Find authenticated court documents without watermarks at docketalarm.com.

This material may be protected by Copyright law (Title 17 U.S. Code)

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Role of Glutaredoxin-3 and Glutaredoxin-4 in the Iron Regulation of the Aft1 Transcriptional Activator in *Saccharomyces cerevisiae**

Received for publication, March 7, 2006, and in revised form, April 24, 2006 Published, JBC Papers in Press, April 28, 2006, DOI 10.1074/jbc.M602165200

Luis Ojeda[‡], Greg Keller[‡], Ulrich Muhlenhoff[§], Julian C. Rutherford[‡], Roland Lill[§], and Dennis R. Winge^{‡1} From the [‡]University of Utah Health Sciences Center, Departments of Medicine and Biochemistry, Salt Lake City, Utah 84132 and [§]Institut fur Zytobiologie und Zytopathologie Philipps-Universitat Marburg, Robert-Koch-Strasse 6, 35033 Marburg, Germany

The transcription factors Aft1 and Aft2 from Saccharomyces cerevisiae regulate the expression of genes involved in iron homeostasis. These factors induce the expression of iron regulon genes in iron-deficient yeast but are inactivated in iron-replete cells. Iron inhibition of Aft1/Aft2 was previously shown to be dependent on mitochondrial components required for cytosolic iron sulfur protein biogenesis. We presently show that the nuclear monothiol glutaredoxins Grx3 and Grx4 are critical for iron inhibition of Aft1 in yeast cells. Cells lacking both glutaredoxins show constitutive expression of iron regulon genes. Overexpression of Grx4 attenuates wild type Aft1 activity. The thioredoxin-like domain in Grx3 and Grx4 is dispensable in mediating iron inhibition of Aft1 activity, whereas the conserved cysteine that is part of the conserved CGFS motif in monothiol glutaredoxins is essential for this function. Grx3 and Grx4 interact with Aft1 as shown by two-hybrid interactions and co-immunoprecipitation assays. The interaction between glutaredoxins and Aft1 is not modulated by the iron status of cells but is dependent on the conserved glutaredoxin domain Cys residue. Thus, Grx3 and Grx4 are novel components required for Aft1 iron regulation that most likely occurs in the nucleus.

Iron, an indispensable nutrient in cell physiology, is used in ironsulfur clusters, hemes, and diiron-oxo metal centers in enzymes. *Saccharomyces cerevisiae*, a model organism in metal metabolism, maintains iron homeostasis largely through the regulation of iron uptake and storage. In this yeast, survival under low iron conditions is ensured through the utilization of the iron-responsive transcriptional activators Aft1 and Aft2 (1–3). These factors are activated in iron-deficient cells and induce the expression of more than 20 genes that are referred to as the iron regulon (4–7). This regulon includes genes whose products function in ionic iron acquisition, iron siderophore uptake, and vacuolar iron utilization. Activated Aft1 also induces the expression of *CTH2* that encodes an RNA-binding protein. Cth2 mediates the degradation of transcripts of some iron-requiring enzymes to conserve iron in the cell (8).

Aft1 is localized to the nucleus under low iron conditions and to cytoplasm under iron-sufficient conditions (9). Under iron-sufficient conditions, Aft1 remains inactive due to its cytoplasmic localization where it is unable to drive transcription (9). Aft1 has two nuclear local-

ization sequences and a nuclear export sequence (NES),² which map to its N-terminal DNA binding domain (9, 10). Mutations of two leucines within the NES result in retention of Aft1 within the nucleus and constitutive transcriptional activity regardless of iron levels. In addition, Aft1 contains a functionally important conserved ²⁹¹CXC²⁹³ sequence motif adjacent to the DNA binding domain and 190 residues downstream of the NES. Cys to Phe substitutions at either Cys within this motif in Aft1 result in constitutive transcriptional activation in ironreplete cells (9, 11). As expected, the constitutively active C291F Aft1 variant (Aft1-1^{up}) is retained within the nucleus. Thus, iron-regulation of Aft1 is dependent on its cycling between the nuclear and the cytoplasmic compartments.

