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Monothiol CGFS Glutaredoxins and BolA-like Proteins: [2Fe-2S] Binding Partners in Iron Homeostasis

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Abstract

Monothiol glutaredoxins (Grxs) with a signature CGFS active site and BolA-like proteins have recently emerged as novel players in iron homeostasis. Elegant genetic and biochemical studies examining the functional and physical interactions of CGFS Grxs in the fungi *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have unveiled their essential roles in intracellular iron signaling, iron trafficking, and the maturation of Fe-S cluster proteins. Biophysical and biochemical analyses of the [2Fe-2S]-bridging interaction between CGFS Grxs and a BolA-like protein in *S. cerevisiae* provided the first molecular-level understanding of the iron regulation mechanism in this model eukaryote, and established the ubiquitous CGFS Grxs and BolA-like proteins as novel Fe-S cluster-binding regulatory partners. Parallel studies focused on *E. coli* and human homologues for CGFS Grxs and BolA-like proteins have supported the studies in yeast and provided additional clues to their involvement in cellular iron metabolism. Herein we review recent progress in uncovering the cellular and molecular mechanisms by which CGFS Grxs and BolA-like proteins help regulate iron metabolism in both eukaryotic and prokaryotic organisms.

Iron Homeostasis and Disease

The unique chemical properties of iron render it an essential but potentially toxic protein cofactor. Iron is capable of performing redox chemistry that is required for basic cellular functions, such as respiration, photosynthesis, DNA biosynthesis, and nitrogen fixation, yet this same property can also lead to the production of reactive oxygen species that damage cellular components. Thus, maintaining optimal intracellular levels of this transition metal is critical for cell survival. Due to the low bioavailability of iron, iron deficiency is the most common and widespread nutritional disorder in the world (1). At the other extreme, iron overload diseases are common systemic iron disorders primarily caused by mutations in proteins that sense, regulate, or mediate iron absorption from the gastrointestinal tract (for reviews see (2–4)). Increased iron absorption and storage leads to excessive iron accumulation in various organs (mainly liver, heart, and pancreas) causing progressive organ damage and increased mortality as a consequence of elevated oxidative stress. In addition to systemic iron disorders, numerous human diseases have also been linked with iron dysregulation at the cellular level. For example, specific defects in Fe-S cluster biogenesis factors lead to Friedreich's ataxia, X-linked sideroblastic anemia, sideroblastic-like microcytic anemia, and myopathy (for reviews see (5, 6)). Significant progress in identifying proteins involved in iron metabolism has provided a glimpse at the elaborate control mechanisms required to regulate this essential metal and revealed critical insight into the pathophysiology of iron-associated genetic disorders. However, there are substantial gaps in

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our fundamental understanding of iron regulation mechanisms at both the cellular and molecular level that require further study.

The fungi *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have proven to be effective models for studying eukaryotic iron homeostasis at the cellular level. Despite their relative simplicity, biochemical and genetic studies in yeast have been critical for identifying proteins required for iron uptake, intracellular iron transport and mobilization, and heme and Fe-S cluster biogenesis in higher eukaryotes (7–10). Furthermore, genome-wide studies in yeast have revealed how eukaryotic cells adapt to both iron deficiency (reviewed in (11)) and iron overload (12, 13). In addition, yeast studies have been pivotal in defining the pathophysiology of human diseases of iron metabolism, such as Friedreich's ataxia and aceruloplasminemia (14–18). Current knowledge on iron acquisition and storage systems in yeast is provided in several recent reviews (19–21) and will not be discussed here. The purpose of this review is to summarize recent developments in our understanding of iron sensing and regulation at the molecular level and highlight the roles of the CGFS monothiol Grxs and BolA-like proteins in these regulation pathways. We will emphasize the involvement of these two protein families in iron regulation in yeast model systems and draw comparisons to parallel studies in other organisms.

CGFS Monothiol Glutaredoxin Family

Glutaredoxins (Grxs) were initially identified as members of the thioredoxin (Trx)-fold family that catalyze thiol-disulfide exchange reactions in a glutathione (GSH)-dependent manner via a conserved CPY/FC active site (22). Classical dithiol Grxs that utilize a dithiol mechanism to reduce intramolecular disulfide bonds require both cysteines for catalytic activity, while Grxs that catalyze glutathionylation/deglutathionylation reactions via a monothiol mechanism require only the N-terminal active site Cys (23). With the increasing number of sequenced genomes, several Grxs with divergent active site sequences have been identified, thus requiring an updated phylogenetic classification for the Grx family (24). The most widespread Grxs present in both prokaryotes and eukaryotes are grouped in Class I, which includes the classical dithiol Grxs, and in Class II, which are defined as monothiol Grxs with a conserved CGFS active site. CGFS-type monothiol Grxs can be further classified into two groups: single domain CGFS Grxs and multidomain CGFS Grxs with an N-terminal Trx-like domain and one or more Grx-like domains (Figure 1).

Unlike Class I Grxs, CGFS Grxs have little or no thiol-disulfide oxidoreductase activity when tested with standard Grx model substrates (25–30). However, the CGFS active site is required for a different purpose: coordination of a [2Fe-2S] cluster. Both single and multidomain CGFS Grxs form $[2Fe\text{-}2S]^{2+}$-bridged homodimers with all-cysteinyl ligation provided by the two CGFS active sites and two GSH molecules (30–37). Formation of this Fe-S complex is supported by studies in *S. cerevisiae* demonstrating that iron binding to CGFS Grxs *in vivo* requires the CGFS motif, sufficient cellular GSH levels, and the mitochondrial Fe-S assembly machinery (38). The first crystal structure of a [2Fe-2S]-bridged CGFS Grx homodimer was published for *E. coli* Grx4 in 2009, confirming that two GSH molecules are covalently linked to the cluster, but held in place by non-covalent interactions with the GSH binding pocket of each Grx4 monomer (33) (Figure 2). A recent crystal structure for human Glrx5 reveals a similar coordination environment for the [2Fe-2S] cluster; however, in this structure, two [2Fe-2S]-bridged homodimers interact to form a tetramer (30). There is currently no published crystal structure available for a [2Fe-2S]-bound multidomain CGFS Grx, thus the orientation of the Trx-like domain in relation to the [2Fe-2S]-bridged Grx-like domain(s) is unknown.

In eukaryotes, single domain CGFS Grxs (e.g., yeast Grx5 and human Glrx5, see Figure 1) are localized to mitochondria or chloroplasts and have been implicated in the maturation of Fe-S cluster proteins (for recent reviews see (5, 39, 40)). In contrast, multidomain CGFS Grxs (e.g., yeast Grx3/4 and human Glrx3) display cytosolic/nuclear localization where they are proposed to play dual roles in cytosolic iron trafficking and iron regulation (38, 41–44). The Fe-S biogenesis function of single domain CGFS Grxs as well as the trafficking and regulatory functions of multidomain CGFS Grxs in yeast are all dependent on the presence of the conserved Cys in the CGFS active site, suggesting that coordination of the [2Fe-2S] cluster is essential to these functions (38, 42, 44–47).

Bioinformatics Studies Provide a Link between CGFS Grxs and BolA Proteins

Bioinformatics analyses provided the initial link between CGFS Grxs and another widely distributed protein family, the BolA-like proteins (48). A physical interaction between members of these two protein families was first detected via genome-wide yeast two-hybrid assays for *S. cerevisiae* and *D. melanogaster* (49, 50) and high-throughput affinity capture studies in *S. cerevisiae* and *E. coli* (51–53). In addition, gene clustering and co-occurrence analyses also predicted a functional interaction between CGFS Grxs and BolA-like proteins in both eukaryotes and prokaryotes (24, 48, 54). Finally, an *E. coli* synthetic genetic array study demonstrated that deletion mutants for *grxD* and *yrbA*, genes encoding a CGFS Grx and BolA-like protein, respectively, both display aggravating genetic interactions with mutations in the Isc (iron sulfur cluster) operon, which encodes components of the housekeeping Fe-S cluster assembly pathway. These studies suggested that Grx4 and YrbA function together in an alternate pathway for Fe-S cluster assembly in *E. coli* (55).

BolA-like proteins are generally grouped into three subfamilies designated BolA1-, BolA2-, and BolA3-like proteins. BolA1-like proteins are found in both prokaryotes and eukaryotes and include the eponymous member of the family, namely *E. coli* BolA. *E. coli* BolA, so named because of the round, bolus morphology exhibited by *bolA* overexpression strains, is a putative transcriptional regulator that plays a role in stress response via control of genes involved in maintenance of cell morphology (56–58). The other BolA-like protein encoded by the *E. coli* genome, namely YrbA, is grouped in a separate clade from the BolA1-3 subfamilies in the BolA-like protein phylogenetic tree (59). YrbA is genetically linked to iron metabolism as mentioned above; however, its specific function is unknown. Unlike BolA1 proteins, BolA2- and BolA3-like proteins are found exclusively in eukaryotes (60).

