



Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster.

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► **To cite this version:**

Ulrich Mühlenhoff, Sabine Molik, José R Godoy, Marta A Uzarska, Nadine Richter, et al.. Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster.. Cell Metab, 2010, 12 (4), pp.373-85. <10.1016/j.cmet.2010.08.001>. <hal-00630763>

HAL Id: hal-00630763

<https://hal.archives-ouvertes.fr/hal-00630763>

Submitted on 10 Oct 2011

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Elsevier Editorial System(tm) for Cell Metabolism
Manuscript Draft

Manuscript Number: CELL-METABOLISM-D-10-00122R2

Title: Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster

Article Type: Research Article

Keywords: Mitochondria, iron homeostasis, metallo proteins, glutathione, sulfur metabolism, ribonucleotide reductase.

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Abstract: 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 which functions both as an iron sensor and in intracellular iron delivery. Collectively, our study uncovers a novel role of monothiol glutaredoxins in cellular iron metabolism with a surprising connection to cellular redox and sulfur metabolisms.

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Opposed Reviewers:



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Marburg, 10.06.2010

Submission of revised manuscript CELL-METABOLISM-D-10-00122R1: Mühlenhoff et al.

Dear Dr. Emambokus,

Thank you very much for your kind e-mails concerning our revised manuscript CELL-METABOLISM-D-10-00122R1 (Mühlenhoff et al.) on the role of monothiol glutaredoxins in iron metabolism.

We were pleased to learn that all three Reviewers appreciated our response to their valuable suggestions, and that they considered our manuscript as being much improved, with two Reviewers recommending the revised version for publication. We now have once again amended our manuscript to satisfy the remaining minor concern of Reviewer 1. Our detailed response to this Reviewer is attached. We also have carefully followed your technical instructions to finalize the manuscript.

We thank you and the Reviewers again for the helpful and constructive comments on our manuscript, and hope that with these improvements our manuscript is now acceptable for publication in *Cell Metabolism*.

With best regards,

Uli Mühlenhoff

Christopher Horst Lillig

Roland Lill

Response to the Comments of Reviewer 1:

We are pleased to read that all three Reviewers appreciated our response to their valuable suggestions and that they felt that our manuscript has been improved, with two Reviewers recommending the revised manuscript for publication. We have addressed the minor remaining concern of Reviewer 1 in detail below. We also have technically modified our manuscript as requested by the Editor.

Reviewer #1:

In the first review this Reviewer had suspected that our data may be explained by a complete overlap of the phenotypes/functions of Aft1/2 and Grx3/4. We are glad to learn that the data collected from published papers in our Response letter for the revised version of our manuscript (Table 1 and Fig. R1) convinced the Reviewer to accept that Grx3/4 perform a role additional to its Aft1/2-related function in iron uptake regulation (... *argue towards additional rather than an epistatic function of these glutaredoxins with regards to Aft function*). Nevertheless, s/he suggests to provide additional experimental evidence to support this point. First, the Reviewer asked for expression information for three specific genes (*YAP5*, *CTH1* and *CTH2*) in Aft1up versus grx3/4Δ cells. As mentioned in our first response letter, such information is already available, since genome-wide DNA array studies have been performed for these mutant cells. The differences for the three genes mentioned by the Reviewer are minor (fold change in Aft1up versus Grx3/4-deleted cells is [CTH2: 11.2 (grx3/4Δ) vs. 5.1 (Aft1-up); CTH1 0.78 (grx3/4Δ) vs. 1.5 (Aft1-up); YAP5: 1.9 (grx3/4Δ) vs. 1.9 (Aft1-up)]). This is not surprising because they all belong to the Aft1/2-regulated genes, i.e. a set of genes where the functions of Aft1/2 and Grx3/4 indeed overlap. However, comparison of the full data sets shows severe gene expression differences between the two cells types which – as we report in our manuscript – are imaged by respective biochemical alterations. This can only be explained on the basis of an additional function of Grx3/4.

The second suggestion of the Reviewer, i.e. repeating experiments of Fig. 1 in the aft1/2 delta background is interesting, even though not really related to the content of our manuscript. Nevertheless, we have performed such an experiment (attached as Fig. R2, for Reviewer only). Part A shows that both aconitase and Leu1 activities are hardly changed in aft1/2Δ cells. The slight effect (20% decrease) is due to the limited iron uptake in these cells (as already discussed by the Reviewer). A direct comparison of these data to results presented in our manuscript (e.g., Figs. 1A-B, S1A) and shown in Fig. R2B below demonstrate that Grx3/4-depleted cells completely loose aconitase, Leu1, and catalase activities. These cells, in contrast to aft1/2Δ cells, have a huge iron overload (Fig. 3A). Notably, Fig. R2B (middle bars) also provides data on a Grx4 mutant protein (Mut1) which specifically activates Aft1/2-dependent *FET3*, but still fully supports Fe/S protein maturation and heme biogenesis. This clear separation of the two functions of Grx3/4, iron uptake regulation and the novel function in iron trafficking, is also evident from the analysis of the site-directed mutants of Grx4 in Fig. 6 and is discussed in our manuscript. This data fully satisfies the request of the Reviewer. Since the requested information is either published (first part) or redundant to data presented in our manuscript, we do not think they warrant the space to become integrated into the manuscript. However, to meet the concern of the Reviewer we have rewritten the 1st paragraph of Discussion to make this point (phenotypical difference of Aft1/2up and Grx3/4-depleted cells) absolutely clear.

We hope that, with these alterations, our manuscript is suitable for publication in *Cell Metabolism*.

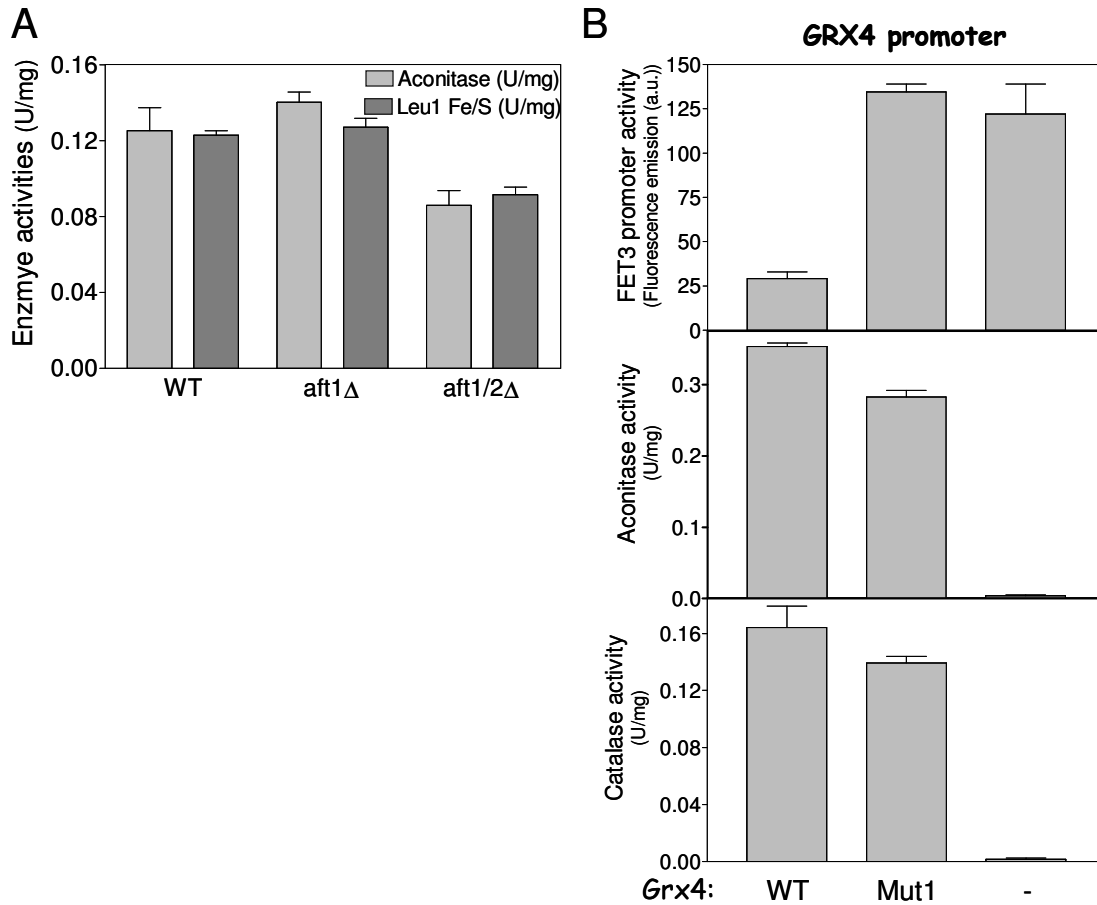


Figure R2: The functions of Aft1/2 and Grx3/4 can be distinguished.

(For reviewers only.)

Legend: (A) Enzyme activities of the Fe/S proteins aconitase and Leu1 were determined in cell extracts of wild-type, *aft1Δ*, and *aft1/2Δ* cells. Cells were cultivated in minimal SD medium supplemented with 50 μM Fe, and the indicated enzyme activities were determined. (B) Gal-GRX4 cells lacking (-) or expressing wild-type (WT) Grx4 or a Grx4 mutant protein (Mut1) under the control of the *GRX4* promoter from vector pCM189 were cultivated in minimal SD medium. After 64 h, cells were analyzed for *FET3* promoter activities, or aconitase and catalase enzyme activities.

**Cytosolic monothiol glutaredoxins function
in intracellular iron sensing and trafficking
via their bound iron-sulfur cluster**

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Word count: Text: 54,982 characters

Summary: 150 words

Abstract

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 which 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.

Highlights

Glutaredoxins Grx3/4 are essential for iron insertion into proteins and cofactors

Deficiency in Grx3/4 functionally impairs all iron-requiring processes

Grx3/4-deficient cells accumulate iron but cannot biologically use it

The Fe/S center of Grx3/4 is critical for both iron trafficking and sensing

Keywords

Mitochondria, iron homeostasis, metallo proteins, glutathione, sulfur metabolism, ribonucleotide reductase.

Introduction

Iron is essential for virtually all organisms, since 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 into various subcellular compartments. A soluble, low molecular-mass form of iron was described in the 1970s, but ever since the discovery of this 'labile iron pool' its physiological importance and composition has been under debate (Crichton and Charloteaux-Wauters, 1987; Richardson and Ponka, 1997). Presumably, iron may also be bound to dedicated proteins assuring 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.

Since iron is not only essential but also toxic at higher levels, cells have developed sophisticated systems for assuring a tightly regulated iron homeostasis (Hentze et al., 2004; Kaplan and Kaplan, 2009). While in mammals this process is executed by iron-regulatory proteins in a post-transcriptional fashion, the yeast *S. 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).

