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Article

Cytosolic Monothiol Glutaredoxins Function in Intracellular Iron Sensing and Trafficking via Their Bound Iron-Sulfur Cluster

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SUMMARY

Iron is an essential nutrient for cells. It is unknown how iron, after its import into the cytosol, is specifically delivered to iron-dependent processes in various cellular compartments. Here, we identify an essential function of the conserved cytosolic monothiol glutaredoxins Grx3 and Grx4 in intracellular iron trafficking and sensing. Depletion of Grx3/4 specifically impaired all iron-requiring reactions in the cytosol, mitochondria, and nucleus, including the synthesis of Fe/S clusters, heme, and di-iron centers. These defects were caused by impairment of iron insertion into proteins and iron transfer to mitochondria, indicating that intracellular iron is not bioavailable, despite highly elevated cytosolic levels. The crucial task of Grx3/4 is mediated by a bridging, glutathione-containing Fe/S center that functions both as an iron sensor and in intracellular iron delivery. Collectively, our study uncovers an important role of monothiol glutaredoxins in cellular iron metabolism, with a surprising connection to cellular redox and sulfur metabolisms.

INTRODUCTION

Iron is essential for virtually all organisms, because it functions as a cofactor in central cellular processes such as respiration, DNA synthesis and repair, ribosome biogenesis, and metabolism. Research over the past decade has uncovered sophisticated systems facilitating the specific transport of iron across the plasma and various intracellular membranes (Hentze et al., 2004; Kaplan and Kaplan, 2009; Philpott and Protchenko, 2008; Vergara and Thiele, 2008). Despite its central metabolic function, little is known about the passage of iron through the eukaryotic cytosol to become incorporated into proteins and transported the discovery of this "labile iron pool," its physiological importance and composition have been under debate (Crichton and Charloteaux-Wauters, 1987; Richardson and Ponka, 1997). Presumably, iron may also be bound to dedicated proteins ensuring specific delivery and insertion into iron-requiring sites. A metallo-chaperone function has been worked out for insertion of copper and nickel into respective metal-containing enzymes (Finney and O'Halloran, 2003; Lyons and Eide, 2007), but proteins performing a general role in iron trafficking or insertion are unknown. An iron donor function has been suggested for frataxin in mitochondrial Fe/S cluster biosynthesis (Bencze et al., 2006; Lill, 2009). In humans, the poly (rC) binding protein 1 (PCBP1) was shown to specifically deliver bound iron to ferritin, the major iron storage protein in higher eukaryotes (Shi et al., 2008). The apparently specific role of the PCBP1 iron chaperone and the fact that both ferritin and PCBP1 are not universally conserved leave open the possibility that other proteins with a general importance for iron trafficking exist. Clearly, the mode of specific iron delivery within the eukaryotic cytosol remains one of the fundamental unresolved problems of iron homeostasis.

Because iron is not only essential but also toxic at higher levels, cells have developed sophisticated systems for ensuring a tightly regulated iron homeostasis (Hentze et al., 2004; Kaplan and Kaplan, 2009). In mammals this process is executed by iron-regulatory proteins in a posttranscriptional fashion, and the yeast *Saccharomyces cerevisiae* uses the iron-sensing transcription factors Aft1 and Aft2. Under iron deprivation, Aft1-Aft2 activate transcription of genes of the iron regulon encoding cell-surface iron transporters and proteins involved in intracellular iron utilization (Kaplan and Kaplan, 2009; Philpott and Protchenko, 2008). Sensing of intracellular iron by Aft1 also requires the regulatory proteins Fra1-Fra2 and the cytosolic-nuclear monothiol glutaredoxins Grx3 and Grx4, which are essential for the nuclear export of Aft1 in response to iron sufficiency (Kumanovics et al., 2008; Ojeda et al., 2006; Pujol-Carrion et al., 2006). The regulatory role of Grx3/4 is functionally conserved in fungi that utilize iron-regulated transcription systems unrelated to those from *S. cerevisiae* (Haas et al., 2008; Kaplan and Kaplan, 2009; Mercier and Labbe, 2009).

