

Fre1p Cu²⁺ Reduction and Fet3p Cu¹⁺ Oxidation Modulate Copper Toxicity in *Saccharomyces cerevisiae**

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Fre1p is a metalloreductase in the yeast plasma membrane that is essential to uptake of environmental Cu²⁺ and Fe³⁺. Fet3p is a multicopper oxidase in this membrane essential for high affinity iron uptake. In the uptake of Fe³⁺, Fre1p produces Fe²⁺ that is a substrate for Fet3p; the Fe³⁺ produced by Fet3p is a ligand for the iron permease, Ftr1p. Deletion of *FET3* leads to iron deficiency; this deletion also causes a copper sensitivity not seen in wild type. Deletion of *FTR1* leads to copper sensitivity also. Production in the *ftr1Δ* strain of an iron-uptake negative Ftr1p mutant, Ftr1p(RAGLA), suppressed this copper sensitivity. This Ftr1p mutant supported the plasma membrane targeting of active Fet3p that is blocked in the parental *ftr1Δ* strain. A ferroxidase-negative Fet3p did not suppress the copper sensitivity in a *fet3Δ* strain, although it supported the plasma membrane localization of the Fet3p-Ftr1p complex. Thus, loss of membrane-associated Fet3p oxidase activity correlated with copper sensitivity. Furthermore, *in vitro* Cu¹⁺ was shown to be an excellent substrate for Fet3p. Last, the copper sensitivity of the *fet3Δ* strain was suppressed by co-deletion of *FRE1*, suggesting that the cytotoxic species was Cu¹⁺. In contrast, deletion of *CTR1* or of *FET4* did not suppress the copper sensitivity in the *fet3Δ* strain; these genes encode the two major copper transporters in laboratory yeast strains. This result indicated that the apparent cuprous ion toxicity was not due to excess intracellular copper. These biochemical and physiologic results indicate that at least with respect to cuprous and ferrous ions, Fet3p can be considered a metallo-oxidase and appears to play an essential role in both iron and copper homeostasis in yeast. Its functional homologs, e.g. ceruloplasmin and hephaestin, could play a similar role in mammals.

There is a well recognized link between copper and iron metabolism. This link is illustrated by the copper dependence of high affinity iron uptake in the yeast *Saccharomyces cerevisiae* (1). This dependence is due to the role that copper plays as cofactor for the ferroxidase, Fet3p (2). Fet3p and the iron permease, Ftr1p, form a complex in the yeast plasma membrane (3). Genetic and biochemical studies show that in high affinity iron uptake Fet3p catalyzes the oxidation of Fe²⁺ to Fe³⁺;

Ftr1p then transports the Fe³⁺ into the cell (3–7). In humans, the Fet3p ortholog, ceruloplasmin, also catalyzes the oxidation of Fe²⁺ for iron release into circulation (see Ref. 8 and references therein). Hephaestin, a membrane-bound ceruloplasmin paralog, is required for iron efflux from the placenta and enterocytes in mammals (9). Thus, the copper-dependent ferroxidase activity characteristic of these members of the multicopper oxidase family has a conserved role in iron metabolism.

Studies of *FET3* suggest that this ferroxidase might also play a role in cellular defense against copper toxicity; that is, a yeast mutant carrying a disruption of the *FET3* gene was found to be sensitive to high concentrations of Cu²⁺ added to the medium. One explanation for this observation was that Fet3p functioned in copper detoxification by binding free copper, thus suppressing the pro-oxidant activity that this metal ion exhibits in the generation of oxygen free radicals (10). This copper-sensitive growth phenotype of *fet3Δ* cells did correlate with a higher intracellular copper concentration than that observed in wild type cells (11).

