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Iron homeostasis in the fission yeast *Schizosaccharomyces pombe*

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Abstract *Schizosaccharomyces pombe* has acquisition processes for iron, an essential nutrient. One pathway consists to produce, excrete, and capture siderophore–iron complexes. A second pathway requires enzymatic reduction of ferric iron at the cell surface prior to uptake by a permease–oxidase complex. Genes encoding proteins involved in iron assimilation are transcriptionally regulated as a function of iron availability. Under high iron conditions, the GATA-type regulator Fep1 represses the expression of iron uptake genes. The repressor function of Fep1 requires the presence of the Tup11 or Tup12 transcriptional co-repressor. Under low iron conditions, two regulatory mechanisms occur. First, the iron transport genes are highly induced. Second, there is a transcription factor cascade implicating the heteromeric CCAAT-binding complex that turns off a set of genes encoding iron-utilizing proteins, presumably to avoid a futile expenditure of energy in producing iron-using proteins that lack the necessary cofactor to function. Thus, collectively, these regulatory responses to variations in iron concentrations ensure that iron is present within cells for essential biochemical reactions, yet prevent the accumulation of iron or iron-using proteins to deleterious levels.

Keywords Iron · Siderophore · Reductive iron transport · Fep1 · Tup11/12 · CCAAT-binding factor · Fission yeast

Abbreviations

AD	Activation domain
$K_{d,app}$	Apparent dissociation constant
bp	Base pair(s)
BPS	Bathophenanthrolinedisulfonic acid
DBD	DNA-binding domain
Dip	2,2′ Dipyridyl
Fep1	Fe protein 1
ORF	Open reading frame
ZF1	N-terminal zinc finger
ZF2	C-terminal zinc finger

Introduction

The trace metal iron is an essential co-factor for a wide variety of cellular enzymes (Hentze et al. 2004; Kaplan et al. 2006). Iron exists in two

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oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}), which can donate and accept electrons, respectively. Paradoxically, the redox active nature of iron may become detrimental for the cell. Via the Fenton reaction, excess ferrous iron (Fe^{2+}) can convert hydrogen peroxide to hydroxyl radical, which is reactive and damaging (Halliwell and Gutteridge 1992). Biologic targets of this oxidant are membrane lipids, proteins, and nucleic acids. Given the potential for iron toxicity, different mechanisms and molecules for the transport and storage of iron have evolved to minimize opportunities for the generation of free iron. Another problem that organisms in aerobic zones encounter with the biological chemistry of iron is its low bio-availability. Although iron is abundant in the environment, this one is usually present as insoluble ferric hydroxides (Philpott 2006). In order to warrant a supply of iron, organisms have developed different strategies for iron acquisition including secretion and utilization of siderophores, ferrireductase activity at the cell surface, and heme-iron assimilation inside the cell (Dancis et al. 1990, 1992; Georgatsou and Alexandraki 1994; Haas 2003; Kosman 2003; Lesuisse and Labbé 1989; Mazmanian et al. 2003; Winkelmann 2002). Although an organism may have more than one way to acquire iron, its transport within cells must be under tight regulatory control. This review focuses on the mechanism and regulation of iron assimilation by fission yeast cells and the recent discoveries that have identified the molecules involved.

Iron acquisition

Nonreductive iron transport

To acquire iron, the fission yeast *Schizosaccharomyces pombe* has two separate iron uptake systems. The first one relies on the hydroxamate-type siderophore ferrichrome (Schrettl et al. 2004). Ferrichrome is a low-molecular-mass organic compound that specifically associates ferric iron with exceptionally high affinity (Haas 2003; Neilands 1995; Winkelmann 2002). Data base analysis revealed that two siderophore biosynthetic genes in *S. pombe* could be involved in ferrichrome biosynthesis (Schrettl et al. 2004). The two genes *SPAC23G3.02c* and *SPAC23G3.03* encoding Sib1 (also denoted CAB72227) and Sib2 (also denoted CAB72228), respectively, are located on chromosome I of fission yeast. Sib1 exhibits 16% amino acid sequence identity with the ferrichrome peptide synthetase Sid2 from *Ustilago maydis* (Yuan et al. 2001). Although this percentage of identity is low, like Sid2, Sib1 is predicted to possess several copies of a repeated unit containing an adenylation domain, a condensation domain, and a peptidyl carrier domain that are typically found in peptide synthetases. Furthermore, as observed for Sid2 and the peptide synthetase SidC of *Aspergillus nidulans* (Eisendle et al. 2003), Sib1 is an exceptionally large protein with a modular organization (Schrettl et al. 2004).

The putative ornithine N^5-monooxygenase Sib2 protein is 34% identical to *A. nidulans* SidA on the amino acid level (Eisendle et al. 2003). Based upon this sequence homology to SidA, Sib2 is predicted to catalyze the first enzymatic step in siderophore biosynthesis. As in SidA, Sib2 contains canonical peptide signatures. First, Sib2 has a putative flavine adenine dinucleotide (FAD)-binding domain. Second, Sib2 contains a putative nicotinamide adenine dinucleotide phosphate (NADP)-binding domain. Third, Sib2, like other monooxygenases involved in siderophore biosynthesis, has a domain predicted to be involved in substrate binding (Schrettl et al. 2004). Interestingly, *SPAC23G3.02c* and *SPAC23G3.03* genes are adjacent on chromosome I. Furthermore, computer algorithm analysis predicts that they are divergently transcribed from a common promoter. The fact that their expression may be controlled by a common upstream region argues in favour of an involvement of the gene products in a common pathway.