glutaredoxin (Grx) domain (Herrero and de la Torre-Ruiz, 2007; Lillig et al., 2008). Although the Grx3/4 subfamily of multidomain monothiol glutaredoxins is conserved in eukaryotes, no universal function has been assigned to this family so far. In contrast to most members of the Grx protein family that catalyze dithiol-disulfide redox reactions, monothiol Grx proteins rarely possess oxidoreductase activity. Instead, after in vitro reconstitution or upon overexpression in *Escherichia coli*, they are capable of binding a bridging [2Fe-2S] cluster utilizing the active-site cysteine residue of the Grx domain and glutathione (GSH) as ligands (Li et al., 2009; Picciocchi et al., 2007). The existence of this unusual Fe/S center under physiological conditions, however, has not been demonstrated, and its functional role has remained unclear.

Here, we have used yeast as a model to define an essential role of Grx3/4 in intracellular iron trafficking. Depletion of Grx3/4 led to functional impairment of virtually all iron-dependent processes, including heme biosynthesis, mitochondrial and cytosolic Fe/S protein biogenesis, and the formation of di-iron centers in mitochondria and the cytosol, eventually leading to the loss of cell viability. We provide evidence for the in vivo binding of a bridging Fe/S cluster to Grx3/4 and we assign a crucial physiological function to this cofactor both in cytosolic iron trafficking and as an iron sensor. Thus, the conserved cytosolic monothiol glutaredoxins use their bound Fe/S cofactor for a general role in intracellular iron trafficking.

RESULTS

Deficiency in Grx3/4 Is Associated with Defects in Iron-Dependent Enzymes

To facilitate the functional analysis of Grx3/4, we constructed a regulatable yeast strain (Gal-GRX4; strain background W303-1A; see Table S1 available online) in which *GRX3* was deleted and *GRX4* was expressed under the control of the glucose-repressible *GAL-L* promoter. Upon Grx4 depletion, Gal-GRX4 cells failed to grow on both fermentable and nonfermentable carbon sources (Figure 1A; see below). Likewise, double deletion of *GRX3-GRX4* was lethal in the W303 strain background, distinguishing these cells from strain BY4742 grx3/4Δ, which shows only severely retarded growth (Figure 1A) (Ojeda et al., 2006). The strong effect of Grx3/4 deficiency on cell viability is not explained by their role in iron regulation, since Aft1 is not essential under iron-replete conditions (Kaplan and Kaplan, 2009). These data and the general conservation of Grx3/4 in eukaryotes suggest that these proteins perform a so far unknown, important function.

Gal-GRX4 cells were used to investigate the immediate consequences of Grx3/4 deficiency. Gal-GRX4 cells were cultivated in minimal medium supplemented with glucose and iron chloride to gradually deplete Grx4 (Figures 1B and 1C). A strong activation of the Aft1-dependent *FET3* gene was observed using a *FET3* promoter-*GFP* fusion as a reporter (Figure 1B) (Ojeda et al., 2006). Surprisingly, the activities of the mitochondrial Fe/S protein aconitase and cytosolic catalase, a heme-containing protein, drastically decreased, despite the presumed sufficient cellular iron supply. These effects resemble those upon depletion of Ssq1 (strain Gal-SSQ1). Grx3/4 deficiency was associated with a severe activity loss of respiratory complexes II (succinate dehydrogenase) and IV (cytochrome oxidase) but was fully complemented by expression of *GRX4* from a plasmid (Figure 1D). Likewise, low activities of both aconitase and respiratory complexes III and IV were observed in BY4742 grx3/4Δ cells (Figure S1A), consistent with our earlier observation of an impaired ^{55}Fe/S cluster incorporation into aconitase (Ojeda et al., 2006). Immunostaining of cell extracts from Grx4-depleted Gal-GRX4 cells and BY4742 grx3/4Δ cells further revealed changes in the levels of several iron-containing proteins, including the aconitase-type Fe/S proteins Aco1 and Leu1, ferrochelatase Hem15, and the core mitochondrial ISC assembly protein Isu1 (Figure 1C). These changes of protein levels correlate with those of the transcriptome of both iron-deprived and ISC machinery–compromised cells (Hausmann et al., 2008; Shakoury-Elizeh et al., 2004). In contrast, other iron-dependent proteins, such as succinate dehydrogenase subunit 2 (Sdh2) and the ubiquinone biosynthesis enzyme Coq7, were hardly changed and behaved similarly to the noniron proteins mitochondrial cytochrome oxidase subunit 4 (Cox4) and porin (Por1), cytosolic Hsp70, and ribosomal subunit Rps3. Together, these findings indicate that Grx3/4-deficient cells develop severe defects in several mitochondrial and cytosolic iron-dependent proteins, despite the induction of the Aft1-dependent iron uptake system. Notably, these global iron-related defects are not detected upon the constitutive activation of Aft1 (Hausmann et al., 2008; Ihrig et al., 2010), suggesting that these consequences of Grx3/4 deficiency occur independently of Aft1 and a deregulated iron homeostasis.

