5 minutes at room temperature and washed twice in 5 mM EDTA and once in milliQ water. Test flasks with no added Mn were inoculated with washed cells at a density of 5 x 10\(^4\) cells/mL, and grown for 7 days with air bubbling.
For cell size analysis, a Cellometer Auto M10 (Nexcelom Bioscience) was used. Samples from three independent experiments were collected at mid-log growth phase and cell diameter was determined from 1,100 cells per sample.

**Chlorophyll content and Chlorophyll Fluorescence Measurements**

Chlorophyll was extracted from whole cells with an acetone/methanol (80/20, v/v) mixture. Total chlorophyll (Chl a and b) content was estimated from the absorbance at 646.6 and 663.6 nm measured on a PerkinElmer LAMBDA 25 UV/visual spectrometer with absorbance at 750 nm as a reference to remove background from cell debris, as described (68). Each measurement was done in two technical replicates collected from three independent experimental replicates.

Minimum chlorophyll fluorescence (F0) and maximum quantum yield of photosystem II (Fv/Fm) were determined in cells after 10 min dark adaptation under a saturating pulse with IMAGING-PAM MAXI Chlorophyll Fluorometer and ImagingWin software (Heinz Walz, Effeltrich, Germany).

**Elemental Content Measurements**

1×10⁸ cells from all cultures were collected by centrifugation at 3,500 ×g for 3 min and washed three times with 1 mM Na₂-EDTA, pH 8.0 to remove metals associated with the cell surface and twice with Milli-Q water. After removing remaining water by brief centrifugation, cell pellets were digested with 70% nitric acid at room temperature overnight and at 65 °C for 2 h. Digested samples were diluted with Milli-Q water to a final nitric acid concentration of 2% (v/v). To measure metal content of culture medium, aliquots of the medium were treated with nitric acid and diluted in a 1:1 mixture of Epon 812 and propylene oxide for 2 h and in a 2:1 mixture for 2 h. Finally, the previous mixture was infiltrated in pure Epon 812 overnight and cured in an oven at 60 °C for 48 h. Sections of 200-nm thickness (gray interference color) were cut on an ultramicrotome (RMC MTX) using a diamond knife and deposited on 100 mesh carbon-coated molybdenum grids (G100-Mo, Electron Microscopy Sciences).

For nanoscale secondary ion mass spectrometry (nanoSIMS) imaging of the intracellular distribution of bound P, Ca, and Mn, we used the LLNL CAMECA NanoSIMS 50 (Gennevilliers, France) (70–72). Sectioned cells were first located and imaged by a JEOL 7401 field emission electron microscope in scanning transmission electron microscopy (STEM) mode. Then they were relocated and analyzed in the NanoSIMS 50. A focused negative oxygen ion primary beam was scanned over the sample to generate secondary ion images. The secondary ion mass spectrometer was tuned for ~3500 mass resolving power (M/ΔM, 1.5x correction) (73) and ¹²C⁺, ³¹P⁺, ⁴⁰Ca⁺, and ⁵⁵Mn⁺ were detected simultaneously by electron multipliers in pulse
counting mode. The correct metal ion peaks were identified using NBS610 glass (National Institute of Standards and Technology, USA). The analysis areas were first sputtered with high current (~1.2 nA O⁻, 55 x 55 µm², 256 x 256 pixels, 1 ms/pixel, 11 cycles, depth ~30 nm) (70) to establish sputtering equilibrium, followed by moderate spatial resolution analysis (~500 nm, 200 pA O⁻, 50 x 50 µm², 256 x 256 pixels, 1 ms/pixel, 50 cycles). Higher spatial resolution images were also collected (~200nm, 30 pA, 50 x 50 µm², 512 x 512 pixels, 1 ms/pixel, 50 cycles).

