Investigating the Role of Iron-Sulfur Signaling in Yeast Transcriptional Regulation

Malini Gupta

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INVESTIGATING THE ROLE OF IRON-SULFUR SIGNALING IN YEAST TRANSCRIPTIONAL REGULATION

by

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DEDICATION

I would like to dedicate this thesis to my grandmother, my “Didu”, the late Mrs. Parbati Sen. She was a constant source of affection, courage, admiration during my growing up years. She instilled in me the importance of a good education as well as nurtured my creative abilities. A fighter, yet a kindhearted helpful human being she inculcated in me the humane values. I am aware if she was still around, her eyes would light up and she would be so proud of me for successfully completing my PhD.
ACKNOWLEDGEMENTS

Completing graduate school was quite a journey! Last six years not only helped me evolve as a scientist but also a more evolved human being. As in life, journey through my PhD was full of ups and downs and thanks to my strong network of personal support system, my parents back in India, Mr. Tarun Ranjan Gupta and Mrs. Manikuntala Gupta my uncle Mr. Chanchal Sengupta, my husband Mr. Tirthankar Bandyopadhyay, my in-laws Mr. Ram Jiban Bandyopadhyay and Mrs. Rita Banerjee, I could emerge victorious.

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Graduate school was both a professional as well as an emotional journey for me. Besides training me to troubleshoot science problems it helped me realize important life lessons. It taught me the importance of failures, and how we value success more when we must struggle to earn it. The importance of self-care and a solid mental health is another
valuable realization from this journey. As I now embark upon a new journey, I would like to take these learnings with me and be prepared to conquer newer challenges.
ABSTRACT

As an essential cofactor in a myriad of cellular processes, uptake and mobilization of iron must be tightly controlled. Iron homeostasis in fungi involves balancing iron uptake and storage with iron utilization to achieve adequate, non-toxic levels of this essential nutrient. Extensive work in the non-pathogenic yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe have uncovered unique iron regulation networks for each organism that control iron metabolism via distinct molecular mechanisms. The activities of all fungal iron-sensing transcription factors characterized to date are regulated via iron-sulfur cluster signaling.

In Saccharomyces cerevisiae, expression of iron uptake, utilization and storage genes is primarily regulated by transcriptional activators Aft1 (and its parologue Aft2) and Yap5. Upon iron repletion, a mitochondrially generated Fe-S cluster is delivered to Aft1/Aft2 by a cytosolic complex comprising Grx3/4 and Fra2, leading to the dimerization and eventual export of Aft1/2 to the cytosol. Genetic studies suggest that the protein Fra1 may also have a role in regulating Aft1/2 activity; however, its specific role is unknown. Deletion of either Fra1 or Fra2 in addition to deletion of the vacuolar iron importer Ccc1 restores iron-responsive control of the iron regulon despite the absence of Fra2 or Fra1. Before testing for possible alternate mechanisms for this restoration, we wanted to understand if the mitochondrial iron-sulfur (Fe-S) biogenesis machinery was contributing
to inhibition of Aft1/2 in the strains in question. β-galactosidase reporter assay results show that in the absence of a functional mitochondrial iron-sulfur cluster biogenesis machinery (isu1Δ mutants), the iron regulon is constitutively active in all strains except for ccc1Δfra1Δisu1Δ, suggesting that the mitochondria generated inhibitory cluster is not required for inhibition of Aft1 and Aft2 in this mutant strain.

We employed a genetic screen approach to corroborate the in vitro evidence of inhibitory cluster transfer from the cytosolic complex comprising Grx3-Fra2 to Aft1 and Aft2. With the help of error-prone PCR, we successfully created mutated pools of FRA1 and FRA2. Our goal was to isolate point mutations in each of these genes which would result in constitutive inhibition of the iron regulon via Aft1 and Aft2, and once identified, to test the effect of those mutations on inhibitory cluster transfer to Aft1 and Aft2 in vitro. The isolated FRA1 and FRA2 mutants were non-informative non-sense or missense mutations that led to probable defective protein yielding false positive phenotypes in the genetic screen.

Iron homeostasis in Schizosaccharomyces pombe is maintained via transcriptional repression of iron uptake and iron utilization genes. The GATA-type transcriptional repressor Fep1 binds to the promoters of iron uptake and transport genes under iron replete conditions, turning off their expression to avoid iron overload. Fep1 function is controlled at the post-translational level by the cytosolic CGFS glutaredoxin Grx4 and its binding partner Fra2. Biochemical and spectroscopic characterization of Fep1 suggests that it bears a [Fe-S] cofactor and forms a [2Fe-2S]-bound heterocomplex with Grx4 as well as Grx4-Fra2. Monothiol glutaredoxins along with BolA like proteins (Fra2) have been repeatedly
shown to be associated with the delivery and transfer of Fe-S clusters to proteins and thus effective communicators of the cellular iron status to iron-responsive transcription factors.
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LIST OF ABBREVIATIONS

Aft1/2 ................................................................. Activator of ferrous uptake

AP ...................................................................................... As purified

BPS ...................................................... Bathophenanthroline disulfonic acid

DBD ........................................ DNA binding domain

DTT .................................................... Dithiothritol

FAS .................................................... Ferrous ammonium sulfate

FL ....................................................... Full Length

GRX .......................................................... Glutaredoxin

GSH ............................................................ Glutathione

MBP .................................................. Maltose Binding protein

REC ......................................................... Reconstituted

TCEP .................................................. Tris(2-carboxyethyl) phosphine

TRX ................................................... Thioredoxin

WT ........................................................... Wild type
CHAPTER 1

INTRODUCTION AND SCOPE OF THESIS

Regulation of intracellular iron metabolism is crucial for the survival of almost all living organisms. As a cofactor for a wide variety of essential proteins and enzymes, the uptake and mobilization of iron is tightly controlled. However, excess iron must be minimized or safely stored to avoid the generation of damaging oxygen radicals by labile iron as well as the mismetallation of non-iron metalloproteins. The single-celled eukaryotes *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have served as excellent model systems to tease out these intracellular regulation pathways and compare different strategies for adapting to both high iron and low iron environments. In each case, iron homeostasis pathways employ a network of proteins working in concert to control the uptake, utilization, and storage of iron in response to changes in intracellular iron levels. A unifying theme in these regulation pathways is the central role of iron-sulfur (Fe-S) clusters as sensors of iron bioavailability. Even though the transcription factors that control iron metabolism are not conserved between these two divergent yeast species, they have all been shown to bind Fe-S clusters that regulate their protein-protein interactions and/or DNA binding activity. Furthermore, *in vivo* and *in vitro* studies have established that [2Fe-2S]-binding cytosolic glutaredoxins (Grxs) with a Cys-Gly-Phe-Ser (CGFS) active site post-translationally regulate the activity of these transcription factors via direct binding and/or Fe-S cluster transfer [1-3]. The same principle of Fe-S cluster-dependent signaling
has been seen in the transcription factors that control iron metabolism in pathogenic fungi. They share homology with either *S. cerevisiae* or *S. pombe* transcription factors, providing the framework to decipher their molecular mechanisms. Importantly, these regulation pathways are often essential for virulence, providing potential targets for the development of anti-fungal compounds. A deeper understanding of iron regulation pathways by connecting information gained across species may help in the development of novel strategies for treating iron-related diseases and combatting microbial invasions.

**ACQUISITION OF EXTRACELLULAR IRON**

Acquisition of extracellular iron is a challenge since iron is abundant in the environment as sparsely soluble Fe$^{3+}$ ion (reported solubility $10^{-18}$ M). As a result, most organisms employ high affinity uptake systems for efficient uptake and reduction of iron [4]. Some yeast, including *S. pombe* and *P. pastoris*, also produce high affinity iron chelators called siderophores that help bind Fe$^{3+}$ in aqueous solutions. For opportunistic pathogens like *Candida albicans*, iron acquisition is trickier since the human host makes iron acquisition difficult for the pathogen. Iron is regulated in humans both systemically and at the cellular level thus providing a certain “nutritional immunity”. Depending less on dietary iron uptake, sequestering iron within specific proteins like hemoglobin, transferrin, and ferritin and favoring more recycling of iron from senescent erythrocytes and macrophages serves the dual purpose of restricting iron bioavailability for pathogens as well as protecting the cells from the toxicity of free circulating iron. Similar to the other yeasts, *Candida* has a high affinity ionic iron uptake system, a siderophore uptake system and an additional hemoglobin-iron uptake system. The high affinity iron uptake systems identified in *S. cerevisiae*, *S. pombe*, and more recent reports in *C. albicans* confirm a
similar approach of first recruiting plasma membrane ferrireductases for reducing ferric iron (Fe$^{3+}$) to a more soluble ferrous state (Fe$^{2+}$). Potentially toxic ferrous iron then is acted upon by a multicopper oxidase/permease system to ensure transport into the cells. Like S. cerevisiae, C. albicans is incapable of synthesizing siderophores but produce Sit1/Arn1 siderophore transporters for uptake of iron-binding ferrichrome or ferroxamine siderophores generated by other yeast species. CFEM or fungal extracellular membrane proteins Rbt5, Pga7, Pga10 and Csa2 enable iron uptake from hemoglobin or hemin in C. albicans [2, 3, 5, 6].

**MONOTHIOL GRXS AND BOLA-LIKE PROTEINS FUNCTION IN IRON METABOLISM**

Grxs are a highly conserved family of proteins and can be classified into two groups based on their active site sequence – CXXC dithiol Grxs and CGFS monothiol Grxs. Originally characterized to be proteins involved in thiol-disulfide exchange, CGFS-type Grxs have little to no oxidoreductase activity, and instead have been shown to have roles in iron homeostasis, Fe-S cluster assembly, and signal transduction. Biochemical characterization of Grx4 from S. cerevisiae and S. pombe by our group and others have shown that these proteins exist in a monomeric apo form or a [2Fe-2S]-bridged dimeric holo form. This property is conserved between both eukaryotic and prokaryotic monothiol Grxs [7-9]. Both S. cerevisiae and S. pombe have three CGFS-type Grxs involved in iron homeostasis including ScGrx3, ScGrx4, ScGrx5 and SpGrx3, SpGrx4, and SpGrx5 (Figure 1.1). In the former, Grx3 and Grx4 are paralogous proteins possessing both an N-terminal thioredoxin (Trx)-like domain and a C-terminal Grx-like domain. These paralogues share up to 60% sequence identity and has been shown that cytosolic Grx3 and Grx4 perform
Figure 1.1. Domain organization of selected monothiol glutaredoxins from *S. cerevisiae*, *S. pombe* and *H. sapiens*. Designated in bold yellow letters are the conserved cysteines in the TRX and CGFS motif of the GRX domains. The mitochondrial targeting sequence is shown in pink at the N-terminus of Grx5 proteins.
redundant functions, since deletion of each gene singly has little effect on iron regulation, while a grx3Δgrx4Δ double mutant exhibits constitutive expression of the iron regulon. The CGFS motif in the Grx-like domain of ScGrx3/4 is specifically required for interaction with Aft1 and regulation of its activity. ScGrx5 is a mitochondrial monothiol Grx which has a single Grx-like domain and plays an essential role in the early steps of mitochondrial Fe-S cluster biogenesis. *S. cerevisiae* also contain two cis-Golgi-localized monothiol Grxs, Grx6 and Grx7; however, they do not possess the CGFS motif and have been suggested to be involved into the oxidative stress response, similar to dithiol Grxs. In *S. pombe*, however, Grx4 is primarily localized to the nucleus and contains the similar modules as ScGrx3/4, an N-terminal Trx-like domain plus a C-terminal Grx-like domain. SpGrx3, on the other hand, is not a CGFS-type Grx but bears a CPYS active site and is localized in the nuclear rim and ER and might play a role in oxidative stress response. SpGrx5 shares high sequence similarity with ScGrx5, resides in the mitochondria, and plays a crucial role in iron regulation [8]. In humans, there are two monothiol Grxs, Grx3 and Grx5 (HsGrx3 and HsGrx5). Grx3, also known as PICOT for PKC-interacting cousin of Trx, has a Trx-like domain and two Grx-like domains and HsGrx5 is mitochondrial as its counterparts in other organisms. Deficiency of Grx5 leads to microcytic anemia thus directly connecting its role in human iron regulation.

One of the major known interacting partners of monothiol Grxs are the widely conserved BolA-like proteins. BolA proteins in *E. coli* were identified as morphogens that regulate functions of certain cell wall proteins but later was found to be highly conserved in both prokaryotes and eukaryotes. There are three non-redundant BolA paralogues in fungi and mammals of which BolA1-like proteins are mostly uncharacterized while BolA2-
and BolA3-like proteins are localized in the cytosol/nucleus and and BolA3), human BolA1 shares the highest sequence similarity with *E. coli* BolA (46% identity, 58% similarity), while human BolA2 is most similar to yeast Fra2 (37% identity, 59% similarity) (Figure 1.2) [10, 11]. Monothiol Grxs from humans, *S. cerevisiae* as well as *S. pombe* (unpublished data from our group) form [2Fe-2S] bridged heterotrimeric or heterodimers respectively aiding in iron trafficking and regulation [8, 12]. In humans, the [2Fe-2S]-Grx3-BolA2 complex has recently been shown to transfer [2Fe-2S] clusters to a member of the cytosolic Fe-S cluster assembly system called Ciapin1. In humans, the-[2Fe-2S]-Grx3-BolA2 complex represents a reservoir of [2Fe-2S] clusters which can be transferred to various apo proteins thus acting as a [2Fe-2S] chaperone [12]. Recently our group also reported that both *E. coli* BolA and YrbA form a distinct [2Fe-2S]-bridged cluster complexes with Grx4, thus indicating different functional roles for each of these heterodimers [13].

In humans, the mitochondrial BolA3 has been associated with multiple mitochondrial dysfunction syndrome 2 which is linked with decreased functions of respiratory complexes I and II as well as lipoic acid dependent enzymes [14]. Also, deficiency of mitochondrial BolA1 in humans results in increased mitochondrial protein thiol oxidation and altered mitochondrial morphology. Collectively known as the mBols, these proteins have relatives in yeast called Bol1 and Bol3. The mBols in humans have been shown to work in the latter half of the mitochondrial Fe-S biogenesis pathway and supposedly in conjunction with Grx5 [15]. Both *S. cerevisiae* and human mBols have a major function of inserting [4Fe-4S] clusters into LIAS (Lipoyl synthase) and respiratory complexes I and II [14]. The mitochondrial BolA homologue in *S. pombe*, Uvi31, is
Figure 1.2. Domain organization of BolA like proteins from *S. cerevisiae*, *S. pombe* and *H. sapiens*. Designated in the figure are the conserved His residues which act as Fe-S ligands in the BolA like domain (green box). The pink boxes designate mitochondrial targeting sequences found in the BolA3 proteins.
induced under UV irradiation and has been suggested to control the progress of the cell cycle after DNA damage [16].