Grx3/4 belong to the large thioredoxin (Trx) fold family, and are composed of an N-terminal Trx and a C-terminal monothiol glutaredoxin (Grx) domain (Herrero and de la Torre-Ruiz, 2007; Lillig et al., 2008). Although the Grx3/4 subfamily of multi-domain 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 *E. 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; Table S1) 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 non-fermentable carbon sources (Figs. 1A and 3C). 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 (Fig. 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 (Figs. 1B and 1C). A strong activation of the Aft1-dependent *FET3* gene was observed using a *FET3* promoter-*GFP* fusion as a reporter (Fig. 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, a component the iron-sulfur cluster (ISC) assembly machinery, even though the changes occurred later due to slower 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 (Fig. 1D). Likewise, low activities of both aconitase and respiratory complexes III and IV were observed in BY4742 *grx3/4Δ* cells (Fig. S1A), consistent with our earlier observation of an impaired ⁵⁵Fe/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 (Fig. 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 non-iron 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., 2009), 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 ⁵⁵Fe radiolabelling 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 (Fig. 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 (insert). 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 ⁵⁵Fe 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 ⁵⁵Fe incorporation upon Grx4 depletion (Figs. 2B and 2C). Protein levels of Bio2, Ilv3, and Isu1 did not change upon depletion of Grx4 (inserts). These findings indicate a general impairment in the *de novo* assembly of Fe/S proteins upon depletion of Grx3/4.

In principle, the defect of Fe/S protein maturation upon Grx3/4 depletion could be explained by a primary mitochondrial Fe/S protein assembly defect, since mitochondria are involved in generation of all cellular Fe/S proteins (Lill and Muhlenhoff, 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 the capacity of mitochondrial detergent extracts to support Fe/S cluster insertion into the apoform of purified Yah1, a [2Fe-2S] ferredoxin (Molik et al., 2007). Apo-Yah1 was incubated with mitochondrial lysates and ⁵⁵Fe, bound to Q-

spharose, and the amount of bound radioactivity was quantified by scintillation counting. Remarkably, extracts derived from Grx3/4-depleted mitochondria were even more competent in synthesizing the Fe/S cluster on Yah1 than Grx4-complemented Gal-GRX4 cells (Fig. 2D). This finding strongly suggests that the mitochondrial 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 to wild-type cells or Gal-GRX4 cells complemented by *GRX4* (Fig. 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 (Hausmann et al., 2008; Ihrig et al., 2009).

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, since these cells are expected to accumulate iron due to a constitutively activated cellular iron uptake system (Ojeda et al., 2006). To verify this, we measured the cellular iron content by ICP-MS analysis of wild-type and Grx4-depleted Gal-GRX4 cells grown in minimal medium supplemented with 100 μM FeCl_3 . Total cellular iron increased 6-fold upon depletion of Grx4 (Fig. 3A). The level of chelatable iron increased similarly and was mainly present as Fe^{2+} (Fig. 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 to wild-type (Fig. 3B). Mitochondria from BY4742 *grx3/4* Δ 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, as cells with mitochondrial Fe/S protein assembly defects usually display strongly elevated mitochondrial iron levels (Lill and Muhlenhoff, 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, 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 did not increase the low enzyme activities of aconitase and catalase (Fig. 3C, top panel and Fig. 3D). Similarly, overproduction of Ccc1 failed to restore growth (Fig. 3C, middle). These data suggest that the accumulated iron is not stored in the vacuole.

Grx4-depleted Gal-GRX4 cells failed to grow under anaerobic conditions (Fig. 3C, bottom). 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* (Fig. 3E). Together, these results and the predominant presence of iron in its ferrous form (Fig. S2) indicate that reducing conditions prevail in Grx3/4-deficient cells.

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. Since 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 (Fig. S3). Nevertheless, the specific activity of Rnr was 6-fold lower compared to wild-type cells (Fig. 4A). This was likely due to inefficient metallation, since ⁵⁵Fe insertion into Rnr *in vivo* (followed by immunoprecipitation of subunit Rnr2) was 5-7-fold decreased in Grx4-depleted cells (Fig. 4B). As a second di-iron protein, we analyzed the mitochondrial mono-oxygenase Coq7 which catalyses the hydroxylation of demethoxyubiquinol (DMQ₆), the penultimate reaction of ubiquinone (CoQ₆) biosynthesis (Tran et al., 2006). HPLC analysis of mitochondrial lipid extracts revealed diminished CoQ₆ and increased DMQ₆ levels upon Grx4 depletion (Fig. 4C). This effect was reversed to wild-type ratios by expression of *GRX4* from a plasmid (Fig. 4D). The simultaneous accumulation of the substrate (DMQ₆) and decrease of the product (CoQ₆) of the enzyme Coq7 indicates a diminished activity of this di-iron mono-oxygenase upon depletion of Grx3/4. Collectively, these data demonstrate that a Grx3/4 deficiency causes a severe defect in cellular di-iron enzymes.

Are the observed defects in Grx3/4-depleted cells specific for iron-dependent proteins? This question was addressed by analyzing the activities and protein levels of several metal-dependent enzymes. The in-gel activities of the endogenous Cu/Zn- and Mn-dependent superoxide dismutases (Sod1 and Sod2, respectively) remained unchanged in Grx4-depleted

Gal-GRX4 cells (Fig. 4E). In marked contrast, both the level and activity of ectopically expressed iron-only superoxide dismutase (FeSod) from *E. coli* strongly declined. Further, the Zn-dependent alcohol dehydrogenase (ADH) was 3-fold more active upon Grx4 depletion (Fig. S1B). This increased ADH activity is characteristic for a switch towards fermentative metabolism and is typically observed upon iron deprivation. The normal function of several metal-reliant enzymes indicates that the described defects in Grx3/4-depleted cells are specific for iron-related processes. We conclude that Grx3/4 perform an essential role in cellular iron trafficking, in addition and independently of their non-essential function in iron uptake regulation.

Grx3/4 assemble a bridging Fe/S cluster independently of the CIA machinery

For a function of monothiol glutaredoxins in cellular iron trafficking, iron-binding may be a necessary prerequisite. Recent *in vitro* studies have shown that various glutaredoxins, including yeast Grx3/4, can bind a bridging, GSH-liganded [2Fe-2S] cluster (Bandyopadhyay et al., 2008; Berndt et al., 2007; Johansson et al., 2007; Li et al., 2009). We asked whether this unusual Fe/S cofactor is of physiological relevance and can be observed in a native environment using the ⁵⁵Fe radiolabelling assay. Significant amounts of ⁵⁵Fe were co-immunoprecipitated with anti-Grx4-antibodies from cell extracts derived from wild-type cells (strain BY4742), while only background levels were observed in *grx3/4Δ* cells (Fig. 5A). Similar amounts of ⁵⁵Fe were co-immunoprecipitated from both *grx3Δ* and *grx4Δ* cells, indicating that the homologous Grx3 and Grx4 bind iron with similar efficiency and independently of each other. The amount of Grx4-bound ⁵⁵Fe was unchanged both in wild-type cells of our standard strain W303-1A, and, remarkably, under anaerobic or oxidative stress conditions prevailing after addition of H₂O₂ or upon deletion of *SOD1* (*sod1Δ* cells) indicating that iron binding to Grx3/4 was insensitive to oxidative stress (Fig. 5B).

To analyze whether the iron bound to Grx3/4 is part of an Fe/S cluster, we investigated the requirement of ⁵⁵Fe binding for components of Fe/S protein biogenesis (Lill and Muhlenhoff, 2008). We first analyzed the role of the cysteine desulfurase complex Nfs1-Isd11 which serves as the sulfur donor for Fe/S protein biogenesis. For regulated depletion of Nfs1 or Isd11 we used Gal-NFS1 or Gal-ISD11 cells, respectively. ⁵⁵Fe binding to Grx4 (Fig. 5C) and to the cytosolic Fe/S protein Leu1 as a control (Fig. S4) declined to background levels upon depletion of either Nfs1 or Isd11. The same result was seen in Gal-ISU1Δ*isu2* cells depleted for the core ISC scaffold proteins Isu1/2. No recovery of iron binding to Grx3/4 was observed, when a cytosolic-nuclear version of Nfs1 (ΔN-Nfs1) was produced in Gal-NFS1

cells lacking mitochondrial Nfs1 (Fig. 5C). We found during these experiments that the Grx4 protein levels decreased upon Nfs1 depletion, while the Grx3 levels remained unchanged. While the reason for this specific decrease is unknown, we note that the remaining Grx3 did not bind ^{55}Fe above background levels, unlike in *grx4* Δ cells (cf. Fig. 5A). The dependence of iron binding to Grx3/4 on core components of the mitochondrial ISC assembly system demonstrates that Grx3/4 bind an Fe/S cluster *in vivo*.

Does incorporation of the Fe/S cluster into Grx3/4 also require the cytosolic iron-sulfur protein assembly (CIA) system (Lill, 2009)? Depletion of the essential CIA components Nbp35 and Dre2 in the respective *GAL* promoter-exchange mutants significantly increased rather than impaired ^{55}Fe binding to Grx3/4 (Fig. 5D), while hardly any ^{55}Fe binding was observed to Leu1 as a control (Fig. S4). This surprising finding suggests that incorporation of the Fe/S cluster into Grx3/4, while requiring mitochondrial Nfs1-Isd11-Isu1, does not depend on the cytosolic CIA machinery.

The non-canonical pathway used for Fe/S cluster assembly on Grx3/4 may be due to the special nature of this cofactor, with a conserved Cys and GSH serving as ligands of a [2Fe-2S] cluster bridged between two Grx monomers, as observed after *in vitro* reconstitution or after overexpression in *E. coli* (Li et al., 2009; Picciocchi et al., 2007). We therefore sought to obtain *in vivo* evidence for this configuration. First, we determined which ligands might coordinate the Fe/S cluster. The importance of the conserved active-site cysteine residues C171 in the C-terminal Grx domain and C37 in the N-terminal Trx domain (Fig. 5E, top) for ^{55}Fe binding was tested in wild-type cells expressing a plasmid-encoded Myc-tagged Grx4 (Grx4-Myc) in which these residues were mutated to serine (mutant proteins C171S and C37S) or alanine (C171A). Myc-tagged wild-type Grx4 was fully functional (see below), and was produced at similar levels as the mutant proteins (Fig. 5E, bottom). Mutant proteins C171S and C171A did not bind ^{55}Fe above background levels (Fig. 5E, middle). In contrast, mutant protein C37S bound wild-type amounts of ^{55}Fe suggesting that iron is bound via C171. Second, the importance of GSH for ^{55}Fe binding to Grx3/4 *in vivo* was tested using the GSH synthesis-deficient mutant *gsh1* Δ that can be depleted for GSH upon growth in media lacking GSH. While significant amounts of ^{55}Fe were bound to Grx4-Myc in the presence of exogenously added GSH, only background levels of ^{55}Fe were found in GSH-deprived cells (Fig. 5F). Together, these findings suggest that the active-site C171 of the Grx domain and GSH serve as ligands of the Grx3/4-bound Fe/S cluster.

