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Minireview

# A genetic approach to elucidating eukaryotic iron metabolism

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**Abstract** Studies of mutants of the yeast *Saccharomyces cerevisiae* have led to the identification of genes required for high affinity iron uptake. Reduction of iron (III) outside the cell is accomplished by means of reductases encoded by *FRE1* and *FRE2*, homologues of the gp91-*phox* component of the oxygen reductase of human granulocytes. High affinity iron (II) transport from the exterior to the interior of the cell occurs by means of a transport system that has not been molecularly characterized. However, the transport process requires the activity of a copper-containing oxidase encoded by *FET3*. The amino acid sequence of this protein resembles other multi-copper oxidases, including mammalian ceruloplasmin. High affinity copper uptake mediated by the copper transport protein encoded by *CTR1* is required to provide the *FET3* protein with copper, and thus copper uptake is indirectly required for ferrous iron uptake. These genetic elements of yeast and their relationships may be conserved in complex eukaryotic organisms.

*Key words:* Iron; Copper; Transport; Ferric reductase; *Saccharomyces cerevisiae*

## 1. Introduction

The universal requirement that organisms have for iron presents a variety of challenges, many of which are manifestations of the problems produced by oxygen. The insolubity of iron (III) and the potential toxicity of iron (II) in the presence of oxygen demand elaborate biological responses to provide organisms with sufficient iron while preventing against iron toxicity [1]. In this review, we will focus on recent work characterizing specific mechanisms for iron acquisition in the yeast *Saccharomyces cerevisiae*. The oxidation of iron (II) by molecular oxygen results in a decrease in solubility at neutral pH of sixteen orders of magnitude to levels that are likely to be incompatible with the needs of cells [2]. Organisms have resolved this problem by utilizing two distinct strategies (Fig. 1). The first, used by most prokaryotes and some eukaryotes, involves the synthesis and secretion of low molecular weight ferric chelators, or siderophores, which effectively solubilize environmental iron (III) [3]. The ferric-siderophore complexes are specifically transported into the cell. Release of the iron from the siderophore may occur by a variety of mechanisms often involving iron (III) reduction inside the cell [4,5]. Many eukaryotes, including *S. cerevisiae*, do not synthesize siderophores [2] but rather appear to transport ferrous iron from the environment across the plasma membrane to the cell interior. In order to achieve this, an organism must have a mechanism to reduce environmental iron (III) to iron (II). The role of ferric reductases in iron uptake has long been postulated [6] and surface reductases have been assayed in a variety of cells, including *Saccharomyces cerevisiae* [6–10]. The description of an externally directed ferric reductase activity in this organism which could be repressed by excess iron further suggested a connection between this activity and regulated iron assimilation [11]. Demonstration of the link between physiological iron uptake and the measured biochemical activity required both the molecular identification of one or more surface reductases and genetic experiments to establish the importance of such reductases in iron assimilation. *S. cerevisiae* has proved to be a tractable eukaryotic organism amenable to the genetic analysis of biochemical pathways, and recent efforts have reinforced this approach for iron metabolism.

## 2. Identification of a ferric reductase

Using a colorimetric assay capable of detecting surface reductase activity of single colonies of *S. cerevisiae*, a mutant was identified that lacked 90% of such surface ferric reductase activity [12]. The gene responsible for the defect in the mutant, termed *FRE1*, was cloned by complementation and characterized. Deletion of the *FRE1* gene in several strain backgrounds resulted in a decreased level of cell-surface reductase activity, similar to the defect in the original mutant. Importantly, the *FRE1* null strains were also severely defective in the capacity to assimilate radioactive iron presented to the cells as a ferric salt. Furthermore, expression of *FRE1* was found to be transcriptionally controlled by the concentration of iron in the growth medium. Induction by iron deprivation and repression by iron sufficiency was mediated, in large part, through a small region of the 5′ flanking region of the *FRE1* gene [13]. Thus, *FRE1* was required for ferric iron uptake and its expression was regulated in a manner consistent with a homeostatically controlled component of an iron uptake system.

