Manganese toxicity and *Saccharomyces cerevisiae* Mam3p, a member of the ACDP (ancient conserved domain protein) family

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Manganese is an essential, but potentially toxic, trace metal in biological systems. Overexposure to manganese is known to cause neurological deficits in humans, but the pathways that lead to manganese toxicity are largely unknown. We have employed the bakers' yeast *Saccharomyces cerevisiae* as a model system to identify genes that contribute to manganese-related damage. In a genetic screen for yeast manganese-resistance mutants, we identified *S. cerevisiae* *MAM3* as a gene which, when deleted, would increase cellular tolerance to toxic levels of manganese and also increased the cell's resistance towards cobalt and zinc. By sequence analysis, Mam3p shares strong similarity with the mammalian ACDP (ancient conserved domain protein) family of polypeptides. Mutations in human ACDP1 have been associated with urofacial (Ochoa) syndrome. However, the functions of eukaryotic

ACDPs remain unknown. We show here that *S. cerevisiae* *MAM3* encodes an integral membrane protein of the yeast vacuole whose expression levels directly correlate with the degree of manganese toxicity. Surprisingly, Mam3p contributes to manganese toxicity without any obvious changes in vacuolar accumulation of metals. Furthermore, through genetic epistasis studies, we demonstrate that *MAM3* operates independently of the well-established manganese-trafficking pathways in yeast, involving the manganese transporters Pmr1p, Smf2p and Pho84p. This is the first report of a eukaryotic ACDP family protein involved in metal homeostasis.

Key words: Mam3p, manganese, *Saccharomyces cerevisiae*, toxicity, urofacial syndrome, vacuole.

INTRODUCTION

Manganese is an essential trace element in biological systems. Manganese-dependent enzymes are found within diverse locations in the cell, including the Golgi, mitochondria and cytoplasm. However, high concentrations of manganese are potentially toxic. In humans, exposure to this metal can lead to manism, a Parkinson's disease-like neurological disorder with characteristic syndromes of mental difficulties and impairments in motor skills [1,2]. Although the importance of manganese in health and disease is widely recognized, very little is known regarding the factors that contribute to toxicity from this metal.

Saccharomyces cerevisiae, the bakers' yeast, has proven to be an excellent model system for studying manganese homeostasis [3]. A number of players have been successfully identified through genetic studies and virtually all have homologues in humans. Two Nramp (natural resistance-associated macrophage protein) family members, Smf1p and Smf2p, contribute to high-affinity manganese transport in *S. cerevisiae*. The role of Smf1p is to transfer manganese across the cell surface [4,5]. Smf2p is localized at intracellular vesicles and is required to deliver manganese to secretory pathway enzymes and to mitochondrial superoxide dismutase 2 [6]. Except under manganese starvation conditions, Smf1p and Smf2p levels are maintained at a low level to prevent high intracellular accumulation of manganese and other metals. This down-regulation of Smf1p and Smf2p under manganese surplus conditions involves targeting these proteins to the vacuole via a mechanism involving Bsd2p and ubiquitination in *S. cerevisiae* [5,7–9].

Under manganese toxicity conditions, pathways other than Smf1p/Smf2p mediate uptake of manganese into the cell [3]. We recently found that the cell surface phosphate transporter in yeast, Pho84p, has an additional role as a low-affinity manganese transporter [10]. Pho84p is responsible for manganese or manganese/phosphate complex uptake under manganese toxicity conditions. Strains containing a deletion in *PHO84* exhibit low manganese accumulation and resistance to manganese toxicity [10]. Detoxification of manganese in yeast is largely controlled by Pmr1p, a Golgi-localized P-type ATPase transporter for manganese and calcium [11–13]. This transporter helps to eliminate toxic manganese from the cell by pumping it into the Golgi, where manganese is then exported to the cell surface via the secretory pathway [12,14]. *pmr1*Δ mutants are highly sensitive to manganese and accumulate high levels of the metal [15]. But *PMR1* is also important for normal manganese homeostasis and is required to deliver manganese, as well as calcium, into the secretory pathway where these ions are required for protein processing and secretion [11–14]. Mutations in the human homologue of *PMR1* gene cause Hailey–Hailey disease [16,17], which is an inherited skin blistering disorder.

