Manganese Homeostasis in *Saccharomyces cerevisiae*

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1. Introduction

Manganese, whose name is derived from a Greek word for magic, is an essential trace element that is required by organisms across every kingdom of life.[1] The magic of manganese lies not only in its ability to transform itself into an effector for a diverse range of redox and nonredox functions but also in its ability to appear and disappear from a variety of locations within a cell. Regarding the former, decades of chemical, biochemical, and biophysical characterization of manganese-containing complexes have revealed much about how primary and secondary coordination spheres temper the reactivity and function of the metal center.[2,3] However, regarding the latter, the cellular transport and trafficking of manganese, much of the mystery persists.[4−6]

A diverse array of metalloproteins require manganese for function, including oxidoreductases, DNA and RNA polymerases, peptidases, kinases, decarboxylases, and sugar transferases, which are present in a variety of cellular locales, such as the nucleus, mitochondria, cytosol, Golgi, and vacuole.[3] Yet it is not completely clear how cells manage to import appropriate amounts of environmental manganese, transport the metal to the correct intracellular compartments, and distribute it to relevant biomacromolecules.[4−6] Matters may be further complicated by environmental stresses that could lead to under- or overexposure of cells to manganese; too little manganese may inactivate manganese-requiring biological processes, whereas too much manganese is toxic.[3−6] The latter is underscored by manganism, a Parkinson's disease-like condition in which overexposure to manganese leads to severe neurological damage.[7−12] In such instances where cells are manganese stressed, either through deficiency or surplus, living systems are obligated to respond through the concerted regulation of manganese cell surface and intracellular transporters, as well as any putative manganese chaperones, so as to maintain healthy intracellular concentrations of the metal and correctly appropriate manganese to its cognate protein ligands.[4−6]

Much of our current, albeit limited understanding of manganese homeostatic mechanisms has been elucidated through molecular genetic studies of the budding yeast, *Saccharomyces cerevisiae*. As such, this review will largely focus on global manganese homeostatic pathways operative in the eukaryotic cell of *S. cerevisiae*, with references being made to analogous pathways in metazoans where applicable. Specifically, this review will highlight the mechanisms by

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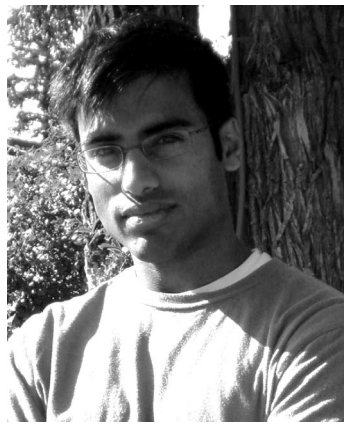
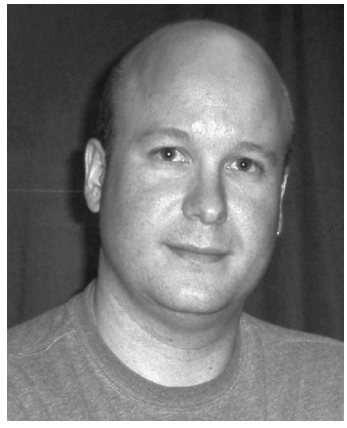


Amit R. Reddi was born in 1981, in Silver Spring, MD. He attended Carleton College (B.A. with Honors, 2003), where he earned the Finholt Prize in Inorganic Chemistry and graduated with distinction in chemistry. He went on to study metalloprotein design at Columbia University (Ph.D. with Distinction, 2008) under Professor Brian R. Gibney. His thesis research on the thermodynamic characterization of designed metalloproteins was recognized with the Pegram Award for excellence in graduate research. In addition, Amit was honored with the Miller Award for outstanding graduate teaching. Since May 2008, Amit has been pursuing his postdoctoral training with Professor Valeria Culotta at Johns Hopkins University, where he is researching the role of manganese in cellular oxidative stress protection.



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which cell surface and intracellular manganese transporters import and distribute manganese, as well as how these homeostatic mechanisms respond to manganese deficiency or surplus.

The manner in which cells mediate the uptake and distribution of manganese is dependent on the exposure of environmental manganese to the cell. As depicted in Figure 1, manganese exposure lies on a continuum between two environmental extremes, manganese deficiency and surplus, with manganese sufficiency occupying a place in the continuum between the two extremes.[6] The range of intracellular manganese levels that constitute manganese sufficiency is quite large, nearly 2 orders of magnitude. In various studies done, yeast were seen to accumulate between 2−100 nmol of manganese/(10×10^9 cells), or 0.04−2.0

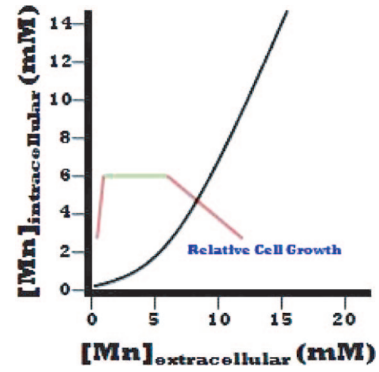

Figure 1. Approximation of the intracellular accumulation of manganese in yeast as a function of varying concentrations of manganese supplemented to a minimal medium. Superimposed on the diagram is a growth isotherm of yeast cells as a function of manganese supplementation. The red bars on the growth isotherm correspond to metal-induced toxicity due to manganese deficiency or surplus, while the green bar corresponds to growth under conditions of sufficient, i.e., nontoxic, manganese.

mM manganese (assuming a single yeast cell has a volume of 50 femtoliters), without any impact on cell growth.[13−18] However, at levels below or above this, the stresses of manganese deficiency and manganese toxicity ensue, setting off a series of responses aimed at normalizing manganese levels. In general, cells respond to such manganese stress by upregulating or downregulating cell surface and intracellular transport systems. As described in detail below, regulation of manganese transport in yeast does not involve any known transcriptional pathway,[6,13,19] such as those described for transporters of copper, zinc, and iron.[20] Instead, all the known manganese regulatory pathways in yeast occur at the post-translational level through changes in transporter protein localization and turnover. In this review, we shall provide an overview of the various manganese transport systems in *S. cerevisiae*, how they function under diverse cellular conditions, and how certain transporters are regulated in response to manganese stress.

