Functional studies of hephaestin in yeast: evidence for multicopper oxidase activity in the endocytic pathway

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Hephaestin is a mammalian gene that encodes a predicted multicopper oxidase required for intestinal iron export. To examine if hephaestin can act as a ferroxidase, we studied yeast strains transformed with plasmids containing both a full-length hephaestin and a hephaestin lacking a transmembrane domain. Yeast with a deletion in *FET3*, which encodes a cell-surface multicopper oxidase, cannot grow on low-iron media. Expression of full-length hephaestin could complement the low-iron growth phenotype of a Δ *fet3* strain. Complementation of Δ *fet3* cells by hephaestin required genes that encode proteins necessary for the copper loading of Fet3p, including *CCC2* and *GEF1*. Expression of hephaestin in Δ *fet3* cells led to an increase in both iron transport

and oxidase activity. These results demonstrate that hephaestin is a copper-dependent protein. In contrast with Fet3p, which is found on the cell surface, hephaestin was co-localized with Pep12p-containing vesicles. Inhibition of endocytosis or deletion of both the vacuolar iron transporters (*SMF3* and *FET5/FTH1*) prevented hephaestin from complementing the low-iron growth phenotype of Δ *fet3* cells, suggesting that hephaestin is functioning within the endocytic apparatus.

Key words: endocytosis, hephaestin, iron, multicopper oxidase, yeast.

INTRODUCTION

Multicopper oxidases play an important role in iron metabolism in eukaryotes as diverse as yeast and humans. In three species of yeast, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans*, a plasma membrane multicopper oxidase is required for high-affinity iron transport [1]. The multicopper oxidase (Fet3p in *S. cerevisiae*, *fio1*⁺ in *S. pombe* and CaFet3p in *C. albicans*) appears to form a tight complex with a multitopic protein (Ftr1p in *S. cerevisiae*, *fip1*⁺ in *S. pombe* and CaFtr1p in *C. albicans*) that functions as a transmembrane iron permease [1,2]. The multicopper oxidase acts as a ferroxidase converting Fe²⁺ into Fe³⁺ [3]. The permease is thought to bind Fe³⁺ and transport it across the plasma membrane. Genetic studies indicate that the oxidase and permease are the only two plasma membrane proteins required for high-affinity transport of Fe²⁺ across the cell membrane [1].

Whereas multicopper oxidases affect iron uptake into yeast, genetic and physiological studies indicate that the mammalian homologues of *FET3*, namely ceruloplasmin and hephaestin, facilitate iron transport out of cells [4–6]. Humans or mice that lack ceruloplasmin gradually accumulate iron in the liver, pancreas, retina and basal ganglia. Introduction of ceruloplasmin into mice with a targeted disruption of the ceruloplasmin gene (*Cp*[−]/*Cp*[−]) results in the immediate release of iron from tissues [7]. The iron is redistributed via transferrin, a plasma protein that binds ferric iron, to the cells that express high levels of transferrin receptors, such as rapidly multiplying immature red blood cells in the bone marrow. Similarly, the absence of hephaestin, as in *sla* (sex-linked anaemia) of mouse [8], impairs iron mobilization from the intestine. In this disorder, newborn mice show a severe anaemia owing to a defect in the entry of iron into plasma, with iron accumulating in the villus cells of the intestinal

mucosa. Hephaestin has high sequence identity and similarity to ceruloplasmin and contains all the structural domains and copper-binding motifs required for a multicopper oxidase. Hephaestin differs from ceruloplasmin in its primary site of expression (small intestine versus liver) and, similar to Fet3p, contains an additional C-terminal putative transmembrane domain. The predicted structural features of hephaestin and the phenotype of the *sla* mouse suggest that hephaestin may enable intestinal iron export through its ferroxidase activity.

In the present study, we provide genetic and biochemical data demonstrating that hephaestin has multicopper oxidase activity. We expressed hephaestin in *S. cerevisiae* and demonstrated that the expressed molecule complements the low-iron growth defect of Δ *fet3* strains, mediates ⁵⁹Fe uptake, and oxidizes the substrate p-PD (*p*-phenylenediamine dihydrochloride). We show that hephaestin is localized to the endocytic apparatus and promotes iron transport from the endocytic apparatus to the cytosol. These results indicate that hephaestin is a multicopper oxidase. The present study provides evidence that the direction of multicopper-oxidase-mediated iron transport is not specified by the catalytic activity of the oxidase, but by the iron carrier or transporter.