Protection against manganese toxicity in *S. cerevisiae* can also be facilitated by sequestration of the metal in the vacuole. The yeast vacuole participates in both the storage and detoxification of manganese and the metal appears concentrated in the vacuole compared with cytosol [18]. Yeast cells with mutations that affect vacuolar structure or H⁺-ATPase are sensitive to a wide spectrum of metal ions. In addition to manganese [19], zinc [20], cobalt [21] and copper [22,23] are also affected by such mutations.

Abbreviations used: ACD, ancient conserved domain; ACDP, ACD protein; COX4, cytochrome oxidase subunit IV; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; SD, minimal synthetic dextrose medium supplemented with the required amino acids; UFS, urofacial syndrome; YPD, yeast extract and peptone-based medium supplemented with 2% glucose; YPEG, yeast extract and peptone-based medium with 2% ethanol and 2% glycerol.

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Golgi and vacuolar sequestration of manganese are effective at guarding against metal toxicity to a certain extent. But under manganese surplus conditions, these detoxification pathways become overwhelmed and metal damage ensues. Other than Pho84p operating at the cell surface, virtually nothing is known regarding the pathways that lead to manganese toxicity under these conditions. To this end, we conducted a search for *S. cerevisiae* genes which, when deleted, would result in increased resistance to manganese toxicity; such genes should represent factors that contribute to manganese damage. One gene identified in this regard was *S. cerevisiae* *MAM3*. Mutations in *MAM3* confer very high levels of manganese resistance on yeast and also increase resistance to zinc and cobalt. Mam3p is a member of the ACDP (ancient conserved domain protein) family of proteins that are conserved from bacteria to humans [24]. Human ACDP1 has been linked to a genetic disease UFS (urofacial syndrome) or Ochoa syndrome [25,26], although the function of ACDP1 is not known. We show here that yeast Mam3p functions at the site of the vacuolar membrane to affect manganese toxicity through a novel mechanism that does not involve vacuolar sequestration of the metal or the manganese homeostasis pathways engaged by Pho84p, Pmr1p or Smf2p. Studies on Mam3p provide the first evidence for a eukaryotic ACDP being involved in metal homeostasis.

EXPERIMENTAL

Yeast strains, plasmids, media and culture conditions

Most of the strains used in this study were derived from parental strain BY4741 (*MATa*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *his3Δ1*). The *pho84Δ::kanMX4* (6524), *pmr1Δ::kanMX4* (4534) and *smf2Δ::kanMX4* (1878) mutant derivatives of BY4741 and the homozygous diploid pool of yeast deletion strains were purchased from ResGen. Disruptions of *MAM3* in strains BY4741, *pho84Δ*, *pmr1Δ* and *smf2Δ* were generated with the *mam3Δ::URA3* plasmid pMY001, resulting in strain MY001 (*mam3Δ*), MY003 (*pho84Δmam3Δ*), MY006 (*pmr1Δmam3Δ*) and MY002 (*smf2Δmam3Δ*). Gene deletions were verified by PCR. Strain MY010 (Rho⁻) was obtained by growing BY4741 on enriched YPD (yeast extract and peptone-based medium supplemented with 2% glucose) medium containing ethidium bromide (80 mg/l) and by monitoring for formation of petite colonies that failed to grow on medium containing ethanol and glycerol as carbon sources. The *pep4Δ* (XL126) and its isogenic wild-type strain YR98 have been described previously [9]. The strains SM2188 (*MATa his4 leu2 ura3 bar1-1*) and SM2186 (SM2188 *end3^{ts}*) were gifts from Dr S. Michaelis (Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.) [27].

Yeast transformations were performed using the lithium acetate procedure [28]. Cells were propagated at 30°C (unless specified otherwise) either in an enriched yeast extract, YPD or YPEG (yeast extract and peptone-based medium with 2% ethanol and 2% glycerol), or in SD (minimal synthetic dextrose medium supplemented with the required amino acids).

