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Phosphate disruption and metal toxicity in *Saccharomyces cerevisiae*: Effects of RAD23 and the histone chaperone HPC2

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Abstract

In cells, there exists a delicate balance between accumulation of charged metal cations and abundant anionic complexes such as phosphate. When phosphate metabolism is disrupted, cell-wide spread disturbances in metal homeostasis may ensue. The best example is a yeast *pho80* mutant that hyperaccumulates phosphate and as result, also hyperaccumulates metal cations from the environment and shows exquisite sensitive to toxicity from metals such as manganese. In this study, we sought to identify genes that when over-expressed would suppress the manganese toxicity of *pho80* mutants. Two classes of suppressors were isolated, including the histone chaperones *SPT16* and *HPC2*, and *RAD23*, a well-conserved protein involved in DNA repair and proteosomal degradation. The histone chaperone gene *HPC2* reversed the elevated manganese and phosphate of *pho80* mutants by specifically repressing *PHO84*, encoding a metal-phosphate transporter. *RAD23* also reduced manganese toxicity by lowering manganese levels, but *RAD23* did not alter phosphate nor repress *PHO84*. We observed that the *RAD23*-reversal of manganese toxicity reflected its role in protein quality control, not DNA repair. Our studies are consistent with a model in which Rad23p partners with the deglycosylating enzyme Png1p to reduce manganese toxicity through proteosomal degradation of glycosylated substrate(s).

Keywords

Manganese; Phosphate; Yeasts; Metals

1. Introduction

Phosphate is regulated in yeast cells through the Pho85p–Pho80p cyclin dependent kinase–cyclin complex [1]. When phosphate is abundant, this complex phosphorylates the Pho4p transcription factor and prevents the factor from activating phosphate metabolism genes. Conversely, when phosphate is limiting, IP_7 inositol pyrophosphate inhibits the Pho85p–Pho80p kinase complex, and the dephosphorylated Pho4p factor is free to enter the nucleus and activate the transcription of a wide assortment of genes that mediate the uptake, storage and metabolism of phosphate (summarized in cartoon of Fig. 4) [2–5].

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One of many targets of Pho4p regulation is *PHO84* which encodes the cell surface high affinity transporter for phosphate [4–7]. Compared to other gene targets of Pho4p, *PHO84* exhibits a lower threshold for induction and is rapidly activated with relatively small changes in intracellular phosphate [8]. Interestingly the substrate for the Pho84p phosphate transporter is a phosphate–divalent metal ion complex, such as manganese–phosphate [9,10]. In this manner, Pho84p not only controls cellular phosphate but can also influence the uptake and toxicity of divalent metals. We have previously shown that cells lacking *PHO84* are resistant to toxicity from manganese, zinc and cobalt [10]. Moreover, activation of *PHO84* gene expression, through a mutation in the aforementioned Pho80–Pho85 kinase, leads to elevated uptake of manganese from the environment [11]. However, this elevated manganese uptake by Pho84p was by itself not sufficient to explain all the manganese toxicity in *pho80* (or *pho85*) mutants. There are other targets of the Pho4p transcription factor that cause increased sensitivity to metals when phosphate regulation is lost [11]. The nature of this Pho84-independent manganese toxicity is not understood.

In an attempt to better understand the changes in metal sensitivity associated with loss of phosphate control, we conducted a dosage suppressor screen for genes that alleviate the manganese sensitivity of a *pho80Δ* mutant. This screen led to the identification of two histone chaperones *HPC2* and *SPT16*, as well as *RAD23*, involved in nucleotide excision repair and proteosome function. The histone chaperones were found to suppress metal toxicity by repressing transcription of the metal-phosphate transporter gene, *PHO84*. By comparison *RAD23* reversed manganese toxicity without changes in *PHO84* gene activity. Our mechanistic studies implicate a role for *RAD23* in proteosomal degradation of a certain glycosylated protein that controls manganese accumulation and manganese toxicity.

2. Materials and methods

2.1. Strains and growth conditions

All the strains in this study are isogenic to BY4741 (*Mata*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *his3Δ1*). Commercially available deletion strains described throughout (Open Biosystems) were verified using DNA sequencing. *PHO84* was disrupted in BY4741 and in the *pho80Δ::KanMX4* strain to create LR122 (*pho84Δ::LEU2*) and LR154 (*pho80Δ::KanMX4 pho84Δ::HIS3*) using the previously published pLJ246 [10] and pLJ089 [12] *pho84* deletion plasmids. LR237 (*pho80Δ::LEU2*), LR359 (*rpn10Δ::KanMX4 pho80Δ::LEU2*) and LR329 (*png1Δ::KanMX4 pho80Δ::LEU2*) were constructed using the pLR001 deletion plasmid [11] in wild type BY4741, or the respective isogenic single gene deletion strains from the Open Biosystems collection. *RAD4* was disrupted in BY4741 using the LR01 deletion plasmid (described below) in *pho80Δ::KanMX4* to generate the LR222 (*pho80Δ::KanMX4 rad4Δ::LEU2*) strain.