The mechanism by which Aft1 and Aft2 sense cellular iron levels has been a topic of interest and intense research. Clues on the mechanism of iron sensing came from the observation that cells defective for Fe-S cluster biogenesis within the mitochondrial matrix exhibited constitutive expression of the iron regulon (12). Since disruption of Fe-S cluster biogenesis results in mitochondrial iron accumulation, it was initially thought that Aft1 was constitutive by virtue of depletion of cytosolic iron (12). It was later shown that disruption of Fe-S cluster biogenesis by diminution in the levels of the cysteine desulfurase (Nfs1) or the frataxin homologue (Yfh1) did not decrease cytosolic iron (13). This is an indication that Aft1 becomes constitutive due to impairment of a signal created by the mitochondrial Fe-S biosynthetic machinery and not to an indirect effect of alteration in iron compartmentalization.

In *S. cerevisiae* mitochondria are required for maturation of Fe-S proteins both inside and outside of the organelle (14). For synthesis of cytosolic and nuclear Fe-S proteins, mitochondria export a still unknown compound via the mitochondrial inner membrane transporter Atm1 (15). Other components of this export machinery are the mitochondrial intermembrane space sulfhydryl oxidase Erv1 as well as glutathione (15, 16). Depletion of glutathione activates Aft1 (11, 17). After export to the cytosol, the cytosolic Fe-S protein assembly machinery (CIA) matures Fe-S clusters and inserts them into target proteins (14). The CIA machinery includes the proteins Nar1, Cfd1, Nbp35, and Cia1 (18–20).

Iron sensing by Aft1 and Aft2 requires proper mitochondrial Fe-S cluster biosynthesis as well as a functional export to the cytoplasm. However, it does not require the CIA machinery (11), demonstrating that iron sensing by Aft1/Aft2 is not linked to the maturation of cytosolic 4Fe-4S clusters.

Since the CIA complex is not required to mediate iron inhibition of Aft1 function, we predicted that other proteins may be involved in sensing the iron inhibitory signal extruded by Atm1. One attractive candi-

JUNE 30, 2006 • VOLUME 281 • NUMBER 26

33300

Toxicol.

12

^{*} This work was supported by National Institutes of Health Grant CA61286 (to D. R. W.) and grants of the Sonderforschungsbereich 593, Deutsche Forschungsgemeinschaft (Gottfried Wilhelm Leibniz program), Fonds der chemischen Industrie, and the European Commission (to R. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. ¹ To whom correspondence should be addressed. Tel.: 801-585-5103; Fax: 801-585-5469; E-mail: dennis.winge@hsc.utah.edu.

² The abbreviations used are: NES, nuclear export sequence; CM, complete synthetic medium; TAP, tandem affinity purification; CIA, cytosolic Fe-S, protein assembly; BPS, bathophenanthroline sulfonate.

Glutaredoxins Modulate Aft1 Function

TABLE 1

Strains used in the present study

Strain	Description
Wild type	BY4742 $Mat\alpha$, his3 $\Delta 1$, leu2 $\Delta 0$, lys2 $\Delta 0$, ura3 $\Delta 0$
$\Delta grx3$	BY4742 Matα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, grx3::KanMX
$\Delta grx4$	BY4742 Mat α , his3 $\Delta 1$, leu2 $\Delta 0$, lys2 $\Delta 0$, ura3 $\Delta 0$, grx4::KanMX
$\Delta grx3, \Delta grx4$	BY4742 Matα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, grx3::LEU2 grx4::KanMX
$\Delta glr1$	BY 4742 $Mat\alpha$, his 3 $\Delta 1$, leu 2 $\Delta 0$, lys 2 $\Delta 0$, ura 3 $\Delta 0$, glr 1::Kan MX
$\Delta a ft 1$	BY4742 Matα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, aft1::KanMX
Wild type	CY4 Mat a ura3-52, leu2-3, 112 trp1-1 ade2-1, his3-11 can1-100
$\Delta trr1$	CY4 Mat a ura3-52, leu2-3, 112 trp1-1 ade2-1, his3-11 can1-100 trr1:: <i>HIS3</i>
YM4271	Mata, ura3-52, his3-A200, ade2-101, lys2-801, leu2-3,112, trp1-901 tyr1-501 gal4-A512 gal80-A538, ade5::hisG
$\Delta a ft1$ (YM4271)	Mata, ura3-52, his3-Δ200, ade2-101, lys2-801, leu2-3,112, trp1-901 tyr1-501 gal4-Δ512 gal80-Δ538, ade5::hisG, aft1::HIS3