To construct the *MAM3* disruption plasmid pMY001, *MAM3* upstream sequences (−853 to −257) and downstream sequences (+2102 to +2701) were amplified by PCR using primers introducing *Bam*HI and *Hind*III or *Hind*III and *Sal*I sites respectively. The *MAM3* PCR products were digested with the indicated enzymes and ligated in a trimolecular reaction to the *Bam*HI- and *Sal*I-digested *URA3* integrating vector pRS306, resulting in pMY001. Transformation of yeast strains with pMY001 linearized with *Hind*III, resulted in deletion of chromosomal *MAM3*

sequences from −256 to +2101. Plasmid pMY003 (multicopy *MAM3*) contains *MAM3* sequences −694 to +2701 amplified by PCR and inserted at the *Bam*HI and *Hind*III sites of pRS425, a *LEU2* 2-micron based vector. The Mam3–GFP (green fluorescent protein) fusion plasmid pAP003 (given by A. Pledge and E. Luk, Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, U.S.A.) was constructed in the pAA1-based vector (*LEU2-CEN*) [29], and contains *MAM3* coding sequences and promoter up to −847 fused at the Mam3p C-terminus to GFP. The plasmid pHS12 [30] (COX4–GFP) was a gift from Dr R. Jensen (Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.) and is a *LEU2-CEN* plasmid containing one copy of GFP fused at the N-terminus to the pre-sequence of mitochondria COX4 (cytochrome oxidase subunit IV).

Screen for manganese-resistant mutants of *S. cerevisiae*

A frozen aliquot of the ResGen homozygous diploid deletion collection pool was thawed and cultured in liquid YPD medium for 5 h to a *D*₆₀₀ of 0.5 and spread at a density of 10⁷ cells/plate on to solid YPD medium supplemented with 10 mM MnCl₂. After incubating at 30°C for three days, manganese-resistant colonies were isolated and the genes affected in twenty independent colonies were identified by standard PCR and DNA sequencing analysis [31]. Each colony was subjected to two PCR reactions. The primers used in each reaction were either the pair U1 (5′-GAT GTC CAC GAG GTC TCT-3′) and KanB (5′-CTG CAG CGA GGA GCC GTA AT-3′), giving a 300-bp PCR product, or the pair D1 (5′-CGG TGT CGG TCT CGT AG-3′) and KanD (5′-TCG CCT CGA CAT CAT CTG C-3′), giving a 188-bp PCR product. The unique 20-bp tags integrated into the ORF (open reading frame) disruption cassettes were identified by DNA sequencing analysis of the PCR products (Johns Hopkins Genetic Resources Core Facility) using KanB and KanD as sequencing primers. Gene deletions were identified by matching the sequence results of the tags to the yeast deletion database (http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.htm).

Fluorescence microscopy, cell fractionation and immunodetection techniques

For fluorescence microscopy studies, strains BY4741 (wild-type parent), MY001 (*mam3Δ*), YR98 (*PEP4⁺*), XL126 (*pep4Δ*), SM2186 (*end3^{ts}*) and SM2188 (*END3⁺*) were transformed with pHS12 (COX4–GFP), pAP003 (Mam3–GFP *CEN* plasmid) or pRS415 (vector control). The transformants were grown under aerobic conditions to a *D*₆₀₀ of 1.0 in selective SD medium. Cells were washed or, where indicated, further fixed with 37% formaldehyde and stained with DAPI (4,6-diamidino-2-phenylindole). Cells (live or fixed) were monitored by fluorescence and Normarski DIC (differential interference contrast) microscopy on a Zeiss Axiovert 135TV microscope (microscopy facility, Johns Hopkins Medical Institutions) at a magnification of ×1000.

To monitor the membrane localization of Mam3–GFP, cell lysates were prepared as described previously [32] from 500 ml of cells grown in liquid SD medium to a *D*₆₀₀ of 2.0; 1 ml aliquots of cell lysates were supplemented with 0.5 M NaCl, 0.1 M Na₂CO₃ or 1% Triton X-100, followed by separation of membrane and soluble fractions by centrifugation at 100 000 *g* as described previously [32]. Each pellet fraction was resuspended in 1 ml of lysis buffer (50 mM Tris/HCl, pH 7.5, 200 mM sorbitol, 5 mM MgCl₂, 100 mM NaCl, 500 μM PMSF, 1:100 protease inhibitor cocktails) containing 1% Triton X-100. All fractions were then subjected SDS/PAGE (12% gel) and analysed

by Western blotting using an antibody directed against GFP (1:1000 dilution; Molecular Probes) and a secondary anti-rabbit IgG (1:125 000 dilution; Amersham). Detection employed the enhanced chemiluminescence (ECL[®]) kit (Amersham) according to the manufacturer's instructions.