2. Nramp Manganese Transporters, Smf1p and Smf2p

2.1. Overview of the Nramp Family of Metal Ion Transporters

The Nramp family constitutes a large class of metal-ion transporters that are widely conserved from bacteria to humans.[21] Nramp transporters, which are localized either at the cell surface or in intracellular vesicles, drive the translocation of a wide range of divalent metal substrates, including manganese, iron, cobalt, copper, zinc, and cadmium, across membranes toward the cytosol by coupling the flow of protons and metals.[4,6,21−32] The first of these to be mechanistically studied was mammalian DMT1 (divalent metal transporter). When expressed in oocytes, Dmt1 was found to cotransport Fe^{2+} and H^+ with a stoichiometry of 1:1 at pH 7.0 and physiological membrane potentials of −90 to −30 mV, demonstrating that Nramp transporters are indeed proton-metal cotransporters.[32] DMT1 was also seen to exhibit "driving force slippage", where at increased membrane potentials or proton concentrations metal and proton uptake becomes decoupled, resulting in significant deviations (slippage) from metal/proton stoichiometries of 1:1.[32] Driving force slippage is thought to "put the brakes"

on excessive metal ion uptake and prevent toxic metal ion overload under conditions when protons are in excess.[24,25] Although it is unusual for cations (protons) to drive the translocation of other cations (divalent transition metals), Nelson and colleagues observed an influence of anions on the currents generated by DMT1.[24,27] Their results indicated that metal transport is also dependent on Cl^- or other small anions and that the divalent metals may in fact be cotransported with Cl^- due to its abundance.[27]

All Nramp transporters possess a highly conserved core of 10 transmembrane domains, with yeast and mammalian Nramps possessing 11 and 12 transmembrane domains, respectively, as well as a highly conserved metal transport motif in a cytoplasmic loop between transmembrane domain 8 and 9.[25] The current lack of high-resolution structural information has made it difficult to delineate the pathway and residues critical for metal and proton translocation. However, mutagenesis studies of bacterial, yeast, and mammalian Nramps, which have been extensively reviewed elsewhere,[25] are beginning to uncover the key residues that enable metal binding/translocation, proton coupling, and substrate slippage.

Mn^{2+} is a relatively hard Lewis acid, and based solely on Pearson's Hard−Soft Acid Base Theory, one would expect the Nramp transporters to contain hard bases such as proteinaceous carboxylates to coordinate metal.[3] Indeed, the Nramp transporters possess a number of conserved glutamate and aspartate-based carboxylate ligands that, when mutated, abrogate transport activity of the protein. For example, three anionic and highly conserved residues, D93, E154, and D192 in transmembrane domains 1, 3 and 4, respectively, are essential for metal transport activity in human DMT1, and the same is true for the analogous residues in bacterial and yeast Nramp transporters.[25,33]

2.2. Identification of *S. cerevisiae* Smf1p and Smf2p as Nramp Transporters

S. cerevisiae Smf1p and Smf2p (suppressor of mitochondria import function) were originally identified in 1992 as multicopy suppressors of a mitochondrial protein processing defect with no known connection to metals or transport.[34] Around the same time, in 1993, a Smf-like protein was identified in mice as being critical for host immune defense, and this protein was given the name Nramp1 for natural resistance associated macrophage protein.[35] In 1995, when Philip Gros defined Nramp as a large family of divalent metal transporters,[21] the yeast Nramp Smf1p was described by Nathan Nelson as a high-affinity manganese transporter.[29] The previously denoted role for Smf1p in mitochondrial import simply reflected manganese activation of a step in mitochondrial protein processing.

Like other members of the Nramp family, Smf1p is not specific for one metal.[4,6,21−31] The transporter has the capacity to uptake a variety of metals, including cadmium, cobalt, copper, iron, manganese, and zinc, when overexpressed in yeast and in *Xenopus* oocyte systems.[24,26−32] Smf1p also cotransports divalent metals with protons, but unlike its mammalian counterparts, there is no proton slippage. Instead, a Na^+ slippage phenomenon has been described whereby high extracellular sodium, as would be encountered during desiccation stress in yeast, inhibits proton binding and metal ion translocation.[26]

Upon completion of the yeast genome in the late 1990s, it became obvious that *S. cerevisiae* expresses three Nramp transporters: the previously identified Smf1p and Smf2p manganese transporters, as well as a third that was denoted Smf3p.[13,24] In spite of its name, Smf3p has no obvious impact on mitochondrial protein processing or manganese transport of any kind. Smf3p functions in the vacuolar export of iron atoms, and as expected, the transporter is regulated by the iron status of the cell.[13] For the purposes of this review, we shall restrict our discussions on Nramp to the two manganese transporters, Smf1p and Smf2p.

2.3. Disparate Roles of Smf1p and Smf2p in Manganese Uptake and Trafficking

Smf1p and Smf2p (as well as Smf3p) share 50% identity (67% similarity)[6] and 30% identity (50% similarity) to their mammalian homologues,[35] Nramp1 and DMT1, respectively. Despite similarities in their primary structure, a number of studies have shown that Smf1p and Smf2p are not functionally redundant.[6,13,15,19,36−38]

Smf1p is by far the more abundant of the two,[13] and originally much emphasis focused on the role of this major Nramp transporter for manganese. Smf1p has been localized at the cell surface and intracellular vesicles (apparently endosomes), although at steady state, very little of this transporter can be seen at the plasma membrane.[6,19,29,39−41] Moreover, genetic studies have cast doubt as to whether this transporter actually functions in yeast manganese transport. When *SMF1* is deleted under nonstressed manganese-sufficient conditions, not only is the concentration of cellular manganese unchanged relative to the wild-type strain,[29] but also there are no apparent aberrations in the activity of manganese-dependent enzymes such as manganese-dependent sugar transferases and SOD2, the mitochondrial superoxide dismutase.[38] Thus, it was concluded in 2001 that the primary Nramp transporter of yeast Smf1p does not play a role in manganese uptake and distribution.[38] Nevertheless, this role has been ascribed to the less abundant Nramp transporter Smf2p.[38]

As with Smf1p, the bulk of steady-state Smf2p resides in intracellular Golgi-like vesicles, presumably endosomes.[6,13,38] Despite the lack of a clear cell surface localization, Smf2p was seen to have a significant impact on manganese homeostasis. *smf2*Δ deletion strains accumulate very low levels of manganese relative to wild-type.[38] Furthermore, the activity of manganese-dependent enzymes such as sugar transferases in the Golgi, SOD2 in the mitochondria, and a cytosolic Mn-SOD are attenuated, demonstrating a cell-wide deficiency in manganese levels.[38,42] Genetically, Smf2p exhibited all the hallmarks of a cell-surface manganese transporter, yet attempts to demonstrate a cell-surface localization for this transporter have all failed.[6,13,38] This apparent paradox may be explained by an examination of manganese transport kinetics. The rate of manganese uptake in yeast is only $\sim 10^3$ ions/cell/min, and since turnover rates of 10^2 molecules/s have been reported for other proton-coupled transporters, Pelham has proposed that a small number of cell-surface Nramp molecules could account for all the manganese uptake of yeast.[41] Such a low number of Smf2p molecules could easily escape detection by conventional microscopy or cell-fractionation techniques. In any case, minimizing the number of Smf2p molecules at the cell surface may help protect against toxicity of other metals such as cadmium and lead.

