The Fe(II) permease Fet4p functions as a low affinity copper transporter and supports normal copper trafficking in *Saccharomyces cerevisiae*

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The plasma-membrane of *Saccharomyces cerevisiae* contains high affinity permeases for Cu(I) and Fe(II). A low affinity Fe(II) permease has also been identified, designated Fet4p. A corresponding low affinity copper permease has not been characterized, although yeast cells that lack high affinity copper uptake do accumulate this metal ion. We demonstrate in the present study that Fet4p can function as a low affinity copper permease. Copper is a non-competitive inhibitor of ^{55}Fe uptake through Fet4p ($K_i = 22\ \mu$M). Fet4p-dependent ^{67}Cu uptake was kinetically characterized, with K_m and V_{max} values of 35 μM and 8 pmol of copper/min per 10^6 cells respectively. A *fet4*-containing strain exhibited no saturable, low affinity copper uptake indicating that this uptake was attributable to Fet4p. Mutant forms of Fet4p that exhibited decreased efficiency in $^{55/59}$Fe uptake were similarly compromised in ^{67}Cu uptake, indicating that similar amino acid residues in Fet4p contribute to both uptake processes. The copper taken into the cell by Fet4p was metabolized similarly to the copper taken into the cell by the high affinity permease, Ctr1p. This was shown by the Fet4p-dependence of copper activation of Fet3p, the copper oxidase that supports high affinity iron uptake in yeast. Also, copper-transported by Fet4p down-regulated the copper sensitive transcription factor, Mac1p. Whether supplied by Ctr1p or by Fet4p, an intracellular copper concentration of approx. 10 μM caused a 50 % reduction in the transcriptional activity of Mac1p. The data suggest that the initial trafficking of newly arrived copper in the yeast cell is independent of the copper uptake pathway involved, and that this copper may be targeted first to a presumably small 'holding' pool prior to its partitioning within the cell.

Keywords: copper uptake, copper trafficking, metal interactions, yeast.

INTRODUCTION

The pathway and protein components of high affinity iron and copper uptake in the budding yeast, *Saccharomyces cerevisiae*, have been elucidated over the past few years. Both uptake processes require an initial one-electron reduction of the high valency forms, Fe(III) and Cu(II), to their respective lower valency states [1–5]. This reduction is catalysed by plasma-membrane reductases. Several of these have been genetically characterized; the two most important reductases for copper and iron uptake are encoded by the *FRE1* and *FRE2* genes [3,4,6–9]. The uptake of both metals also requires high-affinity transporters. The iron transporter is encoded by *FTR1* [10], and the copper transporters are encoded by *CTR1* [11,12] and *CTR3* [13]. Ctr3p is inactive in most laboratory strains of yeast, because of a transposon insertion mutation. An additional plasma-membrane component is required for high affinity iron uptake [1,10,14]. This is a multinuclear copper oxidase [15], Fet3p, an enzyme that catalyses a ferroxidase reaction, e.g. $4Fe(II) + O_2 + 4H^+ \rightarrow 4Fe(III) + 2H_2O$. The Fet3p-dependent reoxidation of the Fe(II) produced by the reductase reaction and Ftr1p-dependent iron uptake are tightly coupled mechanistically and physically: Fet3p and Ftr1p are assembled in a vesicular compartment prior to delivery to the plasma-membrane [10,16]. The essential role that copper-dependent Fet3p plays in high affinity iron uptake makes iron uptake reliant on the copper status of the yeast cell. Copper deficient yeast exhibit reduced or absent high affinity iron uptake and a concomitant respiration deficiency that blocks growth on non-fermentable carbon sources, such as glycerol and ethanol.

S. cerevisiae exhibits low affinity uptake of both metals as well. The K_m values for these processes are approx. 40-fold (for copper) to 400-fold (for iron) greater than for the high affinity uptake systems (high affinity K_m values are 2 μM and 0.2 μM for copper and iron respectively) [3,6,17]. The low affinity uptake pathway for iron has been characterized in some detail; it is due to the action of the *FET4* gene product [18,19]. Fet4p has six potential transmembrane domains and is localized in the plasma-membrane. Like high affinity Ftr1p-dependent uptake, iron transport by Fet4p requires prior reduction of Fe(III) to Fe(II). In contrast to Ftr1p-mediated uptake, however, the low affinity pathway is Fet3p-independent. Thus this pathway can supply iron to the cell when Fet3p activity is absent.

As noted, one of the conditions in which Fet3p activity is limiting or absent is cellular copper deficiency. Thus copper-limited cells can become iron-deficient due to the lack of the holo-Fet3p required for high affinity iron uptake [1,11,12]. Clearly, there is a cellular advantage in having additional ways, such as a separate low affinity pathway, to accumulate the copper that is essential for the uptake of iron. Indeed, the copper-dependent iron deficiency in a *ctr1ctr3*-containing mutant strain can be overcome if the medium is supplemented with sufficient copper ($> 20\ \mu$M) indicating the presence of a Ctr1p/Ctr3p-independent, low affinity copper uptake pathway [13,20–22]. This putative uptake pathway has not been characterized.

Some indirect evidence indicates that in yeast, Smf1p can support copper uptake [23,24]. However, this member of the Nramp transporter family [5] is found in the plasma-membrane only under manganese-depleted conditions, and its cellular

Abbreviations used: BCS, bathocuproine disulphonic acid; YPD, 1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose.

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localization is not significantly responsive to copper or most other transition metal ions [24]. In contrast, Fet4p may have a broader metal ion specificity, in that iron uptake through Fet4p is inhibited by cobalt, cadmium and nickel [18]. Therefore in the present study we test the hypothesis that the low affinity iron transporter, Fet4p, can also serve as a low affinity copper transporter. We characterize low affinity copper uptake in detail and show it to be Fet4p-dependent. We also show that copper taken into the cell via Fet4p is biologically comparable with the copper taken into the cell through the high affinity copper transporters. Fet4p most likely serves as the dominant copper transporter in yeast grown under conditions of elevated copper concentrations. The apparently normal metabolism of this copper will help to determine how newly arrived copper is targeted to the cohort of copper chaperones responsible for efficient and specific delivery of the metal ion to apo-copper metalloproteins.

