Regulation of Manganese Antioxidants by Nutrient Sensing Pathways in *Saccharomyces cerevisiae*

Amit R. Reddi and Valeria C. Culotta[1]
Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland 21205

ABSTRACT In aerobic organisms, protection from oxidative damage involves the combined action of enzymatic and nonproteinaceous cellular factors that collectively remove harmful reactive oxygen species. One class of nonproteinaceous antioxidants includes small molecule complexes of manganese (Mn) that can scavenge superoxide anion radicals and provide a backup for superoxide dismutase enzymes. Such Mn antioxidants have been identified in diverse organisms; however, nothing regarding their physiology in the context of cellular adaptation to stress was known. Using a molecular genetic approach in Bakers' yeast, *Saccharomyces cerevisiae*, we report that the Mn antioxidants can fall under control of the same pathways used for nutrient sensing and stress responses. Specifically, a serine/threonine PAS-kinase, Rim15p, that is known to integrate phosphate, nitrogen, and carbon sensing, can also control Mn antioxidant activity in yeast. Rim15p is negatively regulated by the phosphate-sensing kinase complex Pho80p/Pho85p and by the nitrogen-sensing Akt/S6 kinase homolog, Sch9p. We observed that loss of either of these upstream kinase sensors dramatically inhibited the potency of Mn as an antioxidant. Downstream of Rim15p are transcription factors Gis1p and the redundant Msn2/Msn4p pair that typically respond to nutrient and stress signals. Both transcription factors were found to modulate the potency of the Mn antioxidant but in opposing fashions: loss of Gis1p was seen to enhance Mn antioxidant activity whereas loss of Msn2/4p greatly suppressed it. Our observed roles for nutrient and stress response kinases and transcription factors in regulating the Mn antioxidant underscore its physiological importance in aerobic fitness.

ADAPTATION to life in oxygen has entailed evolution of numerous enzymatic and nonenzymatic methods for detoxifying reactive oxygen species and repairing damage. Much is known about the widely spread superoxide dismutase (SOD) enzymes that disproportionate superoxide anion into oxygen and hydrogen peroxide (McCord and Fridovich 1969; Abreu and Cabelli 2010). Less understood is the class of small molecule nonproteinaceous manganese (Mn) containing complexes that can substitute for SOD in a variety of organisms. So-called "Mn antioxidants" were first identified in *Lactobaccilus plantarum* that lacks SOD enzymes, but is aerobically viable due to intracellular accumulation of millimolar Mn (Archibald and Fridovich 1981a,b, 1982a,b; Archibald and Duong 1984). High levels of Mn have also been shown to substitute for SOD in strains of *Escherichia coli* (Al-Maghrebi *et al.* 2002), *Neisseria gonorrheae* (Tseng *et al.* 2001), and the yeast *Saccharomyces cerevisiae* (Chang and Kosman 1989; Sanchez *et al.* 2005; Reddi *et al.* 2009), engineered to lack SOD enzymes. Elegant work by Daly and colleagues has shown that tolerance to radiation and oxidative stress in a variety of bacterial species is due to accumulation of high levels of intracellular Mn, but low Fe (Daly 2006, 2009; Daly *et al.* 2007, 2010; Gross 2007; Granger *et al.* 2011). In higher organisms, Mn treatment can prolong the life span and oxidative stress resistance in the simple metazoan, *Caenorhabditis elegans*, and also defend against reactive oxygen species (ROS) in the cryopreservation of sperm (Lin *et al.* 2006; Bansal and Kaur 2009; Cheema *et al.* 2009).

The biochemical nature of the Mn antioxidant has been subject to much investigation. It has been defined as a Mn-dependent but SOD-independent superoxide scavenging activity in bacterial and yeast lysates. This activity is EDTA sensitive, dialyzable, heat and protease resistant, and was proposed to represent Mn complexes of small molecule cellular metabolite(s) (Archibald and Fridovich 1981a; Chang and Kosman 1989). A number of Mn–carboxylato complexes (Archibald and Fridovich 1982b), as well as Mn-orthophosphate

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[1]Corresponding author: Department of Biochemistry and Molecular Biology, Johns Hopkins University, E8030 Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205. E-mail: vculotta@jhsph.edu

(Pi) (Barnese *et al.* 2008), have been shown to exhibit superoxide scavenging activity *in vitro*, and the extreme radioresistance of *Deinococcus radiodurans* is associated with certain Mn–Pi and -peptide complexes (Daly *et al.* 2010). Moreover, our *in vivo* studies in the Baker's yeast have demonstrated a role for Mn–Pi as an important backup for Cu/Zn containing SOD (SOD1) (McNaughton *et al.* 2010).

Using ENDOR spectroscopy to monitor Mn speciation in intact yeast cells, we observed a close correlation between the cellular level of Mn–Pi in various yeast strains and their resistance to oxygen toxicity (McNaughton *et al.* 2010; Szuromi 2010). Of the strains tested, the most severe oxygen toxicity was observed when phosphate control was disrupted through mutations in the phosphate-sensing kinase complex Pho80p/Pho85p (Kaneko *et al.* 1982; Ogawa *et al.* 2000; Wykoff and O'shea 2001; Carroll and O'shea 2002; Lee *et al.* 2007; Wykoff *et al.* 2007). Deletion of either *pho80* or *pho85* rendered *sod1*Δ mutants lacking Cu/Zn SOD1 inviable in air and cells seemed nearly devoid of Mn antioxidant protection (Reddi *et al.* 2009). By ENDOR spectroscopy, Mn–Pi levels were lowered in this strain (McNaughton *et al.* 2010), although not to a degree expected for the profound oxygen toxicity observed. Ablation of Pho80/Pho85p inhibited Mn antioxidant activity through a mechanism other than simple loss of Mn–Pi.

In the context of cellular physiology, what dictates formation of Mn antioxidants? Are these spontaneously formed complexes of Mn that incidentally scavenge superoxide, or does the cell tightly regulate Mn antioxidant activity in concert with protein-based antioxidants? Using the Bakers' yeast *S. cerevisiae* as a model organism, we provide evidence for the latter. Namely, we find that the ability of cells to utilize Mn as an antioxidant can fall under control of Rim15p, a PAS-kinase that integrates phosphate, nitrogen, and carbon-nutrient sensing (Pedruzzi *et al.* 2003; Cameroni *et al.* 2004; Roosen *et al.* 2005; Wanke *et al.* 2005; Swinnen *et al.* 2006; Smets *et al.* 2010; Yang *et al.* 2010). Hyperactivation of Rim15p from genetic ablation of its Pho80p/Pho85p (phosphate sensing) or Sch9p (nitrogen sensing) upstream regulators results in strong downregulation of the Mn antioxidant, helping to explain the profound oxygen toxicity in the *pho80 (or pho85) sod1* null mutants. In addition, transcription factors downstream of Rim15p, which include Gis1p and the redundant pair Msn2p and Msn4p (hereafter referred to as Msn2/4p) that typically regulate stress-response factors, likewise play a role in titrating the activity of the Mn antioxidant. Overall, this work demonstrates that the Mn antioxidant is controlled by the same pathways that sense nutrients and respond to stress.

Materials and Methods

Yeast strains, plasmids, and growth conditions

All yeast strains for this study were derived from BY4741 (MAT**a**, *leu2*Δ*0*, *met15*Δ*0*, *ura3*Δ*0*, *his3*Δ*1*). A full listing, including descriptions on their source or construction, is included in Supporting Information, File S1. Details on the construction of the *msn2*::*HIS3* disruption plasmid, pAR007, and the *sch9*::*HIS3* disruption plasmid, pAR009, are in File S1. All strains were verified by PCR and DNA sequencing.

In general, yeast cells were grown at 30° in enriched yeast extract, peptone, dextrose medium (YPD), or synthetic complete (SC) medium lacking lysine as needed (Sherman 1991). For anaerobic growth, YPD or SC media was supplemented with 15 mg/liter ergosterol and 0.5% Tween-80 (YPDE or SCE, respectively). All experiments employed cells freshly obtained from frozen stocks and cultured on YPDE in oxygen-depleted, CO_2-enriched culture jars (GasPak, Becton-Dickinson). Growth tests to assay oxygen resistance in SC complete medium were conducted under well-aerated (shaking at 220 rpm) conditions as described (Reddi *et al.* 2009); tests for aerobic lysine auxotrophy and Mn toxicity were conducted under micro-aerobic conditions (not shaking) as described (Reddi *et al.* 2009; Rosenfeld *et al.* 2010).

Biochemical assays

For all biochemical assays including phosphate and manganese measurements, superoxide scavenging activity and Fe/S enzyme assays, cells were pregrown in triplicate cultures in SCE media ≈16 hr under an anaerobic N_2 atmosphere prior to dilution to $o.d._{600nm}$ = 0.25 and growth for 6 hr in air, shaking at 220 rpm. Cells were then harvested, washed three times in ice-cold TE buffer (10 mM Tris-hydrochloride and 1 mM EDTA, pH 8.0), and three times in ice-cold MiliQ water prior to further analyses.

Total cellular phosphates (orthophosphate and polyphosphates) were measured using the molybdate reactivity method (Ames 1966; Reddi *et al.* 2009; McNaughton *et al.* 2010). Cells were lysed by glass bead homogenization in 500 ml of 0.1% Triton X-100. Total phosphate was measured from boiling 3–30 μg of whole-cell lysates for 10 min in 1 N H_2SO_4. Phosphate was quantified using a calibration curve of 0–300 μM phosphoric acid. Total cellular manganese was measured using atomic absorption spectroscopy (AAS) as described previously (Reddi *et al.* 2009).

For superoxide scavenging-activity assays, cells were lysed in 50 mM MES, 100 mM KCl, 0.1% Triton X-100, pH 7.0 by glass bead homogenization and 10–300 μg of cell lysate protein was subject to the xanthine/xanthine oxidase/XTT (3′-1-(phenylamino)-carbonyl-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay for superoxide generation and detection (Ukeda *et al.* 1997). The increase in absorbance at 470 nm over 60 min upon addition of xanthine oxidase, corresponding to the rate of reduction of XTT by superoxide, was read in a Biotek HT Synergy plate reader or in a Beckman-Coulter UV/vis spectrophotometer. The rate of XTT reduction by lysates in the absence of xanthine oxidase was subtracted from all measurements. One unit of superoxide scavenging activity was defined as a 50% decrease in the rate of XTT reduction.

