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ORIGINAL RESEARCH COMMUNICATION

The Multidomain Thioredoxin-Monothiol Glutaredoxins Represent a Distinct Functional Group

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Abstract

Monothiol glutaredoxins (Grxs) with a noncanonical CGFS active site are found in all kingdoms of life. They include members with a single domain and thioredoxin-Grx fusion proteins. In *Saccharomyces cerevisiae*, the multidomain Grx3 and Grx4 play an essential role in intracellular iron trafficking. This crucial task is mediated by an essential Fe/S cofactor. This study shows that this unique physiological role cannot be executed by single domain Grxs, because the thioredoxin domain is indispensable for function *in vivo*. Mutational analysis revealed that a CPxS active site motif is fully compatible with Fe/S cluster binding on Grx4, while a dithiol active site results in cofactor destabilization and a moderate impairment of *in vivo* function. These requirements for Fe/S cofactor stabilization on Grx4 are virtually the opposite of those previously reported for single domain Grxs. Grx4 functions as iron sensor for the iron-sensing transcription factor Aft1 in *S. cerevisiae*. We found that Aft1 binds to a conserved binding site at the C-terminus of Grx4. This interaction is essential for the regulation of Aft1. Collectively, our analysis demonstrates that the multidomain monothiol Grxs form a unique protein family distinct from that of the single domain Grxs. *Antioxid. Redox Signal.* 15, 19–30.

Introduction

Glutaredoxins (Grxs) are members of the highly conserved thioredoxin (Trx) fold superfamily (20, 22, 36). Classical Grxs are small glutathione (GSH): disulfide-oxidoreductases that primarily catalyze glutathionylation and deglutathionylation of target proteins (7, 19, 20, 22, 36, 42, 55, 60). Classical Grxs usually contain a dithiol CP(Y/F)C motif in their active site. By contrast, monothiol Grx with only one cysteinyl side chain in a CGFS active site motif do rarely possess oxidoreductase activity (18, 36, 55). Instead, they are capable of binding a (2Fe/2S) cluster that bridges two Grx monomers and that is coordinated by the active site cysteine residues and two molecules of GSH (18, 36, 51). This capability to bind iron–sulfur (Fe/S) clusters is shared by several dithiol Grxs with noncanonical dithiol C(S/G)(Y/F)C active site motifs such as poplar GrxC1 and human Grx2 (14, 37, 58). In photosynthetic organisms, further potential Fe/S cofactor-binding Grx variants exist (11, 55). To date, two physiological roles for monothiol Grxs with Fe/S cofactors have been identified. They are involved in the biogenesis of cellular Fe/S proteins (57) and they play a central role in cytosolic iron trafficking (47). For the identification of these physiological functions, the analysis in baker's yeast *Saccharomyces cerevisiae* has been fundamental.

S. cerevisiae harbors two classical cytosolic dithiol Grxs (Grx1 and Grx2) that are involved in protection against oxidative stress (19, 20, 39), an unusual dithiol Grx (Grx8) (13), three monothiol Grxs (Grx3, Grx4, and Grx5) with a canonical CGFS-active site (53) and two with a noncanonical CSYS- (Grx6) and CPYS-active site motifs (Grx7) (26, 41). The latter are cis-Golgi localized monothiol Grxs that are capable of catalyzing dithiol-disulfide redox reactions similar to dithiol Grxs.

The mitochondrial monothiol Grx5 is conserved throughout the eukaryotes and most likely of bacterial origin (18). In *S. cerevisiae*, Grx5 plays a crucial role in the protection of mitochondria against oxidative stress (19, 53). In addition, its deletion is associated with low activities and assembly defects of Fe/S cofactor containing enzymes, indicating that Grx5 is involved in the maturation of cellular Fe/S proteins (35, 46, 53, 63). This function is conserved in vertebrates (8, 64, 66). Grx5 and other monothiol Grxs with CGFS active site motifs bind a labile 2Fe/2S cofactor that can be mobilized and inserted into other Fe/S proteins *in vitro* (4, 25, 28, 51, 55, 57). Grx5 can be functionally replaced by other Fe/S cluster-binding Grxs, collectively suggesting that the mitochondrial monothiol Grxs may function as scaffolds for the *de novo* synthesis of Fe/S clusters or as transfer protein that mediate the insertion of Fe/S cofactors into recipient apo-proteins (3, 4, 45, 57).

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The cytosolic monothiol glutaredoxins Grx3 and Grx4 (Grx3/4) of *S. cerevisiae* are highly homologous proteins that belong to a subgroup of the Grxs consisting of an N-terminal Trx domain and one (fungi), two (vertebrates) or three (plants) C-terminal monothiol Grx domains (1, 18, 55). Monothiol Grxs with this multidomain architecture are restricted to eukaryotes. Low levels of Grx3/4 induce the loss of function of, virtually, all cellular Fe-dependent pathways that eventually results in cell death. This loss of function is caused by a general failure in the insertion of iron into iron-dependent proteins and mitochondria despite high intracellular iron levels, indicating that *S. cerevisiae* Grx3/4 play a central role in cytosolic iron trafficking (47). Grx3/4 bind iron in form of an Fe/S-cofactor in their native environment *in vivo*. Defects in iron-binding induce the same lethal phenotype as cells lacking Grx3/4 altogether. *In vivo* iron binding has been demonstrated for human Grx3 (PICOT), suggesting that the role of the multidomain Grxs in iron trafficking is likely conserved (16).

Grx3 and Grx4 play a central role in the regulation of cellular iron homeostasis of *S. cerevisiae*. They directly interact with the iron-responsive transcription factor Aft1 that plays a central role in the adaptation to iron-depriving conditions (29, 50, 59). The majority of genes controlled by Aft1 encode products that function in cellular iron uptake or iron transport across intracellular membranes. Aft1 shuttles between the cytosol and nucleus in an iron-responsive manner and acts as transcriptional activator (29, 50, 59, 65). In the absence of Grx3/4 or its bound Fe/S cofactor, Aft1 is constitutively activated, indicating that the Grx3/4 bound Fe/S cofactor functions as the cytosolic iron sensor for Aft1. The regulatory role of the cytosolic monothiol Grx in cellular iron homeostasis is functionally conserved in fungi that utilize iron regulatory systems that are unrelated to those from *S. cerevisiae* (27, 31, 40).

Here we report a detailed *in vivo* structure/function analysis of the cytosolic monothiol glutaredoxin Grx4 in *S. cerevisiae*. Mutational analysis of CGFS active site residues of Grx4 revealed different requirements for the stabilization of the Fe/S cofactor on Grx4 *in vivo* than previously reported for single domain Grxs. In addition, we show that single domain Grxs cannot functionally complement the multidomain Grx4, because the N-terminal Trx domain is essential for *in vivo* function. Collectively, our analysis strongly suggests that the multidomain monothiol Grx represent a unique subclass within the Trx protein family that is functionally distinct from that of the single domain Grxs.

Materials and Methods

Yeast wild type strain W303-1A, grx5Δ, Gal-GRX4/grx3Δ (W303-1A, *pGRX4::GAL-L-natNT2; grx3::LEU2*), and Gal-GRX4/AFT1-lux cells (Gal-GRX4/grx3Δ, *AFT1-luc2::HIS3)* were cultivated in minimal medium containing all recommended supplements (synthetic complete medium [SC]), 2% w/v glucose (synthetic complete medium with glucose) or 3% w/v glycerol (61). Iron-replete media were supplemented with 50 μM $FeCl_3$. Minimal medium lacking $FeCl_3$ was obtained from Formedium. Gal-GRX4 cells were depleted for Grx4 to critical levels by cultivation in synthetic complete medium with glucose for 64 h prior to analysis. For growth assays, cells were depleted for 40 h, 1:5 serial dilutions were spotted to SC plates supplemented with glucose, galactose, or glycerol and incubated for 2–3 days. *S. cerevisiae* and *S. pombe* Grx4 were expressed from vector pCM189 under the control of the $tetO_7$ promoter or the endogenous *GRX4* promoter. *S. cerevisiae* Grx4 was expressed with a C-terminal Myc-tag. Site-directed mutants were generated by PCR-mediated nucleotide exchange using appropriate primers (67), chimeric constructs by PCR-driven overlap extension (17). Human Grx3 and *S. cerevisiae* Grx5 were expressed from vector p416MET25 and *D. melanogaster* Grx3 from p426TDH3 (Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/ars)

In vivo radio-labeling of yeast with $^{55}FeCl_3$ (Perkin-Elmer) and measurement of Fe-55 incorporation into proteins by immunoprecipitation was carried out as described (43). Antibodies were raised in rabbits against recombinant proteins. Antibodies against C-Myc or HA were obtained from Santa-Cruz. Enzyme activities of aconitase and catalase, and the promoter strength of the *FET3* promoter using *FET3*-GFP reporter plasmids were determined as described (43). *In vitro* Fe/S cluster reconstitutions were performed according to (6). For analysis of Aft1-Grx4 interactions, Myc-tagged Grx4 was expressed in Gal-GRX4/grx3Δ/AFT1-Lux cells that harbor the endogenous *AFT1* gene fused to a C-terminal luciferase gene. Cells were depleted for endogenous Grx4, Grx4-Myc was immunoprecipitated with α-Myc antibodies as described (43) and coimmunoprecipitated luciferase-derived luminescence was quantified in a multiplate reader (Infinite M200, TECAN). Error bars represent the standard error of the mean ($n > 4$).

Results

Analysis of the Grx active site residues in iron sensing and iron delivery by Grx3/4

Previous work on the cytosolic Trx–Grx fusion protein Grx3/4 in *S. cerevisiae* showed that these proteins harbor an Fe/S cofactor that is bound on active site of the Grx domain and is essentially required for the passage of iron throughout the cytosol (47). The fact that iron-binding to *S. cerevisiae* Grx3/4 can be followed in the endogenous host *in vivo* and is associated with an essential cellular function represents a unique opportunity to systematically study structure–function relationships in monothiol Grxs. Our first aim was to precisely define the role of the Fe/S cofactor binding site in Fe/S cluster stabilization and cytosolic iron handling of Grx4 *in vivo.* For this, the amino acid residues adjacent to the conserved cysteine of the CGFS active site were systematically substituted by other naturally occurring amino acid residues and the modified proteins were expressed in yeast under the control of the native *GRX4* promoter (Table 1).