The genomes of *S. cerevisiae, S. pombe,* and *H. sapiens* each encode three BolA homologues, one for each of the three subfamilies (Figure 3). Human BolA1 is an ortholog of Yal044w-a in *S. cerevisiae* and Uvi31 in *S. pombe,* which are evolutionarily closest to *E. coli* BolA. Human BolA2 is an ortholog of Fra2 in *S. cerevisiae* and SPAC8C9.11 in *S. pombe*, while human BolA3 is grouped with Aim1 in *S. cerevisiae* and SPCC4B3.11c in *S. pombe* (59, 60). NMR structures for mouse BolA1 (1V60) and *E. coli* BolA (2DHM) are available in the Protein Data Bank, and the solution structure of mouse BolA2 (1V9J) was published in 2004 (60). The mouse BolA2 structure reveals a fold with structural similarities to nucleic acid binding proteins, including a helix-turn-helix motif (60). The yeast and human BolA3 homologues shown in Figure 3 all have 91–95% probability for mitochondrial targeting based on analysis of their N-terminal sequences using the MitoProt II program (61). In fact, mitochondrial localization of human BolA3 has been verified in human fibroblast cells (62). Roles for *S. cerevisiae* Fra2 (a BolA2 homologue) and human BolA3 in iron homeostasis have been established and are highlighted in this review. However, there are no published studies exploring analogous functions for the BolA protein family in *S. pombe* or for the other BolA homologues in *S. cerevisiae* and humans.

Roles for Grx3/4 and Fra2 in Iron Homeostasis in *S. cerevisiae*

A function for both CGFS Grxs and BolA-like proteins in iron regulation was first revealed by genetic studies in the budding yeast *S. cerevisiae.* The expression of iron uptake and storage genes in *S. cerevisiae* is primarily controlled by the iron-responsive transcription factor Aft1 and its paralog Aft2 (63, 64). Aft1 and Aft2 activate gene expression in iron-deficient conditions by binding to the same promoter elements, presumably via a similar mechanism (65–67). Aft1 is considered the primary regulator of iron homeostasis since *aft1Δ* mutants exhibit a stronger iron-deficiency phenotype than *aft2Δ* mutants. However, an *aft1Δ aft2Δ* double mutant is more sensitive to iron-deficient conditions than an *aft1Δ* single mutant, indicating that Aft2 can partially compensate for loss of Aft1 in iron regulation (63, 64, 66). Aft1 is proposed to continuously cycle between the nucleus and cytosol, favoring cytosolic localization under iron replete conditions (Figure 4A). Under iron-depleted conditions, Aft1 accumulates in the nucleus where it binds to and activates genes involved in iron uptake, transport, and storage, known collectively as the iron regulon (Figure 4B) (68–73). A high-throughput yeast two-hybrid study demonstrating an interaction between Aft1 and Grx3 (74) led researchers to test whether Grx3 and Grx4 influenced Aft1 regulation of iron homeostasis. Deletion of *GRX3* or *GRX4* singly has little or no phenotypic consequence, while *grx3Δ grx4Δ* double mutants are severely growth impaired or inviable (depending on the strain background), and exhibit intracellular iron overaccumulation resulting from constitutive activation of the iron regulon (38, 42, 43). The two cytosolic *S. cerevisiae* CGFS Grxs thus perform essential but redundant functions in iron metabolism as suggested by their high degree of sequence similarity (67% identical, 79% similar), and will be referred to jointly as Grx3/4 in this review. Interestingly, recent studies demonstrate that a variety of iron-dependent cytosolic and mitochondrial enzymes that bind Fe-S clusters, heme, and non-heme iron exhibit dramatically reduced *in vivo* iron incorporation and enzymatic activity upon disruption of Grx3/4 expression, indicating that intracellular iron is not bioavailable in *grx3grx4* mutants despite high levels (38, 75). Furthermore, *grx3grx4* mutants exhibit decreased mitochondrial iron accumulation (as well as decreased copper and zinc) implicating a role for these proteins in iron delivery to mitochondria (38). Thus, in addition to regulation of Aft1/2 activity, the cytosolic, multidomain CGFS Grxs are suggested to play an essential role in intracellular iron trafficking.

A role for BolA-like proteins in iron regulation was discovered via a genetic screen for altered iron metabolism in *S. cerevisiae*. A deletion mutant for the BolA-like protein encoded by *YGL220W* exhibited phenotypes suggestive of misregulation of iron metabolism, including accumulation of mitochondrial iron, constitutive siderophore and ferrous iron uptake, and constitutive ferrireductase activity (76). A subsequent study established that Ygl220w (renamed as Fra2 for Fe repressor of activation-2) is part of a signaling pathway that includes the mitochondrial Fe-S cluster biosynthesis machinery, Grx3/4, and an aminopeptidase P-like protein named Fra1 (Fe repressor of activation-1) (41). Under Fe replete conditions, the Fra1/Fra2/Grx3/Grx4 signaling pathway is proposed to interpret and transmit an as-yet unidentified Fe-S-dependent mitochondrial inhibitory signal to Aft1/2 that induces its multimerization, which it turn favors export of Aft1/2 from the nucleus via interaction with the exportin Msn5 (Figure 4A) (41, 77). The specific compartment in which the Fra-Grx inhibitory complex interacts with Aft1/2 is unclear. An initial study using overexpressed, GFP-tagged Grx3 suggested that this protein is primarily localized to the nucleus (45). However, natively expressed, untagged Grx3 was found to exhibit mainly cytosolic localization. In addition, restriction of Grx4 to the cytosol via tethering to the mitochondrial outer membrane was shown to have little effect on inhibition of Aft1 activity in response to iron, suggesting that the Fra-Grx inhibitory complex acts in the cytosol (41). Under low iron conditions or upon disruption of mitochondrial Fe-S cluster

biogenesis, the Fra-Grx iron-signaling pathway is deactivated, allowing Aft1 (and presumably Aft2) to accumulate in the nucleus and activate the iron regulon (Figure 4B). This signaling pathway is mediated by specific protein-protein interactions between Aft1 and Grx3/4, Grx3/4 and Fra2, and Fra1 and Fra2 as detected by yeast two-hybrid and co-immunoprecipitation interaction studies (41–43). In each case, the protein pairs interact in an iron-independent manner *in vivo*, thus the specific iron-dependent molecular mechanism for inhibiting Aft1 and Aft2 is unclear.

The regulatory functions of the low iron-sensing transcriptional activators Aft1/Aft2 are also complemented by a high iron-sensing transcriptional activator named Yap5 (reviewed in (19)). Under high iron conditions, Yap5 activates expression of the vacuolar iron transporter *CCC1* resulting in increased iron transport into the vacuole, which effectively lowers cytosolic iron levels (78). A role for the Grx-Fra signaling pathway in regulation of Yap5 activity has not been established; however, a specific connection with Grx4 function was recently established by the fact that Grx4 is upregulated by Yap5 under high iron conditions (79). It is possible that increased Grx4 levels help reduce iron toxicity by sequestering cytosolic iron in the form of [2Fe-2S] Grx4 homodimers or increasing intracellular iron trafficking to facilitate iron usage. Additional studies are required to test this hypothesis.

Molecular Basis of Grx3/4 and Fra2 Regulation of Iron Homeostasis in *S. cerevisiae*

A combination of biochemical, genetic, and spectroscopic studies has started to uncover the molecular details of the protein-protein and metal-protein interactions that govern regulation of Aft1/2 activity. As mentioned earlier, Grx3/4 forms $[2Fe\text{-}2S]^{2+}$-bridged homodimers via the CGFS active sites in the Grx domains (32, 35). Mutation of this Cys residue disrupts both the iron regulation and iron trafficking functions of Grx3/4, prevents iron incorporation and Grx4 homodimer formation *in vivo*, and abolishes the interaction between Grx3/4 and Aft1 (38, 42). Taken together, these data suggest that Fe-S coordination is essential to the function of Grx3/4. In addition to the CGFS active site, the functionalities of other regions of Grx3/4 have been mapped out by *in vivo* and *in vitro* mutagenesis studies. By replacing the C-terminal 16 amino acids of *S. cerevisiae* Grx4 with the corresponding region of *S. pombe* Grx4, Hoffmann and coworkers demonstrated that this specific sequence of *S. cerevisiae* Grx4 is essential for the *in vivo* interaction between Grx4 and Aft1. Interestingly, replacement of this sequence did not disrupt the iron trafficking functions of Grx3/4, indicating that the trafficking and regulation functions of Grx3/4 are independent (80). The role of the less well-conserved Trx domain has also been studied, although its specific function still remains elusive. Mutation of a conserved Cys in the Trx domain of Grx3/4 was found to have little impact on Fe-S binding or homodimer formation *in vitro* (35), as well as iron binding, iron trafficking, and Aft1/2 iron regulation *in vivo* (38). Removal of this domain does not impact Fe-S binding to the Grx domain *in vivo* and *in vitro* (35, 80), although the Trx domain is essential for Grx3/4 trafficking and regulation functions *in vivo* (80). Based on these results, the Trx domain is proposed to mediate specific protein-protein interactions with Grx3/4 binding partners; however, further studies are required to test this hypothesis.