For aconitase (Aco1p) and isopropylmalate isomerase (Leu1p) activity assays, cells were subjected to glass bead

lysis in 50 mM MES, 100 mM KCl, 0.1% Triton X-100, pH 7.0 under a nitrogen atmosphere in a COY chamber. Aco1p and Leu1p activity was determined spectrophotometrically using a Biotek HT Synergy plate reader (Wallace *et al.* 2004). The assay mixture contained 50–300 μg of lysate protein in 200 μl of a buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, and 100 mM NaCl and supplemented with either 0.5 mM *cis*-aconitate (Aco1p activity) or 0.5 mM citraconitate (Leu1p activity). Activities were determined by monitoring the disappearance of *cis*-aconitate (Aco1p) or citraconitate (Leu1p) at 240 or 235 nm, respectively, over the course of 3 min. These species were quantified by generating calibration curves of standardized concentrations of *cis*-aconitate or citraconitate. In both cases, 1 U of activity is defined as 1 nmol of substrate consumed per minute per milligram of protein.

Results

Phosphate signaling through Pho4p is not responsible for the aerobic lethality of sod1Δ pho80Δ mutants

A loss of phosphate control in *S. cerevisiae* through disruption of *PHO80* or *PHO85* renders *sod1Δ* cells inviable in air and unable to effectively utilize Mn for oxidative stress suppression (Reddi *et al.* 2009; McNaughton *et al.* 2010; Rosenfeld *et al.* 2010). As seen in Figure 1A, Mn supplements to the growth medium only partly restore aerobic growth to a *sod1Δ pho80Δ* double mutant. The limited efficacy of Mn in this regard might be explained by the intolerance of *pho80* mutants to Mn toxicity (Figure 1B). These mutants accumulate very high Mn (Figure 1C, note log scale) due to uncontrolled uptake of Mn–Pi by the Pho84p transporter (Wykoff and O'shea 2001; Jensen *et al.* 2003; Reddi *et al.* 2009; Rosenfeld *et al.* 2010). To remove effects of Mn toxicity, we disrupted *PHO84*, which reversed both the high Mn accumulation (Figure 1C) and Mn sensitivity (Figure 1B) of the *sod1Δ pho80Δ* mutant. However, the *pho84* mutation did not restore aerobic growth and most striking, Mn supplementation was ineffective at reversing the aerobic lethality of a *sod1Δ pho80Δ pho84Δ* mutant (Figure 1A). Hence, *sod1Δ pho80Δ* cells require massive accumulation of Mn to rescue any oxygen toxicity, consistent with the low efficacy of Mn as an antioxidant in this strain.

The Pho80p/Pho85p kinase pair negatively regulates the Pho4p transcription factor for the induction of phosphate uptake and storage genes such as *PHO84* (Kaneko *et al.* 1982; Ogawa *et al.* 2000; Wykoff and O'shea 2001; Lee *et al.* 2007; Wykoff *et al.* 2007). We addressed whether hyperactive Pho4p in *pho80* mutants accounts for the severe oxygen toxicity of *sod1Δ pho80Δ* cells. As seen in Figure 2A, deletion of *PHO4* did not reverse the aerobic lethality of *sod1Δ pho80Δ* cells, despite lowering phosphate (Figure 2B) and intracellular (Mn) (Figure 2C) to levels that approximated the *sod1Δ* control. Hence, hyperactivated Pho4p cannot account for the aerobic lethality of *sod1Δ pho80Δ* cells and another downstream effector molecule must be responsible.

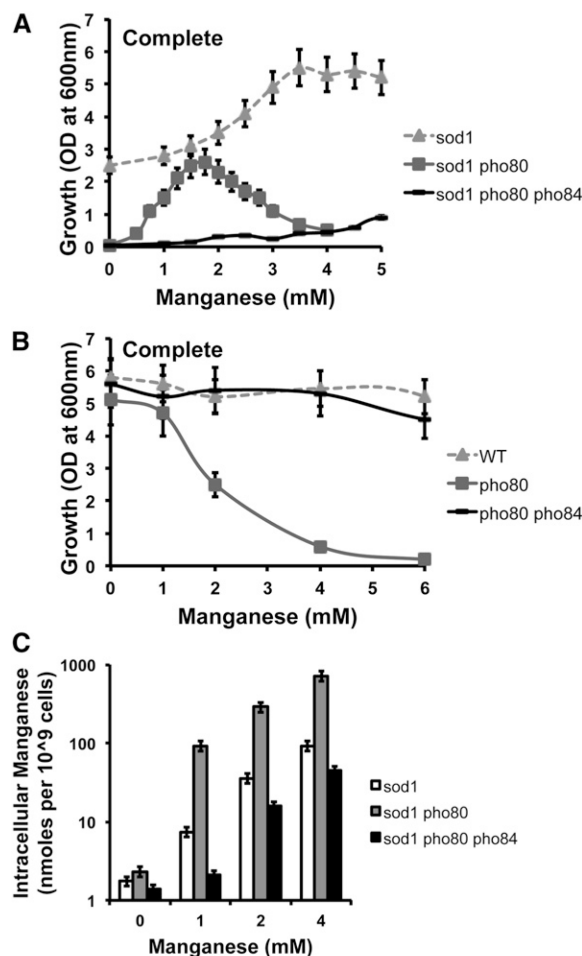

Figure 1 Loss of the Mn–phosphate transporter Pho84p enhances oxygen toxicity in a *sod1Δ pho80Δ* strain. (A) The indicated *sod1Δ* strains were seeded in synthetic complete (SC) media at an optical density at 600 nm ($o.d._{600nm}$) of 0.05, and grown shaking for 16 hr with the indicated concentrations of $MnCl_2$. Total growth was determined by measuring the $o.d._{600nm}$. (B) Manganese toxicity of the indicated strains was determined by growing cells in SC medium supplemented with the indicated concentrations of Mn as described in A under nonshaking conditions. (C) Manganese accumulation of the indicated *sod1Δ* strains was determined by atomic absorption spectroscopy (AAS) with cells grown in SC medium treated for 6 hr with the indicated concentrations of $MnCl_2$ as described in *Materials and Methods*. Manganese content is represented as nanomoles per 10^9 cells. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: WT, BY4741; *pho80Δ*, LR237; *pho80Δ pho84Δ*, LR154; *sod1Δ*, AR203; *sod1Δ pho80Δ*, LR156; *sod1Δ pho80Δ pho84Δ*, LR178.

Rim15p as a regulator of Mn antioxidant activity

A second target of the Pho80p–Pho85p cyclin–CDK complex is the PAS-kinase, Rim15p (Wanke *et al.* 2005; Swinnen *et al.* 2006). As with Pho4p, Rim15p is hyperactivated in cells lacking *pho80* or *pho85*. A deletion of *rim15* had no effect on the elevated phosphate (Figure 3B) or manganese (Figure 3C) of a *sod1Δ pho80Δ* mutant. Nevertheless, loss of Rim15p restored aerobic viability to these cells (Figure 3A). Thus, hyperactive Rim15p appears responsible for the severe oxidative stress of *sod1Δ pho80Δ* mutants under atmospheric oxygen.

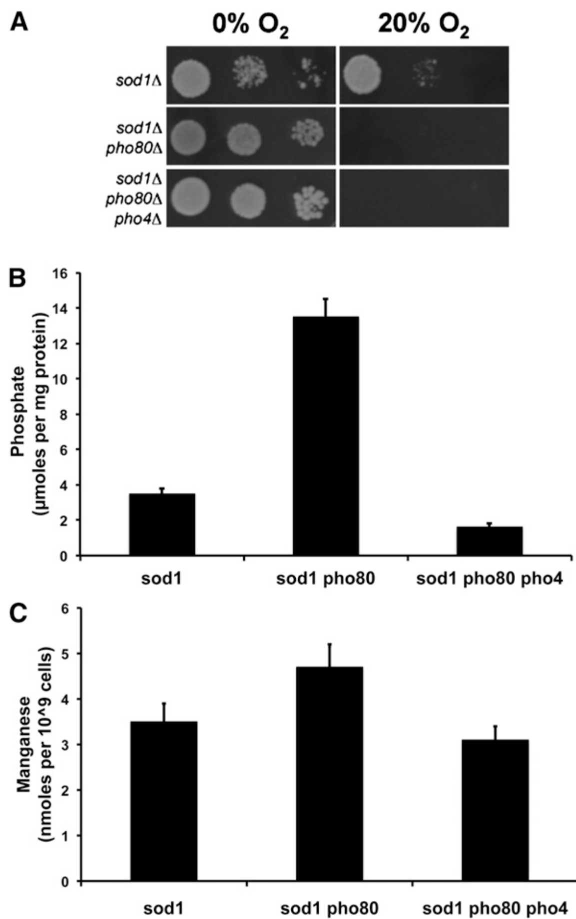

Figure 2 Activation of the Pho4p transcription factor is not responsible for the aerobic lethality of *sod1*Δ *pho80*Δ strains. (A) The effect of a *pho4*Δ mutation on the aerobic lethality of *sod1*Δ *pho80*Δ cells was tested by spotting 10^4, 10^3, and 10^2 cells of the indicated strains onto SCE plates and by growing in air or anaerobically for 3 days. (B) Phosphate content of the indicated strains was measured by molybdate reactivity of the indicated strains grown in SC medium as described in *Materials and Methods*. (C) Manganese content of the indicated strains was measured by AAS precisely as described in Figure 1C. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: *sod1*Δ, AR203; *sod1*Δ *pho80*Δ, LR156; *sod1*Δ *pho80*Δ *pho4*Δ, AR106.