The nanoSIMS ion image data were processed quantitatively using custom software (L’Image, L.R. Nittler, Carnegie Institution of Washington, USA). The ion images were corrected for detector dead time and image shifts between scans, and then used to produce ion images. Regions of interest (ROIs) were defined using an automated algorithm to subdivide the analyzed area into hexagons (Fig. S4). Hexagons that were not on cells were deleted. Ion ratios (³¹P⁺/¹²C⁺, ⁴⁰Ca⁺/¹²C⁺, and ⁵⁵Mn⁺/¹²C⁺) for each ROI were calculated by averaging the ratios over replicate scans. These data provide relative quantitative composition, but they were not standardized to provide concentration data.

**Confocal Microscopy**

Chlamydomonas cells were cultured to early stationary phase and collected by centrifugation (3500 x g, 3 min) and washed twice with 10 mM sodium phosphate, pH 7.5. All fluorescent dyes were diluted in 10 mM sodium phosphate pH 7.5 to a final concentration of 2 µM. Cells were treated with Lysosensor DND189, Lysosensor DND167 or M1 Mn probe and mounted on glass slides for immediate visualization. Cells treated with DAPI were first fixed for 10 min with 2% glutaraldehyde, followed by permeabilization with 40 µM digitonin for 10 min before mounting on glass slides for visualization. Microscopy was performed on a Zeiss LSCM Airyscan 880 (Figure 6C-D, Figure S6) equipped with a 63X/1.4 oil immersion objective or a Zeiss Elyra using Lattice SIM with a 100X/1.46 oil objective (Figure 6A-B). Images were recorded using filter sets or spectral mode as indicated in the figure legends. All aspects of image capture were controlled via Zeiss ZEN Black software, including fluorescent emission signals from probes and/or chlorophyll.

**X-ray Absorption Spectroscopy**

Multiple independent samples of 5x10⁸ cells from cultures treated with excess manganese were collected by centrifugation (3,500 x g, 5 min), and washed twice with 1 mM EDTA and twice with Milli-Q water to remove metals associated with cell-surface, and subsequently washed with 10 mM sodium phosphate. After removing the liquid, cells were suspended in 30% glycerol (v/v, estimated from volume of cell pellet) and loaded with a Hamilton syringe into a Kapton-wrapped lucite XAS sample cell and flash-frozen in liquid nitrogen. Samples were stored in liquid nitrogen until beam exposure. XAS data were collected at the Stanford Synchrotron Radiation Lightsource on beamline 9-3, which is equipped with a Si(220) double-crystal monochromator, equipped with a focusing mirror that also provides harmonic rejection. Fluorescence spectra were collected using a 100-element Ge solid state detector from Canberra. During data collection, the Oxford Instruments continuous-flow liquid helium cryostat was stabilized at 10 K. Manganese data were collected using a 3 µm Cr filter placed between the cryostat and the detector to reduce unassociated scattering. Mn foil spectra were collected simultaneously with the protein data for energy calibration. The first inflection point for Mn was set at 6543.3 eV. Manganese XAS spectra were recorded using 5 eV steps in the pre-edge regions and 0.25 eV steps in the edge regions and 0.05 Å⁻¹ increments in the extended X-ray absorption fine structure (EXAFS) region (to k=13 Å⁻¹), integrating from 1-20s in a k³-weighted manner. An average of 6 scans were collected and averaged for each sample. Each scan lasted approximately 45 minutes. Each spectrum was closely monitored for X-ray induced radiation damage.

XAS spectra were processed and analyzed using the EXAFSPAK program suite written for Macintosh OSX. Fluorescence scans corresponding to each channel were examined for anomalies. A Gaussian function was used in the pre-edge region and a three-region cubic spline was used in the EXAFS region. EXAFS data were converted to k space using E₀ values of 6560.00 eV. Spectra were simulated using single and multiple scattering amplitude and phase functions generated using the Feff v8 software package integrated within EXAFSPAK. Single
scattering models were calculated for oxygen, nitrogen, phosphorous and carbon to simulate possible manganese ligand environments. Calibrated scale factors (Sc) and model $E_0$ values were not allowed to vary during fitting; the Sc for manganese samples was 0.95. Mn data were fit out to a $k$ value of 13.0 Å$^{-1}$. Calibration from Mn (II) and Mn(III) theoretical model compounds were used to determine the fit $E_0$ and Sc parameters. $E_0$ values for Mn-O, Mn-C were set at -10 and Mn-P was set at -12. EXAFS spectra were simulated using both filtered and unfiltered data, however simulation results were presented for only fits to raw (unfiltered) data. Simulation protocols and criteria for determining the best fit were as described previously (56).