Finally, we tried to find *in vivo* evidence for the presence of a bridging Fe/S cluster on Grx3/4. A yeast strain was constructed that simultaneously expressed C-terminally HA- and

Myc-tagged Grx4. Cell extracts were subjected to immunoprecipitation with anti-HA or anti-Myc immunobeads followed by immunoblotting. Immunoprecipitation with anti-HA antibodies led to co-isolation of Grx4-HA and a smaller amount of Grx4-Myc (Fig. 5G). The same result, yet with inversed intensities was observed using anti-Myc beads, while no cross-reacting bands were visible in wild-type cells. In cells expressing a C171S mutant Grx4-Myc and wild-type Grx4-HA, co-immunoprecipitation was far less efficient, but still detectable. These observations document the importance of residue C171 for efficient Grx4 dimer formation, and thus are consistent with the idea of a bridging Fe/S cluster between two Grx monomers. Collectively, our findings suggest that under physiological conditions Grx3/4 bind a bridging Fe/S cluster that is coordinated by the active-site cysteine and GSH consistent with the structure of glutaredoxins reconstituted *in vitro*.

The Grx3/4-bound Fe/S cluster is important for iron metabolism

Is the Grx3/4-bound Fe/S cluster important for the function of Grx3/4 in iron trafficking and is this cofactor also required for iron sensing? As shown above the C171S and C171A Grx4-Myc mutant proteins have lost the ability to stably bind the Fe/S cluster (Fig. 5E). Grx4-depleted Gal-GRX4 cells expressing these mutant proteins from the endogenous *GRX4* promoter failed to grow, whereas wild-type and C37S Grx4-Myc that retained normal iron binding supported wild-type growth (Fig. 6A, top). Aft1-dependent transcription of *FET3* was fully activated and virtually no aconitase and catalase activities were observed in Gal-GRX4 cells producing C171S and C171A Grx4-Myc instead of wild-type Grx4 (Fig. 6B). In contrast, wild-type and C37S Grx4-Myc-expressing Gal-GRX4 cells showed wild-type signals in these assays. All Grx4-Myc proteins were present at similar levels, with the exception of C171A which apparently was less stable (Fig. 6A, bottom). These results demonstrate the crucial role of residue C171 and thus the Grx3/4-bound Fe/S cluster for both iron regulation and iron trafficking.

Formally, it is possible that the active-site C171 performs its essential role in Grx4 via thiol-dependent redox chemistry (Herrero and de la Torre-Ruiz, 2007; Lillig et al., 2008) rather than by coordination of the Fe/S cluster. Such a function can be excluded from the observation that overexpression (from a *Tet* promoter) of the C171S (but not C171A) Grx4-Myc mutant protein in Grx4-depleted Gal-GRX4 cells restored wild-type growth (Fig. 6C). We noted that Grx4 overexpression generally diminished *FET3* induction (Fig. 6D). The corresponding C171S cells still displayed strong *FET3* activation, yet wild-type aconitase and strongly increased catalase activities. The restored iron loading of these and presumably other

iron-dependent proteins may explain why the C171S cells grow normally, despite a still disturbed iron regulation. Consistent with this interpretation, C171A cells did not show any recovery of enzyme activities. This striking result for C171S Grx4-Myc may be explained on the basis of earlier observations showing that, in some cases, Ser (but not Ala) may function as a (weak) ligand for iron (da Silva and Williams, 2001; Johnson et al., 2005). We therefore suppose that the C171S Grx4 still binds an Fe/S cluster, thus stabilizing the protein and maintaining its function in iron trafficking at high protein levels (Figs. 6A and 6C). However, the Fe/S cluster is bound in a fashion too labile to be isolated by our co-immunoprecipitation method, and to function properly in iron regulation. In summary, these data indicate that the essential function of residue C171 cannot be explained by thiol-dependent redox reactivity but rather by coordination of the bridging Fe/S cluster which was characterized here to perform an essential function in both intracellular iron trafficking and sensing.

Discussion

Our analysis of the cytosolic-nuclear monothiol glutaredoxins Grx3 and Grx4 provides strong evidence for an essential and general role of these proteins in intracellular iron trafficking, a so far poorly understood process (Fig. S5). This function of Grx3/4 is additional to their involvement in Aft1-dependent iron sensing, since the phenotypes of Grx3/4-depleted and Aft1-activated cells differ markedly in that only the former cells display global and severe biochemical defects in multiple iron-dependent enzymes (Hausmann et al., 2008; Ihrig et al., 2009). For instance, the function of cellular Fe/S-, heme- and other iron-containing proteins is strongly impaired upon depletion of Grx3/4, but not affected when Aft1 is activated. Many of these proteins are involved in essential processes such as DNA synthesis and ribosome function, explaining why Grx3/4-depleted cells eventually lose their viability. The two functions of Grx3/4 in iron trafficking and sensing are mediated by their crucial cofactor, a GSH-liganded and bridging [2Fe-2S] center. Destruction of this Fe/S center elicits a highly similar phenotype as the depletion of the Grx proteins. The observed biochemical consequences of Grx3/4 depletion are specific for iron, since the functions of other metal-dependent enzymes such as Cu,Zn- and Mn-dependent superoxide dismutases were not affected. Together, our findings demonstrate a crucial role of Grx3/4 in intracellular iron delivery from the cytosolic labile iron pool to virtually all iron-binding proteins or pathways in the cell (Fig. S5). Hence, Grx3/4 represent long-sought general factors that facilitate the proper assembly of various iron centers in proteins and cofactors. Grx3/4 are unlikely to make

specific and direct contacts with the potential iron-dependent targets. Therefore, they may not function as classical metallo-chaperones that deliver their metal specifically to individual targets (Finney and O'Halloran, 2003; Lyons and Eide, 2007). Rather, the iron activated by Grx3/4 may be utilized by dedicated assembly factors such as components of the mitochondrial ISC or cytosolic CIA systems (Lill and Muhlenhoff, 2008) or the cytosolic ferritin-specific iron chaperone PCBP1 (Shi et al., 2008) for proper insertion into recipient apo-proteins.

Several observations make it unlikely that the severe iron trafficking defect in the absence of Grx3/4 is related to oxidative stress damage, even though, in principle, Fenton chemistry is possible in Grx3/4-depleted cells due to their massive accumulation of intracellular ferrous iron. First, the lethal phenotype of Grx3/4-depleted cells was not cured under anaerobic conditions, and the activities of iron-dependent enzymes were not recovered. Second, BY4742 *grx3/4*Δ cells display only weak signs of oxidative stress, possibly because the cytosolic redox balance in yeast is maintained by the dithiol glutaredoxins Grx1 and Grx2 (Rodriguez-Manzanique et al., 1999). Third, binding of the essential Fe/S center to Grx3/4 was rather insensitive to oxidative stress. Finally, Grx3/4-depleted cells show hyper-accumulation of reduced GSH with no signs for an increase in oxidized GSSG.

The observed cytosolic iron trafficking defect in Grx3/4-deficient cells cannot be explained by low levels of iron in the cytosol. Rather, these cells accumulate high amounts of chelatable iron which in all likelihood is predominantly present in the cytosolic compartment. An iron efflux into the vacuole, the main storage organelle for excess iron in plants and fungi (Kaplan and Kaplan, 2009; Philpott and Protchenko, 2008), is unlikely since the deletion of the vacuolar divalent metal transporter gene *CCCI* did not rescue any of the defects of Grx3/4-depleted cells. Mitochondria, which accumulate iron under conditions of hampered Fe/S protein biogenesis (Lill and Muhlenhoff, 2008), displayed diminished iron levels in Grx3/4-depleted cells. These data suggest that the surplus iron in Grx3/4-deficient cells is not bioavailable for efficient funneling into iron-dependent processes.

Our ⁵⁵Fe radiolabelling experiments demonstrate that the Fe/S cluster of monothiol glutaredoxins, as initially characterized *in vitro* or after overexpression in *E. coli* (Li et al., 2009; Picciocchi et al., 2007), is also assembled in its native environment and is of utmost physiological relevance. The essential involvement of the core components of the mitochondrial ISC assembly system provides strong evidence that the bound iron is part of an Fe/S cluster, and that the sulfide ions of the [2Fe-2S] cluster are derived from the mitochondrial cysteine desulfurase complex Nfs1-Isd11. Our *in vivo* results support the

unusual bridging character of the Fe/S cluster, which is coordinated by the Grx domain active-site Cys171 and GSH. Hence, GSH performs a dual role in cellular iron metabolism. As part of the Grx3/4 complex it is central for intracellular iron delivery and sensing, and as a component of the ISC export machinery it cooperates with the mitochondrial ABC transporter Atm1 both in the assembly of cytosolic-nuclear Fe/S proteins and in mitochondrial-cytosolic iron homeostasis (Lill, 2009; Sipos et al., 2002). Surprisingly, the Grx3/4 Fe/S cofactor is assembled independently of the CIA system, an observation which differs from all other known, non-scaffold cytosolic Fe/S proteins analyzed so far (Lill and Muhlenhoff, 2008). This unique assembly mode is likely related to the special type of Fe/S cluster on Grx3/4. The CIA machinery-independent maturation of Grx3/4 is consistent with earlier observations that this machinery is not important for cellular iron regulation in yeast (Hausmann et al., 2008; Kaplan and Kaplan, 2009).

While it is clear that the bridging Fe/S cluster of Grx3/4 is crucial for iron trafficking, the question remains how it may function. Currently, it cannot be discerned whether the iron moiety of the Fe/S cluster is mobilized and used for insertion into iron-dependent proteins, or whether the Fe/S cluster plays a more indirect role by activating cytosolic iron for specific trafficking. In any case, it is well-known for Fe/S clusters that they can reversibly lose one of their iron ions, the most prominent example being aconitase (Beinert et al., 1997). Thus, the Fe/S cluster of Grx3/4 may not be fully disassembled during its action in iron delivery. The role of the Grx3/4 Fe/S center in iron trafficking is clearly distinct from the proposed function of other monothiol glutaredoxins as Fe/S scaffold (or Fe/S carrier) proteins facilitating Fe/S cluster transfer to target apoproteins (Bandyopadhyay et al., 2008; Picciocchi et al., 2007). Thus, monothiol glutaredoxins may use diverse mechanisms for maturation of iron-containing proteins.

In principle, the essential requirement of the Grx active-site C171 for iron homeostasis could be explained by thiol-dependent redox chemistry. However, with few exceptions, monothiol glutaredoxins, including yeast Grx3/4, are inactive in GSH-dependent redox reactions *in vitro*, and there is little evidence that monothiol glutaredoxins carry out specific catabolic functions (Herrero and de la Torre-Ruiz, 2007). In addition, BY4742 *grx3/4Δ* cells display wild-type glutathione reductase activity (Rodriguez-Manzaneque et al., 1999). Most convincingly, the high-copy suppressor phenotype of the C171S mutation in the Grx domain excludes impaired thiol-related (redox) chemistry as an explanation for the functional defects prevailing in Grx3/4-depleted cells. High levels of the Cys171 mutant protein, but not the corresponding Ala variant, supported wild-type growth, and almost fully restored the

activities of iron-dependent proteins. This suggests that the C171S protein is still capable of coordinating the Fe/S co-factor, as this is known for other proteins with Cys to Ser mutations (da Silva and Williams, 2001; Johnson et al., 2005). However, iron binding to the C171S mutant protein may be more labile thus precluding its detection by our *in vivo* ⁵⁵Fe labeling and immunoprecipitation assay. As a consequence the amount of bound Fe/S cluster may be too low to support Grx4 function in Aft1 sensing. Consistent with this interpretation, in reconstituted human Grx2 and poplar GrxC1, the [2Fe-2S] cluster is coordinated in a binding pocket at the interface of two Grx monomers by the two active-site Cys residues and two non-covalently bound GSH molecules (Berndt et al., 2007; Johansson et al., 2007; Rouhier et al., 2007). The replacement of cysteine with serine may still provide a low-affinity iron binding site.