MATERIALS AND METHODS

Cells and media

The wild-type yeast strains used in the present study were DY150 and DY1457, which were derived from a W303 background. The genotypes of the strains used here are outlined in Table 1. Strains with deletions in *FTH1*, *FET5* and *SMF3* were generated either by double-fusion PCR to delete these genes directly in the Δ *fet3* strain or by cross-mating strains with single or double deletions and dissecting the diploids to generate strains with

Abbreviations used: BPS, bathophenanthroline disulphonate; endo H, endoglycosidase H; p-PD, *p*-phenylenediamine dihydrochloride; RT, reverse transcriptase; *sla*, sex-linked anaemia; *tHEPH*, hephaestin lacking the transmembrane domain.

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Table 1 Yeast strains

Strain	Genotype	Source
DY150	<i>MATα</i> <i>ade2 can1 ura3 leu2 his3 trp1</i>	[9]
DY1457	<i>MATα</i> <i>ade6 can1 ura3 leu2 his3 trp1</i>	[9]
DEY1397-6A	<i>MATα</i> <i>ade2 can1 ura3 leu2 his3 trp1 fet3Δ::HIS3</i>	[9]
DEY1453	<i>MATα</i> <i>ade2 can1 ura3 leu2 his3 trp1 fet3Δ::HIS3 fet4Δ::LEU2</i>	[21]
DEY1530	<i>MATα</i> <i>ade2 lys2 ura3 leu2 his3 trp1 fet3Δ::HIS3 fet4Δ::LEU2 ftr1Δ::TRP1</i>	[21]
DEY1531	<i>MATα</i> <i>ade6 can1 ura3 leu2 his3 trp1 end4::LEU2</i>	[25]
JK723	<i>MATα</i> <i>ade2 can1 ura3 leu2 his3 trp1 fet3Δ::HIS3 fth1Δ::LEU2</i>	This study
JK492	<i>MATα</i> <i>ade2 can1 ura3 leu2 his3 trp1 fet3Δ::HIS3 end4Δ::LEU2</i>	This study
MM19 (<i>ccc2-1</i>)	<i>MATα</i> <i>ade2 can1 ura3 leu2 his3 trp1 fet4Δ::LEU2</i> & a mutation in <i>ccc2</i>	[17a]
JY185 (<i>gef1-1</i>)	<i>MATα</i> <i>ura3 leu2 lys2</i> a mutation in <i>gef1</i> gene	[26]
JK897	<i>MATα</i> <i>ade2 can1 ura3 leu2 his3 trp1 fet3Δ::HIS3 fet5Δ::HIS3 smf3Δ::KanMX</i>	This study
JK905	<i>MATα</i> <i>ade2 can1 ura3 leu2 his3 trp1 fet3Δ::HIS3 fet5Δ::LEU2</i>	This study

multiple deletions. The primers for $\Delta fth1$ were 5'-GGACTCC-TGACTACTTCTGT-3', 5'-GTCGTGACTGGGAAAACCCTG-GCGAATCCTCAAACGCCATGGTG-3', 5'-TCCTGTGTGAA-ATTGTTATCCGCTTAGCACATCAGAGCCAGACA-3' and 5'-ATTGAAGCAGGTCAAAGGGCT-3'. The primers for $\Delta fet5$ were 5'-GCGGGATCCATGGATTACAAGGATGACGATGACAAG-GGTGGTATGTTGTTCTACTCGTTCGTGTGGT-3', 5'-GTCG-TGACTGGGAAAACCCTGGCGAGCAGTATTGTCTTGAA-AGCCGTTAG-3', 5'-TCCTGTGTGAAATTGTTATCCGCTTC-GGTCTTTATTGAGGCACCC-3' and 5'-GCGGGATCCTTTCT-TGTTTAATTAGCCGCGAGA-3'. The primers for $\Delta smf3$ were 5'-CGACATCCTGTTGTCATGCG-3', 5'-TTAATTAACCCGG-GGATCCGACCAACCACTAGTGG-3', 5'-GTTTAAA-CGAGCTCGAATTCCCACTCCATTGAACCTCAAG-3' and 5'-GTGTCAGCGGTAGTGAATC-3'.