**Electron Paramagnetic and Electron Nuclear Double Resonance**

$2 \times 10^8$ cells from cultures were collected by centrifugation (3,500 × g, 5 min), and metals associated with the cell surface were removed by washing twice with 1 mM EDTA, pH 8 and twice with Milli-Q water. After a subsequent wash with 50 mM HEPES, pH 7.0, cells were resuspended in 30% glycerol in 50 mM HEPES, pH 7.0, and loaded into an ENDOR sample tube, flash-frozen in liquid nitrogen, and stored at −80˚C until analysis (21). 35 GHz CW EPR spectra were recorded using a lab-built 35 GHz EPR Spectrometer (74). As described in (21), the absorption-display EPR spectra (collected from cellular Chlamydomonas and Mn-metabolites by using continuous-wave (CW) “rapid passage” methods at 2K) are characteristic of an $S = 5/2$ ion with small zero-field splitting (ZFS), with the principal ZFS parameter, $D$, much less than the microwave quantum ($h\nu$). Such spectra show a central 55Mn ($I = 5/2$) sextet arising from hyperfine interactions, $A \sim 91$ G, that is associated with transitions between the $m_s = +1/2$ and −1/2 electron-spin substrates. These features “ride on” and are flanked by significantly broader signals from the four “satellite” transitions involving the other electron-spin substrates ($m_s \pm 3/2 \leftrightarrow \pm 1/2$). The net absorption spectrum is the sum of the five envelopes of these five transitions among substrates.

Pulsed ENDOR/ESEEM spectra were recorded using a lab-built 35 GHz pulsed EPR spectrometer (75). All spectra were recorded at 2K which is achieved using immersion helium cryostat. $^{31}$P, $^1$H Davies ENDOR spectra were recorded using the pulse sequence ($\pi - T - \pi/2 - \tau - \pi - \tau - \text{echo}$), where $T$ is the time interval for which the radio-frequency (RF) pulse is randomly hopped. ENDOR of a paramagnetic metal-ion center such as Mn$^{2+}$ provides an NMR spectrum of the nuclei that is hyperfine-coupled to the electron spin (76), and thus can be used to identify and characterize coordinating ligands (77, 78). The frozen-solution spectrum of an I =1/2 nucleus, such as $^{31}$P and $^1$H, coupled to Mn$^{2+}$ comprises a set of doublet features centered at the nuclear larmor frequency and split by multiples of the electron-nuclear hyperfine coupling ($A$). The primary doublet is associated with the $m_s = \pm 1/2$ electron spin sublevels of Mn$^{2+}$ and is split by $A$; weaker satellite doublets associated with the $m_s = \pm 3/2$ and $\pm 5/2$ sublevels are split by 3A and 5A. All spectra in this study display $^1$H signals that can be assigned to the protons of bound water. In addition, all the spectra, except for the aqueous solution, show a sharp $m_s = \pm 1/2$ $^{31}$P doublet from a phosphate moiety bound to Mn$^{2+}$ center. The intensities of $^{31}$P and $^1$H signals differ significantly among the spectra, and analysis of these intensities provide a means of assessing cellular Mn$^{2+}$ speciation (21).

3-Pulse ESEEM spectra were recorded using the pulse sequence, $\pi/2 - \tau - \pi/2 - T - \pi/2 - \tau - \text{echo}$ where $T$ is the time varied between second and third microwave pulses, with four-step phase cycling to suppress unwanted Hahn and refocused echoes (79). A $^{14}$N nuclei ($I = 1$) directly coordinated with $^{55}$Mn creates modulation in the electron spin echo decay, which is dominated by $^{14}$N hyperfine interaction. To quantitate $^{14}$N ESEEM responses from cellular Mn$^{2+}$, we chose as a standard the $^{14}$N response from the Mn-imidazole complex, which binds one Imidazole and (presumably) five waters.
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**FOOTNOTES**