Grx3/4 play a role in iron-uptake regulation by cooperating with the iron-responsive transcription factor Aft1, the major regulator of cellular iron uptake in yeast (Philpott and Protchenko, 2008). In fact, the physical interaction between Grx3/4 and Aft1 was the initial evidence linking Grx3/4 and cellular iron homeostasis (Ojeda et al., 2006; Pujol-Carrion et al., 2006). Our study shows that the Grx3/4-bound Fe/S cluster functions as the long-sought iron sensor which communicates the cytosolic iron status to Aft1 (Fig. S5). Removal of the bound Fe/S cluster, e.g., by mutation of the active-site Cys of the Grx domain or by depletion of GSH abolished the ability of Grx3/4 to regulate Aft1. The iron-sensing role of Grx3/4 appears to be conserved in other fungi that utilize iron-responsive transcription factors, even if they are structurally unrelated to *S. cerevisiae* Aft1 (Kaplan and Kaplan, 2009; Mercier and Labbe, 2009).

The crucial function of Grx3/4 and its unique Fe/S center in intracellular iron trafficking and sensing opens an additional chapter of the intimate connection between the iron metabolism and Fe/S protein biogenesis. Not only is the iron metabolism regulated by (mitochondrial) Fe/S protein biogenesis (Kaplan and Kaplan, 2009), but conversely iron insertion into Fe/S proteins requires its activation by the Fe/S cluster-containing Grx3/4. The mutual dependence of these two important pathways is further linked to the cellular redox balancing systems via GSH which is crucial for both iron insertion into iron-dependent factors and regulation of cellular iron homeostasis (Lill and Muhlenhoff, 2008). These considerations reserve a central role for monothiol glutaredoxins Grx3/4 and their bound Fe/S center in cellular metabolism. The central function of Grx3/4 in intracellular iron trafficking may provide the explanation for the conservation of this protein family in eukaryotes. The

discovery of this glutaredoxin function now paves the way towards mechanistic investigations of how iron is specifically activated for incorporation into iron-requiring processes.

Experimental procedures

Yeast strains, cell growth and plasmids- Yeast strains used in this study are listed in Table S1. If not stated otherwise, the strain background is W303-1A. Cells were cultivated in minimal medium containing all recommended supplements (SC), 2% w/v glucose (SD) or 3% w/v glycerol (Sherman, 2002). Iron-replete media were supplemented with 50 μ M FeCl₃. Media for anaerobic growth were supplemented with Tween80, ergosterol and methionine. Gal-GRX4 cells were depleted for Grx4 to critical levels by cultivation in SD medium for 40 to 64 h prior to analysis. Repression of other conditional Gal-strains was performed as described (Table S2). *gsh1* Δ cells were cultivated in the presence or absence of 1 mM GSH for 64 h prior to analysis (Sipos et al., 2002). Wild-type and site-directed mutant Grx4 proteins were expressed in yeast with a C-terminal Myc-tag from plasmid pCM189 (Ojeda et al., 2006) under the control of the *tetO*₇ or the endogenous *GRX4* promoter (Table S2).

Biochemical analyses- *In vivo* radiolabelling of yeast cells with ⁵⁵FeCl₃ (Perkin-Elmer) and measurement of ⁵⁵Fe incorporation into proteins by immunoprecipitation and into heme was carried out as described (Pierik et al., 2009). Antibodies were raised in rabbits against recombinant proteins expressed in *E. coli*. ⁵⁵Fe-labelling of added apo-ferredoxin in detergent extracts of isolated mitochondria was carried out as described (Molik et al., 2007). DMQ₆ and CoQ₆ were quantified according to (Tran et al., 2006), GSH and GSSG according to (Elledge and Davis, 1987).

Miscellaneous methods- The following published methods were used: manipulation of DNA and PCR (Sambrook and Russel, 2001); preparation of yeast mitochondria (Molik et al., 2007), immunological techniques (Harlow and Lane, 1988); *FET3* promoter assays (Molik et al., 2007); determination of cellular and mitochondrial metal contents by ICP-MS (Muhlenhoff et al., 2003); enzyme activities of iron proteins, ADH, and MDH (Molik et al., 2007), ribonucleotide reductase (Wang et al., 2009), and superoxide dismutase (Flohe and Otting, 1984). Error bars represent the standard error of the mean (SEM) (n \geq 4).

Acknowledgments

We are grateful to Dr. D.R. Winge for generous help and discussion. We acknowledge financial support from Deutsche Forschungsgemeinschaft (SFB 593, Gottfried-Wilhelm Leibniz program, and GRK 1216), von Behring-Röntgen Stiftung, Max-Planck Gesellschaft,

Fonds der chemischen Industrie, NIH GM29295 (to J.S.), Ministerio de Ciencia e Innovacion (CSD2007-0020; to E.H.), Région Rhône-Alpes program CIBLE 2009 (to F. P.).

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Figure legends

Fig. 1: Deficiency in Grx3/4 is associated with defects in iron-dependent enzymes.

(A) Wild-type (WT), Gal-GRX4, *grx3* Δ (strain background W303-1A), and *grx3/4* Δ (strain background BY4742) were grown in SD medium for 40 h. Tenfold serial dilutions were spotted onto YPEG plates and incubated for 2 days at 30°C. (B) WT, Gal-GRX4 and Gal-SSQ1 strains harbouring 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, and cell extracts were assayed for aconitase and catalase activities, or (C) analyzed for the indicated proteins by immunostaining. (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 h and 64 h, and from Gal-GRX4 cells expressing *GRX4* from vector pCM189 (+Grx4). Error bars indicate the SEM ($n \geq 4$).

Fig. 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 μ Ci ^{55}Fe for 2 h. 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 h, and 64 h (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 h or 64 h 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 h 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 ($n \geq 4$).

Fig. 3: Deficiency in Grx3/4 results in cytosolic iron and GSH accumulation.

The metal content of (A) wild-type (WT) and Gal-GRX4 cells (depleted for 64 h) and (B) mitochondria isolated from the indicated strains was determined by ICP-MS. (C) The indicated strains lacking (*ccc1* Δ) or overproducing Ccc1 (*Ccc1* \uparrow) were cultivated in SD medium for 40 h.

Tenfold serial dilutions were spotted onto SC agar plates containing glycerol (Glyc) or glucose (Glc), and cultivated at 30°C under aerobic (+O₂) or anaerobic (-O₂) conditions. **(D)** WT, Gal-GRX4 and Gal-GRX4/*ccc1Δ* cells were grown in SD medium for 64 h, 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 h or 64 h) and Gal-GRX4 cells expressing *GRX4* from a plasmid (+Grx4).

Fig. 4: Deficiency in Grx3/4 leads to functional impairment of di-iron enzymes.

(A) Permeabilised wild-type (WT) and Grx4-depleted Gal-GRX4 cells were assayed for specific ribonucleotide reductase activity (see also Fig. S3). **(B)** WT and Gal-GRX4 cells were grown in SD medium for 40 and 64 h, radiolabeled, and ⁵⁵Fe binding to Rnr2 was analyzed by immunoprecipitation and scintillation counting. Protein levels of Rnr2 and Por1 were determined by immunoblotting (insert). **(C)** The substrate (demethoxyubiquinol DMQ₆) and product (ubiquinone CoQ₆) of mitochondrial mono-oxygenase Coq7 were analyzed by electrochemical detection coupled to HPLC separation of mitochondrial lipid extracts from WT and Grx4-depleted Gal-GRX4 cells. CoQ₄ is a commercial standard. **(D)** Ratio of DMQ₆ and CoQ₆ levels in mitochondria isolated from WT, Gal-GRX4 cells (depleted for 40 h or 64 h) containing either vector pCM189 or pCM189-GRX4 (+Grx4). **(E)** WT and Grx4-depleted Gal-GRX4 cells overproducing Fe-only superoxide dismutase from *E. coli* (FeSod) were analyzed for superoxide dismutase in-gel activities and FeSod by immunoblotting.

Fig. 5: Grx3/4 assemble a bridging Fe/S cluster independently of the CIA machinery.

(A) ⁵⁵Fe binding to Grx3/4 was determined in wild-type (WT), *grx3Δ*, *grx4Δ* and *grx3/4Δ* cells (strain background BY4742). Grx3/4 protein levels were assessed by immunostaining. Por1 served as a loading control. The anti-Grx4 antiserum recognizes an unspecific band, in addition to Grx3/4. **(B)** ⁵⁵Fe binding to Grx3/4 was determined in WT and *sod1Δ* cells (strain background W303-1A). Where indicated, cells were pretreated with 1 mM H₂O₂ for 10 min or examined under anaerobic (-O₂) conditions. **(C, D)** ⁵⁵Fe binding to Grx3/4 was determined in WT, Gal-NFS1 (with and without a plasmid expressing a cytosolic version of Nfs1 (+ΔN-NFS1)), Gal-ISD11, Gal-ISU1 **(C)** or Gal-NBP35 and Gal-DRE2 cells **(D)** after growth in SD medium for 40 h. BY4742 *grx3/4Δ* cells served as a background control. Grx3/4 and Por1 levels were assessed by immunostaining. **(E)** Top: Schematic presentation of the domain structure of Grx4. Middle: C-terminally Myc-tagged WT, C37S, C171S, or C171A mutant Grx4 were expressed in WT cells from vector pCM189, and ⁵⁵Fe binding to Grx4 was

determined by radiolabelling and immunoprecipitation with α -Myc beads. Bottom: Grx4-Myc and Por1 levels were assessed by immunostaining. **(F)** WT and *gsh1* Δ cells overproducing Grx4-Myc were cultivated in the presence or absence of 1 mM GSH for 64 h (Sipos et al., 2002). ^{55}Fe binding to Grx4-Myc was determined by immunoprecipitation with α -Myc beads. **(G)** Extracts of WT cells expressing C-terminally HA-tagged Grx4 and wild-type or C171S Grx4-Myc as indicated were subjected to immunoprecipitation (IP) with α -Myc or α -HA-antibodies. The precipitate was analyzed by immunostaining with α -HA or α -Grx4 antibodies. The arrow (\uparrow) indicates a lane with a molecular mass marker which is stained by the α -HA antiserum.