At first, we studied the effect of the substitution of glycine to proline, since recombinant Grxs with a CPFS or CPYS active site have been shown previously to virtually lose the ability to bind Fe/S cofactors *in vitro* (6, 58, 62). Grx4 proteins with CPFS, CPYS, or CGYS active sites were expressed with a C-terminal Myc-tag from a plasmid under the control of the endogenous *GRX4* promoter in the regulatable yeast strain Gal-GRX4/grx3Δ. In this strain (termed Gal-GRX4), *GRX3* is deleted and *GRX4* is expressed under the control of the glucose-repressible *GAL-L* promoter. To define the effect of these mutations on cell viability, Gal-GRX4 cells were depleted for endogenous Grx4 by cultivation in glucose containing SC minimal medium and serial dilutions were spotted onto SC

Table 1. Natural Occurrence of Glutaredoxin Active Site Motifs Used in This Study

Motif	*Example*	*Localization*	*Source*
CGFS	Grx4	Cytosol	*Saccharomyces cerevisiae*
CPFS	α-Glutathione transferase A1-1	Cytosol	*Homo sapiens*
CGYS	Nonnatural		—
CPYS	Grx7	Cis-golgi	*S. cerevisiae*
CGFC	GrxC	Cytosol	*Roseovarius*. sp. *TM1035*
CPFC	Grx C2	Cytosol	*P. trichocarpa*
CSYC	Grx2	Cytosol	*H. sapiens*
CPYC	classical dithiol	Cytosol	*Ubiquitous*

Residues that deviate from the Grx4 active site are underlined. [For an overview see ref. (11).]
Grx, glutaredoxin.

minimal media supplemented with the nonfermentable carbon source glycerol. While Grx4-depleted Gal-GRX4 cells failed to grow on this medium, the three modified Grx4 proteins supported growth of Gal-GRX4 cells at virtually the same rates as wild type Grx4 expressed from the same plasmid (Fig. 1B). Consistent with this observation, Gal-GRX4 cells expressing these mutants showed activities of the mitochondrial Fe/S protein aconitase and of the cytosolic heme protein catalase that were similar to those found in cells that expressed wild type Grx4 (Fig. 1C). To analyze iron binding to these Grx4 variants, the proteins were overproduced with a C-terminal Myc-tag under the control of the strong $tetO_7$ promoter in wild type yeast cells and radio-labeled with Fe-55 *in vivo*. Subsequently, the proteins were immunoprecipitated with α-Myc antibodies and the amount of coprecipitated Fe-55 was quantified by scintillation counting. Remarkably, the CPFS, CPYS, and CGYS active site variants displayed iron binding levels very similar to that of wild type Grx4 (Fig. 1E). Since the proteins were expressed at comparable levels, these data demonstrate that a CPxS active site motif does neither significantly affect the co-ordination of an Fe/S cofactor, nor

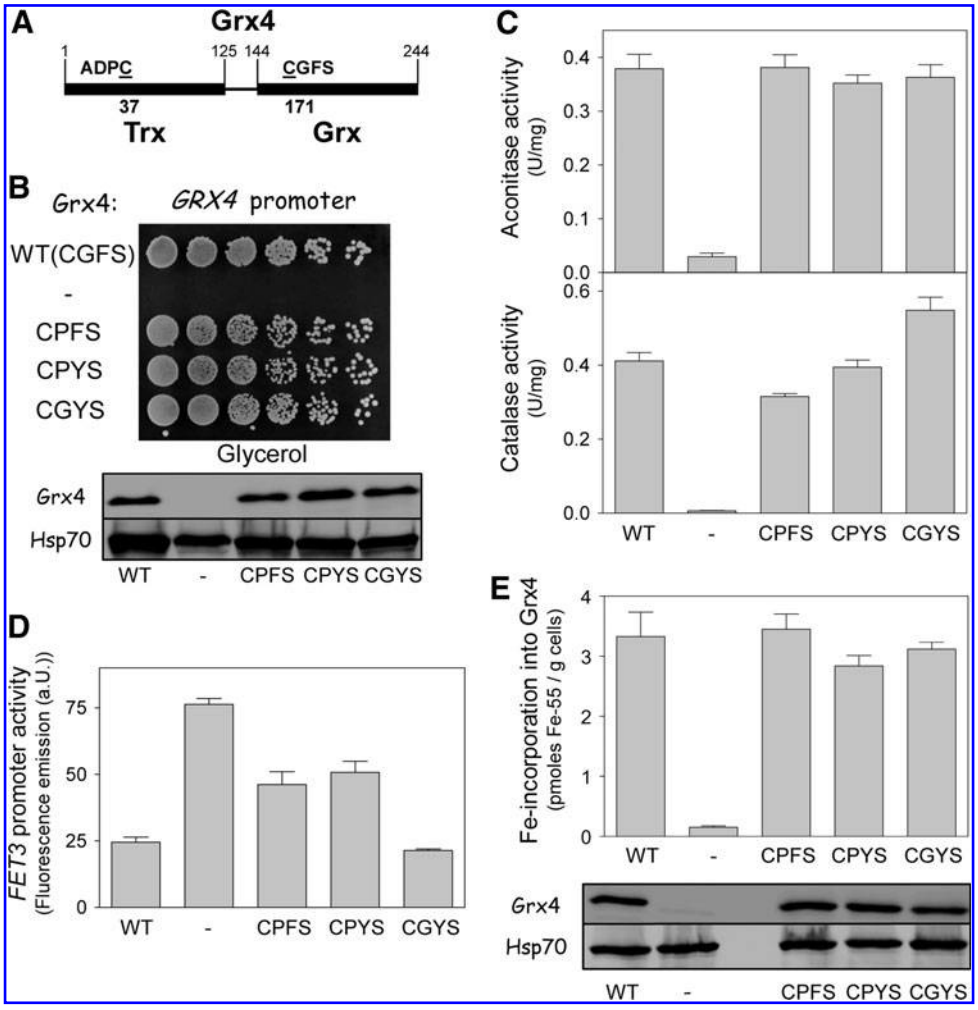

FIG. 1. A proline in the glutaredoxin (Grx) active site does not affect iron handling by Grx4. (A) Schematic presentation of the domain structure of Grx4. **(B–D)** Gal-GRX4 cells expressing wild type and the indicated Grx4 mutants under the control of the *GRX4* promoter were grown in synthetic complete medium with glucose (SD) to deplete endogenous Grx4. **(B)** After 40 h, fivefold serial dilutions were spotted onto glycerol-containing synthetic complete minimal medium and cultivated at 30°C. Grx4 levels were assessed by immunostaining with α-Myc. Cytosolic Hsp70 served as a loading control. **(C)** After 64 h, enzyme activities of aconitase and catalase of cell extracts and **(D)** the activity of the *FET3* promoter were determined. **(E)** W303-1A wild type cells expressing wild type Grx4 and the indicated site-directed mutants under the control of the $tetO_7$ promoter were cultivated in iron-poor minimal SD medium for 16 h and radiolabeled with 10 μCi Fe-55 for 2 h. Grx4 proteins were immunoprecipitated from cell extracts with α-Myc beads and coprecipitated Fe-55 was quantified by scintillation counting. Grx4 and Hsp70 levels were assessed by immunostaining. Cells harboring the empty vector pCM189 (–) served as controls. Error bars indicate the standard error of the mean (SEM) ($n > 4$).

the function of Grx4 in cytosolic iron handling. This finding distinguishes the cytosolic multidomain Trx–Grx fusion proteins from the single-domain Grxs that display a destabilization of the Fe/S cluster upon insertion of a proline into the Grx active site (6, 58, 62).

In *S. cerevisiae*, the iron bound to Grx3/4 serves as iron sensor for the iron-responsive transcription factor Aft1 that activates the expression of the genes involved in cellular iron uptake upon iron limitation (47, 48, 52). To study the effect of mutations in the active site on the role of Grx4 in iron sensing, we determined the promoter strength of the Aft1-dependent *FET3* gene in Gal-GRX4 cells using a *FET3* promoter-*GFP* fusion construct as a reporter (43). While the *FET3* promoter was fully activated in Grx4-depleted cells, Gal-Grx4 cells that expressed the CGYS mutant showed a basal expression of the *FET3* promoter similar to those expressing the wild type protein (Fig. 1D). By contrast, cells expressing the CPFS and CPYS variants from the endogenous *GRX4* promoter displayed a partial activation of the *FET3* promoter that is indicative for a partially deregulated Aft1. Apparently, a proline in the Grx active site affects the function of Grx4 in iron sensing, despite the fact that this residue does not significantly affect iron binding. Apparently, subtle changes in the environment of the Fe/S cofactor are already sufficient to partially disturb iron sensing by Aft1.