As expected, biochemical and physiological experiments indicated that *S. pombe* produces ferrichrome (Schrettl et al. 2004). In the presence of exogenous iron, the cellular ferrichrome pool was found in the ferri-form, while under conditions of iron deficiency, the desferri-form was primarily detected (Schrettl et al. 2004). Surprisingly, the amount of extracellular

ferrichrome excreted did not change significantly between iron-replete and low iron conditions. Furthermore, even during iron deficiency only small amounts of desferrichrome was found extracellularly, with the majority of siderophores detected within cells, suggesting that *S. pombe* uses ferrichrome as iron storage molecule, as well as iron carrier system to extract the metal from the environment. The ability of fission yeast cells to produce and excrete ferrichrome is consistent with the existence of *S. pombe* Str1, which confers ferrichrome uptake when ectopically expressed in a *Saccharomyces cerevisiae fet3*Δ *arn1-4*Δ mutant strain (Pelletier et al. 2003). The *str1*$^{+}$ open reading frame encodes a protein of 612 amino acids harbouring 14 potential membrane-spanning regions (Pelletier et al. 2003). The sequence and predicted topology of Str1 exhibit significant similarities to four previously identified siderophore transporters: the Arn1 (31%), Arn2/Taf1 (30%), Arn3/Sit1 (29%), and Arn4/Enb1 (29%) proteins from *S. cerevisiae* (Heymann et al. 1999, 2000a, b; Lesuisse et al. 1998; Yun et al. 2000a, b). The sequence homology with the *S. cerevisiae* proteins is noteworthy, especially within the putative transmembrane domains of Str1. Two other proteins found in the *S. pombe* proteome exhibit sequence similarity to Str1. These proteins were denoted Str2 and Str3 (Pelletier et al. 2003). The amino acid identity between Str1 and the putative siderophore transporters Str2 and Str3 is 29% and 25%, respectively. A hallmark of Str1, Str2, and Str3 is that all exhibit very similar hydropathy profiles. Complementation experiments in *S. cerevisiae fet3*Δ *arn1-4*Δ mutant cells suggested that *str2*$^{+}$ is involved in ferrioxamine B-iron uptake, and to a lesser extent in ferrichrome-iron uptake (Pelletier et al. 2003). However, a *fet3*Δ *arn1-4*Δ strain harbouring the *str3*$^{+}$ allele fails to grow when ferrichrome- or ferrioxamine B-iron was the iron source (Pelletier et al. 2003). Although Str3 may participate in the mobilization of iron bound to siderophores, its substrate specificity has not been determined. Based on these observations, it appears that *S. pombe* has three siderophore transporters, which are different in terms of substrate preference.

Reductive iron transport

In *S. pombe*, a second pathway that necessitates a reductive transport mechanism is used to solubilize ferric iron. A fission yeast mutant deficient in ferric reductase activity was isolated (*frp1-314*) (Roman et al. 1993), and the wild-type allele of the gene affected in this mutant was subsequently cloned (*frp1*$^{+}$) (Roman et al. 1993). In addition to being deficient in ferric reductase activity, a *frp1*Δ mutant fails to acquire iron when delivered in the ferric form (Roman et al. 1993). Furthermore, the mutant strain showed impaired growth in an iron-deficient medium (Roman et al. 1993). Complementation of the mutation by the single open reading frame *frp1*$^{+}$ corrected the defects in reductase activity, ferric iron uptake, and cell growth (Roman et al. 1993). The Frp1 protein shares 27% amino acid identity with the Fre1 protein from *S. cerevisiae* (Dancis et al. 1992; Roman et al. 1993). Moreover, Frp1 bears sequence identity (20%) with the gp91^{phox} subunit of cytochrome b_{558}, a human phagocyte oxidoreductase (Finegold et al. 1996; Roman et al. 1993). Kyte-Doolittle hydropathy profiles of Frp1, Fre1, and gp91^{phox} reveal multiple hydrophobic regions that are consistent with transmembrane domains. Greatest homology is seen in the C-terminal parts of the three sequences, where there are several clusters of amino acids common to all three proteins. For gp91^{phox}, three of these motifs have been assigned functions: one in flavin adenine dinucleotide (FAD) binding and the other two in nicotinamide adenine dinucleotide phosphate (NADPH) binding (Segal et al. 1992). Consistently, when a partially purified yeast fraction of plasma membrane ferric reductase was prepared, it has been shown that the preparation required FAD and NADPH for activity (Lesuisse et al. 1990), therefore supporting the relationship between yeast reductase and gp91^{phox} proteins. The homology of the yeast reductase proteins with the gp91^{phox} subunit, which is part of a complex responsible for transporting electrons, is particularly significant, because, in the yeast system, electrons must be transferred from a cytoplasmic donor to extracellular ferric iron. Furthermore, the similarities between gp91^{phox} and the yeast

reductase components support the model that the Frp1 and Fre1 proteins are structural components of their respective reductase complexes with a role in electron transfer at the cell surface.