1 Throughout the text, we use the elemental symbol (Fe, Mn) as an abbreviation of the word when we are referring generically to all species of a given element; we only use the ionic forms when specifically referring to a specific ionic form.
**TABLES**

Table 1. **Summary of Mn EXAFS fitting analysis for 1000μM Mn Chlamydomonas cell samples.** EXAFS fitting analysis was done for two independent sample replicates: 1000μM Mn(1) and 1000μM Mn(2). Long-range scattering could be best fit using carbon scattering (Solution #1) with F’ values of 0.85 and 1.02 for each replicate, or including phosphorus scattering (Solution #2) with F’ values of 0.78 and 1.02.

| Sample          | Nearest-neighbor ligand environment\(^a\) | Long-range ligand environment\(^a\) |
|-----------------|------------------------------------------|-----------------------------------|
|                 | Atom\(^b\)  | R(Å)\(^c\) | C.N.\(^d\) | σ\(^2\)\(^e\) | Atom\(^b\)  | R(Å)\(^c\) | C.N.\(^d\) | σ\(^2\)\(^e\) | F’\(^f\) |
|                 | O/N         | 2.18      | 5          | 4.93         | C           | 3.22      | 4          | 2.52         | 0.85    |
| 1000μM Mn(1)    | C           | 3.94      | 3          | 1.86         | C           | 4.45      | 4          | 1.66         | 1.02    |
|                 |             |           |            |              |             |           |            |              |         |
| 1000μM Mn(2)    | C           | 3.22      | 4          | 1.82         | C           | 3.97      | 4          | 1.26         | 1.02    |
|                 |             |           |            |              |             |           |            |              |         |
|                 | C           | 4.46      | 3          | 2.48         |             |           |            |              |         |
|                 |             |           |            |              |             |           |            |              |         |
|                 | P           | 3.34      | 1          | 1.59         | C           | 3.95      | 2          | 1.01         | 0.78    |
| 1000μM Mn(1)    | C           | 4.46      | 4          | 2.47         |             |           |            |              |         |
| 1000μM Mn(2)    | P           | 3.34      | 1          | 1.53         | C           | 3.98      | 4          | 2.03         | 1.02    |
|                 |             |           |            |              |             |           |            |              |         |
|                 | C           | 4.49      | 3          | 2.08         |             |           |            |              |         |

\(^a\) Independent metal-ligand scattering environment.

\(^b\) Scattering atoms: N (nitrogen), O (oxygen), C (carbon), P (phosphorus).

\(^c\) Average metal-ligand bond length, ± 0.13 Å⁻¹.

\(^d\) Average metal-ligand Coordination Number (CN), ± 1.0.

\(^e\) Average Debye-Waller factor (Å x 10³).

\(^f\) Number of degrees of freedom weighted mean square deviation between data and fit.
Figure 1. *Chlamydomonas* cells accumulate Mn under excess. Cultures were inoculated from a 6 µM MnEDTA pre-culture at an initial cell density of $10^4$ cells ml$^{-1}$ and grown in medium supplied with the indicated amount of MnEDTA (PFD, ~100 µmol m$^{-2}$s$^{-1}$). (A) Growth was measured by counting cells every 24 h in a hemocytometer. (B) Intracellular Mn content was quantified via ICP-MS from 4 independent experiment replicates. The horizontal bars represent the mean for each sample, ± standard deviation.
**Figure 2.** Correlative quantification of $^{12}$C$^+$-normalized $^{55}$Mn$^+$ with $^{31}$P$^+$ and $^{40}$Ca$^+$ shows the co-localization of Mn with Ca and P.