Deficiency in Grx3/4 Impairs the De Novo Synthesis of Cellular Fe/S Clusters and Heme

We asked whether the decreased Fe/S protein activities in Grx3/4-depleted cells might be explained by an impaired de novo synthesis of their Fe/S clusters and addressed this problem by using an established ^{55}Fe radiolabeling and immunoprecipitation assay (Molik et al., 2007). First, the essential cytosolic Fe/S proteins Rli1, Dre2, and Nar1 were analyzed by expressing these proteins from a high-copy vector in wild-type and Gal-GRX4 cells. Fe/S cluster insertion into all three Fe/S protein targets was decreased 4–10-fold upon Grx4 depletion (Figure 2A). The amount of Dre2 in Gal-GRX4 cells was comparable to that in wild-type cells, indicating a specific Fe/S cluster assembly defect (Figure 2A, inset). In the case of Rli1 and Nar1, protein levels were diminished, likely suggesting that the apoforms of these Fe/S proteins were degraded. Similar apoprotein instability is frequently observed upon strong defects in Fe/S protein biogenesis (Balk et al., 2004). Analysis of ^{55}Fe incorporation into the mitochondrial Fe/S proteins Bio2 (biotin synthase) and Ilv3 (dihydroxyacid dehydratase) and the essential mitochondrial ISC scaffold protein Isu1 revealed an up to 4-fold lower ^{55}Fe incorporation upon Grx4 depletion (Figures 2B and 2C). Protein levels of Bio2, Ilv3, and Isu1 did not change upon depletion of Grx4 (Figures 2B and 2C, insets). These findings indicate a general impairment in the de novo assembly of Fe/S proteins upon depletion of Grx3/4.