MATERIALS AND METHODS

Yeast strains and culture conditions

All of the strains used were derived from the parental wild-type W303 strain, and were isogenic except where indicated [18,19,25]. These strains were: DDY0100 (*MAT*a *can1 his3 leu2 trp1 ura3 fet3::HIS3 fet4::LEU2 trp1::GAL1FET4*); DY1457 (*MAT*α *ade6 can1 his3 leu2 trp1 ura3*); ARY1457 (DY1457 *ctr1::URA3*); DEY1422 (DY1457 *fet4::LEU2*); ARY1422 (DEY1422 *ctr1::URA3*); DEY1422T2 (DEY1422 *trp1::GAL1FET4*); and 1457T2c (DY1457 *ctr1::URA3 ccc2::LEU2 trp1::GAL1FET4*). A *fre1fre2*-containing strain [7] was used for one experiment to demonstrate the reductase-dependence of Fet4p-mediated copper uptake. All strains were Mac1p-plus and Aft1p-plus, but Ctr3p-minus. Mac1p and Aft1p are copper- and iron-responsive transcription factors that regulate the expression of genes encoding the components of their respective high affinity uptake pathways, e.g. *CTR1* [9,13], and *FTR1* and *FET3* [8,10]. The expression of *FET4* is Mac1p- and Aft1p-independent [3,19]. The strains containing *trp1::GAL1FET4* produce Fet4p when grown in galactose but are Fet4p-minus when grown in glucose [18,19]. Cultures were typically grown in a completely defined, synthetic medium that was based on the components of yeast nitrogen base ('YNB') as described in the Difco Manual (1984, 10th edn, pp. 1135–1141; Difco Laboratories, Detroit, MI, U.S.A.). Copper and iron were omitted from the medium; in addition, the medium was Chelex-treated prior to the addition of a copper- and iron-free trace metal stock solution. The medium was then re-supplemented with copper and iron as indicated [3,17]. Severe copper depletion was achieved by growing the cells in the presence of 100 μM bathocuproine disulphonic acid (BCS).

Metal ion uptake kinetic measurements

^{67}Cu uptake measurements were made at 30 °C in 0.1 M Mes (pH 6.0) containing 20 mM citrate and 2% (w/v) glucose as described previously [3,17]. The ^{67}Cu was carrier-free and was obtained from Brookhaven National Laboratory (Upton, NY, U.S.A.). When present, ascorbate was added at a concentration of 1 mM. Fe(II) uptake assays were performed at 30 °C as described previously [6], using either ^{55}Fe or ^{59}Fe. Both were carrier-free and were obtained from New England Nuclear (Boston, MA, U.S.A.). Cell-associated radioactivity due to ^{55}Fe uptake was measured by liquid-scintillation counting; ^{59}Fe was measured by gamma-counting. The specific radioactivity of the metal isotopes varied over the course of the experiments described herein due to their relatively short half-lives. The amount of isotope used was adjusted to compensate for differences in specific activity such that total cell-associated counts were consistently at least 50-fold above background (> 1000 c.p.m./cell sample). The uptake values reported in the present study are initial rates determined in 15 min reactions. All uptake velocities were from the linear portions of the time versus metal ion accumulation curves. Velocity data for copper inhibition of ^{55}Fe uptake was graphically analysed using the method of Dixon [26]. The lines in Figure 1 were generated by linear least-squares analysis using InStat software from GraphPad (San Diego, CA, U.S.A.). Non-linear fits of the data to the Michaelis–Menten equation were highly significant (results not shown) and yielded (apparent) V_{max} and K_m values. ^{67}Cu uptake data were similarly fitted; the lines in Figure 2 are constructed using the fitted kinetic constants for copper uptake in the various strains. The standard errors in these fitted constants ranged from 6–12%. This analysis was performed using Enzfitter software from BioSoft (Cambridge, U.K.).

Fet3p analyses

The amount of the soluble, secreted Fet3p produced in the experiment described in Table 3 was determined by Western blot analysis. This Fet3p was produced from plasmid pDY148, which is a high-copy vector that carries a recombinant *FET3* gene effectively truncated at nucleotide +1666 (i.e. at amino acid residue 555) to which is appended the sequence encoding the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). Lacking the C-terminal, membrane-spanning domain, this Fet3 protein is secreted directly into the growth medium [15]. This medium was clarified by centrifugation and filtration (through a 0.22 μm membrane) and then concentrated 10-fold. Samples were fractionated by SDS/PAGE, electroblotted, and the blots were probed with an anti-FLAG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Immunocomplexes were visualized using chemiluminescent detection (Pierce, Rockford, IL, U.S.A.), employing the appropriate horseradish peroxidase-conjugated second antibody and Kodak BIOMAX™ MR film. Fet3p oxidase activity was used as a measure of the fraction of this Fet3 protein that was in the copper-replete, holo form. This activity was measured using *o*-dianisidine as the substrate, as described previously [27].

Copper concentration dependence of Mac1p activity

Yeast transformants carrying a *CTR1* promoter::*lac*Z reporter plasmid [9,25] were grown in Chelex-treated/copper-supplemented defined medium for at least five doublings until the cultures reached mid-exponential phase (D_{660} = 1.5–2.0). Samples (2×10^7 cells) were assayed in triplicate for β-galactosidase activity, which was expressed in Miller units in the standard fashion [1]. Samples of these cells were digested in HNO_3 and then analysed for copper content by flameless atomic absorption spectrophotometry on a Perkin Elmer Model 1100 instrument equipped with a Model 700 graphite furnace. The data in Figure 3 are from three independent experiments and the mean values were determined with the use of InStat software. The S.E.M. values ranged from ±4–9% of the mean values.