Cells were grown in YPD medium [1.0% (w/v) yeast extract/2.0% (w/v) peptone/2.0% (w/v) glucose] and CM (a synthetic medium of yeast nitrogen base, amino acids and glucose) or in YPD/CM, iron-limited by the addition of BPS (bathophenanthroline disulphonate) [9]. DNA transformations of *Escherichia coli* and *S. cerevisiae* were performed by standard procedures [10,11].

Construction of *Heph* (hephaestin gene) and truncated *Heph* for expression in yeast

Mouse *Heph* cDNA was obtained by reverse transcriptase-PCR of mRNA isolated from the small intestine of C57/BL6J mice. The full-length open reading frame for *Heph* cDNA was inserted into a yeast multicopy shuttle vector, namely pTF63. At the *ArvII* site of the 5'-end of the *Heph* open reading frame, 291 bp of *Heph* leader sequences were replaced by 153 bp of yeast *FET3* leader sequences along with 500 bp of the *FET3* promoter region. This fragment was generated by PCR. The resulting protein would have the first 51 amino acids of Fet3p, for which the primers were 5'-AAGGAAAAAAGCGCCGCTGCCTTGGCTTGCTATTTC-3' and 5'-CAGTGGTCTAGGAAGTGGCCATTACAGGTG-AT-3'. A FLAG epitope (DYKDDDDK) was added to the C-terminus of *Heph*. The PCR primers used for this purpose were 5'-CAGAGGAGAGGTTGGTGATA-3' and 5'-CCGCTC-GAGTTATCATCTGTGTCATCCTTGTGAATCACCACCTTGCTTGAGAGAGAGAAGCTT-3'. The plasmid containing this construct is termed pHEPH. A truncated version of *Heph* that lacked the transmembrane domain was generated by removing 192 bp of transmembrane domain DNA at the 3'-end of the *Heph* cDNA, replacing it with a sequence that encoded the FLAG tag (ptHEPH2). The PCR primers were 5'-CAGAGGAGAGG-TTGGTGATA-3' and 5'-CCGCTCGAGTTATCACTTGTGTCATC-

GTCATCCTTGTAATCACCACCCCTTCACATTGTCACCTCC-AAT-3'. All constructs were sequence-verified.

Western-blot analysis, endo H (endoglycosidase H) digestion and immunofluorescence microscopy

All samples were prepared for SDS/PAGE and Western-blot analysis as described in [11]. Endo H was used according to the manufacturer's instructions (New England Biolabs, Beverly, MA, U.S.A.). Subcellular fractionation was performed using a 15% (v/v) Percoll gradient as described previously [12]. Localization of the expressed protein was done by immunofluorescence microscopy as described previously [13]. The following are some of the antibodies used in the present study: mouse anti-FLAG antibody (Sigma) used at a 1:5000 dilution for Western-blot analysis and a 1:500 dilution for immunofluorescence microscopy; mouse anti-pep12 antibody (Molecular Probes, Eugene, OR, U.S.A.) used at a 1:200 dilution for Western-blot analysis; mouse anti-porin antibody (Molecular Probes) used at a 1:1000 dilution for Western-blot analysis; and mouse anti-(carboxypeptidase Y) antibody (Molecular Probes) used at a 1:4000 dilution for Western-blot analysis.