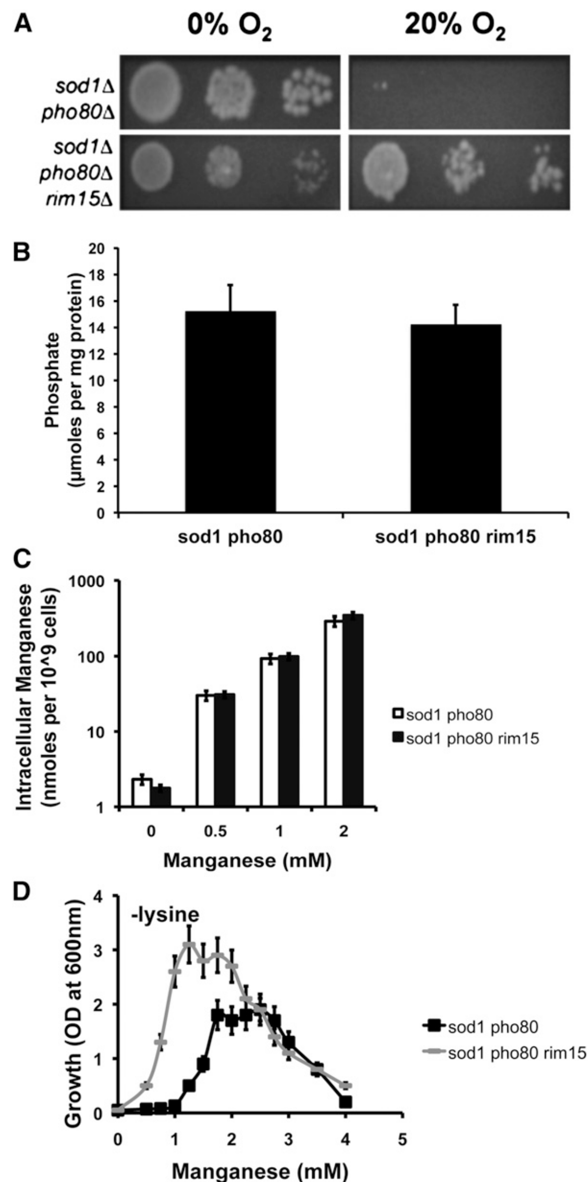

Figure 3 *RIM15* is responsible for the aerobic lethality and poor utility of Mn as an antioxidant in *sod1*Δ *pho80*Δ strains. Shown are the effects of a *rim15* mutation on (A) aerobic lethality of *sod1*Δ *pho80*Δ strains, (B) phosphate accumulation, and (C) Mn accumulation, as described in Figure 2, A, B, and C, respectively. (D) Manganese rescue of the *sod1*Δ-linked aerobic lysine auxotrophy was tested in SC medium lacking lysine and supplemented with the indicated concentrations of $MnCl_2$. Cells were grown as in A except under nonshaking conditions. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: *sod1*Δ *pho80*Δ, LR156; *sod1*Δ *pho80*Δ *rim15*Δ, AR110.

Another marker of *sod1*Δ-linked oxidative stress is an aerobic lysine auxotrophy resulting from superoxide damage to lysine biosynthetic enzyme(s) (Bilinski *et al.* 1985; Wallace *et al.* 2004). This defect can be reversed by Mn supplementation (Chang and Kosman 1989; Sanchez *et al.* 2005; Reddi *et al.* 2009). In comparing the dose response to Mn, the aerobic lysine auxotrophy of the *sod1*Δ *pho80*Δ *rim15*Δ triple mutant was more effectively rescued by Mn than the *sod1*Δ *pho80*Δ double mutant (Figure 3D), despite accumulating identical levels of the metal (Figure 3C). Thus, deletion of *RIM15* in *sod1*Δ *pho80*Δ cells not only promotes aerobic growth, but also lowers the intracellular dose of Mn required to protect against oxidative damage.

Rim15p is negatively regulated by a number of kinases downstream of nutrient sensing and signaling circuits. In addition to the aforementioned Pho80p–Pho85p cyclin–CDK pair (phosphate sensing), these kinases include Tor1p and the yeast Akt/S6K homolog, Sch9p, which are both involved in nitrogen sensing, and PKA, which senses carbon sources (Pedruzzi *et al.* 2003; Cameroni *et al.* 2004; Roosen *et al.* 2005; Wanke *et al.* 2005; Swinnen *et al.* 2006; Smets *et al.* 2010; Yang *et al.* 2010). We sought to determine if ablating the activities of any of these kinases in *sod1* cells

phenocopied the effects of a *pho80*Δ mutation. Toward this end, we deleted *TOR1*, *RAS2*, a positive regulator of PKA activity, and *SCH9* in *sod1*Δ cells. Of these, the *sch9* mutation conferred a severe aerobic growth defect to *sod1*Δ cells that was particularly profound in liquid cultures and was poorly rescued by Mn supplements (Figures 4A and 4B). This *sch9*-growth defect was indeed due to oxygen toxicity as it was abolished under anaerobic conditions or in strains with WT *SOD1* (Figure 4C). Moreover, *sch9*Δ conferred oxygen sensitivity to *sod1*Δ nulls without global changes in cellular phosphates or Mn (Figure S1A and Figure S1B). The ability of *sch9* mutations to phenocopy *pho80* mutations in terms of *sod1*Δ oxidative stress supports a role for Rim15p in negatively regulating the Mn antioxidant.

The downstream transcription factors Gis1p and Msn2/4p work in opposite to regulate the Mn antioxidant as a scavenger of superoxide in the cytosol

The downstream targets of Rim15p activation include the transcription factors Gis1p and the redundant pair Msn2/4p. All three transcription factors, which share ~95% of their gene targets, are thought to work in concert to activate cellular stress defense pathways, as well as metabolic adaptations to nutrient deficiency (Cameroni *et al.* 2004; Swinnen *et al.* 2006; Smets *et al.* 2010). We tested whether Gis1p and/or Msn2/4p activation was responsible for the aerobic lethality of the *sod1*Δ *pho80*Δ strain. As shown in Figure 5A, deletion of *gis1* rescued the aerobic lethality of *sod1*Δ *pho80*Δ cells, while *msn2/4* deletions did not. Moreover, in a dose-response study, extracellular Mn was more effective in suppressing the aerobic growth defect (Figure S2A) and aerobic lysine auxotrophy (Figure 5C) of *sod1*Δ *pho80*Δ cells when *gis1* was deleted, even though intracellular Mn (Figure 5B) and phosphate (Figure S2B) levels were unchanged. At equivalent intracellular concentrations of ~90 nmol Mn per 10^9 cells achieved when *sod1*Δ cells are treated with 4 mM Mn or when *sod1*Δ *pho80*Δ *gis1*Δ cells are treated with 1 mM Mn (Figure 5B), the aerobic lysine auxotrophy of both strains is rescued by Mn to nearly the same degree (Figure 5C). Overall, the deletion of *gis1* phenocopies the effect of a *rim15* deletion in *sod1*Δ *pho80*Δ cells in terms of reversing the aerobic lethality and promoting Mn antioxidant capacity.

In stark contrast to a *gis1* deletion, the *msn2/4* mutations dramatically inhibited the ability of Mn to promote aerobic growth. Mn supplements were completely ineffective in suppressing the aerobic lysine auxotrophy of *sod1*Δ *pho80*Δ *msn2/4*Δ cells (Figure 5C) and also poorly reversed the aerobic lethality of *sod1*Δ *pho80*Δ *msn2/4*Δ in complete medium (Figure S2A). The differential effects of *gis1 vs. msn2/4* mutations on the efficacy of Mn as an antioxidant were not due to differences in Mn accumulation (Figure 5B) or Mn toxicity (Figure S2C) or intracellular phosphate concentrations (Figure S2B). As a potential caveat to these studies, *sod1*Δ *pho80*Δ *msn2/4*Δ cells also grow poorly under anaerobic conditions (Figure 5A), due to a synthetic defect of combining *pho80* mutations with *msn2*Δ *msn4*Δ. However, our other studies (see Figures 5D and 6) demonstrate that the inhibitory effects of *msn2*Δ *msn4*Δ mutations on the Mn antioxidant are also visible in a *PHO80*+ strain.

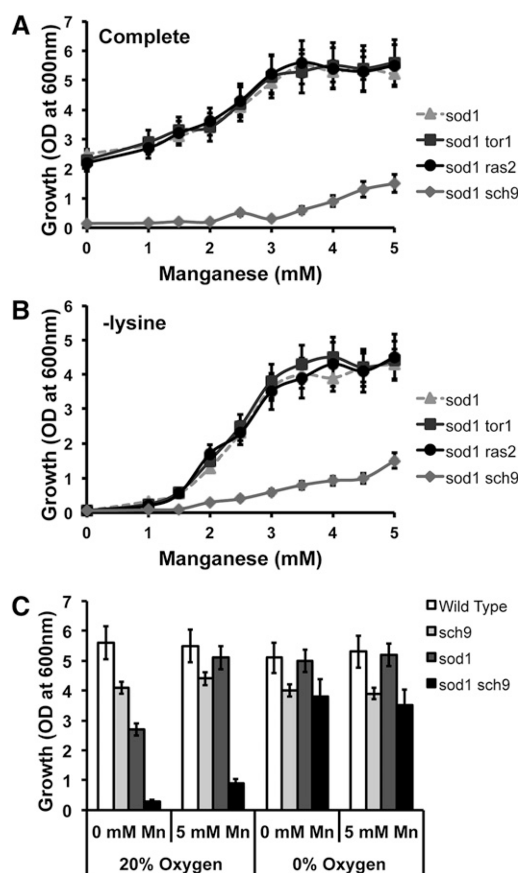

Figure 4 Deleting *sch9* renders *sod1*Δ cells oxidatively stressed and unable to utilize Mn for antioxidant protection. (A and B) The effect of *sch9*Δ, *tor1*Δ, and *ras2*Δ deletions on Mn promotion of aerobic growth was tested in (A) well-aerated SC media and (B) media lacking lysine, as was done as in Figures 1A and 3D, respectively. (C) The effect of *sch9*Δ, *sod1*Δ, and *sod1*Δ *sch9*Δ mutations on growth in SCE media in the absence and presence of oxygen and manganese was determined by measuring the o.d.$_{600nm}$ values of shaking cultures after 16 hr of growth, as described in *Materials and Methods*. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: *sod1*Δ, AR203; *sod1*Δ *sch9*Δ, AR163; *sch9*Δ, AR164. *sod1*Δ *tor1*Δ, AR120; *sod1*Δ *ras2*Δ, AR158.