Cells were maintained by growth at 30 °C in either enriched YPD (1% bacto-yeast extract, 2% bacto-peptone, 2% dextrose), or SC (synthetic complete) media with 2% agarose added for solid media [13]. For metal toxicity tests, 10^5, 10^4, 5×10^2 and ~25 cells were spotted onto solid YPD medium supplemented with defined metal concentrations and allowed to grow at 30 °C for 48 h.

2.2. Plasmids and genomic library screening

To isolate multi-copy suppressors of *pho80* manganese toxicity, the LR801 *pho80Δ::KanMX4* strain was transformed with a genomic *URA3/2μ* library derived from pRS202 [14]. Approximately 100,000 colonies were tested for growth on SC medium lacking uracil and supplemented with 40 mM $MnCl_2$. Plasmids conferring manganese resistance were isolated and sequenced, and through a series of sub-cloning experiments, the

manganese-resistance loci identified. In total, the complementing *PHO80* gene was isolated four times, *MNR2* and *RAD23* isolated four and five times respectively; *HPC2* was isolated seven times and a single isolate of *SPT16* was obtained. All subsequent analyses of *SPT16* involved the original library isolate pS3 that represents a Sau3A/Sau3A genomic DNA fragment from chromosome VIII sequences 98003 to 104554. The p3LΔEcoRI plasmid for expressing *HPC2* (spanning chromosome II sequences 653413–657032) is a derivative of the original genomic library clone in which an EcoRI/EcoRI fragment outside of the *HPC2* containing region was deleted. The p1FΔKpnI plasmid for expressing *RAD23* was derived from the original genomic library clone by deleting a Kpn1/KpnI fragment outside of the *HPC2* containing region. The *RAD4* disruption plasmid, LR01, was generated by PCR amplification of upstream (−610 to −98) and downstream (2665–3465) sequences introducing BamHI and NotI or PstI and BamHI sites, respectively. The *RAD4* PCR products were digested with the indicated enzymes and ligated in a trimolecular reaction into pRS305 (*LEU2*) [15] digested with SalI and NotI. Transformation with pLR01 digested with BamHI resulted in the deletion of chromosomal *RAD4* from to −98 to +2665.

2.3. Biochemical assays

For measurements of phosphate and for atomic absorption spectroscopy (AAS) analysis of manganese, cells were seeded at an OD_{600} = 0.05 in YPD medium and grown for 18 h at 30 °C. In the case of metals analysis, the growth media was supplemented with either 70 μM (*pho80Δ* single mutants) or 400 μM (*pho80Δ pho84Δ* double mutants) $MnCl_2$ to monitor metal accumulation under manganese toxicity conditions. The *pho80Δ pho84Δ* is far more resistant to manganese than the single *pho80Δ* mutant, hence the need for higher levels of the metal. Whole cell manganese analysis by AAS was carried out on a PerkinElmer Life Sciences AAnalyst 600 graphite furnace atomic absorption spectrometer according to the manufacturer's specifications, as described [10]. Inorganic phosphate was assayed using molybdate reactivity as described [12]. Polyphosphate was detected using polyacrylamide gel electrophoresis and toluidine blue staining as described [12].

For quantitative PCR, triplicate cultures of WT and *pho80* mutants were grown in YPD + 75 μM $MnCl_2$ to an OD_{600} = 1.0. Manganese was added to cultures to maintain selective advantage of 2μ plasmids in *pho80Δ* cells. Cells were harvested, RNA extracted using the hot phenol method [16] and analysis of *PHO84* expression by quantitative PCR carried out precisely as previously done using *ACT1* as control [11].

3. Results and discussion

3.1. Isolation of manganese resistant suppressor genes

We screened a multi-copy yeast genomic library for genes that would reverse the manganese sensitivity of *pho80Δ* mutants. This screen resulted in the identification of five genes that when overexpressed, permitted growth of *pho80Δ* mutants on high manganese. These genes included the complementing *PHO80* locus, the vacuolar magnesiumand cobalt transporter *MNR2* that also confers resistance to manganese [17], the DNA repair and ubiquitin receptor protein *RAD23* [18], and two genes involved in chromatin remodeling, namely *SPT16* and *HPC2*. Roles for *SPT16, HPC2* and *RAD23* in modulating manganese toxicity had not been previously documented and these genes were selected for further study.