Since Grx3/4 are essential for viability in *S. cerevisiae*, the function of the cytosolic monothiol Grxs cannot be taken over by their cytosolic dithiol counterparts (47, 49). This likely suggests that a dithiol active site may be incompatible with a function in iron handling. We therefore constructed Grx4 variants with the CSYC active site of human GRX2, a CGFC motif, and the classical dithiol CPFC and CPYC motifs (Table 1). All four dithiol Grx4 variants supported growth of Gal-GRX4 cells at virtually the same rates as the wild type protein (Fig. 2A). Activities of the mitochondrial Fe/S protein aconitase were reduced by 25%–50% in Gal-GRX4 cells expressing these mutant proteins, while catalase activities were affected more strongly, especially by Grx4 variants with dithiol motifs that also harbored a proline in position 2 (Fig. 2B). Strikingly, all dithiol Grx4 variants displayed a strong decline in Fe-55-binding in our *in vivo* radio-labeling assay (Fig. 2D). This effect was generally stronger in mutants with an active site proline (up to ~12-fold reduced in comparison to wild type) than in those with a CSYC or CGFC motif (2.6–5.5-fold reduced). In addition, Gal-Grx4 cells expressing any of the dithiol Grx4 variants displayed a full activation of the *FET3* promoter, indicating that the dithiol Grx4 variants are unable to regulate the Aft1 transcription factor (Fig. 2C). Apparently, Aft1 discriminates between monothiol and dithiol Grxs and cannot be regulated by dithiol Grxs. Whether Aft1 directly recognizes the second cysteine residue or responses to the diminished amount of Fe/S cofactor on these Grx4 mutants cannot be distinguished. Taken together, the introduction of a dithiol active site motif has a noticeably stronger effect on iron binding to the cytosolic Trx–Grx fusion proteins and their function in iron delivery and iron sensing than the introduction of a proline in position 2. By contrast, single-domain Grxs can efficiently bind Fe/S cofactors, despite the fact that they

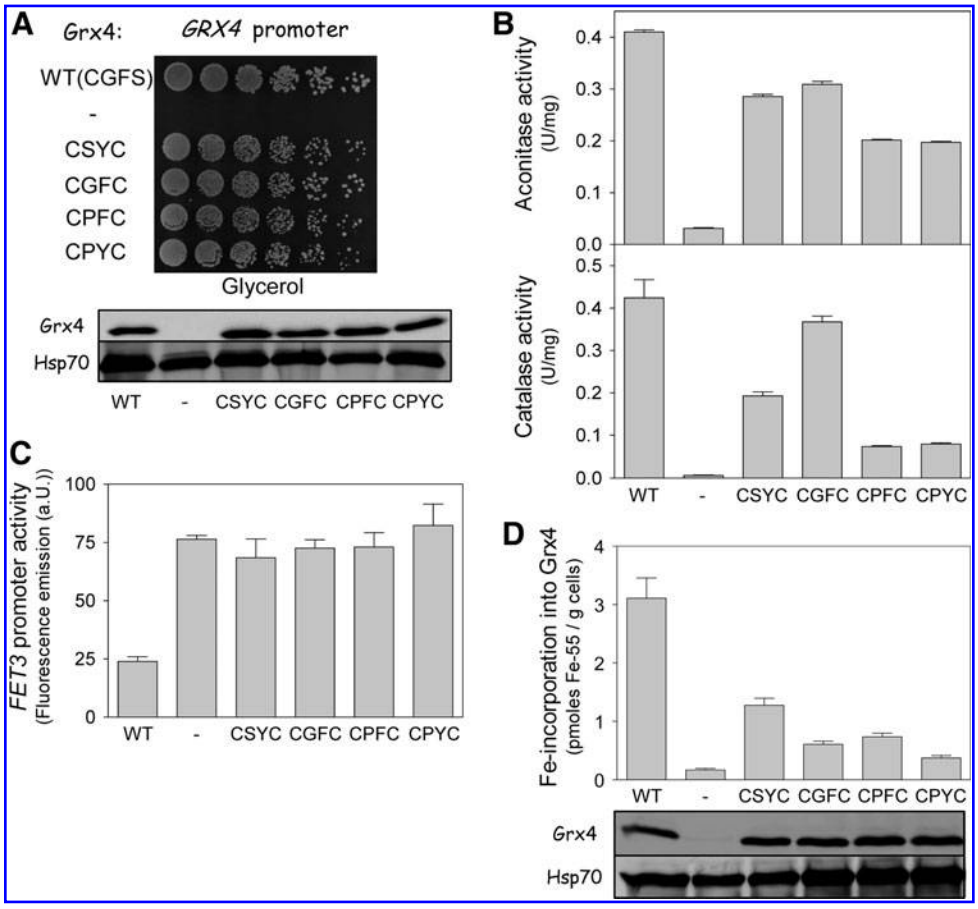

FIG. 2. A dithiol Grx active site affects iron handling and impairs iron sensing by Grx4. Gal-GRX4 cells expressing wild type and the indicated Grx4 variants under the control of the *GRX4* promoter were grown in SD medium. **(A)** After 40 h, fivefold serial dilutions were spotted onto synthetic complete medium with glycerol and levels of Grx4 and cytosolic Hsp70 were assessed by immunostaining. **(B)** After 64 h, cells were analyzed for aconitase and catalase enzyme activities and *FET3* promoter activities **(C)**. **(D)** Binding of Fe-55 to the Grx4 proteins was determined by Fe-55 radio-labeling of W303-1A wild type cells expressing the indicated Grx4 proteins from the $tetO_7$ promoter. Grx4 and Hsp70 levels were assessed by immunostaining. Cells harboring the empty vector (–) served as controls. Error bars indicate the SEM ($n > 4$).

may contain a natural dithiol active site, as is the case for human Grx2, but are generally more sensitive to the introduction of a proline residue (14, 37, 58).

Similar to single domain monothiol Grxs, *in vitro* reconstitution assays showed that GSH serves as a ligand for the Fe/S cofactor of Grx4 (Supplementary Fig. S1). To test the role of the noncovalently bound GSH for *in vivo* function, two mutations into the GSH binding site, T211R and D226R, were introduced that critically affect GSH binding (6). Consistent with the *in vitro* data, the T211R and D226R mutant displayed a ~2-fold lower iron binding when expressed from a strong promoter in wild type cells (Supplementary Fig. S1). Nevertheless, both proteins fully complemented the growth defects and low enzyme activities of Grx4-depleted Gal-GRX4 cells when expressed from the endogenous *GRX4* promoter (Supplementary Fig. S1). Apparently, a reduced affinity for the Fe/S cluster is not critical for Grx4 function *in vivo*. A catalytic function of the Trx domain was previously excluded since the catalytic cysteine of the Trx active site was not required for Grx4 function *in vivo* (47). In support of this conclusion, a W33D mutant in which the conserved tryptophan adjacent to the Trx active site was replaced by aspartate displayed wild type iron binding and fully rescued Gal-GRX4 cells (Supplementary Fig. S1B).

Dissection of the role of the Trx domain

The analysis above showed that only the conserved cysteine of the CGFS active site motif is absolutely essential for function of Grx3 and Grx4 in *S. cerevisiae* (47). Hence, the fact that the cytosolic monothiol Grxs are essential and can thus not be functionally replaced by their cytosolic dithiol counterparts Grx1 and Grx2 is not explained by differences in their Grx active site alone. To define whether this lack of functional complementation by dithiol Grxs was restricted to differences in the Grx domain, we investigated, whether the mitochondrial single domain monothiol Grx5 from *S. cerevisiae* can take over the function of Grx3/4. Grx4 has been shown to complement grx5Δ cells when targeted to the mitochondria, demonstrating a high degree of functional conservation (44). Surprisingly, a cytosolic version of Grx5 that lacks the N-terminal 27 amino acid residues that constitute the mitochondrial targeting sequence (ΔN-Grx5) failed to rescue the growth defects of Gal-GRX4 cells on both respiratory growth conditions in the presence of glycerol and under the less harsh fermentative growth conditions in the presence of glucose when over-expressed from the strong *MET25* promoter (Fig. 3A and data not shown). Consistent with this, Gal-GRX4 cells expressing ΔN-Grx5 displayed low aconitase and catalase activities and showed a fully activated *FET3* promoter (Fig. 3B). Sub-cellular fractionation experiments confirmed that ΔN-Grx5 was expressed in the cytosol (Fig. 3C). Apparently, the single domain monothiol Grx5 cannot functionally replace the cytosolic multidomain Grx proteins. These data suggest that the Trx domain of the cytosolic multidomain Grx proteins may carry an essential function. To demonstrate this conclusion, we expressed the Grx and the Trx domain of Grx4 separately in yeast cells. Indeed, both domains failed to rescue Gal-GRX4 cells with respect to cell growth and function of iron-dependent enzymes, despite the fact that they were

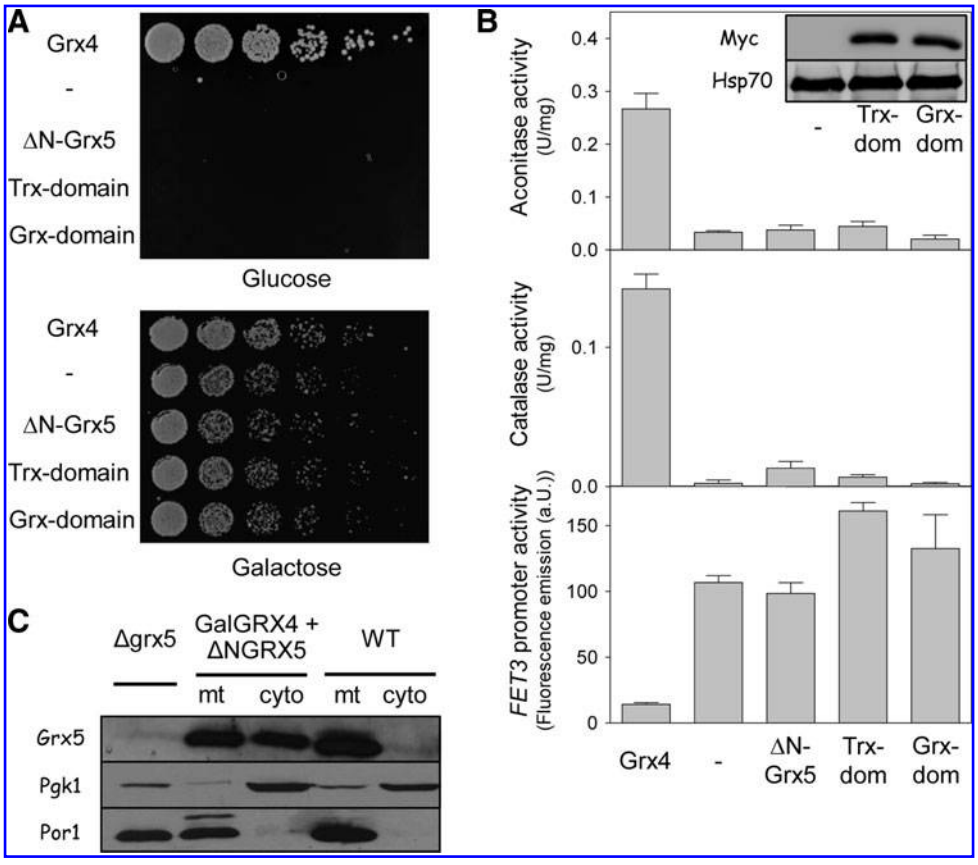

FIG. 3. Single domain monothiol Grxs do not support Grx4 function. (A) Gal-GRX4 cells over-expressing wild type Grx4, a cytosolic version of *Saccharomyces cerevisiae* Grx5 (ΔN-Grx5), the N-terminal thioredoxin (Trx) domain or the C-terminal Grx domain of Grx4 or under the control of the $tetO_7$ or *MET25* promoters were analyzed for growth on minimal media supplemented with glucose or galactose as described in Figure 1B. **(B)** Gal-GRX4 cells expressing the indicated proteins were analyzed for aconitase and catalase enzyme activities and *FET3* promoter activities. **(C)** Gal-GRX4 cells expressing ΔN-Grx5 were grown in SD minimal medium, mitochondria (Mito) and postmitochondrial supernatant (PMS) were isolated and immunostained with antisera against Grx5, mitochondrial Por1, and cytosolic 3-phosphoglycerate kinase (Pgk1). *grx5*Δ cells served as control.

highly expressed (Fig. 3A, B). Taken together, these observations demonstrate that the multidomain monothiol Grxs are functionally distinct from their single domain counterparts in that the N-terminal Trx domain is essential for function.