We next assessed whether the effects of *gis1* and *msn2/4* mutations on the Mn antioxidant were evident without hyperactivation of Rim15p, *i.e.*, in a *PHO80*+ strain. For these studies, *sod1*Δ *gis1*Δ double and *sod1*Δ *msn2*Δ *msn4*Δ triple mutants were generated. As seen in Figure 5D, the ability of Mn to support *sod1*Δ aerobic growth on media lacking lysine was significantly enhanced with a *gis1*Δ mutation and repressed with *msn2/4*Δ mutations without global changes in cellular phosphates or manganese (Figure S3). Even in the absence of hyperactivation conditions, these factors control the efficacy of Mn as an antioxidant.

To more directly study the differential effects of Gis1p and Msn2/4p on the Mn antioxidant, we used a biochemical assay to probe Mn-dependent superoxide scavenging activity. Figure

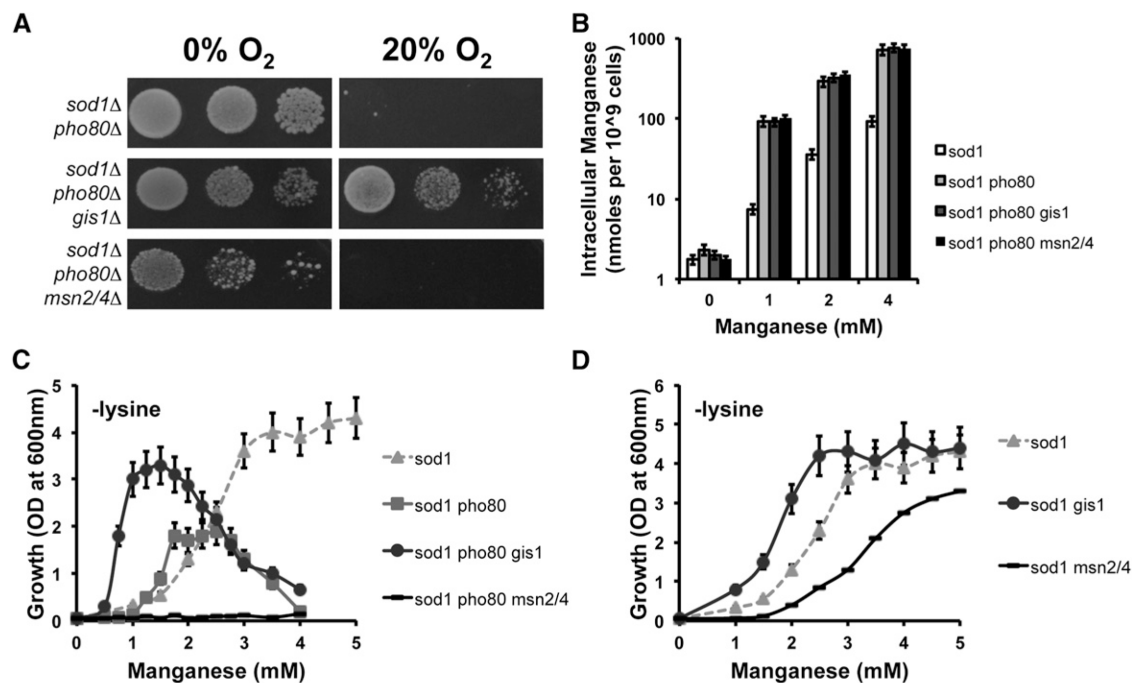

Figure 5 *GIS1* and *MSN2/4* have opposing affects on Mn antioxidant protection. The effect of *gis1* and *msn2/4* mutations on the (A) aerobic lethality of *sod1Δ pho80Δ* cells, (B) Mn accumulation in *sod1Δ pho80Δ* cells, and (C and D) Mn-mediated rescue of the *sod1Δ*-linked aerobic lysine auxotrophy in *sod1Δ pho80Δ* (C) and *sod1Δ* (D) cells were tested precisely as in Figure 3, A, C, and D, respectively. Details on culture conditions and growth are described in *Materials and Methods*. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: *sod1Δ*, AR203; *sod1Δ pho80Δ*, LR156; *sod1Δ pho80Δ gis1Δ*, AR138; *sod1Δ pho80Δ msn2/4Δ*, AR300; *sod1Δ gis1Δ*, AR121; *sod1Δ msn2/4Δ*, AR155.

6A shows the superoxide scavenging activity of cell lysates prepared from WT, *sod1Δ*, *sod2Δ*, and *sod1Δ sod2Δ* cells, based on the xanthine/xanthine oxidase/XTT assay for *in vitro* superoxide generation and detection (Ukeda *et al.* 1997). By comparing results from the different *sod* null strains, we estimated the activities of Sod1p and Sod2p to be ~154 and ~20 U/mg protein, respectively, as well as SOD-independent superoxide scavenging (SISS) activity as ~5 U/mg protein. Previous studies have implicated this SISS to be that of the "Mn antioxidant" (Chang and Kosman 1989). Indeed, consistent with previous studies, we determined SISS activity in the background of cells lacking the cytosolic Cu/Zn SOD1 and the mitochondrial Mn–SOD2 is specific for Mn and is EDTA sensitive and heat resistant (Figures 6B and 6C). We observed that the *gis1* and *msn2/4* mutations differentially affect Mn-dependent SISS activity *in vitro*. As shown in Figure 6D, the *gis1* mutation endows cell extracts with approximately twofold greater superoxide scavenging activity (~11 U/mg protein), whereas the *msn2/4* mutations reduce it by nearly the same factor (~2 U/mg protein). In addition, Mn supplemented to cells during growth is more effectively utilized by the *gis1* mutant and poorly utilized by the *msn2/4* mutant for superoxide scavenging (Figure 6D).

Finally, we sought to determine the cellular localization of Gis1p and Msn2/4p-dependent Mn antioxidant activity. To address this, we took advantage of the fact that Fe/S cluster proteins are specific targets of superoxide toxicity (Wallace *et al.* 2004) and examined activity of two highly homologous dehydratase Fe/S enzymes that are differentially localized, namely aconitase (Aco1p, mitochondrial matrix) and isopropylmalate isomerase (IPMI) (Leu1p, cytosol). Figures 7A and 7B show that deletion of the SOD encoding genes result in ~30% and 75% reductions in aconitase and IPMI activities, respectively, and these losses in activities are fully restored by Mn supplements. Although *gis1* and *msn2/4* mutations had no effect on the Mn rescue of mitochondrial aconitase (Figure 7A), these same mutations had a significant impact on Mn-mediated protection of cytosolic IMPI activity (Figure 7B). The *gis1* mutation enhanced IPMI activity at 2 mM Mn, whereas the *msn2/4* mutations reduced IPMI activity at all Mn levels tested. Hence, *gis1* and *msn2/4* mutations affect Mn antioxidant activity in the cytosol.

Discussion

Intracellular Mn has long been known to suppress oxidative stress in a variety of organisms. In organisms that do not express SODs and/or naturally accumulate high Mn and low Fe (*e.g.*, *L. plantarum* and *D. radiodurans*), there is good evidence for nonproteinaceous complexes of Mn such as Mn–Pi serving as physiological antioxidants, primarily by protecting against protein oxidation (Daly *et al.* 2004, 2007, 2010; Daly 2006, 2009; Fredrickson *et al.* 2008). Mn may also act as an antioxidant by functionally substituting for some Fe enzymes, thereby mitigating the potential for deleterious Fenton