date protein was the nuclear glutaredoxin-3 (Grx3), which was reported to interact with Aft1 in a global yeast two-hybrid interaction study (21). Glutaredoxins are glutathione-dependent thiol-disulfide oxidoreductases that function in maintaining the cellular redox homeostasis. S. cerevisiae has two dithiol glutaredoxins (Grx1 and Grx2) and three monothiol glutaredoxins (Grx3, Grx4, and Grx5) (22-24). The monothiol glutaredoxins are believed to reduce mixed disulfides formed between a protein and glutathione in a process known as deglutathionylation. In contrast, dithiol glutaredoxins can participate in deglutathionylation as well as in the direct reduction of disulfides (25). Grx5, the most studied monothiol glutaredoxin, is localized to the mitochondrial matrix, where it participates in the maturation of Fe-S clusters (24). Grx3 and Grx4 are predominantly localized to the nucleus (26). These proteins can substitute for Grx5 when overexpressed and targeted to the mitochondrial matrix (23); no information on their natural function has been reported. In addition to the reported interaction between Grx3 and Aft1, iron inhibition of Aft1 requires glutathione (11). Based on these clues, we evaluated the role of Grx3 and Grx4 in the iron inhibition of Aft1 and show presently that iron sensing is dependent on the presence of the redundant Grx3 and Grx4 proteins.

MATERIALS AND METHODS

DOCKE

Yeast Strains and Culture Conditions-The yeast strains used in this study are listed in Table 1. BY4742, $\Delta grx3$, $\Delta grx4$, and $\Delta glr1$ strains were obtained from Research Genetics. A PCR-created LEU2 cassette was integrated by homologous recombination at the GRX3 locus in a $\Delta grx4$ cell to create the $\Delta grx3\Delta grx4$ strain. The $\Delta trr1$ and YM4271 strains were previously described (27). A PCR-created HIS3 cassette was integrated by homologous recombination at the AFT1 locus in a YM4271 strain to create the $\Delta \mathit{aft1}$ strain used in yeast two-hybrid experiments. Cells were grown at 30 °C either in YPD medium, containing yeast extract, tryptone, and dextrose, or in complete synthetic medium (CM) or incomplete synthetic medium lacking, for example, uracil (CM-Ura) or leucine (CM-Leu). For several experiments, the growth medium was supplemented with 0.1 mM bathophenanthroline sulfonate (BPS) as a ferrous iron chelator to lower the availability of iron or supplemented with 0.1 mM FeCl_2. Doxycycline was added to the medium at a 5 μ g/ml final concentration for the indicated periods of time to modulate expression from the tetO₇ promoter (28). All cells were harvested during log phase.

Plasmids—All plasmid constructs were confirmed by DNA sequencing. Full-length wild type *GRX3* and *GRX4* coding sequences as well as their mutant forms (*GRX3 C211S* and *GRX4 C171S*) were tagged at the 3'-end with one Myc epitope. In addition, full-length wild type *GRX4* as well as its mutant forms (*GRX4 C171S* and *GRX4* GPm) were also tagged at the 3'-end with His₆ epitopes. All of these constructs were cloned in the YCp pCM189 and YEp pCM190 plasmids under the control of the doxycycline-regulated *tetO₇* promoter (28). In the previous cloning procedures, *GRX3* constructs were cloned between NotI/PstI sites, and *GRX4* constructs were cloned at BamHI/PstI sites.

For the two-hybrid experiments, the plasmid pBG4D-1, which contains the *ADH1* promoter and the *GAL4* (codons 1–147) DNA binding domain, was used. The *GRX3* and *GRX4* coding sequences were amplified and ligated into pBG4D-1, resulting in *GRX3* and *GRX4* 3'-ends being fused in frame with the *GAL4* DNA binding domain. The VP16 activation domain fused to the *CYC1* terminator (5' to 3' orientation) was amplified and ligated into pRS416. The *AFT1* promoter and open reading frame was PCR-amplified with SpeI/BgIII sites and ligated into cut pRS416 VP16-*CYC1* plasmid, resulting in *AFT1* being fused in frame with VP16. The resulting *AFT1* VP16 was used as a template for PCR mutagenesis of C291F and C293F. The previous *AFT1* constructs are under the control of the *AFT1* promoter.