Next, we investigated whether homologous multidomain monothiol Grxs from other species can, in contrast to their single domain cousins, functionally replace *S. cerevisiae* Grx4. Human Grx3 (PICOT), *D. melanogaster* Grx4, and *S. pombe* Grx4 were overproduced under the control of the *TDH3* promoter in Gal-GRX4 cells. Of these, *S. pombe* Grx4 was able to partially complement the growth defect of Gal-GRX4 cells, as it supported fermentative growth on glucose-containing, but not respiratory growth on glycerol-containing media (Fig. 4A). When *S. pombe* Grx4 was expressed at endogenous levels under the control of the *S. cerevisiae GRX4* promoter, the complementation was less efficient (Fig. 4A). Consistent with this, the overexpression of *S. pombe* Grx4 induced a moderate recovery of aconitase and catalase enzyme activities to 20%–30% of the rates observed with *S. cerevsiae* Grx4 (Fig. 4B). The high-copy suppressor phenotype of *S. pombe* Grx4 demonstrates that the eukaryotic multidomain monothiol Grx proteins are functional orthologs. Nevertheless, significant species-specific differences exist that preclude functional complementation studies in *S. cerevisiae*. Since the mitochondrial single domain Grx5 can be functionally replaced by a large variety of foreign Grxs, this lack of complementation is significant (4). Remarkably, *S. pombe* Grx4 bound ~3-fold more iron than *S. cerevisiae* Grx4 upon over-expression in wild type cells (Fig. 4C). Apparently, the lack of full complementation by *S. pombe* Grx4 is caused by an impaired iron delivery from Grx4, rather than defects in Fe/S cofactor assembly.

To positively demonstrate the essential role of the Trx domain, we constructed Grx4 chimeras in which the *S. cerevisiae* Grx or Trx domains were substituted by the corresponding domains from *S. pombe* Grx4 or human Grx3 (Fig. 5A). All construct retained the poorly conserved linker of *S. cerevisiae* Grx4 and were expressed under the control of the *S. cerevisiae GRX4* promoter. Chimeric proteins with Grx or Trx domains from *S. pombe* rescued the growth defect of Gal-GRX4 cells on both fermentative and nonfermentative carbon sources as efficient as *S. cerevisiae* wild type Grx4 (Fig. 5B and data not shown). The chimera with the more distantly related human Trx domain failed to rescue Grx4-depleted cells when expressed from the endogenous *GRX4* promoter, but showed a partial complementation upon overproduction (Fig. 5B). Both *S. pombe-S. cerevisiae* Grx4 chimeras improved the enzyme activities of aconitase to ~70% and that of catalase to 30%–70% of those observed with *S. cerevsiae* Grx4 (Fig. 5C). Consistent with this observation, both chimeras bound iron with similar affinities as *S. cerevisiae* Grx4 when overproduced in wild type cells (Fig. 5D). Together, these data show that the Trx or Grx domains of *S. cerevisiae* Grx4, which are nonfunctional when expressed alone (Fig. 3), gain function when fused to foreign Trx or Grx domains and that the degree of complementation depends on the evolutionary distance. These data therefore demonstrate that the Trx and the Grx domain are both essential for the function of the multidomain monothiol Grxs.

Identification of the Aft1 interaction site

Aft1-like transcription factors are restricted to a group of ascomycetes yeast (2) (Supplementary Fig. S2). In the majority

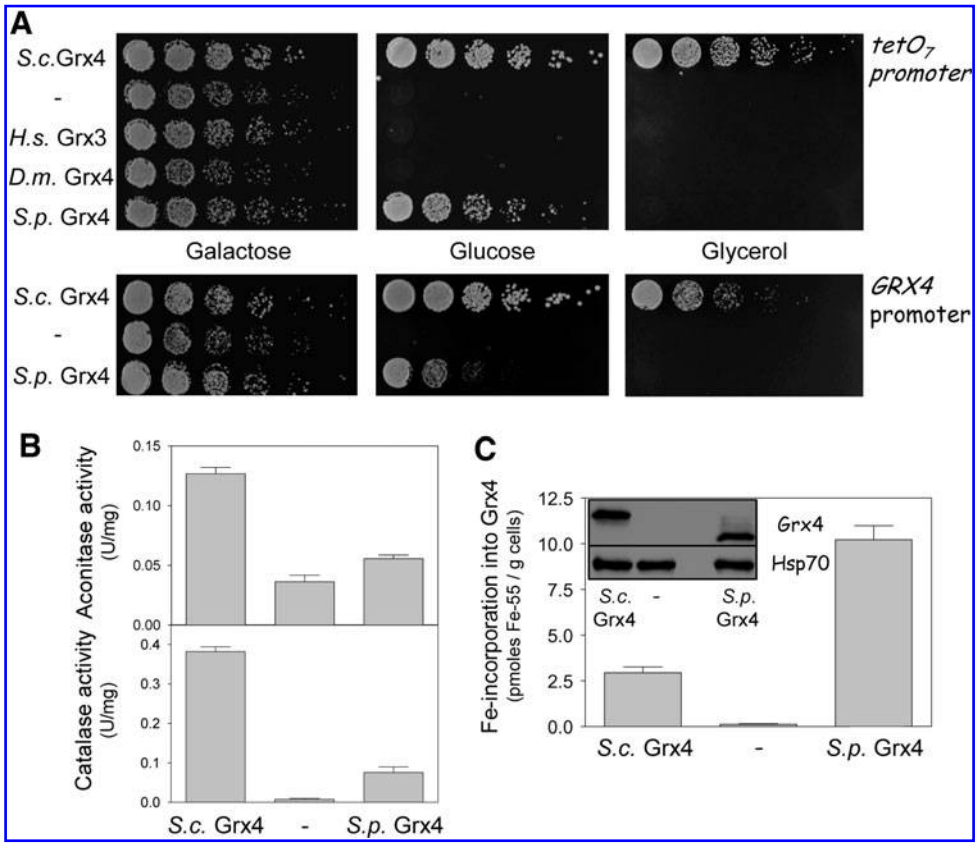

FIG. 4. *S. pombe* Grx4 partially rescues the growth defects of depleted Gal-GRX4 cells. (A) Gal-GRX4 cells expressing human Grx3, *D. melanogaster* or *S. pombe* Grx4 under the control of the *TDH3 or MET25* promoters or *S. pombe* Grx4 under the control of the *S. cerevisiae GRX4* promoter were analyzed for cells growth. **(B)** Cells expressing *S. pombe* Grx4 were analyzed for aconitase and catalase activities. **(C)** Fe-55 binding to *S. pombe* and *S. cerevisiae* Grx4 was determined by Fe-55 radiolabeling of wild type cells expressing the proteins with a C-terminal Myc-tag from a $tetO_7$ promoter. Grx4 and Hsp70 levels were determined by immunostaining with antisera against Myc and Hsp70. Cells harboring the empty vector (−) served as controls.