(A) Correlated STEM and nanoSIMS imaging of cells grown in medium containing 1000 µM Mn. Sections of fixed Chlamydomonas cells were imaged in positive secondary ion mode. Scale bar = 5 µm. (B, C) Correlative quantification of $^{12}$C$^+$-normalized $^{55}$Mn$^+$ with $^{31}$P$^+$ and $^{40}$Ca$^+$ from nanoSIMS imaged cells grown in 1000 µM (3 replicates) and 6 µM MnEDTA (2 replicates). The sub-cellular correlation of regions of interest (ROI) for $^{55}$Mn/$^{12}$C vs. $^{40}$Ca/$^{12}$C is shown in panel B and $^{55}$Mn/$^{12}$C vs. $^{31}$P/$^{12}$C in panel C. Arrows indicate regions of high ($\rightarrow$) and low ($\downarrow$) concentration of Mn, Ca, and P in the NanoSIMS image for each element; the same areas of interest are shown as ● (high) and ○ (low), respectively in the correlation plots in panel B and C.
Figure 3. *vtc1-1* mutant defective in polyphosphate and Ca accumulation does not accumulate Mn under excess Mn condition. Cultures of the complemented strain (*VTC1*) and *vtc1-1* mutant were inoculated into medium containing 6 or 600 μM MnEDTA with an initial cell density of 10⁴ cells ml⁻¹ (PFD, ~100 μmol m⁻² s⁻¹). (A) Growth was measured by counting cells every 24h in a hemocytometer. Total Mn (B), P (C), and Ca (D) content was measured via ICP-MS from 4 independent experiment replicates. The horizontal bars represent the mean for each sample, ± standard deviation.
**Figure 4. vtc1-1 mutant cells contain an acidic compartment.**
The low pH dye Lysosensor DND-189 can detect low pH compartments in both (A) *VTC1* and (B) mutant *vtc1-1* cells using Zeiss Elyra Lattice SIM in channel mode. (C) *VTC1* and (D) mutant *vtc1-1* cells exposed to replete (6μM) or high (600μM) Mn were stained with phosphate dye (DAPI) to observe intracellular polyphosphate accumulation (appears yellow when present). Chlorophyll auto fluorescence is shown in green. Confocal images were collected on a Zeiss 880 microscope using Airyscan in channel mode, and exposures were adjusted as needed to observe intracellular staining. Scale bar = 5μm. Images are representative of one replicate for each condition.
Figure 5. Hyper-accumulated Mn can be used as a reservoir under low Mn condition. Pre- and test cultures of the vtc1-1 mutant and complemented strain (VTC1) were grown in a minimal growth medium (–acetate) with air bubbling. Pre-test cultures were grown with 6 or 600 µM Mn for one week. Test cultures were inoculated with EDTA-washed cells with different Mn concentration (no added Mn, 6 and 600 µM MnEDTA) and grown for 7 days. (A) Representative photographs of flasks for VTC1 and vtc1-1 after 6 days of growth in absence of added Mn, after pre-treatment with 6 or 600 µM MnEDTA. (B) Total Mn content of VTC1 and vtc1-1 cells shown in panel A. The horizontal bars represent the mean for each sample, ± standard deviation (n = 3). The experiment was repeated three times with similar results.
Figure 6. **Over-accumulated Mn is a Mn²⁺ species predominantly associated with oxygen and nitrogen.**