If all the essential manganese for the cell is provided by Smf2p, then why do cells express a second manganese

Nramp transporter (Smf1p) and at such abundant levels? For 15 years since its initial identification, the function of yeast Smf1p remained elusive. It was not until 2009 that an effect of *smf1* mutations on yeast growth became obvious.[15] Specifically, Smf1p plays a critical role in oxidative stress resistance.[15]

In bacteria and yeast, manganese has long been known to serve as a protective anti-oxidant, particularly as a back-up for superoxide dismutase.[15,43−53] Inside cells, manganese forms certain nonproteinacous complexes (still of an unknown nature) that can react with superoxide or other reactive oxygen species.[15,43−53] In yeast cells lacking SOD1, raising intracellular manganese levels to ~100 nmol/(10×10^9 cells) by treatment with manganese salts will reverse all symptoms of oxidative stress.[15,48,49,66] Moreover, physiological levels of manganese (~2 nmol/(10×10^9 cells)) sustains aerobic viability of these *sod1*Δ cells.[15] Our very recent studies have pinpointed Smf1p as the primary source of this manganese anti-oxidant.[15] For example, deletion of *SMF1*, but NOT *SMF2*, in the background of *sod1*Δ cells will attenuate the cell's ability to protect against oxidative damage when supplemented with exogenous manganese salts.[15] Furthermore, as discussed in greater detail below, genetic suppressors of *sod1*Δ oxidative stress, i.e., the *BSD2* and *PMR1* genes, are not nearly as effective in protecting cells against oxidative damage when *SMF1* is deleted.[15] Thus, *in toto*, the data illustrates the biological necessity of having dual manganese Nramp transporters. As summarized in Figure 2, while Smf2p provides the essential manganese for manganese-requiring enzymes, Smf1p is more of the stress-responsive Nramp transporter that is particularly critical under conditions of oxidative stress.

2.4. Regulation of Smf1p and Smf2p by Metal Ions

Because under- or overexposure to essential transition metals can be detrimental, metal transporters must be regulated in a dynamic fashion to respond quickly and efficiently to changes in environmental stresses. For many transition metal transporters, including copper, iron, and zinc translocators, regulation occurs at the level of gene transcription.[20] However, regulation of the manganese Nramp transporters, Smf1p and Smf2p, is notably different in that it predominantly occurs post-translationally. Under conditions of sufficient and physiologic metal levels, most but not all of the high-affinity manganese transporters are targeted to the vacuole for degradation, presumably to limit uptake of toxic amounts of metals. When cells are starved for manganese, these transporters become quite abundant, concentrating cellular manganese so as to compensate for its environmental scarcity.[13,19,39,40] At the opposite extreme of toxic metal excess, vacuolar degradation of the Nramp transporters is enhanced, virtually eliminating Smf1p from the cell.[13,19,39,40] In total, four distinct pathways have been identified for regulating Smf1p and Smf2p in response to changes in environmental metals, and these shall be discussed independently.

2.5. Bsd2p Control of Smf1p and Smf2p Under Physiological Manganese

Under physiologic conditions when manganese is neither deficient nor in toxic excess, the vast majority (~90%) of newly synthesized Smf1p and Smf2p are directly targeted

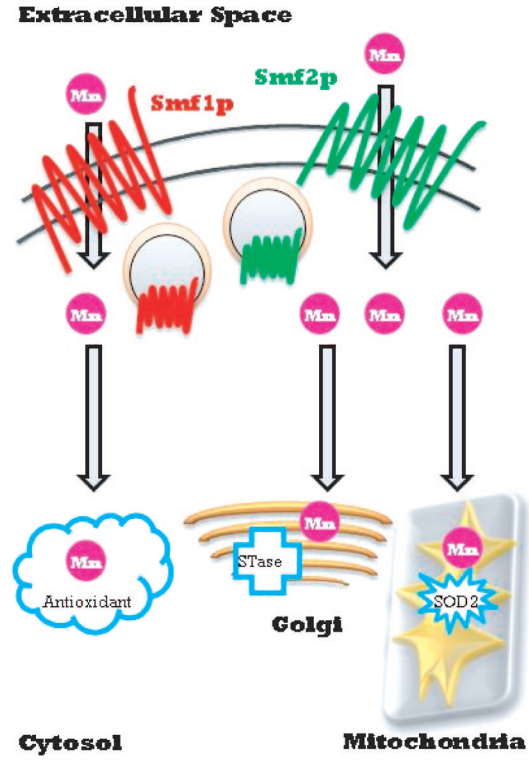

Figure 2. Disparate roles of the Nramp transporters, Smf1p and Smf2p. Smf1p (in red), which has been found to localize both at the cell surface and in intracellular vesicles (depicted as circles), is primarily responsible for providing manganese to a nonproteinaceous manganese-requiring anti-oxidant that can substitute for Cu/Zn SOD1.[15] Smf2p (in green), which is found primarily in intracellular vesicles, but is also thought to reside at the cell surface in minute quantities, is responsible for total cellular manganese accumulation. The manganese transported by Smf2p is bioavailable to manganese-requiring enzymes such as sugar transferases (STase) and Sod2p, which are present in the Golgi and mitochondria, respectively.

to the vacuole for degradation. This constitutive degradation maintains Smf1p and Smf2p at steady-state levels that are just enough to supply the cell with essential manganese. While most cell surface transporters are targeted to the vacuole through endocytosis, vacuolar degradation of Smf1p and Smf2p with physiologic manganese occurs in the exocytic pathway with Golgi-derived newly synthesized molecules. As Smf1p and Smf2p transit the Golgi, they are marked for vacuolar degradation by ubiquitination, then enter multivesicular bodies (MVB) where membrane proteins are internalized as a prerequisite to vacuolar entry.[40,41,54] Ubiquitination of Smf1p and Smf2p is mediated by Rsp5p, a HECT domain E3 ligase that recognizes PY motifs on target proteins. However, PY motifs are absent on Smf1p and Smf2p, and ubiquitination requires adaptor proteins, one of which is Bsd2p.[40,41]

We originally identified *S. cerevisiae BSD2* (bypass SOD1 defect) in 1994 as one of two genes that, when mutated, would reverse oxidative damage in yeast cells lacking Cu/Zn SOD1, with the other gene being *BSD1*, which is discussed in the section on Pmr1p.[55] The anti-oxidant protection of *bsd2* was correlated with increased levels of the Smf1 and Smf2 polypeptides.[19,31] In *bsd2*Δ cells, the steady-state levels of Smf1p and Smf2p rise ~10-fold and cells hyperaccumulate manganese anti-oxidants that circumvent the need for SOD1.[13,15,19,31] As Nramp transporters are fairly promiscuous, cells lacking *bsd2* also hyperaccumulate