Iron transport assay

Exponentially growing yeast cells (mid-log) were transferred to CM-Ura with 80 μ M BPS and grown for 12 h. The cells were washed and assayed for iron uptake by incubation at specified times at 30 °C in 0.6 μ M 59 Fe in the presence of 1.0 mM ascorbate. Cells were washed and the radioactivity was determined as described previously [9].

p-PD oxidase activity assay

Oxidase activity was performed by the method of Yuan et al. [14] with some modifications. Cells grown in CM-Ura medium to an attenuation of 1.0 were washed and transferred to iron-limited CM-Ura medium (80 μ M BPS/200 μ M CuSO₄/50 mM Tris acetate, pH 6.5). Cells were homogenized as described previously [15]. Membrane proteins were isolated by centrifugation of the homogenate at 100 000 g for 30 min. The membrane pellet was resuspended in 100 mM sodium acetate (pH 5.7) and 0.1% Triton X-100. For the p-PD assay, 20 μ l containing 40 μ g of protein was mixed with 200 μ l of buffer containing 0.1% p-PD. The reaction product was determined by measuring the absorbance at 570 nm. Protein determinations were performed with the bicinchoninic acid reagent (Pierce, Rockford, IL, U.S.A.), using BSA (Sigma Fraction V) as a standard.

Figure 1 Expression of hephaestin in yeast as shown by Western-blot analysis

A $\Delta fet3$ strain was transformed with constructs containing either a plasmid (p) containing FLAG-tagged hephaestin (pHEPH) or a FLAG-tagged truncated hephaestin (ptHEPH) lacking the transmembrane domain. Cells were incubated in low-iron media to induce expression of hephaestin. The cells were harvested and extracts were prepared and examined by Western-blot analysis. Aliquots were also treated with endo H before electrophoresis. Molecular-mass markers are indicated on the left.

RESULTS

Expression of hephaestin in yeast and its ability to complement the low-iron phenotype of $\Delta fet3$

Initially, a cDNA for *Heph* was cloned into a yeast expression vector under the control of the *GALI* promoter. No protein was observed by Western-blot analysis in transformed cells grown in galactose media. We replaced the leader sequence of hephaestin with the *FET3* leader sequence and expressed the modified hephaestin under the control of the *FET3* promoter (pHEPH). The full-length hephaestin containing the first 51 amino acids of Fet3p, which encompasses the leader sequence, is synthesized

and glycosylated, as evidenced by a decrease in molecular mass when treated with endo H (Figure 1). We also expressed a form of hephaestin lacking the transmembrane domain (ptHEPH), which was expected to be secreted. This molecule is also expressed and glycosylated; however, for unknown reasons it was not secreted.

We examined the ability of $\Delta fet3$ cells expressing either the full-length or truncated hephaestin to grow on low-iron media. As expected, the $\Delta fet3$ strain, transformed with a control plasmid, showed no growth on low-iron media (Figure 2A). Cells transformed with a plasmid containing the full-length hephaestin protein (pHEPH) showed growth on low-iron media. The degree of growth was always less than that of wild-type cells. In contrast, the truncated *Heph* construct (ptHEPH) did not permit growth of $\Delta fet3$ strains. *S. cerevisiae* can also accumulate iron through a low-affinity iron transport system encoded by *FET4* [16]. Under normal conditions, the *FET4* transport system does not permit growth on low-iron media. Overexpression of *FET4*, however, will permit growth on low-iron media. To rule out the possibility that expression of hephaestin was activating the low-affinity iron transport system, a $\Delta fet3 \Delta fet4$ strain was transformed with pHEPH. Expression of hephaestin permitted the double-deletion strain to grow on low-iron media (Figure 2B).

The Fet3p-dependent transport system requires normal copper homeostasis [9,17]. Defects in *CCC2*, a Golgi copper transporter, result in an inability to grow on low-iron medium, owing to a defect in the copper loading of apoFet3p in post-Golgi compartments [14]. Transformation of pHEPH did not permit a strain with a mutation in *CCC2* to grow on low-iron media (Figure 2C). *GEF1* is a gene encoding an intracellular chloride channel that is also required for the copper loading of Fet3p [17a,17b]. Strains with a

Figure 2 Ability of pHEPH-transformed $\Delta fet3$ cells to grow on low iron

Wild-type (WT) cells, and the cells with designated deletions or mutations, were transformed with either the pHEPH construct, containing an intact hephaestin, or the ptHEPH construct, which encodes a hephaestin lacking the transmembrane and cytosolic domains. The cells were plated on media that had been made iron-limited by the addition of the iron chelator BPS. The plates were incubated at 30 °C for 3 days and then examined for growth. Each panel shows a specific mutant (A, $\Delta fet3$; B, $\Delta fet3 \Delta fet4$; C, *ccc2-1*; D, *gef1-1*). A WT strain was streaked on each plate as a positive control.