## 3. A family of plasma membrane reductases

Yeast strains with complete deletion of the *FRE1* open reading frame exhibited residual surface ferric reductase activity [13]. This residual activity varied considerably depending on the strain background and growth conditions, but its existence raised the possiblity that there were additional ferric reductase gene(s) in *S. cerevisiae*. That this residual activity, not attributable to *FRE1*, might also be involved in physiologic iron uptake was suggested by the fact that the activity was regulated by iron in a manner similar to the regulation of *FRE1* [13]. Recently, in the course of the sequencing of chromosome XI of *S. cerevisiae*, an open reading frame was identified with low but signif-

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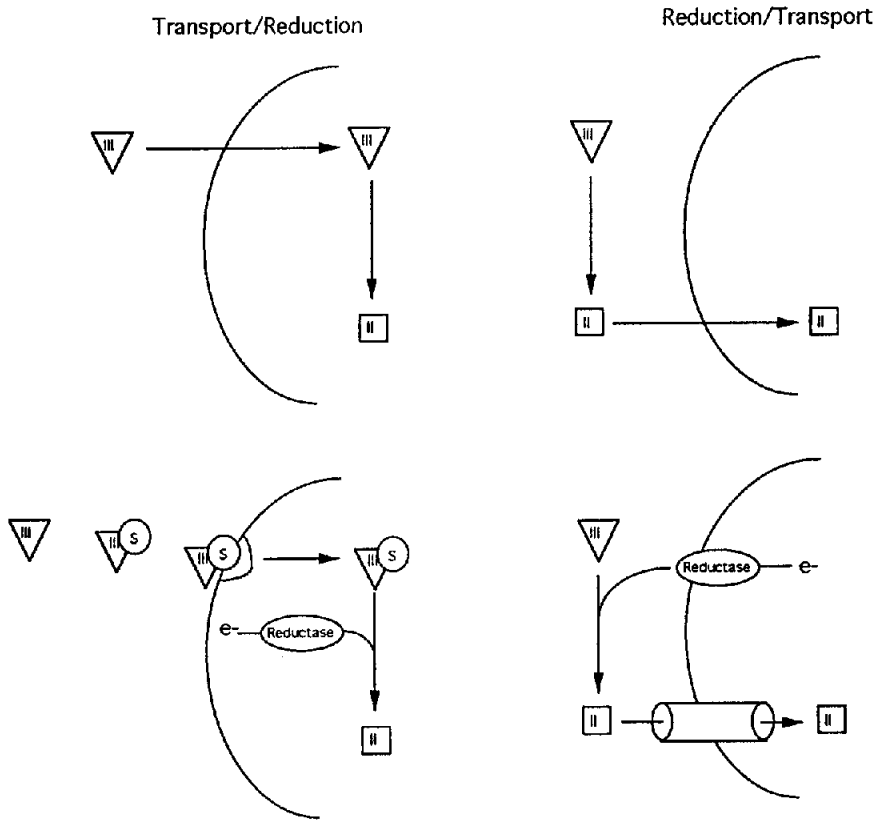


Fig. 1. Transport/reduction. Bacteria and some fungi are capable of transporting ferric iron (triangles with the symbol III) from the enviroment to the cell interior prior to reduction to ferrous iron (squares with the symbol II) which occurs inside the cell. The lower diagram depicts the siderophore (circle with S) binding to iron (III) outside the cell and being transported to the cell interior via the siderophore receptor. A cytosolic reductase (oval labeled reductase) is required to complete the iron delivery, stripping the iron from the siderophore and yielding cytosolic iron (II). Reduction/transport: In the yeast *Saccharomcyces cerevisiae*, iron (III) is reduced outside the cell by a membrane bound externally directed reductase (oval labeled reductase) and transported to the cell interior by means of a molecularly distinct ferrous transporter (cylinder).

icant amino acid homology with *FRE1* [14]. The gene, termed *FRE2*, was found to be regulated by iron in a manner similar to *FRE1*, although there were some differences between *FRE1* and *FRE2* in the kinetics of the regulation and the effects of growth phase. Deletion of *FRE2* and *FRE1* resulted in a mutant strain with essentially undetectable iron regulated surface reductase activity. Furthermore, the double deletion strain, while viable, displayed a severely impaired ability to grow in low-iron media conditions [15]. The fission yeast *Schizosaccharomyces pombe* is distantly related to *Saccharomyces cerevisiae* and yet appears to assimilate iron through a mechanism analogous to *S. cerevisiae*. In *S. pombe*, a homologue of *FRE1*, termed *frp1*$^+$, was identified by following an approach similar to the approach used in *S. cerevisiae*. The colony assay was used to identify a reductase-negative mutant, and the mutant was complemented with a genomic library. The gene *frp1*$^+$ was found to be required for ferric iron assimilation and was shown to be regulated by iron, like *FRE1* and *FRE2* [16].