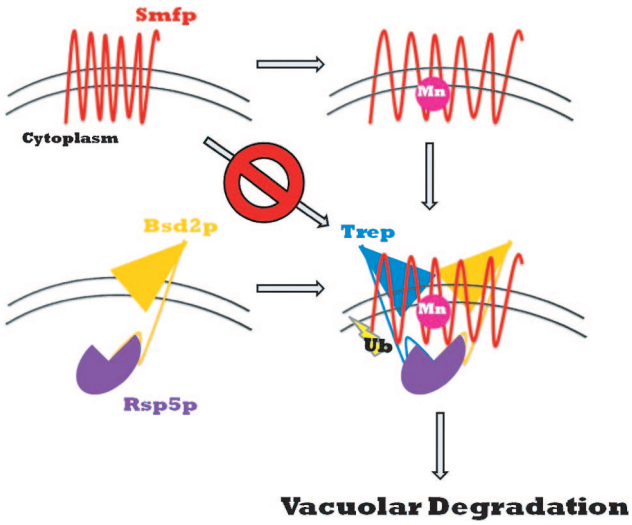

Figure 3. Bsd2p regulation of the Smf proteins. When manganese is sufficiently available to cells, the Smf proteins (combination of Smf1p and Smf2p, in red) in the active metal bound state are ubiquitinated (Ub, yellow) by Rsp5p (purple) in a Bsd2p (gold) and Trep1 and Trep2 (collectively shown here as Trep; blue) dependent manner and directed to the vacuole for degradation. First, Rsp5p and Bsd2p interact through their PY domains, forming a complex. This complex next recognizes the PY domain of Trep via a recognition site on Rsp5p. The Trep/Bsd2p/Rsp5 complex then mediates the ubiquitination of the manganese-bound Smf proteins, leading to their targeting to the vacuole for degradation.[39−41,56]

toxic levels of copper, cadmium, and cobalt.[31] We proposed that Bsd2p mediates vacuolar degradation of Smf1p and Smf2p,[19,39] although the mechanism remained elusive until 2004 when Hugh Pelham described Bsd2p as an adaptor protein for Rsp5p.[56]

Bsd2p contains two PY domains needed for Rsp5p recognition and thereby links Smf1p and Smf2p to Rsp5p. In a similar fashion, Bsd2p facilitates targeting of native vacuolar enzymes to the vacuole, including carboxypeptidase S and the polyphosphatase, Phm5p.[40,41,56] However, unlike these native vacuolar enzymes, Rsp5p-ubiquitination of Smf1p and Smf2p requires two additional adaptor proteins, namely, Tre1p and Tre2p (transferin receptor-like).[41] Deletion of both *TRE1* and *TRE2* prevents the degradation of Smf1p and Smf2p, increasing manganese uptake and cadmium sensitivity much like a *bsd2*Δ mutant. Tre1p and Tre2p each contain one PY domain, yet this is insufficient to link the Smf transporters to Rsp5. In a model proposed by Pelham and colleagues, and shown schematically in Figure 3, Bsd2p and Rsp5p first form a complex that is then bound by the Tre proteins with Smf1p.[40] Subsequently, the ubiquitinated proteins are directed through the MVB pathway for their eventual degradation in the vacuole.

2.6. Upregulation of Smf1p and Smf2p Under Manganese Starvation

Manganese starvation conditions in yeast are achieved by growing cells in a special defined medium that has been depleted of manganese through ion exchange.[19] The most notable effect of such manganese depletion is complete loss of Bsd2p and Tre control of Smf1p and Smf2p. The polypeptides are diverted from the vacuole and localize to the cell surface (Smf1p) and intracellular vesicles (Smf2p).[13,19,39,40] The trans-acting factors involved in relocating the transporters are still unknown, yet our very recent studies with Smf1p have indicated that the transporters themselves may be the sensors for manganese starvation. Smf1p mutants defective in manganese transport are not properly regulated by low manganese.[33] Moreover, using yeast mutants defective in Golgi manganese transport, we observed that manganese within the Golgi lumen is sensed during manganese starvation, and it is loss of Golgi manganese that signals movement of Smf1p to the cell surface.[33] The Golgi lumen is an ideal site for sensing manganese starvation, as a number of essential manganese sugar transferases are housed in the Golgi.[38,57,58]

2.7. Downregulation of Smf1p Under Manganese Toxicity

When intracellular levels of manganese exceed ~100 nmol/(10×10^9 cells), yeast cell toxicity ensues. We recently observed that, under these conditions, all the Smf1p transporter is subject to degradation in the vacuole. Compared to physiologic manganese conditions where ~10% of Smf1p escapes vacuolar degradation, virtually all of the transporter is eliminated with toxic manganese.[33] We shall refer to these separate conditions as "basal" and "stimulated" degradation of Smf1p at physiologic and toxic manganese, respectively. As with basal degradation, stimulated degradation of Smf1p is manganese-specific and involves vacuolar entry through the MVB pathway, although stimulated degradation is not dependent on Bsd2p and Tre proteins.[33]

We observed that stimulated degradation of Smf1p by toxic manganese occurs in two phases. First, cell-surface Smf1p is subject to rapid endocytosis and movement to the vacuole. This phase is dependent on the ubiquitin ligase Rsp5p and the N-terminus of Smf1p. Second, intracellular Smf1p undergoes a relatively slow movement toward the vacuole that only occurs with prolonged exposures to toxic manganese. This slow response to toxic manganese does not require Rsp5p or other known ubiquitin ligases. Furthermore, sequences other than the N-terminal region of Smf1p are required for this sensing of manganese under chronic exposures. The very slow response of Smf1p to metal toxicity is quite unusual but may reflect the role of Smf1p in accumulating the "Mn anti-oxidant" as described above. Smf1p transport of manganese can be beneficial under oxidative stress, and the immediate degradation of this transporter during high manganese exposures may not always be advantageous.

2.8. Regulation of Smf1p by Cadmium

Nramp transporters are notoriously indiscriminate in their choice of metals, and if presented with a nonessential toxic ion such as cadmium, Smf1p will readily translocate the metal. Indeed, cadmium toxicity in yeast directly correlates with Smf1p transporter levels.[31,59] Very recently, Hugh Pelham and colleagues have uncovered a novel form of Smf1p regulation that occurs during exposure to cadmium but not to the native substrate manganese.[59] Specifically, cadmium induces endocytosis of cell-surface Smf1p and degradation of the transporter in the vacuole. As with toxic manganese (above), cadmium treatment triggers Smf1p ubiquitination by Rsp5p as a prerequisite to vacuolar targeting. Yet the response to cadmium differs from the endocytic response to manganese in that two arrestin-like proteins, namely, yeast Ecm21p or Csr2p, are used as adaptors for Rsp5p and Smf1p, as well as two lysines in the

N-terminus.[59] By comparison, manganese-stimulated endocytosis of Smf1p does not rely on these arrestin proteins nor these specific lysines.[33]

These studies with Smf1p can be compared and contrasted to post-translational regulation of the cadmium efflux pump Pca1p in yeast.[60,61] Cadmium also regulates this transporter at the level of protein turnover, but in the case of Pca1p, turnover occurs via ERAD (endoplasmic reticulum associated degradation) rather than the vacuolar degradation.[60,61] Moreover, cadmium *prevents* ERAD-mediated turnover of Pca1p and allows the transporter to accumulate at the cell surface where cadmium extrusion can take place.[60,61] Therefore, the metal can act at multiple cellular locations to both down-regulate toxic cadmium uptake (via Smf1p) and upregulate cadmium efflux (via Pca1p).