According to the currently accepted concept of iron uptake, after reduction, ferrous iron is oxidized to ferric iron by the plasma membrane multicopper oxidase Fio1 (Askwith and Kaplan 1997). The ferric iron produced is then taken up by a second component, Fip1, which is the permease that transports iron across the plasma membrane (Askwith and Kaplan 1997). Sequence comparisons of the *S. pombe* Fio1 and Fip1 to the *S. cerevisiae* Fet3 and Ftr1, respectively, support the conclusion that these proteins are highly similar. Moreover, it has been shown that simultaneous expression of both *S. pombe fio1*$^+$ and *fip1*$^+$ genes in an *S. cerevisiae fet3*Δ disruption strain is sufficient to reconstitute a functional iron transport system (Askwith and Kaplan 1997). Based on properties of Fet3 (Askwith et al. 1994; De Silva et al. 1995; Hassett et al. 1998; Solomon et al. 1996), it is predicted that the requirement for Fio1 is dictated by its ability to catalyze four single-electron oxidations of substrate (ferrous iron ions), an action that is followed by a four-electron reduction of O_2 to produce two molecules of H_2O. To do so, such activity requires four copper atoms that are associated by a group of highly conserved amino acid ligands, which are found in Fio1 as well as in Fet3 (Askwith and Kaplan 1997; Blackburn et al. 2000; Taylor et al. 2005). Within its N terminus, Fio1 has a putative signal peptide sequence, which may direct the protein to the plasma membrane. Moreover, like Fet3, Fio1 is predicted to possess one transmembrane domain within its C-terminal region. The latter observation that Fio1 contains only a single transmembrane domain suggested that this protein may be only one subunit of a heteromeric protein complex. The second subunit of the iron uptake system was first found in *S. cerevisiae* with the discovery of *FTR1* (Stearman et al. 1996). In a wild-type strain, the Ftr1 protein appeared on the cell surface, but in a *fet3*Δ deletion mutant, Ftr1 was present in an internal compartment, most likely the endoplasmic reticulum (Stearman et al. 1996). Similarly, it was found that in the absence of Ftr1, the *FET3* gene product was retained within the secretory pathway (Stearman et al. 1996). The necessity of co-expression of both the Fet3 and the Ftr1 protein for localization to the plasma membrane suggested that assembly of a Fet3/Ftr1 complex is required for either protein to proceed through the secretory pathway to the plasma membrane where it becomes competent for iron transport. The iron permease Fip1, like Ftr1, has six (possibly seven) transmembrane domains (Askwith and Kaplan 1997). As in Ftr1, Fip1 harbours two REXLE motifs that have been demonstrated (for Ftr1) to be involved in iron uptake by site-directed mutagenesis (Askwith and Kaplan 1997; Severance et al. 2004; Stearman et al. 1996). A third potential iron-binding motif, D(A/M)XE, has also been proposed for iron mobilization (Severance et al. 2004). Although genes similar to *FET3* and *FTR1* were identified in other eukaryotic organisms, functional complementation of a defective high-affinity iron transport system in *S. cerevisiae* cells lacking either Fet3 or Ftr1 is only partial with Fet3 or Ftr1 from *Candida albicans* and ineffective with the *S. pombe* orthologues (Askwith and Kaplan 1997; Ramanan and Wang 2000). As a matter of fact, expression of *S. pombe fio1*$^+$ alone in an *S. cerevisiae fet3*Δ deletion strain failed to mediate iron transport (Askwith and Kaplan 1997). This result may indicate that the *S. pombe* multicopper oxidase, while functionally homologous to the *S. cerevisiae* multicopper oxidase, does not have sufficient similarity to associate with the *S. cerevisiae* permease. As mentioned above, only expression of both *fio1*$^+$ and *fip1*$^+$ in an *S. cerevisiae fet3*Δ mutant strain reconstitutes iron transport (Askwith and Kaplan 1997). Thus, it appears that despite a general conservation of a high-affinity iron uptake oxidase–permease complex in eukaryotes, more information must be obtained to elucidate the precise mechanisms whereby the iron transport machinery must be properly assembled before reaching the cell surface.

A critical issue for cells is the necessity to control iron concentrations in order to be able to rapidly respond to changes in extracellular iron levels. Interestingly, exposure of *S. pombe* to high iron results in a dramatic decrease in the rate of high-affinity iron uptake (Askwith and Kaplan

1997). Based on this observation, it was proposed that the rapidity of the change in transport rate in fission yeast may be explained by the existence of a post-translational mechanism that might lead to the degradation of the Fio1–Fip1 transport system (Askwith and Kaplan 1997). Unfortunately, such process by which Fio1 and Fip1 may be regulated remains unexplored. Interestingly, however, in *S. cerevisiae*, the Fet3–Ftr1-based iron transport system was found to be regulated post-translationally (Felice et al. 2005). In iron-limited cells, both Fet3 and Ftr1 were stable at the cell surface. Exposure to high levels of extracellular iron triggered a loss of Fet3–Ftr1 uptake activity and proteins. This inactivation occurs through iron-induced internalization of the Fet3–Ftr1 complex and its subsequent degradation in the vacuole (Felice et al. 2005).

A second oxidase–permease complex is present in *S. cerevisiae*. It is constituted of the Fet5 and Fth1 proteins (Spizzo et al. 1997; Urbanowski and Piper 1999). Although Fet5 is closely related to Fet3, it localizes on the membrane of vacuoles (Urbanowski and Piper 1999). Likewise, Fth1, a paralog of Ftr1 (Stearman et al. 1996), is visualized surrounding the vacuole (Urbanowski and Piper 1999). Furthermore, it has been shown that Fet5 associates with Fth1, and deletion of the *FET5* gene results in the accumulation of Fth1 in the endoplasmic reticulum (Urbanowski and Piper 1999). A proposed model for the Fet5–Fth1 transport system is that the vacuole can act as a store for iron and that the Fet5–Fth1 complex serves as a means to mobilize stored iron under low iron conditions to replenish the cytosol (Urbanowski and Piper 1999). Curiously, *S. pombe* does not have Fet5 and Fth1 homologues[1]. Perhaps, when grown under low iron conditions, *S. pombe* may mobilize stored iron from the vacuole by using an alternative mechanism. Therefore, *S. pombe* mobilization of intracellular iron stores may be carried on by proteins that are not present in *S. cerevisiae*.

[1] B. Pelletier, A. Mercier, and S. Labbé, unpublished data.