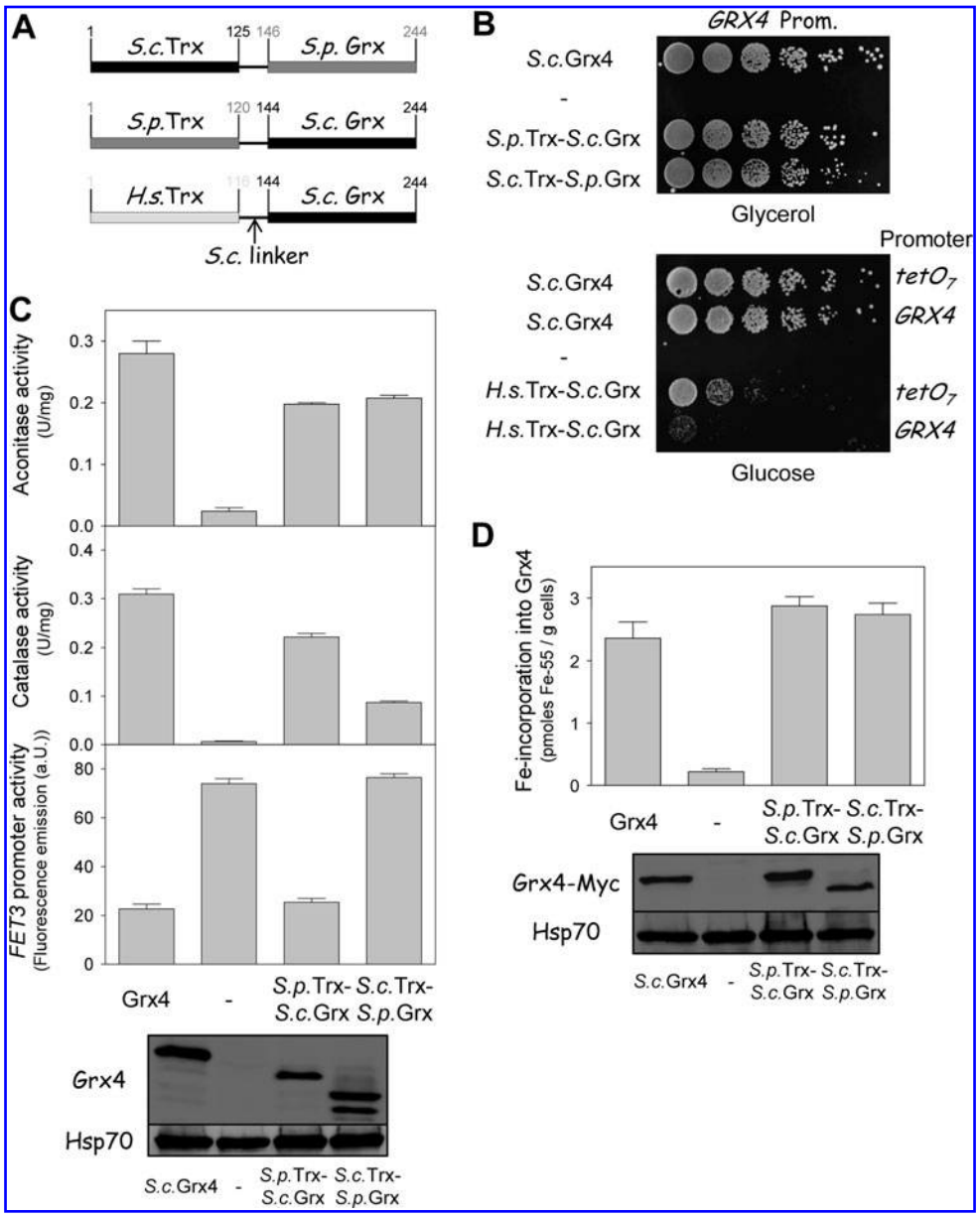

FIG. 5. The N-terminal Trx domain is essential for Grx4 function. (A) Schematic presentation of the domain structures of chimeric Grx4 proteins analyzed. Gal-GRX4 cells expressing the indicated Grx variants under the control of the *S. cerevisiae GRX4* promoter or the strong $tetO_7$ promoter as indicated were analyzed for cells growth **(B)**, enzyme activites of aconitase and catalase, and *FET3* promoter strength **(C)** as described in Figure 1. **(D)** Fe-55 binding to the indicated Grx4 variants was determined by radio-labeling of wild type cells expressing the proteins with a Myc-tag from a $tetO_7$ promoter. Grx4 and Hsp70 levels were determined by immunostaining with antisera against Myc and Hsp70. Error bars indicate the SEM ($n > 4$). Cells harboring the empty vector (–) served as controls.

of fungi, including *S. pombe*, cellular iron homeostasis is regulated by an interplay of an iron responsive repressors that are unrelated to Aft1 (15, 23, 31). Surprisingly, the two *S. pombe–S. cerevisiae* Grx4 chimeras behaved diametrically different with respect to their ability to regulate Aft1 (Fig. 5C). The chimera harboring the *S. cerevisiae* Grx domain reduced the expression of the Aft1-dependent *FET3* promoter in Gal-GRX4 cells to similar levels as those that expressed *S. cerevisiae* wild type Grx4. By contrast, cells that expressed the chimera harboring the *S. pombe* Grx domain displayed the full induction of the *FET3* promoter, which is indicative for a deregulated Aft1 (Fig. 5C). Since both chimeras bound iron and rescued the iron-handling defects of Gal-GRX4 cells, this observation suggests that the regulation of Aft1 likely involves a regulatory part on the Grx domain that is missing in the corresponding domain of *S. pombe* Grx4. The amino acid sequence of the Grx domains of the cytosolic Grx4 proteins from these two fungi is highly conserved. It was therefore remarkable that the immediate C-termini of the Aft1-regulating *S. cerevisiae* proteins Grx3 and Grx4 differed considerably from that of *S. pombe* Grx4 (Fig. 6A). We therefore constructed a *S. cerevisiae* Grx4 mutant, *S.p.*-C-term, in which the last 16 residues were replaced by the corresponding 14 C-terminal residues from *S. pombe* Grx4 (Fig. 6A). This substitution mutant indeed failed to suppress the induction of *FET3* when expressed in Gal-GRX4 cells (Fig. 6C, top). The protein otherwise rescued the growth defects and low enzyme activities of depleted Gal-GRX4 cells as efficiently as wild type Grx4 and did not display any alterations in Fe-55 binding (Fig. 6B–D). Collectively, these observations demonstrate that the C-terminus of *S. cerevisiae* Grx4 is essential for the regulation of Aft1.

In the simplest scenario, the C-terminus of Grx4 represents a specific binding site for the Aft1 transcription factor. Due to its endogenous low expression levels, the interaction of Aft1 with other proteins is difficult to analyse by conventional

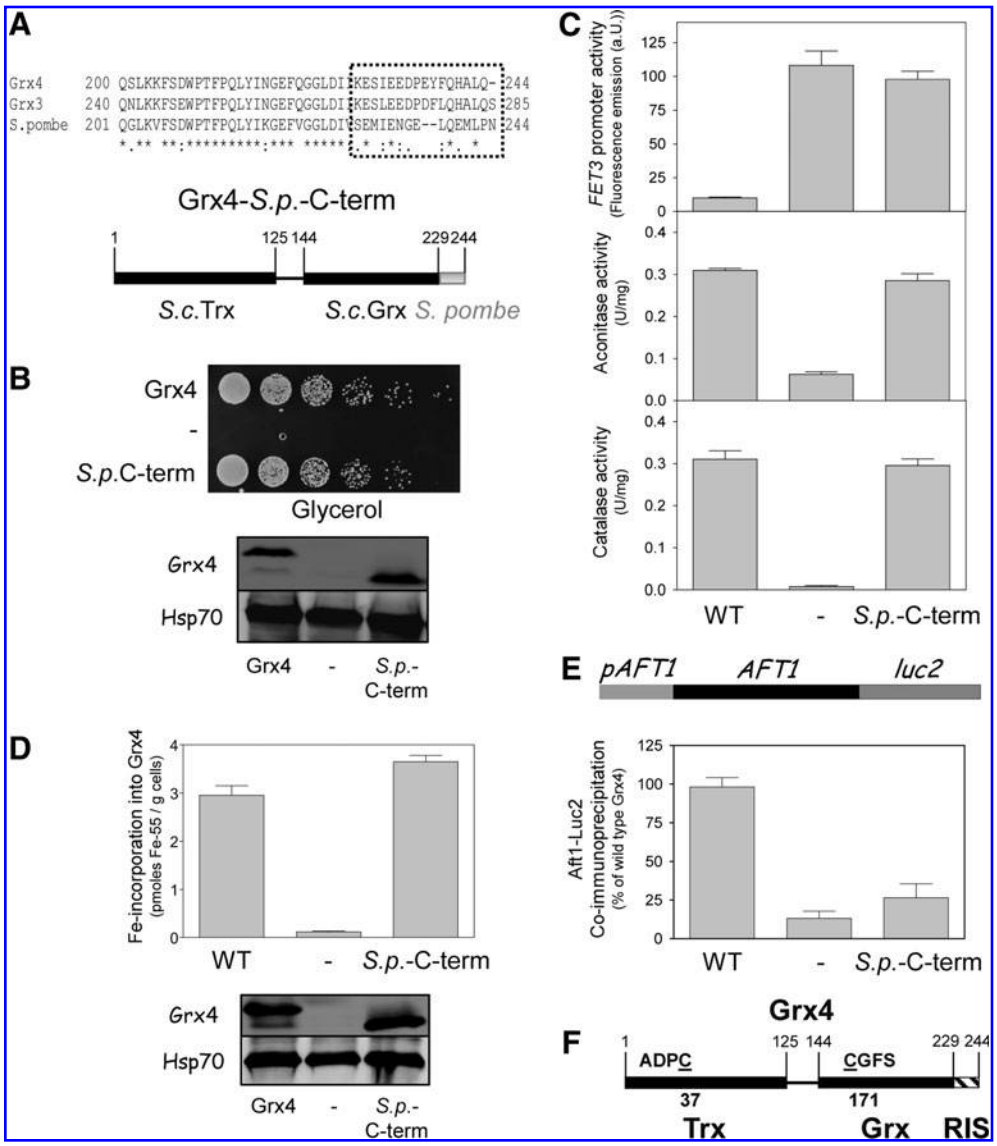

FIG. 6. Aft1 binds to the C-terminal end of Grx4. **(A)** Sequence alignment of the C-termini of Grx3 and Grx4 from *S. cerevisiae* and *S. pombe* Grx4. The rectangle indicated the C-terminal amino acids that are not conserved between *S. cerevisiae* and *S. pombe* Grx4. Gal-GRX4 cells expressing *S. cerevisiae* wild type Grx4 and a Grx4 exchange mutant harboring the C-terminus of *S. pombe* Grx4 (S.p.-C-term) as indicated in **A** from the *GRX4* promoter were analyzed for cells growth and Grx4 levels **(B)**, *FET3* promoter activities, and enzyme activites of aconitase and catalase **(C)**. **(D)** Fe-55 binding to wild type Grx4 and Grx4 S.p.-C-term was determined by radio-labeling and immunoprecipitation of wild type cells over-expressing the proteins from the $tetO_7$ promoter. Grx4 and Hsp70 levels were determined by immunostaining with antisera against Myc and Hsp70. **(E)** Upper part: Schematic presentation of the *AFT1* locus in strain Gal-GRX4/*grx3*Δ/*AFT1*-Lux that harbors the endogenous *AFT1* gene fused to a C-terminal luciferase gene. Wild type Grx4 and the exchange mutant S.p.-C-term were expressed with a Myc-tag from a $tetO_7$ promoter in Gal-GRX4/*grx3*Δ/AFT1-Lux cells. Cells were depleted for endogenous Grx4, Grx4-Myc was immunoprecipitated with α-Myc antibodies and the amount of coimmunoprecipitated Aft1 was quantified from the luciferase activity associated with the immunobeads. Error bars indicate the SEM ($n > 4$). Cells harboring the empty vector (−) served as controls. **(F)** Domain structure of *S. cerevisiae* Grx4. RIS: Regulator interacting sequence.

immunological techniques. To increase the sensitivity of detection, the genomic copy of the *AFT1* open reading frame in Gal-GRX4 cells was fused at its C-terminus with the open reading frame of firefly luciferase (Fig. 6E, top). The corresponding Aft1-luc2 fusion protein retained function, as it was able to confer an iron-responsive expression of the *FET3* gene (data not shown). The resulting Gal-GRX4/AFT1-luc2 cells were transformed with plasmids over-expressing wild type and the C-terminal exchange mutant GRX4 *S.p.*-C-term with a Myc-tag. Cells were depleted by cultivation in galactose-free media, the Grx4 proteins were immunoprecipitated with α-Myc antibodies from cell lysates and the amount of coimmunoprecipitated Aft1-luc2 protein was quantified from the luciferase activity associated with the immuno-beads. Significant amounts of luciferase activity coimmunoprecipitated with wild type Grx4 by this approach (Fig. 6E). However, ~4-fold less luciferase activity was coimmunoprecipitated with the C-terminal exchange mutant and these levels were only ~2-fold above background. Since both Grx4 proteins were expressed at comparable levels (Fig. 6D), these data demonstrate that the iron-responsive transcription factor binds to the extreme C-terminal end of the cytosolic monothiol Grx3/4 proteins *in vivo* and that this interaction is essential for the regulation of cellular iron homeostasis in *S. cerevisiae*.