RESULTS

Fet4p-dependent ^{55}Fe uptake is inhibited by copper

Except where noted, all of the experiments were carried out using strains of yeast that lacked high affinity copper and/or iron

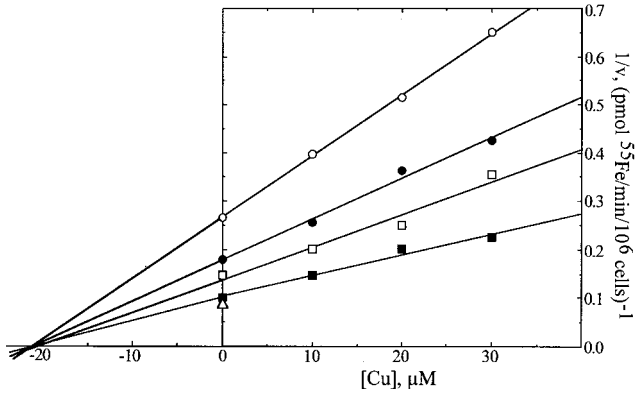

Figure 1 Copper inhibition of ^{55}Fe uptake in yeast

The *Δfet3GAL1::FET4*-containing strain DDY0100 was grown in galactose and then used in ^{55}Fe uptake assays as described in the Materials and methods section. Copper was included in the uptake buffer as indicated. The ^{55}Fe uptake velocity data are presented as a Dixon plot [26]. The fact that the lines intersect on the axis indicates that copper is a simple non-competitive inhibitor of iron uptake; the abscissa value of the point of intersection of the lines gives the K_i value for copper inhibition, which is 22 ± 3 μM. The concentrations of ^{55}Fe used were: 15 μM (open circles); 30 μM (closed circles); 60 μM (open squares); 150 μM (closed squares). The lines were generated by linear least-squares analysis. $1/V_{max}$ for ^{55}Fe uptake is indicated by the open triangle. This value came from a direct, non-linear fit of the velocity data obtained in the absence of copper to the Michaelis–Menten equation.

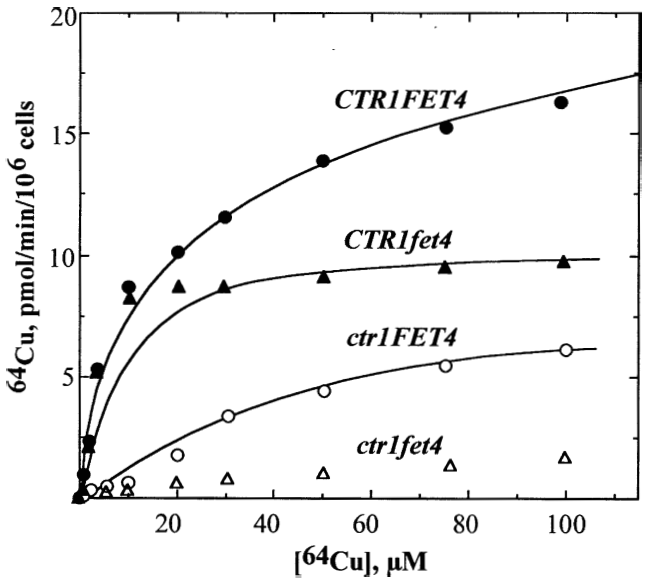

Figure 2 ^{67}Cu uptake mediated by Fet4p

Strains with the indicated genotypes were used in ^{67}Cu uptake assays, which were carried out as described in the Materials and methods section. Ascorbate (1 mM) was included. The lines for the *CTR1Δfet4* and *Δctr1FET4* strains were generated by direct, non-linear fitting of the corresponding velocities to the Michaelis–Menten equation. The line for the *CTR1FET4* strain was calculated using the kinetic constants for Ctr1p- and Fet4p-mediated uptake (from the preceding analyses) and the velocity equation for two transporters with different kinetic constants independently supporting the same uptake process [26]. These kinetic constants and their standard errors are given in the text.

uptake, i.e. *Δctr1*- and *Δfet3*-containing mutants. (All of the strains used were defective for the second high affinity transporter encoded by *CTR3*.) In addition, Fet4p was overproduced in some experimental regimens so as to maximize the measurable low affinity iron (metal ion) uptake and thereby provide more precise kinetic data. The regulated overexpression of *FET4* was achieved by integrating a *GAL1* promoter–*FET4* chimaera (at the *TRP1* locus) and growing the resulting strain on galactose [18,19]. Furthermore, this galactose-dependent overproduction demonstrated the correlation between Fet4p amount and metal ion uptake.

Two types of kinetic experiments were carried out to demonstrate that Fet4p could utilize copper as a substrate in metal ion uptake. In the first experiment, copper was tested as an inhibitor of the low affinity iron uptake activity that has been well documented for this transporter [18,19]. The strain used for these experiments, DDY0100, carried a deletion of *FET3* so that all ^{55}Fe uptake was attributable to Fet4p. Fet4p was overproduced in the cells used in order to provide more precise kinetic data for the inhibition analysis. These inhibition data are displayed in Figure 1 as a Dixon plot, in which the reciprocal of the velocity of ^{55}Fe accumulation is plotted as a function of copper concentration at different concentrations of total iron. This analysis demonstrated that copper was a relatively strong inhibitor of ^{55}Fe uptake through Fet4p, with a K_I of 22 ± 3 μM. This value can be compared with the K_m value for high affinity copper uptake of 2 μM [17], and of low affinity iron uptake through Fet4p of 35 μM [2,3]. The behaviour of the velocity data analysed in this fashion (Figure 1) suggested that copper was a simple non-competitive inhibitor of iron uptake through Fet4p. This type of inhibition was indicated by the close intersection of the lines on the abscissa axis [4]. Regardless of the precise kinetic mechanism of this inhibition, the results are consistent with the hypothesis that copper, as well as iron, interacts with Fet4p, and could serve as a substrate for this metal ion permease.