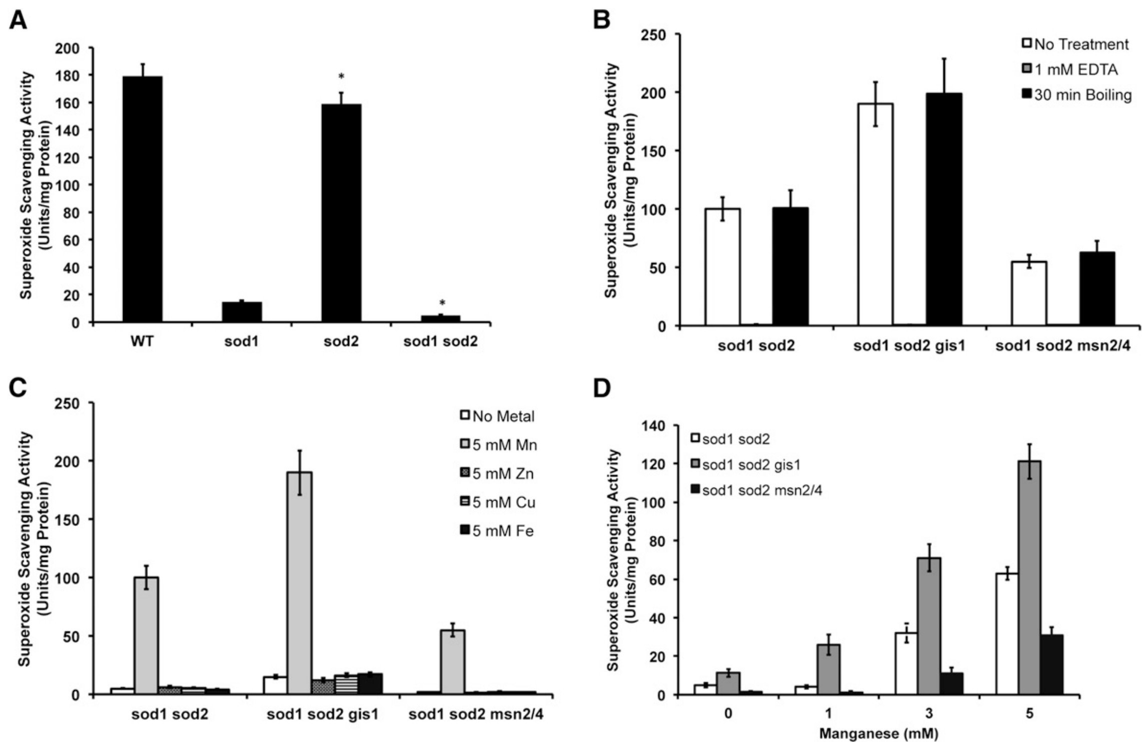

Figure 6 *GIS1* and *MSN2/4* differentially affect manganese-dependent superoxide scavenging activity in SOD-deficient cells. The indicated cells were grown for 6 hr in SC medium that was either (A) not supplemented with metals, or supplemented with (B) 5 mM $MnCl_2$, (C) 5 mM of $MnCl_2$, $ZnCl_2$, $CuCl_2$, or $FeCl_2$, or (D) the indicated concentrations of $MnCl_2$. Whole-cell lysates were prepared and analyzed for total cellular superoxide scavenging activity by the xanthine/xanthine oxidase/XTT assay described in *Materials and Methods*. One unit is defined as a 50% decrease in the rate of XTT reduction per milligram lysate protein. (A) The asterisk denotes a $P < 0.05$ and reflects the statistical significance in loss of activity upon a *sod2*Δ mutation. (B) Where indicated, lysates were incubated for 30 min at room temperature with 1 mM EDTA or at 100° prior to superoxide scavenging activity assays to demonstrate metal chelator sensitivity and heat resistance of SISS activity. Details on culture conditions and the superoxide scavenging assays are described in *Materials and Methods*. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: WT, BY4741; *sod1*Δ, AR203; *sod1*Δ *gis1*Δ, AR121; *sod1*Δ *msn2/4*Δ, AR155; *sod1*Δ *sod2*Δ, AR142; *sod1*Δ *sod2*Δ *gis1*Δ, AR161; *sod1*Δ *sod2*Δ *msn2/4*Δ, AR160.

reactions at enzyme active sites (Anjem *et al.* 2009; Sobota and Imlay 2011). Regardless of the mechanism, it was unclear whether the protective effect of Mn is part of the cell's tightly regulated battery of antioxidant responses or is a passive unregulated process. Using *S. cerevisiae* as a model organism, we provide the first line of evidence that the Mn antioxidant is

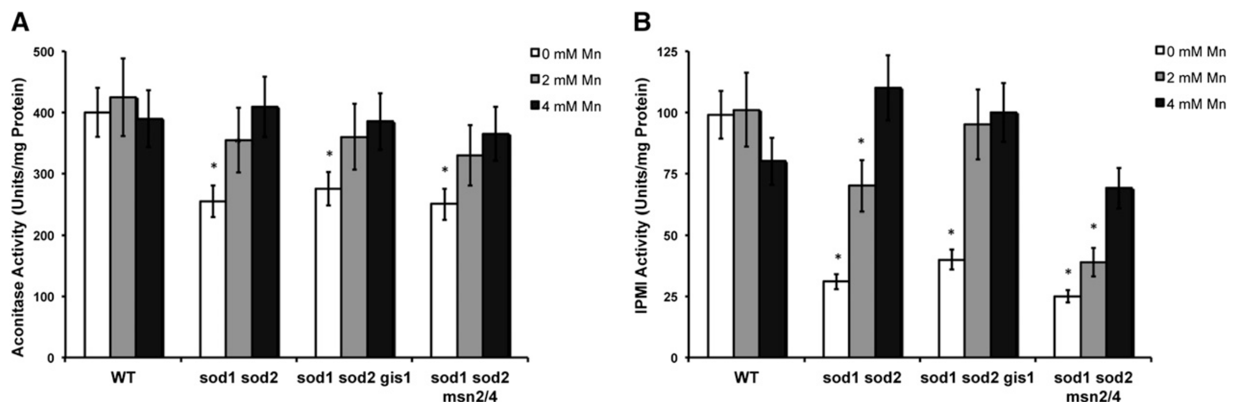

Figure 7 *GIS1* and *MSN2/4* differentially affect the ability of manganese to protect cytosolic but not mitochondrial Fe/S proteins. The indicated strains were grown in SC medium that was supplemented for 6 hr with the designated concentrations of $MnCl_2$ prior to cell lysate preparation and analysis of (A) aconitase and (B) isopropylmalate isomerase (IPMI) activity as described in *Materials and Methods*. Asterisk denotes a $P < 0.05$ and reflects the statistical significance between 4 mM Mn treatment and 0 or 2 mM Mn treatment. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: WT, BY4741; *sod1*Δ *sod2*Δ, AR142; *sod1*Δ *sod2*Δ *gis1*Δ, AR161; *sod1*Δ *sod2*Δ *msn2/4*Δ, AR160.

a component of the oxidative stress defense that is regulated through nutrient sensing pathways. Specifically, the nutrient signaling kinases Pho80p, Sch9p, and Rim15p as well as downstream regulators Msn2/4p and Gis1p can all regulate the efficacy of the Mn antioxidant in yeast cells.

We show herein that loss of *gis1* promotes the Mn antioxidant whereas mutations in *msn2/4* inhibit it; hence Gis1p and Msn2/4p negatively and positively regulate the potency of Mn as an antioxidant, respectively. Traditionally, these transcription factors are known for their regulation of genes involved in the response to stress and nutrients as well as genes for promoting longevity in response to calorie restriction (Reinders *et al.* 1998; Pedruzzi *et al.* 2000, 2003; Hasan *et al.* 2002; Cameroni *et al.* 2004; Fabrizio *et al.* 2004, 2001; Roosen *et al.* 2005; Swinnen *et al.* 2006; Medvedik *et al.* 2007; Wei *et al.* 2008; Zhang *et al.* 2009; Smets *et al.* 2010). The Mn antioxidant can now be added to this list of stress resistance and metabolism factors that are governed by such nutrient and stress signaling pathways.

Gis1p and Msn2/4p are reported to share a bulk of their gene targets (Cameroni *et al.* 2004; Swinnen *et al.* 2006; Smets *et al.* 2010); thus it is surprising that they have opposing effects on activity of the Mn antioxidant. It is possible that Gis1p and Msn2/4p negatively and positively regulate the same downstream target or coordinately regulate two distinct targets that have opposing actions on Mn as an antioxidant. While we still do not know the identity of the downstream factor(s), our data tentatively point to cytosolic target(s) for oxidative stress suppression. Specifically, *gis1* and *msn2/4* mutations affect the ability of Mn to protect the activity of the cytosolic Fe/S protein, Leu1p but not the homologous mitochondrial Fe/S protein, Aco1p. It is possible that the nature of the Mn antioxidant in the cytosol is distinct from that in the mitochondria and Gis1p and Msn2/4p signaling pathways only regulate the former.

Since Mn–Pi has been implicated as a key Mn antioxidant in yeast cells (Barnese *et al.* 2008; McNaughton *et al.* 2010) it is possible that Gis1p and Msn2/4p affect cellular phosphate interactions with Mn. However, we observed that *gis1* or *msn2/4* mutations have no effect on total phosphate or Mn. Moreover, we previously proposed that the lowering of Mn–Pi in *sod1*Δ *pho85*Δ mutants could not by itself explain the extreme oxidative stress of this strain (McNaughton *et al.* 2010). The activation of Rim15p and Gis1p in this mutant appears to inhibit the Mn antioxidant independent of Mn–Pi effects. Mn complexes to carboxylates (*e.g.*, lactate, succinate) can also act as antioxidants (Archibald and Fridovich 1982b) and it is possible that such compounds are targets for regulation by Rim15p, Gis1p, and Msn2/4p. This study also raises the intriguing possibility that phosphate can promote Mn antioxidant activity through Pho80p-mediated inhibition of Rim15p and Gis1p independently of phosphate-dependent chemical scavenging of superoxide by Mn–Pi complexes.

In conclusion, we demonstrate that Rim15p, Gis1p, and Msn2/4p, which are downstream of conserved nutrient sensing pathways, can regulate cytosolic Mn-mediated superoxide scavenging activity. Unlike previous studies that have implicated Gis1p and Msn2/4p working in parallel gene activation pathways (Cameroni *et al.* 2004; Swinnen *et al.* 2006; Smets *et al.* 2010), we provide evidence that these factors can work in opposite with regard to the Mn antioxidant: Gis1p negatively regulates Mn antioxidant activity whereas Msn2/4p positively regulates it. In this manner, the two factors provide checks and balances for one another and together titrate the precise degree of oxidative stress protection required.

Acknowledgments

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Literature Cited

Abreu, I. A., and D. E. Cabelli, 2010 Superoxide dismutases-a review of the metal-associated mechanistic variations. Biochim. Biophys. Acta 1804: 263–274.

Al-Maghrebi, M., I. Fridovich, and L. Benov, 2002 Manganese supplementation relieves the phenotypic deficits seen in superoxide-dismutase-null Escherichia coli. Arch. Biochem. Biophys. 402: 104–109.

Ames, B. N., 1966 Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. 8: 115–118.

Anjem, A., S. Varghese, and J. A. Imlay, 2009 Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. Mol. Microbiol. 72: 844–858.

Archibald, F. S., and M. N. Duong, 1984 Manganese Acquistion by *Lactobacillus plantarum*. J. Bacteriol. 158: 1–8.

Archibald, F. S., and I. Fridovich, 1981a Manganese and defenses against oxygen toxicity in *Lactobacillus plantarum*. J. Bacteriol. 145: 422–451.

Archibald, F. S., and I. Fridovich, 1981b Manganese, superoxide dismutase and oxygen tolerance in some lactic acid bacteria. J. Bacteriol. 146: 928–936.

Archibald, F. S., and I. Fridovich, 1982a Investigations on the state of the manganese in Lactobacillus plantarum. Arch. Biochem. Biophys. 215: 589–596.

Archibald, F. S., and I. Fridovich, 1982b The scavenging of superoxide radical by manganous complexes *in vitro*. Arch. Biochem. Biophys. 214: 452–463.

Bansal, A. K., and A. R. Kaur, 2009 Cooperative functions of manganese and thiol redox system against oxidative stress in human spermatozoa. J. Hum. Reprod. Sci. 2: 76–80.