(A) Mn XANES were obtained from cells grown in medium containing 1000 µM MnEDTA. Spectra with a pre-edge feature at 6541.1 eV (shown in pink) correspond to the 1s-3d transition. Comparisons of two sample duplicates (black) to a MnSO₄ standard (gray) represent Mn²⁺ species. The excess Mn samples were off-set from the standard. (B) k³-weighted EXAFS data (left panels) and phase shifted Fourier transform (right panels) are shown for representative scans of raw unfiltered data (black) and best-fit simulations (green) from Mn loaded cells. Near neighbor ligand environments are primarily constructed of O/N, while mixed scattering was observed in the long-range ligand environment for both P and C. EXAFS data for the long-range ligand environment corresponding to P is shown in panel B (top) and for C in panel B (bottom).
Figure 7. The final Mn ‘storage’ ligand is not polyP in wild-type Chlamydomonas cells.
EPR (A) and ENDOR (B, C) spectra were collected from wild type cells (genotype CC-4532) grown in 6 µM or 600 µM MnEDTA. x1, x10 represents magnification of EPR spectra. B presents ENDOR spectra for known Mn metabolite complexes as reference. The braces represent ENDOR responses of $^{31}\text{P}$, $^1\text{H}$ nuclei. The ENDOR peaks highlighted with gray highlights are used as to measure absolute $^{31}\text{P}$ and $^1\text{H}$ ENDOR responses, which are in turn used for calculating fractional contribution of different Mn-metabolites to the cellular Mn-ENDOR spectra. ENDOR spectra in C are magnified x2. The symbol (*) identifies the third harmonic peak of a $^{55}\text{Mn}$ ENDOR response at ~40 MHz (not shown). (D, E) $^{31}\text{P}$ signals from wild type cells grown in 6 (D) or 600 (E) µM MnEDTA. The $\nu_s$ peaks of the $^{31}\text{P}$ ENDOR spectra shown here are centered around the $^{31}\text{P}$ larmor frequency ($\nu_l$) indicated by the (triangle) symbol, and shifted to higher frequency by half the hyperfine coupling, $A/2$ as indicated for Mn-Phy and Mn-Pi metabolites. Note that the concentration of Mn$^{2+}$ in all samples was adjusted to be equal to facilitate comparisons of peak heights for $^1\text{H}$ and $^{31}\text{P}$ features. Inset: original scale of spectra shown in D and E. Panels F, G: Fraction of Mn bound to phytate (Phy) or orthophosphate (Pi) in wild type cells grown in normal or excess Mn conditions (F), and associated Mn concentrations (G). For panel F, The error bars are from the least square fit of the ENDOR data. For panel G, the horizontal bars represent the mean for each sample, ± standard deviation ($n = 3$). CW EPR conditions: MW frequency 34.8 GHz, $T = 2K$, magnetic field scan rate 2 kG/min, modulation amplitude 1G. Davies ENDOR conditions: magnetic field ~ 12.5 kG, $\tau_{21} = 60$ ns, $\tau = 400$ ns, $T_{r} = 160\mu$s, repetition time 10ms.
Figure 8. Time-course analysis of Mn-ligand formation and disappearance.

A, B. Chlamydomonas cells were first grown in 6 µM MnEDTA before being transferred to fresh medium supplied with 600 µM MnEDTA. Duplicate samples were collected over 48 h, their Mn content determined (A), as well as the fraction of Mn-metabolites bound to phytates (Phy) or pyrophosphate (Pi) (B).

C, D. In the reciprocal experiment, cells were first grown in Mn excess conditions in 600 µM MnEDTA before transfer to Mn-free medium. Again, Mn content (C) and the fraction of Mn-Phy and Mn-Pi (D) detected in samples over time was measured. The error bars in panels B and D are from the least square fit of the ENDOR data.
Figure 9. **Imidazole compounds as opportunistic Mn ligand in vtc1-1.**

ENDOR (A, B) and ESEEM (C) spectra were collected from CC-4532, VTC1 and vtc1-1 cells grown in 6 µM or 600 µM MnEDTA. Note that the concentration of Mn in all samples was adjusted to be equal and therefore facilitate comparisons of peak heights for $^1$H and $^{31}$P features.

**A.** Full ENDOR spectra for vtc1-1, VTC1 cells grown in 6 µM, and 600 µM MnEDTA.

**B.** $^{31}$P nu+ features from vtc1-1, and VTC1 cells grown in 6 µM or 600 µM Mn, with individual contributions from Mn-phytate and Mn-Pi metabolites indicated above each set of spectra as in Figure 7. Lower inset: original scale of spectra shown in B.

**C.** ESEEM spectra for Mn-imidazole (Mn-Imi), VTC1 and vtc1-1 cells grown in 6 µM MnEDTA. The magnitude of $^{14}$N time-wave modulation of Mn-imi complex is multiplied by 1/5 to match that from vtc1-1; both have the same Mn$^{2+}$ concentration. **ENDOR Conditions:** as in Figure 7. **ESEEM Conditions:** magnetic field ~ 12.5 kG, $t_{\pi/2} = 50$ ns, $\tau = 400$ ns, repetition time 10ms.
Manganese co-localizes with calcium and phosphorus in Chlamydomonas acidocalcisomes and is mobilized in Mn-deficient conditions
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