In vitro biochemical and biophysical studies have been used to probe the interactions between *S. cerevisiae* Grx3/4 and its binding partner Fra2. Our group recently demonstrated that both Grx3 and Grx4 form $[2Fe\text{-}2S]^{2+}$-bridged heterodimers with Fra2 (35, 81). Fra2-Grx3/4 heterodimers exhibit significant differences in cluster stability and coordination environment in comparison to Grx3/4 homodimers. The Fe-S cluster in the Grx3/4 homodimer is sensitive to both O_2 oxidation and reduction with dithionite, while the Fe-S cluster in the Fra2-Grx3/4 heterodimer is both reductively and oxidatively stable as isolated.

In addition, the [2Fe-2S] Grx3 homodimer can be rapidly converted to a [2Fe-2S] Grx3-Fra2 heterodimer by titration with apo-Fra2, demonstrating that formation of the heterodimer is thermodynamically and kinetically favored. Spectroscopic and mutagenesis studies confirmed that the iron ligands in the Fra2-Grx3/4 heterodimer are provided by the Grx active site Cys of Grx3/4, a conserved His in Fra2 (His103), and a Cys from GSH. The identity of the fourth iron ligand is unknown; however, an additional His ligand is ruled out by the spectroscopic data (35, 81). This ligand arrangement is somewhat unusual as [2Fe-2S] clusters are typically bound in Cys_4 or Cys_2His_2 coordination environments, and are not often found at the interface of two binding partners (82). Removal of the Trx domain had no effect on heterodimer Fe-S cluster binding or Fra2-Grx3/4 complex formation (35). In addition, mutation of His103 in Fra2 does not interfere in heterodimer formation and cluster binding *in vitro*, although EPR and EXAFS analyses suggest that the Fe-S cluster stability is compromised upon replacement of His103 with Cys or Ala (81). However, His103 in Fra2 is indispensable for inhibition of Aft1 *in vivo*, suggesting that a stable and/or redox-active cluster may be important for Fra2-Grx3/4 function (81). Furthermore, this residue is well conserved in both prokaryotic and eukaryotic BolA homologues (see Figure 3), therefore it may play an important structural or functional role for other members of this protein family. Although recombinant [2Fe-2S]-bound Grx3/4 homodimers and Fra2-Grx3/4 heterodimers are apparently assembled by the *E. coli* Fe-S biogenesis machinery during overexpression, it remains a mystery how the [2Fe-2S] clusters are assembled on Grx3/4 homodimers and Fra2-Grx3/4 heterodimers in *S. cerevisiae* under physiological conditions. The yeast cytosolic iron-sulfur assembly (CIA) system is not required for *in vivo* iron incorporation into Grx3/4 (38) or iron-dependent inhibition of Aft1/2 activity (65). Thus, an unidentified parallel pathway must exist for Fe-S loading into Grx3/4 and Fra2-Grx3/4 complexes.

Mutagenesis studies of Aft1/2 have also provided some insight into the molecular details of Aft1/2 inhibition in response to iron. The N-terminal DNA binding domains of Aft1 and Aft2 are homologous to the WRKY-GCM1 superfamily of eukaryotic transcriptional factors. Both Aft1 and Aft2 have conserved Cys/His residues in this domain that are zinc finger ligands in some WRKY-GCM1 family members (83). Aft1 and Aft2 also share a CDC motif that is required for interaction with Grx3/4 and the exportin Msn5, as well as Aft1/2 oligomerization and translocation in response to iron (Figure 5A). Mutation of either Cys residue in the CDC motif (called $Aft1/2^{up}$ mutations) leads to constitutive nuclear localization of Aft1/2 and activation of the iron regulon (65, 68, 77).

Despite the significant progress in defining the molecular interactions between several components in this iron signaling pathway, some key aspects of the iron sensing and regulation mechanism still remain unresolved. Most importantly, what is the specific molecular mechanism for inhibiting Aft1/2 activity in response to iron? The essential role of the CDC motif in iron-dependent Aft1/2 inhibition, taken together with the dependence of Grx3/4 and Fra2 function on [2Fe-2S] cluster binding, strongly suggests that thiol redox chemistry and/or Fe-S or Fe binding to Aft1/2 may drive multimerization and translocation of Aft1/2 to the cytosol under iron replete conditions. Fra1 is also required for Aft1-mediated iron signaling and interacts with Fra2 *in vivo,* but how is it specifically involved in the signaling pathway? It is also worthwhile to note that the Fra-Grx signaling pathway may not be the only method for inhibiting Aft1/2 activity under iron replete conditions. Transcriptional reporter assays of Aft1/2-regulated genes indicate that the iron regulon is not fully activated in iron-sufficient medium in *fra1Δ* or *fra2Δ* mutants or upon disruption of mitochondrial Fe-S assembly pathways, suggesting that a separate signal may partially inhibit Aft1/2 activity in these mutants (41). These possibilities must be examined in order to tease out the molecular details of the regulation mechanism and provide a more complete picture of iron regulation in *S. cerevisiae* at the cellular and molecular level.

Roles for Grx4 in Iron Homeostasis in *S. pombe*

Due to significant evolutionary divergence, the genome of *S. pombe* does not encode homologues of *S. cerevisiae* Aft1 and Aft2. Instead, iron homeostasis in *S. pombe* is primarily modulated by two repressors, Fep1 and Php4, that are responsible for controlling iron acquisition and iron utilization, respectively (19, 21). Fep1 is a GATA-type transcription factor that binds to GATA-containing sequences in promoters of iron uptake and transport genes in iron replete conditions, thereby repressing their expression to avoid iron overload (Figure 6A, *left*). Repression by DNA-bound Fep1 requires recruitment and binding of the co-repressors Tup11 and Tup12 in an iron-independent manner, and deletion-mapping studies have delineated the Tup11 interaction domain in the C-terminus of Fep1 (Figure 5B) (84). The C-terminus of Fep1 also contains a dimerization domain that is required for efficient repression of Fep1 target genes (85). When iron levels are low, Fep1 is released from the promoter region and the Fep1 regulon is activated via an unknown mechanism to promote iron acquisition (Figure 6B, *left*) (86).

The transcriptional repressor Php4 also controls iron homeostasis in *S. pombe* by regulating expression of genes involved in iron-dependent metabolic pathways. Php4 binds to a heterotrimeric protein complex composed of Php2, Php3, and Php5. Under iron replete conditions, Php4 is not expressed and the Php2/Php3/Php5 complex activates expression of its target genes by binding to CCAAT sequences in their promoters (Figure 6A, *left*). Php2/Php3/Php5-regulated genes encode proteins involved in iron-dependent metabolic pathways such as iron-sulfur cluster biogenesis, heme biosynthesis, the mitochondrial electron transport chain, and the tricarboxylic acid cycle (87). Under low iron conditions, Php4 is expressed and binds to the Php2/Php3/Php5 complex, causing it to switch from an activator to a repressor (Figure 6B, *left*). Thus, iron-utilizing pathways are downregulated as an iron-sparing response to lower bioavailable iron.

Php4 is itself regulated at the transcriptional level by Fep1 since the *php4*$^{+}$ gene contains GATA elements within its promoter (87). When iron levels are high, Fep1 binds to the promoter of *php4*$^{+}$ and inactivates its transcription (Figure 6A, *right*). When iron levels are low, Fep1 is unable to inhibit *php4*$^{+}$ transcription and Php4 is subsequently expressed, allowing it to repress iron-utilizing pathways via the Php2/Php3/Php5 complex (Figure 6B, *right)*. The Php2/Php3/Php5 complex itself is not directly responsive to iron levels since Php2, Php3 and Php5 are constitutively synthesized (87). A genome-wide DNA microarray study also revealed that the *fep1*$^{+}$ gene contains CCAAT *cis*-acting elements in its promoter region and is downregulated in a Php4-dependent manner in response to iron deprivation (Figure 6B, *right*) (88). This reciprocal regulatory loop between two iron-responsive repressors through mutual control of each other's expression allows direct crosstalk between iron acquisition and iron utilization pathways to fine tune iron homeostasis in *S. pombe*.