Discussion

In recent years a growing number of Grxs with non-canonical active site motifs have been described (10, 11, 18, 26, 36, 41, 56). For most of these, their physiological roles are unknown and the functional relationships between the individual Grxs and their degree of interchangeability within a cell are unclear. Monothiol Grxs with a CGFS active site represent a well-conserved protein family that is found in all

kingdoms of life (18). They include members with a single Grx domain and multidomain proteins consisting of an N-terminal Trx domain and one to three C-terminal monothiol Grx domains (1, 18, 55). In fungi and vertebrates, single domain monothiol Grxs are found in mitochondria where they play an auxiliary role in iron–sulfur cluster assembly (32, 46, 54). This function can be taken over by other monothiol Grxs, including multidomain monothiol Grxs and Grxs with noncanonical active site motifs, indicating that this function tolerates a considerable degree of structural variations (4, 44, 55). The physiological relevance of this interchangeability, however, is not clear and hampers the identification of specific physiological roles for the noncanonical Grx. Recently, the multidomain monothiol Grxs Grx3/4 of *S. cerevisiae* have been shown to play an essential role in the passage of iron from the cytosolic labile iron pool to its sites of utilization (47). Similar to single domain monothiol Grxs, *S. cerevisiae* Grx3/4 harbor an Fe/S cofactor at the active-site of the Grx domain, and this cofactor is essential for the generation and/or transfer of intracellular iron in a bioavailable form (33, 47). This novel physiological role raises the question anew in how far the monothiol Grxs indeed form a coherent group of functionally orthologous members with Fe/S cofactors. The fact that iron-binding to *S. cerevisiae* Grx3/4 can be followed in the endogenous host *in vivo* and is associated with an essential cellular function represented an unique opportunity to answer this question by a systematic study of structure/function relationships in multidomain monothiol Grxs. Our analysis showed that the unique physiological role multidomain monothiol Grxs can not be executed by single domain Grxs, because the Trx domain is indispensable for *in vivo* function. In addition, it allowed the identification of structural differences at the Grx active site that contribute further to the *in vivo* functional incompatibilities between the Fe/S cofactor-binding single domain and multidomain Grxs.

In contrast to most classical dithiol Grx with a CPYC active site motif, monothiol Grxs, including the multidomain Grxs from *S. cerevisiae*, are capable of binding a bridged Fe/S cofactor at the Grx active-site (4, 12, 33, 47, 51, 62). The change of the second glycine of the canonical monothiol CGFS active site motif into a proline as found in the active site of classical dithiol Grxs is likely responsible for this lack of Fe/S cofactor coordination (6, 58, 62). In contrast to these conclusions based on the analysis of single-domain Grxs, we found that variants of the multidomain Grx4 with CP(Y/F)S active site motifs bind iron with virtually wild type affinities in their native host and were fully functional. Further, the introduction of a second cysteine into the active site affected the stability of the Fe/S cofactor on Grx4 *in vivo* and its function in iron handling. This observation is again in contrast to single domain Grxs such as human Grx2 that may accommodate Fe/S cofactors in a di-thiol active site (21, 37, 58). Just recently, also the first Fe/S cluster coordinating dithiol Grx with a CPYC active site has been described (9). The introduction of a canonical dithiol CP(Y/F)C active site motif caused a further decline in Grx4 function, but still did not fully abolish its function. Apparently, the *in vivo* role of the monothiol Grx in cytosolic iron trafficking tolerates a considerable variation in its active site. Nevertheless, despite the clear differences in the requirements for Fe-cluster binding of single domain and multidomain Grxs, only the conserved first cysteine of the CGFS active site is absolutely essential for *in vivo* function of all monothiol Grxs (5, 47, 48). Hence, the fact that the function of the cytosolic monothiol Grxs cannot be taken over by their cytosolic dithiol counterparts is not explained by differences in their Grx active site alone.

Despite the apparent flexibility of the Grx active site with respect to Grx4 function, the single domain monothiol Grx5 was unable to substitute Grx4 and the Grx domain of Grx4 was alone not functional. This observation contradicts previous results obtained with *S. cerevisiae* Grx3 (48). However, this previous work employed a strain background in which Grx3/4 are not essential and the Grx-domain construct used included the complete linker domain of Grx3 plus a part of the Trx domain. Thus, a major conclusion of our work is that the N-terminal Trx domain is absolutely required for *in vivo* function of the multidomain Grxs. Apparently, the fusion of a N-terminal Trx domain with a C-terminal Grx domain represents not only a structural difference between the multidomain monothiol Grx and their single domain counterparts, but is an indispensable and decisive part for the execution of a novel, specialized function. The fact that the Grx domain of *S. cerevisiae* Grx4 gained function when fused to Trx domains from other species is consistent with this conclusion. Further, the surprising observation that Grx4 can not be efficiently rescued by multidomain Grxs from other species clearly distinguishes the multidomain monothiol Grx from their single domain cousins that can be efficiently substituted by a large variety of different Grxs (4). These species-specific differences, however, are not confined to the Trx or Grx domain alone and preclude further functional complementation studies in *S. cerevisiae*. What could be the function of the Trx domain? A Trx-related enzymatic role can be excluded, since the conserved cysteine of the Trx active site and the adjacent conserved tryptophan are not required for function. It is therefore more likely that this domain may function as a docking-site for interacting partners involved, for instance, in Fe/S cluster assembly on the cytosolic monothiol Grxs. Since Trxs interact directly with a variety of target proteins, this docking-site model is attractive (7, 22, 38). Bona-fide interaction partners, however, are currently unknown, except for the BolA protein Fra2 and the iron-responsive transcription factor Aft1 (30, 33, 48, 52).

The physical interaction between Grx3/4 and Aft1 was the first evidence that linked the multidomain Grxs to cellular iron metabolism (48, 50, 52). The cooperation between Grx3/4 and Aft1 is a central aspect in the regulation of cellular iron-uptake as the Grx3/4-bound Fe/S cluster serves as iron sensor that communicates the cytosolic iron status to Aft1 (47). Our analysis revealed that the regulation of Aft1 by Grx3/4 is generally more sensitive to changes in the Grx active site than their function in iron delivery. Strikingly, the sensing mechanism of Aft1 transcription factor is incompatible with a dithiol Grx active site, explaining how Aft1 discriminates between the monothiol and dithiol Grxs present in the yeast cytosol. With respect to the high levels of cytosolic dithiol Grxs in *S. cerevisiae*, this discrimination makes perfect sense.

We found that Aft1 binds directly to the immediate C-terminus of Grx4. Since the loss of interaction results in the constitutive activation of Aft1-dependent transcription, a direct physical interaction between Grx3/4 and Aft1 is essential for signal transmission from Grx3/4 to Aft1. The same constitutive activation was seen when the Grx3/4-bound cluster is destroyed (47). Whether the Grx3/4-bound Fe/S cofactor is

also directly involved in Aft1 binding is not known. Remarkably, except for the de-regulation of Aft1, the loss of interaction between Aft1 and Grx4 has no effect on cellular iron-dependent pathways, as cells expressing a Grx4 variant with a modified C-terminus that did not support the regulation of Aft1 displayed wild type activities of iron-dependent enzymes and showed no growth defects. Hence, the function of the monothiol Grxs in cytosolic iron delivery is independent from their function in the regulation of cellular iron homeostasis and can be physically separated.

Aft1-like transcription factors are restricted to a group of ascomycetes yeast (2). The immediate C-terminal end of the multidomain Grxs is highly conserved in all Aft1-containing fungi (Supplementary Fig. S2). This observation is significant, as the C-termini of multidomain Grxs is poorly conserved in eukaryotes. It is therefore likely that the C-terminus of the multidomain-Grxs constitutes a specific binding-site for Aft1-like transcription factors that can be recognized by a conserved signature motif (Supplementary Fig. S2). In *S. pombe*, a direct interaction between Grx4 and the iron-responsive regulator Php4 is essential for the regulation of Php4 (40). Aft1 and Php4 are structurally unrelated, and the C-terminal ends of *S. pombe* Grx4 and Grx3/4 from *S. cerevisiae* differ considerable (Fig. 6A) (2, 29, 31, 40). It is therefore tempting to speculate that Php4 may bind to the C-terminal end of *S. pombe* Grx4. Php4 is functional, but not structurally, related to the iron-responsive repressor HapX that plays a central role in the regulation of iron homeostasis in a majority of fungi (15, 23, 31). Remarkably, phylogenetic analysis of the C-termini of multidomain Grxs reveal that the C-termini of the multidomain Grxs of fungi with HapX repressors form two coherent branches that are separated from Aft1-containing yeast (Supplementary Fig. S2). One group includes only ascomycetes yeasts with a C-terminal Grx motif relatively similar to that of Aft1-containing yeasts. The other branch includes only ascomycetes with HapX repressors. These display a conserved C-terminal Grx motif that is clearly distinct from that of Aft1-containing yeasts. Despite subtle differences, the C-termini of the multidomain Grxs of HapX and Aft1-containing fungi are clearly related but differ completely from those of higher eukaryotes (Supplementary Fig. S2). Collectively, these observations suggest that the C-terminus of the cytosolic multidomain Grxs serves as a binding-site for iron-responsive transcriptional regulators throughout the fungal kingdom (Fig. 6F).