Fig. 6: The active-site cysteine of the Grx domain is essential for iron metabolism. Gal-GRX4 cells lacking (-) or expressing wild-type (WT) Grx4-Myc or the Grx4-Myc mutant proteins C37S, C171S or C171A under the control of the *GRX4* **(A)** or *tetO₇* **(C)** promoter from vector pCM189 were cultivated in SD medium. After 40 h, tenfold serial dilutions were spotted onto SC medium with glycerol. The levels of Grx4-Myc, endogenous Grx4, and cytosolic Hsp70 were assessed by immunostaining. **(B, D)** After 64 h cells from A and C were analyzed for *FET3* promoter activities, or aconitase and catalase enzyme activities.

Fig. 1; Mühlenhoff et al.

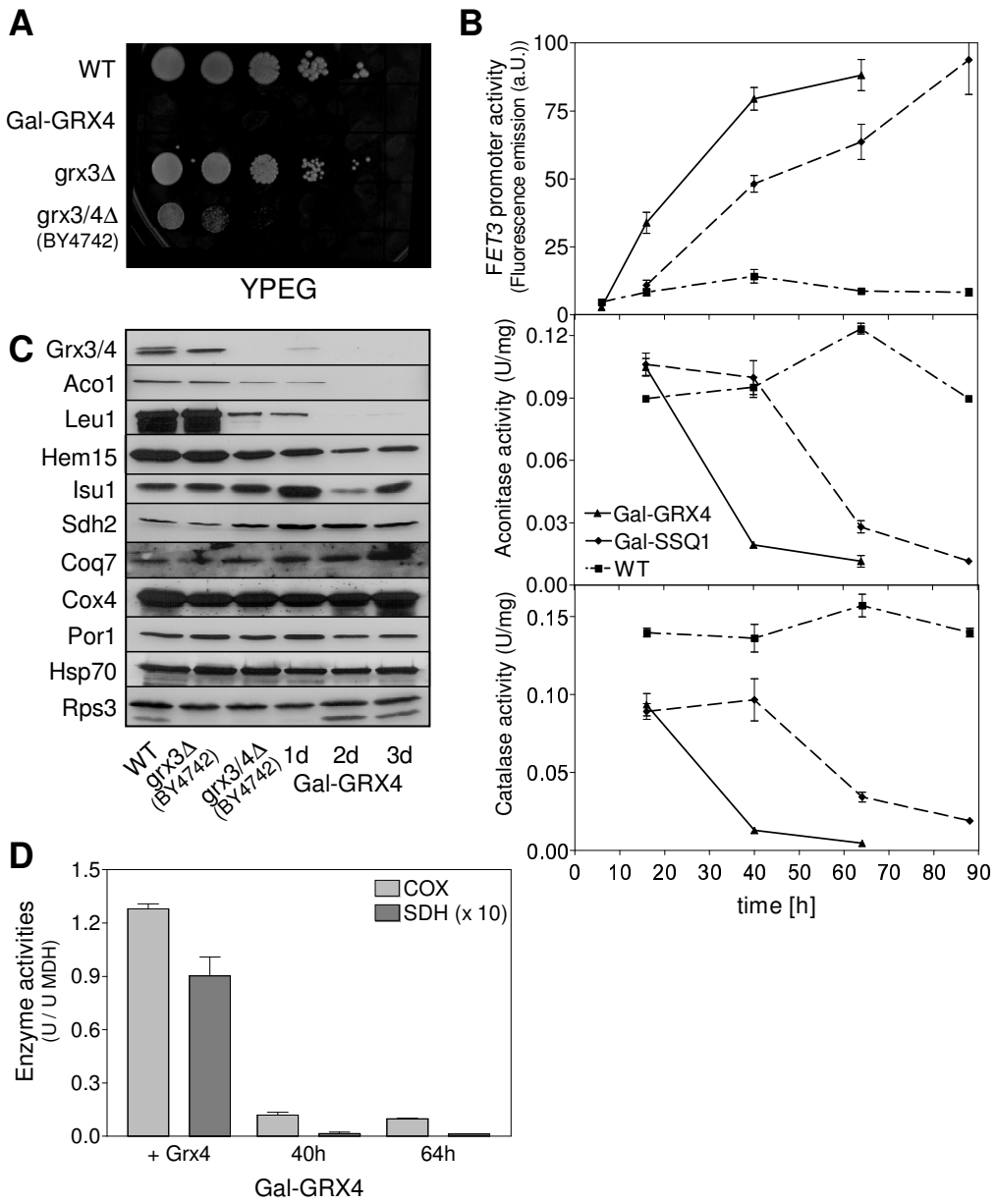


Fig. 2; Mühlhoff et al.

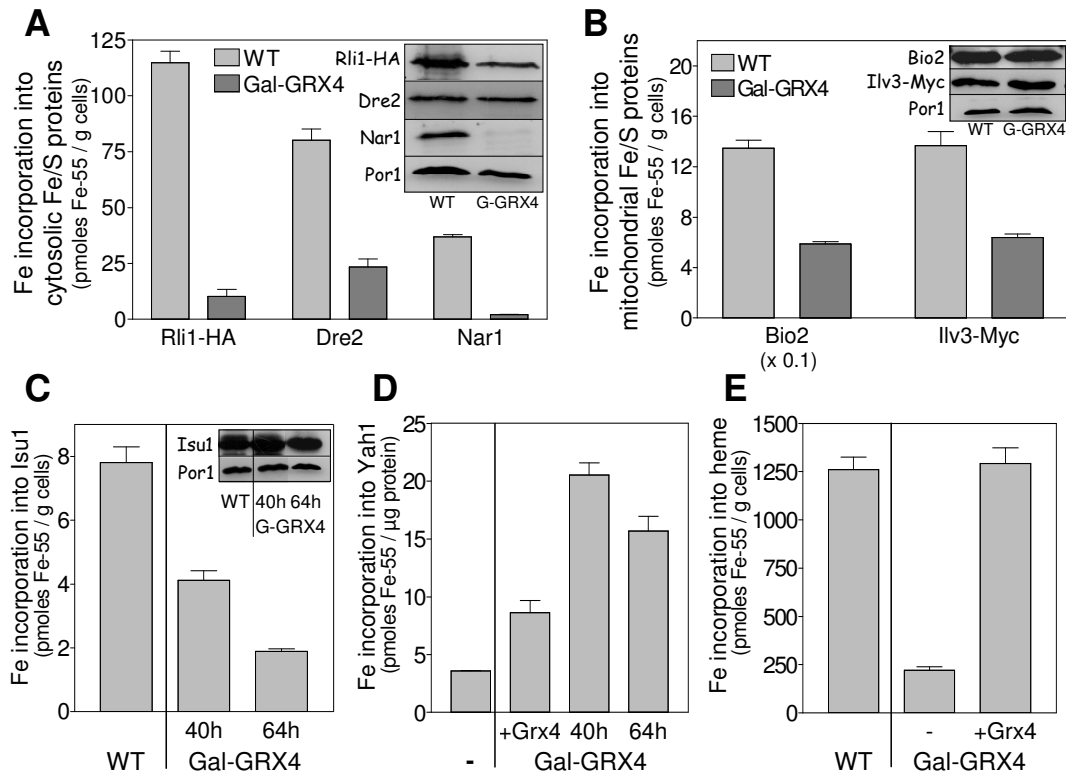


Fig. 3; Mühlhoff et al.

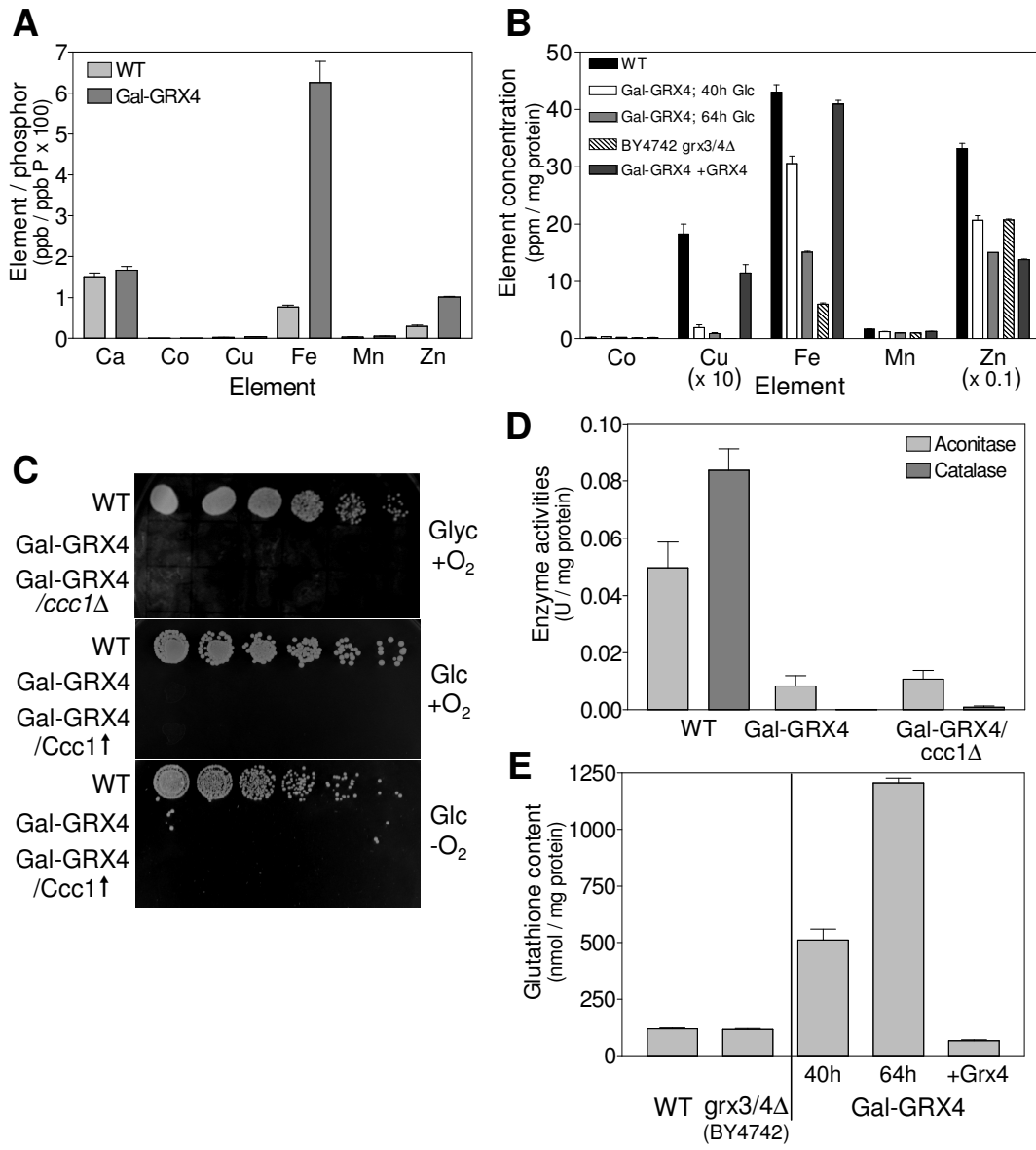


Fig. 4; Mühlhoff et al.