In addition to iron-dependent cross-regulation at the transcriptional level, the activities of both Fep1 and Php4 are controlled at the post-translational level by *S. pombe* Grx4, a member of the multidomain CGFS Grx subfamily. Thus, it is interesting to note that although Fep1 and Php4 do not share significant sequence identity with Aft1 or Aft2 and use different regulation mechanisms, multidomain CGFS Grxs still mediate iron regulation in these evolutionarily divergent systems. While *S. cerevisiae* has two cytosolic CGFS Grxs (Grx3/4), *S. pombe* only has one (Grx4) (see Figure 1) (89). Although a role for Grx4 in iron trafficking has not been explored in *S. pombe*, it is clear that Grx4 is required to regulate the activity of both Fep1 and Php4 through specific protein-protein interactions. Deletion of *grx4* leads to constitutive repression of both Php4- and Fep1-regulated genes and constitutive nuclear localization of Php4 (44, 47, 90). Yeast two-hybrid and bimolecular fluorescence complementation experiments established that Grx4 physically interacts with

Php4 regardless of cellular iron levels. However, under iron-replete conditions Grx4 promotes Php4 export to the cytosol by facilitating direct interaction with the nuclear exportin Crm1 (Figure 6A). Nevertheless, nucleocytoplasmic shuttling is not the primary mechanism for control of Php4 activity since Php4-regulated genes are still activated in iron-replete conditions upon inhibition of Crm1 nuclear export activity. These results suggest that in addition to promoting nuclear export of Php4, Grx4 must also prevent Php4 from switching the Php2/Php3/Php5 complex from activator to repressor under iron replete conditions (90). Two recent studies have shown that Fep1 is also regulated through Grx4 at the post-translational level (44, 47). Grx4 was shown to constitutively interact with Fep1; however, the Fep1-Grx4 complex resides in the nucleus regardless of iron status inside the cell, unlike the iron-dependent nucleocytoplasmic shuttling of the Php4-Grx4 complex. When iron is limited, Grx4 inhibits Fep1 function, which leads to dissociation of Fep1 from chromatin and derepression of the Fep1 regulon (Figure 6B, *left*), including the *php4*$^{+}$-encoded repressor that will then further inhibit the expression of *fep1*$^{+}$ (Figure 6B, *right*). When iron is abundant, Grx4 is unable to inhibit Fep1 repressor function, although it still physically interacts with Fep1 (Figure 6A, *left*) (44, 47).

Molecular Basis of Grx4 Regulation of Iron Homeostasis in *S. pombe*

A combination of yeast two-hybrid, co-immunoprecipitation, and bimolecular fluorescence complementation studies have helped map out the specific domains and residues that govern protein-protein interactions between Grx4 and Fep1 or Php4 and provide a molecular-level understanding of the iron-dependent mechanisms for inhibiting the activity of these transcriptional repressors. These studies demonstrated that the N-terminal Trx domain of Grx4 invariably and strongly interacts with the C-terminus of Fep1, whereas the C-terminal Grx domain of Grx4 weakly interacts with the N-terminus of Fep1 only in iron-depleted conditions. The association between the N-terminus of Fep1 and the Grx domain of Grx4 requires Grx4 Cys172 located in the CGFS motif, while the interaction between the C-terminus of Fep1 and the Trx domain of Grx4 is dependent on Cys35 located in the WAxxC motif (see Figure 1) (44). Analysis of Fep1 function upon expression of C35A or C35S Grx4 in *grx4* mutant strains indicated that Cys35 in the Trx domain is dispensable for Grx4-dependent inhibition of Fep1 activity. In contrast, mutation of Cys172 in the Grx domain of Grx4 led to constitutive repression by Fep1, demonstrating that the weak interaction between the Grx domain of Grx4 and the N-terminal domain of Fep1 is critical to the inhibition mechanism (44, 47). It is interesting to note that *S. pombe* Fep1 and other iron-dependent GATA transcription factor homologues in siderophore-producing fungi have a conserved cysteine-rich region sandwiched between two zinc finger domains in the N-terminal DNA binding domain (Figure 5B) (21, 91). The four conserved Cys residues in this region are required for high affinity DNA binding and have been implicated in Fe^{3+} binding (87, 91). The ability of this cysteine rich region to bind an Fe-S cluster has not yet been tested, although this is in fact a strong possibility since the UV-visible absorption spectrum of as-purified recombinant SRE (92), a Fep1 homologue from *N. crassa*, is reminiscent of proteins that bind a $[2Fe\text{-}2S]^{2+}$ cluster (93). Nevertheless, *in vivo* studies suggest that iron binding is not absolutely necessary for Fep1 DNA binding since Fep1 remains bound to DNA and represses its target genes in *grx4* mutants under both low and high iron conditions (44, 47). It is also important to note that inhibition of Fep1 activity via interaction with Grx4 occurs under low iron conditions in *S. pombe*, in contrast to the situation in *S. cerevisiae* where Grx3/4-mediated inhibition of Aft1/2 occurs under iron-replete conditions. This suggests that the CGFS Grx-dependent inhibition mechanisms for *S. cerevisiae* Aft1/2 and *S. pombe* Fep1 are quite distinct.

In contrast to Fep1, *S. pombe* Php4 is inhibited by Grx4 in iron-replete conditions and undergoes iron-dependent nucleocytoplasmic shuttling similar to *S. cerevisiae* Aft1/2. The

Php4 nuclear export signal is located in the N-terminal domain along with the putative interaction site for the Php2/3/5 complex (Figure 5B) (87, 90). Interestingly, Php4 and its orthologs in other fungi have one to three conserved cysteine rich regions in the C-terminal domain that are proposed to bind iron (94). *S. pombe* Php4 has only two conserved cysteines in this region, Cys221 and Cys227 (Figure 5B). A recent report by Vachon and coworkers demonstrates that these two conserved cysteines as well as Grx4 Cys172 located in the CGFS [2Fe-2S] binding site are essential for the iron-dependent interaction between the Php4 C-terminal domain (residues 152–254) and the Grx domain of Grx4 (95). In addition, transcriptional analysis of Php4-regulated genes upon expression of C172S/A Grx4 mutants demonstrates that Cys172 in the CGFS domain is absolutely required for Grx4-dependent inhibition of Php4 activity (47, 95). Based on these findings, Php4 and Grx4 are proposed to form an Fe-S binding complex that inhibits Php4 activity in iron replete conditions (Figure 6A) (95).

The interaction between Php4 and Grx4 is also partially mediated by the Trx domain of Grx4. Yet, in contrast to the Grx domain, the Trx domain of Grx4 binds to Php4 in an iron-independent manner. The Trx domain interaction site on Php4 has been mapped to two regions between residues 55–218 and 255–295 (Figure 5B). Grx4 Cys35 is required for the strong interaction between the Trx domain and Php4 (95); however, unlike the conserved Cys in the Grx domain, mutation of the conserved Cys in the Trx domain has no effect on Php4 activity *in vivo* (47). This interaction pattern between Grx4 and Php4 parallels the Grx4-Fep1 interaction: the Trx domain Cys35 facilitates a strong, iron-independent interaction with the repressor that is dispensable for repressor inhibition, while the Grx domain Cys172 maintains a weak, iron-dependent interaction that is essential for repressor inhibition. However, one key difference is that the Grx domain of Grx4 binds to Php4 only under iron replete conditions *in vivo*, but interacts with Fep1 exclusively under low iron conditions.

Overall, *S. cerevisiae* and *S. pombe* utilize different mechanisms for regulation of iron homeostasis, given the fact that Aft1 and Aft2 are transcriptional activators while Fep1 and Php4 are transcriptional repressors. However, the available data strongly suggest that Fe-S binding via multidomain CGFS Grxs (Grx3/4 for *S. cerevisiae* and Grx4 for *S. pombe*) is an important factor required for inhibition of *S. cerevisiae* Aft1/2 as well as *S. pombe* Php4 and Fep1. In each case, mutation of the CGFS motif in Grx3/Grx4 disrupts Fe-dependent inhibition of the transcriptional regulators, leading to constitutive activation by Aft1/Aft2 or constitutive repression by Php4 and Fep1. In addition, GSH depletion in both *S. cerevisiae* and *S. pombe* leads to a similar loss of iron-dependent inhibition of Aft1/Aft2 and Php4, presumably stemming from the requirement for GSH as a [2Fe-2S] cluster ligand in CGFS Grx homodimers (65, 88, 96). Nevertheless, there are some key differences between the regulation mechanisms. For instance, Aft1/Aft2 regulation involves two additional proteins to some extent, the BolA homologue Fra2 and the aminopeptidase P-like protein Fra1. Roles for either of these proteins in *S. pombe* iron homeostasis have not been reported. Another important distinction is that Aft1/Aft2 and Php4 activities are inhibited by cytosolic CGFS Grxs under iron replete conditions, while Fep1 activity is inhibited under low iron conditions. Thus, if ligation of a [2Fe-2S] by Grx3/Grx4 is a key factor, FeS cluster binding must promote the inhibitive interaction between Aft1/Aft2/Php4 and Grx3/Grx4 while it prevents the inhibitive interaction between Fep1 and Grx4. Additional studies are required to reveal the inhibition mechanisms for each transcriptional factor at the molecular level.