Barnese, K., E. B. Gralla, D. E. Cabelli, and J. S. Valentine, 2008 Manganous phosphate acts as a superoxide dismutase. J. Am. Chem. Soc. 130: 4604–4606.

Bilinski, T., Z. Krawiec, L. Liczmanski, and J. Litwinska, 1985 Is hydroxyl radical generated by the fenton reaction in vivo? Biochem. Biophys. Res. Commun. 130: 533–539.

Cameroni, E., N. Hulo, J. Roosen, J. Winderickx, and C. De Virgilio, 2004 The novel yeast PAS kinase Rim 15 orchestrates G0-associated antioxidant defense mechanisms. Cell Cycle 3: 462–468.

Carroll, A. S., and E. K. O'shea, 2002 Pho85 and signaling environmental conditions. Trends Biochem. Sci. 27: 87–93.

Chang, E. C., and D. J. Kosman, 1989 Intracellular Mn(II)-associated superoxide scavenging activity protects Cu,Zn superoxide dismutase-deficient *Saccharomyces cerevisiae* against dioxygen stress. J. Biol. Chem. 264: 12172–12178.

Cheema, R. S., A. K. Bansal, and G. S. Bilaspuri, 2009 Manganese provides antioxidant protection for sperm cryopreservation that may offer new consideration for clinical fertility. Oxid. Med. Cell Longev. 2: 152–159.

Daly, M. J., 2006 Modulating radiation resistance: insights based on defenses against reactive oxygen species in the radioresistant bacterium *Deinococcus radiodurans*. Clin. Lab. Med. 26: 491–504 x.

Daly, M. J., 2009 A new perspective on radiation resistance based on *Deinococcus radiodurans*. Nat. Rev. Microbiol. 7: 237–245.

Daly, M. J., E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, M. Zhai *et al.*, 2004 Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. Science 306: 1025–1028.

Daly, M. J., E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, M. Zhai *et al.*, 2007 Protein oxidation implicated as the primary determinant of bacterial radioresistance. PLoS Biol. 5: e92.

Daly, M. J., E. K. Gaidamakova, V. Y. Matrosova, J. G. Kiang, R. Fukumoto *et al.*, 2010 Small-molecule antioxidant proteome-shields in *Deinococcus radiodurans*. PLoS ONE 5: e12570.

Fabrizio, P., F. Pozza, S. D. Pletcher, C. M. Gendron, and V. D. Longo, 2001 Regulation of longevity and stress resistance by Sch9 in yeast. Science 292: 288–290.

Fabrizio, P., S. D. Pletcher, N. Minois, J. W. Vaupel, and V. D. Longo, 2004 Chronological aging-independent replicative life span regulation by Msn2/Msn4 and Sod2 in *Saccharomyces cerevisiae*. FEBS Lett. 557: 136–142.

Fredrickson, J. K., S. M. Li, E. K. Gaidamakova, V. Y. Matrosova, M. Zhai *et al.*, 2008 Protein oxidation: Key to bacterial desiccation resistance? Isme J. 2: 393–403.

Granger, A. C., E. K. Gaidamakova, V. Y. Matrosova, M. J. Daly, and P. Setlow, 2011 Effects of Mn and Fe levels on *Bacillus subtilis* spore resistance and effects of Mn2+, other divalent cations, orthophosphate, and dipicolinic acid on protein resistance to ionizing radiation. Appl. Environ. Microbiol. 77: 32–40.

Gross, L., 2007 Paradox resolved?: the strange case of the radiation-resistant bacteria. PLoS Biol. 5: e108.

Hasan, R., C. Leroy, A. D. Isnard, J. Labarre, E. Boy-Marcotte *et al.*, 2002 The control of the yeast H2O2 response by the Msn2/4 transcription factors. Mol. Microbiol. 45: 233–241.

Jensen, L. T., M. Ajua-Alemanji, and V. C. Culotta, 2003 The *Saccharomyces cerevisiae* high affinity phosphate transporter encoded by PHO84 also functions in manganese homeostasis. J. Biol. Chem. 278: 42036–42040.

Kaneko, Y., A. Toh-E, and Y. Oshima, 1982 Identification of the genetic locus for the structural gene and a new regulatory gene for the synthesis of repressible alkaline phosphatase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 2: 127–137.

Lee, Y. S., S. Mulugu, J. D. York, and E. K. O'shea, 2007 Regulation of a cyclin-CDK-CDK inhibitor complex by inositol pyrophosphates. Science 316: 109–112.

Lin, Y. T., H. Hoang, S. I. Hsieh, N. Rangel, A. L. Foster *et al.*, 2006 Manganous ion supplementation accelerates wild type development, enhances stress resistance, and rescues the life span of a short-lived *Caenorhabditis elegans* mutant. Free Radic. Biol. Med. 40: 1185–1193.

McCord, J. M., and I. Fridovich, 1969 Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244: 6049–6055.

McNaughton, R. L., A. R. Reddi, M. H. Clement, A. Sharma, K. Barnese *et al.*, 2010 Probing in vivo Mn2+ speciation and oxidative stress resistance in yeast cells with electron-nuclear double resonance spectroscopy. Proc. Natl. Acad. Sci. USA 107: 15335–15339.

Medvedik, O., D. W. Lamming, K. D. Kim, and D. A. Sinclair, 2007 MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in *Saccharomyces cerevisiae*. PLoS Biol. 5: e261.

Ogawa, N., J. Derisi, and P. O. Brown, 2000 New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol. Biol. Cell 11: 4309–4321.

Pedruzzi, I., N. Burckert, P. Egger, and C. De Virgilio, 2000 *Saccharomyces cerevisiae* Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1. EMBO J. 19: 2569–2579.

Pedruzzi, I., F. Dubouloz, E. Cameroni, V. Wanke, J. Roosen *et al.*, 2003 TOR and PKA signaling pathways converge on the protein kinase Rim15 to control entry into G0. Mol. Cell 12: 1607–1613.

Reddi, A. R., L. T. Jensen, A. Naranuntarat, L. Rosenfeld, E. Leung *et al.*, 2009 The overlapping roles of manganese and Cu/Zn SOD in oxidative stress protection. Free Radic. Biol. Med. 46: 154–162.

Reinders, A., N. Burckert, T. Boller, A. Wiemken, and C. De Virgilio, 1998 *Saccharomyces cerevisiae* cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase. Genes Dev. 12: 2943–2955.

Roosen, J., K. Engelen, K. Marchal, J. Mathys, G. Griffioen *et al.*, 2005 PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability. Mol. Microbiol. 55: 862–880.

Rosenfeld, L., A. R. Reddi, E. Leung, K. Aranda, L. T. Jensen *et al.*, 2010 The effect of phosphate accumulation on metal ion homeostasis in *Saccharomyces cerevisiae*. J. Biol. Inorg. Chem. 15: 1051–1062.

Sanchez, R. J., C. Srinivasan, W. H. Munroe, M. A. Wallace, J. Martins *et al.*, 2005 Exogenous manganous ion at millimolar levels rescues all known dioxygen-sensitive phenotypes of yeast lacking CuZnSOD. J. Biol. Inorg. Chem. 10: 913–923.

Sherman, F., 1991 Getting started with yeast. Methods Enzymol. 194: 3–21.

Smets, B., R. Ghillebert, P. De Snijder, M. Binda, E. Swinnen *et al.*, 2010 Life in the midst of scarcity: adaptations to nutrient availability in *Saccharomyces cerevisiae*. Curr. Genet. 56: 1–32.

Sobota, J. M., and J. A. Imlay, 2011 Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese. Proc. Natl. Acad. Sci. USA 108: 5402–5407.

Swinnen, E., V. Wanke, J. Roosen, B. Smets, F. Dubouloz *et al.*, 2006 Rim15 and the crossroads of nutrient signalling pathways in *Saccharomyces cerevisiae*. Cell Div. 1: 3.

Szuromi, P., 2010 Manganese sightings. Science 329: 1442.

Tseng, H. J., Y. Srikhanta, A. G. McEwan, and M. P. Jennings, 2001 Accumulation of manganese in *Neisseria gonorrhoeae* correlates with resistance to oxidative killing by superoxide anion and is independent of superoxide dismutase activity. Mol. Microbiol. 40: 1175–1186.

Ukeda, H., S. Maeda, T. Ishii, and M. Sawamura, 1997 Spectrophotometric assay for superoxide dismutase based on tetrazolium salt 3′-1-(phenylamino)-carbonyl-3, 4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate reduction by xanthine-xanthine oxidase. Anal. Biochem. 251: 206–209.

Wallace, M. A., L. L. Liou, J. Martins, M. H. Clement, S. Bailey *et al.*, 2004 Superoxide inhibits 4Fe-4S cluster enzymes involved in amino acid biosynthesis. Cross-compartment protection by CuZn-superoxide dismutase. J. Biol. Chem. 279: 32055–32062.

Wanke, V., I. Pedruzzi, E. Cameroni, F. Dubouloz, and C. De Virgilio, 2005 Regulation of G0 entry by the Pho80-Pho85 cyclin-CDK complex. EMBO J. 24: 4271–4278.

Wei, M., P. Fabrizio, J. Hu, H. Ge, C. Cheng *et al.*, 2008 Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS Genet. 4: e13.

Wykoff, D. D., and E. K. O'shea, 2001 Phosphate transport and sensing in *Saccharomyces cerevisiae*. Genetics 159: 1491–1499.

Wykoff, D. D., A. H. Rizvi, J. M. Raser, B. Margolin, and E. K. O'shea, 2007 Positive feedback regulates switching of phosphate transporters in *S. cerevisiae*. Mol. Cell 27: 1005–1013.

Yang, Z., J. Geng, W. L. Yen, K. Wang, and D. J. Klionsky, 2010 Positive or negative roles of different cyclin-dependent kinase Pho85-cyclin complexes orchestrate induction of autophagy in *Saccharomyces cerevisiae*. Mol. Cell 38: 250–264.

Zhang, N., J. Wu, and S. G. Oliver, 2009 Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during transition into stationary phase in yeast. Microbiology 155: 1690–1698.