3.2. The chromatin remodeling factors and metal toxicity in pho80 mutants

Spt16p and Hpc2p are among a class of proteins known as histone chaperones that facilitate the assembly and disassembly of nucleosomes. Spt16p is a component of the FACT nucleosome remodeling complex that acts on H2A/H2B histones [19], while Hpc2p is part of the HIR complex that operates on histones H3/H4 [20]. As seen in Fig. 1A, over-

expression of *HPC2* and *SPT16* not only reduced manganese toxicity in *pho80Δ* mutants, but also suppressed toxicity from cobalt and copper. The effects of *HPC2* and *SPT16* were not limited to metals. Mutants of *pho80Δ* are known to hyperaccumulate phosphate [6,12] and over-expression of either *HPC2* or *SPT16* helped reduce levels of orthophosphate (Fig. 1B) and polyphosphate (Fig. 1C) in the *pho80Δ* mutant. In all these assays, the effects of over-expressed *HPC2* were more pronounced than that of *SPT16* and we therefore focused on *HPC2* for further analysis.

We tested whether the *HPC2*-resistance to manganese was unique to cells with disrupted phosphate control or was independent of phosphate. As seen in Fig. 2A, over-expression of *HPC2* did not increase manganese resistance in a wild type strain, nor in a *pmr1* mutant that is highly sensitive to manganese toxicity without changes in phosphate [21,22]. By comparison, *HPC2* did increase manganese resistance of a *pho85* mutant that like *pho80* strains, cannot control phosphate uptake (Fig. 2A bottom) [12,22]. Hence, the effects of *HPC2* on manganese are specific to strains with disruptions in phosphate metabolism.

Manganese toxicity of *pho80* mutants correlates with increased uptake of manganese from the growth medium [11]. As seen in Fig. 2B, *pho80* mutants accumulate ≈4-fold higher levels of manganese than WT cells exposed to manganese. Over-expression of *HPC2* substantially reversed this high accumulation of manganese (Fig. 2B), indicating that *HPC2* reverses manganese toxicity by inhibiting manganese uptake. We tested whether *HPC2* was working through *PHO84*, encoding the metal-phosphate transporter [10,11]. By quantitative PCR, *PHO84* mRNA levels increased more than 2-fold in *pho80Δ* mutants (Fig. 2C). Notably, HPC2 overexpression decreased *PHO84* mRNA to near-control levels. To determine if this lowering of *PHO84* mRNA was by itself responsible for *HPC2* suppression of manganese toxicity, we created a *pho80 pho84* double mutant. As seen in Fig. 2D, over-expressed *HPC2* could not suppress manganese toxicity in this strain lacking Pho94p (Fig. 2D). Together these results indicate that the *HPC2* is reversing metal toxicity of *pho80* mutants specifically by repressing transcription of the *PHO84* metal-phosphate transporter, presumably through chromatin remodeling effects.

Activation of Pho4-target genes in yeast is known to involve chromatin remodeling. As a histone chaperone, Hpc2p could conceivably repress (or activate) the chromatin of any number of Pho4-targets, but the studies of Fig. 2D demonstrate that repression of *PHO84* is the only target relevant to the manganese resistance observed. Compared to other Pho4-target genes, the chromatin of *PHO84* in particular has a particularly low threshold for activation by low phosphate [23]. Rapid activation of *PHO84* involves histone eviction through the remodelers Snf2 and Ino80 and the histone acetyltransferase Gcn5 [23] as well as the binding of topoisomerase II to the gene promoter [24]. However, nothing has been reported for factors that mediate repression of *PHO84* chromatin when phosphate is abundant, other than the histone deactylases Hda1p and Hda2p [25]. Based on our findings here with Hpc2p, the H3/H4 histone chaperones of the HIR complex are likely candidates for repressing *PHO84*.

It is noteworthy that the histone chaperone, Spt16 is also a multicopy suppressor of *pho80* metal toxicity and phosphate accumulation (Fig. 1). Spt16 is a member of the FACT complex that is best known for its role in histone eviction during elongation, but can also re-assemble nucleosomes after the wake of RNA polymerase II [19]. Based on a synthetic lethality of *spt16* and *hpc2* mutations, it was proposed that Spt16 and Hpc2 play parallel roles in nucleosome deposition [26]. Like Hpc2p, Spt16p may facilitate repression of *PHO84* chromatin when phosphate is abundant.