**IRON-DEPENDENT TRANSCRIPTIONAL REGULATION IN *S. CEREVISIAE***

As mentioned earlier, both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are excellent model eukaryotes for iron regulation studies. While a unifying theme in these regulation pathways is the importance of Fe-S clusters as iron sensors, the transcriptional regulation occurs in opposite manners in these organisms. Aft1/2 and Yap5 are the transcriptional activators which are responsible for sensing low and high iron in *S. cerevisiae*. Whereas in *S. pombe*, transcriptional regulation occurs via repression through the action of the low iron sensing CCAAT-type repressor Php4 and the GATA-type repressor Fep1. Interestingly, both Grx4 and Fra2 are still the cytosolic mediators of this transcriptional regulation [3, 6]. A brief overview of each type of regulation is described in the following paragraphs. The iron regulon comprises a set of genes which express proteins involved in iron acquisition at the plasma membrane, iron import into the mitochondria, Fe-S cluster biosynthesis, and vacuolar iron export in *S. cerevisiae*. The mechanism for iron-dependent regulation mediated by the paralogous transcriptional activators Aft1 and Aft2 has been illuminated from numerous in vivo and in vitro studies stretching over the past three decades [2, 3]. Under iron starved conditions, Aft1 accumulates in the nucleus and activates the iron regulon (Figure 1.3) [17, 18]. Once iron is replete, cytosolic Grx3, and Grx4 accompanied by Fra1 and Fra2 transfers a mitochondria-dependent inhibitory [2Fe-2S] cluster to Aft1 (assumed to be true for Aft2 as well) (Figure 1.3) [19]. Interaction with Grx3 and Grx4 results in the dissociation of Aft1 from the DNA [7, 20, 21]. The cluster ligands in these heterocomplexes are provided
Figure 1.3. Model for iron regulation in *S. cerevisiae* (top) under iron deficient conditions, (bottom) iron replete conditions (green arrows indicate active processes whereas red arrows indicate inhibited processes.)
by Grx via the Cys in the CGFS active site, a Cys from glutathione (GSH), and two residues provided by Fra2 (His and possibly a Cys) [8, 22]. Fe-S cluster assembly/delivery to these cytosolic trafficking factors, in turn, is dependent on the mitochondrial iron-sulfur cluster (ISC) assembly pathway (but not the cytosolic assembly pathway), GSH, and the mitochondrial ABC transporter Atm1 [23]. Export from the nucleus upon iron replete conditions is mediated by the exportin Msn5 [24]. Control of DNA binding activity and nuclear-cytosolic trafficking of Aft1 and Aft2 in response to iron is dependent on a conserved CDC motif located just downstream of the DNA binding Zn finger domain. Replacement of one or both Cys in this motif abolishes Aft1/2 inhibition in response to iron causing constitutive activation of the iron regulon. This motif was recently shown to be essential for binding a [2Fe-2S] cluster that regulates the DNA binding affinity of Aft2 (and presumably Aft1) [25, 26]. Iron independent post-translational regulation of Aft1 and Aft2 has also been ascribed in literature, including phosphorylation by Hog1 protein kinase in response to changes in sphingolipid metabolism [27]. Aft1/2 activity is also linked to and complemented by the mRNA binding proteins Cth1 and Cth2, which are included in the Aft1/2 iron regulon [28]. Their expression under low iron conditions facilitates the degradation of mRNA targets that encode proteins involved in non-essential, iron-consuming pathways (e.g. respiration, TCA cycle, heme biosynthesis) as an additional mechanism to conserve iron for essential functions. Aft1 and Aft2 orthologs exist in other yeast species as well however, in some cases, iron-responsive transcriptional activity is not conserved. For example, in the opportunistic pathogen Candida albicans and methylotrophic yeast Pichia pastoris, Aft1/2 orthologs possess a Zn-finger DNA binding domain that is homologous to ScAft1 but lack the CDC iron-responsive motif [29, 30].
Consequently, these transcriptional regulators have little or no impact on iron metabolism in these organisms. But in *Candida glabrata*, several recent reports have confirmed that Aft1 is the primary regulator of the iron starvation response [30]. Yeasts and plants have no mechanism of getting rid of excess iron nor do they have an iron binding ferritin-like storage protein. Thus, excess iron needs to be stored and regulated to avoid toxicity. The basic leucine zipper (bZIP) transcription factor Yap5 orchestrates the response to high iron conditions in *S. cerevisiae* (Figure 1.4) [31, 32]. Yap5 mounts this defense against high iron by activating expression of the vacuolar iron importer Ccc1, the Cu-binding metallothionein Cup1, and the cytosolic Fe-S binding proteins Grx4 and Tyw1 [33]. Upregulation of *CCC1* by Yap5 plays a major role in tackling high iron conditions as it leads to clearance of excess iron from the cytosol to the vacuole [34]. Also, upregulation of *GRX4* during iron excess may be connected to the Grx3/4-Fra2 mediated inhibition of Aft1/2. Yap5 is constitutively bound to its target promoter but undergoes conformational changes upon binding two [2Fe-2S] clusters in vitro via two cysteine rich domains [35]. The Fe-S binding motif in one of these Cys-rich domains (CGFCX5CXC) is well conserved among fungal Yap5 orthologs and is reminiscent of the Fe-S binding site in the CGFS Grxs that regulate Aft1/Aft2 activity [32]. Yap5 activation in vivo is dependent on the mitochondrial Fe-S synthesis machinery (ISC pathway) but not the cytosolic/nuclear machinery (CIA pathway) similar to Aft1/2.

**IRON-DEPENDENT TRANSCRIPTIONAL REGULATION IN *S. POMBE***

The fission yeast *Schizosaccharomyces pombe* maintains iron homeostasis via transcriptional repression of iron uptake and iron utilization genes. Under iron limitation, the transcriptional regulator Php4 interacts with the heteromeric CCAAT binding complex
Figure 1.4. Model for iron regulation in *S. cerevisiae* under high iron conditions (green arrows indicate active processes).
composed of Php2, Php3, and Php5 and represses iron utilization genes to economize iron usage. On the other hand, when iron is replete, the GATA-type transcriptional repressor Fep1 binds to the promoters of iron uptake and transport genes that contain an ATC(A/T)GATA(A/T) motif, turning off their expression to avoid iron overload (Figure 1.5). In addition, Fep1 and Php4 have been shown to reciprocally regulate expression of each other, thus allowing tight control of intracellular iron levels.

Php4 regulates expression of proteins involved in iron-dependent metabolic pathways such as the TCA cycle, mitochondrial respiration, amino acid biosynthesis, heme biosynthesis, and Fe-S cluster assembly. Php4 is needed for iron conservation: when cellular iron is limiting, expression of genes encoding iron-containing proteins is repressed. Php4 is only induced under low iron conditions and binds to the constitutively expressed CCAAT-binding complex (Php2/3/5). Binding of Php4 converts the complex from an activator to a repressor preventing expression of proteins involved in iron-rich metabolic pathways [36]. While expression of Php4 is controlled by Fep1, its localization is controlled by the exportin Crm1 and the cytosolic CGFS-type Grx4. Php4 is localized to the nucleus under low iron conditions, whereas when iron is sufficient, it is exported to the cytoplasm by the exportin Crm1 [37]. The high iron response in S. pombe is mediated by transcriptional repression via Fep1, a GATA-type repressor that binds a 5′-(A/T)GATAA-3′ sequence to facilitate transcriptional repression of iron uptake or siderophore synthesis when iron is abundant [38]. Most fungal GATA type regulators have two Cys2/Cys2-type zinc fingers within their N-termini (ZF1 and ZF2) and a highly conserved intervening Cys-rich region located between the zinc fingers. The N-terminal domain of S. pombe Fep1 that contains the aforementioned zinc fingers and Cys-rich region is essential for this iron-
Figure 1.5. Model for iron regulation in *S. pombe* under (top) iron deficient conditions and (bottom) iron replete conditions (green arrows indicate active processes whereas red arrows indicate inhibited processes).
dependent DNA association, while the C-terminal region mediates interaction with the corepressor Tup11 [38, 39]. An early report suggested that purified recombinant Fep1 bound iron alone, rather than an Fe-S cluster, whereas a separate study demonstrated that both as-purified and chemically reconstituted Fep1 binds a mixture of different Fe-S clusters [40, 41]. In *P. pastoris* it was recently shown that Fep1 can bind a [2Fe-2S] cluster either by Cys65 and Cys68 of the ZF1 domain or the central cysteine residues depending on the presence of oxygen [42]. This group primarily investigated the function of these Cys-rich domains by mutating all four conserved Cys in the Cys-rich domain to Ser (4S variant), resulting in decreased DNA binding affinity when compared to WT protein. Also, according to this study, the presence or absence of oxygen via an unidentified mechanism guides whether the Fe-S cluster will bind the ZF1 residues or the central four cysteine residues. Anaerobic studies on Fep1 and the 4S mutants suggested that the central Cys was the preferred binding site since Fe-S cluster binding to the ZF1 domain decreased the repression function of Fep1 by partial depression of the target genes. To summarize, the cysteine residues in the DNA binding domain of Fep1 act as switches to regulate the repressor activity of Fep1 in response to cellular iron conditions; however, the type of bound cofactor, and the molecular mechanism for regulation of Fep1 activity is still under active investigation.

In addition to iron-dependent transcriptional regulation by Php4 and the CCAAT-binding complex, Fep1 function is also controlled at the post-translational level by the cytosolic CGFS Grx4 and its binding partner Fra2. Grx4 is reported to be involved in both high and low iron regulation by Fep1 and Php4 [43, 44]. Grx4 forms a [2Fe-2S] bridged dimer that interacts with the low iron-sensing transcriptional repressor Php4 in *S. pombe*.
The GRX domain of Grx4 associates with Fep1 under low iron conditions and associates with Php4 under high iron conditions, thereby acting as a major link between the high and the low iron regulation systems. In grx4Δ strains, Fep1 is insensitive to iron levels thus acting as a constitutive repressor of iron uptake genes. This result indicates that Grx4 must play a role in communicating the cellular iron status to Fep1 in order to inactivate its repressor activity. Fep1 and Grx4 have been shown to associate constitutively via the C-terminal region of Fep1 and N-terminal TRX domain of Grx4, whereas a weaker and iron-dependent interaction happens within the N-terminal DNA binding domain of Fep1 and the C-terminal GRX domain of Grx4 only under low iron conditions. Since both the N-terminal DNA binding domain of Fep1 and the C-terminal GRX domain of Grx4 contain the cysteine ligands necessary to ligate Fe-S clusters, an involvement of such a switch seems possible.

The Bol2 homologue in S. pombe, Fra2, has been shown to not be involved in the Php4 mediated iron sparing response; however, it has been implicated in the regulation mediated by the high iron sensing repressor Fep1 [16]. In vivo results suggest the existence of a heteromeric complex between Grx4, Fra2 and Fep1 independent of iron levels in the cell [40]. The possible reason behind the formation of such a complex most likely depends on the iron sensing and cluster transfer characteristics of Grx4 and Fra2 proteins as evident from previous findings in S. cerevisiae [13, 19, 22, 45].

Mapping experiments also revealed that Php4 interacts with the N-terminal (thioredoxin-like, TRX) and C-terminal (glutaredoxin-like, GRX) regions of Grx4 [46]. Association with the TRX domain is constitutive and requires Cys35 in the TRX-like WAAPCK sequence, while association with the GRX domain is dependent on iron and
requires Cys172 of the CGFS active site. The GRX domain alone is adequate for iron-dependent inhibition of Php4 and is required for relocalization of the Php4-Grx4 complex from the nucleus to the cytosol. In addition, Cys221 and Cys227 in the C-terminus of Php4 are required for interaction with the GRX domain and may be involved in iron binding. Recently our lab and collaborators determined that \textit{S. pombe} Grx4 binds a [2Fe-2S] cluster similar to other monothiol Grxs. They also reported that recombinant Php4 does not purify with bound iron or an Fe-S cluster when expressed alone, but it does bind a [2Fe-2S] cluster in complex with Grx4. Grx4 C172 and Php4 C221/C227 are required for Fe-S cluster binding and aid in the interaction between Php4 and Grx4, thus showing that Grx4 regulates Php4 function at the protein level through [2Fe-2S] cluster binding [9].

**SCOPE OF THESIS**

Iron homeostasis in fungi involves balancing iron uptake and storage with iron utilization to achieve adequate, non-toxic levels of this essential nutrient. Extensive work in the non-pathogenic yeasts \textit{Saccharomyces cerevisiae} and \textit{Schizosaccharomyces pombe} have uncovered unique iron regulation networks for each organism that control iron metabolism via distinct molecular mechanisms. However, common themes have emerged from these studies. The activities of all fungal iron-sensing transcription factors characterized to date are regulated via Fe-S cluster signaling. Furthermore, Grxs play a key role in relaying the intracellular iron status to these DNA-binding proteins. This thesis outlines in vivo and in vitro approaches to characterize the Fe-S signaling and elucidate the specific roles played by Grxs and BoIa proteins in both \textit{S. cerevisiae} and \textit{S. pombe}.
In the second chapter, an \textit{in vivo} approach was used to comment on the role of mitochondrial Fe-S cluster biogenesis machinery in certain yeast mutants. Deletion of either Fra1 or Fra2 had been shown in the past to activate Aft1/2 despite sufficient intracellular iron, which was consistent with the established model that Fra2 is required to transfer an inhibitory Fe-S cluster to Aft1/2. However, an additional deletion of the vacuolar iron importer Ccc1 reversed this effect, restoring iron-responsive control of the iron regulon despite the absence of Fra2 or Fra1. Thus, it was possible that when cytosolic iron is high (as in a \textit{ccc1\Delta} strain), Fra1 and Fra2 are not required for Aft1/2 inhibition and alternate pathways might be active in the absence of these proteins. Before diverging to possible alternate mechanisms, we first wanted to understand if the mitochondrial Fe-S biogenesis machinery was contributing to inhibition of Aft1/2 in the strains in question. With the help of $\beta$-galactosidase reporter assays we are able to show that in the absence of functional mitochondrial Fe-S cluster biogenesis (\textit{isu1\Delta} mutants), the iron regulon is constitutively active in all strains except for \textit{ccc1\Delta fra1\Delta isu1\Delta}, suggesting that the mitochondria generated inhibitory cluster is not required for inhibition of Aft1 and Aft2 in this mutant strain.

In the third chapter, we wanted to corroborate previous findings on the importance of Grx3/4, Fra1 and Fra2 by identifying the specific residues in each of these proteins which were important for the Fe-S mediated regulation of Aft1 and Aft2. We took a genetic screen approach to investigate the key residues and with the aid of error-prone PCR we were successful in creating mutated pools of \textit{FRA1} and \textit{FRA2}. The goal of the genetic screen experiment was to isolate point mutations in each of these genes which would result in constitutive inhibition of the iron regulon via Aft1 and Aft2, and once identified to test the
effect of those mutations on inhibitory cluster transfer to Aft1/Aft2 \textit{in vitro}. We were able to isolate a few \textit{fra1} and \textit{fra2} mutants which were non-informative nonsense or suppressor mutations beyond the open reading frames, that led to either a defective protein or yielded false positive phenotypes in the genetic screening.