Vacuoles were prepared according to published methods [18] from 300 ml of cells grown to a D_{600} of 2.0 in YPD liquid medium or YPD medium supplemented with MnCl_2 . Cells were washed twice with TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8) and once with de-ionized water prior to generation of spheroplasts and preparation of cell lysates as described previously [18]. Vacuoles were isolated by Ficoll gradient centrifugation [18], and vacuolar purity was monitored by Western blot analysis using antibodies directed against CPY (carboxypeptidase Y) and PGK1 (phosphoglycerate kinase 1), which are markers for the vacuole and cytosol respectively.

Metal measurements

For analysis of metal contents in whole cell, vacuolar or cytosolic fractions, cells were grown in liquid YPD medium, or the same medium supplemented with 500 μM MnCl_2 for either 16 or 1.5 h. Analysis of whole-cell manganese content was carried out as described previously [10] using a PerkinElmer Life Sciences AAnalyst 600 graphite furnace atomic absorption spectrophotometer according to manufacturer's instructions. In the case of cell fractions, 0.05–0.15 μg of proteins from vacuolar or cytosolic lysate fractions (generated as described above) were subjected to analysis.

Sequence alignments and topology

Alignments of Mam3p against human ACDP1 (NP_065081), ACDP2 (NP_951058), ACDP3 (NP_06093) and ACDP4 (NP_064569) were obtained using the ClustalW application (<http://www.ch.embnet.org/software/ClustalW.html>). Topology of Mam3p was predicted using TopPred 2 (<http://www.sbc.su.se/~erikw/toppred2>).

RESULTS

Manganese resistance in *S. cerevisiae* is associated with mutations in *MAM3*, encoding a member of the ACDP family of proteins

To identify yeast mutants that are resistant to manganese toxicity, the ResGen homozygous diploid collection of mutants was screened for mutants that survived on medium supplemented with 10 mM MnCl_2 . Manganese-resistant mutants occurred at a frequency of approx. 1×10^{-4} . A sampling of these revealed that more than half of the manganese-resistant mutants harbour mutations in *S. cerevisiae* *MAM3*.

S. cerevisiae Mam3p is a member of the ACDP family of proteins that contain an ACD ('ancient conserved domain') [24]. Whereas *S. cerevisiae* appears to express a single ACD containing protein (Mam3p), humans express four such proteins (ACDP1–ACDP4) [24].

ACDP proteins have received recent attention because human ACDP1 has been linked to UFS, a rare genetic disorder that affects both facial expression and the urinary tract [33]. The role of human ACDP1 in UFS is not understood, largely because the function of ACDP family members remains elusive. Our identification of *S. cerevisiae* *mam3* Δ as a manganese-resistant mutant suggests a possible role for eukaryotic ACDP proteins in metal homeostasis.

As seen in Figure 1(A), amino acids 241–402 of Mam3p represent the ACD sharing extensive similarity with human ACDPs [24]. Yet the similarity is not limited to this previously reported ACD region, and extends upstream to Mam3p amino acid ~70. Overall, the region spanning residues 71–402 shares 39 % identity and 59 % similarity to human ACDP2 and ACDP4, and 27 % homology and 47 % similarity to ACDP3, whereas Mam3p residues 192–402 share 35 % identity and 58 % similarity to human ACDP1 (Figure 1A). Mam3p is predicted to harbour three potential transmembrane domains (amino acids 66–86, 144–164 and 177–197) (Figure 1A and illustrated in Figure 1B), suggesting that Mam3p may be a membrane protein. The three potential transmembrane domains are also predicted for human ACDP2–ACDP4.

MAM3 gene dosage and resistance to metal toxicity in *S. cerevisiae*

To confirm that *MAM3* alone was responsible for affecting manganese resistance, we engineered a *mam3* Δ gene deletion in a haploid strain (BY4741), and found that, like the diploid mutants, the *mam3* Δ haploid was manganese resistant on both minimal medium (Figure 2A) and enriched medium (Figure 2B). Whereas deletion of *MAM3* caused manganese resistance, over-expression of *MAM3* was associated with increased manganese sensitivity compared with wild-type, as shown in Figure 2(A). There is a good correlation between *MAM3* gene dosage and sensitivity towards manganese toxicity.