CGFS Grxs, BolA-like Proteins, and Iron Homeostasis in *E. coli*

The *E. coli* genome encodes only one CGFS-type monothiol Grx (Grx4) that contains a single Grx-like domain (see Figure 1). Direct involvement of Grx4 in iron regulation via

interaction with the global iron regulator Fur has not been demonstrated. However, the gene encoding Grx4 (*grxD)* is induced under iron-depleted conditions and the induction level is significantly higher in *fur*⁻ strains, suggesting that Fur may be involved in regulation of *grxD* expression either directly or indirectly (29). In addition, *grxD* deletion mutants are sensitive to iron limitation, further emphasizing the functional involvement of Grx4 in iron metabolism. As mentioned earlier, a *grxD* mutant exhibits synthetic lethality when combined with mutations in several key components in the *isc* operon, indicating that Grx4 may function in a parallel pathway for Fe-S biogenesis. In *E. coli,* this parallel pathway is the Suf (sulfur formation) Fe-S assembly system that functions primarily under oxidative stress and iron deficient conditions (97). A deletion mutant for the BolA paralog YrbA displayed a similar aggravating genetic interaction with the Isc system, suggesting that it also operates in the Suf pathway with Grx4. Interestingly, a *bolA-isc* genetic interaction was not reported in this synthetic genetic array analysis, suggesting that the BolA and YrbA do not have redundant functions. This is not surprising given that the paralogs only share 22% sequence identity and 36% sequence similarity. Further genetic analysis of a *bolA yrbA* double mutant is necessary to help determine whether they have overlapping roles in *E. coli*.

A physical interaction between *E. coli* Grx4 and BolA was recently identified from both *in vitro* and *in vivo* studies (36, 53). Similar to the *S. cerevisiae* Grx3/4-Fra2 interaction (Figure 7, *middle*), *E. coli* Grx4 forms [2Fe-2S]-bridged heterodimers with BolA in addition to [2Fe-2S]-bridged homodimers (Figure 7, *top*). Moreover, [2Fe-2S] Grx4 is proposed to act as a Fe-S scaffold protein for *de novo* Fe-S cluster biosynthesis since it transfers its cluster to apo-ferredoxin with reasonable efficiency. In contrast, cluster transfer from [2Fe-2S] BolA-Grx4 to apo-ferredoxin is significantly slower, suggesting different functional roles for the [2Fe-2S]-bridged homo- and heterodimeric complexes *in vivo* (36). Although it is clear that the CGFS Grx-BolA interaction is conserved in both prokaryotes and eukaryotes, more work has to be done to resolve their specific roles in iron metabolism and overall cellular function. In addition, is not known whether YrbA and Grx4 directly interact in a similar manner to BolA and Grx4. These open questions as well as the specific connections with Fur and Fe-S cluster assembly systems are of particular interest.

CGFS Grxs, BolA-like Proteins, and Iron Homeostasis in *H. sapiens*

Iron-responsive transcription factors that are homologous to *S. cerevisiae* Aft1/Aft2 or *S. pombe* Php4/Fep1 have not been identified in humans. Instead, human cellular iron homeostasis is post-transcriptionally regulated by two mRNA binding proteins, IRP1 and IRP2 (reviewed in (98–100)). Under iron-depleted conditions, IRP1 and IRP2 bind to iron response elements (IREs) located in the 5′ or 3′ untranslated regions of mRNAs encoding proteins involved in iron homeostasis, which in turn leads to translational blocking or stabilization of the mRNA, depending on the location of the IRE. The net result is an increase in iron uptake and a decrease in iron storage/sequestration or utilization. Under iron replete conditions, IRP1 binds a [4Fe-4S] cluster that facilitates a conformation change that precludes mRNA binding. Thus, regulation of IRP1 activity is based on assembly (iron-replete) and disassembly (iron-depleted) of a [4Fe-4S] cluster in IRP1, which in turn is dependent on both mitochondrial and cytosolic Fe-S cluster assembly machineries (99). As such, the mitochondrial CGFS Grx (human Glrx5, see Figure 1) has been shown to be essential for iron-dependent regulation of IRP1 activity, via its role as a component of the mitochondrial Fe-S assembly machinery (37). Thus, CGFS Grxs play an equally important role on human iron metabolism compared to yeast metabolism, albeit through a somewhat different mechanism.

As opposed to IRP1, IRP2 does not bind an Fe-S cluster under iron replete conditions, but is rapidly ubiquinated by FBXL5, a component of an E3 ubiquitin ligase complex, which leads

to proteasomal degradation of IRP2. FBXL5 is stabilized under iron replete conditions via ligation of a diiron center, but is itself ubiquinated and degraded under iron-depleted conditions, in a reciprocal manner to IRP2. IRP1 is also a target for FBXL5, thus providing an additional mode of regulation for this iron sensor. [4Fe-4S] binding to IRP1 presumably limits access to the target sequence for FBXL5 binding, thereby preventing degradation of the protein in addition to inhibiting its mRNA binding activity (101). Unlike IRP1, a role for CGFS Grxs in regulation of IRP2 activity has not yet been demonstrated.

In addition to the mitochondrial single-domain CGFS Grx (Glrx5), the human genome encodes a cytosolic multidomain CGFS Grx (Glrx3), which has various alternative names in the literature including PICOT (for PKC-interacting cousin of thioredoxin) and TXNL2 (for thioredoxin-like 2). Human Glrx3 is analogous to yeast Grx3/Grx4 but has two tandem Grx-like domains rather than one (Figure 1). It is expressed in a wide variety of organs and tissues (102) and has been implicated in a number of signaling pathways involved in immune cell response, cell cycle progression during embryogenesis, cancer cell growth and metastasis, and regulation of cardiac hypertrophy (103–107). The specific function of Glrx3 in these pathways is unknown, but it is proposed to have a redox-dependent signaling role as demonstrated for other Trx and Grx oxidoreductases. However, as with other members of the CGFS Grx family, a specific enzymatic activity for Glrx3 has not been demonstrated. Nevertheless, Glrx3 performs an essential role in mammalian cells as *Glrx3*$^{-/-}$ mice die during embryogenesis (104, 105).

Recent studies have unveiled several intriguing connections between human Glrx3 and iron homeostasis. ^{55}Fe co-immunoprecipitation studies in Jurkat cells demonstrated that Glrx3 binds iron *in vivo* and forms [2Fe-2S]-bridged homodimers *in vitro* in an analogous manner to its yeast and *E. coli* homologues (34). However, while the yeast Grx3/4 homodimer has one bridging [2Fe-2S] cluster, the human Glrx3 homodimer has two tandem Grx domains that each form a [2Fe-2S] bridge with the other Glrx3 monomer (Figure 7, *bottom*) (34, 108). Another connection with iron metabolism is the finding that human Glrx3 physically interacts with the Fe-S binding protein CIAPIN1, also known as anamorsin (109), which is an essential component of the cytosolic iron-sulfur assembly (CIA) system (110). Interestingly, the *S. cerevisiae* homologue of CIAPIN1, called Dre2, also binds to yeast Grx3/Grx4, providing evidence for evolutionary conservation of this interaction (111). The interaction between Glrx3 and CIAPIN1 in humans suggests the possible involvement of Glrx3 in the CIA pathway and/or regulation of IRP1. In addition to specific roles in Fe-S cluster assembly, a recent study demonstrates that *S. cerevisiae* Dre2 and Grx3/4 are also required for *in vivo* formation of the diferric tyrosyl radical in the enzyme ribonucleotide reductase (75). A role for human Glrx3 in assembly of iron cofactors in mammalian cells has not been demonstrated, but is a strong possibility given the conservation of sequence and functional interactions found in these yeast and human iron homeostasis factors.