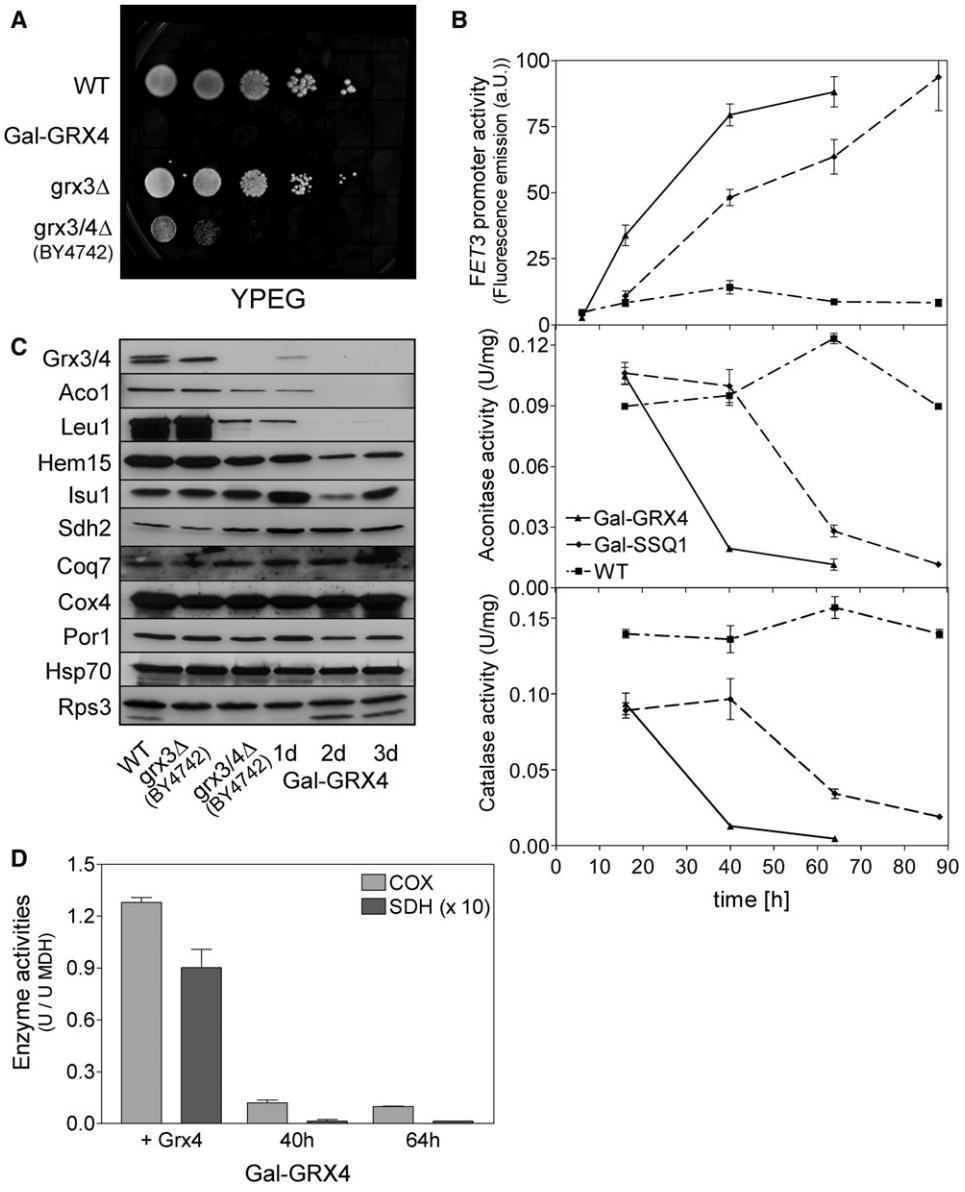

Figure 1. Deficiency in Grx3/4 Is Associated with Defects in Iron-Dependent Enzymes

(A) Wild-type (WT), Gal-GRX4, grx3Δ (strain background W303-1A), and grx3/4Δ (strain background BY4742) were grown in SD medium for 40 hr. Ten-fold serial dilutions were spotted onto YPEG plates and incubated for 2 days at 30°C.

(B and C) WT, Gal-GRX4, and Gal-SSQ1 strains harboring plasmid pFET3-GFP were grown in SD minimal medium. At the indicated times, *FET3* promoter activities were determined by measuring the GFP-specific fluorescence emission of cells (B), and cell extracts were assayed for aconitase and catalase activities, or were analyzed for the indicated proteins by immunostaining (C).

(D) Enzyme activities of respiratory complexes II (SDH) and IV (COX) were determined relative to malate dehydrogenase (MDH) in mitochondria isolated from Gal-GRX4 cells cultivated in rich glucose medium for 40 hr and 64 hr, and from Gal-GRX4 cells expressing *GRX4* from vector pCM189 (+Grx4). Error bars indicate the SEM ($n \geq 4$).

protein assembly defect, because mitochondria are involved in generation of all cellular Fe/S proteins (Lill and Mühlenhoff, 2008). However, we note that the observed effects were less severe in mitochondria compared to the cytosol. To directly test the functionality of the mitochondrial ISC assembly machinery, we made use of an anaerobic in vitro system analyzing ferredoxin (Molik et al., 2007). Apo-Yah1 was incubated with mitochondrial lysates and ^{55}Fe. The radiolabeled Yah1 holoprotein was bound to Q-sepharose, and the amount of radioactivity associated with holo-Yah1 was quantified by scintillation counting. Remarkably, extracts derived from Grx3/4-depleted mitochondria were even more competent in synthesizing the Fe/S