The fourth chapter delved into characterization of the high iron transcriptional repressor Fep1 from \textit{S. pombe}. With the help of biochemical and spectroscopic tools, the work in this chapter aimed to define the interaction of this protein with Grx4 and Fra2, and how these proteins regulated the repressor activity of Fep1. The roles of Grx4 and Fra2 had been previously described in \textit{S. cerevisiae} by our lab and also how Grx4 controls the low iron sensing Php4 in \textit{S. pombe} was recently unveiled by our lab and collaborators. This chapter thus added onto the already established functions of Grx4 in \textit{S. pombe} and also commented on the importance of Grx4-Fra2 to be universally involved in Fe-S cluster trafficking in most organisms.

Taken together, these studies explore the involvement of Fe-S clusters as a common theme for iron-responsive transcriptional regulation and also the importance of Grx4-Fra2 in yeast iron regulation.
REFERENCES


CHAPTER 2

TESTING THE ROLE OF IRON SULFUR CLUSTER BIOGENESIS IN YEAST

IRON REGULATION

ABSTRACT

Iron is an important micronutrient for almost all living organisms. To maintain optimum concentration of iron in cells, iron homeostasis is strictly regulated in both prokaryotes and eukaryotes. *S. cerevisiae*, a model eukaryote, employs multiple routes to incorporate iron, although the molecular mechanisms for iron sensing and trafficking are still being identified. This simple eukaryote serves as an ideal tool for researchers to fill in the underlying gaps in our understanding of iron homeostasis and signaling. *S. cerevisiae* responds to iron starvation conditions via activation of the iron regulon by the paralogous low iron-sensing transcription factors Aft1 and Aft2, which leads to increased iron uptake into the cell [1]. Our group recently unveiled the molecular mechanism for inhibition of Aft1/2, demonstrating that the cytosolic glutaredoxins Grx3/Grx4 and accessory protein Fra2 transfer an Fe-S cluster to Aft2 (and presumably Aft1) leading to its decreased DNA binding affinity [2]. Genetic studies suggest that the protein Fra1 may also have a role in regulating Aft1/2 activity; however, its specific role is unknown [3]. Deletion of either Fra1 or Fra2 was shown to activate Aft1/2 despite sufficient intracellular iron, which is consistent with the established model that Fra2 is required to transfer an inhibitory Fe-S cluster to Aft1/2 [3]. However, an additional deletion of the vacuolar iron importer Ccc1...
reversed this effect, restoring iron-responsive control of the iron regulon despite the absence of Fra2 or Fra1. Thus, it is possible that when cytosolic iron is high (as in a ccc1Δ strain), Fra1 and Fra2 are not required for Aft1/2 inhibition and alternate pathways might be active in the absence of these proteins. To investigate these questions, we assayed the expression of Aft1/2 responsive genes in different iron regulon mutants and determined if the mitochondrial Fe-S cluster biogenesis machinery is required for signaling iron bioavailability to Aft1/2 under these conditions. We are able to show that in the absence of a functional mitochondrial Fe-S cluster biogenesis machinery (isu1Δ mutants), the iron regulon is not constitutively active in all the strains suggesting some of them, like fra1Δccc1Δisu1, the mitochondria generated inhibitory cluster is not required for inhibition of Aft1 and Aft2 in these strains. Our study would be complete and more thorough if we obtained the isu1Δ, fra2Δccc1Δ and the fra2Δccc1Δisu1Δ strains for comparison.

INTRODUCTION

Iron is important in biology as a redox element. The ease of accepting and donating electrons also makes iron a potential cytotoxic element, requiring tight control of iron uptake and metabolism. Iron is mostly present in the Fe^{2+} or Fe^{3+} form but its bioavailability is limited by its pH dependence and solubility. The biologically relevant Fe^{3+} occurs as insoluble Fe^{3+} oxides and must be solubilized in order to be taken up by the cell. The iron regulon in *S. cerevisiae* encodes genes whose products are involved in the uptake, transport, metabolism and storage of iron [4]. Strict regulation of this process is desirable since excess of iron can generate lethal free radicals via the Fenton reaction or cause mismetallation of non-iron metalloproteins.
Iron is required for the biosynthesis of the iron-sulfur cluster (ISC) cofactors in mitochondria. One of the main components of ISC assembly pathway in the mitochondria is the scaffold protein Isu1 that serves as the site of de novo synthesis of the nascent iron sulfur (Fe-S) cluster [5, 6]. Other proteins in this assembly pathway include an iron and electron donors and a cysteine desulfurase enzyme that mobilizes sulfur for incorporation into the Fe-S cluster [7]. There is an array of accessory proteins that help in subsequent transfer of the nascent cluster from the Isu1 scaffold to respective apo proteins. Recent findings suggest that Atm1, the mitochondrial ABC transporter, exports a glutathione polysulfide, containing glutathione and persulfide (GS-S-SG) for cytosolic Fe-S cluster synthesis [8]. Aft1 translocate into the nucleus under iron-starved conditions and shuttles back into the cytosol under iron replete conditions [9, 10]. Published studies suggest that Aft1 senses an inhibitory signal relayed from the mitochondrial Fe-S cluster machinery [11]. Deletion of ISC assembly genes results in constitutive activation of the iron regulon, which is not suppressed by addition of external iron [3, 11]. The current model of Aft1/2 inhibition suggests that under iron replete conditions, cytosolic Grx3/4 and Fra2 transfer an Fe-S cluster to Aft1/2 that results in their dissociation from target promoters [2]. The cytosolic glutaredoxins Grx3/4 are [2Fe-2S]-binding proteins that require the participation of the mitochondrial ABC transporter Atm1 for insertion of its clusters.

Yeasts have no mechanism of excreting excess iron and lack a ferritin-like iron storage protein. Thus, excess iron needs to be stored intracellularly in a membrane-enclosed vacuole and regulated to avoid toxicity. Vacuolar iron stores can be deployed at times of low cytosolic iron via vacuolar iron exporters such as Smf3 and the Fet5/Fth1 system [12, 13]. The vacuolar iron importer Cce1, which plays a major role in tackling
high iron conditions, is regulated at the transcriptional level by Yap5 and the post-
transcriptional level by the mRNA-binding proteins Cth1/Cth2 [14]. Upregulation of 
*CCC1* by Yap5 under high iron conditions has been shown to result in the clearance of 
excess iron from the cytosol [14]. How Yap5 senses intracellular iron levels is under 
speculation though recent reports suggest that under high iron conditions, Yap5-mediated 
transcription is activated by a conformational change upon receiving [2Fe-2S] clusters. 
However, the donor of this cluster remains unidentified since, unlike Aft1/2, Yap5 activity 
is not affected by deletion of the cytosolic glutaredoxins Grx3 and Grx4 [15].

According to the established model of Aft1/2 inhibition it has been suggested that 
under iron replete conditions, cytosolic Grx3/4 and Fra2 transmit an inhibitory signal to 
Aft2 (and presumably Aft1), which results in its dissociation from its target promoter [2]. 
It has also been established that deletion of ISC assembly genes results in constitutive 
activation of the iron regulon, which is not suppressed by addition of iron externally [3,
11]. However, it has been observed by our collaborators that additional deletion of the 
vacuolar iron importer Ccc1 reversed this effect by restoring iron-responsive control of the 
iron regulon despite the absence of Fra2 or Fra1 (J. Kaplan, unpublished data). These 
results suggest that when cytosolic iron is high (as in a *ccc1Δ* strain), Fra1 and Fra2 are not 
required for Aft1/2 inhibition. Since our model indicates that Fra2 is involved in Fe-S 
transfer to Aft1/2, we set out to test whether Aft1/2 may bind Fe as an inhibitory signal 
under these conditions, rather than an Fe-S cluster, since previous studies suggested that 
Aft2 can bind Fe(II) *in vitro* [2]. In such a scenario, the mitochondrial ISC machinery 
would be dispensable for iron signaling to Aft1/2. In order to test this hypothesis, we made 
*isu1Δ* strains and introduced an iron-dependent reporter plasmid. Expression of Aft1/2
regulated pFET3-\textit{LacZ} reporter construct would directly reflect the iron responsiveness of the deletion strains. With the help of β-galactosidase assays we have been able to show that under high cytosolic iron as in \textit{ccc1}\textDelta strain, and in the absence of Fra1 or Fra2, mitochondrial Fe-S cluster biogenesis machinery is not required for the inhibition of Aft1/2 in certain strains. This would weaken the established roles of Grx3/4, Fra1 and Fra2 in the delivery of the mitochondria derived inhibitory cluster transfer to Aft1/2.

**MATERIALS AND METHODS**

*Yeast strains and growth media.* \textit{Saccharomyces cerevisiae} strains used in this study are listed in Table 2.1. Strains were grown in yeast extract-peptone-based media supplemented with 2% glucose (YPD) or synthetic complete (SC) media supplemented with 2% glucose and the appropriate amino acids.

*Construction of plasmids.* Construction of a deletion plasmid for generating \textit{isu1}\textDelta strains was accomplished via PCR amplification of the flanking regions of the \textit{ISU1} ORF using the respective upstream and downstream primer pairs shown in Table 2.2. The two PCR products were cloned into the yeast integrating plasmid pRS402 digested with BamHI and SalI (NEB) [16]. The ligated circular yeast expression plasmid containing the flanking regions was then linearized with restriction enzyme EcoRI (NEB), followed by transformation into the WT yeast strain DY150, \textit{ccc1}\textDelta, \textit{fra1}\textDelta, \textit{fra2}\textDelta, \textit{ccc1}\textDelta\textit{fra1}\textDelta strains obtained from Jerry Kaplan [3]. \textit{ISU1} in the yeast genome was replaced by the selectable marker \textit{ADE2} via homologous recombination into the genome thereby creating \textit{isu1}\textDelta strains. The deletion was confirmed by colony screening PCR using the primers shown in Table 2.3. A plasmid expressing the \textit{LacZ} gene under the control of the \textit{FET3} promoter (pFC-W) was a kind gift from Dr. Dennis Winge (Univ. Utah) [17].
Lithium Acetate Transformation of *S. cerevisiae*. All yeast transformations were done using the lithium acetate procedure. Briefly, yeast cultures were grown on YPD plates for 2-3 days in anaerobic chambers at 30 °C. Cells were transferred to 5 mL of YPD media and grown at 30°C for 4-5 hours followed by harvesting and resuspension in 800 μL of autoclaved water, 100 μL 10x TE (100 mM Tris, 10 mM EDTA, pH 8.0), 100 μL 1M-lithium acetate (LiAc, pH 7.4) solutions. The transformation reaction mixture contained 100 μL yeast cells, 10 μL salmon sperm DNA and 5 μL plasmid / 10 μL linear DNA (0.4 mg/mL) or 10 μL H₂O for the negative control. All samples were vortexed and 500 μL PEG plate solution (80% 45% polyethylene glycol, 10% 1 M LiAc, and 10% 10X TE) was added followed by inverting 4-6 times. Samples were then incubated at 30 °C for 1 hour. Next, 5 μL of DMSO was added and cells were heat shocked at 42 °C for 10 min. Cells were immediately kept on ice for 2 min and then centrifuged at 13000 rpm for 10 s. PEG was aspirated and the pellet was resuspended in 150 μL of sterile water. The cells were then plated on appropriate plates and grown in anaerobic chambers for 2-3 days at 30 °C.

β-galactosidase assays. Strains were transformed with the *FET3*-LacZ reporter plasmid, pFC-W. Strains were grown in SD media at 30 °C to an OD of 1 and were divided into 3-mL aliquots for induction. The cultures were restarted with an OD ~0.25 and then induced with 100 μM BPS (low iron), 100 μM FeSO₄ (high iron) and sterile water (normal iron) as a control and grown for 4 hours at 30 °C until they reached an OD of 0.8-1.0. Cells were harvested and washed with cold Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol (BME), then resuspended in 200 μL of Z buffer with 10 μL of 100 mM PMSF and 100 μL of glass beads (Sigma). Cells were lysed with two 2-minute cycles in a Mini-Bead beater, extracts were centrifuged (13000
Table 2.1. Strains used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT DY150</td>
<td>ura3-52, leu2-3, 112, trp1-1, his3-11, ade2-1, can1-100(oc)</td>
<td>L. Li 2001[18]</td>
</tr>
<tr>
<td>ccc1Δ</td>
<td>ura3, leu2, trp1-1, his3, ade2, can1, ccc1Δ::HIS3</td>
<td>L. Li 2001[18]</td>
</tr>
<tr>
<td>fra1Δ</td>
<td>ura3-52, leu2-3, 112, trp1-1, his3-11 ade2-1, can1-100(oc) fra1Δ::HIS3</td>
<td>J. Kaplan unpublished</td>
</tr>
<tr>
<td>fra2Δ</td>
<td>ura3-52, leu2-3, 112, trp1-1, his3-11 15, ade2, can1-100(oc) fra2Δ::HIS3</td>
<td>J. Kaplan unpublished</td>
</tr>
<tr>
<td>fra1Δccc1Δ</td>
<td>ura3-52, leu2-3, 112, trp1-1, his3-11, 15, ade2, can1-100(oc) fra2Δ::kanMX, ccc1Δ::HIS3, ho:FET3LacZ</td>
<td>J. Kaplan unpublished</td>
</tr>
<tr>
<td>ccc1Δisu1Δ</td>
<td>ura3, leu2, trp1, his3, ade2, can1, ccc1Δ::HIS3, isu1Δ::ADE2</td>
<td>This study</td>
</tr>
<tr>
<td>fra1Δisu1Δ</td>
<td>ura3-52, leu2-3, 112, trp1-1, his3-11 ade2-1, can1-100(oc) fra1Δ::HIS3, isu1Δ::ADE2</td>
<td>This study</td>
</tr>
<tr>
<td>fra2Δisu1Δ</td>
<td>ura3-52, leu2-3, 112, trp1-1, his3-11 15, ade2, can1-100(oc) fra2Δ::HIS3, isu1Δ::ADE2</td>
<td>This study</td>
</tr>
<tr>
<td>fra1Δccc1Δisu1Δ</td>
<td>ura3-52, leu2-3, 112, trp1-1, his3-11, 15, ade2, can1-100(oc) fra2Δ::kanMX, ccc1Δ::HIS3, isu1Δ::ADE2</td>
<td>This study</td>
</tr>
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Table 2.2 Primers used for cloning ISU1 gene.