We tested whether a *mam3* Δ mutation affected resistance towards other metal ions. As shown in Figure 2(B), disruption of *MAM3* also conferred resistance to cobalt and zinc. However, there was no increased resistance to toxicity from copper (results not shown) and cadmium (Figure 2B). If anything, *mam3* Δ mutants were associated with increased sensitivity towards cadmium (Figure 2B). Therefore, *mam3* Δ mutations affect resistance to a specific subclass of heavy metal ions.

mam3 Δ mutants are not defective in mitochondrial morphology or function

MAM3 was originally identified as an *S. cerevisiae* mutant exhibiting mitochondrial absent morphology. Through a systemic functional analysis of yeast gene-deletion mutants, *mam3* Δ mutants were reported to have short and round mitochondria and exhibit growth defects on non-fermentable carbon sources [34]. It was curious as to how a mitochondrial defect of this type could be associated with resistance to metal toxicity.

To examine mitochondrial morphology in our *mam3* Δ ::*URA3* haploid strain, we employed COX4-GFP as a fluorescence marker for the mitochondria [35]. Contrary to previous reports [34], we failed to detect any major differences in mitochondrial morphology between a *mam3* Δ mutant and its isogenic wild-type control, and both exhibited long tubular-like mitochondria (Figure 3A). Furthermore, respiratory chain function appears normal in *mam3* Δ mutants, as these strains exhibited no growth defect on glycerol/ethanol plates (Figure 3B). Therefore, the reported mitochondrial defect of *mam3* Δ mutants is not reproducible in other strain backgrounds.

Mam3p is a vacuolar membrane protein

To address the intracellular localization of Mam3p, a Mam3–GFP fusion was created in which one copy of GFP was fused to the soluble C-terminus of Mam3p expressed on a *CEN* plasmid. This Mam3–GFP chimera was capable of complementing the manganese-resistance phenotype of a *mam3* Δ mutant (results not shown). Production of Mam3–GFP was analysed by

Figure 3 *mam3Δ* mutants exhibit no obvious defect in mitochondrial morphology or function

(A) Strains BY4741 (WT, wild-type) and MY001 (*mam3Δ*) transformed with COX4-GFP were prepared and examined by fluorescence microscopy as described in the Experimental section. (B) The indicated strains were tested for growth, as described in Figure 2, on YPD or YPEG plates at 30 °C for 3 days. Strains utilized: wild-type BY4741 (WT), MY010 (Rho⁻) and MY001 (*mam3Δ*).

release of Mam3-GFP from the membrane fraction (Figure 4B, lane 7). These observations strongly indicate that Mam3p is an integral membrane protein.

To determine the intracellular localization of Mam3p, live cells expressing Mam3-GFP were subjected to fluorescence microscopy. Figure 4(C) ('GFP') shows that Mam3-GFP localizes on the periphery/membrane of the vacuole, but not the vacuolar lumen. The vacuole can be visualized as indentations under Nomarski optics (Figure 4C; 'DIC'). The localization of Mam3p is consistent with a high throughput analysis of yeast protein localization that was published while the present study was in progress [37].

Since Mam3p is localized at the vacuole, we addressed whether the bulk of Mam3-GFP is actually targeted to the vacuolar lumen for degradation. Mam3-GFP was expressed in *pep4Δ* mutant, in which vacuolar proteases are inactive [38]. An intense lumen staining was not observed (Figure 4C, middle panel). Therefore, Mam3p does not appear to be degraded in the vacuole lumen.

Although there was no obvious cell surface staining of Mam3p, a transient localization was still possible. In yeast, certain plasma membrane proteins, such as Ste6p, are rapidly internalized and recycled through endocytosis [27]. To test the possibility that Mam3p transiently localizes in the plasma membrane, we expressed Mam3-GFP in *end3^{ts}* mutants, in which endocytosis is blocked at the non-permissive temperature (37 °C). At the non-permissive temperature, *end3^{ts}* cells expressing Mam3-GFP exhibited no rim staining predicted for a plasma membrane protein (Figure 4C, right-hand panel), whereas the control Ste6-GFP moved to the cell surface under these conditions (results not shown). Thus Mam3p does not reside at the cell surface.