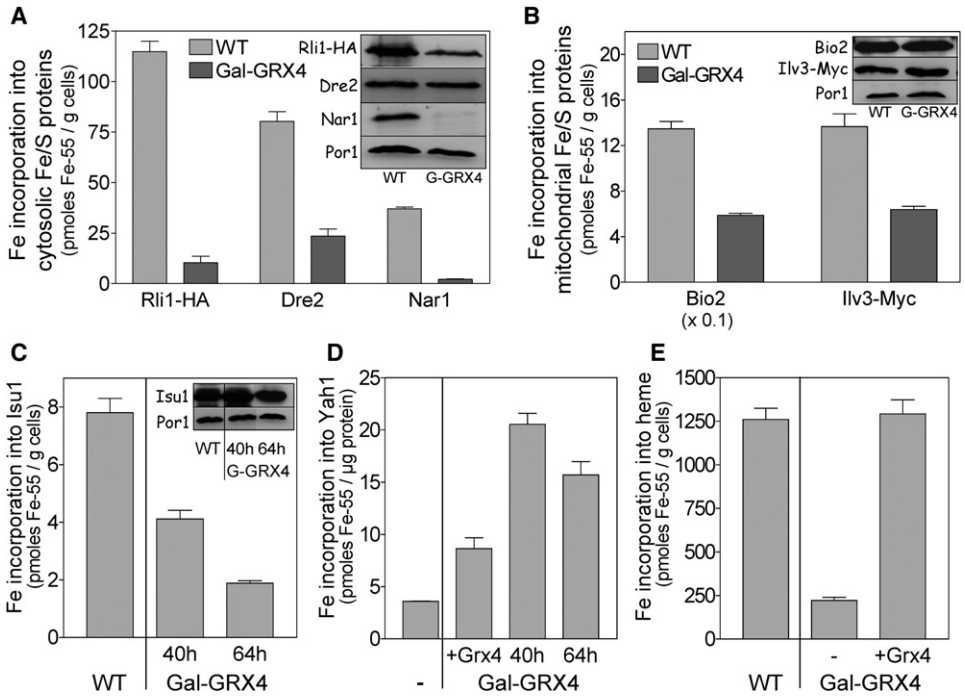

Figure 2. Deficiency in Grx3/4 Impairs the De Novo Synthesis of Cellular Fe/S Clusters and Heme

(A–C) Wild-type (WT) and Grx4-depleted Gal-GRX4 cells overproducing the cytosolic Fe/S proteins Rli1-HA, Dre2 or Nar1 (A), mitochondrial Bio2, Ilv3-Myc (B), or Isu1 (C) were radiolabeled with 10 µCi ^{55}Fe for 2 hr. The Fe/S proteins were immunoprecipitated, and bound ^{55}Fe was quantified by scintillation counting. Protein levels were assessed by immunostaining. Porin (Por1) served as a loading control. Gal-GRX4 cells were depleted for Grx4 by growth in SD medium for 40 hr and 64 hr (in case of Isu1).

(D) Purified apo-Yah1 was incubated under anaerobic conditions in the presence of ^{55}Fe and cysteine either alone (-) or with detergent extracts of mitochondria isolated from 40 hr or 64 hr depleted Gal-GRX4 cells or Gal-GRX4 cells overproducing Grx4 (+Grx4). ^{55}Fe/S cluster reconstitution on re-isolated Yah1 was quantified by scintillation counting.

(E) WT and Gal-GRX4 cells (40 hr depletion) harboring either vector pCM189 (-) or pCM189-GRX4 (+Grx4) were radiolabeled with ^{55}Fe. ^{55}Fe-heme was extracted with butyl-acetate and quantified by scintillation counting. Error bars indicate the SEM ($n \geq 4$).

ISC assembly system is functional in Grx3/4-depleted cells, rendering it likely that the decreased Fe/S cluster incorporation into apoproteins is explained by impaired iron supply.