In light of these observations we considered whether changes in iron status affected overall copper homeostasis. Thus, we investigated the molecular etiology of the copper sensitivity of a *fet3Δ* mutant. We now have found that deletion of *FTR1* causes a comparable copper-sensitive phenotype. Expression in an *ftr1Δ* strain of an Ftr1p mutant protein that supports Fet3p-Ftr1p processing, including the activation of Fet3p and its plasma membrane localization but which is iron uptake-negative, suppressed the copper sensitivity of this mutant strain. On the other hand, localization of a ferroxidase-negative Fet3p to the plasma membrane failed to suppress this copper sensitivity. Biochemical studies using purified Fet3p showed that Fet3p catalyzes the oxidation of Cu¹⁺ to Cu²⁺. Last, deletion of the plasma membrane Cu²⁺ reductase *FRE1* suppressed the copper sensitivity exhibited by the *fet3Δ* strain, whereas suppression of copper uptake activities did not. These results show that the role Fet3p plays in the cellular defense against copper toxicity is most likely associated with a novel cuprous oxidase activity acting in the extracellular space. Reasonably, the other multicopper oxidases that are ferroxidases, such as ceruloplasmin, hephaestin, and the bacterial CueO protein (12–14), share this additional metaloxidase activity and thereby support a comparable Cu¹⁺ detoxification pathway in their respective organismal milieu.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Wild type strain BY4741 and BY4741*fet3Δ* and BY4741*ftr1Δ* mutant strains were kindly provided by D. R. Winge and G. Hartzog (the parental strain is described in Rutherford *et al.* (15)). Strain 42C (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ftr1Δ::TRP1*) was a gift from A. Dancis (3). Isogenic strains DEY1457 (*MATα can1 his3 leu2 trp1 ura3 ade6*), DEY1394 (*MATα can1 his3 leu2 trp1 ura3 ade6 fet3::HIS*),

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DEY1457*fre1Δ* (*MATα can1 his3 leu2 trp1 ura3 ade6 fre1::URA3*), DEY1394 *fre1Δ* (*MATα can1 his3 leu2 trp1 ura3 ade6 fre1::URA3 fet3::HIS3*) were used for analyzing the suppressive effect of *FRE1* deletion on the copper sensitivity of the *fet3Δ* mutant. Strains ZY126 (*MATα can1 his3 leu2 trp1 ura3 ade6 fet4::LEU2 fet3::HIS3 Δctr1::URA3*) and ZY127 (*MATα can1 his3 leu2 trp1 ura3 ade6 fet4::LEU2 fet3::HIS3 Δctr1::URA3*) were used for analyzing the effect of *FET4* and *CTR1* deletions on suppressing copper sensitivity of the *fet3Δ* strain. ZY126 and ZY127 were derived by one-step homologous recombination at the *CTR1* locus in DEY1422 and DEY1422*fet3Δ*, respectively. Strain AMR76 was derived from DEY1394; it contained *ftr1::TRP1* as well as *fet3::HIS3* and was used in the *in situ* confocal fluorescence co-localization studies. The parental strains, DEY1457 and DEY1394, were provided by D. Eide (16). The *CTR3* locus in all of these strains was non-functional due to a Ty2 transposon insertion (17). Plasmids YEp351, YEp351FTR1, and YEp351(RAGLA) were generously provided by A. Dancis (3). pDY133FET3 and pDY133FET3(T1D) were derived from pDY134, a low copy plasmid that contains the *FET3* transcription unit (6).

Analysis of Copper Resistance—The various wild type and mutant strains were streaked onto agar containing synthetic complete (SC)¹ medium without or with CuSO₄ or with both CuSO₄ and (NH₄)₂Fe(SO₄)₂. The cells were incubated at 30 °C for 3 days and photographed. Transformants of strain 42C carrying YEp351, YEp351FTR1, or YEp351FTR1(RAGLA) were streaked onto agar containing the appropriate drop-out synthetic medium (–Leu). The copper resistance of these cells was determined as above.

Kinetic Analysis of Metallo-oxidation by Fet3p, ⁵⁹Fe Uptake, and Cell Localization—Soluble forms of wild type Fet3p and of Fet3p(T1D) were purified as described (6, 18, 19). Cuprous ion oxidation was determined by oxygen consumption using the Oxygraph from Hansatech (www.hansatech-instruments.co.uk) as described (6, 19). Stock solutions of cuprous chloride (Alfa) (in acetonitrile) and ferrous ammonium sulfate (Sigma) were freshly prepared in nitrogen-purged buffers; all transfers from this stock solution were done using gas-tight syringes. The buffer used for O₂ uptake measurements was air-saturated, pH 5.0, 0.1 M MES containing 5% (v/v) acetonitrile (final volume percent). ⁵⁹Fe uptake was performed in strain DEY1394 (*fet3::HIS3*) transformed with empty vector, pDY133FET3, or pDY133FET3(T1D) as described (19). Localization of Fet3p:GFP in live cells (transformants of AMR76) producing wild type Ftr1p and the Ftr1p(RAGLA) mutant or of Ftr1p:YFP in cells producing wild type Fet3p or Fet3p(T1D) was imaged using a Bio-Rad MRC 1024 confocal system equipped with a 15-megawatt krypton/argon laser and operating on a Nikon Optiphot upright microscope and an oil immersion 60× 1.4NA objective. Optical sections were acquired at 0.5 μm, and the XY resolution was set at ≥0.2 μm using the Bio-Rad Lasersharp V3.0 software and later processed using Confocal Assistant V4.02. Fet3p:GFP was produced from plasmid pDY133FET3:GFP, and Ftr1p:YFP was produced from plasmid p703FTR1:YFP (19).