Regulation of iron transport gene expression

Cis-acting elements in transcriptional control

A feature of iron acquisition systems in *S. pombe* is their ability to respond to variations in iron requirements. When iron is scarce, the capacity to absorb iron is increased to meet metabolic needs, and when iron is plentiful, the uptake systems are repressed to prevent the metal accumulating to toxic levels. A hallmark of the genes encoding components of the iron uptake systems including *frp1*$^+$, *fio1*$^+$, *fip1*$^+$, *str1*$^+$, *str2*$^+$, and *str3*$^+$ is the fact that they are inversely regulated by iron at the transcriptional level (Fig. 1). The transcription of these genes is induced under conditions of iron deprivation, whereas their inactivation occurs under iron-replete conditions (Askwith and Kaplan 1997; Labbé et al. 1999; Pelletier et al. 2002, 2003; Roman et al. 1993). The ability of *frp1*$^+$, *fio1*$^+$, *fip1*$^+$, *str1*$^+$, *str2*$^+$, and *str3*$^+$ genes to be repressed by iron is controlled in *cis* by a short DNA sequence that is present in nonidentical copies in the 5′ flanking region of these genes (Pelletier et al. 2002; Pelletier et al. 2003). Site-directed mutagenesis and deletion of the *fio1*$^+$–*fip1*$^+$ intergenic promoter region as well as the 5′ untranslated region of *str1*$^+$ demonstrated that the conserved core sequence, 5′-(A/T)GATAA-3′, is crucial for repression by iron (Pelletier et al. 2002, 2003). At least the presence of one GATA-type regulatory sequence is required for iron-dependent repression of *fio1*$^+$ and *str1*$^+$ mRNA levels (Pelletier et al. 2002, 2003). When two copies of GATA elements are present, iron-mediated repression of target gene expression is more pronounced (Pelletier et al. 2002). We also observed that when two copies of the 5′-(A/T)GATAA-3′ site are present in the promoter regions of target genes, these elements can be arranged either in inverted or tandem orientation.[1] Furthermore, when the target DNA contained two adjacent GATA elements, it has been shown that they have different transcriptional efficiencies (Pelletier et al. 2002). Moreover, we observed that when the GATA element contains the following nucleotides, 5′-ATC(A/T)GATA(A/T)-3′, the magnitude of the iron-regulatory response is the strongest.[1]

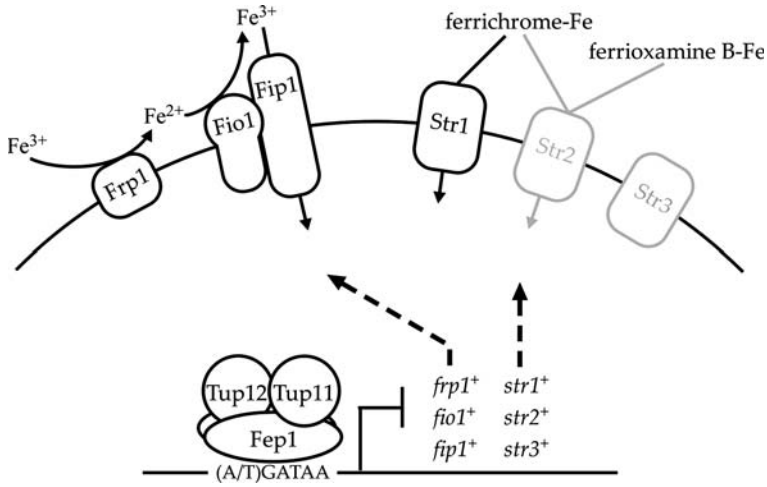

Fig. 1 Iron transport systems of *S. pombe*. The components of the reductive iron acquisition system are Frp1, a plasma membrane Fe^{3+} reductase, Fio1, a cell surface multicopper oxidase, and Fip1, an iron permease. Prior to uptake, Fe^{2+} is generated by the activity of the Fe^{3+} reductase Frp1. This Fe^{2+} is oxidized to Fe^{3+} by the Fio1 ferroxidase subunit of the high-affinity transporter complex and then passed across the plasma membrane via the Fip1 permease subunit. The nonreductive system of iron acquisition includes siderophore–iron transporters Str1, Str2, and Str3. Of the three siderophore transporter proteins identified, Str1 mobilizes iron bound to ferrichrome. Str2 is able to take up both ferrichrome- and ferrioxamine B-iron, whereas substrate specificity has not been ascertained for Str3. Regulation of these genes encoding components of both systems is under the control of Fep1. During high iron conditions, Fep1 binds to DNA sequences containing 5′-(A/T)GATAA-3′ and forms a complex with Tup11 and possibly Tup12, which act as co-repressors to inhibit gene expression

Consistent with the base pair composition of the regulatory sequence 5′-(A/T)GATAA-3′, the fission yeast transcription factor for regulating the expression of genes encoding components involved in iron uptake has been determined to be a member of the GATA factor protein family, designated Fep1 (for iron protein 1) (Pelletier et al. 2002).

The iron sensor Fep1

fep1$^+$ encodes an iron-responsive transcriptional repressor with two Cys_2/Cys_2-type zinc fingers. We referred to these motifs as the N-terminal zinc finger (ZF1), and the C-terminal zinc finger (ZF2), according to their relative positions in the GATA-type protein (Pelletier et al. 2002, 2005). Furthermore, within its N terminus, Fep1 harbours a set of four cysteines in the region comprised between the two zinc fingers. The production of the N-terminal 241 amino acids region of Fep1 in *E. coli* demonstrated that this polypeptidic segment encompasses the DNA-binding domain (Pelletier et al. 2002). Furthermore, in vitro DNA-binding activity of recombinant Fep1 1-241 is dependent on the protein being expressed in cells that are grown in the presence of iron before extract preparation and purification (Pelletier et al. 2002). Importantly, this region of Fep1 (amino acids 1-241), which contains ZF1, the four cysteine residues, and ZF2, is highly similar to the N-terminal regions of the Urbs1, SRE, SREA, and Sfu1 proteins that have been shown to play a central role in the regulation of iron-responsive genes of *Ustilago maydis*, *Neurospora crassa*, *Aspergillus nidulans*, and *Candida albicans*, respectively (An et al. 1997a, 1997b; Haas et al. 1999; Harrison and Marzluf 2002; Lan et al. 2004; Oberegger et al. 2001, 2002; Zhou et al. 1998). In vitro binding experiments revealed that the *S. pombe* Fep1 protein can bind 5′-(A/T)GATAA-3′ motifs using only ZF2 and not ZF1 (Pelletier et al. 2005). The requirement of only the ZF2 for DNA binding is reminiscent to the situation in *U. maydis* where only the second finger is sufficient for specific Urbs1 DNA binding (An et al. 1997b). However, as opposed to Urbs1, Fep1 requires both ZF1 and