In studies published elsewhere, human ACDPs were reported to be localized in the nucleus [24], although mouse Acdp1 was localized to the plasma membrane [39]. To verify that Mam3p does not localize to the nucleus, cells expressing Mam3-GFP were fixed and permeabilized to enhance staining of the nuclei. As seen in Figure 4(D), there was no overlapping staining of

Mam3-GFP with the round nuclei marked by staining with DAPI. Overall, these studies demonstrate that Mam3-GFP is an integral membrane protein of the vacuole.

Metal ion accumulation in *mam3Δ* mutants

In an attempt to address the mechanism of metal resistance associated with *mam3Δ*, we analysed metal ion accumulation by atomic absorption spectrophotometry. Under standard growth conditions, we failed to detect any significant changes in total cell accumulation of manganese (Figure 5A), or of cobalt (results not shown). The only effect seen with *mam3Δ* mutations was a somewhat lowered manganese accumulation under metal toxicity conditions. In the experiment of Figure 5(C), cells were cultured for 16 h in the presence of 500 μM MnCl₂, a condition in which growth of wild-type cells was inhibited by approx. 50%. A 2-fold reduction in manganese accumulation was seen in *mam3Δ* mutants. When cells were treated with manganese for a short period (1.5 h), the effect of *mam3Δ* mutations seemed somewhat more pronounced (Figure 5B), yet even this decrease in manganese accumulation may not account for the strong resistance to manganese toxicity seen in *mam3Δ* mutants (see below).

Since Mam3p is vacuolar, and as the vacuole is a known site for metal ion storage and detoxification [18–23,40,41], we tested whether metal accumulation in vacuoles was specifically affected. Vacuoles were isolated from *mam3Δ* and isogenic wild-type cells by Ficoll gradient centrifugation [18] and analysed for metal contents. Under normal growth conditions, there were no noticeable differences in vacuolar manganese of *mam3Δ* versus wild-type strains (results not shown). The same was observed for other metals tested, including iron and zinc, as well as copper and sodium (results not shown). Under manganese toxicity conditions, where *mam3Δ* mutants accumulate lower levels of whole-cell manganese (as in Figures 5B and 5C), the vacuoles likewise contained lower levels of manganese (Figure 5D). However, this same decrease was seen in the cytosol as well (Figure 5D). Therefore, Mam3p does not specifically control vacuolar compartmentalization of metals and may work indirectly to influence manganese toxicity.

Manganese detoxification in *mam3Δ* mutants is distinct from the well-established Pho84p, Pmr1p and Smf2p pathways

We tested whether Mam3p operates through known pathways for manganese homeostasis to effect manganese damage. Under manganese toxicity conditions, yeast cells take up manganese by way of the cell surface phosphate and manganese transporter Pho84p [10]. Toxic manganese can be eliminated from the cell by pumping the metal into the secretory pathway via Pmr1p, a Golgi-localized transporter for manganese and calcium [6,14,42]. The high-affinity manganese transport pathway involving Smf1p and Smf2p does not normally contribute to manganese toxicity; however, if these proteins are over-expressed, metal toxicity can ensue [43]. Hence it was possible that Mam3p affects manganese toxicity by having an impact on the metal transport activities of Smf1p/Smf2p, Pmr1p or Pho84p.

To address this, we tested whether *mam3Δ* mutations can still confer manganese resistance on yeast strains containing either *smf2Δ*, *pmr1Δ* or *pho84Δ* mutations. As seen in Figure 6, *mam3Δ* mutations were able to increase manganese resistance in an *smf2Δ* mutant (Figure 6A) and also helped reverse the very strong manganese sensitivity of a *pmr1Δ* mutation (Figure 6B). Furthermore, *mam3Δ* mutations still improved manganese resistance in strains lacking Pho84p (compare the + Mn growth of *pho84Δ* versus *mam3Δ pho84Δ* strains, Figure 6C). In agreement