<table>
<thead>
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<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Primer A _ISU1</td>
<td>5’-CCGAAGTTCCGGAGGCCTTTATCTCC -3’</td>
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<tr>
<td>PRIMER B _ISU1</td>
<td>5’-GTTATGCTGGATCCTCTCAAAAACC-3’</td>
</tr>
<tr>
<td>PRIMER C _ISU1</td>
<td>5’-CAGATCAAAGTCGACGACTCTACTG-3’</td>
</tr>
<tr>
<td>PRIMER D _ISU1</td>
<td>5’-AAACAAATATATAAGGGGGAAGTGG -3’</td>
</tr>
</tbody>
</table>
Table 2.3 Primers used for colony screening PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer A_ISU1_cPCR</td>
<td>5’-GGCGTAGAGAATTCGTTCCCTTAAC-3’</td>
</tr>
<tr>
<td>PRIMER B_ISU1_cPCR</td>
<td>5’-GTTATGCTGGATCCTCTCTCAAAACC-3’</td>
</tr>
<tr>
<td>PRIMER C_ISU1_cPCR</td>
<td>5’-CAGATCAAAGTCGACGACTCTCTACTG-3’</td>
</tr>
<tr>
<td>PRIMER D_ISU1_cPCR</td>
<td>5’-CCCTGGAGAATTCTATCTTAGGAAG-3’</td>
</tr>
<tr>
<td>PRIMER E_ISU1_cPCR</td>
<td>5’-GCTGGCCTTTTTGCTCACATGTTCTTTCC -3’</td>
</tr>
</tbody>
</table>
rpm, 10 min) and the supernatants were collected for assaying. Extract dilutions were made with Z buffer. The protein concentration of the extracts was determined by the Bradford method (Bio-Rad). The assay was carried out in 1-mL polystyrene cuvettes (VWR), where each 1-mL reaction contained Z buffer with 0.7 mg/ml o-nitrophenyl β-D-galactopyranoside (ONPG) and cell extract. Reactions were incubated at 30 °C until a faint yellow color appeared, then 300 μL of 1 M Na₂CO₃ was added to stop the reaction and the reaction time was documented (min). The absorbance at 420 nm was recorded, and β-galactosidase activity using the following equation where 1.3 corrects for the reaction volume and 0.0045 is the OD of a 1 nmol/mL solution of ONPG, with units of nmol ONPG converted per min per mg protein.

$$\text{β-galactosidase units} = \frac{A_{420} \times 1.3 \times \text{dilution factor}}{0.0045 \times \text{protein concentration} \times \text{time} \times \text{volume}}$$

**RESULTS**

**fra1Δ and fra2Δ mutants are not responsive to iron levels in the cell.**

Transcriptional regulation of *FET3* is controlled by the transcription factors Aft1 and Aft2, which are general regulators of proteins involved in iron homeostasis. Thus, testing transcription of *FET3* is a way to determine if regulation by Aft1/2 is functional. When WT cells are grown under high or normal iron conditions, β-galactosidase activity should be low, while cells grown under low iron conditions should have a high activity.

When we compared the β-galactosidase activity between WT (DY150) and the single deletion mutants fra1Δ and fra2Δ, β-galactosidase activity was high irrespective of the iron levels for the two deletion strains (Fig. 2.1). This is in agreement with earlier published findings as Fra1 and Fra2 are proteins involved in the transfer of an inhibitory cluster to Aft1/Aft2 along with Grx3/4 *in vivo* [3], so their loss results in an uninhibited
Aft1/Aft2 activity leading to high expression of the reporter construct irrespective of iron levels in the cell.

*Deletion of CCC1 in addition to Fra1/Fra2 has a WT phenotype.* CCC1 encodes a vacuolar iron importer that is under the control of the high iron sensing transcriptional activator Yap5 in *S. cerevisiae*. Under low iron conditions, CCC1 transcription is negligible which ensures availability of iron for cytosolic and mitochondrial iron-dependent processes. When iron levels are high, Yap5 enhances the transcription of this gene resulting in removal of excess free iron into the vacuole thus protecting the cell from the high iron mediated toxicity. In comparison with the WT cells, β-galactosidase activity of ccc1Δ mutants bearing the *FET3*-LacZ reporter construct would be similar. Since the iron regulon is constitutively active in fra1Δ and fra2Δ mutants, these cells are likely to have a higher accumulation of intracellular iron levels compared to WT cells. The iron regulon responds to a mitochondrially generated inhibitory [2Fe-2S] cluster which is delivered to Aft1/2 by cytosolic Grx3/4 and Fra2 and possibly accompanied by Fra1. Interestingly, in the same strains, an additional deletion of CCC1 results in reversion to the WT phenotype (Fig. 2.2).

As mentioned earlier, high iron sensing Yap5 is responsible for activating the expression of iron utilization or storage genes including CCC1 which encodes the vacuolar iron importer. Since an additional deletion of CCC1 in fra1Δ or fra2Δ (not shown) somehow reverts to the WT phenotype, the importance of these two gene products and specifically Fra2 in the inhibitory cluster transfer to Aft1/2 under iron replete conditions became questionable. This indicates that under iron replete conditions, cytosolic Grx homodimers could deliver the mitochondrially derived cluster to Aft1/2 in the absence of Fra2 or Fra1. Alternatively, the excess iron could directly bind Aft1/2 or induce disulfide
Figure 2.1. Absence of fra1 or fra2 leads to constitutive activation of the iron regulon

WT, fra1Δ and fra2Δ were transformed with the FET3-LacZ reporter plasmid, pFC-W and grown in triplicate in SD media at 30 °C. Upon reaching 1 OD, cultures were divided into 3-mL aliquots for induction with 100 μM BPS (low iron: pink bars), 100 μM FeSO₄ (high iron: red bars) and sterile water (normal iron: orange bars) as a control, at a starting OD of 0.25 and grown for 4 hours at 30 °C until they reached an OD of 0.8-1.0. Strains tested were WT, fra1Δ and fra2Δ.
formation on Aft1/2, leading to eventual dimerization and export from the nucleus thereby turning off the iron regulon. Collectively, these possibilities suggest alternate regulation mechanisms that do not involve Fra1 or Fra2.

**Inhibition of Aft1/2 is still dependent on the mitochondrial Fe-S biogenesis machinery in fra1Δccc1Δ.** Before diverging to possible alternate mechanisms, we first wanted to understand if the mitochondrial Fe-S biogenesis machinery was contributing to inhibition of Aft1/2 in these strains. *ISU1* deletion was introduced in these strains which encodes the mitochondrial scaffold protein for the synthesis of Fe-S clusters. The absence of Isu1 would result in defective Fe-S cluster production in the mitochondria thus creating a false impression of low iron in the system due to reduced mitochondria generated inhibitory cluster delivery to Aft1/Aft2. In the *isu1Δ* single mutant, we expect constitutive expression of Aft1/Aft2-regulated genes observed as high *FET3*-LacZ activity irrespective of the iron conditions, as it is known that a decrease in mitochondrial Fe-S cluster biogenesis results in increased expression of the iron regulon [19]. Comparison of the β-galactosidase activities between the *isu1* mutants showed that inhibition of Aft1/Aft2 was still under the control of the mitochondrial Fe-S cluster biogenesis machinery in *ccc1Δisu1* and *fra1Δisu1* but apparently not for *fra2Δisu1* (Figure 2.3). For *fra1Δccc1Δisu1*, deletion of Isu1 did not show much different result than *fra1Δccc1Δ* (Figure 2.2). This was not entirely in accordance with the published model that under iron replete conditions, a mitochondrion generated Fe-S cluster was responsible for the inactivation and dimerization of Aft1/Aft2 leading to eventual export into the cytoplasm. In the absence of *ISU1*, there *fra1Δccc1Δisu1* and *fra1Δccc1Δ*, it seems that the lack of the inhibitory cluster generation is not important for the regulation of Aft1/2. But it is also required to test the *fra2Δccc1Δ*,
Figure 2.2 Deletion of \textit{ccc1} in addition to \textit{fra1} reverts to WT phenotype. WT, \textit{ccc1}\(\Delta\), \textit{fra1}\(\Delta\) and \textit{fra1}\(\Delta\) \textit{ccc1}\(\Delta\) were transformed with the \textit{FET3}-\textit{LacZ} reporter plasmid, pFC-W and grown in triplicate in SD media at 30 °C. Upon reaching 1 OD, cultures were divided into 3-mL aliquots for induction with 100 μM BPS (low iron: pink bars), 100 μM FeSO\(_4\) (high iron: red bars) and sterile water (normal iron: orange bars) as a control, at a starting OD of 0.25 and grown for 4 hours at 30 °C until they reached an OD of 0.8-1.0. Strains tested were WT, \textit{ccc1}\(\Delta\), \textit{fra1}\(\Delta\) and \textit{fra1}\(\Delta\)\textit{ccc1}\(\Delta\).
Figure 2.3 Only ccc1Δisu1Δ and fra1Δisu1Δ have constitutively active iron regulon. WT, ccc1Δisu1Δ, fra1Δisu1Δ, fra2Δisu1Δ, fra1Δccc1Δisu1Δ were transformed with the FET3-LacZ reporter plasmid, pFC-W and grown in triplicates in SD media at 30 °C. Upon reaching 1 OD, cultures were divided into 3-mL aliquots for induction with 100 μM BPS (low iron: pink bars), 100 μM FeSO₄ (high iron: red bars) and sterile water (normal iron: orange bars) as a control, at a starting OD of 0.25 and grown for 4 hours at 30 °C until they reached an OD of 0.8-1.0.
was a deficit in the generation of the inhibitory cluster in these strains but for fra2Δ ccc1Δ isu1Δ in comparison with fra2Δccc1Δ and fra2Δ before further commenting on any deviation from the published model since according to the model cytosolic Grx3/4, Fra1 and Fra2 aid in the delivery of the mitochondria derived Fe-S to Aft1/2 under iron replete conditions.

DISCUSSION

According to the established model of Aft1/2 inhibition it has been shown that under iron replete conditions, cytosolic Grx3/4 and Fra2 transmit an inhibitory signal to Aft2 (and presumably Aft1), which results in its dissociation from its target promoter [2]. It has also been established that deletion of ISC assembly genes results in constitutive activation of the iron regulon, which is not suppressed by addition of iron externally [3, 11]. The perplexing observation by our collaborators that additional deletion of the vacuolar iron importer Ccc1 reversed the constitutive activation of iron regulon effect by restoring iron-responsive control despite the absence of Fra2 or Fra1 (J. Kaplan, unpublished data). This led to the possibility of an alternative mechanism independent of Fra2 or Fra1. Before diverging into alternative pathways which might occur in these strains, we were interested in finding out if the mitochondria derived inhibitory cluster was still transferred to Aft1 and Aft2 in these strains.

To answer this question, we created isu1Δ strains to disrupt the mitochondrial Fe-S cluster biogenesis machinery and gauge the effect on expression of the iron regulon with the help of the FET3-LacZ reporter plasmid pFC-W. We were able to show that the iron regulon was constitutively active in the isu1Δ strains tested here. In addition to the strains described here, our study would be complete and more thorough if we obtained the isu1Δ,
fra2Δccc1Δ and the fra2Δccc1Δisu1Δ strains for comparison. A systematic analysis of these strains side-by-side with the other single, double and triple mutants described in this study would further help in bolstering our conclusion that Aft1 and Aft2 are inhibited by an Fe-S cluster delivered by Grx3/4 and Fra1/Fra2.

But what could be happening in the fra1Δccc1Δ and fra2Δccc1Δ strains? In these strains, there will be an increased accumulation of iron which might result in direct binding of iron leading to dimerization and eventual export of Aft1 or Aft2 into the cytoplasm. Measuring the whole cell iron levels and investigating if excess iron induces formation of disulfide bonds in Aft1/2 thus can be investigated. Direct binding of iron by Aft2 has been suggested in vitro but in order to investigate the same in vivo, pull-down experiments with Fe55 on Aft1/2 might be helpful [2]. Iron induced disulfide formation on Aft1 or Aft2 could be studied using thiol modification assays teamed with non-reducing SDS PAGE. It is also possible that under iron replete conditions, as in the strains, the levels of Grx4 increase which, in the absence of its binding partner Fra2 could mediate an inhibitory cluster transfer to Aft1/2. Measuring both mRNA and protein levels of Grx3 and Grx4 will help us determine if their expression is upregulated in the absence of Fra1/Fra2 to facilitate transfer of the inhibitory cluster from Grx3/4 homodimers to Aft1/2.

It is also important to note that yeast is a facultative anaerobe and might possess distinct phenotype under aerobic and anaerobic conditions [20]. The β-galactosidase assay using FET3-LacZ reporter plasmid was performed aerobically as under anaerobic conditions expression of FET3 is downregulated due to presence of repressors Tup1p and Hda1p [21]. In order to investigate whether fra1Δccc1Δ, fra2Δccc1Δ had a similar response to different iron conditions, β-galactosidase assay using ARN1-LacZ reporter could also be
informative. *ARN1* is a siderophore iron uptake protein and it is under the transcriptional control of Aft1 and Aft2 in the absence of heme or under anaerobiosis [21].

Finding answers to how the iron-responsive phenotype is rescued in *fra2Δccc1Δ* and *fra1Δccc1Δ* strains would help re-establish the roles of Fra1 and Fra2 in the inhibitory network or may indicate the existence of an alternative mechanism for the iron regulation in these particular strains.
REFERENCES


CHAPTER 3

SCREENING FOR FRA2-GRX3-FRA1 MUTATIONS THAT IMPACT IRON REGULATION IN VIVO

ABSTRACT

Iron is an important micronutrient for almost all living organisms. To maintain optimum concentration of iron in cells, iron homeostasis is strictly regulated in both prokaryotes and eukaryotes. S. cerevisiae, a model eukaryote, employs multiple routes to incorporate iron, although the molecular mechanisms for iron sensing and trafficking are still being identified. This simple eukaryote serves as an ideal tool for researchers to fill in the underlying gaps in our understanding of iron homeostasis and signaling. S. cerevisiae responds to iron starvation conditions via activation of the iron regulon by the paralogous low iron-sensing transcription factors Aft1 and Aft2, which leads to increased iron uptake into the cell [1]. Our group recently unveiled the molecular mechanism for inhibition of Aft1/2, demonstrating that the cytosolic glutaredoxins Grx3/Grx4 and accessory protein Fra2 transfer an Fe-S cluster to Aft2 (and presumably Aft1) leading to its decreased DNA binding affinity [2]. Genetic studies suggest that the protein Fra1 may also have a role in regulating Aft1/2 activity; however, its specific role is unknown [3]. Deletion of either Fra1 or Fra2 was shown to activate Aft1/2 despite sufficient intracellular iron, which is consistent with the established model that Fra2 is required to transfer an inhibitory Fe-S cluster to Aft1/2 [3]. Although in vitro evidence suggested inhibitory cluster transfer from
the cytosolic complex comprising Grx3-Fra2 to Aft2, we wanted to corroborate these findings by identifying the specific residues in each of these proteins which were important for the Fe-S mediated regulation of Aft1 and Aft2. We took a genetic screen approach to investigate the key residues and with the aid of error-prone PCR we were successful in creating mutated pools of *FRA1* and *FRA2*. The goal of the genetic screen experiment was to isolate point mutations in each of these genes that would result in constitutive inhibition of the iron regulon controlled by Aft1 and Aft2. The mutations isolated in this study were sent for Sanger sequencing (Genewiz) and were identified to be non-informative non-sense or insertion/deletion resulting in frameshift leading to defective protein accompanied by spontaneous suppressor mutations.

**INTRODUCTION**

As a redox-active metal, iron is a protein cofactor that can easily accept and donate electrons, serving an important role in a wide variety of crucial metabolic processes such as respiration, DNA metabolism, and photosynthesis. Although this redox-active property is advantageous for these cellular processes, it also makes iron a major risk factor leading to oxidative stress. When in excess, iron can combine with hydrogen peroxide in the cell resulting in the production of harmful reactive oxygen species. Uptake and mobilization of iron must therefore be tightly controlled to avoid both iron deficiency and iron overload. Regulation of iron homeostasis involves a network of proteins working in concert to control the uptake, utilization and storage of iron in response to fluctuations in intracellular iron levels [4].