Overall, as summarized in Figure 4, multiple tiers exist for regulating the Nramp metal transporter Smf1p at the post-translational level in response to changes in environmental metals. Under physiologic manganese, the only known function for Smf1p is protection against oxidative stress, and here 90% of the transporter is degraded in the vacuole through the Bsd2p-pathway. Nevertheless, the residual 10% is quite abundant compared to the other manganese Nramp transporter Smf2p. When faced with metal toxicity, the cell clears this residual 10% Smf1p through additional vacuolar degradation. At the opposite extreme, under conditions of manganese starvation, virtually all of the Smf1p protein escapes vacuolar degradation and the transporter abundantly accumulates at the cell surface, presumably to assist Smf2p in providing essential manganese for the cell. Furthermore, when confronted with heavy metal cadmium toxicity, Smf1p is endocytosed for degradation in the vacuole in an Rsp5p-dependent manner. However, the arrestin-like proteins, Ecm21p or Csr2p, serve as adapters for Rsp5p, instead of Bsd2p and Trep.

3. Phosphate-Manganese Connection: Pho84p-Mediated Uptake of Manganese

The Smf1p and Smf2p transporters are largely responsible for uptake of essential manganese that is needed to activate manganese enzymes and for oxidative stress protection. However, under manganese surplus conditions, the metal can enter the cell by an additional route, specifically through the high-affinity cell surface phosphate transporter Pho84p.[62]

Pho84p is one of at least six transporters that contribute to cellular accumulation of phosphate.[62−64] Pho84p belongs to a family of phosphate/proton symporters and is a member of the major facilitator superfamily (MFS).[65] Its K_m for phosphate is ∼1−15 μM, and it transports 2−3 H^+ per phosphate. Pho84p, which is 587 amino acids in length, is predicted to consist of 12 transmembrane segments.[65] On the basis of homology modeling, hydropathy analysis, recent three-dimensional structural determinations of two other MFS proteins, and electron paramagnetic resonance (EPR) line-shape analysis of spin probes placed throughout Pho84p, Pho84p can be visualized as two homologous sequence segments each containing six transmembrane domains that are separated by a large central loop.[65]

Through reconstituted liposome studies in 1999, Persson and colleagues observed that the substrate of Pho84p transport is actually a divalent metal complex of phosphate ($MeHPO_4$) with a preference for manganese and cobalt.[66] In vivo, the likely substrate for Pho84p is magnesium phosphate rather than manganese phosphate due to the greater abundance of Mg^{2+} versus Mn^{2+} in yeast growth media. However, when surplus manganese is added to cultures, a switch in Pho84p substrate can occur toward $MnHPO_4$.

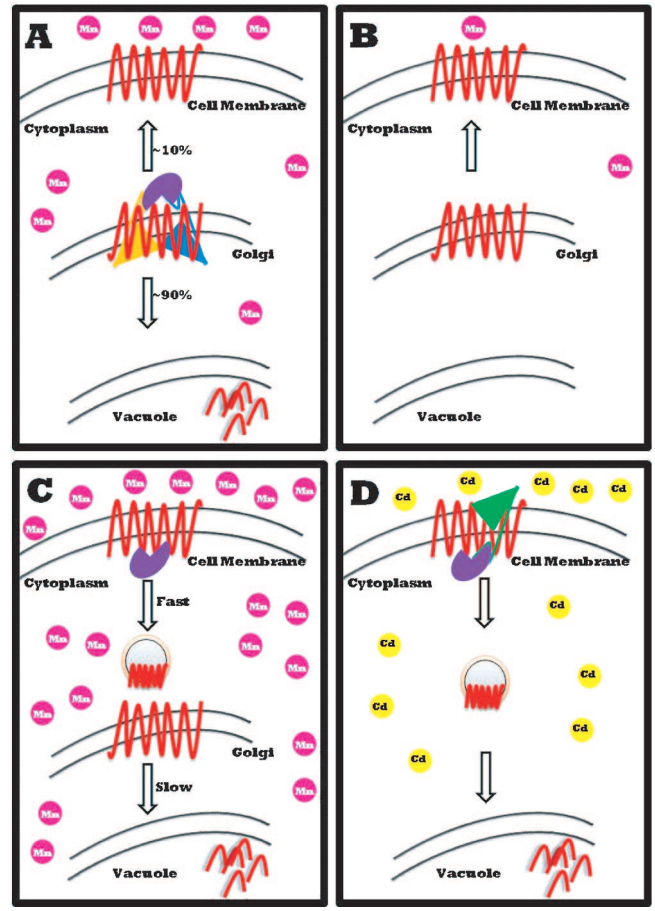

Figure 4. Multiple tiers of regulation of the Smf proteins. (A) When manganese is sufficiently available to cells, under nontoxic concentrations, ∼90% of Smf1p (red), is targeted to the vacuole immediately after synthesis by Rsp5p (purple) mediated ubiquitination. This ubiquitination is facilitated by the Trep (blue)−Bsd2p (gold) adapter complex. However, the remaining Smf1p is sorted to either the cell surface or intracellular vesicles.[13,19,39−41,56] (B) When cells are starved for manganese, Smf1p (red) is not sent for degradation to the vacuole but is instead sorted to the cell surface.[13,19,31,39] (C) When cells are exposed to excess toxic levels of manganese, the degradation of Smf1p (red) is biphasic. In the fast step, cell-surface Smf1p is subject to rapid endocytosis and movement to the vacuole in a Rsp5p-dependent manner. In the slow step, intracellular Smf1p undergoes a relatively slow Rsp5p-independent movement toward the vacuole that only occurs with prolonged exposures to toxic manganese.[33] (D) When cells are exposed to toxic cadmium, but not manganese, cell surface Smf1p (red) is endocytosed to the vacuole for degradation.[59] This occurs in a Trep−Bsd2p independent fashion, instead requiring Ecm21p or Csr2p (green) as adapters for Rsp5p (purple).