Figure 4 Mam3p is a permanent member of the vacuolar membrane

(**A**) Lysate protein (40 μ g) prepared from strain BY4741 transformed with vector pRS415 (–) or pAP003 (Mam3–GFP; +) were analysed by Western blotting with a rabbit anti-GFP antibody. The arrow marks the position of the Mam3–GFP fusion protein. Protein molecular-mass markers (in kDa) run in parallel are indicated. (**B**) Extracts prepared from BY4741 transformed with pAP003 (Mam3–GFP) were treated with 0.5 M NaCl, 0.1 M Na₂CO₃ or 1% Triton X-100 respectively, followed by centrifugation at 100 000 *g*, as described in the Experimental section. Supernatant and pellet fractions (20 μ l) were analysed by Western blotting using an anti-GFP antibody. P, pellet; S, supernatant. (**C**) Strain PEP4⁺ (YR98), pep4Δ (XL126) and end3^{ts} (SM2186) transformed with Mam3–GFP or vector were grown in SD medium to a *D*₆₀₀ of 1.0, examined as live cells by fluorescence microscopy ($\times 1000$ magnification) and viewed by Normarski DIC (differential interference contrast) microscopy. (**D**) Strains END3⁺ (SM2188) transformed with Mam3–GFP, were fixed with formaldehyde, stained with DAPI and examined by fluorescence microscopy ($\times 1000$ magnification) and viewed by DIC. The images in green correspond to GFP fluorescence, grey are viewed DIC and blue correspond to cells stained with DAPI for DNA.

with the idea that Mam3p does not control Pho84p, we observed no effect of *mam3* mutations on the expression of the *PHO84* gene (results not shown) nor on the phosphate transport role of Pho84p, as monitored by polyphosphate accumulation (results not shown). Overall, these genetic studies demonstrate that Mam3p does not operate through Smf2p, Pmr1p or Pho84p to affect manganese toxicity.

In the course of these studies, we noted a discordance between manganese resistance and total cellular levels of manganese. For example, when *pho84*Δ and *mam3*Δ single mutants were compared, *mam3*Δ mutants were considerably more manganese resistant (Figure 6C), however, *pho84*Δ mutants showed much lower cellular accumulation of manganese (Figure 5B). Furthermore, even though *mam3*Δ *pho84*Δ mutants were more resistant to manganese toxicity than single *pho84*Δ mutants (Figure 6C), these double mutants were not further reduced in manganese accumulation (compare *pho84*Δ with *mam3*Δ *pho84*Δ in Figure 5B). The lack of correlation between metal accumulation and metal toxicity indicates that the somewhat lowered manganese accumulation in *mam3* mutants (Figures 5B and 5C) is not the underlying basis for metal resistance. In this regard, it is noteworthy that *mam3*Δ mutants only exhibit reduced manganese accumulation when the wild-type control strain is confronted

with manganese toxicity. Hence, the effects seen may represent differences in vigor of the wild-type versus *mam3*Δ strains under these conditions.

Collectively, these studies demonstrate that *S. cerevisiae* Mam3p can contribute to manganese toxicity through a pathway that does not involve vacuolar sequestration of metals, Golgi pumping of manganese via Pmr1p or regulated manganese transport by Pho84p and Smf2p. Although the precise mechanism is still elusive, these studies establish a role for a eukaryotic ACDP protein in metal homeostasis.

DISCUSSION

The goal of this study was to identify factors that contribute to manganese toxicity in a eukaryotic cell. Through a genetic screen, *S. cerevisiae* *mam3* was identified as a mutant conferring strong resistance to manganese toxicity. Mutations in *MAM3* also increased cells' tolerance to cobalt and zinc. *S. cerevisiae* *MAM3* is a member of the ACD family of proteins. In humans, ACDP1 has been linked to UFS, a rare genetic disorder affecting facial expression and urinary tract function. The underlying etiology for UFS is completely unknown, largely due to a lack of

Figure 5 Manganese accumulation in *mam3Δ* mutant cells

The indicated strains were grown to mid-log phase in YPD medium without (A) or with (B) supplementation with 500 μ M $MnCl_2$. (C, D) Cells were grown as in (A), then supplemented with 500 μ M $MnCl_2$ for 1.5 h. (D) Vacuole and crude cytosol fractions were isolated by Ficoll gradient centrifugation. Manganese content of whole cells, isolated vacuole and crude cytosol were monitored by atomic absorption spectroscopy. The results are expressed as the means $\pm$ S.D. for at least two independent experiments. Strains used: wild-type BY4741 (WT), MY001 (*mam3Δ*), 6524 (*pho84Δ*) and MY003 (*mam3Δ pho84Δ*).