Table 2 Iron transport and p-PD activity in $\Delta fet3$ cells transported with *pHEPH*

Iron transport activity was assayed in $\Delta fet3$ cells transformed with either a control plasmid or plasmids containing the full-length hephaestin (*pHEPH*). Cells were grown in low-iron media for 12 h, washed and incubated with ^{59}Fe for the specified time periods. The amount of cell-associated radioactivity and p-PD activity in extracts of membrane fractions were determined as described in the Materials and methods section.

	Iron uptake (fmol $\cdot$ min $^{-1}$ $\cdot$ (10 6 cells $^{-1}$))	p-PD assay [$\Delta 570 \cdot \text{min}^{-1} \cdot (\text{g of protein}^{-1})$]
$\Delta fet3$	9.74 $\pm$ 2.95	7.56 $\pm$ 0.001
$\Delta fet3/\text{Heph}$	26.71 $\pm$ 2.27	10.56 $\pm$ 0.003

deletion or mutation in *GEF1* cannot grow on low-iron medium, again due to an inability to copper-load apoFet3p. No growth on low-iron media was observed in *gef1-1* cells transformed with the *pHEPH*-containing plasmid (Figure 2D). These results suggest that apo-hephaestin, similar to apoFet3p, is copper-loaded in a post-Golgi vesicle. These results confirm that growth of the *pHEPH*-transformed strain on low iron was dependent on the gene products required to make a functional multicopper oxidase.

Strains of *S. cerevisiae* with a deletion in *FET3* are not capable of growing on low-iron media [9], because of an inability to acquire iron. As expected, a low level of iron transport activity was seen in $\Delta fet3$ cells incubated with a low concentration of ^{59}Fe (0.6 μM ; Table 2). Incubation of $\Delta fet3$ cells transformed with *pHEPH* showed a low, but measurable, level of ^{59}Fe transport activity. Cells transformed with the *ptHEPH*, which expresses the truncated protein, showed transport activity similar to that seen in $\Delta fet3$ cells transformed with the vector (results not shown). Growth on low-iron media and iron transport assays together suggest that hephaestin mediates iron uptake in $\Delta fet3$ cells.

Multicopper oxidases are capable of oxidizing organic substrates such as p-PD. Extracts of $\Delta fet3$ cells that had been transformed with *pHEPH* showed consistently higher levels of p-PD activity when compared with $\Delta fet3$ cells (Table 2). The level of p-PD activity was consistent with the degree of hephaestin-mediated iron transport and low-iron growth. No increase in p-PD activity above background levels was detected in cells transformed with the truncated form of hephaestin (results not shown), which is used as a negative control. These observations indicate that expression of hephaestin results in increased iron transport and multicopper oxidase activity.

Hephaestin mediates iron uptake in an intracellular vesicle

The fungal multicopper oxidases convert Fe^{2+} into Fe^{3+} catalytically, which is then transported across the membrane by a membrane-spanning protein. Each of the yeast multicopper oxidases has a specific partner transporter: *FTR1* in *S. cerevisiae*, *fpl1* in *S. pombe* and *CaFTR1* in *C. albicans*. In the absence of the partner, the multicopper oxidase cannot effect transmembrane iron transport. For Fet3p, the presence of the transporter is required for the proper membrane localization of the multicopper oxidase. In the absence of Ftr1p, Fet3p does not localize to the cell surface. To determine whether Ftr1p is the transporter that effects hephaestin-mediated iron transport, we transformed *pHEPH* into a $\Delta fet3\Delta fet4\Delta ftr1$ strain (Figure 3A). Surprisingly, deletion of *FTR1* did not affect the ability of yeast expressing hephaestin to grow on low-iron media. We then determined whether hephaestin might interact with Fth1p, an *FTR1* homologue that is located on the vacuole and is thought to be a partner to *FET5*,

Figure 3 Genetic evidence that hephaestin effects iron transport within intracellular vesicles

Plasmids containing either the full-length *Heph* construct or the truncated *Heph* construct were transformed into cells that had the designated genes deleted. The cells were plated on media containing low iron and cell growth was assessed after 3 days.

a vacuolar multicopper oxidase [18]. To determine if Fth1p was the transmembrane permease for hephaestin, we generated a $\Delta fet3\Delta fth1$ strain, which will not grow on low iron. Transformation with *pHEPH* permitted the strain to grow on low-iron media, suggesting that Fth1p is not an obligate partner for yeast-expressed hephaestin (results not shown).