3.3. The effects of RAD23 on manganese toxicity in pho80 mutants

Manganese toxicity in *pho80Δ* mutants is also reversed by overexpression of *RAD23*. As with *HPC2*, the suppression of manganese toxicity by *RAD23* was specific to cells with disrupted phosphate control (Fig. 2A) and correlated with a reduction in cellular manganese levels (Fig. 2B). However, unlike the histone chaperones *HPC2* and *SPT16*, *RAD23* seemed relatively specific to manganese. *RAD23* afforded no increased resistance to other metals including copper and cobalt (Fig. 1A) and did not significantly alter phosphate levels (Fig. 1B, C). These results would suggest that *RAD23* does not work to repress the metal-phosphate transporter Pho84p. Consistent with this, over-expression of *RAD23* did not reduce mRNA levels of the *PHO84* gene (Fig. 2B). Moreover, *RAD23* still lowered manganese accumulation in a strain lacking *pho84* (Fig. 3A). *RAD23* works independent of Pho84p to reverse manganese toxicity.

Rad23p functions in both nucleotide excision DNA repair and the proteasomal pathway for protein degradation [18]. We sought to determine which of these roles was responsible for the *RAD23*-reversal of manganese toxicity. When DNA is damaged, Rad23p helps to repair the lesion through a complex with Rad4p, the yeast homolog of human Xeroderma pigmentosum group C complementing protein (XPC) [18]. To determine whether this Rad4-dependent DNA repair pathway was relevant to suppression of manganese toxicity, we created a *pho80 rad4* double mutant. As seen in Fig. 3B, *RAD23* was still capable of suppressing manganese toxicity in the absence of Rad4p. To test whether the proteosomal function of *RAD23* was involved, we introduced a mutation in *RPN10* encoding a component of the 19S proteasomal lid [27]. As seen in Fig. 3C, loss of *RPN10* inhibited the ability of *RAD23* to reverse manganese toxicity. Hence *RAD23* attenuates the manganese toxicity of *pho80* mutants through a mechanism that involves its role in the ubiquitin-proteosome system, not DNA repair.

Rad23p functions as an adaptor protein to transport ubiquitinated misfolded proteins to the proteosome for degradation [18]. Rad23p has numerous substrates, many of which are products of ERAD or endoplasmic reticulum (ER)-associated degradation. One such class of Rad23 substrates are *N*-glycosylated proteins that were misfolded in the ER. Rad23p recognizes these substrates through a partnership with Png1p, the primary deglycosylating enzyme in the cytosol [28,29]. We observed that *RAD23* is not capable of reversing manganese toxicity in a *pho80* strain that also lacks Png1p (Fig. 3D). These studies strongly indicate that *RAD23* is reversing manganese toxicity by mediating the proteosomal degradation of a glycosylated protein in the secretory pathway.

What is the target of *RAD23* that affects manganese toxicity? Because *RAD23* over-expression reduces the accumulation of cellular manganese (Figs. 2B and 3A), a membrane transporter for manganese is likely. Moreover, this transporter should be regulated by phosphate and the Pho80/Pho85 pathway because *RAD23* only reverses manganese toxicity in strains that have lost phosphate control (Fig. 2A). To date, Pho84p is the only manganese transporter known to be regulated by phosphate in yeast. We previously published that loss of *pho80* increases manganese toxicity through two mechanisms: one resulting from increased manganese–phosphate uptake by Pho84p and a second that is independent of Pho84p [11]. Although the identity of this Pho84p-independent manganese transport system is still unknown, our studies indicate that this secondary pathway for manganese is a target for quality control of glycosylated proteins through Rad23p.

3.4. Overview

These findings underscore the complex ways in which loss of phosphate control can impact on manganese uptake in a eukaryotic cell. On one hand, activation of the metal-phosphate

transporter Pho84p results in the uncontrolled uptake of toxic levels of manganese from the growth medium. Epigenetic effects on *PHO84* must be a major contribution to the metal toxicity because the repression of *PHO84* by the histone chaperone Hpc2p was in itself sufficient to restore much of the manganese resistance to *pho80Δ* cells (Fig. 4). Yet *PHO84* is not the entire story: loss of phosphate control also activates a secondary pathway for manganese uptake that is still of unknown nature [11]. We provide evidence here that this secondary pathway represents a phosphate regulated manganese transport system that is subject to quality control by *RAD23* proteosomal degradation (Fig. 4).