Figure 6 Manganese detoxification in *mam3Δ* mutant is distinct from the well-established *Smf2p*, *Pmr1p* and *Pho84p* pathways

The indicated strains of yeast were tested for growth as described in Figure 2 on YPD supplemented where indicated with 5.0 mM (A, C) or 100 μ M (B) $MnCl_2$ (+Mn). Cells were allowed to grow at 30°C for 2 days. Strains used: 1878 (*smf2Δ*); MY002 (*smf2Δ mam3Δ*); 4534 (*pmr1Δ*); MY006 (*mam3Δ pmr1Δ*); wild-type BY4741 (WT); MY001 (*mam3Δ*); 6524 (*pho84Δ*); MY003 (*mam3Δ pho84Δ*).

understanding of ACDP function. An extensive analysis of yeast *MAM3* might therefore provide insight as to the role of eukaryotic ACDP proteins.

Mutants of *MAM3* (mitochondria aberrant morphology) were first identified on the basis of defects in mitochondrial morphology and respiration [34]. However, such mitochondrial anomalies were not observed in our *mam3Δ* mutants. The difference may reflect strain backgrounds or possible occurrence of a petite mutation in the previously described *mam3Δ* mutant [34]. In any case, the metal resistance of *mam3Δ* cells cannot be readily attributed to the mitochondria.

S. cerevisiae Mam3p is a vacuolar membrane protein. Murine ACDP1 has also been localized to a membrane, in this case the plasma membrane [39]. Although human ACDPs were first reported to be nuclear [24], it was subsequently suggested that this finding reflected the non-specific nature of the antibody employed [39]. Hence both human and murine ACDP1 are likely to be membrane-associated, even though these particular ACDP family members lack the N-terminal hydrophobic domain of mammalian ACDP2–ACDP4 and of *S. cerevisiae* Mam3p. Perhaps all ACDPs function at the site of a membrane, as either membrane-associated or as integral membrane proteins.

The yeast vacuole is an important site for the storage and detoxification of essential metals, such as manganese [18,19,40], iron [18,41], zinc [20], cobalt [21] and copper [22,23]. The vacuole also plays a role in detoxifying non-essential toxic metals, such as cadmium [44,45]. Although the spectrum of metals affected by a *mam3Δ* mutation show some overlap with the aforementioned ions (for example, manganese, cobalt and zinc), not all the vacuolar metals are affected in *mam3Δ* mutants (for example, copper). In addition, *mam3Δ* mutants exhibited no noticeable change in vacuolar morphology, vacuolar acidification (results not shown) or vacuolar partitioning of manganese, or of other ions including zinc, iron and sodium (results not shown). It is therefore unlikely that Mam3p has an impact globally on vacuolar function.

One possibility is that Mam3p may be a transporter for the vacuole, but since we detected no change in vacuolar partitioning of metals, Mam3p may instead act on non-metal substrates, such as metal-liganding molecules. Although GSH seems a likely candidate, because GSH–metal conjugates can be transported into the vacuole [44,45], we noted no obvious changes in vacuolar GSH content of *mam3Δ* mutants (results not shown). Yet it is possible that Mam3p might transport metal binding substrates other than GSH. Mam3p might also facilitate transport of molecules that indirectly affect metals. For example, mitochondrial Yhm1p in yeast has a dramatic impact on iron homeostasis [46], but is a mitochondrial transporter for GTP/GDP [47]. A similar scenario may be true for Mam3p.

We tested whether *MAM3* operates through known metal metabolism pathways to cause manganese toxicity. Our results demonstrate that mutations in *MAM3* lead to manganese resistance through mechanisms that do not involve Golgi sequestration of manganese via *Pmr1p* [11,13] or the uptake of toxic manganese via *Pho84p* [10]. We have likewise excluded a requirement for the high-affinity manganese transport system involving *Smf2p* [6]. Hence, the role of Mam3p in manganese metabolism appears unique to previously reported pathways, although the exact mechanism is still unknown. In any case, these studies have exposed a role for a eukaryotic ACDP in metal metabolism.

As further evidence for the role of ACD-containing proteins in metal homeostasis, a bacterial ACDP-like polypeptide, known as CorC from *Salmonella typhimurium* [24], has been implicated in magnesium and cobalt homeostasis [48,49]. Mutations in *corC* are associated with cobalt resistance [49], however, there is no

evidence to date that CorC itself is a metal transporter. Hence, both *S. cerevisiae* *MAM3* and bacteria *corC* influence metal ion homeostasis through mechanisms that may not involve direct membrane transport of the ions. We hypothesize that human ACDP1 may function in a similar way in metal metabolism.

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