Ctr1p-deficient yeast exhibits Fet4p-dependent low affinity ^{67}Cu uptake

The ability of Fet4p to transport copper was directly assessed by measuring the Fet4p-dependence of ^{67}Cu uptake in strains that lacked both high affinity copper transporters (see Figure 2). In this experiment, copper accumulation was measured as a function of total copper concentration in wild-type strain DY1457 and in congenic *Δctr1FET4*, *CTR1Δfet4* and *Δctr1Δfet4*-containing mutants [19]. Note that all of the strains used were based on the parental W303 strain, see the Materials and methods section. What these plots show qualitatively is that a low affinity, saturable copper uptake activity is detectable in either the wild-type or *Δctr1FET4* strains that is absent in the *CTR1Δfet4* and *Δctr1Δfet4* backgrounds. Although some cellular copper accumulation was exhibited by this latter strain, it did not conform to a simple saturation function, indicative of facilitated transport over the concentration of copper used. On the other hand, elimination of the apparent saturable, low affinity uptake process, as in the *CTR1Δfet4* strain, resulted in an excellent fit of the velocity data to the kinetic equation describing high affinity copper uptake alone.

Fitting of the low affinity copper uptake data obtained for the *Δctr1FET4* strain to the Michaelis–Menten equation was limited in statistical significance by fact that the maximum concentration of copper used was only 2-fold greater than the fitted K_m value of 35 ± 7 μM. Significant non-saturable accumulation of copper in the yeast cells was detectable at copper concentrations exceeding 100 μM. The K_m value (35 μM), however, can be compared favourably with the K_i for copper inhibition of iron uptake (22 μM) given the acknowledged limitations in these

Table 1 Galactose-inducible iron and copper uptake in *GAL1::FET4*-containing strains

Yeast strains containing the deletions indicated were used as backgrounds for integration of the *GAL1::FET4* expression cassette. The *Δfet3FET4* (iron uptake) and *Δctr1FET4* (copper uptake) controls were included to demonstrate the effect of galactose growth on the expression of the endogenous *FET4* and the resulting metal ion uptake activity produced. The cultures were grown in either glucose or galactose, and were then used in either ^{59}Fe or ^{67}Cu uptake assays. The total metal ion concentration used was 30 μM in the presence of 1 mM ascorbate. The values represent means ± S.D.

	Uptake velocity (pmol/min per 10^6 cells)			
	Iron uptake		Copper uptake	
Strain genotype	+ Glucose	+ Galactose	+ Glucose	+ Galactose
Δfet3FET4	0.22 ± 0.04	0.16 ± 0.02	–	–
Δfet3Δfet4 GAL1::FET4	0.08 ± 0.02	1.12 ± 0.08	–	–
Δctr1FET4	–	–	3.2 ± 0.4	2.8 ± 0.2
Δctr1Δfet4 GAL1::FET4	–	–	0.07 ± 0.02	16.2 ± 0.5

Table 2 ^{59}Fe and ^{67}Cu uptake by wild-type and mutant Fet4 proteins

Uptake velocities ± S.E.M. ($n = 3$) were determined at metal ion concentrations of 30 μM. Metal ions were added as chloride salts, in the presence of ascorbate. Expression of the *FET4* alleles (present in single-copy and integrated at the *TRP1* locus) was under the control of the *GAL1* promoter, as described in the Materials and methods section. All strains had a *Δctr1Δfet3Δfet4*-containing background so that iron and copper uptake were dependent on this heterologously expressed Fet4 protein.

	Uptake velocity (pmol/min per 10^6 cells)	
Fet4p	Iron uptake	Copper uptake
Wild-type	2.2 ± 0.2	15.8 ± 0.7
Y276A	0.3 ± 0.1	6.3 ± 0.5
Y352A	0.2 ± 0.1	5.1 ± 0.5
D400A	0.0	0.8 ± 0.3

kinetic data (also see the Discussion). In addition, this fit gave a V_{max} value of 8 ± 2 pmol of copper/min per 10^6 cells, comparable with the maximum velocity of Ctr1p-mediated uptake of 10 ± 2 pmol of copper/min per 10^6 cells (Figure 2 and [17]). This V_{max} value indicated that the low affinity uptake of copper can provide levels of cellular copper comparable with that afforded by Ctr1p when the extracellular copper concentration is near or greater than 35 μM. The kinetic constants obtained from the independent fit of the data sets obtained from the *CTR1Δfet4* and *Δctr1FET4* strains were then used to generate the theoretical curve that is overlaid on the velocity data for the *CTR1FET4* wild-type strain. The close correspondence of the experimental data and this theoretical curve indicates that the high and low affinity processes can account for all of the kinetically saturable, facilitated copper uptake by a wild-type strain of *S. cerevisiae*.

Low affinity ^{67}Cu uptake correlates with the amount of Fet4p

The potential of Fet4p to act as a copper transporter was tested in a third kinetic experiment. ^{67}Cu uptake was measured as a function of the level of expression of *FET4*, where this gene was under control of the galactose-inducible *GAL1* promoter, as used to determine the copper inhibition of iron uptake (Figure 1). As above, a *ctr1*-containing background was used so that any galactose-inducible copper uptake observed could be attributed to Fet4p [18]. As a positive control for this copper uptake assay, the galactose-inducible low affinity iron uptake that has been well-established [18,19] was first confirmed using a congenic pair that carried a deletion of *FET3* and so lacked high affinity iron uptake. The corresponding galactose-inducible iron and copper uptake data are presented in Table 1.