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Abbreviations

YPD	yeast extract peptone dextrose medium
SC	synthetic complete medium
AAS	atomic absorption spectrometry
PCR	polymerase chain reaction
ER	endoplasmic reticulum

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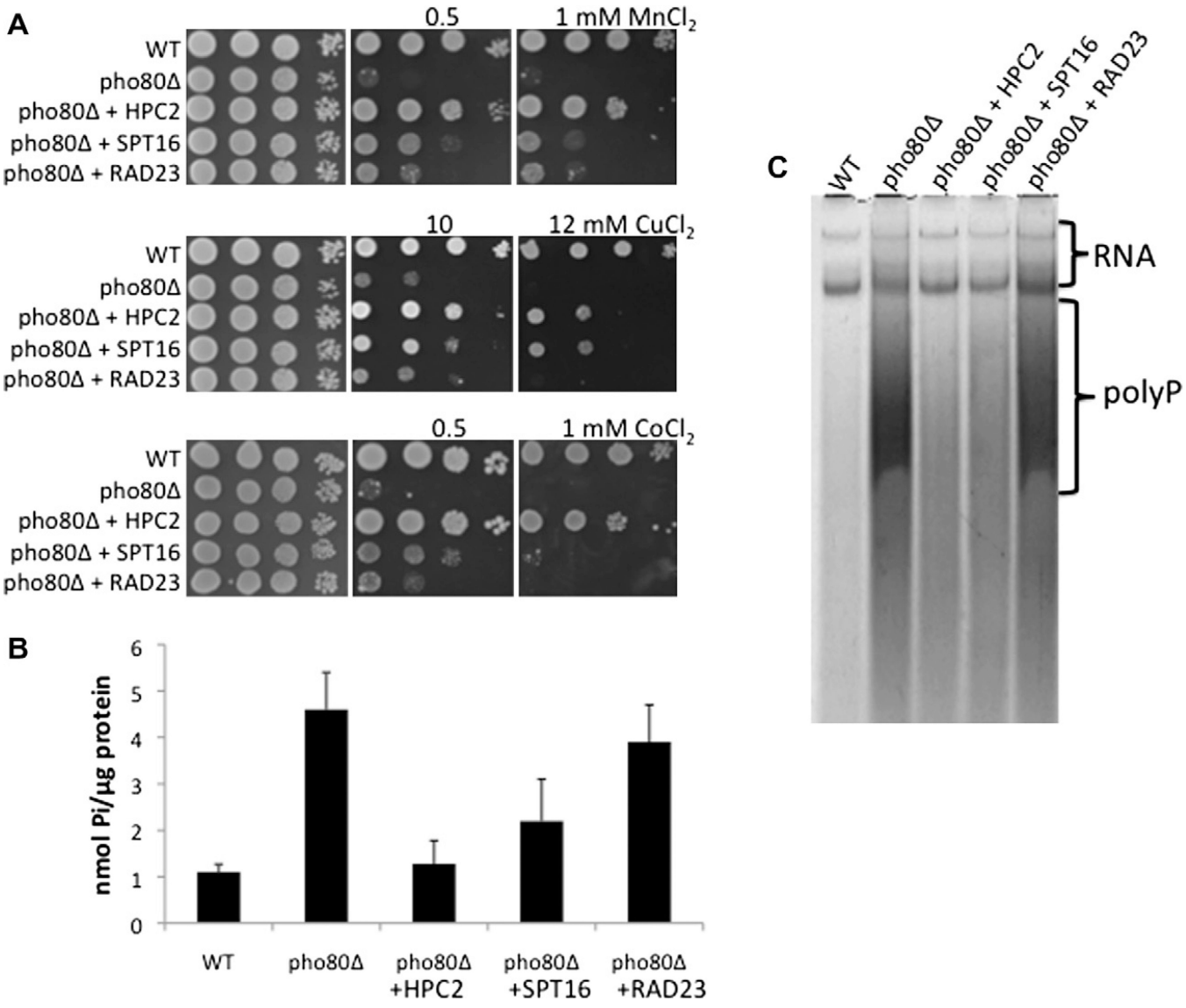

Fig. 1.

Effects of over-expressing *HPC2*, *SPT15* and *RAD23* on metal toxicity and phosphate accumulation of *pho80Δ* mutants. (A) Metal toxicity was measured by spotting 1×10^5, 1×10^4, 5×10^2 and 25 cells of the indicated strains onto YPD medium supplemented with the designated concentrations of metal salts and by allowing growth for 2 days at 30 °C. (B) Whole cell orthophosphate analysis of cell lysates was carried out using the molybdate detection method. Values represent the averages of two independent cultures from independent experimental trials; error bars represent standard deviation. (C) Polyphosphate analysis of cells was conducted by polyacrylamide gel electrophoresis and toluidine blue staining as described in Section 2. The positions of RNA and large polyphosphate molecules are shown. Strains used: WT, BY4741; *pho80Δ*, LR801. Multi-copy yeast plasmids harboring genomic copies of the designated genes were as follows: HPC2, p3LΔEcoRI; SPT16, pS3; RAD23, p1FΔKpnI.