To date, three iron-responsive transcription factors from *S. cerevisiae* are known, Aft1, its paralog Aft2 and Yap5. The latter plays a central role in the regulation of vacuolar iron storage (34). All three transcription factors are de-regulated in cells lacking Grx3 and Grx4, suggesting that these monothiol Grxs are likely directly involved in their regulation (48). Indeed, we could show that Grx4 interacts with Aft2 when both proteins are overproduced in wild type cells (Supplementary Fig. S3). Whether Yap5 also directly interacts with Grx3/4 or is activated in response to defects in iron delivery caused by low levels of these cytosolic monothiol Grxs is unknown. In addition, *S. cerevisiae* harbors several iron-responsive transcription factors that do not response directly to iron but to metabolites that are synthesized in an iron-dependent manner, such as Leu3 and Hap1 (24). Their de-regulation in cells lacking Grx3/4 is clearly related to defects in cytosolic iron handling caused by low levels of Grx3/4 (47, 48). In vertebrates, post-transcriptional regulation *via* iron-regulatory proteins IRP1 and IRP2 is central for cellular iron homeostasis (2, 29, 31, 40). However, whether multidomain monothiol Grxs such as PICOT are involved in the regulation of IRP1 and IRP2 is currently unknown.

Taken together, our study defines the multidomain monothiol Grxs as a functionally separate subgroup within the Trx family. This subgroup is distinct from that of the single domain monothiol Grxs, despite the fact that both groups contain members that may harbor Fe/S cofactors, because the Trx domain is essential for *in vivo* function. The single domain monothiol Grxs are found in all kingdoms of life and include members that play an auxiliary role in cellular Fe/S protein maturation in eukaryotes. By contrast, multidomain monothiol Grxs are found only in eukaryotes where they are involved in cytosolic iron delivery and in the regulation of cellular iron homeostasis in fungi.

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Author Disclosure Statement

No competing financial interests exist.

References

1. Alves R, Vilaprinyo E, Sorribas A, and Herrero E. Evolution based on domain combinations: the case of glutaredoxins. *BMC Evol Biol* 9: 66, 2009.
2. Babu MM, Iyer LM, Balaji S, and Aravind L. The natural history of the WRKY-GCM1 zinc fingers and the relationship between transcription factors and transposons. *Nucleic Acids Res* 34: 6505–6520, 2006.
3. Bandyopadhyay S, Chandramouli K, and Johnson MK. Iron-sulfur cluster biosynthesis. *Biochem Soc Trans* 36: 1112–1119, 2008.
4. Bandyopadhyay S, Gama F, Molina-Navarro MM, Gualberto JM, Claxton R, Naik SG, Huynh BH, Herrero E, Jacquot JP, Johnson MK, and Rouhier N. Chloroplast monothiol glutaredoxins as scaffold proteins for the assembly and delivery of [2Fe-2S] clusters. *EMBO J* 27: 1122–1133, 2008.
5. Belli G, Polaina J, Tamarit J, De La Torre MA, Rodriguez-Manzaneque MT, Ros J, and Herrero E. Structure-function analysis of yeast Grx5 monothiol glutaredoxin defines essential amino acids for the function of the protein. *J Biol Chem* 277: 37590–37596, 2002.
6. Berndt C, Hudemann C, Hanschmann EM, Axelsson R, Holmgren A, and Lillig CH. How does iron-sulfur cluster coordination regulate the activity of human glutaredoxin 2? *Antioxid Redox Signal* 9: 151–157, 2007.
7. Berndt C, Lillig CH, and Holmgren A. Thioredoxins and glutaredoxins as facilitators of protein folding. *Biochim Biophys Acta* 1783: 641–650, 2008.
8. Camaschella C, Campanella A, De Falco L, Boschetto L, Merlini R, Silvestri L, Levi S, and Iolascon A. The human counterpart of zebrafish shiraz shows sideroblastic-like microcytic anemia and iron overload. *Blood* 110: 1353–1358, 2007.
9. Ceylan S, Seidel V, Ziebart N, Berndt C, Dirdjaja N, and Krauth-Siegel RL. The dithiol glutaredoxins of african trypanosomes have distinct roles and are closely linked to the

unique trypanothione metabolism. *J Biol Chem* 285: 35224–35237, 2010.
10. Comini MA, Rettig J, Dirdjaja N, Hanschmann EM, Berndt C, and Krauth-Siegel RL. Monothiol glutaredoxin-1 is an essential iron-sulfur protein in the mitochondrion of African trypanosomes. *J Biol Chem* 283: 27785–27798, 2008.
11. Couturier J, Jacquot JP, and Rouhier N. Evolution and diversity of glutaredoxins in photosynthetic organisms. *Cell Mol Life Sci* 66: 2539–2557, 2009.
12. Couturier J, Koh CS, Zaffagnini M, Winger AM, Gualberto JM, Corbier C, Decottignies P, Jacquot JP, Lemaire SD, Didierjean C, and Rouhier N. Structure-function relationship of the chloroplastic glutaredoxin S12 with an atypical WCSYS active site. *J Biol Chem* 284: 9299–9310, 2009.
13. Eckers E, Bien M, Stroobant V, Herrmann JM, and Deponte M. Biochemical characterization of dithiol glutaredoxin 8 from Saccharomyces cerevisiae: the catalytic redox mechanism redux. *Biochemistry* 48: 1410–1423, 2009.
14. Feng Y, Zhong N, Rouhier N, Hase T, Kusunoki M, Jacquot JP, Jin C, and Xia B. Structural insight into poplar glutaredoxin C1 with a bridging iron-sulfur cluster at the active site. *Biochemistry* 45: 7998–8008, 2006.
15. Haas H, Eisendle M, and Turgeon BG. Siderophores in fungal physiology and virulence. *Annu Rev Phytopathol* 46: 149–187, 2008.
16. Haunhorst P, Berndt C, Eitner S, Godoy JR, and Lillig CH. Characterization of the human monothiol glutaredoxin 3 (PICOT) as iron-sulfur protein. *Biochem Biophys Res Commun* 394: 372–376, 2010.
17. Heckman KL and Pease LR. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc* 2: 924–932, 2007.
18. Herrero E and de la Torre-Ruiz MA. Monothiol glutaredoxins: a common domain for multiple functions. *Cell Mol Life Sci* 64: 1518–1530, 2007.
19. Herrero E, Ros J, Belli G, and Cabiscol E. Redox control and oxidative stress in yeast cells. *Biochim Biophys Acta* 1780: 1217–1235, 2008.
20. Herrero E, Ros J, Tamarit J, and Belli G. Glutaredoxins in fungi. *Photosynth Res* 89: 127–140, 2006.
21. Hoff KG, Culler SJ, Nguyen PQ, McGuire RM, Silberg JJ, and Smolke CD. *In vivo* fluorescent detection of Fe-S clusters coordinated by human GRX2. *Chem Biol* 16: 1299–1308, 2009.
22. Holmgren A, Johansson C, Berndt C, Lonn ME, Hudemann C, and Lillig CH. Thiol redox control via thioredoxin and glutaredoxin systems. *Biochem Soc Trans* 33: 1375–1377, 2005.
23. Hortschansky P, Eisendle M, Al-Abdallah Q, Schmidt AD, Bergmann S, Thon M, Kniemeyer O, Abt B, Seeber B, Werner ER, Kato M, Brakhage AA, and Haas H. Interaction of HapX with the CCAAT-binding complex—a novel mechanism of gene regulation by iron. *EMBO J* 26: 3157–3168, 2007.
24. Ihrig J, Hausmann A, Hain A, Richter N, Hamza I, Lill R, and Muhlenhoff U. Iron regulation through the back door: iron-dependent metabolite levels contribute to transcriptional adaptation to iron deprivation in *Saccharomyces cerevisiae*. *Eukaryot Cell* 9: 460–471, 2010.
25. Iwema T, Picciocchi A, Traore DA, Ferrer JL, Chauvat F, and Jacquamet L. Structural basis for delivery of the intact [Fe2S2] cluster by monothiol glutaredoxin. *Biochemistry* 48: 6041–6043, 2009.
26. Izquierdo A, Casas C, Muhlenhoff U, Lillig CH, and Herrero E. Saccharomyces cerevisiae Grx6 and Grx7 are monothiol glutaredoxins associated with the early secretory pathway. *Eukaryot Cell* 7: 1415–1426, 2008.
27. Jbel M, Mercier A, Pelletier B, Beaudoin J, and Labbe S. Iron activates *in vivo* DNA binding of Schizosaccharomyces pombe transcription factor Fep1 through its amino-terminal region. *Eukaryot Cell* 8: 649–664, 2009.
28. Johansson C, Roos AK, Montano SJ, Sengupta R, Filippakopoulos P, Guo K, von Delft F, Holmgren A, Oppermann U, and Kavanagh KL. The Crystal structure of human GLRX5: iron sulphur cluster coordination, tetrameric assembly and monomer activity. *Biochem J* 433: 303–311, 2010.
29. Kaplan CD and Kaplan J. Iron acquisition and transcriptional regulation. *Chem Rev* 109: 4536–4552, 2009.
30. Kumanovics A, Chen OS, Li L, Bagley D, Adkins EM, Lin H, Dingra NN, Outten CE, Keller G, Winge D, Ward DM, and Kaplan J. Identification of FRA1 and FRA2 as genes involved in regulating the yeast iron regulon in response to decreased mitochondrial iron-sulfur cluster synthesis. *J Biol Chem* 283: 10276–10286, 2008.
31. Labbe S, Pelletier B, and Mercier A. Iron homeostasis in the fission yeast Schizosaccharomyces pombe. *Biometals* 20: 523–537, 2007.
32. Lee DW, Kaur D, Chinta SJ, Rajagopalan S, and Andersen JK. A disruption in iron-sulfur center biogenesis via inhibition of mitochondrial dithiol glutaredoxin 2 may contribute to mitochondrial and cellular iron dysregulation in mammalian glutathione-depleted dopaminergic cells: implications for Parkinson's disease. *Antioxid Redox Signal* 11: 2083–2094, 2009.
33. Li H, Mapolelo DT, Dingra NN, Naik SG, Lees NS, Hoffman BM, Riggs-Gelasco PJ, Huynh BH, Johnson MK, and Outten CE. The yeast iron regulatory proteins Grx3/4 and Fra2 form heterodimeric complexes containing a [2Fe-2S] cluster with cysteinyl and histidyl ligation. *Biochemistry* 48: 9569–9581, 2009.
34. Li L, Bagley D, Ward DM, and Kaplan J. Yap5 is an iron-responsive transcriptional activator that regulates vacuolar iron storage in yeast. *Mol Cell Biol* 28: 1326–1337, 2008.
35. Lill R and Muhlenhoff U. Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases. *Annu Rev Biochem* 77: 669–700, 2008.
36. Lillig CH, Berndt C, and Holmgren A. Glutaredoxin systems. *Biochim Biophys Acta* 1780: 1304–1317, 2008.
37. Lillig CH, Berndt C, Vergnolle O, Lonn ME, Hudemann C, Bill E, and Holmgren A. Characterization of human glutaredoxin 2 as iron-sulfur protein: a possible role as redox sensor. *Proc Natl Acad Sci U S A* 102: 8168–8173, 2005.
38. Lindahl M and Kieselbach T. Disulphide proteomes and interactions with thioredoxin on the track towards understanding redox regulation in chloroplasts and cyanobacteria. *J Proteomics* 72: 416–438, 2009.
39. Luikenhuis S, Perrone G, Dawes IW, and Grant CM. The yeast Saccharomyces cerevisiae contains two glutaredoxin genes that are required for protection against reactive oxygen species. *Mol Biol Cell* 9: 1081–1091, 1998.
40. Mercier A and Labbe S. Both Php4 function and subcellular localization are regulated by iron via a multistep mechanism involving the glutaredoxin Grx4 and the exportin Crm1. *J Biol Chem* 284: 20249–20262, 2009.
41. Mesecke N, Mittler S, Eckers E, Herrmann JM, and Deponte M. Two novel monothiol glutaredoxins from Saccharomyces cerevisiae provide further insight into iron-sulfur cluster binding, oligomerization, and enzymatic activity of glutaredoxins. *Biochemistry* 47: 1452–1463, 2008.
42. Mieyal JJ, Gallogly MM, Qanungo S, Sabens EA, and Shelton MD. Molecular mechanisms and clinical implications of