The low affinity iron uptake exhibited by the *Δfet3FET4*-containing cells grown in glucose was slightly greater than those cells grown in galactose. In contrast, glucose-grown cells with the *Δfet3Δfet4GAL1::FET4* genotype exhibited a strongly reduced iron uptake overall since they expressed neither iron transport system. Growing these cells in galactose resulted in an iron uptake rate of 1.12 pmol of ^{59}Fe/min per 10^6 cells. This represented an 8-fold induction in comparison with galactose-grown cells expressing *FET4* from its own promoter, rather than the *GAL1* promoter, and confirmed what has been observed previously [18,19].

A similar galactose-dependent difference was observed when ^{67}Cu uptake was measured in the congenic *Δctr1*-containing strains when grown under these two conditions. With a total copper concentration of 30 μM, copper uptake increased from 2.8 pmol ^{67}Cu/min per 10^6 cells in the galactose-grown *Δctr1FET4*-containing cells to 16.2 pmol ^{67}Cu/min per 10^6 cells in the galactose-grown *Δctr1Δfet4GAL1::FET4* cells (a 6-fold increase). This parallel increase of both copper and iron uptake in the cells overproducing Fet4p is strong evidence that both uptake processes are dependent on this protein. It should also be noted that overexpression of Fet4p from the *GAL1* promoter made wild-type cells more sensitive to the growth inhibitory effects of moderate levels of copper in the growth medium (results not shown).

Low affinity ^{67}Cu uptake correlates with the activity of Fet4p

In a last kinetic experiment, designed to link Fet4p activity to copper uptake, mutant *FET4* alleles were expressed in a *Δfet4*-containing background so that the low affinity copper uptake was dependent on the mutant Fet4p alone. Several of these mutant proteins have been characterized, and three were selected for this experiment based on their behaviour with respect to iron uptake [19]. Table 2 gives the velocities of iron uptake by these Fet4p proteins under V/K conditions of iron concentration. The effect of these mutations on copper uptake is shown by the comparable values in Table 2. Qualitatively, there is a close parallel between these two data sets in that each mutation has a similar effect on iron and copper uptake efficiency. That there is not an exact quantitative correlation is reasonable given the different electronic and co-ordination properties of ionic copper and iron.

Cu(I) is the substrate for Fet4p-mediated uptake

Copper uptake by Ctr1p, and iron uptake by either Ftr1p or Fet4p uses the lower valence state of the two metal ions, Cu(I) and Fe(II) respectively. Consequently, these uptake processes are dependent on the action of the plasma-membrane reductases, Fre1p and/or Fre2p. The dependence on these reductases can be bypassed if uptake experiments are performed in the presence of a reducing agent, such as ascorbate. Indeed, all of the experiments described above were carried out in this way so that the data were strictly reductase-independent. This fact alone indicates that Cu(I) is the substrate for the apparent Fet4p-mediated uptake, but this inference was tested directly by carrying out ^{67}Cu

Table 3 Activation of soluble Fet3p in wild-type and *Δctr1*-containing strains

Yeast strains were grown in either glucose or galactose as indicated in medium containing a limiting copper concentration of 500 nM. Strains contained a high-copy plasmid that encoded a soluble secreted form of Fet3p that was tagged with the FLAG epitope [11,18]. Growth medium was assayed for Fet3p oxidase activity using *o*-dianisidine as the substrate [27]. The amount of Fet3p in the sample was determined by Western blot analysis using an anti-FLAG antibody. The activity data (presented as means ± S.D.) are therefore given as the specific activity of the Fet3p present. The strain denoted 'wild-type (−copper)' contained 100 μM BCS; it was the negative control that gave the residual activity in a culture producing only the apo form of Fet3p. *CCC2* encodes the Cu-ATPase that targets copper to apo-Fet3p in the cell.

	Fet3p oxidase activity (μmol/min per mg of Fet3p)	
Strain genotype	+ Glucose	+ Galactose
Wild-type	3.05 ± 0.15	2.75 ± 0.10
Wild-type (−copper)	0.15 ± 0.08	0.10 ± 0.05
Δctr1 GAL1::FET4	0.45 ± 0.08	2.60 ± 0.10
Δctr1Δccc2 GAL1::FET4	0.60 ± 0.08	0.55 ± 0.10

uptake experiments in a *Δctr1*-containing strain that included *Δfre1* and *Δfre2* alleles in its background [7]. The results of this experiment were that in the absence of ascorbate, copper uptake by the *Δctr1FET4* strain (copper concentration of 30 μM) was reduced by 90% in the *Δfre1Δfre2*-containing background in comparison with the wild-type reductase (0.25 pmol of ^{67}Cu/min per 10^6 cells versus 2.7 pmol of ^{67}Cu/min per 10^6 cells). This difference in low affinity copper uptake in the double reductase mutant was completely suppressed by the inclusion of ascorbate in the uptake buffer (results not shown). As noted, this requirement parallels the need for Fe(III) reduction prior to Fet4p-mediated low affinity iron uptake [18].

Copper accumulated by Fet4p can be used to activate apo-Fet3p

The rationale for this work was based on the observation that the respiration (iron) deficiency exhibited by a strain lacking Ctr1p activity, e.g. a *Δctr1*-containing mutant, can be suppressed by addition of copper at concentrations greater than 20 μM. This fact suggested that the copper taken into such strains was available for trafficking to the post-Golgi vesicle where apo-Fet3p becomes copper-activated [16,21]. It also placed constraints on potential mechanistic models of this trafficking process. We carried out two experiments to directly demonstrate this apparent physiological trafficking of Fet4p-derived intracellular copper. In the first, we took advantage of the expression system designed for the production of a secreted form of Fet3p. This allele lacks the DNA sequences encoding the C-terminal, plasma-membrane-anchoring domain in this protein; this protein is otherwise processed normally [15]. Correct copper trafficking to the apo form of this truncated Fet3p can be simply determined by measuring the oxidase activity in the growth medium due to the presence therein of the copper-activated form of this multinuclear copper oxidase.