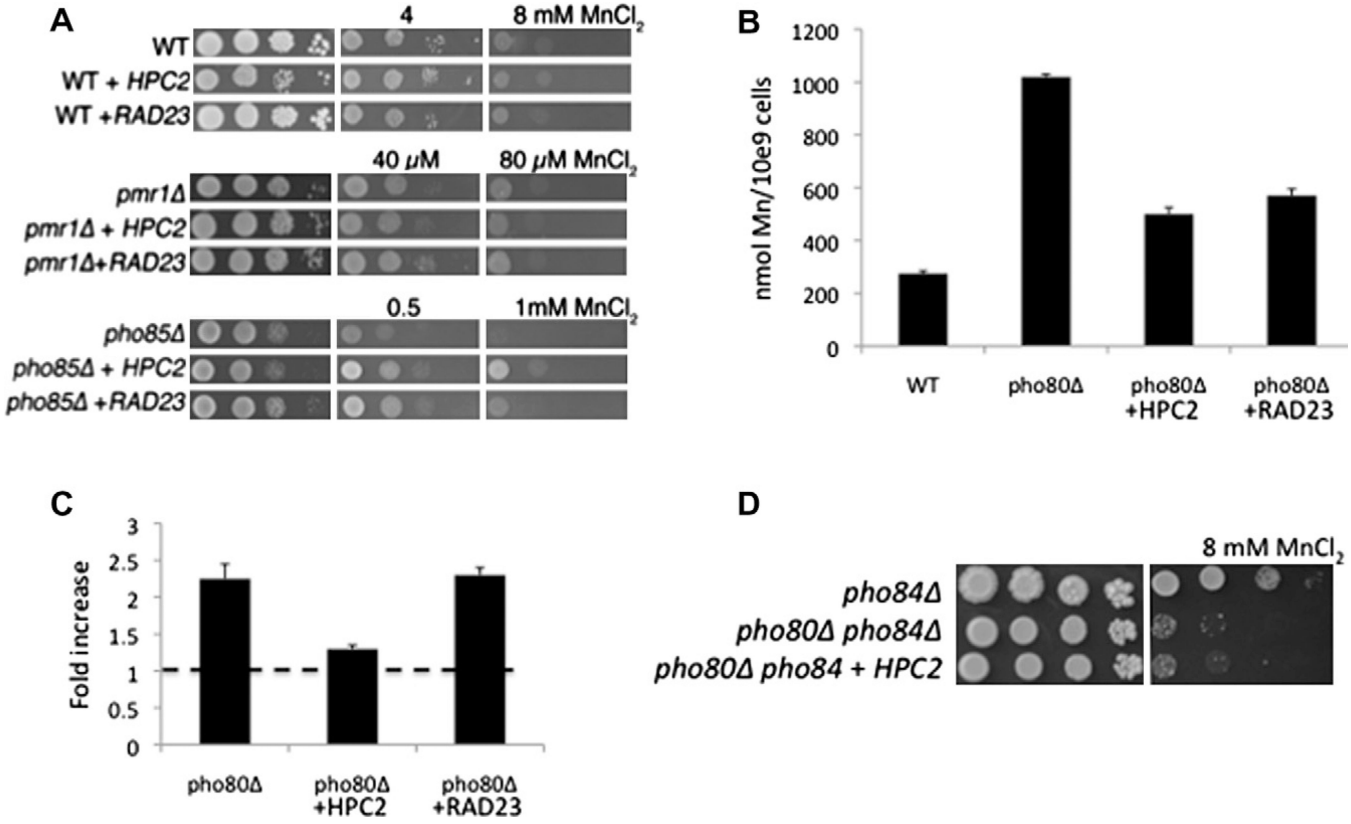

Fig. 2.

HPC2 and *RAD23* lower manganese accumulation in *pho80* mutants by distinct methods. (A) The indicated strains were tested for manganese toxicity as in Fig. 1A. The different levels of manganese salts represent the differing sensitivities of the parental strains to the metal where WT strains are most resistant and *pmr1Δ* mutants are most sensitive to manganese toxicity. (B) The indicated strains were tested for whole cell manganese by AAS as described in Section 2. Values are averages of duplicate measurements from two independent cultures and error bars indicate standard deviation. (C) Real time PCR analysis of *PHO84* mRNA in the indicated strains was carried out as described in Section 2 and previously published [11]. Fold change represents the increase in *PHO84* mRNA over the WT control. Dotted line shows value of 1.0 assigned to WT control. Values are averages of duplicate measurements from three independent cultures; error bars indicate standard deviation. (D) The indicated strains were tested for manganese toxicity as in Fig. 1A. The *pho84Δ* mutation reverses much of the manganese toxicity of a *pho80Δ* strain; hence the higher level of manganese used in toxicity testing. Strains used: (A) WT, BY4741; *pmr1Δ* and *pho85Δ* are *KanMX4* deletion derivatives of BY4741; (B and C) as described in Fig. 1; (D) *pho84Δ*, LR122; *pho80Δ pho84Δ*, LR154. The indicated plasmids for expressing *HPC2* and *RAD23* are as described in Fig. 1.