Consistent with this idea, ^{55}Fe insertion into heme was 5.5-fold lower in Grx4-depleted Gal-GRX4 cells, compared with wild-type cells or Gal-GRX4 cells complemented by *GRX4* (Figure 2E). This diminished heme synthesis activity may explain the loss of function of heme-dependent enzymes, such as catalase and cytochrome oxidase, upon depletion of Grx3/4 (see above). In summary, Grx3/4-depleted cells are strongly impaired in both cellular Fe/S protein maturation and heme biosynthesis. Such defects are not observed in Aft1-activated cells (Haussmann et al., 2008; Ihrig et al., 2010).

Deficiency in Grx3/4 Leads to Impairment of Di-Iron Enzymes Despite Cytosolic Iron Overload

The strong decrease of cellular Fe/S clusters and heme in Grx3/4-deficient cells is somewhat paradoxical, because these cells are expected to accumulate iron as the result of a constitutively activated cellular iron uptake system (Ojeda et al., 2006). To verify this idea, we measured the cellular iron content by ICP-MS analysis of wild-type and Grx4-depleted Gal-GRX4 cells grown (Figure 3A). The level of chelatable iron increased similarly and was mainly present as Fe^{2+} (Figure S2). Cellular levels of other metals, with the exception of Zn (3-fold higher), were hardly changed. In contrast, mitochondrial iron levels were up to 2.3-fold lower in Grx4-depleted Gal-GRX4 cells compared with wild-type levels (Figure 3B). Mitochondria from BY4742 grx3/4Δ cells contained even 7.5-fold less iron. Mitochondrial Mn, Co, and Zn levels were hardly altered, but Cu changed in parallel to iron. The decrease in mitochondrial iron levels in Grx3/4-depleted cells is the more remarkable, because cells with mitochondrial Fe/S protein assembly defects usually display strongly elevated mitochondrial iron levels (Lill and Mühlenhoff, 2008). The fact that this was not observed, despite high levels of total cellular iron, indicates a defective delivery of iron to mitochondria in Grx3/4-deficient cells.

A reasonable explanation for these general defects in iron handling in the absence of Grx3/4 may be a sequestration of iron into the vacuole, the major iron storage compartment in fungi (Kaplan and Kaplan, 2009; Philpott and Protchenko, 2008). To test this idea, we varied the amount of Ccc1, the major importer of divalent metals into the vacuole. Deletion of *CCC1* did not restore growth of Grx4-depleted Gal-GRX4 cells and