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Regulation of Manganese Antioxidants by Nutrient Sensing Pathways in *Saccharomyces cerevisiae*

Amit R. Reddi and Valeria C. Culotta

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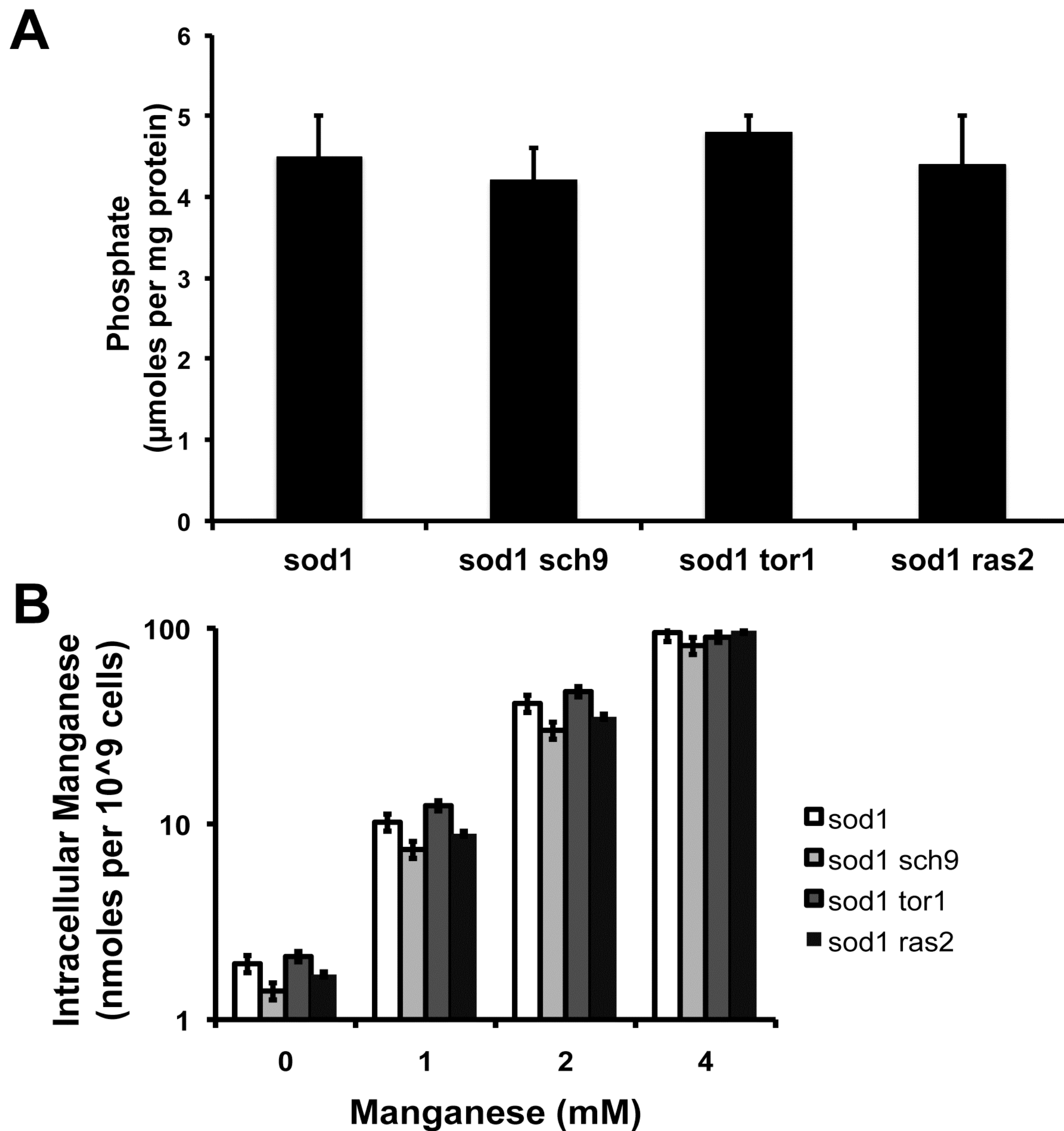

Figure S1 The effect of *sch9*, *tor1*, and *ras2* deletions on phosphate and manganese accumulation in *sod1* cells. **(A, B)** The effect of *sch9*, *tor1*, and *ras2* deletions on the **(A)** phosphate content of *sod1* cells and **(B)** Mn content of *sod1* cells,tested precisely as described in Figure 2B and 1D, respectively. Details on culture conditions and growth are described in the Materials and Methods. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: *sod1*Δ, AR203; *sod1*Δ *sch9*Δ, AR163; *sch9*Δ, AR164. *sod1*Δ *tor1*Δ, AR120; *sod1*Δ *ras2*Δ, AR158.

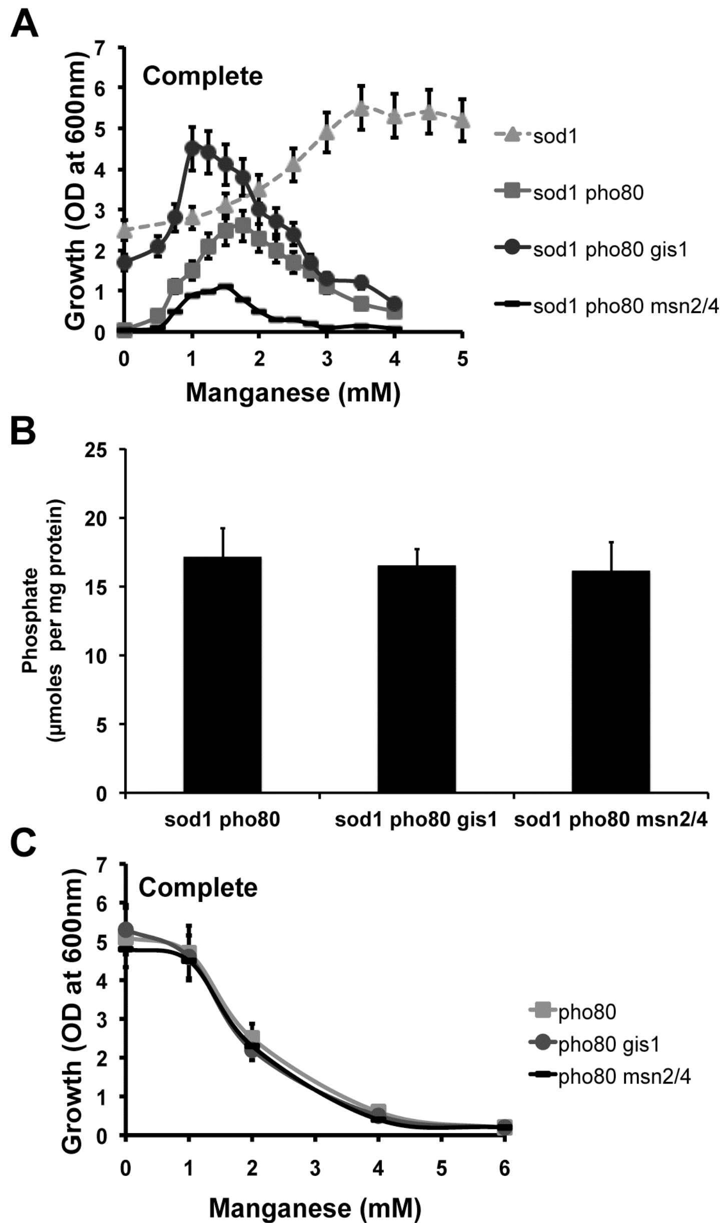

Figure S2 The effect of *gis1* and *msn2/4* mutations on aerobic growth, phosphate accumulation, and manganese toxicity in *sod1 pho80* cells. **(A-C))** The effect of *gis1 and msn2/4* mutations on **(A)** aerobic growth as a function of Mn supplementation to SC media in *sod1*Δ *pho80*Δ cells, **(B)** phosphate content of *sod1*Δ *pho80*Δ cells and **(C)** Mn-toxicity of *pho80*Δ mutants grown in complete media. Experiments depicted in Figure S2 were performed precisely as described in Figures 1A, 2B, and 1C, respectively. Details on culture conditions and growth and analytical techniques are described in the Materials and Methods. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: *sod1*Δ, AR203; *sod1*Δ *pho80*Δ, LR156; *sod1*Δ *pho80*Δ *gis1*Δ*,* AR138; *sod1*Δ *pho80*Δ *msn2/4*Δ, AR300; *pho80*Δ, LR237; *pho80*Δ *gis1*Δ, AR123; *pho80*Δ *msn2/4*Δ, AR156.