The first evidence for Pho84p acting as a manganese transporter came from our genome-wide analysis of manganese-resistant mutants of yeast. Two mutants involved in phosphate metabolism, namely, *pho84* and *vtc4*, were associated with manganese resistance and a lowering of cellular manganese, particularly when cells were supplemented with surplus manganese.[15,62] Pho84p operates to take up environmental $MnHPO_4$ under manganese toxicity conditions, but this transporter contributes little if any manganese to manganese-requiring enzymes[62] or to accumulation of manganese anti-oxidants as a backup for Cu/Zn SOD.[15] The manganese transported by Pho84p is biologically active as

a toxicant but not as an essential nutrient. Vtc4p, on the other hand, while not a manganese transporter, is still able to effect manganese metabolism by limiting the uptake of cellular manganese.[15] It was very recently determined that Vtc4p is a Mn^{2+}-dependent polyphosphate polymerase.[67] It appears that loss of polyphosphate synthesis in *vtc4* mutants somehow signals cells to inhibit uptake of phosphate or manganese phosphate complexes by Pho84p. The mechanism for this feedback regulation is currently unknown.

Pho84p is strongly regulated at the transcriptional level by phosphate. A well-characterized pathway involving the Pho80/Pho85 cyclin/CDK partnership and the Pho4p downstream transcription factor are responsible for controlling expression of yeast *PHO* genes involved in phosphate transport and metabolism.[64,66] Pho80/Pho85 negatively regulate Pho4p, and in *pho80* or *pho85* mutants, genes such as *PHO84* are expressed at high levels. We have observed that these same *pho80* or *pho85* mutants hyperaccumulate manganese and are exquisitely sensitive toward manganese toxicity, largely due to uncontrolled expression of *PHO84* (Rosenfeld and Culotta, unpublished results). These findings underscore the role of Pho84p in mediating manganese toxicity through phosphate uptake.

4. Golgi Manganese Transporter, Pmr1p

Pmr1p (for plasma membrane ATPase related), a P-type Ca^{2+}- and Mn^{2+}-transporting ATPase that is localized in the Golgi membrane, transports calcium and manganese from the cytosol into the Golgi lumen.[6,68−71] The calcium and manganese transported by Pmr1p enables the processing and trafficking of proteins moving through the secretory pathway. While calcium is required for protein sorting, manganese serves as an essential cofactor for sugar transferases that help glycosylate proteins in the secretory pathway.[33,67,68,71] In addition, Pmr1p plays an important role in manganese detoxification; surplus manganese in the cell that has the potential to be toxic is transported into the secretory pathway by Pmr1p for excretion.[6,70,71,73] More recently, it was discovered that manganese transported into the Golgi by Pmr1p negatively regulates the target of rapamycin complex (TORC1), a central regulator of cell growth, through an unknown mechanism.[74−77]

Pmr1p is the prototypic member of the SPCA (secretory pathway Ca^{2+}-ATPases) family of transporting ATPases, which are found in a variety of organisms, including fungi, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals.[73,78,79] Pmr1p and its human homologues, SPCA1 and SPCA2, share not only ~50% sequence identity but also functional complementarity in yeast.[73] Its importance in human health and disease is underscored by the fact that mutations in hSPCA1 results in Hailey−Hailey disease, a severe skin blistering disorder that arises due to defects in protein glycosylation.[6,73,80,81] SPCA transporters, which are members of a large superfamily of P-type Ca^{2+}-ATPases that includes plasma membrane Ca^{2+}-ATPases, or PMCA transporters, and sarcoendoplasmic reticulum Ca^{2+}-ATPases, or SERCA transporters, are unique in that they are also able to transport manganese in vivo with high affinity.[72,78]

The factors influencing the relative selectivities of calcium versus manganese have been methodically deciphered by the Rao laboratory.[71,82,83] Their work has paved the way for segregating the roles of calcium and manganese in the secretory pathway. SPCA transporters contain an "EF hand" motif at the N-terminus that is necessary for both calcium and manganese transport activity in vitro. Mutagenesis of key aspartates in the EF hand domain resulted in the alteration of the relative selectivities of manganese and calcium.[79] In addition to the EF hand motif, transmembrane helices 4−6 have been implicated as putative cation translocating regions.[71,82] A phenotypic screen involving the systematic mutagenesis of oxygen-containing ligands revealed that key residues in this region are responsible not only for metal translocation but also for the relative selectivity of calcium versus manganese. For example, a D778A mutation, which is present in transmembrane helix 6, is a loss-of-function mutant, defective for transport of both calcium and manganese.[82] A Q783A mutation, also present in transmembrane helix 6, resulted in the selective loss of manganese transport without any impact on calcium transport.[67,71]

Such structure−function studies of Pmr1p have not only revealed the fundamental engineering requirements of metal-ion selectivity and translocation, but also provide new tools to differentiate between the roles of calcium and manganese in the secretory pathway. Regarding the latter point, Devasahayam et al. used separation of function point mutants of Pmr1p to elucidate the role of manganese and calcium in TOR signaling.[75] The target of rapamycin (TOR) protein kinases, which are present in two complexes, TORC1 and TORC2, are highly conserved regulators of eukaryotic cell growth and metabolism that respond to changes in extracellular nutrient conditions.[74−77] By using D53A, Q783A, and D778A Pmr1p point mutants, which are defective in transport of calcium, manganese, or both, respectively, Devasahayam and co-workers were able to ascribe the influence of Pmr1p on TOR signaling as arising due to manganese in the Golgi.[75] While it is not currently known *how* Golgi manganese regulates TOR signaling, one possibility is that manganese-containing sugar transferases in the secretory pathway are needed for proper trafficking of key downstream targets of TOR.[75]

Molecular genetics studies were the first to uncover the manganese transport activity of Pmr1p. Contemporaneous to the identification of *PMR1* as a Golgi apparatus P-type Ca^{2+} ATPase between 1989 and 1992 by the seminal work of Fink and co-workers,[68,69] we identified in 1992 *BSD1* (for bypass of SOD defects), as a genetic suppressor of the oxidative stress phenotypes of strains lacking superoxide dismutase.[84] It was later determined in 1995 that *BSD1* and *PMR1* were identical alleles.[70] The first clue that implicated *PMR1* in manganese homeostasis was the hyperaccumulation of manganese seen in *pmr1* strains, resulting from the inability to pump toxic manganese into the secretory pathway for excretion.[70,73] Furthermore, *pmr1*Δ strains show a defect in sugar transferase activity that can only be corrected by manganese supplementation.[38,57,68] The *pmr1*Δ strains fully reverse the oxidative stress phenotypes of *sod1*Δ mutants due to the hyperaccumulation of cytosolic manganese, transported largely through Smf1p, which in turn acts as an anti-oxidant as described above.[15,70]

Most recently, it was determined that the combination of Pmr1p and Smf1p is essential for providing the manganese anti-oxidants that serve as a backup to Cu/Zn SOD1.[15] Cells lacking *sod1*Δ are normally viable in 20% oxygen; however, disrupting both Pmr1p and Smf1p in an *sod1*Δ strain results in aerobic lethality that can only be rescued by supplementing manganese salts.[15] These studies demonstrated that the physiologic levels of manganese transported by Smf1p and

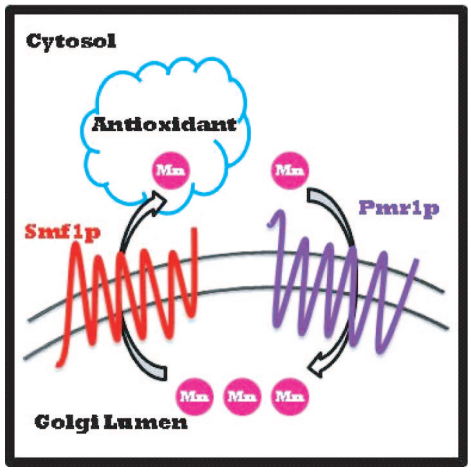

Figure 5. Proposed model for the roles of Pmr1p and Smf1p in providing manganese to the manganese anti-oxidant.[15] Manganese that is provided by Pmr1p (purple) to the Golgi is recycled by Smf1p (red) for its use by the manganese anti-oxidant.