ZF2 for fostering iron-mediated repression of *fio1*$^+$ in vivo (Pelletier et al. 2005). Given the fact that the second zinc finger is necessary for the recognition of 5′-(A/T)GATAA-3′ motifs yet insufficient for iron repression of target gene expression, two potential roles could be envisioned for ZF1. First, ZF1 may form an essential part of a protein–protein interaction domain to facilitate intermolecular repression. Second, whereas the ZF1 of Fep1 cannot bind the 5′-(A/T)GATAA-3′ sequence in isolation, it may play a critical role in increasing the affinity of the Fep1–DNA complexes. Consistent with this latter possibility, we determined that ZF1 plays an important role in increasing the affinity of the Fep1–DNA complex (Pelletier et al. 2005). The fact that mutant versions of Fep1 (lacking ZF1) have much lower affinity (~5–6-fold less) for 5′-(A/T)GATAA-3′ motifs may explain why without ZF1, these derivatives of Fep1 were unable to repress *fio1*$^+$ in vivo in response to excess iron (Pelletier et al. 2005). Similarly, in *N. crassa*, although ZF2 plays a more important role in SRE DNA binding in vitro, results revealed that the presence of both the N- and C-terminal zinc fingers of SRE are required (Zhou et al. 1998). Indeed, mutations affecting either or both SRE zinc fingers blocked the ability of the transcription factor to repress siderophore gene expression in response to high iron concentrations (Zhou et al. 1998). In comparison, the DNA binding domain of *A. nidulans* SREA expressed as a fusion protein in *E. coli* requires the presence of both zinc finger motifs for specific DNA binding (Haas et al. 1999).

Between the two zinc fingers of Fep1, Urbs1, SRE, SREP, SREA, and Sfu1, a conserved N-terminal 27-residue segment with four highly conserved Cys residues is present (Haas et al. 1999; Haas 2003; Pelletier et al. 2002). Fine mapping analysis of the Fep1 conserved Cys residues was carried out by introducing mutations in either the first two, the last two, or all four, and assaying for the ability of mutant proteins to bind DNA and to turn off *fio1*$^+$ transcription (Pelletier et al. 2005). Mutations introduced at the Cys residues, Fep1 C70A/C76A, Fep1 C85A/C88A, or Fep1 C70A/C76A/C85A/C88A revealed a much lower affinity to DNA, which represents ~1 order of magnitude reduction in binding affinity compared with the $K_{d\ (app)}$ observed for the wild-type protein (Pelletier et al. 2005). These experiments suggested that the $K_{d\ (app)}$ of the Cys mutant proteins reflects a much lower affinity for DNA that correlates with failure to repress *fio1*$^+$ transcription (Pelletier et al. 2005). In *N. crassa*, the first two Cys residues located between the finger motifs of SRE have been mutated, resulting in a lower DNA binding affinity for the mutant than the wild-type protein (Harrison and Marzluf 2002). Surprisingly, however, cells expressing the mutant allele exhibited down-regulation of siderophore biosynthesis and ornithine oxygenase enzyme activity under both high- and low-iron conditions. Therefore, it appears that the mutant protein is constitutively active regardless of the iron status. This is unexpected considering that the mutant protein exhibits a lower DNA binding affinity than the unadulterated protein. Perhaps, the last two Cys residues of SRE may counterbalance, thereby masking any potential derepression because of the first two mutated cysteines. Nevertheless, although SRE and Fep1 share the presence of common domains, the possibility that there are marked differences with respect to the amino acid residue requirements concerning their respective functions is reasonable.

Evidence to date suggests that iron-regulatory GATA-type transcriptional repressors bind iron. Recombinant SRE is reddish-brown in color and yields a UV-visible spectrum that is distinctive of iron-containing proteins (Harrison and Marzluf 2002). Furthermore, dithionite-mediated reduction or use of a recombinant SRE protein containing serine residues in place of the first two Cys residues in the Cys-rich region resulted in a complete bleaching of the absorbance spectrum, suggesting that these Cys residues may serve to coordinate one or more iron atoms (Harrison and Marzluf 2002). Furthermore, binding studies with the purified Fep1 1-241 indicated that it requires iron to associate with GATA sequences and that the binding can be abolished by the addition of the chelating agent bathophenanthroline disulfonic acid or 2,2′-dipyridyl (Mercier et al. 2006; Pelletier et al. 2002). Binding activity can be restored by the addition of iron to the chelated Fep1 protein (Mercier et al. 2006). In addition to

demonstrating that iron is important for the formation of an active Fep1–DNA binding complex, our group in collaboration with M. Bisaillon's laboratory used endogenous tryptophan fluorescence to measure the association of iron with Fep1. The interaction between iron and Fep1 fostered a decrease in the intensity of the fluorescence typical of iron-free Fep1. This diminution was quantified and clearly suggested that Fep1 interacts directly with iron[2]. Whether one amino acid residue contributes more than another one in the handling of iron must await a comprehensive dissection of the N-terminal region of Fep1. Interestingly, fungal iron-responsive GATA factors harbour within their second zinc finger region an RXXE motif, which lacks only one glutamic acid residue to be identical to REXXE motifs identified in the *S. cerevisiae* Ftr1 and Fth1 proteins and also found and shown to bind iron within mammalian ferritin light chains (Pelletier et al. 2002; Stearman et al. 1996). Interestingly, within the *U. maydis* Urbs1 protein, a single substitution of the first arginine of the RXXE motif rendered the metalloregulatory factor unable to respond to the presence of iron for repressing gene expression (An et al. 1997b). However, a caveat of this observation is that iron binding and DNA binding functions of Urbs1 could depend on same amino acid residues. Nevertheless, one could envision that ZF2 from the Urbs1 GATA-transcription factor may form a peptide-iron complex that may serve as an iron-responsive domain.