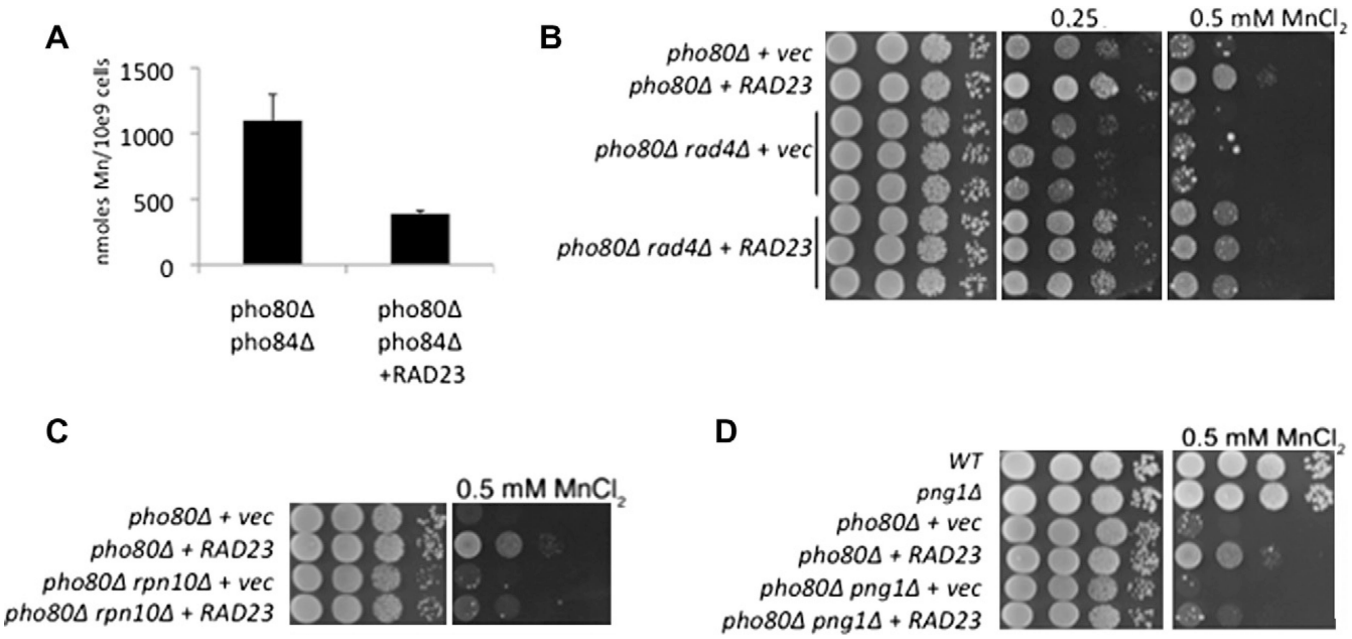

Fig. 3.

RAD23 suppresses manganese toxicity through a mechanism involving the proteosome, not DNA repair. (A) AAS measurements of whole cell manganese in the indicated strains was determined as in Fig. 2B. (B–D) Manganese resistance in the indicated strains was monitored as in Fig. 1A. In part B, results from three independent transformants of the *pho80Δ rad4Δ* mutant are shown. Strains utilized: *pho80Δ pho84Δ*, LR154; *pho80Δ*, LR801; *pho80Δ rad4Δ*, LR222; *pho80Δ rpn10Δ*, LR359; *pho80Δ png1Δ*, LR329. Vec = strains transformed with empty 2μ *URA3* pRS326 plasmid [15]. The *RAD23* expressing plasmid is as described in Fig. 1.