Pmr1p sustain aerobic life in the absence of Cu/Zn SOD1. However, the mechanism by which these two transporters act in concert to provide the manganese anti-oxidant is not completely clear. As Pmr1p and Smf1p are predicted to transport manganese in opposite directions, the former transporting manganese into the secretory pathway and the latter transporting it out, we have recently proposed a model, depicted in Figure 5, in which manganese in the secretory pathway is recycled for use as an anti-oxidant backup for SOD1.[15]

Overall, manganese transport into the Golgi by way of Pmr1p represents a major pathway for the mobilization of intracellular manganese. Not only does Pmr1p provide the Golgi with bioavailable manganese for sugar transferases but it also acts to detoxify excess manganese in cells by transporting it into the secretory pathway for excretion. This latter function of *PMR1* is mirrored by a second human homologue, *SPCA2*.[79] The work of Xiang et al. indicates that SPCA2, which is abundant in the brain, has evolved to detoxify the brain of toxic manganese.[79] In addition, *PMR1* plays an important role in oxidative stress protection by acting in concert with Smf1p to provide manganese used in oxidative stress protection.

5. Vacuolar Manganese Transporter, Ccc1p

Excretion of manganese via Pmr1p and the secretory pathway is not the only means by which the cell detoxifies itself from excess manganese. In *S. cerevisiae*, the vacuole, in addition to its role in the turnover of biomacromolecules, has emerged as a major site for metal-ion storage and detoxification. The first clues implicating the role of vacuoles in metal-ion homeostasis came with genetic studies utilizing yeast strains defective in vacuolar biogenesis and function; these strains exhibited growth defects when exposed to a host of transition metal salts, including cobalt, manganese, iron, nickel, copper, zinc, and cadmium.[85,86] Indeed, manganese is more concentrated in the vacuole than in the cytosol, thereby providing a site with which to sequester toxic excess levels of cellular manganese that can potentially wreak havoc in a variety of cellular compartments.[6,86,87] Thus far, one vacuolar manganese transport pathway has been definitively identified, Ccc1p.[6,88,89] Mutations in this pathway result in increased cellular sensitivity to manganese due to the lack of vacuolar sequestration of the metal.

Ccc1p was originally implicated in calcium metabolism, hence the name cross complementer of csg1, a calcium sensitive mutant.[90] A role in manganese homeostasis was first suggested in 1996 when overexpression of *CCC1* was found to reverse the manganese toxicity of a *pmr1* mutation.[88] Then in 2000, *CCC1* was implicated in iron homeostasis when its overexpression was seen to rescue the iron overload defect of yeast *yfh1* mutants.[91] In 2001, Kaplan and co-workers discovered that Ccc1p is actually a vacuolar transporter of manganese and iron, and the ability of *CCC1* to rescue manganese- and iron-induced toxicity was due to vacuolar sequestration of these ions.[89]

Although manganese can clearly enter the vacuole, it is not known how the metal may be released from this compartment. In the case of iron, two transport systems are known to export iron from the vacuole for use by the cell (Fet5p/Fth1p and Smf3p).[13,92−94] As such, Ccc1p is part of an iron storage system in the vacuole. However, there is no evidence to date that manganese is released from the vacuole; no exporters have been identified. It is possible that Ccc1p merely functions to sequester manganese in the vacuole, thereby detoxifying it, rather than storage and ultimate release of the metal into the cytosol as has been shown for iron.

6. Manganese Metabolism From Yeast to Humans

Because much of the cellular machinery between *S. cerevisiae* and higher metazoans, including humans, is conserved, a large extent of what we have learned in yeast can be extrapolated to humans (Figure 6). However, as a consequence of obvious evolutionary differences, there are some notable differences.

In the case of Nramp transporters, the human homologue Dmt1 has a role in dietary iron and manganese uptake.[25,95] Expressed most prominently in the proximal duodenum, Dmt1 accounts for much of the 3−5% of ingested manganese that is absorbed across the intestinal wall.[95] Mammalian Nramp1 can also function in manganese (as well as iron) transport. This Nramp is expressed in the phagosomes of macrophages, where it acts to transport metals from the lumen of the phagosome into the cytosol, thereby limiting metal ion availability to invading microbes.[25]

Although post-translational regulation of human Nramp proteins by manganese has not yet been demonstrated, mammals express a Bsd2-homologue known as Ndfip1 that also works as an adaptor for an E3-ubiquitin ligase, WWP2.[96] Moreover, Ndfip and WWP2 target the human Nramp transporter Dmt1 for degradation, analogous to the Bsd2p/Rsp5p-mediated degradation of yeast Smf1p and Smf2p.[96] How metal ions such as manganese and iron might influence this Ndfip-mediated degradation of Dmt1 is yet to be determined.

Much of the interest in manganese research has focused on the nervous system, as occupational exposure to high levels of environmental manganese can cause "manganism", a disease that resembles idiopathic Parkinson's disease (PD).[7] A number of models have been put forth regarding the origins of manganese neurotoxicity and have been reviewed extensively elsewhere.[7] For example, manganese can result in mitochondrial dysfunction, perhaps by disrupting the activity of calcium- and magnesium-requiring enzymes in the mitochondria.[7] Manganese has also been proposed to alter the levels of neurotransmitters, such as dopamine (DA), glutamate (Glu), and γ-aminobutyric acid (GABA), possibly by inducing manganese-requiring enzymes involved in neurotrans-