Additional evidence connecting both CGFS Grxs and BolA-like proteins with human iron metabolism is provided by two recent reports (62, 108). In the first report, Cameron and coworkers demonstrate that a mutation in human BolA3, which is localized to the mitochondrial matrix, lead to defects in lipoate biosynthesis and the assembly of some respiratory chain components. The authors propose that the underlying basis of these enzyme deficiencies is a defect in the maturation of Fe-S cluster-containing proteins that are key components of these mitochondrial pathways. Based on the strong physical and functional connection between CGFS Grxs and BolA proteins identified across a wide phylogenetic range, they suggest that BolA3 functions together with Glrx5 in inserting Fe-S clusters into target proteins (62). The second study connecting CGFS Grxs and BolA-like proteins with human iron metabolism was aimed at defining the interaction between cytosolic Glrx3 and another human BolA homologue, BolA2. Given the importance of

[2Fe-2S] Fra2-Grx3/4 in *S. cerevisiae* iron sensing, our group sought to test whether human Glrx3 forms a similar complex with BolA2, which is the human ortholog of yeast Fra2. Biophysical analysis of the recombinant human proteins following co-expression and purification from *E. coli* revealed that Glrx3 forms a [2Fe-2S]-bridged complex with BolA2. This complex is comparable to the [2Fe-2S]-bridged heterodimer formed by yeast Fra2 and Grx3/4; however, since Glrx3 has two tandem Grx domains, it is able to bind two BolA2 molecules forming a heterotrimer with two [2Fe-2S] bridges (Figure 7, *bottom*). Spectroscopic analysis of the [2Fe-2S] clusters in human Glrx3-BolA2 complexes confirmed that the cluster coordination environments are identical to the analogous yeast Fra2-Grx3/4 complex, including the single His ligand (108). Although the specific functions of [2Fe-2S] Glrx3 homodimers and [2Fe-2S] Glrx3-BolA heterotrimers in human cells are unknown, it seems likely that these complexes may play key roles in human iron metabolism given the parallels between the structures of the human and *S. cerevisiae* complexes as well as the extensive genetic and biochemical evidence linking the yeast homologues with iron sensing and regulation.

Concluding Remarks and Perspectives

In the past decade, monothiol CGFS Grxs have been widely investigated from different organisms, revealing their functional involvement in Fe-S cluster biogenesis as demonstrated for single domain family members, as well as iron trafficking and regulation as shown for multidomain CGFS Grxs. These studies have significantly expanded the roles of Grxs beyond the classical dithiol Grxs that catalyze thiol-disulfide redox reactions. In addition, the requirement for GSH in these newly discovered iron-dependent roles uncovers a novel function for this ubiquitous thiol-containing tripeptide as an Fe-S binding ligand and highlights the intimate connection between thiol redox homeostasis and iron metabolism. Formation of a GSH-ligated Fe-S cluster-binding complex is apparently a general feature for all CGFS Grxs, and the cluster is usually essential for their functional engagements. Nevertheless, the specific roles of either single or multidomain CGFS Grxs in Fe-S cluster assembly are still unknown. The regulatory roles of multidomain CGFS Grxs in iron homeostasis are only identified from yeast, and whether participation of CGFS Grxs and BolA-like proteins in iron regulation is a general trait across evolutionarily diverse organisms has to be systematically examined. In addition, despite the detailed biophysical and biochemical analyses published for Grx-BolA complexes from *E. coli*, *S. cerevisiae*, and *H. sapiens*, crystal or NMR structures providing atomic-level views of these [2Fe-2S]-bridging interactions are still lacking. In the future, complementary biophysical, biochemical, and genetic methods are required to methodically tease out the molecular interactions between CGFS Grxs and BolA-like proteins, and characterize their functional relationships with additional interacting partners in the cell. Given the widespread distribution of CGFS Grxs and BolA-like proteins in eukaryotes and prokaryotes, there is much interest in uncovering the fundamental and essential roles of both proteins families in iron metabolism.

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ABBREVIATIONS

Grx	glutaredoxin
Trx	thioredoxin

GSH	glutathione
EPR	electron paramagnetic resonance
EXAFS	extended X-ray absorption fine structure
ENDOR	electron-nuclear double resonance spectroscopy

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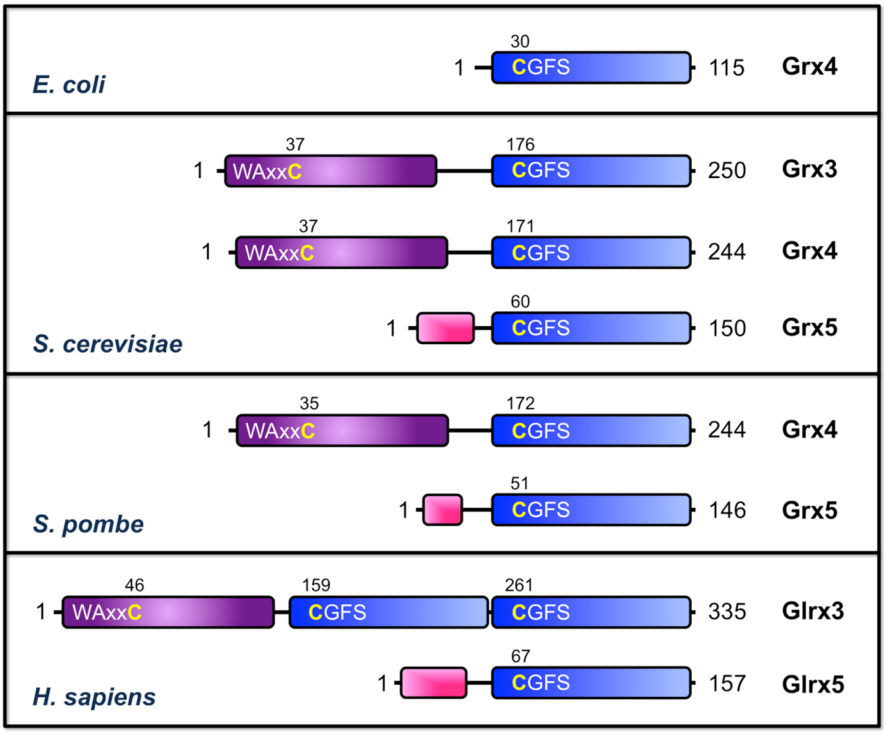

Figure 1.

Domain structure of CGFS monothiol Grxs from *E. coli*, *S. cerevisiae*, *S. pombe*, and *H. sapiens.* The Trx-like domains and Grx-like domains are shown as purple and blue boxes, respectively. The conserved cysteines in the active sites of the Trx and Grx domains are numbered and shown in yellow. Predicted or known mitochondrial targeting signals are shown as pink boxes.