AFT1 was TAP-tagged at its C terminus by homologous recombination in its chromosomal locus (29). The genomic *AFT1*-TAP was later used as a template in a PCR where *AFT1*-TAP was amplified, cut, and ligated into pCM190, where it is under the control of the $tetO_7$ promoter. Wild type *AFT1*, *AFT1*-1^{up}, and *AFT1* L99A were subcloned in plasmid pRS416 under the control of its own promoter.

The C-terminal 375 bp of *GRX4* (including the glutaredoxin domain but excluding the thioredoxin domain) as well as the C-terminal 381 bp of *GRX5* (excluding the mitochondrial target sequence) was PCR-amplified and ligated into pCM190, where they were under the control of the $tetO_7$ promoter.

S1 Nuclease Assays—RNA was extracted from cells grown to midlog phase using the hot acid phenol method, and S1 analysis was performed as previously described (30). For each reaction, $12 \mu g$ of total RNA were hybridized to a ³²P end-labeled DNA oligonucleotide probe before digestion with S1 nuclease and separation on an 8% polyacrylamide, 8 M urea polyacrylamide gel. Dried gels were imaged using a Bio-Rad FX phosphor imager and quantified using Quantity One software prior to autoradiography.

DNA Microarray Analysis—RNA was extracted from wild type BY4742 and $\Delta grx3\Delta grx4$ cells grown in YPD medium supplemented with 200 μ M FeCl₂. Total RNA was isolated using the hot acid phenol method. mRNA was isolated from total RNA by using the Poly(A) Tract mRNA isolation system IV kit from Promega following the manufacturer's instructions. Fabrication of DNA microarray, synthesis of fluorescence-labeled cDNA, hybridization of the microarray, and subsequent scanning were performed in the Huntsman Cancer Institute Microarray Core Facility at the University of Utah.

 β -Galactosidase Assays—Cells were grown to midexponential phase (A_{600} 0.5) in CM–Ura–Leu–His–Trp, 2% glucose either with supplemented iron or in the presence of BPS. β -Galactosidase activity was measured in permeabilized cells as previously described (31) and is expressed in Miller units that are calculated as follows (Δ 420 × 1000)/(min × ml of culture used × absorbance of the culture at 600 nm).

I imr Tri sod tan 4°0 pro and res feri eitł pol mi sec I tec

clo mo clo (usi

res nit des

tio and des use (11

ace 4.2 wa tio

> (34 RE

> > W

tra

in (F le Fł ex ire ty of w bo e? ce su st al in

ti

ge

DOCKET

Immunoprecipitation and Immunodetection—Cellular lysates for immunoprecipitation analysis were prepared by glass beading in 50 mM Tris-Cl, pH 7.5, 150 mM sodium chloride, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, and a protease inhibitor mixture. The supernatant was incubated with a rabbit polyclonal anti-Myc antibody for 1 h at 4 °C. Protein A-agarose was added and incubated overnight at 4 °C. The protein A-agarose was collected by centrifugation, washed three times, and boiled in SDS sample buffer. The immunoprecipitated protein was resolved by SDS-10% polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were blocked and probed with either PAP peroxidase anti-peroxidase (for TAP detection) or rabbit polyclonal anti-Myc. Detection was performed by enhanced chemiluminescence after incubation with a horseradish peroxidase-conjugated secondary antibody.

In addition, an aliquot of the supernatant was used for immunodetection analysis by immunoblotting, using PAP (Sigma), rabbit polyclonal anti-Myc (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-Pgk1 (Molecular Probes), and mouse monoclonal anti-His (Novagen).

Cellular lysates were prepared for immunoblotting by glass beading using 10% trichloroacetic acid in Tris acetate buffer, pH 8. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The membranes were probed with antibodies previously described and detected using chemiluminescence (ECL; Pierce).