Analysis of the amino acid sequence of Fep1 using different computer modeling programs such as COILS (Lupas et al. 1991), GOR4 (Garnier et al. 1996), and MULTICOIL (Wolf et al. 1997) revealed a putative C-terminal amphipathic α-helix that may be involved in protein-protein interactions. The model predicts that two Fep1 molecules may clasp together along a nonpolar face, forming a Fep1–Fep1 intermolecular interaction. Deletion mapping analyses that impaired the ability of Fep1 to self-associate strongly reduced its ability to repress transcription, suggesting that proper assembly of a number of Fep1 molecules at a promoter is required for full repression (Pelletier et al. 2005). The exact biological role of Fep1 self-association is not certain, but there are a number of potential advantages. The most simple effect of self-association is to increase the local concentration of Fep1 and presumably therefore to increase its potency as a transcriptional repressor. It is notable that many iron-responsive promoters contain multiple GATA sites, and it may be that Fep1 self-association is involved in the ordered assembly of higher order complexes at these promoters. Furthermore, Fep1 dimerization may play a specific role in regulating a subset of genes. Another possibility is that Fep1 may be involved in interactions with one or more other proteins that harbour leucine zipper motifs. In *N. crassa* GATA factor SRE, a potential C-terminal coiled-coil motif has also been noticed that may be required for its function (Harrison and Marzluf 2002).

In *S. pombe*, the corepressors Tup11 and Tup12 are important for turning off many genes involved in a wide range of physiological processes (Fagerstrom-Billai and Wright 2005). In *S. cerevisiae*, the protein orthologous to *S. pombe* Tup11 and Tup12, Tup1, forms a protein complex containing four Tup1 subunits that together interact with a single Ssn6 subunit (Smith and Johnson 2000; Varanasi et al. 1996). The Tup1-Ssn6 complex is recruited to the promoters of target genes by interaction with DNA bound transcriptional repressor proteins that recognize specific sequences within target gene promoters (Smith and Johnson 2000). Examples of such repressor proteins include the α2 DNA-binding factor that controls mating-type-specific genes (Komachi and Johnson 1997), the Mig1 repressor that regulates glucose-repressed genes (Treitel and Carlson 1995), and the Rox1 protein, a regulator required for repression of oxygen utilization genes (Balasubramanian et al. 1993). Recently, the general Tup1 corepressor has been shown responsible for transcriptional repression of the high-affinity iron and copper transport systems in response to heme deficiency (Crisp et al. 2006). Down-regulation of *FET3* gene expression requires the recruitment of Tup1 to the Aft1 binding region, suggesting that Aft1

[2] M. Durand, B. Pelletier, M. Bisaillon, and S. Labbé, unpublished data.

could be the Tup1 recruiting transcription factor (Crisp et al. 2006). In *S. pombe*, the *fbp1*$^+$ and *cta3*$^+$ genes encoding fructose 1,6-biphosphatase and a putative P-type ATPase transporter, respectively, have been identified as targets for Tup11–Tup12-mediated repression (Greenall et al. 2002; Janoo et al. 2001). While the Mig1 homolog in *S. pombe*, designated Scr1, has been characterized as a DNA-binding partner of the Tup11–12 corepressor complex, the DNA-interacting protein that recruits the corepressor complex to the *cta3*$^+$ promoter has not been determined (Greenall et al. 2002; Janoo et al. 2001). Genetic analyses with Tup11 and Tup12 have revealed that they both act as negative regulators of *fio1*$^+$ in a redundant manner (Pelletier et al. 2002). Indeed, a mutant strain with deletions in both *tup11*$^+$ and *tup12*$^+$ exhibited a *fio1*$^+$ gene expression that was highly derepressed and unresponsive to down-regulation by iron (Pelletier et al. 2002). Elimination of either Tup11 or Tup12 alone was not sufficient to annihilate the iron-mediated repression of *fio1*$^+$ (Pelletier et al. 2002). Consistently, it has been shown that Tup11 and Fep1 physically interact with each other for transcriptional down-regulation of iron uptake genes (Znaidi et al. 2004). Within its C terminus, Tup11 contains a sevenfold repeated WD40 domain that is predicted to form a seven-bladed β-propeller structure that has an overall donut shape (Znaidi et al. 2004). This latter domain in Tup11 is necessary for its interaction with Fep1 (Znaidi et al. 2004). Furthermore, it has been determined that the Tup11 Tyr^{362} and Leu^{542} residues in the WD40 repeats 2 and 6, respectively, are essential for the association between Tup11 and Fep1 (Znaidi et al. 2004). On the other hand, the presence of a minimal domain encompassing amino acids 405–541 of Fep1 is necessary for physical interaction with Tup11 (Znaidi et al. 2004). Furthermore, when coexpressed in fission yeast, the Tup11 and Fep1 proteins were detected in a heteroprotein complex by coimmunoprecipitation experiments (Znaidi et al. 2004). Although preliminary data have allowed us to detect that Tup12 also interacts with Fep1, the location of the minimal region in Tup12 (or Fep1) that is necessary for Tup12-Fep1 interaction has not been ascertained. Interestingly, iron-mediated regulation of the iron regulon in *C. albicans* requires a corepressor protein orthologous to *S. pombe* Tup11/12 (Knight et al. 2002), supporting the idea that the mechanism involved in down-regulation of iron transport genes is conserved between *C. albicans* and *S. pombe*.

Down-regulation of iron metabolic genes during iron deficiency

The strict requirement for appropriate intracellular iron concentrations suggests that there may be regulatory mechanisms that prevent futile expression of the genes encoding iron-storage and iron-using proteins when iron is limiting. Indeed, in recent years, it has been demonstrated in both prokaryotic and eukaryotic cells that in contrast to genes involved in iron acquisition, iron starvation leads to prevent expression of various genes encoding iron-containing proteins (Kaplan et al. 2006; Massé and Arguin 2005). In *Escherichia coli*, a small noncoding regulatory RNA, designated *ryhB*, is induced under conditions of iron deficiency (Massé and Gottesman 2002). It pairs with its target mRNAs and causes their disappearance by degradation with the aid of RNase E (Massé et al. 2003). RyhB negatively regulates a large percentage of mRNAs encoding iron-using or iron-detoxifying proteins under conditions in which the essential iron co-factor is scarce (Massé et al. 2005). Under iron-replete conditions, the activated Fur repressor binds to the promoter region of *ryhB* and shuts down its transcription (Massé and Gottesman 2002). In these conditions, the *ryhB*-targeted mRNAs are expressed. A similar process has been described for the action of two small RNAs, termed *PrrF1* and *PrrF2*, in the control of iron-regulatable genes in *Pseudomonas aeruginosa* (Wilderman et al. 2004). In *S. cerevisiae*, two transcription factors, Aft1 and Aft2, are iron sensors that activate genes encoding components of iron transport systems in response to iron deprivation (Blaiseau et al. 2001; Rutherford et al. 2001, 2003; Yamaguchi-Iwai et al. 1995, 1996). Cth2, a RNA-binding protein is also synthesized when the availability of iron is limited (Puig et al. 2005). Induction of