Clearly, there has to be some transmembrane transport system to facilitate iron transport from internal organelles to the cytosol. The vacuole contains two iron transport systems capable of transporting iron from the endocytic apparatus to the cytosol. One system comprises Fet5p/Fth1p and the other Smf3p, a member

Figure 4 Subcellular localization of hephaestin

Cells were transformed with either a control plasmid ('Control') or a plasmid containing the epitope-tagged *Heph* construct ('Heph-flag'). They were grown in low-iron media and either (A) examined by immunofluorescence microscopy using an antibody to FLAG or (B) subjected to subcellular localization. Membrane organelles were separated on a Percoll gradient, the gradient was fractionated and samples were subjected to PAGE and Western-blot analysis.

of the NRAMP2 (natural-resistance-associated macrophage protein 2) family of transition metal/ H^+ symporters. Both systems have been shown to transport iron from the vacuole to the cytosol [19]. Expression of hephaestin permits cells with a deletion in $\Delta fet3$ and a deletion in either of the two vacuolar transporters ($\Delta fet3\Delta fet5$ or $\Delta fet3\Delta smf3$) to grow on iron-restricted media. However, transformed cells with deletions in both vacuolar iron-transporter systems ($\Delta fet3\Delta fet5\Delta smf3$) were unable to grow on iron-restricted media (Figure 3B). These results suggest that hephaestin may function in an intracellular compartment. Consistent with this hypothesis is the fact that hephaestin-mediated complementation of the $\Delta fet3$ phenotype requires endocytic activity. Transformation of *pHEPH* into $\Delta fet3$ cells with a deletion of a gene required for endocytosis ($\Delta end4$) did not permit the strain to grow on low iron (Figure 3C).

These results indicate that hephaestin mediates iron transport within an endocytic organelle. This possibility was examined by defining the subcellular localization of epitope-tagged hephaestin in $\Delta fet3$ cells. Immunofluorescence microscopy showed abundant amounts of hephaestin localized to intracellular vesicles (Figure 4A). On Percoll gradients, hephaestin-containing organelles have a distribution similar to that of Pep12p, suggesting that hephaestin may be localized to a late endosomal compartment (Figure 4B). This result suggests that hephaestin mediates iron uptake while being localized to an intracellular compartment.

DISCUSSION

Multicopper oxidases that show ferroxidase activity are required for iron transport in species as diverse as humans and yeast.

The absence of the yeast multicopper oxidase Fet3p leads to an inability to accumulate iron. The absence of the mammalian multicopper oxidase ceruloplasmin leads to an inability to mobilize iron from tissues. *Heph* was identified as a gene which, when defective, results in an anaemia in mice owing to an inability to export iron from the intestine. The sequence of hephaestin indicates that the encoded protein may be a multicopper oxidase with similarities to both ceruloplasmin and Fet3p [8,20]. We took advantage of yeast to examine whether hephaestin had multicopper oxidase activity. Expression of hephaestin in yeast permits $\Delta fet3$ cells to grow on low-iron medium. The use of yeast strains with specific genetic defects demonstrated that complementation required intracellular copper homeostasis, as expected for a multicopper oxidase. Cells transformed with *pHEPH* show a low but increased rate of iron transport and a measurable rate of p-PD activity commensurate with the rate of iron transport and growth promotion. These results suggest that hephaestin is a multicopper oxidase.