Labeling of yeast cells with radioactive iron (⁵⁵Fe) and the determination of iron incorporation into Fe-S proteins by immunoprecipitation and liquid scintillation counting were carried out as previously described (15).

Miscellaneous Procedures—The following published methods were used. The sulfite reductase assay was performed as previously described (11). For aconitase activity assays, cells were lysed by bead beating, and aconitase activity was determined by coupled reaction of aconitase (EC 4.2.1.3) and isocitrate dehydrogenase (EC 1.1.1.42) (32). Mutagenesis was performed by either PCR mutagenesis or by introducing the mutation in the primer followed by homologous recombination (33). Yeast transformation was performed using standard lithium acetate protocol (34).

RESULTS

To evaluate the role of Grx3 and Grx4 in the iron inhibition of Aft1, we quantified the expression of two iron regulon genes, *FET3* and *FIT3*, in cells lacking either Grx3 or Grx4 or in cells lacking both molecules (Fig. 1, *A* and *B*). Gene expression was assessed by quantifying mRNA levels using the S1 nuclease protection assay. Whereas expression of *FET3* and *FIT3* was inhibited in iron-supplemented wild type cells, expression of *FET3*, but not *FIT3*, was elevated 3.5- and 2.5-fold in iron-supplemented $\Delta grx3$ or $\Delta grx4$ cells, respectively, relative to wild type cells. The absence of Grx3 or Grx4 did not affect the full induction of *FET3* observed when the iron bioavailability is limited in cells treated with the iron chelator bathophenanthroline sulfonate (BPS).

Cells lacking both Grx3 and Grx4 exhibited constitutive expression of both *FET3* and *FIT3*. To verify that the major iron regulon genes were expressed in $\Delta grx3\Delta grx4$ cells, DNA microarray analysis was performed comparing wild type and $\Delta grx3\Delta grx4$ cells cultured in YPD medium supplemented with iron (Table 2). The same genes induced by the constitutively active Aft1-1^{up} were highly expressed in $\Delta grx3\Delta grx4$ cells, although the observed induction ratios varied. Such variation in the induction ratios of iron regulon genes is observed under other conditions that activate Aft1 (4–7). The high expression of the Aft2 target gene *MRS4* (7, 35) suggested that both Aft1 and Aft2 are constitutively



FIGURE 1. Aft1 is partially activated in the absence of Grx3 or Grx4 but fully activated in cells lacking both glutaredoxins. Wild type, $\Delta grx3$, $\Delta grx4$, and $\Delta grx3\Delta grx4$ cells were grown in YPD medium in the presence of either 100 μ m BPS or 200 μ m FeCl₂ prior to S1 nuclease analyses of *FET3* (A) and *FIT3* (B) mRNA levels. *CMD1* encoding calmodulin was used as the loading control.

TABLE 2

Genes induced in $\Delta grx3\Delta grx4$ cells compared with Aft1-1^{up}-containing cells

Microarray analysis was conducted on $\Delta grx3\Delta grx4$ cells compared with wild type (WT) cells cultured in YPD containing 0.2 mM FeCl₃. RNA was extracted from these cells, and poly(A) RNA was recovered. The mean from two duplicate experiments is shown. The -fold induction data are compared with transcript profile data of *AFT1-1*^{urp} cells published previously. Only a subset of the iron regulon genes are shown to document that the iron regulon genes are induced in $\Delta grx3\Delta grx4$ cells. In the duplicate experiments with $\Delta grx3\Delta grx4$ cells, the variation in the -fold induction was within 10%.

Gene	$\Delta grx3\Delta grx4$ versus WT (mean $n = 2$)	Aft1-1 ^{up} versus WT (mean $n = 3$)
	-fold induction	
FIT1	38	9
FIT3	19	43
FIT2	12	30
CTH2	11	5
FET3	8	25
SIT1	8	7
ENB1	5.5	7
ISU2	4.7	1.3
CAD1	4.3	1.7
ARN1	4.1	2.5
MRS4	3.8	1.7
COT1	3.7	2.2

active in the $\Delta grx3\Delta grx4$ strain. These data confirm that the iron regulon is induced in the absence of Grx3 and Grx4. The induced expression of iron regulon genes observed in $\Delta grx3\Delta grx4$ cells cultured in iron-replete medium was due to the absence of Grx3 and Grx4, since transformation of the double null cells with *GRX4* under the TET promoter restored inhibition of *FET3* expression (Fig. 2). As expected, the addition of doxycycline to repress *GRX4* expression yielded elevated *FET3* expression.