In *S. cerevisiae*, expression of iron uptake, utilization and storage genes is primarily regulated by the transcriptional activators Aft1, its parologue Aft2, and Yap5 [5-8]. During
iron deprivation, Aft1 and Aft2 activate the expression of the iron regulon, which comprises a set of 27 genes whose products are involved in iron trafficking, metabolism and storage. Iron enters the mitochondria to participate in heme biosynthesis and biogenesis of iron-sulfur (Fe-S) clusters, which are then incorporated into their respective apoproteins. Fe-S clusters are also synthesized in the cytosol by a distinct biosynthetic machinery. *S. cerevisiae* has two distinct pathways for synthesis of mitochondrial and cytosolic Fe-S clusters namely the ISC pathway and the CIA pathway [2, 3, 9, 10].

According to the established model of Aft1/2 inhibition, under iron replete conditions cytosolic Grx3/4 and Fra2 transmit an inhibitory signal to Aft2 (and presumably Aft1), which results in its dissociation from target promoters [2]. Glutaredoxins (Grxs) belong to a highly conserved family of proteins and can be classified into two main groups based on their active site sequence – CXXC containing dithiol Grxs and CGFS motif containing monothiol Grxs. Unlike dithiol Grxs, CGFS type Grxs do not participate in thiol-disulfide exchange reactions, but instead play critical roles in iron homeostasis, Fe-S cluster assembly, and signal transduction [10, 11]. Biochemical characterization of Grx4 from *S. cerevisiae* and *S. pombe* by our lab and collaborators have shown that these proteins exist in a monomeric apo form or a [2Fe-2S]-bridged dimeric holo form and this property is conserved between both eukaryotic and prokaryotic CGFS Grxs [12, 13]. One of the major known interacting partners of CGFS Grxs are the widely conserved BolA-like proteins [13-16]. Grxs and BolA like proteins form both apo and holo [2Fe-2S]-bridged heterodimers which are stable and potent as iron sensing and trafficking complexes. In *E. coli* BolA-like proteins are involved in the regulation of cellular morphology whereas in *S.
and S. pombe, they are involved in iron homeostasis. Mitochondrial BolAs in humans and S. cerevisiae are involved in the maturation of Fe-S clusters.

To identify residues important for inhibitory Fe-S transfer to Aft1 or Aft2 under iron replete conditions in Grx3/4, Fra1, and Fra2, a genetic screen approach using the aminoquinone antibiotic streptonigrin was established. The goal of the screen was to identify point mutations in each of these genes that would constitutively inhibit the iron regulon via interaction with Aft1 and Aft2. Streptonigrin was employed in this screen due to its ability to generate reactive oxygen species in the presence of excess iron and oxygen, thus allowing selection of cells with low intracellular iron [17]. To proceed with the screen, the minimum inhibitory concentration of streptonigrin, that selectively kills WT cells and not the positive controls was first determined. Point mutations in GRX3/4, FRA1, FRA2 were then generated by an error-prone PCR technique, and the randomly mutated pools were then transformed in the respective deletion strains. Positive hits from the genetic screen would favor the growth of only those cells that had a constitutively inhibited iron regulon due to mutations in these target genes. In this a scenario, the mutated proteins may be able to constitutively trap Aft1/2 in the cytosol. But it is important to note that positive hits from this screen could either rise from point mutations leading to mutated version of the proteins that constitutively inhibit Aft1/Aft2, or spontaneous suppressor strain mutations giving rise to a false positive phenotype. Our goal was to narrow down or identify only those mutations that would alter the inhibitory cluster transfer to Aft1/2 and thus dismantle the low iron response. We have been able to successfully establish the screen and determine the working concentration of streptonigrin required, in addition to isolating mutated pools for FRA1 and FRA2. Upon sequencing we were able to recover 5
colonies which were able to survive the screen, but the mutations generated in them were either nonsense or frameshift mutations from insertion/deletion of a base, resulting in prematurely translated or non-functional protein. This suggest that spontaneous mutations beyond the open reading frame of \textit{FRA2} might have enabled the colonies to survive the screen.

\section*{MATERIALS AND METHODS}

\textit{Plasmids, strains and media.} pND104 (pRS415-\textit{Grx3}) and pND140 (made by Dr. Nin Dingra) were used to obtain \textit{GRX3} and \textit{FRA2} for the error prone PCR technique [18]. pND104 was digested with SalI (NEB) and NotI (NEB) and then \textit{GRX3} was sub-cloned into pRS411 (\textit{CEN MET15}). \textit{GRX4} and \textit{FRA1} was amplified from BY4741 genomic DNA and cloned into pRS413 (\textit{CEN HIS3}) and pRS315 (\textit{CEN LEU2}) backbones using the same restriction enzymes [19]. WT, \textit{grx3Δ, grx4Δ, fra1Δ} and \textit{fra2Δ} deletion strains were obtained from the BY4741 deletion library (Research Genetics). Bacterial transformations were done in chemically competent DH5\textsubscript{α} cells and grown in LB media with appropriate antibiotics. Yeast transformations were performed using standard lithium acetate transformation protocol. WT yeast strains were grown in synthetic complete (SC) while the mutants in synthetic drop out (SD) media supplemented with appropriate amino acids and either 100 µM BPS (Sigma) for low iron or 100 µM ferrous ammonium sulfate (Sigma) for high iron conditions. Both these types of growth media have known chemical compositions compared to undefined or rich media but in case of the synthetic drop-out certain amino-acid are omitted based on the type of selection required by a strain. Streptonigrin (Sigma) stock was made in HCl (1 mg/mL) and added to the respective media at the required concentrations.
Creation of the mutant pool. Error-prone PCR was performed using the Genemorph II EZ clone domain mutagenesis kit (Agilent Cat# 200552) on the constructs. The kit contained special Mutazyme II DNA polymerase for introducing mutations during PCR with a more uniform mutational spectrum compared to other error-prone PCR enzymes. The optimized reaction buffer included in the kit ensures that the only variable to be changed for the error-prone PCR was the DNA amounts which would directly reflect the mutational frequency. The constructs (mentioned in the previous section) were used as templates for creation of the mutated target genes with the low fidelity Mutazyme II using primers listed in Table 3.1. These mutated PCR products then served as megaprimer for the subsequent EZ clone step of the error-prone PCR process where they were annealed to the original plasmid eventually amplifying the whole plasmid using a high-fidelity DNA polymerase (Figure 3.1), then isolated using the Wizard Plus DNA purification system (Promega Cat #A1330). This unusual eight-hour growth in selective liquid media instead of overnight growth on selection plates was performed to reduce excessive growth of the cells thereby minimizing risks of any extraneous mutations.

The mutagenized pool was then transformed into the respective yeast deletion strains (e.g. FRA1 plasmid pool transformed into fra1∆ strain) but instead of plating, the transformed cells were selected in liquid media at 30 °C. The following morning when cells reached an OD\textsubscript{600} of 0.25, the iron regulon was induced by addition of 100 µM BPS, an extracellular iron chelator, and grown for an additional 8 hours at 30 °C. Post induction, we proceeded with a fixed amount (200 cells) for controls and test strains. Cells were then washed twice with media and treated with 300 µM ferrous ammonium sulfate and 12 µg/mL streptonigrin for 45 minutes at 30 degrees. Next, cells were harvested, washed with
Figure 3.1. Schematic representation of error-prone PCR. EZ clone domain mutagenesis Kit (Agilent Technologies Cat # 200552).
Table 3.1 Primers used for this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grx4-forward</td>
<td>GATTAAGACTGCAGAAGTAATAAAAGG</td>
<td>This study</td>
</tr>
<tr>
<td>Grx4-reverse</td>
<td>GTTGAGAAGCGGAGTCGACGGTG</td>
<td>This study</td>
</tr>
<tr>
<td>Fra1-forward</td>
<td>GAAAGGCGGTCTAGAGTAAATGATG</td>
<td>This study</td>
</tr>
<tr>
<td>Fra1-reverse</td>
<td>GAAAATCCTGCTCGAGCTCCATGGAAC</td>
<td>This study</td>
</tr>
<tr>
<td>Fra2-forward</td>
<td>CGGTTATATATACATATATG</td>
<td>This study</td>
</tr>
<tr>
<td>Fra2-reverse</td>
<td>AATAGAACATATCATCCCTTTCA</td>
<td>This study</td>
</tr>
</tbody>
</table>
media to ensure removal of any iron or streptonigrin and resuspended in 200 µL media. These cells were streaked on plates with high iron in the presence or absence of streptonigrin and grown anaerobically for three days at 30 °C.

Recovery of mutated plasmid from yeast and identification of mutations. Colonies from the high iron and streptonigrin plates were grown overnight in 2 mL selection media at 30 °C. The following day, the overnight culture was pelleted down and resuspended in buffer containing 1.2 M sorbitol, 100 mM potassium phosphate pH 7.5, and 400 µg/mL zymolyase and incubated at 37 °C for 2 hours to break open the yeast cell wall. The cells were then lysed using the Promega Wizard plus SV DNA isolation kit lysis solution followed by heating for 10 minutes at 65 °C. The rest of the protocol was similar to the standard plasmid isolation protocol and the isolated plasmid was eluted in 10 µL warm water.

RESULTS

Mutant megaprimers and mutagenized pool for Fra1 and Fra2. For analyzing protein structure/function relationship, the Genemorph kit (Agilent Technologies Cat # 200552) suggested a low to medium mutation frequency which would lead to mutation of one or two nucleotides per ORF to ensure one amino acid change. The desired mutational frequency could either be achieved by changing the amount of template DNA or changing the number of PCR cycles (Table 3.1). For our experiments, we wanted a low mutational frequency so that it would result in a single amino acid change. The single amino acid change could either result in a functional defect or untranslated or mistranslated protein. We were successful in performing error-prone PCR on FRA1, FRA2, GRX3, GRX4 which would eventually serve as megaprimers resulting in a mutagenized pool for the genetic
screen (Figure 3.2). We initially proceeded with \textit{FRA1} and \textit{FRA2} for the following steps and successfully created the mutagenized pool to be used for the genetic screen (Figure 3.3).

\textbf{Determination of the minimum inhibitory concentration of streptonigrin.} \textit{aft1Δ} or \textit{fet3Δ} were used as control strains for this experiment since in \textit{aft1Δ}, the iron regulon would not respond to low iron levels due to the absence of Aft1. Fet3 is a multicopper oxidase in \textit{S. cerevisiae} which oxidizes Fe\(^{2+}\) to Fe\(^{3+}\) for high affinity iron uptake during iron deficient conditions. \textit{FET3} is a member of the iron regulon which is under the transcriptional regulation of Aft1/2. In the absence of Fet3 the high affinity iron uptake system would not be able to acquire iron and thus both in \textit{aft1Δ} and \textit{fet3Δ} strains, the iron content of the cells would be lower than WT cells. Low levels of iron would then make them more resistant to the toxic effect of streptonigrin, thus serving as positive controls for the screen (Figure 3.4). Keeping the iron concentration fixed at 300 µM ferrous ammonium sulfate and upon increasing streptonigrin concentrations, 12 µg/mL was chosen to be the working concentration for the genetic screen experiment that would selectively kill normal WT cells vs. cells defective in iron import from the media (Figure 3.5).

\textbf{Genetic Screen for constitutive inhibition of Aft1.} For both \textit{FRA1} and \textit{FRA2}, we were able to recover only about 5% plated cells on high iron streptonigrin plates. The colonies were petite and pink, dissimilar to those on the plates having no streptonigrin. Since these cells were exposed to extreme conditions, spontaneous suppressors might have led to such texture. Nevertheless, the resistant colonies were then collected and grown in SD (-Leu) media to isolate the respective plasmids. Recovery of these plasmids from yeast was poor since these were low copy plasmids and thus were re-transformed into
Table 3.2 Mutational frequency vs. initial target quantity for error prone PCR (EZ clone domain mutagenesis Kit (Agilent Technologies Cat # 200552)).

<table>
<thead>
<tr>
<th>Mutation rate</th>
<th>Mutation frequency (mutations/kb)(^a)</th>
<th>Initial target amount (ng)(^b,c)</th>
<th>Recommended fold amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0–4.5</td>
<td>500–1000</td>
<td>1.5–10</td>
</tr>
<tr>
<td>Medium</td>
<td>4.5–9</td>
<td>100–500</td>
<td>10–100</td>
</tr>
<tr>
<td>High</td>
<td>9–16</td>
<td>0.1–100</td>
<td>100–10,000</td>
</tr>
</tbody>
</table>
Figure 3.2. Agarose gels showing mutant mega primers. a) and b) agarose gel electrophoresis showing correct sizes for mutated GRX3, GRX4, FRA2 and FRA1 using Genemorph EZ clone domain mutagenesis kit. The 1.1 kb gel standard was provided with the aforementioned kit for comparing intensities of the PCR product. Also, to demonstrate the effectiveness of the process a 3.0 kb control plasmid was advised to be included simultaneously with the PCR reactions. These PCR products were used as mutant megaprimers for the next step.
Figure 3.3. **Agarose gels showing mutagenized PCR pool.** Agarose gels showing the pre and post DpnI treated mutagenized pool for *FRA1* (a, Lanes 1 and 2) and *FRA2* (b, Lanes 2 and 3). The mutated genes (Figure 3.2 a, b) were used as mutant megaprimers with pRS315-Fra2 and pND140 as template plasmids following manufacturer’s protocol. DpnI enzyme was used to digest the template plasmid from the mutated PCR pool, which could then be used for downstream *in vivo* experiments.
Figure 3.4. Comparison of growth between WT, \(aft1\Delta\) and \(fet3\Delta\) under regular vs high iron conditions. a) and b) WT, \(aft1\Delta\) and \(fet3\Delta\) strains containing vector backbones were serially diluted and spotted on SD (-Leu) media without extra Fe or 300 µM Fe (ferrous ammonium sulphate) and grown anaerobically at 30 °C.
Figure 3.5. Determination of minimum inhibitory concentration of streptonigrin. a) and b). WT, aft1Δ and fet3Δ strains containing vector backbones were serially diluted and spotted on SD (-Leu) media with increasing concentrations of streptonigrin (SNG) keeping iron concentration fixed at 300 µM Fe (ferrous ammonium sulfate) and grown anaerobically at 30 ºC.
DH5α for enhanced yield. Upon isolating the mutant plasmids from these bacteria, they were sent for sequencing (Genewiz) in order to identify the mutations. If such mutations resulting in amino acid changes could be identified, those mutant proteins would then be overexpressed in specialized bacterial strains and biochemically characterized. Comparison with the WT versions would then enable us to determine the effect of those residues in the delivery of the cluster to Aft1/2. Upon sequencing we were able to shortlist 5 colonies in which error prone PCR was successful. In FRA1, a point mutation led to premature termination of the open reading frame, or we generated a non-sense mutation along with 2 other mutations outside the ORF which spontaneous suppressor mutations would be. For FRA2, we were able to generate 4 colonies out of which 3 had insertion/deletion of a base resulting in frameshift causing truncated version of the functional protein. For the fourth one, there was no mutation detected in the open reading frame of the protein suggesting there was a spontaneous suppressor in the genome. Nonfunctional or defective Fra1 and Fra2 proteins would lead to constitutive activation of the iron regulon according to the published model [3]. Thus, such strains must have high iron similar to fra1Δ and fra2Δ and would not be able to survive in the presence of streptonigrin. Since we were able to isolate 5 colonies which survived in the presence of 300 μM ferrous ammonium sulfate and 12 μg/ mL streptonigrin, it is highly likely these strains developed spontaneous suppressor mutations in the genome which aided in their survival strategy. Identification of such a suppressor would require whole genome sequencing of these strains in question. For our screen, our expected outcome would be to recover one or more missense mutations, which would result in substitution of the original amino acid with one which might confer the ability of the protein to constitutively inhibit
Aft1/2 by trapping it in the cytosol. Optimizing the screen conditions to recover more colonies would greatly enhance the chances of recovering more hits.