Cth2 is mediated by increased transcript levels, and the Aft1 and Aft2 transcription factors play an essential role in this process (Puig et al. 2005). In response to low environmental iron levels, Puig et al. (2005) have shown that Cth2 binds to specific mRNAs and triggers them for degradation. The AU-rich elements (AREs) through which Cth2 exerts its function are found in the 3′ untranslated regions of targeted mRNAs, which encode several iron-containing components including enzymes involved in the tricarboxylic acid (TCA) cycle, Fe–S cluster assembly, and heme biosynthesis (Puig et al. 2005). In contrast, in the presence of iron, *CTH2* expression is severely repressed, allowing reaccumulation of the target mRNAs.

Recently, our group has uncovered a novel homeostatic mechanism whereby the fission yeast *S. pombe* regulates gene expression in response to iron deficiency. In contrast to the bacterial systems using small RNAs or the budding yeast *S. cerevisiae* using mRNA turnover through a RNA-binding protein, the regulation in *S. pombe* functions through a heteromeric DNA-binding complex that acts at the transcriptional level (Mercier et al. 2006). In the presence of the iron chelator Dip, a set of fission yeast genes (*pcl1*$^+$, *sdh4*$^+$, and *isa1*$^+$) were found to be negatively regulated in response to iron deprivation (Mercier et al. 2006). These gene products are predicted to play a role in iron storage (*pcl1*$^+$), the TCA cycle (*sdh4*$^+$), and Fe-S cluster assembly (*isa1*$^+$). Promoter deletion and site-directed mutagenesis showed that the *pcl1*$^+$ promoter requires a CCAAT-type regulatory sequence for expression and regulation by iron starvation (Mercier et al. 2006). Consistently, CCAAT boxes were also identified in the *sdh4*$^+$, and *isa1*$^+$ promoters. Several reports reveal that there is evidence for a conserved multimeric CCAAT-binding factor, which recognizes the CCAAT element in a wide variety of organisms (Maity and de Crombrugghe 1998; Mantovani 1998). The first complex identified was the Hap complex of *S. cerevisiae*, which consists of four subunits, Hap2, Hap3, Hap4, and Hap5 (Forsburg and Guarente 1989; McNabb et al. 1995; Olesen and Guarente 1990; Pinkham and Guarente 1985). Hap2, Hap3, and Hap5 are essential for the formation of a DNA binding complex, while Hap4 is not directly involved in DNA binding but carries a functional activation domain (Forsburg and Guarente 1989; Xing et al. 1993). The Hap complex is required for the expression of genes involved in oxidative phosphorylation in response to growth on nonfermentable carbon sources (McNabb and Pinto 2005; Pinkham and Guarente 1985).

Conserved homologues of Hap2, Hap3, and Hap5 have been found in *S. pombe*, and they were designated Php2, Php3, and Php5, respectively (McNabb et al. 1997; Olesen et al. 1991; Xing et al. 1993). Consistently, ectopic expression of *S. pombe php2*$^+$, *php3*$^+$, and *php5*$^+$ functionally complements *S. cerevisiae hap2*Δ, *hap3*Δ, and *hap5*Δ mutants, respectively, for growth on respiratory carbon sources (McNabb et al. 1997; Olesen et al. 1991; Xing et al. 1993). In fission yeast, following glucose depletion, a protein complex comprising the Php2, Php3, and Php5 proteins positively regulates many of the genes involved in oxidative phosphorylation including *cyc1*$^+$, which encodes the cytochrome *c* oxidase protein (McNabb et al. 1997). Until recently, a Hap4 homologue was not reported in fission yeast. However, a recent analysis of genomic DNA sequence from the *S. pombe* Genome Project revealed one locus (*SPBC16E9.01c* or *php4*$^+$) that was predicted to encode a putative Hap4 homologue (Sybirna et al. 2005). *php4*$^+$ encodes a protein of 295 amino acids harbouring a conserved 16-amino acid region, ^{26}RVSKQWVVPPRPKPGR^{41}. The conserved motif, which is also present in Hap4, is known to be required for the association of Hap4 with Hap2/Hap3/Hap5 (McNabb and Pinto 2005). Although Php4 bears this N-terminal 16-amino acid motif found within *S. cerevisiae* Hap4 as well as other Hap4 homologues (Sybirna et al. 2005), the rest of Php4 exhibits a limited overall sequence homology with Hap4 (8.5% identity; 12.8% similarity). Importantly, four notable differences exist between the *S. pombe* Php4 and *S. cerevisiae* Hap4 products. First, under glucose conditions, *php4*$^+$ mRNA is readily detectable in fission yeast. Furthermore, under glucose conditions and in the absence of Fep1, although *php4*$^+$ mRNA was unregulated by cellular iron status, its expression was clearly observed (Mercier et al. 2006). This contrasts with the situation in