Examination of the amino acid sequences of Fre1p and and Fre2p revealed a marked similarity of their hydropathy plots [15]. Each has an N-terminal leader sequence and multiple predicted membrane spanning domains. As originally described for Fre1p and Frp1p [16], Fre2p demonstrates amino acid sequence similarity of a low but significant level to the gp91-*phox* subunit of the respiratory burst oxidase of human granulocytes (reviewed in [17]). The latter functions in the transfer of reducing equivalents from cytoplasmic NADPH across the plasma membrane to molecular $O_2$ as the acceptor. The human oxygen reductase and the yeast ferric reductases resemble one another in the direction of movement of reducing equivalents from cytoplasm to an extracellular acceptor and the single-electron nature of the reduction. Moreover, contained within the primary sequences of these reductases are conserved amino acid motifs that may be critical to their function, including predicted NADPH and flavin adenine nucleotide binding sites [18,19]. These amino acid homologies, coupled with the genetic analysis, suggests that Fre1p, Fre2p and Frp1p are likely to be structural elements of surface reductases.

## 4. The transport of ferrous iron

Evidence has accumulated suggesting that organisms may be capable of acquiring iron (II) from the environment without the mediation of a siderophore [20]. The identification of the *S. cerevisiae FRE1* gene made possible the demonstration of a distinct ferrous uptake mechanism acting downstream of the reductase in the uptake pathway of environmental iron. Complete deletion of the *FRE1* gene in several genetic backgrounds resulted in severely deficient uptake of iron (III) but unimpaired (and even elevated) uptake of iron (II). Biochemical