**DISCUSSION**

As it has been already established that under iron deprivation, the high affinity iron uptake system comprising Aft1 and its parologue Aft2 activate the expression of the iron regulon in *S. cerevisiae*. Upon activation of iron uptake genes, iron enters the mitochondria to participate in heme biosynthesis and biogenesis of Fe-S clusters, which are then incorporated into their respective apoproteins. Upon iron replete conditions, a mitochondria derived Fe-S is exported via Atm1 and delivered to Aft1/2 by a cytosolic complex comprising Grx3/4, Fra2 and Fra1 leading to dimerization and eventual export into the cytoplasm thus inhibiting the iron regulon [2-4, 9, 10].

In this study we were interested in identifying key residues in each of these cytosolic proteins involved in this inhibitory Fe-S cluster transfer to Aft1 and Aft2. To identify residues, a genetic screen approach using the aminoquinone antibiotic streptonigrin was used in a screen to identify point mutations in each of these genes that would constitutively inhibit the iron regulon via altered interaction with Aft1 and Aft2. The positive controls used in this study were *aft1Δ* and *fet3Δ*, which would mimic the constitutively inhibited phenotype of the mutants and would survive in the working concentration of streptonigrin used in the screen. Positive hits from the genetic screen would favor the growth of only those cells which due to mutations in the respective genes had a constitutively inhibited iron regulon, a scenario where they were perhaps able to trap Aft1/2 in the cytosol. It is important to note that the positive controls used in this screen were *aft1Δ* and *fet3Δ*. The *aft1Δ* strain still had an active Aft2 and since they have
overlapping functions, $aft1\Delta aft2\Delta$ could also be added as an extra control which would nullify any compensating effect of Aft2 in the $aft1\Delta$ strain. Similarly, $AFT1-1^{up}$ could be a good choice for negative control since in this strain, overaccumulation of iron occurs due to constitutive binding of Aft1 to its target promoter resulting in enhanced iron acquisition. $AFT1-1^{up}$ harbors Cys291Phe mutation, which is a part of the iron sensing Cys-Asp-Cys motif and this mutation leads to insensitivity of Aft1 to iron level changes. Due to constitutive expression of the iron regulon genes, this strain would over accumulate iron resulting in low survival in the presence of streptonigrin [20]. Thus, in the working condition of streptonigrin determined for the screen (12 µg/mL), WT would cease to grow and so would an $AFT1-1^{up}$ strain but growth of $aft1\Delta$, $fet3\Delta$, and $aft1\Delta aft2\Delta$ would be favored. But it is important to note that positive hits from this screen could either rise from point mutations leading to mutated versions of the proteins that constitutively inhibit Aft1/Aft2 or spontaneous suppressor mutations giving rise to a false positive phenotype.

Our goal was to narrow down or identify only those mutations that would alter the inhibitory cluster transfer to Aft1/2 and thus dismantle the low iron response. Although we succeeded in employing the screen and isolated mutated pools for $FRA1$ and $FRA2$, we were unable to retrieve a functional point mutation. Upon sequencing mutated pools for $FRA1$ and $FRA2$, we were only able to detect non-sense mutations thus leading to functionally defective Fra1 and Fra2 and false positive hits. The false positive hits comprising strains with the ability to grow in the presence of 300 µM ferrous ammonium sulfate and 12 µg/mL streptonigrin indicated spontaneous suppressor mutations in the genome. Such mutations could be characterized with the help of whole genome sequencing of the strains in question, but such mutations may or may not be directly related to the
pathways in question. For us, our goal was to identify mutations in Fra1, Fra2 and Grx3/4 that would constitutively inhibit Aft1/2, and thus we employed a genetic screen which would help detect such mutations. But additional spontaneous mutations might complicate results and the genes containing these mutations might not always be directly related to the pathways in question. Nevertheless, it is worth identifying such suppressor mutations which might mimic the genetic screen results to further identifying related pathways which might influence Aft1/2 activity directly or indirectly.

Results like ours are not out of the ordinary for genetic screen experiments and we aim to recover more hits (at least 50%) for the aforementioned as well as Grx3 and Grx4 proteins. In order to carry out a similar experiment for Grx3 and Grx4, we would have to create a grx3Δgrx4Δ strain as these proteins have redundant functions and loss of one can compensate for the other in their respective single deletion strains. With identification of such mutant variants of these proteins, we would be able to determine the specific residues which are involved in the Grx-Fra regulation of Aft1/Aft2.
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CHAPTER 4
INTERACTION BETWEEN FEP1, GRX4 AND FRA2 REGULATE THE LOW IRON RESPONSE IN S. POMBE

ABSTRACT

In the model eukaryote S. pombe, Fep1 is a GATA type transcriptional repressor that is active under iron replete conditions. Previous in vivo results suggest that the cytosolic monothiol glutaredoxin Grx4 acts as an inhibitory partner of Fep1 under iron starvation conditions. The BolA protein Fra2 is also implicated in Fep1 regulation since Fep1 is constitutively active and unresponsive to iron in the absence of Fra2. Fep1, Grx4 and Fra2 have been shown to be a part of a heteromeric complex in vivo. Here, we use biochemical and spectroscopic tools to demonstrate that Grx4-Fra2 participates in two-way iron-sulfur cluster trafficking with Fep1. Furthermore, we provide evidence that Fep1 and Fra2 do not interact in the absence of Grx4, while interaction and Fe-S cluster transfer between Fep1 and Grx4 is independent of Fra2. Taken together, we are able to establish the conserved roles of Grx4-Fra2 in iron sensing and Fe-S cluster trafficking and thus in the regulation of iron responsive transcription factors across species.

INTRODUCTION

Given the critical role of iron in various metabolic pathways, regulation of iron homeostasis is a vital function in most living organisms. In order to maintain a tight balance, this regulation involves a network of proteins working in concert to control the
uptake, utilization, and storage of iron upon sensing intracellular levels. The fission yeast *Schizosaccharomyces pombe* maintains iron homeostasis via transcriptional repression of iron uptake and iron utilization genes. When iron is replete, the GATA-type transcriptional repressor Fep1 binds to the promoters of iron uptake and transport genes that contain an \( ATC(A/T)GATA(A/T) \) motif, turning off their expression to avoid iron overload. On the other hand, under iron limitation, another transcriptional regulator, Php4, interacts with the heteromeric CCAAT-binding complex composed of Php2, Php3, and Php5 and represses iron utilization genes to economize iron usage. In addition, Fep1 and Php4 have been shown to reciprocally regulate expression of each other, thus allowing tight control of intracellular iron levels.

Regulation of iron uptake and transport genes via a GATA-type transcriptional repressor is not unique to *Schizosaccharomyces pombe*. Fep1 orthologs are found in both non-pathogenic and pathogenic fungi, including the methylotrophic yeast *Pichia pastoris* and the pathogens *Candida albicans*, *Cryptococcus neoformans*, *Ustilago maydis*, *Histoplasma capsulatum*, and *Aspergillus* species. However, a GATA-type iron regulator was lost in the *S. cerevisiae* lineage and functionally replaced with the Aft1/Aft2 transcriptional activators [1, 2]. Most fungal GATA-type iron-responsive transcriptional regulators include two class IV zinc finger motifs in their N-termini with a highly conserved intervening Cys-rich region [1]. *S. pombe* Fep1 binds to GATA-containing promoters in vivo under conditions of iron abundance whereas iron starvation abrogates this association [1]. The N-terminal domain of *S. pombe* Fep1 containing the Zn fingers and Cys-rich region is essential for this iron-dependent DNA association, while the C-terminal region mediates interaction with the co-repressor Tup1 [3-5]. In one recent study,
recombinant purified *S. pombe* Fep1 was found to bind iron via Cys-rich region, but no sulfur was detected, leading researchers to suggest that Fep1 binds iron directly and not Fe-S cluster [6]. However, a separate study demonstrated that both as purified and chemically reconstituted Fep1 binds a mixture of different Fe-S clusters [4] To summarize, the Cys-rich regions in the DNA binding domain of Fep1 act as a switch to control the repressor activity of Fep1 in response to cellular iron conditions, yet the specific type of bound cofactor, and the molecular mechanism for controlling Fep1 activity is still under active investigation.

In addition to iron-dependent transcriptional regulation by Php4 and the CCAAT-binding complex, Fep1 function is also controlled at the post-translational level by the cytosolic CGFS glutaredoxin Grx4 and its binding partner Fra2. Grx4 is reported to be involved in both high and low iron regulation by Fep1 and Php4 [7-9]. In the case of the low iron-sensing transcriptional repressor Php4, Grx4 forms a [2Fe-2S]-binding complex with this regulator that promotes its export from the nucleus [9]. While the GRX domain of Grx4 associates with Php4 under high iron conditions, this same domain binds to Fep1 under low iron conditions, thereby acting as a major link between the high and the low Grx4 forms a [2Fe-2S] bridged dimer that interact with the low iron-sensing transcriptional repressor Php4 in *Schizosaccharomyces pombe* [7]. In grx4Δ strains, Fep1 is insensitive to iron levels thus acting as a constitutive repressor of iron uptake genes [7, 8]. This result suggests that Grx4 plays a role in communicating the cellular iron status to Fep1 in order to inhibit the repressor activity. *In vivo* yeast two-hybrid studies have demonstrated that Fep1 and Grx4 associate constitutively via the C-terminal region of Fep1 and the N-terminal TRX domain of Grx4, whereas a weaker and iron-dependent interaction occurs
within the N-terminal DNA binding domain of Fep1 and the C-terminal GRX domain of Grx4 only under low iron conditions [8]. Both the N-terminal DNA binding domain of Fep1 and the C-terminal GRX domain of Grx4 contain the cysteine ligands necessary to ligate Fe-S clusters, suggesting a regulation mechanism that involves these cofactors.

In *S. cerevisiae*, the CGFS Grxs Grx3 and Grx4 form [2Fe-2S]-bridged heterodimers with the BolA-like protein Bol2 (formerly named Fra2), which delivers an Fe-S cluster to the low iron sensing transcriptional activators Aft1 and Aft2 [11]. This inhibitory cluster transfer results in Aft1/2 dimerization, dissociation from the DNA, and cytosolic export, demonstrating that the CGFS Grxs and Bol2 are key regulators of Aft1/Aft2 activity under iron replete conditions [11, 12]. Interestingly, the Bol2 homologue in *S. pombe*, Fra2 has been shown not to be involved in the Php4 mediated iron sparing response, it has been implicated in the regulation mediated by the high iron sensing repressor Fep1. *In vivo* results suggest the existence of a heteromeric complex between Grx4, Fra2, and Fep1 independent of iron levels in the cell [6, 10]. The formation of such a complex may depend on the iron sensing and cluster transfer characteristics of Grx4 and Fra2 proteins, similar to their roles in regulating Aft1/2 activity in *S. cerevisiae* [11].

Here we have used biochemical and spectroscopic tools to characterize the interaction between *S. pombe* Fep1, Grx4, and Fra2. UV-visible and CD absorption spectroscopy have been used to confirm the specific iron cofactor present in Fep1. CD monitored titrations between Fep1 and Grx4-Fra2 suggests transfer of a [2Fe-2S] cluster from Fep1 to apo Grx4 or apo Grx4-Fra2. We also demonstrate that cluster trafficking is reversible between Fep1 and Grx4-Fra2 heterodimer. Such a cluster transfer might posit that under low iron conditions, Grx4 homodimer or Grx4-Fra2 heterodimer might
inactivate Fep1 by abstracting the bound cofactor leading to inhibition of repression or activate Fep1 by donating cluster under iron excess. Previously published results indicate that without the bound cofactor, Fep1 loses its transcriptional activity [16], thus abstraction of the bound cluster might act as a “switching off” mechanism for its repressor activity due to a subsequent conformational change.

MATERIALS AND METHODS

Plasmids. E. coli expression plasmids for S. pombe Fep1-FL and truncated Fep1-DBD, Grx4 and Fra2 were generous gifts from Simon Labbé (University of Sherbrooke) (Table 4.1). All plasmid inserts and any mutagenesis were confirmed by Sanger sequencing (GeneWiz).

Protein Expression and Purification. Overexpression of Fep1-FL was performed in the E. coli strain PK11466 (a generous gift from Dr. Patricia Kiley, University of Wisconsin, Madison). The PK11466 strain was transformed with pET-28a (+) Fep1-6xHis (Fep1-FL, or Fep1 full length) was grown in LB media at 37 °C until A600 reached 0.5-0.7, followed by chilling at 4 °C for 15 minutes before induction with 0.4 mM isopropyl-β-D-thiogalactosidase (IPTG), and grown overnight at 30 °C. Expression of the truncated DNA binding domain (DBD) of Fep1 or 6xHis-Fep1-DBD was optimized in E. coli BL21-CodonPlus(DE3)-RIL (Agilent Technologies) transformed with pRSFDuet-1-His6-Fep1(2-241)-MBP-Grx4 and induced with 1 mM IPTG at 30 °C for 5 hours. Post induction, cells containing Fep1-FL or Fep1-DBD were centrifuged and the brown pellets were resuspended in Buffer A (50 mM Tris-MES, pH 7.9, 300 mM NaCl, 20 mM imidazole, 5% glycerol). Prior to lysis, protease inhibitor tablets (Pierce) or 30 µg/mL PMSF (Sigma Aldrich) and 5 mM GSH were added to Buffer A. After sonication and centrifugation to
remove cell debris, the cell-free extract was filtered and loaded onto 20-mL HisPrep column (GE Healthcare) equilibrated with Buffer A. All subsequent purification steps for Fep1-FL were performed in an anaerobic chamber (O$_2$ < 5 ppm) (Coy Laboratory Products) to maintain the stability of the protein-bound iron cofactors, whereas the purification was done aerobically for the truncated Fep1-DBD. Previous studies demonstrate that both Fep1-FL and Fep1-DBD share the same spectroscopic features, thus we performed biochemical Fe-S cluster reconstitution on the aerobically purified apo Fep1-DBD for spectroscopic studies. Both proteins were eluted using a Buffer B (50 mM Tris MES, pH 7.9, 300 mM NaCl 500 mM imidazole 5 % glycerol) using a 20-500 mM imidazole gradient and the reddish-brown fractions containing Fep1-FL or Fep1-DBD as judged by SDS-PAGE and UV-visible spectroscopy were collected and concentrated prior to storage at -80 °C.