reversible protein S-glutathionylation. *Antioxid Redox Signal* 10: 1941–1988, 2008.
43. Molik S, Lill R, and Muhlenhoff U. Methods for studying iron metabolism in yeast mitochondria. *Methods Cell Biol* 80: 261–280, 2007.
44. Molina MM, Belli G, de la Torre MA, Rodriguez-Manzaneque MT, and Herrero E. Nuclear monothiol glutaredoxins of *Saccharomyces cerevisiae* can function as mitochondrial glutaredoxins. *J Biol Chem* 279: 51923–51930, 2004.
45. Molina-Navarro MM, Casas C, Piedrafita L, Belli G, and Herrero E. Prokaryotic and eukaryotic monothiol glutaredoxins are able to perform the functions of Grx5 in the biogenesis of Fe/S clusters in yeast mitochondria. *FEBS Lett* 580: 2273–2280, 2006.
46. Muhlenhoff U, Gerber J, Richhardt N, and Lill R. Components involved in assembly and dislocation of iron-sulfur clusters on the scaffold protein Isu1p. *EMBO J* 22: 4815–4825, 2003.
47. Muhlenhoff U, Molik S, Godoy JR, Uzarska MA, Richter N, Seubert A, Zhang Y, Stubbe J, Pierrel F, Herrero E, Lillig CH, and Lill R. Cytosolic monothiol glutaredoxins function in intracellular iron sensing and trafficking via their bound iron-sulfur cluster. *Cell Metab* 12: 373–385, 2010.
48. Ojeda L, Keller G, Muhlenhoff U, Rutherford JC, Lill R, and Winge DR. Role of glutaredoxin-3 and glutaredoxin-4 in the iron regulation of the Aft1 transcriptional activator in Saccharomyces cerevisiae. *J Biol Chem* 281: 17661–17669, 2006.
49. Peggion C, Lopreiato R, Casanova E, Ruzzene M, Facchin S, Pinna LA, Carignani G, and Sartori G. Phosphorylation of the Saccharomyces cerevisiae Grx4p glutaredoxin by the Bud32p kinase unveils a novel signaling pathway involving Sch9p, a yeast member of the Akt/PKB subfamily. *FEBS J* 275: 5919–5933, 2008.
50. Philpott CC and Protchenko O. Response to iron deprivation in Saccharomyces cerevisiae. *Eukaryot Cell* 7: 20–27, 2008.
51. Picciocchi A, Saguez C, Boussac A, Cassier-Chauvat C, and Chauvat F. CGFS-type monothiol glutaredoxins from the cyanobacterium Synechocystis PCC6803 and other evolutionary distant model organisms possess a glutathione-ligated [2Fe-2S] cluster. *Biochemistry* 46: 15018–15026, 2007.
52. Pujol-Carrion N, Belli G, Herrero E, Nogues A, and de la Torre-Ruiz MA. Glutaredoxins Grx3 and Grx4 regulate nuclear localisation of Aft1 and the oxidative stress response in Saccharomyces cerevisiae. *J Cell Sci* 119: 4554–4564, 2006.
53. Rodriguez-Manzaneque MT, Ros J, Cabiscol E, Sorribas A, and Herrero E. Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in Saccharomyces cerevisiae. *Mol Cell Biol* 19: 8180–8190, 1999.
54. Rodriguez-Manzaneque MT, Tamarit J, Belli G, Ros J, and Herrero E. Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Mol Biol Cell* 13: 1109–1121, 2002.
55. Rouhier N. Plant glutaredoxins: pivotal players in redox biology and iron-sulphur centre assembly. *New Phytol* 186: 365–372, 2010.
56. Rouhier N, Couturier J, and Jacquot JP. Genome-wide analysis of plant glutaredoxin systems. *J Exp Bot* 57: 1685–1696, 2006.
57. Rouhier N, Couturier J, Johnson MK, and Jacquot JP. Glutaredoxins: roles in iron homeostasis. *Trends Biochem Sci* 35: 43–52, 2010.
58. Rouhier N, Unno H, Bandyopadhyay S, Masip L, Kim SK, Hirasawa M, Gualberto JM, Lattard V, Kusunoki M, Knaff DB, Georgiou G, Hase T, Johnson MK, and Jacquot JP. Functional, structural, and spectroscopic characterization of a glutathione-ligated [2Fe-2S] cluster in poplar glutaredoxin C1. *Proc Natl Acad Sci U S A* 104: 7379–7384, 2007.
59. Rutherford JC and Bird AJ. Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. *Eukaryot Cell* 3: 1–13, 2004.
60. Shelton MD, Chock PB, and Mieyal JJ. Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation. *Antioxid Redox Signal* 7: 348–366, 2005.
61. Sherman F. Getting started with Yeast. *Methods Enzymol* 350: 3–41, 2002.
62. Su D, Berndt C, Fomenko DE, Holmgren A, and Gladyshev VN. A conserved cis-proline precludes metal binding by the active site thiolates in members of the thioredoxin family of proteins. *Biochemistry* 46: 6903–6910, 2007.
63. Tamarit J, Belli G, Cabiscol E, Herrero E, and Ros J. Biochemical characterization of yeast mitochondrial Grx5 monothiol glutaredoxin. *J Biol Chem* 278: 25745–25751, 2003.
64. Wingert RA, Galloway JL, Barut B, Foott H, Fraenkel P, Axe JL, Weber GJ, Dooley K, Davidson AJ, Schmid B, Paw BH, Shaw GC, Kingsley P, Palis J, Schubert H, Chen O, Kaplan J, and Zon LI. Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis. *Nature* 436: 1035–1039, 2005.
65. Yamaguchi-Iwai Y, Ueta R, Fukunaka A, and Sasaki R. Subcellular localization of Aft1 transcription factor responds to iron status in Saccharomyces cerevisiae. *J Biol Chem* 277: 18914–18918, 2002.
66. Ye H, Jeong SY, Ghosh MC, Kovtunovych G, Silvestri L, Ortillo D, Uchida N, Tisdale J, Camaschella C, and Rouault TA. Glutaredoxin 5 deficiency causes sideroblastic anemia by specifically impairing heme biosynthesis and depleting cytosolic iron in human erythroblasts. *J Clin Invest* 120: 1749–1761, 2010.
67. Zheng L, Baumann U, and Reymond JL. An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res* 32: e115, 2004.

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Abbreviations Used

Grx = glutaredoxin
GSH = glutathione
SC = synthetic complete medium
SD = synthetic complete medium with glucose
SEM = standard error of the mean
Trx = thioredoxin

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3. Roland Lill, Bastian Hoffmann, Sabine Molik, Antonio J. Pierik, Nicole Rietzschel, Oliver Stehling, Marta A. Uzarska, Holger Webert, Claudia Wilbrecht, Ulrich Mühlenhoff. 2012. The role of mitochondria in cellular iron–sulfur protein biogenesis and iron metabolism. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1823**:9, 1491-1508. [CrossRef]
4. Haoran Li, Caryn E. Outten. 2012. Monothiol CGFS Glutaredoxins and BolA-like Proteins: [2Fe-2S] Binding Partners in Iron Homeostasis. *Biochemistry* **51**:22, 4377-4389. [CrossRef]
5. Young-Mee Oh, Seung-Keun Hong, Jeong-Tae Yeon, Mee-Kyung Cha, Il-Han Kim. 2012. Interaction between Saccharomyces cerevisiae glutaredoxin 5 and SPT10 and their in vivo functions. *Free Radical Biology and Medicine* **52**:9, 1519-1530. [CrossRef]
6. Haoran Li, Daphne T. Mapolelo, Sajini Randeniya, Michael K. Johnson, Caryn E. Outten. 2012. Human Glutaredoxin 3 Forms [2Fe-2S]-Bridged Complexes with Human BolA2. *Biochemistry* 120210151523008. [CrossRef]
7. Natasha Yeung, Barbara Gold, Nancy L Liu, Ramadevi Prathapam, Harry J. Sterling, Evan R Williams, Gareth P Butland. 2011. The E. coli Monothiol Glutaredoxin GrxD Forms Homodimeric and Heterodimeric FeS Cluster Containing Complexes. *Biochemistry* 110907102245016. [CrossRef]