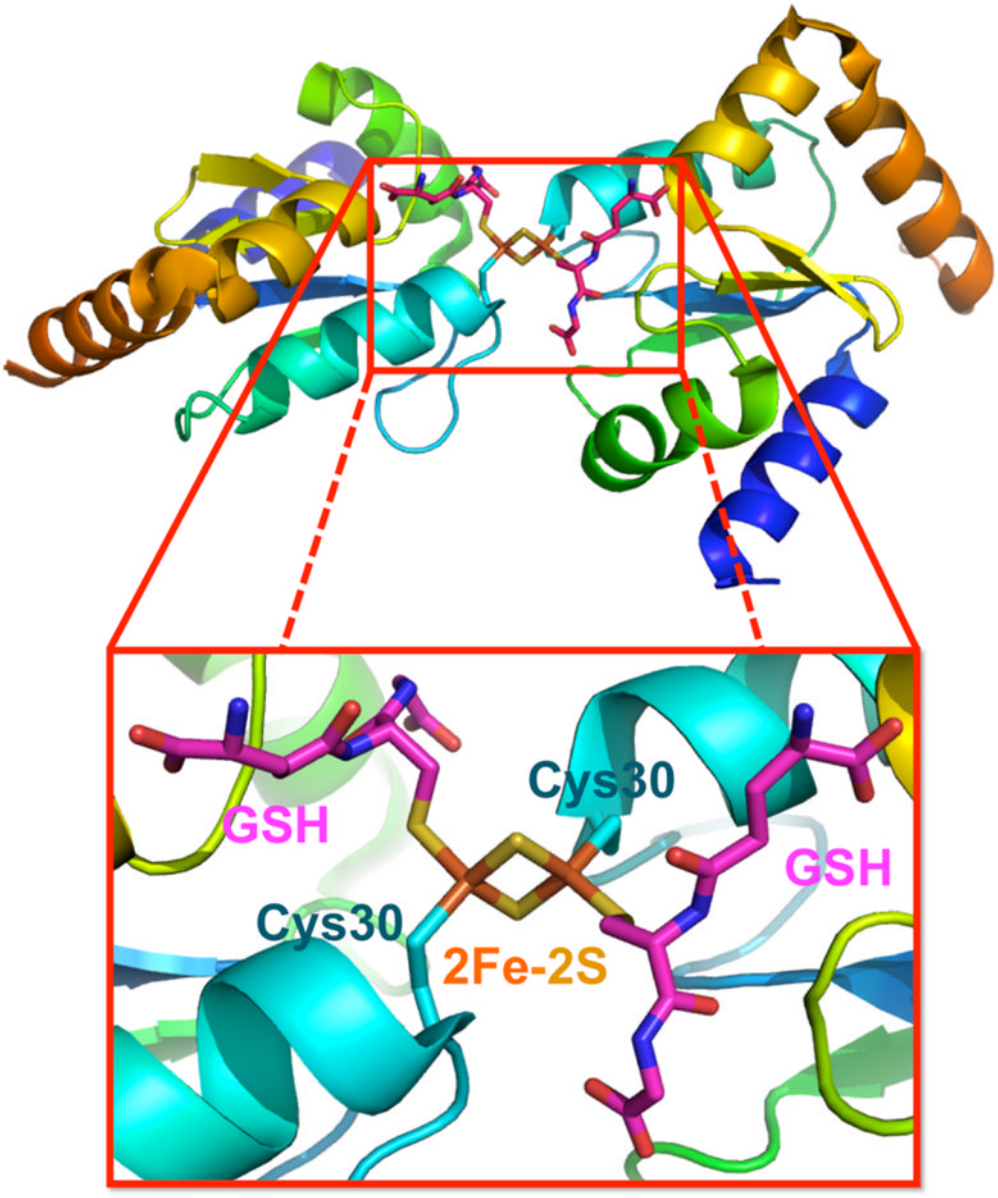

Figure 2.
X-ray crystal structure of *E. coli* Grx4 (PDB 2WCI) (33) with close-up view of the GSH-ligated [2Fe-2S] cluster.

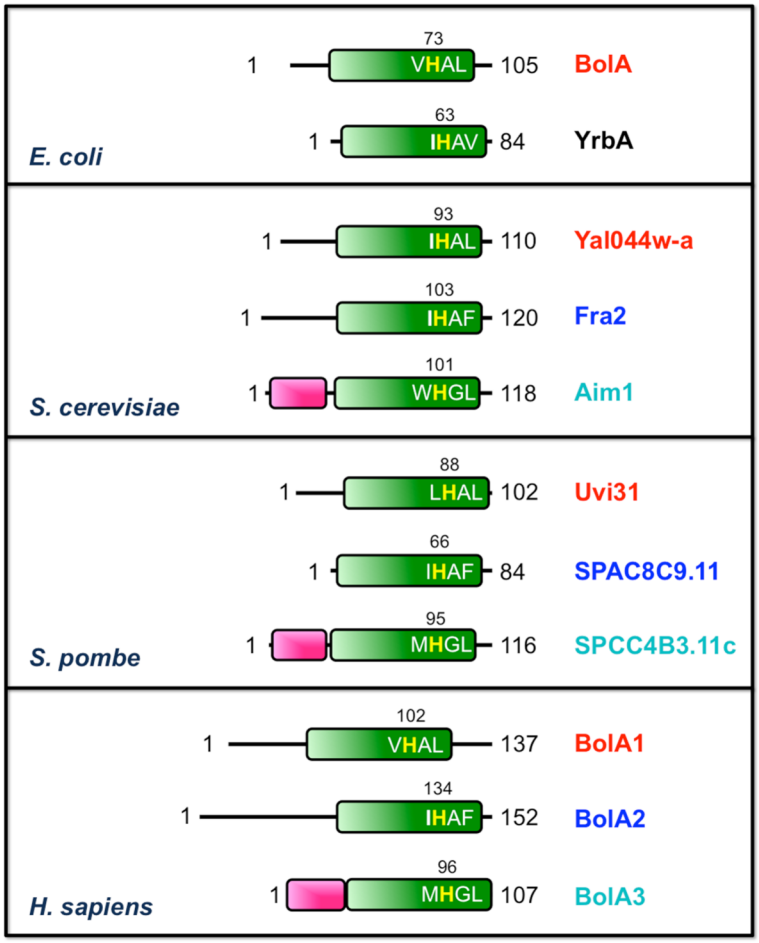

Figure 3.

Domain structure of BolA-like proteins from *E. coli*, *S. cerevisiae*, *S. pombe*, and *H. sapiens.* The protein names of members of the BolA1, BolA2, and BolA3 subfamilies are shown in red, blue, and cyan, respectively. *E. coli* YrbA is not grouped in any of these subfamilies based on phylogenic analysis (59). The BolA-like domain is shown as a green box, while predicted or known mitochondrial targeting signals are shown as pink boxes. The conserved histidines identified as Fe-S ligands in some family members are numbered and shown in yellow.

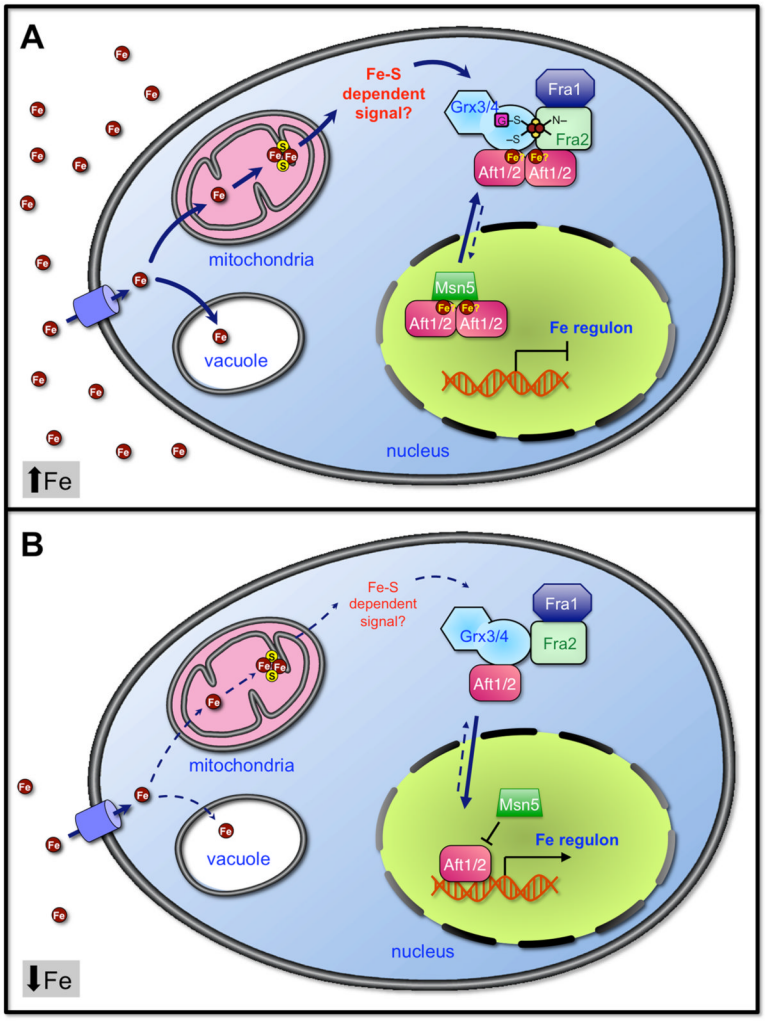

Figure 4.

Proposed model for *S. cerevisiae* iron regulation under iron replete (*A*) and iron depleted (*B*) conditions. Solid and dotted lines indicate active and attenuated pathways, respectively. Co-activators for Aft1/Aft2 are not included in the model for the sake of simplicity. See text for details.