Overexpression of 6xHis-Grx4 was performed in *E. coli* BL21(DE3) strain in LB media at 37 °C. Cells were grown until $A_{600}$ reached 0.5-0.7, chilled to 4°C for 15 minutes before overnight induction with 1 mM IPTG at 25 °C. Post induction, cells overexpressing 6xHis-Grx4 were centrifuged and brown cell pellets were resuspended in Buffer A. Prior to lysis, protease inhibitor tablets (Pierce) or 30 µg/mL PMSF (Sigma Aldrich) and 5mM GSH were added to Buffer A. Post sonication and centrifugation to remove cell debris, the cell-free extract was filtered and loaded onto 20-mL His Prep column (GE Healthcare) equilibrated with Buffer A. All subsequent purification steps were performed in an anaerobic chamber (O$_2$ < 5 ppm) (Coy Laboratory Products) to maintain the stability of the protein-bound iron cofactors. Grx4 was eluted with Buffer B using a 20-500 mM imidazole gradient and the reddish-brown fractions containing holo Grx4 as judged by SDS-
Table 4.1. Plasmids used for the study.

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 pET-28a (+) Fep1-6xHIS (1-564) WT</td>
<td>Simon Labbé (This study)</td>
</tr>
<tr>
<td>2 pRSFDuet1-His6x-Fep1(2-241)-MBP-Grx4</td>
<td>Simon Labbé (This study)</td>
</tr>
<tr>
<td>3 pRSFDuet1-His6x-Grx4</td>
<td>Simon Labbé (This study)</td>
</tr>
<tr>
<td>4 pRSFDuet1-His6x-Fra2</td>
<td>Simon Labbé (This study)</td>
</tr>
<tr>
<td>5 pRSFDuet1-His6x-Fra2-MBP-Grx4</td>
<td>Simon Labbé (This study)</td>
</tr>
<tr>
<td>6 pMAL-p2X-Fep1-DBD</td>
<td>Simon Labbé (This study)</td>
</tr>
</tbody>
</table>
PAGE and UV-visible spectroscopy were collected, concentrated and stored at -80 °C.

Overexpression of 6xHis-Fra2 was performed in *E. coli* BL21(DE3) strain in LB media at 37 °C. Cells were grown until A$_{600}$ reached 0.5-0.7, chilled to 4°C for 15 minutes before overnight induction with 1 mM IPTG at 30 °C. Post induction, cells overexpressing 6xHis-Fra2 were centrifuged and the white cell pellets were resuspended in Buffer A. Post sonication and centrifugation to remove cell debris, the cell-free extract was filtered and loaded onto 20-mL His Prep column (GE Healthcare) equilibrated with Buffer A. Purification of Fra2 was performed aerobically since it does not bind an Fe-S cluster upon overexpression. Fra2 was eluted with Buffer B using 20-500 mM imidazole gradient and the fractions containing Fra2 as judged by SDS-PAGE were collected, concentrated and stored at -80 °C.

Co-expression of MBP-Fep1-DBD, 6xHis-Grx4, and 6xHis-Fra2 was performed in *E. coli* BL21-CodonPlus (DE3)-RIL cells (Agilent) which are specialized for efficient expression heterologous proteins in *E. coli*. Due to insufficient co-expression of these three proteins in BL21DE3 strain, BL21-CodonPlus (DE3)-RIL containing extra copies *argU*, *ileY*, and *leuW* rare tRNAs was used. Cells were grown until A$_{600}$ reached 0.5-0.7, chilled to 4 °C for 15 minutes before induction with 1 mM IPTG and grown at 30 °C overnight. Post induction, cells overexpressing the three proteins were centrifuged and brown cell pellets were collected, split and resuspended in Buffer C for Hi Prep Heparin column (50 mM Tris-MES, pH 7.9, 150 mM NaCl, 5% glycerol) and Buffer A for His Prep respectively. Prior to lysis, protease inhibitor tablets (Pierce) or 30 µg/mL PMSF (Sigma Aldrich) and 5mM GSH were added to the respective Buffers C and A. Post sonication and centrifugation to remove cell debris, the cell-free extracts were filtered and loaded on a 20-
mL Hi Prep Heparin column (GE Healthcare) or a 20-mL His Prep column (GE Healthcare) equilibrated with their respective Buffers C and A. The remaining purification steps were performed in a Coy anaerobic chamber to maintain Fe-S cluster stability. The proteins were eluted with a Buffer D (50 mM Tris MES, pH 7.9, 1M NaCl, 5% glycerol) using a 150-1000 mM NaCl gradient or with Buffer B using 20-500 mM imidazole gradient and the reddish-brown fractions containing Fep1, Grx4 and Fra2 as judged by SDS-PAGE and UV-visible spectroscopy were collected, concentrated, and stored at -80 °C. The identity of the bands from each purification were confirmed by mass spectroscopy.

**Biochemical Analyses.** Protein concentrations were determined by Bradford Assay (Bio-Rad) using bovine serum albumin as protein standard. Iron concentrations were determined using the colorimetric ferrozine assay [13].

**Analytical and Spectroscopic Methods.** Analytical gel filtration analyses were performed on a Superdex 200 10/300 GL column (GE Healthcare), equilibrated with N2-sparged Buffer C and calibrated with gel filtration standards (Sigma Aldrich). Mass spectrometry analysis of purified proteins was determined by using electrospray ionization (ESI). UV-visible absorption and CD spectra were recorded under anaerobic conditions using a Shimadzu UV 1800 spectrophotometer and a Jasco J-815 spectropolarimeter (Jasco, Easton, MD), respectively. For measuring EPR spectra, Bruker EMX plus spectrometer (Bruker, Billerica, MA), equipped with an Oxford ESR900 continuous flow cryostat (Oxford Instruments, Oxfordshire, UK), will be used and quantified under non-saturating conditions by double integration against a 1 mM CuEDTA standard.

**Fe-S cluster reconstitutions.** Fep1 and Grx4 apo proteins were prepared by treating purified proteins with EDTA and potassium ferricyanide in a 1:50:20 ratio for 1 hour on
ice, followed by reduction using 5 mM dithiothreitol (DTT). Apo proteins were then incubated with 20-fold excess of ferrous ammonium sulfate and L-cysteine and catalytic amounts of IscS under strict anaerobic conditions. 5mM GSH was included for both Grx4 and Fep1-FL or Fep1-DBD reconstitutions and left for three hours or overnight at 4 °C. Excess reagents were then removed using a 5 mL Hi Trap Q FF column (GE Healthcare) with a linear gradient of 150 mM -1 M NaCl or a 5 mL Hi Trap Heparin column (GE Healthcare) respectively. The colored fractions were pooled and concentrated.

CD-monitored titrations of Fep1, Grx4 and Fra2. All samples were prepared and scanned under strict anaerobic conditions (O$_2$ < 5 ppm) in a 1-cm cuvette with the [2Fe-2S] kept constant at 50 µM for holo proteins. [2Fe-2S] concentration was estimated from the total iron concentration from colorimetric ferrozine assay. Increasing amounts of apo protein/s were added to fixed quantity of holo protein ([2Fe-2S] = 50µM) to record any spectral changes resulting from cluster transfer and/or complex formation.

RESULTS

Fep1 harbors more than one type of Fe-S cofactor. Previous attempts to spectroscopically characterize S. pombe Fep1 have provided contradicting information on the type of cofactor bound by this transcription factor. In one report, Fep1 is proposed to bind iron [6], whereas a separate report detected a mixture of [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters and mononuclear iron [4]. We tried to characterize the bound cofactor on both Fep1-FL and truncated Fep1-DBD by UV-visible absorption and circular dichroism (CD) spectroscopies. Fep1-FL has a tendency to undergo degradation upon purification into lower molecular weight bands, but this degradation was shown to not interfere with its DNA binding or iron cofactor binding ability [4, 14]. But due to this ongoing degradation
phenomena leading to low yields of the intact protein, it was necessary to include the truncated Fep1-DBD in our studies. This truncated form of Fep1(2-241) contains the two zinc finger domains separated by the invariant cysteine residues that ligate the bound iron in the protein. Both anaerobic and aerobic purification of Fep1-FL yielded the degradation products that were identified as Fep1 fragments by LC/MS. For spectroscopic characterization of holo Fep1-FL or Fep1-DBD, the UV-visible absorption spectra of the as-purified (AP) and reconstituted proteins were compared. The spectra of both forms contained peaks at ~ 320 nm (which was negligible for AP Fep1-DBD) and 418 nm, and a shoulder around 500-600 nm which was similar to previously reported spectra for *S. pombe* Fep1 [4] and MBP-fused DNA binding domain of Fep1 from *P. pastoris* (Figure 4.1. top) [14]. A relatively weak CD signal could be seen for anaerobically purified Fep1-FL and Fep1-DBD but was negligible for reconstituted FL-Fep1 and Fep1-DBD (Figure 4.1. bottom). In order to rule out any experimental artefact concerning the FL-Fep1 CD signature, we expressed and anaerobically purified FL-Fep1 from three independent experiments in different growth conditions and compared their CD spectra, protein and iron per protein yield (Table 4.2). No CD spectra have been reported yet for *S. pombe* FL Fep1 or Fep1-DBD, we report here a weak CD signal with positive bands at 350 nm, 400 nm and 520 nm and a negative band at 450 nm (Figure 4.2). This result may indicate the presence of a mixture of [4Fe-4S], [2Fe-2S] and/or other cluster forms as previously reported from EPR analysis [4]. [4Fe-4S] clusters are usually CD silent thus this necessitates confirmation of the exact cofactor bound by Fep1 by other methods. A previous report on *P. pastoris* Fep1 suggested that this Fep1 homologue binds a [2Fe-2S] In our case, EPR and Mössbauer spectroscopy will be used to further confirm the exact
Figure 4.1. Comparison of the UV-visible absorption and CD spectra of Fep1-FL (blue line) and Fep1-DBD (red line). Spectra were recorded under anaerobic conditions in sealed 1 cm cuvettes for Fep1-FL (blue line) or Fep1 DBD (red line), both as purified (broken blue line) and reconstituted (broken red line) in 50 mM Tris-MES buffer 300 mM NaCl 5mM GSH pH 7.9. ε and Δε values are based on total protein concentrations and 50 μM [2Fe-2S] for UV-Visible and CD respectively.
Figure 4.2. Comparison of the CD spectra of Fep1-FL (blue line) and Fep1-DBD (red line) grown under different conditions. Spectra were recorded under anaerobic conditions in sealed 1 cm cuvettes for Fep1-FL grown in BL21DE3 (blue line) strain in LB media, Fep1-FL in PK11466 strain in LB media (blue broken line), Fep1-FL in PK11466 in LB + 50µM Ferrous Ammonium Sulfate + 20 mM Zinc Sulfate media (blue dotted line) or Fep1 DBD in BL21DE3 in LB media (red line), Δε values are based on 50 µM [2Fe-2S] cluster concentration respectively.
Table 4.2. Protein and iron concentrations of Fep1-FL and Fep1-DBD grown under different conditions.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Protein (mM)</th>
<th>Fe (mM)</th>
<th>Fe / dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fep1-FL, BL, LB</td>
<td>0.976</td>
<td>0.1119</td>
<td>0.229</td>
</tr>
<tr>
<td>Fep1-FL, PK, LB</td>
<td>0.644</td>
<td>0.3431</td>
<td>1.065</td>
</tr>
<tr>
<td>Fep1-FL, PK, LB + Fe + Zn</td>
<td>0.715</td>
<td>0.1432</td>
<td>0.400</td>
</tr>
<tr>
<td>Fep1-DBD, BL, LB</td>
<td>5.22</td>
<td>0.1684</td>
<td>0.064</td>
</tr>
</tbody>
</table>
type/s cluster according to analysis of UV-visible absorption and resonance Raman spectra. of iron cofactor in *S. pombe* Fep1 and also acid labile sulfur assay will be performed to confirm the presence of any sulfur in these proteins [15].

**Grx4 can accept a [2Fe-2S] from Fep1.** Both *in vivo* and *in vitro* evidence show both iron dependent and independent interactions between Grx4 and Fep1 [7, 8]. Upon titrating holo Fep1-FL or Fep1-DBD with increasing amounts of apo-Grx4, we were able to show a gradual transition to [2Fe-2S]-Grx4 homodimer spectra, suggesting -Fe-S cluster trafficking between these two proteins (Figure 4.3., top and bottom). To further confirm our observation, we repeated the titration in the absence of GSH, which is a cluster ligand for Grx4. Under these conditions, Fe-S cluster transfer was abolished, as expected if GSH is required to form the [2Fe-2S]-bridged, GSH-ligated Grx4 homodimer (Figure 4.4). In addition, we observed no changes in the [2Fe-2S]-Grx4 signal upon incubating with apo Fep1, suggesting that the [2Fe-2S]-Grx4 homodimer does not participate in reversible Fe-S cluster transfer to Fep1 (Figure 4.5).

**Fep1 and Fra2 do not interact without Grx4.** After establishing that Fep1/Grx4 are involved in Fe-S cluster exchange, we wanted to know if Fep1 and Fra2 also interacted physically and/or participated in Fe-S cluster transfer. Previous *in vivo* studies have shown iron independent interaction between Fep1 and Fra2 by bimolecular fluorescence complementation assay and additionally co-immunoprecipitation studies in WT cells suggested Fep1, Fra2 and Grx4 form a heteromeric complex [10]. Moreover, *fra2Δ* strains show constitutive repression of Fep1 target genes, thus failing to respond to the change from iron excess to iron depleted conditions. The identified Fe-S ligands in *S. cerevisiae* Fra2, namely His103 and Cys66, are conserved in *S. pombe* Fra2 (His66 and
Figure 4.3. Titration studies of [2Fe-2S] Fep1-FL (top) and [2Fe-2S] Fep1-DBD (bottom) with apo-Grx4 monitored by UV-visible CD spectroscopy. Figure legend ratios are Apo Grx4: [2Fe-2S] Fep1/[2Fe-2S] Fep1-DBD. Δε values are based on 50 µM [2Fe-2S] cluster concentration respectively. Inset Titration fit to change in Δε based on the ratio of Apo Grx4 to [2Fe-2S].
Figure 4.4. Titration studies of between [2Fe-2S] Fep1-FL with apo-Grx4 in the presence and absence of GSH. Titration studies of [2Fe-2S] Fep1-FL with apo-Grx4 monitored by UV-visible CD spectroscopy. Figure legend ratios are Apo Grx4:[2Fe-2S] in presence of 2 mM TCEP or 5 mM GSH. $\Delta \varepsilon$ values are based on 50 $\mu$M [2Fe-2S] cluster concentration respectively.
Figure 4.5. Titration studies of between [2Fe-2S] Grx4 with apo-Fep1. Titration studies of [2Fe-2S] Grx4 with apo-Fep1 monitored by UV-visible CD spectroscopy. Figure legend ratios are Apo Fep1: [2Fe-2S] Grx4 Δε values are based on 50 µM [2Fe-2S] cluster concentration.
Cys29). It is thus highly likely that Fra2 might employ the same residues to exert its effect on Fep1 under iron starvation conditions. Furthermore, taking into account the previous in vivo and current in vitro evidence of the three being part of a heterocomplex might also mean that Fra2 exerts its control on Fep1 via Grx4 [6, 10].