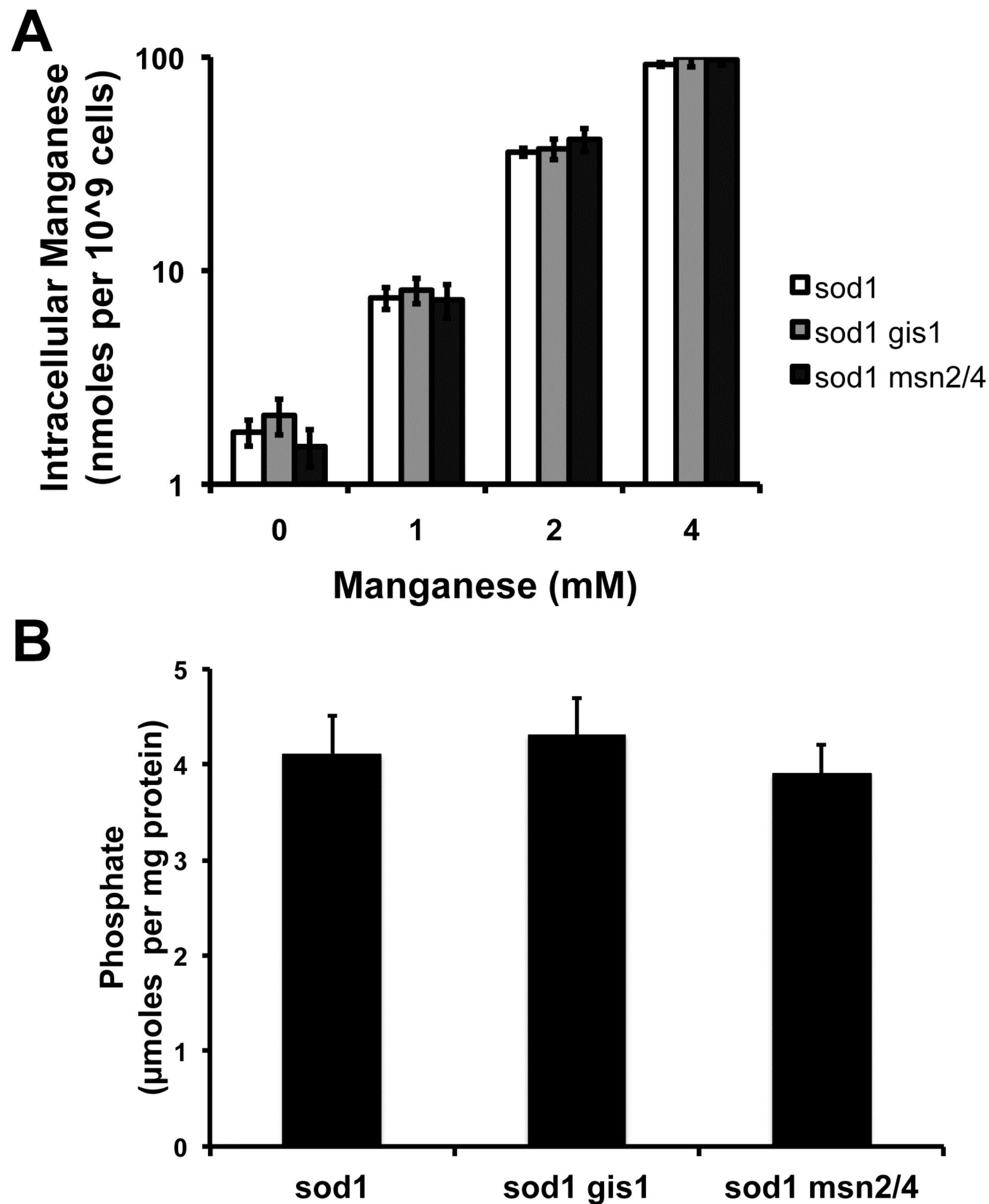

Figure S3 The effect of *gis1 and msn2/4* mutations on manganese and phosphate accumulation in *sod1* cells.
(A,B) The effects of *gis1 and msn2/4* mutations on **(A)** manganese uptake as a function of manganese supplementation to SC media in *sod1*Δ cells and **(B)** phosphate content of *sod1*Δ cells. Experiments depicted in Figure S3 were performed precisely as described in Figures 1D and 2B, respectively. Details on culture conditions and growth and analytical techniques are described in the Materials and Methods. All reported values are averages based on triplicate cultures, with the errors representing the standard deviations. Strains employed: *sod1*Δ, AR203; *sod1*Δ *gis1*Δ, AR121; *sod1*Δ *msn2/4*Δ, AR155.

File S1

List of yeast strains and plasmids used in this study, including descriptions on their source and/or construction.

Strains and plasmids

All yeast strains for this study were derived from BY4741 (MATa*, leu2Δ0, met15Δ0, ura3Δ0, his3Δ1*) and include commercially available (Open Biosystems) *kanMX4* deletion derivatives of *sod1*, *sod2, pho80*, *rim15*, *gis1*, *msn2*, *msn4*, *tor1*, *ras2*, *pho4*, and *pho84*, which were all verified by colony purification and gene sequencing. We also utilized the previously reported strains LR156 (*sod1Δ::LEU2 pho80Δ::kanMX4*), RS001 (*sod1Δ::LEU2 pho84Δ::HIS3*) LR191 (*pho4Δ::LEU2 pho80Δ::kanMX4*), LR154 (*pho84Δ::HIS3 pho80Δ::kanMX4*), LR237 (*pho80Δ::LEU2*), LR122 (*pho84Δ::LEU2*), and LR181 (*pho4Δ::LEU2*) (Reddi *et al.* 2009; Rosenfeld *et al.* 2010). AR108 (*sod1Δ::LEU2 rim15Δ::kanMX4*), AR120 (*sod1Δ::LEU2 tor1Δ::kanMX4*), AR121 (*sod1Δ::LEU2 gis1Δ::kanMX4*), AR140 (*sod1Δ::LEU2 msn2Δ::kanMX4*), AR141 (*sod1Δ::LEU2 msn4Δ::kanMX4*), AR158 (*sod1Δ::LEU2 ras2Δ::kanMX4*), and AR203 (*sod1Δ::LEU2*) were obtained by deleting *SOD1* from the *rim15Δ::kanMX4*, *tor1Δ::kanMX4*, *gis1Δ::kanMX4*, *msn2Δ::kanMX4*, *msn4Δ::kanMX4*, *ras2Δ::kanMX4*, and BY4741 parent strains, respectively, as described previously (Culotta *et al.* 1995). LR178 (*sod1Δ::LEU2 pho84Δ::HIS3 pho80Δ::kanMX4*) was kindly provided by Dr. Leah Rosenfeld and was generated by deleting *SOD1* from LR154 as described previously (Culotta *et al.* 1995). AR142 (*sod1Δ::kanMX4 sod2Δ::URA3*) and AR161 (*sod1Δ::LEU2 sod2Δ::URA3 gis1::KanMX4*) were generated by deleting *SOD2* in the *sod1::KanMX4* and AR121 strains, respectively, as described previously (Culotta *et al.* 1995). AR151 (*msn2Δ::HIS3 msn4Δ::kanMX4*) and AR155 (*sod1::LEU2 msn2::HIS3 msn4::kanMX4*) were generated by deleting *MSN2* using the *msn2::HIS3* deletion plasmid, pAR007. AR160 (*sod1Δ::LEU2 sod2Δ::URA3 msn2::HIS3 msn4::KanMX4*) was generated by deleting *SOD2* in AR155, as described previously (Culotta *et al.* 1995). AR103 (*pho80Δ::LEU2 rim15Δ::kanMX4*), AR123 (*pho80Δ::LEU2 gis1Δ::kanMX4*), and AR156 (*pho80Δ::LEU2 msn2::HIS3 msn4Δ::kanMX4*) were generated by deleting *PHO80* in the *rim15Δ::kanMX4*, *gis1Δ::kanMX4*, and AR151 strains, respectively, as described previously (Rosenfeld *et al.* 2010). AR107 (*pho80Δ::URA3 rim15Δ::kanMX4*), AR134 (*pho80Δ::URA3 gis1Δ::kanMX4*), and AR299 (*pho80Δ::URA3 msn2::HIS3 msn4Δ::kanMX4*) were generated by allelic replacement of the *LEU2* selectable markers of AR103, AR123, and AR156, respectively, with *URA3* by transformation with pRS306 linearized with PvuII. AR110 (*sod1::LEU2 pho80Δ::URA3 rim15Δ::kanMX4*), AR138 (*sod1::LEU2 pho80Δ::URA3 gis1Δ::kanMX4*), and AR300 (*sod1::LEU2 pho80Δ::URA3 msn2::HIS3 msn4Δ::kanMX4*) were constructed by deleting *SOD1* in AR107, AR134, and AR299, respectively, as described previously (Culotta *et al.* 1995). AR010 (*sod1Δ::URA3 pho80Δ::kanMX4*) was generated by allelic replacement of the *LEU2* selectable marker of LR156 with *URA3* by transformation with pRS306 (Sikorski and Hieter 1989) linearized with PvuII. AR105 (*sod1Δ::kanMX4 pho4::LEU2*) and AR106 (*sod1Δ::URA3 pho80Δ::kanMX4 pho4::LEU2*) were constructed by deleting *PHO4* in the *sod1Δ::kanMX4* and AR010 strains, respectively, as described previously (Rosenfeld *et al.* 2010). AR164 (*sch9::HIS3*) was constructed by deleting *SCH9* with pAR009 in BY4741 cells. AR163 (*sod1::LEU2 sch9::HIS3*) was constructed by deleting *SOD1* in AR163, as described previously (Culotta *et al.* 1995).

The *msn2::HIS3* disruption plasmid, pAR007, was generated by first PCR amplifying the upstream (−743 to +27) and downstream (+2264 to +3037) sequences relative to the *MSN2* start site, introducing BamHI and Xho1, and Xba1 and BamHI resctriction sites, respectively. The *MSN2* PCR products were digested with the enzymes indicated and ligated in a trimolecular reaction into the *HIS3* integrating plasmid pRS403 (Sikorski and Hieter 1989) digested with Xba1 and Xho1, resulting in pAR007.

Transformation of yeast strains with pAR007 linearized with BamHI resulted in deletion of *MSN2* sequences from +28 to +2263. The *sch9::HIS3* disruption plasmid, pAR009, was generated by PCR amplifying the upstream (−467 to -138) and downstream (+2485 to +3117) sequences relative to the *SCH9* start site, introducing BamHI and Xho1, and Xba1 and BamHI restriction sites, respectively. The *SCH9* PCR products were digested with the enzymes indicated and ligated in a trimolecular reaction into the *HIS3* integrating plasmid pRS403 (Sikorski and Hieter 1989) digested with Xba1 and Xho1, resulting in pAR009. Transformation of yeast strains with pAR009 linearized with BamHI resulted in deletion of *SCH9* sequences from -137 to +2484. Deletion of *MSN2* or *SCH9* was confirmed by PCR.

Culotta, V. C., H. D. Joh, S. J. Lin, K. H. Slekar and J. Strain, 1995 A physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. J. Biol. Chem. 270**:** 29991-29997.

Reddi, A. R., L. T. Jensen, A. Naranuntarat, L. Rosenfeld, E. Leung *et al.*, 2009 The overlapping roles of manganese and Cu/Zn SOD in oxidative stress protection. Free Radic Biol Med 46**:** 154-162.

Rosenfeld, L., A. R. Reddi, E. Leung, K. Aranda, L. T. Jensen *et al.*, 2010 The effect of phosphate accumulation on metal ion homeostasis in Saccharomyces cerevisiae. J Biol Inorg Chem 15**:** 1051-1062.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces ceresiviae*. Genetics 122**:** 19-27.