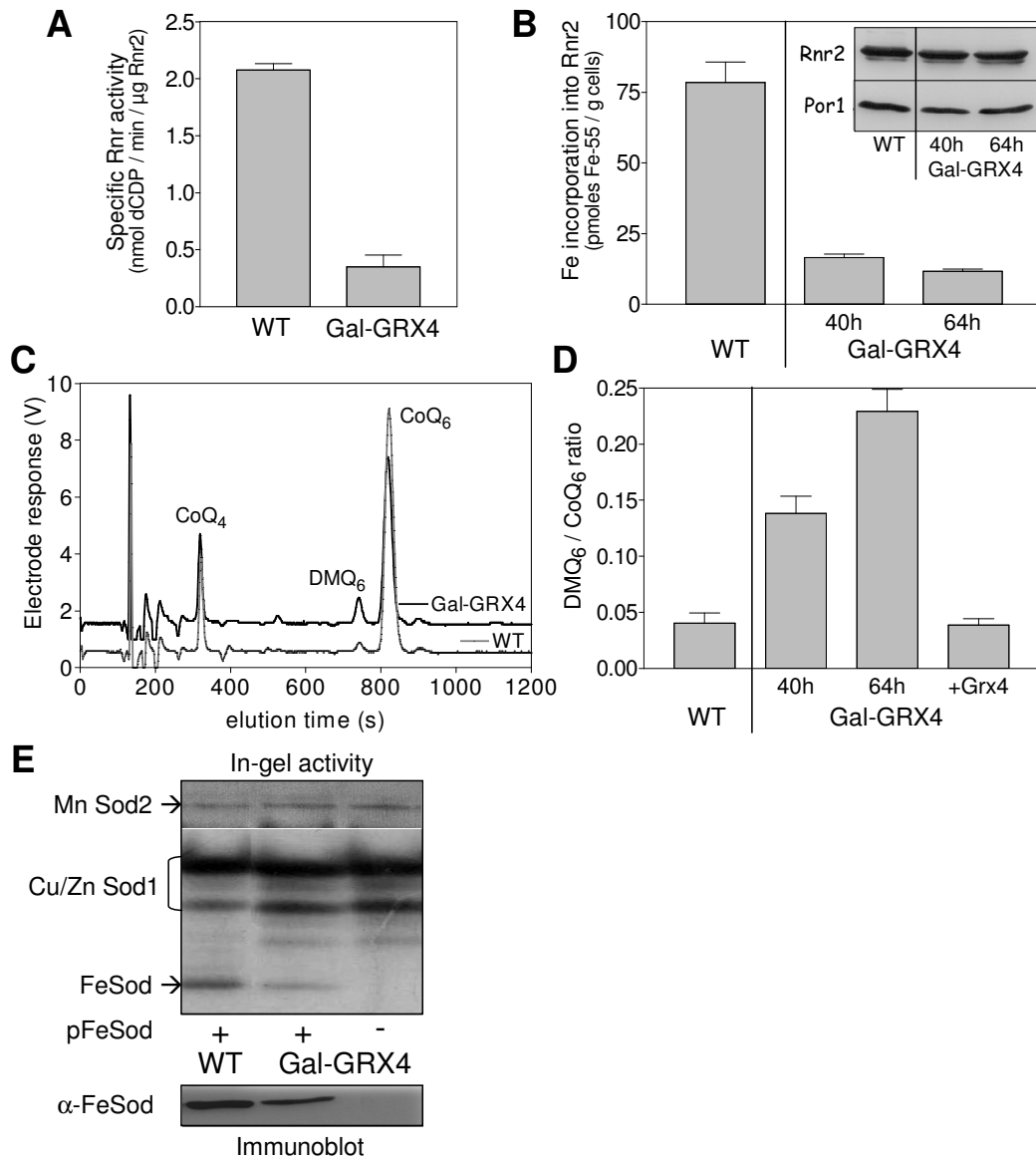


Fig. 5; Mühlhoff et al.

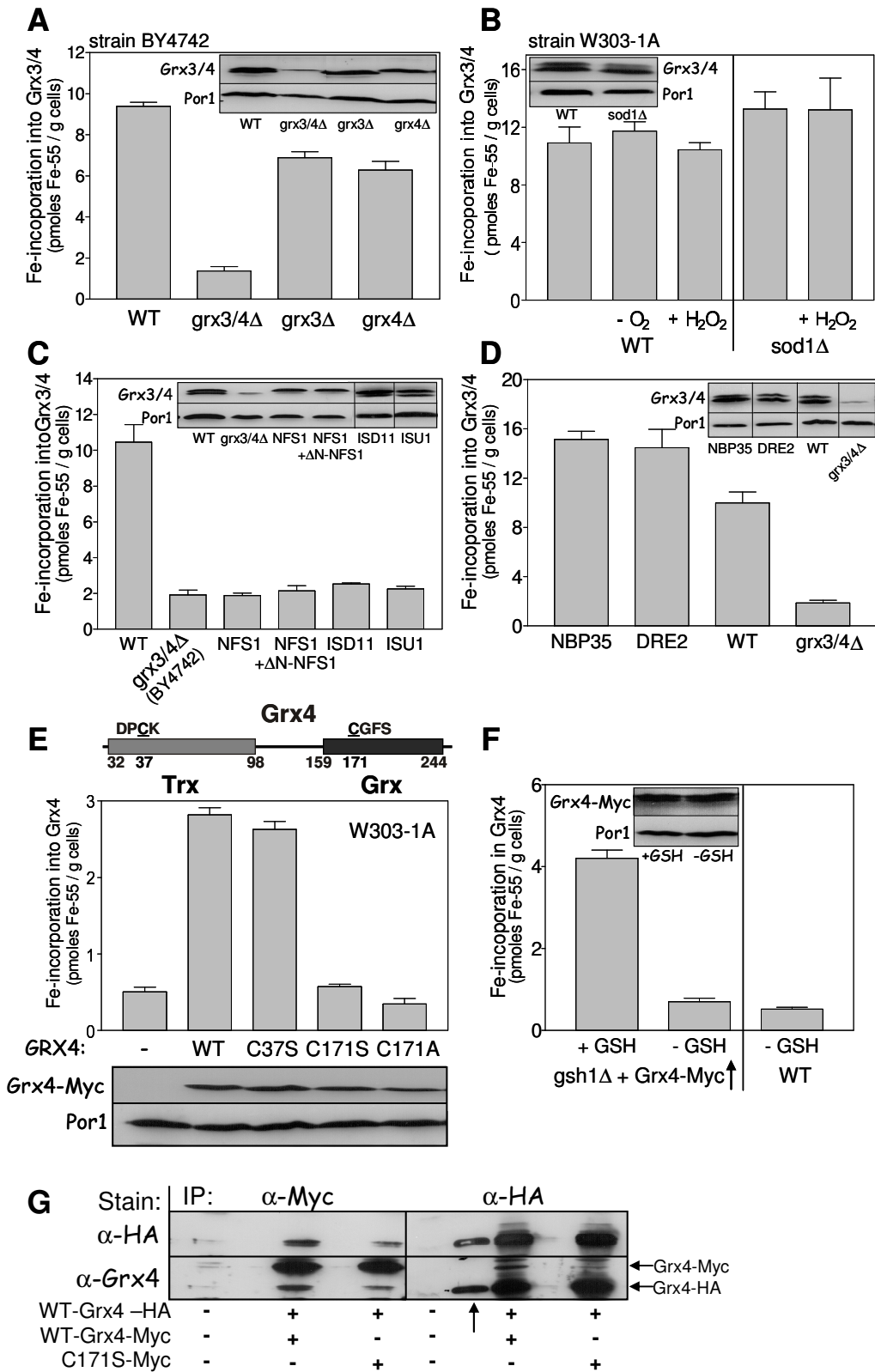
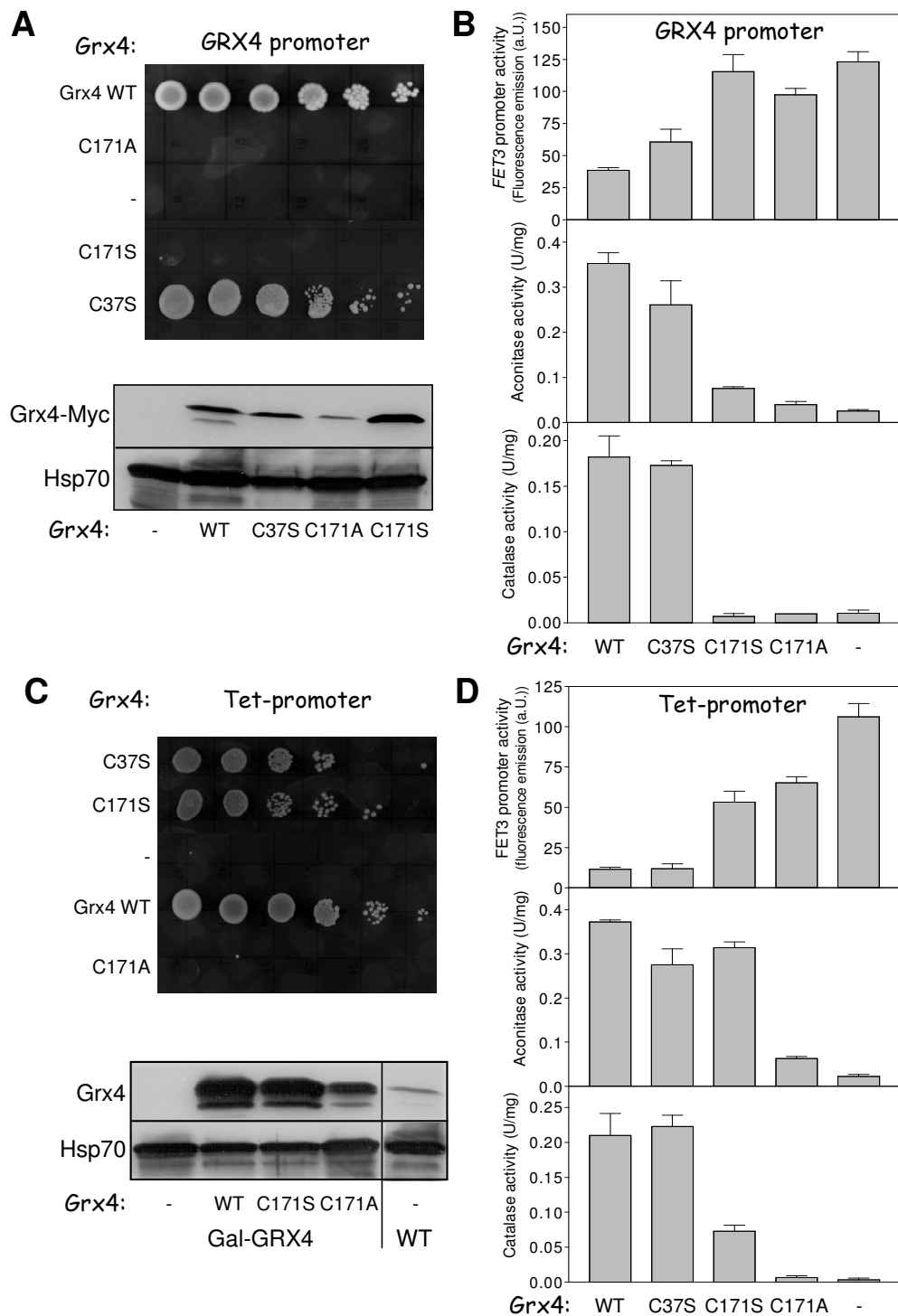


Fig. 6; Mühlhoff et al.