Grx3 and Grx4 appear to be redundant molecules. $\Delta grx4$ cells transformed with a low copy plasmid containing either *GRX3* or *GRX4* restored full iron inhibition of *FET3* transcription (Fig. 3A). Grx3 and Grx4 are members of the monothiol glutaredoxin family and as such possess a single functional cysteinyl residue within a CGFS sequence motif (36). We tested whether the single conserved cysteine residue within the CGFS motif in each protein is essential for iron inhibition of Aft1 activity. Transformation of $\Delta grx4$ cells with a YCp plasmid-borne *GRX4* restored iron inhibition of *FET3* expression, whereas transformants containing a mutant *GRX4* allele encoding a C171S substitution failed to inhibit *FET3* expression (Fig. 3A). In the same way, mutant Grx3 containing a C211S substitution failed to mediate iron inhibition

ling

ıpli-

nds P16 ion) pen nto ume CR are naiter and

rol in

ain

bp

mof log led

ere

ore

Μ

FX

to

pe ed ol ct ursnt

se eas is

)/

6

ay

Glutaredoxins Modulate Aft1 Function



FIGURE 2. The iron regulon induction phenotype seen in $\Delta grx3\Delta grx4$ cells is reversed by introducing a wild type *GRX4* gene. $\Delta grx3\Delta grx4$ cells transformed with a low copy plasmid containing *GRX4* were grown in CM – Ura, 2% glucose for 19 h in the presence or absence of doxycycline (5 μ g/ml). S1 nuclease analysis was used to assess the expression of *FET3* mRNA. *CMD1* encoding calmodulin was used as the loading control.

of *FET3* expression in $\Delta grx3$ cells (Fig. 3*B*). Although the mutant Grx3 and Grx4 proteins were inactive, they were stably expressed (data not shown). Thus, the putative functional cysteinyl residue in each glutaredoxin is important to mediate iron inhibition of Aft1.

Since depletion of Grx3 and Grx4 resulted in constitutive Aft1 activity, the effect of overexpression of *GRX4* on Aft1 function in wild type cells was evaluated. Wild type cells cultured in iron-limited SC medium showed partial *FET3* expression that could be completely inhibited by the addition of iron salts to the culture medium. The overexpression of *GRX4* in cells cultured in this iron-limited medium markedly attenuated *FET3* expression (Fig. 4, A and B, two lanes on the left). The inhibitory effect of *GRX4* overexpression was also seen when Aft1 was fully activated in BPS-supplemented, iron-deficient cells (data not shown). In addition, the C171S substitution in Grx4 partially abrogated the ability of overexpressed Grx4 to attenuate *FET3* expression in wild type cells (Fig. 4A).

Aft1 becomes constitutively active when the ²⁹¹CXC²⁹³ motif or the NES motif is mutated (1, 9). To address whether overexpression of Grx4 can attenuate the function of constitutively active variants of Aft1, the TET-*GRX4*-containing high copy vector was transformed into $\Delta aft1$ cells containing either an *AFT1* allele encoding the C291F variant (Aft1-1^{up}) or the L99A NES variant. Overexpression of Grx4 inhibited wild type Aft1 activity and resulted in a partial, reproducible attenuation of Aft1 (L99A) (Fig. 4, *C* and D) but no significant attenuation of the activity of the Aft1-1^{up} constitutive mutant (Fig. 4*E*).

Monothiol glutaredoxins are believed to function in the deglutathionylation of target proteins (37). In the deglutathionylation reaction, monothiol glutaredoxins are predicted to be transiently glutathionylated themselves (36). We evaluated whether the conserved residues that form the glutathione-binding pocket in other monothiol glutaredoxins are important for Grx4-mediated iron inhibition of Aft1 activity. We tested a double mutant of Grx4 in which the conserved ²⁰⁹WP²¹⁰ was converted to ²⁰⁹DA²¹⁰. The ²⁰⁹DA²¹⁰ mutant Grx4, designated Grx4 GPm for "glutathione pocket mutant," was unable to mediate iron inhibition of *FET3* expression in $\Delta grx4$ cells (Fig. 3*C*). The Grx4 GPm mutant did not affect the full induction of *FET3* by iron deprivation and was shown to be equally stable to the wild type protein by immunoblotting (data not shown). Thus, glutathione binding may be important for Grx4 activity.