In order to determine if there is a direct physical interaction between Fep1 and Fra2, we performed analytical gel filtration column. (Figure 4.6, top and bottom). Also, CD-monitored titrations were performed to confirm if there was a transfer of an active [2Fe-2S] between these proteins and the results indicated no such transfer of cluster from holo Fep1 to apo Fra2 (Figure 4.7, top and bottom). Taken together these results suggest that the interaction between Fep1 and Fra2 is mediated by Grx4 and that Fep1 and Fra2 do not interact on their own. As shown previously, Fra2 probably participates in the de-activation of Fep1 under iron deprivation as a Grx4-Fra2 heterocomplex by abstracting the bound Fe-S cofactor from Fep1.

**Fep1 co-purifies with [2Fe-2S]-Grx4-Fra2.** Grx4 and Fra2 are involved in iron trafficking across species and have key roles in regulation of the low iron sensing transcription factors Aft1 and Aft2 in *S. cerevisiae* [11, 12]. Involvement of these two proteins in an iron sensing pathway in a related yeast thus suggests that Fep1 regulation is mediated by Fe-S cluster binding. Similar to *S. cerevisiae*, CD-monitored titrations between [2Fe-2S]-Grx4 homodimer and apo Fra2 demonstrated formation of a Grx4-Fra2 [2Fe-2S]-bridged heterodimer with similar features to their homologous complex from *S. cerevisiae* (Figure 4.8, bottom). Previous in vivo reports from *S. pombe* suggested that Fep1, Grx4 and Fra2 are part of a heteromeric complex that interacts independent of intracellular iron levels [6, 10]. In order to confirm the formation of such a complex in vitro
Figure 4.6. Gel filtration studies examining the interactions between Fep1-DBD and apo Fra2. Top: Superdex 200 analytical gel filtration chromatograms of Fra2 (green), Fep1-DBD (red). Bottom: SDS-PAGE analysis of the fractions collected. Fra2 does not interact with Fep1-DBD. 50-µL sample was used for analysis and ratios of protein concentration were 2:1 for Fep1-DBD and Fra2.
Figure 4.7. Comparison between CD and UV-visible spectra for titration studies between [2Fe-2S] Fep1-FL with apo-Fra2. Titration studies of [2Fe-2S] Fep1-FL with apo-Fra2 monitored by CD spectroscopy (top) and UV-visible spectroscopy (bottom). Figure legend ratios are Apo Fra2: [2Fe-2S] $\Delta\varepsilon$ and $\varepsilon$ values are based on 50 µM [2Fe-2S] cluster concentration respectively.
we co-expressed these three proteins in *E. coli* and purified them in two steps. CD analysis of this co-purified complex resembled the spectrum of a [2Fe-2S]-Grx4-Fra2 complex and no distinct signature for a hetero complex comprising all three proteins. The CD spectra of copurified Fep1-DBD/Grx4/Fra2 resembles the CD spectra of this [2Fe-2S]-Grx4-Fra2 heterodimer suggesting that Fep1 binds to Grx4-Fra2 but does not directly coordinate the cluster in this heterocomplex (Figure 4.9).

In the co-purified complex between Fep1 and [2Fe-2S]-Grx4-Fra2, Fep1 could be in association with the TRX domain of Grx4 while the GRX domain of Grx4 is involved in a [2Fe-2S]-bridged association with Fra2. Our data suggest that Fep1 is not associated with Fra2 directly, but via Grx4 since co-purification of Fep1-FL and 6xHis-Fra2 on an analytical gel filtration and heparin column (data not shown) did not indicate the presence of a complex.

*Fep1 transfers an Fe-S cluster to apo Grx4 and apo Fra2.* Since co-purified Fep1-DBD, Grx4 and Fra2 yielded a CD spectrum similar to [2Fe-2S]-Grx4-Fra2 heterodimer, we investigated whether Fep1-DBD transfers an Fe-S cluster to these proteins. Consistent degradation of Fep1-FL posed a destabilization effect when the three proteins were mixed together. Upon titration of increasing amounts of apo Grx4-Fra2 with [2Fe-2S] Fep1-DBD, the signal resembled the [2Fe-2S]-Grx4-Fra2 heterodimer complex (Figure 4.10). Due to a consistent precipitation upon mixing these three proteins, the titrations did not yield a gradual increase of the signal, but it clearly resembled a transfer of cluster from [2Fe-2S] Fep1-DBD to apo Grx4-Fra2 (Figure 4.10). This result was consistent with our co-purification experiment where we showed the CD spectra of the complex matched that of the Grx4-Fra2 heterodimer (Figure 4.9). In order to get an of signal of the acceptor
Figure 4.9. Co-purification of Fep1-DBD, Grx4 and Fra2 (top) SDS-PAGE showing the purified proteins (bottom) UV-visible and CD spectra of the co-purified proteins normalized to 100 μM [2Fe-2S] cluster concentration.
Figure 4.10. Titration studies of apo Grx4:Fra2 [2Fe-2S] with Fep1-FL monitored by UV-visible CD spectroscopy. Figure legend ratios are Apo Grx4-Fra2: [2Fe-2S] Fep1-FL. Δε values are based on 50 μM [2Fe-2S] cluster concentration.
protein, the precipitation issue is required to be resolved, since precipitation leads to a change in the ratios of the proteins mixed together.

The mechanism for Grx4-Fra2 regulation of Fep1 activity in *S. pombe* has been proposed in a recent study [4, 6]. Based on observed changes in the UV-visible spectra of purified, recombinant Fep1 mixed with recombinant Grx4-Fra2, these authors suggested that Grx4-Fra2 loses its bridging Fe-S cluster under iron starvation conditions, which initiates iron transfer from Fep1 to Grx4-Fra2. This transfer consequently inactivates the repressor activity of Fep1, leading to de-repression of iron acquisition genes [16]. Our spectroscopic data are consistent with this model, suggesting Fe-S cluster transfer (and not simply iron transfer) from holo-Fep1 to apo Grx4-Fra2.

We next wanted to find out if this cluster transfer was reversible, which would suggest the existence of a two-way Fe-S cluster trafficking between Fep1 and its interacting partners. In such a scenario, under iron excess, a cluster might be donated to Fep1 by Grx4-Fra2 heterodimer in order to activate its repressor activity. Such a possibility was previously suggested in the report which showed that Fep1 harbored a mixture of [2Fe-2S], [2Fe-3S], and [4Fe-4S] clusters [4]. Since Fep1-DBD harbored weak CD spectrum, we relied on the disappearance of the [2Fe-2S]-Grx4-Fra2 signal with increasing amounts of apo Fep1-DBD added (Figure 4.11). Comparison of the UV-visible absorption spectra of [2Fe-2S]-Grx4-Fra2, holo Fep1-DBD, and 1:1 [2Fe-2S]-Grx4-Fra2: apo Fep1-DBD suggests a transition to the holo Fep1-DBD signature when [2Fe-2S]-Grx4-Fra2 is mixed with excess apo Fep1. These UV-visible absorption data thus confirm that the disappearance of [2Fe-2S]-Grx4-Fra2 is not due to Fe-S cluster degradation but rather due to transfer to apo Fep1-DBD (Figure 4.11., top and bottom).
Figure 4.11. Titration studies of apo Fep1-DBD with [2Fe-2S] Grx4-Fra2. (top) Figure legend ratios are Apo Fep1-DBD: [2Fe-2S] Grx4-Fra2. Δε values are based on 50 μM [2Fe-2S] cluster concentration. (bottom) Comparison of UV-visible spectra between [2Fe-2S] Grx4-Fra2, [2Fe-2S] Fep1-DBD, apo Fep1: [2Fe-2S] Grx4-Fra2 1:1, 2:1 respectively. Δε values are based on 50 μM [2Fe-2S] cluster concentration.
Taken together, we are able to demonstrate two-way trafficking of Fe-S clusters between Fep1 and Grx4-Fra2, thus confirming the *in vivo* evidence for iron-dependent regulation of Fep1’s repressor activity [3, 16]. Under iron starvation conditions, Fep1 repressor activity could be inhibited by transfer of the Fep1-bound Fe-S cluster to Grx4-Fra2, possibly leading to conformational changes upon losing the bound cofactor. Acceptance of the cofactor under iron excess could again re-activate the repressor activity. Whether Fep1 receives its Fe-S cofactor only from [2Fe-2S]-Grx4-Fra2 or from other donor proteins in the cell still needs to be elucidated.

**DISCUSSION**

*S. pombe* Fep1 is a GATA type transcription factor that represses expression of iron uptake genes under conditions of iron sufficiency. Upon iron depletion, Fep1 dissociates from its target promoter thereby de-repressing the expression of its target genes [3, 16]. Previous biochemical characterization of Fep1 suggests that it is a dimeric protein with a cysteine rich domain in each monomer that serves as the iron binding site, although the specific iron cofactor bound to Fep1 has been a matter of debate [4, 6]. We have successfully expressed and purified both Fep1-FL and Fep1-DBD and performed UV-visible and CD spectroscopy to evaluate the type of cofactor bound by Fep1. Both as-purified and reconstituted Fep1-FL or Fep1 DBD had very weak or no CD signature, respectively, thus suggesting the existence of a CD-silent [4Fe-4S] type cluster. Interestingly, Fep1 can convert apo Grx4 or apo Grx4-Fra2 into [2Fe-2S]-bridged homodimers or heterodimers, respectively, via Fe-S cluster transfer, thus hinting at the presence of a [2Fe-2S] type cluster, or conversion of [4Fe-4S] to a [2Fe-2S] cluster following transfer. Information from more specialized spectroscopic techniques like
Mössbauer and resonance Raman spectroscopy could add more details to further confirm the exact type or types of cofactors bound by Fep1.

Inactivation of Fep1 is an important step in the regulation of iron homeostasis in *S. pombe*, and this occurs at multiple levels. Firstly, the transcriptional repressor Php4, which is active under iron deficient conditions, binds the Php2/Php3/Php5 heterotrimer and inhibits transcription of *FEP1* [1]. Secondly, Grx4 interacts with Fep1 constitutively in the nucleus where it forms two types of interactions: 1) a strong iron-independent interaction between its N-terminal TRX domain and the C-terminal activation domain of Fep1, and 2) a weaker iron-dependent interaction involving the Cys172 of the CGFS motif in the GRX domain and the N-terminal DNA binding domain (DBD) of Fep1 [7, 8]. Regulation of Fep1 repressor function under iron deprivation is likely to be modulated by the iron-dependent interaction between the GRX domain of Grx4 and the DBD of Fep1 [7, 8]. Thirdly, Fra2 has also been shown to function as a co-inhibitor of Fep1 under conditions of iron deprivation. In the absence of Grx4 or Fra2 or both (as in *grx4Δ* or *fra2Δ* or *grx4Δfra2Δ* strains), Fep1 fails to respond to changes in iron bioavailability, thus leading to constitutive repression of iron uptake genes [8, 10].

Our goal was thus to elucidate the molecular mechanism by which Fep1 was regulated under conditions of iron starvation. We mainly wanted to establish the specific roles played by Grx4 and Fra2 in this aspect. Both Grx4 and Fra2 has been previously shown by our lab and collaborators to be involved in iron sensing and iron trafficking in *S. cerevisiae*, where they regulate the low iron sensing transcriptional activators Aft1 and Aft2 [11, 12]. Aft1/Aft2 activate the iron regulon under iron starvation conditions resulting in expression of iron acquisition genes. Under iron replete conditions, Fe-S cluster transfer
from Grx3/4-Fra2 to Aft1/Aft2 results in DNA dissociation and cytosolic export of Aft1/2, thereby deactivating expression of iron regulon genes [11, 17]. Thus, Grx4 and Fra2 have established roles in iron sensing and delivery, affecting regulation of iron-responsive transcription factors. Contrary to *S. cerevisiae*, in *S. pombe*, these two proteins seem to be inhibiting Fep1 activity under iron-deficient conditions and not iron-replete conditions. The existence of a heteromeric complex between Grx4 and Fra2 with similar spectroscopic signature as its *S. cerevisiae* counterparts was seen in *S. pombe* [12]. Interestingly, co-purified Fep1-DBD, Grx4 and Fra2 also shared similar CD spectra corroborating previous *in vivo* findings of existence of a heterocomplex [6] which was suggested by two independent studies [6, 10]. With the help of CD monitored titrations we were able to confirm a transfer of [2Fe-2S] from Fep1 to apo Grx4-Fra2. Fep1-DBD has very weak CD, the gradual increase in [2Fe-2S] Grx4-Fra2 signal suggested a transition of apo Grx4-Fra2 to [2Fe-2S]-Grx4-Fra2 heterodimer. A reverse transfer between [2Fe-2S] Grx4-Fra2 and apo Fep1-DBD has also been indicated. Since holo Fep1-DBD has no CD signal, we had to depend upon disappearance of the [2Fe-2S] Grx4-Fra2 spectral signature. If [2Fe-2S]-Grx4-Fra2 was indeed transferring its cluster to apo Fep1-DBD, it would be interesting to determine whether the cluster is converted to a CD-silent [4Fe-4S] form with the help of some electron donor, or if Fep1-DBD binds the Fe-S cluster in a somewhat achiral environment resulting in a weak or no CD spectra. We were also able to show unidirectional transfer of [2Fe-2S] from Fep1 to apo Grx4 if GSH was included in the cluster transfer reaction. Grx4 homodimer was unable to transfer its bound cluster to apo Fep1 thus suggesting directionality in this Fe-S transaction. Also, Fep1 and Fra2 failed to interact with each other, both physically and spectroscopically, suggesting that the
previously reported interaction must have occurred in the presence of Grx4 [10]. Alternatively, Fep1 and Fra2 interact in the presence of Grx4.

Taken together, we are able to report that Fep1 binds an Fe-S cluster, although identifying the exact form will require additional spectroscopic analysis. Cluster transfer reactions suggest that Fep1 can donate and probably accept [2Fe-2S] clusters from Grx4-Fra2 heterodimers which might act as a way of regulating Fep1 repressor activity in response to iron availability in the cell. Fep1 can also transfer a [2Fe-2S] unidirectionally to Grx4. We hypothesize that under iron deficient conditions, Grx4-Fra2 might initiate abstraction of a [2Fe-2S] cluster from Fep1, possibly triggering a conformational change that inhibits its repressor function. Upon iron sufficiency, Fep1 might receive an active Fe-S cofactor from [2Fe-2S]-Grx4-Fra2 or some other donor resulting in its re-activation as a transcriptional repressor (Figure 4.12). Consistent in their roles as iron sensing and trafficking proteins, Grx4-Fra2 is thus able to regulate Fep1’s activity in response to iron availability in the system. In order to further establish the regulatory role of Grx4-Fra2 in this context, DNA binding assays in the presence or absence of Grx4-Fra2 will be compared in future studies to report their effect on the transcriptional regulation by Fep1.
Figure 4.12. Working model for interaction between Fep1, Grx4 and Fra2. a) Under iron deficient conditions (left), Grx4-Fra2 loses their bridging cluster thus abstracts the cluster away from Fep1 leading to its de-activation as a repressor. b) Under iron sufficient conditions, Fep1 gains back its cluster from either Grx4-Fra2 or some other un-identified donor leading to its activation as a repressor.
REFERENCES