Supplemental Information to Mühlenhoff et al.

Inventory:

(1) Supplemental Data (5 Figures)

Fig. S1, related to Figure 1.

Fig. S2, related to Figure 3.

Fig. S3, related to Figure 4.

Fig. S4, related to Figure 5.

Fig. S5, Working model of monothiol glutaredoxin function in iron metabolism (related to Discussion)

(2) Supplemental Experimental Procedures

2 Tables listing strains and plasmids used in this study

(3) Supplemental References

Supplemental Figure S1

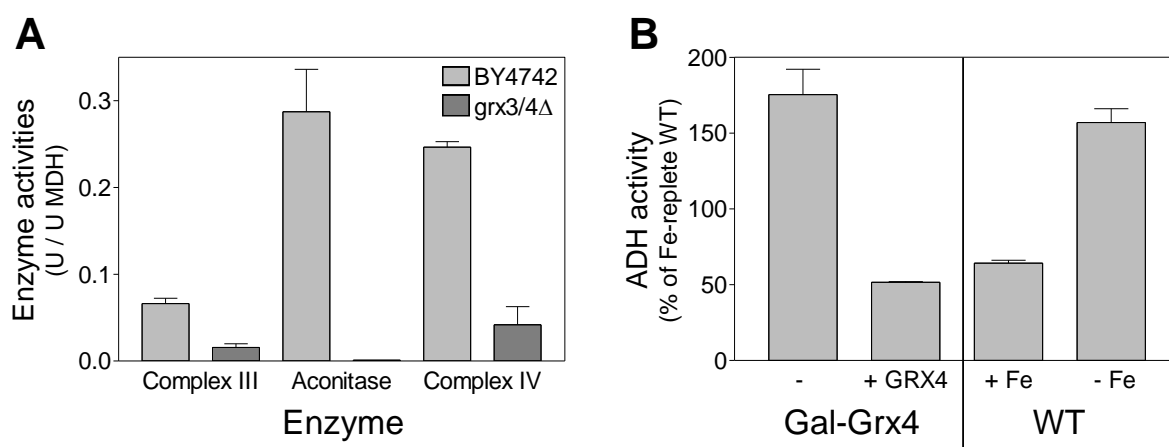


Figure S1. Grx4 depletion induces multiple iron-related defects. (Related to Figure 1 and Figure 3.) (A) Activities of aconitase and respiratory complexes III and IV were determined in mitochondria isolated from BY4742 wild-type and isogenic *grx3/4Δ* cells. (B) Alcohol dehydrogenase (ADH) activities were determined in extracts from Gal-GRX4 cells harboring plasmid pCM189 (-) or pCM189GRX4 (+ GRX4) cultivated for 64 h in iron-replete minimal medium. W303-1A wild type cells (WT) cultivated in iron-replete (+ Fe (50μM)) or iron-depleted minimal medium in the presence of 50μM bathophenanthroline (-Fe) were analyzed in parallel. Error bars indicate the SEM ($n \geq 4$).

Supplemental Figure S2

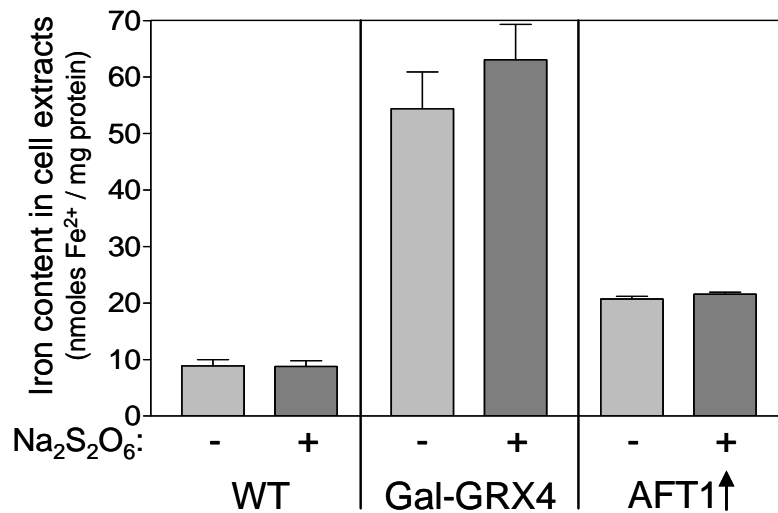


Figure S2. Grx3/4-depleted cells accumulate chelatable, ferrous iron. (Related to Figure 3.) W303-1A wild-type (WT), Gal-GRX4 cells and WT cells overproducing Aft1 from vector p415-MET25-FET3-GFP were cultivated for 40 h in iron-replete minimal SD medium and for 16 h in SD medium supplemented with 100 μ M Fe³⁺. Cell extracts were prepared by mechanical disruption with glass beads under anaerobic conditions on ice and the amount of ferrous Fe²⁺ in the extracts was quantified by the bathophenanthroline method before (-) and after (+) reduction with sodium dithionite (Na₂S₂O₆) (Kispal et al., 1999; Molik et al., 2007). Error bars indicate the SEM (n ≥ 4).

Supplemental Figure S3

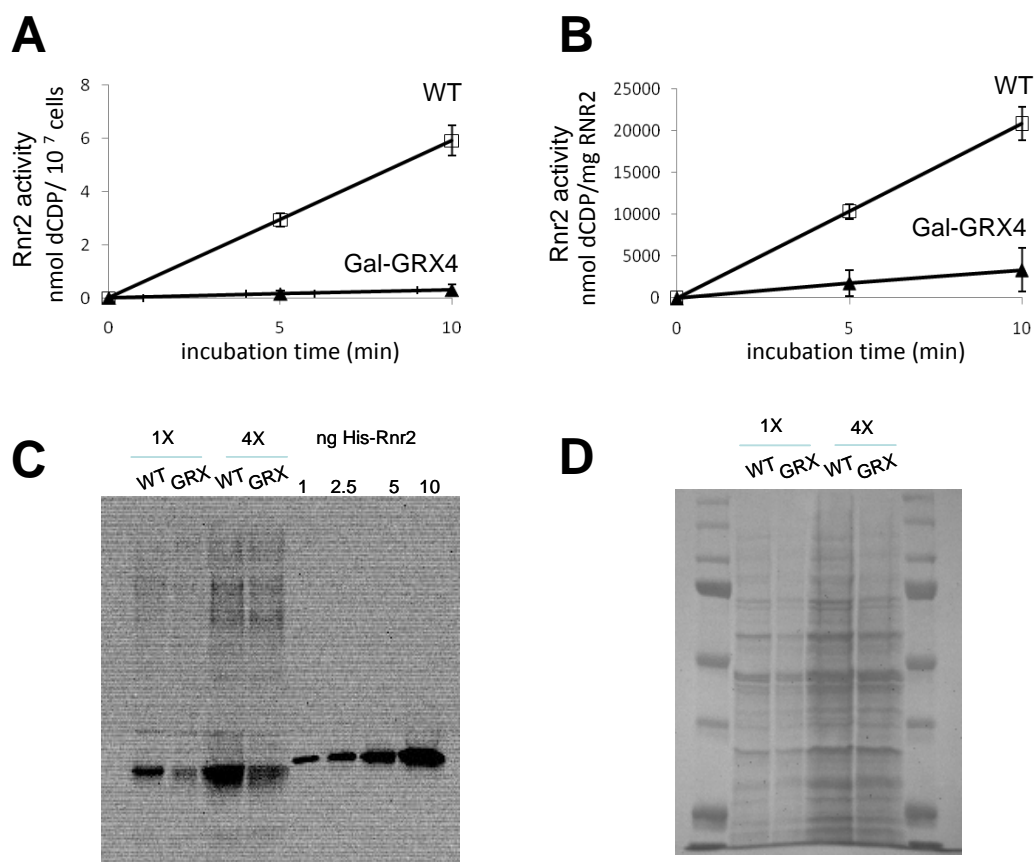


Figure S3. Low levels of Grx3/4 impair the enzymatic function of ribonucleotide reductase. (Related to Figure 4.) W303-1A wild type (WT) and Gal-GRX4 cells (GRX) were grown in 50 ml SD medium for 16 h, diluted into 500 ml of the same medium, and cultivated for 5 h. **(A, B)** Cells were harvested at log phase, permeabilized and the activity of the small subunit of ribonucleotide reductase (Rnr2) was determined. Assay mixtures contained 3.3×10^7 cells, 10 mM NaF, 50 mM DTT, 1 mM ATP, 80 μ g purified recombinant Rnr1 (specific activity 100 nmol min⁻¹ mg⁻¹), 1 mM ³H-CDP (3,400 cpm/nmol). Then, 0.1 M KPO₄ and 0.6 M sorbitol, pH 7.7 was added to give a total volume of 240 μ l. After 0, 5, and 10 min, 80 μ l aliquots were withdrawn, heat inactivated, and dCDP formation was quantified as described (Steeper and Stuart, 1970). Rnr small subunit activities were normalized to cell number **(A)** or Rnr2 protein levels **(B)**. **(C)** Rnr2 protein levels were determined by Western blotting with purified recombinant His-Rnr2 (1-10 ng) as a standard. **(D)** Whole cell protein contents were visualized by amido black staining. Standard deviations were calculated from duplicate measurements of three independent preparations.

Supplemental Figure S4

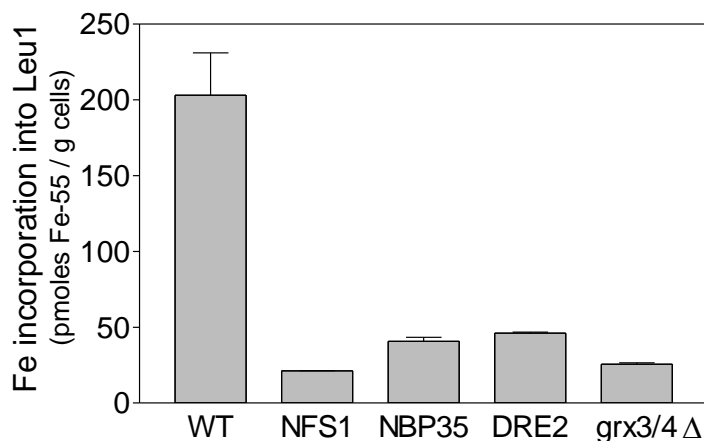


Figure S4. Maturation of the cytosolic Fe/S protein Leu1 iron requires the components of both the ISC and CIA systems and Grx3/4. (Related to Figure 5.) ⁵⁵Fe-binding to endogenous Leu1 was determined in W303-1A wild type (WT), the isogenic Gal-strains Gal-NFS1, Gal-NBP35 and Gal-DRE2 cells after respective protein depletion for 40 h, and in BY4741 grx3/4Δ cells (Molik et al., 2007; Pierik et al., 2009). The analysis was carried out on the same samples that were used for estimating iron binding to Grx4 in Fig. 5. Error bars indicate the SEM (n ≥ 4).