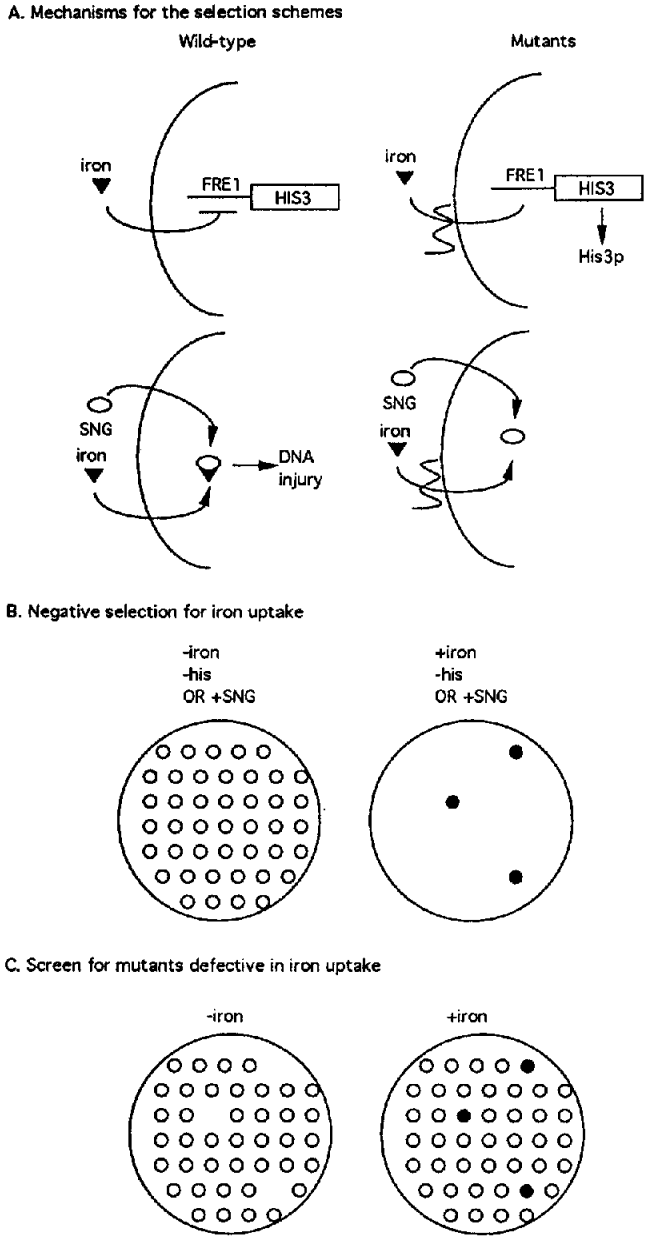


Fig. 2. (A) The objective of the two selection schemes is to prevent the growth of cells able to take up iron. In the *FRE1–HIS3* selection (A, top panel), iron (dark triangle) entering a cell by means of the normal high-affinity uptake pathway leads to repression of the *FRE1* promoter, and histidine auxotrophy results. Since His3 protein is not synthesized in this strain, which lacks an endogenous *HIS3* gene, it will not grow under conditions of sufficient iron and no added histidine. Mutants lacking normal iron uptake abrogate repression of the *FRE1–HIS3* fusion. For the streptonigran (SNG) selection (A, lower panel), the drug is taken into the cell where it may combine with cytosolic iron to create an iron-drug complex injurious to DNA and other macromolecules. Mutants lacking iron uptake will escape the toxic effects of the drug. (B) The selection allows mutants with defects in iron uptake to grow (filled circles) while the wild-type background cells (empty circles) do not grow). (C) When the mutants are evaluated for the ability to grown under conditions of low available iron, many exhibit impaired growth.

studies confirmed the existence of a high-affinity iron (II) uptake system that was saturable and specific for iron. While both the reductase and the ferrous transport system were found to be repressed by iron, the two activities were also distinctly regulated, especially vis-a-vis cell growth [21].

Two recently reported genetic approaches have been used to identify genes involved in mediating iron (II) transport (Fig. 2). In one approach, the iron-regulated *FRE1* promoter was used to drive expression of the His3 protein in a *HIS3*-deleted strain [22]. For this strain, the addition of iron to the medium resulted in auxotrophy for histidine due to the transcriptional repression of the *FRE1–HIS3* fusion gene. Spontaneously arising mutants were isolated that were able to overcome the iron-dependent histidine auxotrophy. Some of these proved to be deficient in{11}high-affinity ferrous iron uptake. An alternative selection scheme [23], employed streptonigrin to select mutants resistant to the toxic effects of the drug. Since the toxicity of this drug is dependent upon the ability of cells to accumulate iron, this approach also identified mutants defective in iron uptake.

Using the *FRE1–HIS3* selection, six distinct genes were identified that when mutated resulted in histidine prototrophy in the presence of iron. The most commonly selected mutations, resulting in the loss of function of the *CTR1* gene, completely abrogated high-affinity iron (II) uptake. The protein product of this gene, predicted to have a core molecular weight of 46 kDa, was found to migrate at 100–105 kDa on an SDS polyacrylamide gel, most likely due to extensive modification by O-linked sugars [24]. The predicted amino acid sequence of the protein suggested that it would traverse the membrane 3 times, and it has been localized to the plasma membrane [22], where it exists as a homooligomer [24]. Its unusual amino terminal domain, which is most likely facing the outside of the cell, contains multiple repeats of an amino acid motif that has recently been identified in a number of copper-binding proteins (MXXM, where X is likely to be a serine or a negatively charged amino acid). This observation prompted the specific assessment of the role of this protein in copper transport. *S. cerevisiae* was found to possess a high-affinity, specific copper uptake system requiring *CTR1*. Thus, mutants of *CTR1* were deficient in the high-affinity uptake of both copper and iron. However, the addition of high concentrations of copper to the medium, presumably able to gain entry to the cell via a low-affinity uptake pathway, resulted in the restoration of high-affinity ferrous iron transport, whereas the converse was not true; iron loading could not restore high-affinity copper uptake [24].

A likely explanation for this effect was provided by the identification of the *FET3* gene [23]. The cloning of the *FET3* gene and examination of the predicted amino acid sequence revealed a protein predicted to traverse the membrane a single time and possessing significant homology to ascorbate oxidase and laccase, multi-copper oxidases found in plants. In addition, *FET3* exhibited more distant but still significant homology to ceruloplasmin, a copper-dependent oxidase present in the plasma of mammals. Included in the sequences conserved among these proteins were motifs believed to be involved in the binding of copper. A mutant in the *FET3* gene was identified using the streptonigrin resistance selection and, like mutants of *CTR1*, was found to lack high-affinity ferrous transport [23]. In contrast to *CTR1* mutants, however, *FET3* mutants exhibited normal copper uptake, and the addition of copper failed to correct the defect in high-affinity iron uptake. A strain carrying mutations of both the *CTR1* and *FET3* genes lacked the copper