The modest degree of complementation on low-iron media, iron transport activity and p-PD oxidase activity does not necessarily reflect the intrinsic multicopper oxidase activity of hephaestin. In yeast, multicopper oxidases require a specific partner transmembrane iron permease. Iron transport across the cell surface requires both Fet3p and Ftr1p on the cell surface [2]. Proper targeting of these proteins requires the simultaneous synthesis of Fet3p and Ftr1p, as these proteins must be co-assembled for proper targeting to the cell surface. The ferroxidase *fio1p* from *S. pombe* cannot substitute for Fet3p in $\Delta fet3$ cells. *Fio1p* cannot form a complex with Ftr1p and is not targeted to the cell surface [1]. Use of a $\Delta ftr1$ strain, however, demonstrated that Ftr1p was not required for *Heph*-mediated complementation. *S. cerevisiae* has a second multicopper oxidase, Fet5p, which is highly homologous with Fet3p, but is localized to the vacuole and forms a complex with Fth1p, an Ftr1p homologue. This complex can export iron from the vacuole [18]. Overexpression of *FET5* will complement the low-iron growth defect of $\Delta fet3$ cells [21]. Expression of hephaestin, however, permits growth of $\Delta fet3$ cells on low-iron media even in the absence of the Fet5p/Fth1p vacuolar iron-transport system. The degree of complementation by hephaestin may be limited by the absence of a specific partner transmembrane permease.

A potential explanation of the ability of hephaestin to overcome the low-iron growth defect of $\Delta fet3$ cells is provided by the observation that hephaestin is located not at the cell surface, but in an intracellular organelle. This vesicle shares sedimentation properties with Pep12p and may be a pre-vacuole. Furthermore, *Heph*-mediated complementation of $\Delta fet3$ strains requires endocytic activity. Therefore hephaestin must facilitate an alternative iron uptake pathway that passes through an intracellular vesicular compartment, consistent with a role for vacuolar iron transporters. Our present hypothesis is that, within the endocytic apparatus, hephaestin catalyses the formation of the less soluble Fe^{3+} from Fe^{2+} . The insoluble ferric ion, precipitated within the endocytic apparatus, is swept into the vacuole by endocytic activity. Within the vacuole, iron can be solubilized by ferric reductases and is now a substrate for the vacuolar iron-transport systems. The observation that hephaestin is unable to complement $\Delta fet3$ cells in the absence of both the Fet5p/Fth1p and Smf3p vacuolar iron-transport systems supports this view.

Both ceruloplasmin and Fet3p are present on the cell exterior. Soluble ceruloplasmin is present in plasma and other plasma biological fluids; glycosylphosphatidylinositol-linked ceruloplasmin is present on the exterior cell surface. Fet3p is localized to the yeast exterior membrane. The active site of these

proteins is located exterior to the cell. Hephaestin, however, may function within cells. Hephaestin transiently expressed in cultured mammalian cells is localized to intracellular vesicles enriched in mannose-6-phosphate receptors (L. Li, unpublished work). Whereas the localization pattern of hephaestin expressed in both yeast and cultured cells can be interpreted as expression artifacts, endogenous hephaestin also has an intracellular localization [22]. Endogenous hephaestin is found in apical absorptive cells of the intestine and not in crypt cells. The protein appears to be localized to intracellular vesicles that have a perinuclear distribution. Previous studies have not identified those vesicles. The results of the studies on yeast suggest that hephaestin can effect iron transport within vesicles, suggesting that, perhaps, within mammalian cells, endogenous hephaestin also functions within the vesicular apparatus and not at the cell surface.

The present study indicates that hephaestin is a multicopper oxidase and can affect cellular iron uptake in yeast. The phenotype of the *sla* mouse clearly indicates that defects in hephaestin result in a decrease in cellular iron export, resulting in cellular iron accumulation [8]. Whereas recent studies indicate that ceruloplasmin may be involved in cellular iron accumulation [23,24], a large body of physiological work as well as recent studies on ceruloplasmin knockout mice [7] demonstrates that ceruloplasmin plays a role in cellular iron export. Fet3p can load iron on to transferrin *in vitro* [3] and, as shown in the present study, hephaestin can effect iron uptake in yeast. These results suggest that multicopper-oxidase-mediated iron transport is intrinsically non-directional and that it is the carrier or transporter that specifies the direction of iron transport, either in or out of cells.

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