The results of this test for the normal metabolism of Fet4p copper are given in Table 3. In these experiments, cells were grown in a defined medium that contained 500 nM copper. This concentration of copper was chosen to limit the amount of copper entering the cell by the non-saturable process indicated by the data in Figure 2. Also, we wished to reduce the background Fet3p activity resulting from the cell-independent activation of apo-Fet3p in the growth medium [16,21]. A wild-type strain was included in these experiments as a positive control. This control demonstrated that Ctr1p-mediated copper uptake at this copper concentration supported activation of Fet3p whether the cells were grown in glucose or galactose. To determine the background level of oxidase activity, the wild-type strain was also grown in the presence of BCS; under this condition of copper starvation, all Fet3p produced was in the apo form. This was indicated by the minimal level of oxidase activity measured. At a copper concentration of 500 nM, a *Δctr1*-containing strain would also be copper-starved and produce predominately apo-Fet3p. This was confirmed by the fact that in the *Δctr1*-containing strain overexpression of *FET4* from the *GAL1* promoter was required for the production of holo-Fet3p that was essentially absent when the *Δctr1GAL1::FET4*-containing cells were grown with glucose as the carbon source. Note that these cells were also expressing the chromosomal *FET4* gene independent of carbon source, i.e. at this limited copper concentration, normal levels of Fet4p were insufficient to physiologically suppress the lack of Ctr1p-mediated copper uptake. Another control used was a strain that lacked the Cu-ATPase, Ccc2p, essential for the uptake of copper into the Fet3p-containing vesicular compartment where the apo-protein is activated [16,21]. In the *Δctr1Δccc2*-containing cells the galactose-dependent activation of Fet3p was suppressed, indicating that the mechanism of this activation was Ccc2p-mediated. Note that the Fet3p protein produced carried a C-terminal FLAG epitope that was used to determine the amount of secreted protein [15]. The specific Fet3p activities given in Table 3 were based on this Western blot analysis (see the Materials and methods section; results not shown).

In parallel with these results on the activation of apo-Fet3p we performed plate assays on the ability of congenic yeast strains to grow on a non-fermentable carbon source (results not shown). The respiration-competence of *S. cerevisiae* is a simple measure of the cells iron status and, since iron uptake requires copper, on cell copper status as well. A strain lacking Ctr1p activity but otherwise wild-type is respiration-deficient; this deficiency can be rescued by supplementation with > 20 μM copper in the medium, but not by supplementation with iron [25]. This rescue by supplementation with copper provides physiological evidence for the epistatic relationship between copper and iron in yeast. For example, a strain carrying a deletion in *FET3* cannot be rescued in this fashion [25]. A galactose-dependence of respiratory capacity could not be tested since galactose is glycolytic. However, we could show that *Δctr1Δfet4*-containing strains consistently grew more slowly even on glucose than either single mutant. Furthermore, the growth deficit in the double mutant strain could not be suppressed unless at least 100 μM copper was added to the growth medium. This result was consistent with the limited copper uptake measured in this background, as shown in Figure 2 and in the *Δctr1Δfet4GAL1::FET4* cells grown in glucose (see Table 1).

Copper accumulated by Fet4p down-regulates the transcriptional activity of Mac1p

The second test of the normal trafficking and utilization of Fet4p-dependent cellular copper was quantification of the intracellular copper concentration that controlled the activity of the copper-dependent transcription factor Mac1p [9,13,25]. We reasoned that if copper delivered by Ctr1p and Fet4p was similarly metabolized, then Mac1p activity should be down-regulated at comparable intracellular copper levels irrespective of how the copper was taken into the cell. The data shown in Figure 3

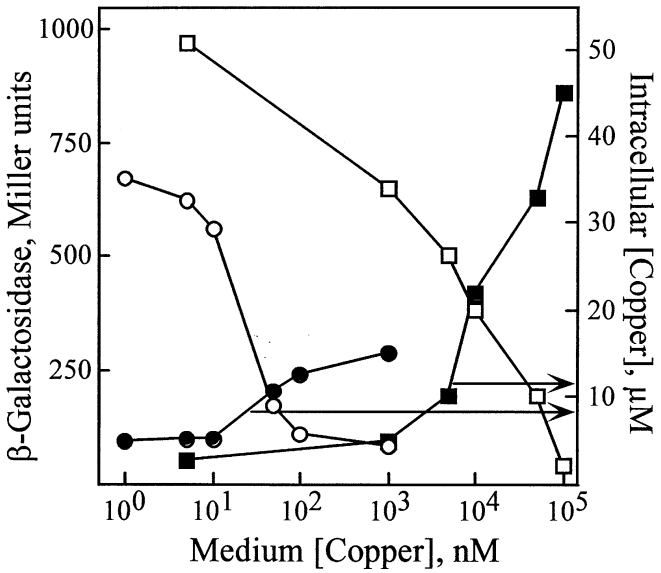

Figure 3 Intracellular copper concentration dependence of Mac1p transcriptional activity

CTR1FET4 wild-type strain DY1457 (circles) and *Δctr1FET4* strain ARY1457 (squares) carried a high-copy *lac*Z reporter plasmid under control of the Mac1p-dependent promoter from *CTR1* [9,25]. Cells were grown in a completely synthetic medium to early-exponential phase in the presence of the extracellular copper concentration indicated. They were then assayed for the amount of β-galactosidase produced as a measure of the transcriptional activity of Mac1p in the particular strain at the indicated extracellular copper concentration (open symbols, values on the left ordinate). Cells were also washed, counted, and digested in HNO_3 and the acid extract analysed for copper by flameless atomic absorption spectrophotometry. These copper mass values were then used to estimate an intracellular copper concentration using the measured cell number and a cell volume of 60 μm^3 [31]. These intracellular copper concentration values are given on the right ordinate, closed symbols (arrows to right). The data points represent the mean values from three experiments. Error bars are not shown in order to avoid cluttering the figure; the S.E.M. values ranged from ±4–9% of the mean.