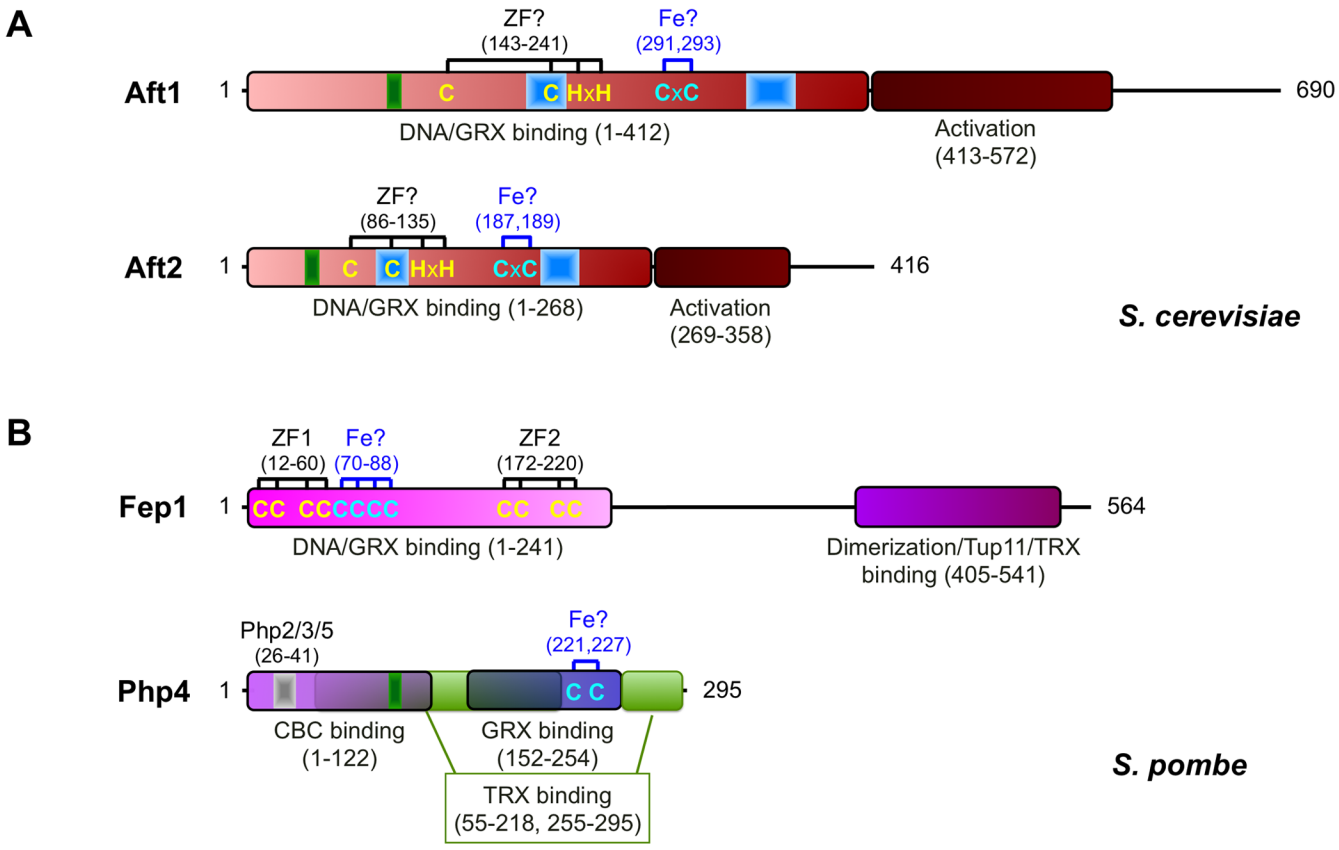

Figure 5.

Domain structure of iron-responsive transcriptional regulators Aft1 and Aft2 from *S. cerevisiae* (*A*) and Fep1 and Php4 from *S. pombe* (*B*). Conserved, putative Zn finger (ZF) ligands are shown in yellow and putative iron-responsive cysteine residues are shown in cyan. The dark green boxes indicate the positions of nuclear export signals, while the light blue boxes show the locations of nuclear import signals. The gray box in the Php4 CCAAT-binding complex (CBC) binding domain depicts the specific location of the Php2/3/5 binding residues.

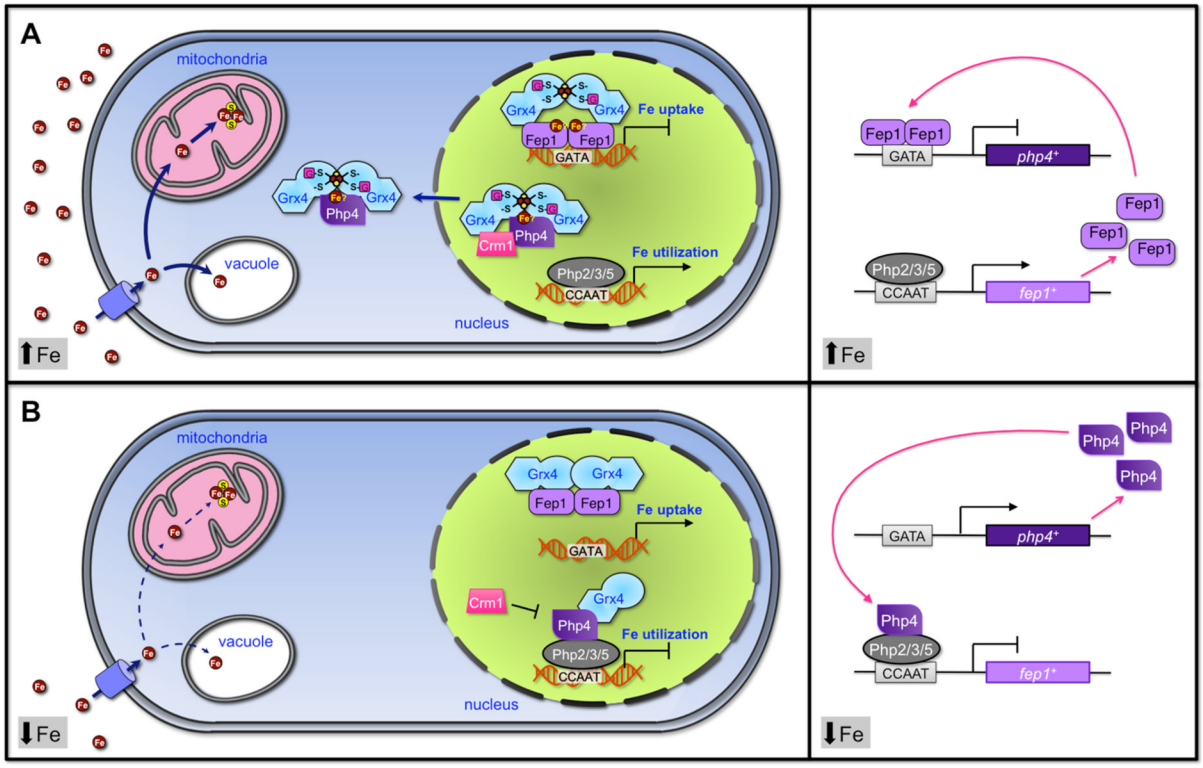

Figure 6.

Proposed model for *S. pombe* iron regulation under iron replete (*A*) and iron depleted (*B*) conditions. Solid and dotted lines on the left panels indicate active and attenuated pathways, respectively. The right panels in *A* and *B* demonstrate the reciprocal transcriptional regulation between Php4 and Fep1 under iron replete and iron depleted conditions. The Fep1 co-repressors Tup11 and Tup12 are not included in the model for the sake of simplicity. See text for details.

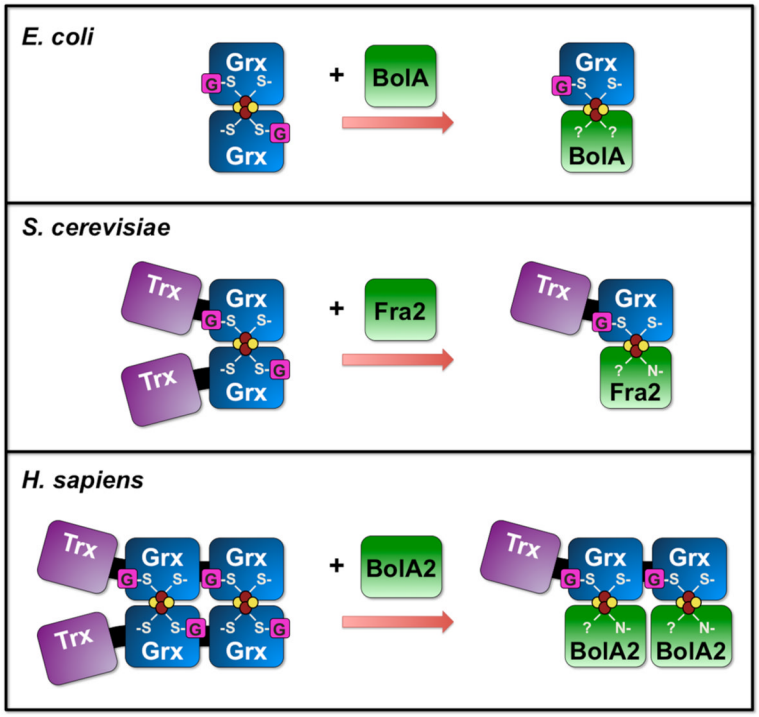

Figure 7.

Models for $[2Fe\text{-}2S]^{2+}$ Grx homodimers (*left*) and $[2Fe\text{-}2S]^{2+}$ Grx-BolA heterocomplexes (*right*) characterized from *E. coli*, *S. cerevisiae*, and *H. sapiens.* In each case, Grx-BolA heterocomplexes can be formed by titration of Grx homodimers with the apo BolA-like protein. In all CGFS Grx homodimers, the active site cysteines in the Grx-like domains and 2 GSH molecules ligate the [2Fe-2S] clusters. For yeast and human Grx-BolA heterocomplexes, each [2Fe-2S] cluster is ligated by one Grx domain active site cysteine, one GSH, a histidine from the BolA-like protein and an unidentified 4th ligand. For the *E. coli* $[2Fe\text{-}2S]^{2+}$ Grx4-BolA heterodimer, the ligands provided by BolA have not been identified.