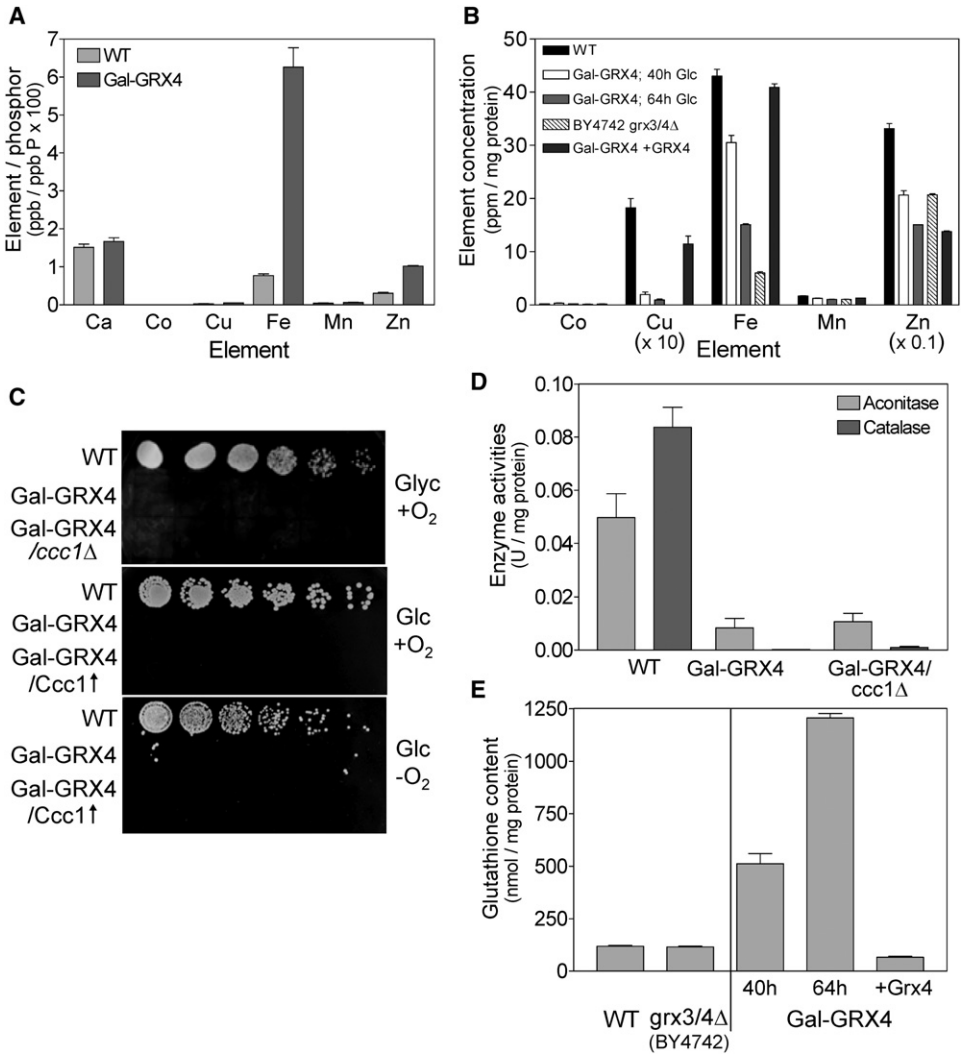

Figure 3. Deficiency in Grx3/4 Results in Cytosolic Iron and GSH Accumulation

(A and B) The metal content of (A) wild-type (WT) and Gal-GRX4 cells (depleted for 64 hr) and (B) mitochondria isolated from the indicated strains was determined by ICP-MS.

(C) The indicated strains lacking (ccc1Δ) or overproducing Ccc1 (Ccc1↑) were cultivated in SD medium for 40 hr. Ten-fold serial dilutions were spotted onto SC agar plates containing glycerol (Glyc) or glucose (Glc), and were cultivated at 30°C under aerobic (+O_2) or anaerobic (−O_2) conditions.

(D) WT, Gal-GRX4 and Gal-GRX4/ccc1Δ cells were grown in SD medium for 64 hr, and aconitase and catalase enzyme activities were determined.

(E) GSH levels were determined in cell extracts from WT, BY4742 grx3/4Δ, Gal-GRX4 (depleted for 40 hr or 64 hr) and Gal-GRX4 cells expressing *GRX4* from a plasmid (+Grx4).

overproduction of Ccc1 failed to restore growth (Figure 3C, middle panel). These data suggest that the accumulated iron is not stored in the vacuole.

Grx4-depleted Gal-GRX4 cells failed to grow under anaerobic conditions (Figure 3C, bottom panel). Thus, reactive oxygen species (due to increased iron levels) are not responsible for the lethal phenotype of Grx3/4-depleted cells. Moreover, oxidized glutathione levels (GSSG; measured under anaerobic conditions) were below the detection limit (not shown). Rather, reduced glutathione (GSH) was strongly elevated in Grx4-depleted Gal-GRX4 cells, but not in BY4742 grx3/4Δ (Figure 3E). Together, these results and the predominant pres-

The experiments presented above showed a maturation defect in cellular Fe/S and heme proteins in Grx3/4-deficient cells despite a cytosolic iron accumulation. Because this indicated a defective delivery of iron, we asked whether other iron-dependent enzymes were affected by Grx3/4 deficiency. First, the iron status of ribonucleotide reductase (Rnr), a cytosolic diferric-tyrosyl radical enzyme essential for deoxyribonucleotide synthesis, was analyzed (Perlstein et al., 2005). Upon depletion of Grx4 in Gal-GRX4 cells, the protein levels of Rnr2 decreased slightly (Figure S3). Nevertheless, the specific activity of Rnr was 6-fold lower compared with wild-type cells (Figure 4A). This was likely due to inefficient metallation, because ^{55}Fe inser-

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