support this prediction at least in part. In this experiment, Mac1p activity was indicated by expression of *lac*Z (β-galactosidase, Miller units) under the control of the promoter from *CTR1* (Figure 3). This episomal chimaeric reporter construct was transformed into Ctr1p-plus and Ctr1p-minus strains. Naturally, Mac1p in the *Δctr1*-carrying strain (which was copper-starved in comparison with the wild-type) exhibited a higher activity overall, and was less sensitive to addition of extracellular copper (Figure 3). However, the intracellular copper concentration-dependence of Mac1p activity was comparable in both Ctr1p-plus and Ctr1p-minus backgrounds (with both containing Fet4p) indicating that the copper accumulated via Fet4p was sensed by Mac1p. For example, the intracellular copper concentration that resulted in a 50% reduction in Mac1p activity in each case was approx. 10 μM (Figure 3). Another close parallel was that at approx. 4 μM intracellular copper, the β-galactosidase activity in the *CTR1* wild-type strain was 625 Miller units and in the *Δctr1*-containing mutant it was 560 Miller units. On the other hand, at higher copper concentrations this correlation became less rigorous, with the Mac1p activity in the *Δctr1*-containing strain retaining greater activity than in the wild-type strain. Nonetheless, the results of these latter two experiments were consistent with the conclusion that copper taken into the cell by Ctr1p-independent pathway(s) could be partitioned and metabolized as if it were transported into the cell by Ctr1p itself.

DISCUSSION

In yeast cells grown in standard rich medium [1% (w/v) yeast extract/2% (w/v) peptone/2% (w/v) glucose; YPD], Ctr1p-mediated, high affinity copper uptake in *S. cerevisiae* exhibits a K_m value for copper of 2 μM and a V_{max} of 10 pmol of copper/min per 10^6 cells [17]. Thus at 0.3 μM copper, which is the average copper concentration in standard YPD medium, the velocity of high affinity copper uptake is approx. 1 pmol of copper/min per 10^6 cells. At this concentration of copper and with normal expression of *FET4*, uptake through the low affinity pathway (K_m = 35 μM and V_{max} = 8 pmol of copper/min per 10^6 cells) is approx. $V_{max}/100$ (i.e. 0.07 pmol of copper/min per 10^6 cells), and does not contribute significantly to total copper accumulation. In contrast, at a copper concentration of 20 μM, i.e. at approx. $K_m/2$ (low affinity), this pathway does contribute greatly to total uptake ($V_{max}/3$, i.e. 2.7 pmol of copper/min per 10^6 cells). Put another way, the total copper uptake of a wild-type strain growing in normal YPD is kinetically equivalent to a strain that lacks high affinity, Ctr1p-mediated uptake growing in YPD supplemented with as little as 20 μM copper. This comparison is consistent with the fact that 20 μM copper is sufficient to suppress the growth defects in a *Δctr1*-containing mutant that result from the loss of copper-dependent high affinity iron uptake.

In the present study we report several kinetic experiments that indicate that low affinity copper uptake can be attributed to Fet4p. First, the K_m value for copper uptake in a Ctr1p-minus/Fet4p-plus strain is similar to the K_i value for copper inhibition of Fet4p-dependent iron uptake. Secondly, deletion of *FET4* abolishes the low affinity copper uptake in a wild-type or a Ctr1p-minus strain (Figure 2). Thirdly, overexpression of *FET4* in a galactose-dependent fashion makes the low affinity uptake of both iron and copper galactose-dependent too (Table 1). Fourthly, rescue of copper deficiency in a strain lacking Ctr1p, as indicated by failure to make holo-Fet3p, is Fet4p-dependent (Table 3).

Our data also suggest not only that Fet4p can support copper uptake, but, more significantly, that the copper taken up via this pathway can be used by the cell. The Fet4p-dependence of the activation of apo-Fet3p was one example of the similar handling of copper transported by Ctr1p and Fet4p. Analysis of the activity of the copper-dependent transcription factor, Mac1p, also indicated that copper accumulated via Fet4p could be metabolized as if it were taken up by Ctr1p. These two results suggest that the trafficking of copper to Atx1p, a copper chaperone [22,29], for delivery to the vesicular system carrying apo-Fet3p [16,21], or to Mac1p, most likely does not involve a direct role for Ctr1p. This inference follows from the fact that Fet4p, which has no sequence similarity to Ctr1p, appears to supply copper to this trafficking process in a quantitatively equivalent fashion. Indirectly, then, the results in the present study suggest that partitioning of newly arrived cell copper occurs subsequent to uptake, and is not directly linked to the transport process itself. That is, one model the data appear to exclude is a ligand exchange process between Ctr1p and a copper chaperone, Atx1p for example, which reasonably would be specific to Ctr1p and not to Fet4p. Such a ligand exchange mechanism for transfer of copper from a copper chaperone to its acceptor protein has been demonstrated [30]. Furthermore, yeast two-hybrid data using domains from Ctr1p as bait and either Atx1p (D. Thiele, personal communication) or Mac1p (A. Joshi and D. Kosman, unpublished work) as prey have been uniformly negative; no interactions have been demonstrated by this technique for any combination of protein fusions involving these two sets of potential partners. Obviously, neither the data in the present

study nor these negative results definitively exclude any mechanism for this copper trafficking let alone suggest one. Nonetheless, the data in the present study are the first to suggest that there may be a general 'holding' area for newly arrived copper that subsequently is the source of metal for the several pathways that target copper to various cellular compartments and/or proteins.