FIGURE 3. The conserved cysteine in the glutaredoxin domain of Grx4 as well as the putative glutathione pocket residues tryptophan and proline are important for the control of Aft1 activity. A, $\Delta grx4$ cells transformed with a low copy plasmid alone (V) or with the same plasmid containing either the wild type GRX3 gene (Grx4), or the GRX4 C1715 mutant gene (Grx4 C1715). B, $\Delta grx3$ cells transformed with a low copy plasmid alone or with the same plasmid containing either the wild type GRX3 gene (Grx4), or the GRX4 C1715 mutant gene (Grx4 C1715). B, $\Delta grx3$ cells transformed with a low copy plasmid alone or with the same plasmid containing either the wild type GRX3 gene or the GRX3 C2115 mutant gene (Grx3 C2115). Cells from A and B were grown in CM—Leu, 2% glucose plus 50 μ M FeCl₂ prior to S1 nuclease analyses of FET3 and CMD1 mRNA levels. C, $\Delta grx4$ cells were grown in CM—Leu, 2% glucose supplemented with the same plasmid containing either the wild type GRX4 gene or the GRX4 GPM). These cells were grown in CM—Leu, 2% glucose supplemented with either 100 μ M BPS or 50 μ M FeCl₂ prior to S1 nuclease analyses of FET3 and CMD1 mRNA levels.

Grx3 and Grx4 differ from the mitochondrial monothiol glutaredoxin Grx5 in that they contain an N-terminal thioredoxin domain in addition to the C-terminal glutaredoxin domain (26). The thioredoxin domain of Grx3 is believed to be responsible for the predominant nuclear localization of this glutaredoxin (26). To test whether the thioredoxin domain is important for iron inhibition of Aft1, we engineered a Grx4 truncate lacking the N-terminal thioredoxin domain. *FET3* expression was iron-inhibited in $\Delta grx3\Delta grx4$ cells harboring a high copy plasmid containing the Grx4 truncate, designated Grx4T (Fig. 5*A*). However, when the Grx4T was expressed in a low copy plasmid, it had no effect (data not shown). A green fluorescent protein fusion of the Grx4 truncate, expressed in a low copy plasmid, was found to have a diffused localization throughout the cell (data not shown), suggesting that insufficient protein existed within the nucleus to mediate iron inhibition of Aft1 activity.

Cells lacking Grx3 and Grx4 exhibit a growth defect in synthetic culture medium (Fig. 5*B*) but are less impaired in rich YPD medium (data not shown). The thioredoxin domain is nonessential for normal cell growth, because expression of Grx4 or Grx4T restored wild type growth (Fig. 5*B*).

Grx5 functions in the mitochondrial Fe-S biogenesis pathway (24). Grx3 and Grx4 were shown to substitute for Grx5 in mitochondrial function when overexpressed and targeted to the mitochondrial matrix (23). Suppression of the $\Delta grx5$ phenotypes requires both the thioredoxin and glutaredoxin domains and the essential glutaredoxin domain Cys residue (23). To address whether the mitochondrial Grx5 can complement $\Delta grx3\Delta grx4$ cells, a truncated *GRX5* construct was engineered that lacked the N-terminal mitochondrial target sequence (designated Grx5 C). Expression of the Grx5 truncate restored wild type growth of $\Delta grx3\Delta grx4$ cells (Fig. 5*B*), yet *FET3* expression was constitutive (Fig. 5*A*), suggesting that the growth defect is unrelated to constitutive Aft1

Find authenticated court documents without watermarks at docketalarm.com.