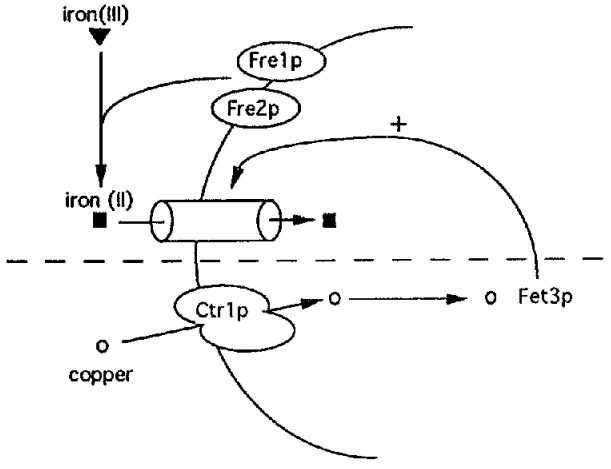


Fig. 3. General scheme for iron uptake in *Saccharomyces cerevisiae*. Iron (III) (filled trianges) in the environment is reduced by externally directed reductases Fre1p and Fre2p (ovals) to iron (II) (filled squares). A ferrous transporter (cylinder) which has been characterized only as an activity transports the ferrous iron to the cellular interior. This step requires copper uptake via the plasma membrane copper transport protein, Ctr1p, and activity of the putative multi-copper oxidase, Fet3p.

mediated restoration of high-affinity ferrous uptake. This suggested that the copper requirement for high-affinity ferrous iron uptake might relate to the need to supply copper to the Fet3 protein.

How might Fet3p, a predicted copper-containing oxidase, be functioning in the high-affinity uptake of ferrous iron? Answering this will require definition of the biochemistry of the Fet3 protein. Is it, in fact, a copper-containing oxidase? If so, what are its physiologic substrates? Critical to any model for the function of this protein will be establishing its intracellular location and its topology with respect to the membrane. It is predicted to have a single transmembrane domain situated near its carboxy terminus and following the copper-binding domain. The absence of an N-terminal leader sequence does not allow us to predict on which side of the membrane the predicted oxidase domain sits. In one model, predicated on the cytosolic location of the oxidase, the iron (II) transport is completed by being coupled to the cytosolic oxidation of the metal back to the ferric form. Alternatively, if the predicted copper-dependent oxidase is either in the lumen of the secretory pathway or outside the cell, the mode of action is more mysterious. One possibility is that the protein provides a signaling system, perhaps sensing oxygen or redox state, that is coupled to iron (II) transport via the regulated expression or function of a ferrous transporter. Such a transporter has not yet been molecularly identified. Whatever the mechanism, these studies point to a central role for this new member of the family of copper-dependent multi-functional oxidases in the ability of cells to accumulate iron via a high-affinity ferrous uptake system (Fig. 3).

## 5. Evidence for the generality of the *S. cerevisiae* model

The literature on iron transport has provided considerable circumstantial evidence suggesting that the mechanisms described for yeast may be conserved in some form in complex eukaryotes. Recently, a ferric reductase activity was localized to the lumenal surface of intestinal epithelial cells in the mouse. This activity was found to correlate with the ability of the cells to assimilate environmental iron [25]. The most complete model for mammalian transmembrane transport of iron has come from the work of Glass and colleagues in their studies on the transport of iron out of reticulocyte endosomes. They proposed that after the acid-dependent release of iron (III) from transferrin bound to the transferrin receptor, there is an obligate NADPH dependent reduction step to produce iron (II), which only then can be transported into the cytoplasm [26–28]. Thus, the reduction of iron (III) by membrane-bound reductases may be a general feature of eukaryotic iron transport. Finally, the connection between copper and iron transport observed for *S. cerevisiae* may have a parallel in mammals. Studies on copper-deficient swine in the 1950s suggested that a deficiency of serum ceruloplasm resulted in the failure to transport iron across membranes [29–31]. In addition, studies with mammalian cells in culture have suggested that copper enhances transferrin-mediated iron delivery into the cytosol [32]. While many of the details of molecular iron metabolism in the simple eukaryote *S. cerevisiae* remain to be worked out, it is hoped that these studies may provide insights into conserved pathways in complex organisms such as man.

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