There is limited experimental data in support of this model, however. Size-fractionation of the cytosol of yeast cells transiently exposed to ^{64}Cu showed that the newly arrived ^{64}Cu was distributed into three pools. Two were associated with known copper proteins, Cu/Zn superoxide dismutase and copper thionein, while the third co-eluted with glutathione and Cu-His_2 [32]. This latter pool contained approx. 5% of the total cytosolic ^{64}Cu and could represent this 'holding' area. On the other hand, a recent estimate of the distribution of copper in the yeast cell indicated there was little if any 'free' (labile) ionic copper that would include copper complexed to low molecular-mass ligands [33]. These apparently conflicting findings suggest that there is some degree of compartmentalization of the copper in the yeast cell, either topologically and/or thermodynamically. Clearly, the copper trafficking model we suggest requires a direct experimental test that the work in the present paper does not provide.

The fact that copper inhibition of iron uptake is best described as simple non-competitive indicates that the initial (substrate) binding of the two metals ions, presumably Cu(I) and Fe(II), to Fet4p is not mutually exclusive. As noted above, this kinetic pattern suggests that Fet4p has ligand arrays for either metal ion that do not overlap but does not, by itself, suggest that these different arrays have inherent metal ion specificity. Another aspect of this inhibition is the K_i value of 22 μM determined from the Dixon plot and its comparison with the Michaelis constant for copper uptake of 35 μM. Strictly, K_i is the dissociation constant of Cu(I) from a Fet4p–Cu(I) binary complex, but in the case of simple non-competitive inhibition, it is also the dissociation constant of Cu(I) from the Fet4p–Fe(II)–Cu(I) ternary complex (one that might be involved in iron uptake). This indicates that Cu(I) binding to Fet4p does not alter the binding of Fe(II), although it does inhibit the subsequent transport of the Fe(II) bound. That is, Cu(I) could bind to a site on Fet4p that is distinct from the substrate binding site for Fe(II) and yet still inhibit iron uptake. On the other hand, copper bound in this way could be transported into the cell. This notion of functionally separate Cu(I) and Fe(II) binding sites is consistent with the data given in Table 2. The degree to which a given point mutation alters the kinetic behaviour of Fet3p towards Cu(I) in comparison with Fe(II) as a substrate for transport would reasonably depend on the degree to which that particular residue contributed to the reaction co-ordinate for the binding and uptake of one versus the other metal ion.

The fact that the *Δctr1Δctr3Δfet4*-containing strain exhibited no saturable copper uptake over the copper concentration range used (Figure 2) indicates that the corresponding metal ion permeases are solely responsible for the measurable facilitated copper uptake in a wild-type strain. Indirectly, these results suggest that other metal ion transporters that might be produced by *S. cerevisiae* play little if any role in the uptake of copper at the plasma-membrane. Thus the role that Smf1p, for example, might play in copper or iron uptake in yeast remains an open question. Production of Smf1p in *Xenopus* oocytes clearly results in a H^+-dependent iron uptake. This saturable uptake has a K_m value for Fe(II) of 2.2 μM [34]. There is no indication of this kinetic process in the iron uptake data in yeast [18,19]. This does not mean that Smf1p cannot or does not support iron (or copper) uptake in yeast, but it does indicate that its contribution, even in the *Δctr1Δctr3Δfet4*-containing strain, must be extremely limited or is expressed under conditions other than those employed in these experiments.

The marked mechanistic differences between the high and low affinity pathways for copper and iron uptake in yeast suggest much about the separable physiological roles of these pathways. In addition to the wide disparities in their K_m values for uptake is the fact that only the high affinity pathways are tightly metal-ion regulated and completely metal-ion specific. Thus the copper-regulated transcription factor, Mac1p, supports transcription of *CTR1* while the iron-regulated factor, Aft1p, supports transcription of *FET3* and *FTR1*. *FET4* expression is not dependent on either transcription factor. These differences are consistent with the likely role that the low affinity pathway plays. Since this latter pathway has such a high K_m value for either metal ion, it contributes only under conditions when a high affinity pathway or pathways are turned off. Furthermore, the fact that low affinity, Fet4p-dependent iron uptake is Fet3p-independent has strong selective advantage, because it uncouples the iron status of the cell from the copper status. This protects against the situation when both metals are in short supply, that is, even in the absence of Fet3p activity (due to low copper) there is still a way, albeit inefficiently, to get iron into the cell. Indeed, this perspective suggests that selection for physiological suppressors of the phenotypes resulting from a deletion in *FET3* (or in the gene encoding the high affinity iron permease, *FTR1*) would identify gene duplications of *FET4*, as is the case for metallothionein loci in many cell types, including yeast [35]. In fact, the *FET4* gene was originally identified as an overexpression suppressor of a *Δfet3* mutant [18].

Thus an overall model of copper and iron handling in yeast is as follows. This organism expresses specific pathways for the high affinity accumulation of the two metals when they are scarce. The components of both of these pathways are regulated by the level of the respective metal ion, with some regulatory overlap where there is functional overlap. Thus *CTR1* expression is regulated specifically by copper, while *FTR1* and *FET3* expression is regulated specifically by iron. This specificity is mediated in each case by a regulatory factor, Mac1p in the case of copper and *CTR1*, and Aft1p in the case of iron and *FTR1* and *FET3*. There is both overlap and specificity with the otherwise apparently redundant reductases in that *FRE1* expression is primarily Mac1p-dependent, while *FRE2* expression is Aft1p-dependent. This design allows for the expression of either pathway independent of the other in response to the specific level of each metal in the environment. However, the respective high affinity uptake pathways are essentially shut-down at medium iron or copper concentrations exceeding 10 μM. This is appropriate since both metals are toxic in excess; suppressing their uptake represents an important defense against these pro-oxidants. Nonetheless, the cells still have a nutritional dependence on both metals in all environmental conditions. Reasonably, it is the role of low affinity pathways like Fet4p to satisfy this need under conditions of nutrient excess.

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