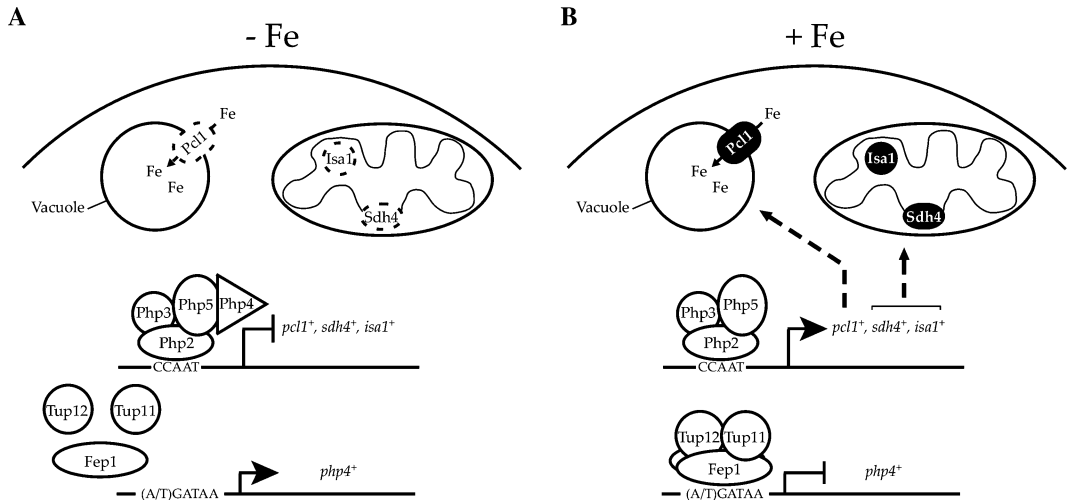

Fig. 2 A proposed transcription model for the regulatory action of *Php4 on pcl1*$^+$*, sdh4*$^+$*, and isa1*$^+$ expression. Under low iron supply conditions, Fep1 cannot bind to DNA and *php4*$^+$ is expressed. Once biosynthesized, Php4 interacts with the Php2/Php3/Php5 heterotrimer to mediate repression of genes that encode iron-storage (Pcl1) and iron-containing (Sdh4 and Isa1) proteins. Conversely, under iron-replete conditions, Fep1 binds DNA and with the aid of Tup11/Tup12 turns off *php4*$^+$ gene expression. This inactivation of *php4*$^+$ enables genes involved in storage and utilization of iron to be expressed via the Php2/Php3/Php5 heterotrimeric CCAAT binding factor

S. cerevisiae where the *HAP4* gene has been shown to be repressed in the presence of glucose and derepressed when cells are grown on nonfermentable carbon sources (Forsburg and Guarente 1989). Second, in *S. pombe*, inactivation of *php4*$^+$ (*php4*Δ) does not affect the ability of cells to grow on nonfermentable carbon sources[3], while *S. cerevisiae hap4*Δ deletion cells are defective in growth on nonfermentable carbon sources (Forsburg and Guarente 1989). Third, in *S. pombe*, we observe that induction of *cyc1*$^+$ expression by a shift to a nonfermentable carbon source is independent of Php4.[3] In *S. cerevisiae* cells containing a *hap4* null allele, genes involved in mitochondrial electron transport such as *CYC1* are not induced when cells are shifted from glucose to a respiratory carbon source (Forsburg and Guarente 1989). Fourth, the *S. pombe* Php4 protein is predicted at neutral pH to have a net charge +9 (pI = 9.9) without any indication of acidic cluster region. As opposed to this situation, the *S. cerevisiae* Hap4 protein is quite hydrophilic with a predicted isoelectric point of 5.2. Furthermore, two regions capable of stimulating transcription have been mapped within Hap4, one between residues 359 and 476, while the other lies between amino acid residues 124–329 (Stebbins and Triezenberg 2004). Taken together, these differences led us to hypothesize that the Php4 protein in *S. pombe* may play a different regulatory function compared to that of the *S. cerevisiae* Hap4 protein.

Recently, we found that in contrast to its activator function in *S. cerevisiae*, the *S. pombe* Php4 protein appeared to function as a negative cofactor that is required for down-regulation of genes encoding iron-using and iron-storage proteins under low iron conditions (Mercier et al. 2006). Consistent with a role for Php4 as a regulatory protein in iron-regulated gene expression, we determined that its expression is under the control of the iron-regulatory transcription factor Fep1 (Mercier et al. 2006). Moreover, as previously determined for the interaction between Fep1 and *fio1*$^+$ promoter GATA sequences (Pelletier et al. 2002), Fep1 interacts with the *php4*$^+$ promoter GATA elements in an iron-mediated manner (Mercier et al. 2006). Based on our results, we proposed a mechanism for regulating genes encoding iron-using proteins as a function of iron availability in fission yeast (Fig. 2). In the absence of iron, an

[3] A. Mercier and S. Labbé, unpublished data.

inactivated Fep1 fails to bind GATA elements in the *php4*$^+$ promoter. Once made, Php4 associates with the Php2/Php3/Php5 heterotrimer already present on the promoters of genes required for iron utilization. As a result, the Php2/Php3/Php5/Php4 complex blocks target gene expression. On the other hand, under conditions of iron excess, Fep1 interacts with GATA elements in the *php4*$^+$ promoter, repressing *php4*$^+$ gene expression. Lack of Php4 allows the CCAAT-binding Php2/Php3/Php5 heterotrimer to activate gene expression. In support to this proposed model, it has been demonstrated that the *C. albicans HAP43* gene (orf19.8298/orf9.681), which encodes a putative transcriptional regulator orthologous to *S. pombe* Php4, is regulated in response to iron status in the same manner as *php4*$^+$ (Lan et al. 2004). Furthermore, it has been shown that the iron-responsive transcriptional control of *HAP43* is mediated by the *C. albicans* Fep1 ortholog, Sfu1 (Lan et al. 2004). Together, these results reveal that the fourth subunit of the heteromeric CCAAT-binding factor from the Archaeascomycete *S. pombe* (and possibly from the filamentous Ascomycete *C. albicans*) plays an important role in coordinating transcriptional down-regulation of genes encoding iron-utilizing proteins under iron-limiting conditions. This suggests a novel function for the heterotetrameric CCAAT-binding factor from *S. pombe* in prioritizing cellular iron use when it is scarce[4].

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[4] In this review, we used the nomenclature for *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* genes and proteins. *S. pombe* wild-type genes are in regular text, italicized, with a superscripted "+" at the end (e.g., *fep1*$^+$). *S. cerevisiae* wild-type genes are capitalized and italicized (e.g., *AFT1*). *S. pombe* and *S. cerevisiae* protein nomenclature is the same and is indicated with a capital letter at the beginning followed by regular letters (e.g., Aft1, Fep1).

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