Supplemental Figure S5

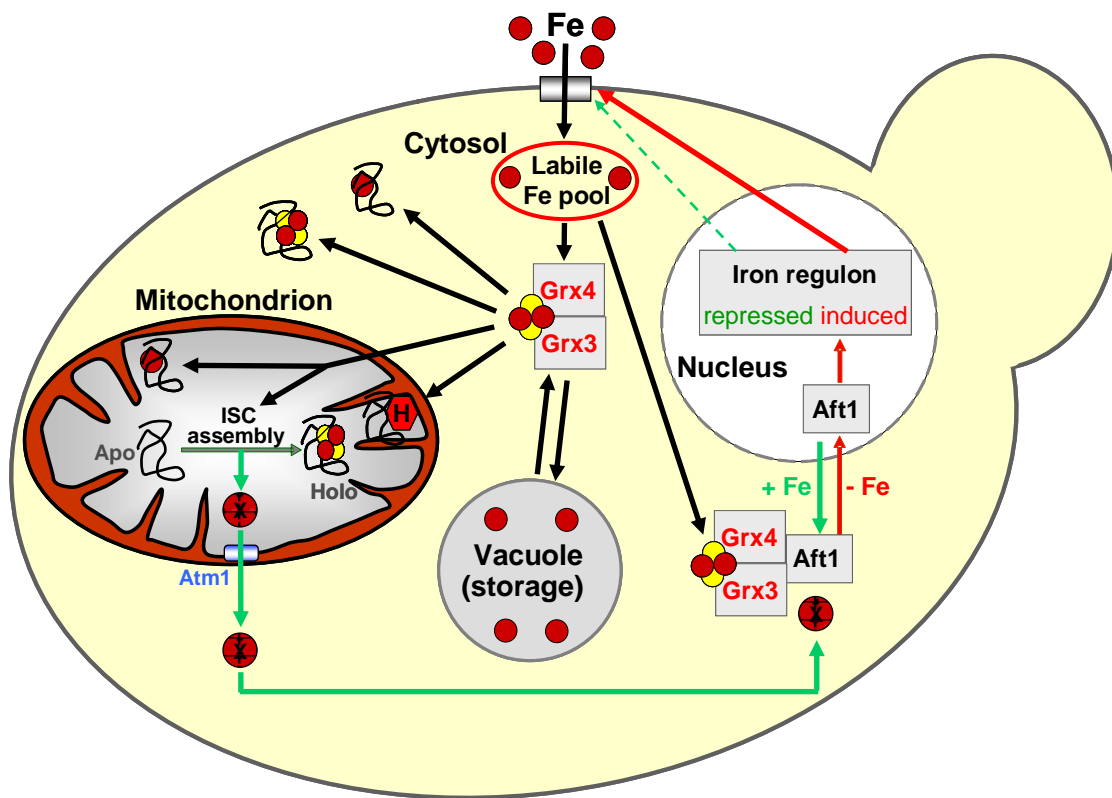


Figure S5. Monothiol glutaredoxins Grx3/4 function in iron trafficking and sensing. (Related to Discussion.) After iron import into the cell's cytosol the monothiol glutaredoxins Grx3/4 mediate the specific trafficking of iron from a hypothetical labile iron pool to iron-dependent enzymes and cell organelles including mitochondria where heme (H) and Fe/S cluster synthesis occurs (black arrows; (Lill, 2009)). The Grx3/4-bound Fe/S cluster (yellow and red circles) is essential for this trafficking function. Regulation of iron uptake and intracellular iron distribution involves the iron-responsive transcription factor Aft1, Grx3/4, and a mitochondrial signaling molecule (X) that is exported by the ABC transporter Atm1 to signal the mitochondrial iron status (red and green arrows). Under iron-limiting conditions, or in the absence of Grx3/4 or X, Aft1 translocates into the nucleus, where it acts as transcriptional activator of the iron regulon encoding proteins involved in iron uptake and distribution. The Fe/S cluster of Grx3/4 functions as the sensor of cytosolic iron levels. See text and (Kaplan and Kaplan, 2009; Lill and Muhlenhoff, 2008) for further details.

Supplemental Experimental Procedures

Supplemental Table I: Yeast Strains Used in This Study

Strain	Genotype	Method of Generation	Source/Reference
W303-1A	<i>MATa; ura3-1; ade2-1; trp1-1; his3-11,15; leu2-3,112</i>		(Mortimer and Johnston, 1986)
BY4742	<i>MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0</i>	obtained from Euroscarf	(Brachmann et al., 1998)
<i>grx3Δ</i>	BY4742, <i>grx3::KanMX4</i>	obtained from Euroscarf	(Ojeda et al., 2006)
W303-1A <i>grx3Δ</i>	W303A, <i>grx3::LEU2</i>	PCR Fragment (pUG73) (Gueldener et al., 2002)	this work
<i>grx4Δ</i>	BY4742, <i>grx4::KanMX4</i>	obtained from Euroscarf	(Ojeda et al., 2006)
<i>grx3/4Δ</i>	BY4742, <i>grx3::LEU2</i> <i>grx4::KanMX4</i>	<i>grx4Δ</i> ; PCR Fragment (pUG73)	(Ojeda et al., 2006)
<i>gsh1Δ</i>	YPH500; <i>gsh1::HIS3</i>	PCR Fragment (pFA6a-HIS3) (Euroscarf)	(Sipos et al., 2002)
<i>sod1Δ</i>	BY4742, <i>sod1::KanMX4</i>	obtained from Euroscarf	
Gal-GRX4 <i>/grx3Δ</i>	W303-1A, <i>pGRX4::GAL-L-natNT2; grx3::LEU2</i>	W303 <i>grx3Δ</i> ; PCR fragment (pYM-N27) (Janke et al., 2004)	this work
Gal-GRX4 <i>/grx3Δ ccc1Δ</i>	W303-1A, <i>pGRX4::GAL1-10-HIS3; grx3::LEU2; ccc1::HIS3</i>	Gal-Grx4 <i>/grx3Δ</i> PCR fragments (pFA6a-HIS3)	this work
Gal-DRE2	W303-1A, <i>pNBP35::GAL-L-natNT2</i>	PCR fragment (pYM-N27) (Janke et al., 2004)	this work
Gal-NBP35	W303-1A, <i>pNBP35::GAL1-10-HIS3</i>	PCR fragment (pFA6a-HIS3-Gal) (Muhlenhoff et al., 2003)	(Hausmann et al., 2005)
Gal-ISU1/ <i>isu2Δ</i>	W303-1A, <i>pISU1::GAL1-10-HIS3, isu2::LEU2</i>	PCR fragments (pFA6a-HIS3-Gal, pUG73)	(Gerber et al., 2004)
Gal-NFS1	W303-1A, <i>pNFS1::GAL1-10-HIS3</i>	PCR fragment (pFA6a-HIS3-Gal)	(Muhlenhoff et al., 2003)
Gal-ISD11	W303-1A, <i>pISD1::GAL1-10-HIS3</i>	PCR fragment (pFA6a-HIS3-Gal)	(Wiedemann et al., 2006)
Gal-SSQ1	W303-1A, <i>pSSQ1::GAL1-10-LEU2</i>	PCR fragment (pTL26)	(Muhlenhoff et al., 2003)

Gene disruptions and promoter exchanges were generated by PCR-based gene replacement and verified by PCR as described previously (Gueldener et al., 2002; Muhlenhoff et al., 2002b). Yeast cells were transformed by the lithium acetate method (Gietz and Woods, 2002).

Supplemental Table II. Plasmid Constructs Used in This Study.

plasmid	ORF	backbone	Source/Reference
p426- <i>BIO2</i>	<i>BIO2</i>	p426- <i>TDH3</i>	(Muhlenhoff et al., 2002a)
p426- <i>DRE2</i>	<i>DRE2</i>	p426- <i>TDH3</i> (Funk et al., 2002)	this work.
p426- <i>ISU1</i>	<i>ISU1</i>	p426- <i>TDH3</i>	(Muhlenhoff et al., 2003)
p426- <i>NAR1</i>	<i>NAR1</i>	p426- <i>TDH3</i>	(Balk et al., 2004)
p416- Δ N- <i>NFS1</i>	<i>NFS1</i> ; N-terminal 33 residues deleted.	p416- <i>MET25</i> (Funk et al., 2002)	(Kispal et al., 1999)
p426- <i>RLI1</i> -HA	<i>RLI1</i> ; C-terminal HA	p426- <i>TDH3</i>	(Kispal et al., 2005)
pFET3- <i>GFP</i>	<i>GFP</i>	p416- <i>MET25</i>	(Hausmann et al., 2008)
pFe-Sod	<i>E.coli sod1</i>	p426- <i>TDH3</i>	this work
pET3d-GRX4	<i>GRX4</i> ; N-terminal His-tag	pET3d	this work
p426- <i>CCC1</i>	<i>CCC1</i>	p426- <i>TDH3</i>	this work
pCM189-Grx4	<i>GRX4</i> ; C-terminal Myc-tag	pCM189 (Gari et al., 1997)	(Ojeda et al., 2006)
pCM189-Grx4-C37S	<i>GRX4</i> ; C-terminal Myc-tag; C37S	pCM189-Grx4	this work
pCM189-Grx4-C171S	<i>GRX4</i> ; C-terminal Myc-tag; C171S	pCM189-Grx4	this work
pCM189-Grx4-C171A	<i>GRX4</i> ; C-terminal Myc-tag; C171A	pCM189-Grx4	this work
pGRX4-Grx4	<i>GRX4</i> ; C-terminal Myc-tag;	pCM189-Grx4; pGRX4 (422bp upstream) in <i>Sma</i> I and <i>Hind</i> III sites	this work
pGRX4-Grx4-C37S	<i>GRX4</i> ; C-terminal Myc-tag; C37S	pGRX4-Grx4	this work
pGRX4-Grx4-C171S	<i>GRX4</i> ; C-terminal Myc-tag; C171S	pGRX4-Grx4	this work
pGRX4-Grx4-	<i>GRX4</i> ; C-terminal Myc-tag;	pGRX4-Grx4	this work

C171A	C171A		
pGRX4-HA	<i>GRX4</i> ; C-terminal HA-tag; TetO7 promoter;	pMM518; Integrative <i>LEU2</i>	(Pujol-Carrion et al., 2006)

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