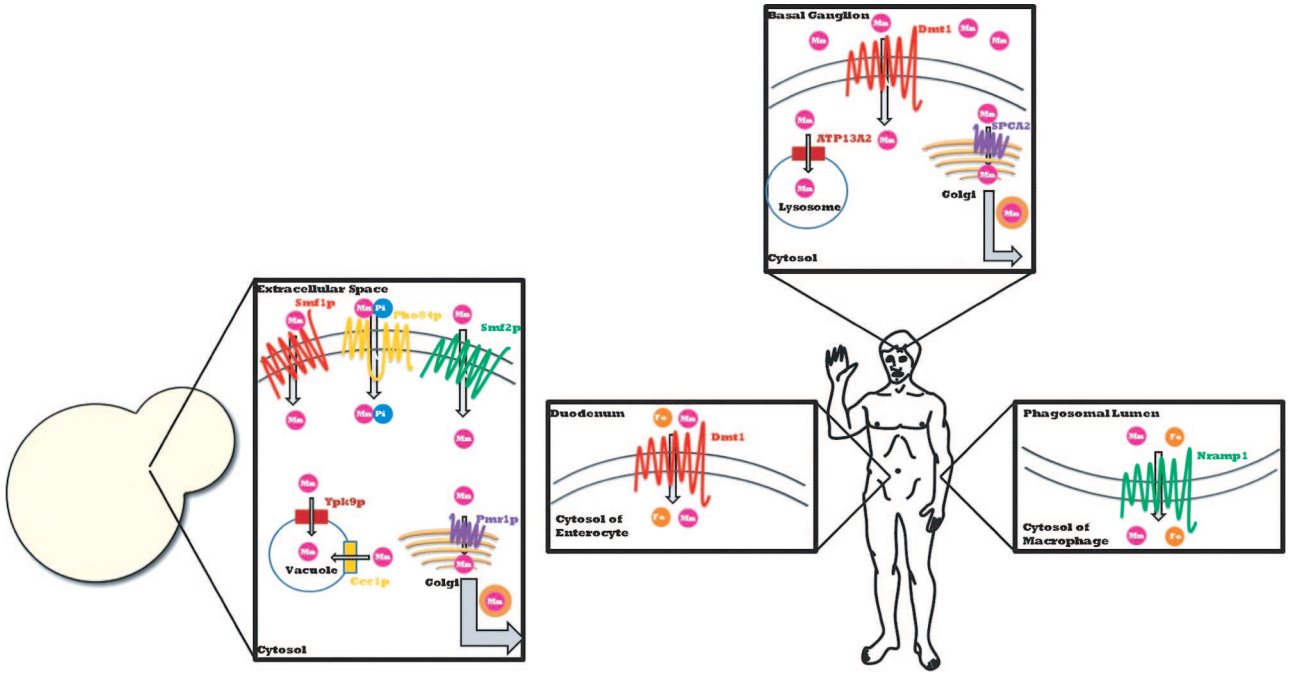

Figure 6. Comparison of manganese transport and trafficking between *S. cerevisiae* (left) and metazoans (right). In yeast, Smf2p (green) provides the bulk of total cellular manganese (pink balls), which is bioavailable to manganese requiring enzymes. Smf1p (red) provides manganese to the manganese anti-oxidant and assists Smf2p import of manganese when cells are manganese starved. Pho84p (gold), the manganese phosphate transporter, is responsible for importing manganese under conditions of high extracellular toxic concentrations of manganese. The vacuolar manganese transporter, Ccc1p (gold rectangle), and the newly identified putative vacuolar manganese importer, Ypk9p (maroon rectangle), help to detoxify toxic manganese. Likewise, manganese pumped into the Golgi via Pmr1p (purple) is detoxified by being exocytosed out of the cell. In metazoans, the Nramp homologue Dmt1 (red), which is expressed in the duodenum, is responsible for dietary iron and manganese intake. The other Nramp homologue, Nramp1 (green), which is expressed in the phagosome of macrophages, serves to pump out iron and manganese that invading microbes require to survive. The basal ganglion is especially sensitive to manganese toxicity due to the expression of Dmt1 in the brain. Like yeast, toxic manganese in metazoans may be detoxified by vacuolar sequestration via the Ypk9p homologue, ATP13A2 (maroon rectangle), or excreted out of the cell by the Pmr1p homologue, SPCA2 (purple).

mitter metabolism (e.g., glutamine synthase).[7] The basal ganglion has relatively high expression levels of Dmt1, which may explain the sensitivity of this region to manganese toxicity and the motor defects associated with manganism.[7,95]

As depicted in Figure 6, the detoxification of manganese in the brain may be analogous to that of yeast in two respects. First, the brain expresses a Pmr1p homologue, SPCA2, that has been suggested to play a major role in manganese sequestration and efflux from the brain.[79] Indeed, rats exposed to manganese exhibit increased expression levels of SPCA2 in the brain.[7] Second, in analogy to vacuolar sequestration of toxic manganese in yeast, very recent work has implicated ATP13A2 as a putative lysosomal manganese transporter (importer).[97,98] Spontaneous mutations in ATP13A2 lead to Kufor−Rakeb syndrome, a form of juvenile Parkinsonism.[97] The yeast homologue of ATP13A2, Ypk9p, has been shown to localize to the yeast vacuole and its deletion augments manganese toxicity, leading to the hypothesis that it is a vacuolar manganese importer.[97,98] However, further studies on the function of Ypk9p/ATP13A2 are needed to define the molecular basis of Kufor−Rakeb syndrome and establish a connection between environmental factors such as manganese exposure and certain forms of Parkinsonism.[97]

7. Conclusion

Because manganese is both essential and potentially toxic, cells must not only transport and traffic sufficient quantities of manganese to appropriate intracellular compartments for their incorporation into manganese requiring biomolecules but also do so without over-/underaccumulating or misincorporating the metal. Any failure to meet these conditions can result in a host of cellular defects. Organisms are particularly susceptible to falling prey to manganese-mediated toxicity when environmental or genetic factors disrupt manganese homeostatic mechanisms, as is the case with manganism in humans exposed to high levels of the metals.[7−12] A full understanding of the mechanisms by which cells maintain manganese homeostasis is critical for our ability to comprehend and treat manganese-related disorders.

Molecular genetics studies in the baker's yeast, *Saccharomyces cerevisiae*, have been instrumental in advancing our knowledge of manganese transport and homeostasis. Thus far, the current understanding of manganese metabolism implicates transporters operating at the cell surface (Smf1p, Smf2p, Pho84p), transporters operating at intracellular sites (Smf1p and Smf2p) to deliver manganese to the cytosol, and transporters that deliver the metal to the Golgi (Pmr1p) and the vacuole (Ccc1p, Ypk9p). Given that manganese is required in essentially every organelle by a variety of proteins and must compete with a host of other metals for transport and trafficking, it is clear that a large number of putative manganese transporters, metallochaperones, and regulatory mechanisms have yet to be identified. For instance, how does manganese cross two membranes to enter the mitochondrial matrix for activation of enzymes such as Mn-superoxide dismustase? Similar questions may be asked of manganese proteins in any number of other organelles. The ease of genetic manipulation and biochemical characterization of *S. cerevisiae*, coupled with the fact that many of the proteins and regulatory mechanisms are conserved between this

simple unicellular eukaryote and higher organisms such as humans, ensures that future work will undoubtedly uncover more details of manganese transport and trafficking relevant to human health and disease.

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