A

С

E

the activity of wild type and NES mutant Aft1. A, wild type cells transformed with a high copy plasmid alone (V) or with the same plasmid containing either the wild type GRX4 gene or the GRX4 C171S mutant gene were grown in CM-Ura, 2% glucose containing the BIO101 low iron nitrogen base. S1 nuclease analysis was used to assess the expression of FET3 mRNA. CMD1 encoding calmodulin was used as the loading control. B, the FET3 mRNA levels in A were quantified and normalized to CMD1 mRNA levels. C, $\Delta aft1$ cells were transformed with two plasmids, a low copy plasmid containing either a wild type AFT1 (Aft1) or a NES mutant AFT1 (Aft1 L99A) gene and a high copy plasmid alone or containing the wild type GRX4 gene. These cells were grown in CM-Ura-Leu, 2% glucose containing the BIO101 low iron nitrogen base. S1 nuclease analysis was used to assess the expression of FET3 mRNA. D, FET3 mRNA levels in C were quantified and normalized to CMD1 mRNA levels. E, cells containing Aft1-1^{up} either in the presence or absence of GRX4 were grown in CM-Ura-Leu, 2% glucose, and FET3 expression was assessed by S1 nuclease analysis. In three independent experiments done in duplicate, the mean FET3 expression in Grx4-overexpressing Aft1-1^{up} cells was 85% of that in control Aft1-1^{up} cells.

s the

r the V) or

type

ans-

r the

nd B

es of

mid

RX4

cose

es of

re

in xin

ant hi-

ed

T3

ру

4).

ad

he

e a

ng

ιi-

ic

m

al

)e

).

al

х

<u>.</u>

n

d

d

f

FIGURE 4. Overexpression of Grx4 decreases



FIGURE 5. The thioredoxin domain of Grx4 is not necessary for complementation of either growth or the control of Aft1 activity in a $\Delta grx3\Delta grx4$ strain. $\Delta grx3\Delta grx4$ cells were transformed with a low copy plasmid containing the wild type GRX4 gene (Grx4) or with a high copy plasmid either alone (V) or containing the GRX4 gene lacking the thioredoxin domain (Grx4 T) or the GXR5 gene lacking the mitochondrial target sequence (Grx5 C). A, cells were grown in CM–Ura, 2% glucose prior to S1 nuclease analyses of FET3 and CMD1 mRNA levels. B, cells from A were plated on CM–Ura, 2% glucose plates. The double null cells have a long lag phase in growth and this manifests in the low growth seen in B.



activity. Since the Grx5 truncate was not stably expressed in the yeast cytoplasm (data not shown), we cannot be certain whether the lack of iron inhibition of *FET3* expression was a result of low protein levels or inactivity in that function.

The previous results are consistent with Grx3 and Grx4 having a direct role in the iron inhibition of Aft1 activity. The original motivation to consider Grx3 was its reported interaction with Aft1 in a global twohybrid study. A two-hybrid assay system for detecting protein-protein interactions was set up to confirm the binding interaction between Grx3 (or Grx4) and Aft1. The Gal4 DNA binding domain was fused to Grx3 and Grx4, generating Grx3/Gal4 and Grx4/Gal4 fusion proteins. The transactivation domain from the herpes simplex VP16 was fused to the C terminus of Aft1. Cells harboring the two fusion proteins and a GAL1/ *lacZ* reporter fusion were assayed for β -galactosidase activity. The combination of either glutaredoxin fusion with Aft1/VP16 resulted in elevated β -galactosidase activity (Fig. 6A). Mutation of the critical Cys in either glutaredoxin abrogated *lacZ* expression consistent with a loss of interaction. The mutant Grx3/Gal4 and Grx4/Gal4 fusion proteins were shown to be equally abundant as the wild type fusion proteins by immunoblotting (data not shown). In addition, the interaction of Grx3 and Grx4 with Aft1 was markedly diminished when the constitutively active Aft1 C291F,C293F mutant variant was used (data not shown). The observed interaction between the two glutaredoxins and Aft1 was not altered by changes in the cellular iron status (Fig. 6B).

To confirm the observed interaction between the glutaredoxins and Aft1, constructs were engineered in which *AFT1* was TAP-tagged and *GRX4* was either Myc-tagged or poly-His-tagged. Cells harboring vec-

ASBUE

DOCKET



Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts



Keep your litigation team up-to-date with **real-time** alerts and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research



With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips



Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.