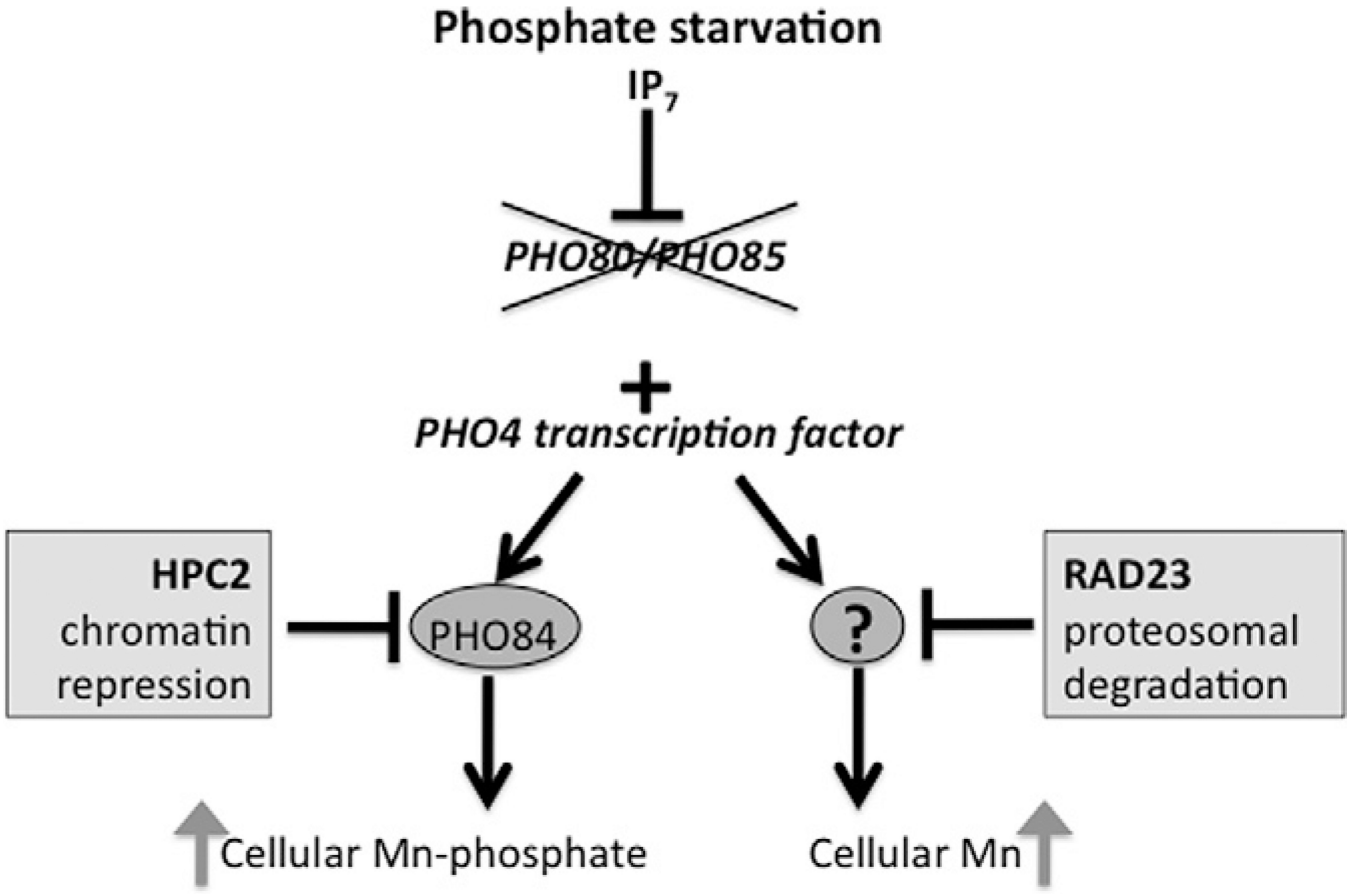

Fig. 4.

The effects of phosphate dysregulation and the *HPC2* and *RAD23* suppressors on manganese accumulation in *S. cerevisiae* Shown is a cartoon depicting the phosphate regulation pathway in *S. cerevisiae*. In phosphate starved cells, IP_7 inositol pyrophosphate inhibits the Pho80p/Pho85p kinase pair which allows for activation of the Pho4p transcription factor [2–5]. Pho4p is also constitutively active in *pho80Δ* or *pho85 Δ* null strains (depicted by X). Pho4p activates expression of the *PHO84* gene encoding the metal-phosphate transporter through chromatin remodeling effects, and as a result, cells hyperaccumulate manganese and phosphate. The Pho84p-increase in manganese toxicity can be reversed by over-expression of the histone chaperone *HPC2* that represses *PHO84* transcription, presumably by nucleosome deposition on the *PHO84* gene promoter. The Pho4p transcription factor also activates a secondary pathway for increasing manganese accumulation and manganese toxicity that is currently of unknown nature (represented by "?") but works independent of *PHO84* and without obvious changes in phosphate [11]. This secondary pathway can be suppressed by over-expression of RAD23 through a pathway involving the proteosomal degradation of glycosylated substrates.