RESULTS

FET3 or FTR1 Deletion Induces a Copper Sensitivity in Yeast—Deletion of *FET3* leads to two discernible phenotypes, at least, iron deficiency (2, 3) and copper sensitivity (10, 11). Reasonably, the copper sensitivity could arise from either the lack of Fet3p activity specifically or from the iron deficiency. To test if iron deficiency was the cause, we examined the copper sensitivity of an *ftr1Δ* strain, since this deletion causes an iron deficiency equivalent to that observed in the *fet3Δ* one (3). We screened first for this iron deficiency by defective growth on medium containing a non-fermentable carbon source (ethanol, YPE), an iron-demanding growth condition (Fig. 1, panel A). As expected, both mutant strains exhibited little if any respiratory growth, although they and the wild type grew equally well on agar containing synthetic complete medium with glucose as SC (panel B). However, on the same medium containing CuSO₄, the *ftr1Δ* strain, like the *fet3Δ* mutant, exhibited a copper-sensitive growth phenotype; the mutant cells, but not the wild type ones, exhibited a complete growth arrest at 800 μM Cu²⁺ as shown in Fig. 1, panel C.

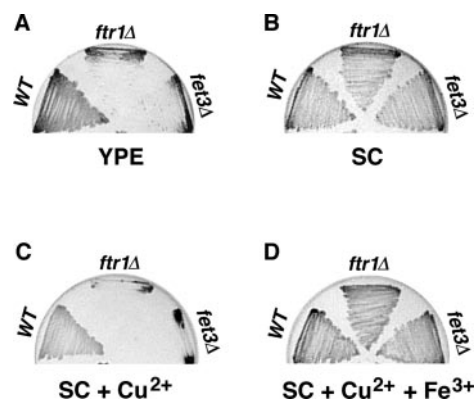


FIG. 1. Cells lacking Ftr1p or Fet3p are respiration deficient and copper sensitive. Isogenic strains BY4741 (WT), BY4741*ftr1Δ*, and BY4741*fet3Δ* were streaked onto agar containing YPE medium alone (panel A), SC medium alone (panel B), and SC medium containing either Cu²⁺ (800 μM) (panel C) or Cu²⁺ (800 μM) and Fe³⁺ (50 μM) (panel D). Cells were incubated at 30 °C for 3 days and photographed.

These data not only confirmed the previous reports with respect the *fet3Δ* strain (10, 11) but demonstrated that deletion of either of the two genes encoding the components of the high affinity iron uptake complex induced a comparable copper sensitivity. Of course this result did not resolve the question of the molecular basis of this sensitivity. For example, if iron deficiency was the cause, iron application should rescue the growth of these two mutant strains in the presence of otherwise cytotoxic levels of copper. Indeed, we found that iron did rescue this growth defect (Fig. 1, panel D). These data parallel a report that showed that iron addition increased the growth rate of *fet3Δ* cells in a liquid medium containing a high concentration of copper (11). On the other hand, these results did not exclude the possibility that the absence of active Fet3p in the two mutant strains contributed to the copper sensitivity. Note that the activation of Fet3p by the addition of its prosthetic group copper atoms requires the co-production and membrane targeting of an Ftr1p protein (3). Thus, both mutant strains were equivalently deficient in Fet3p activity.

Copper Sensitivity Correlates with the Loss of Fet3p Activity—Thus, we directly investigated whether loss of Fet3p activity in the plasma membrane was a contributing factor in the copper sensitivity exhibited by *fet3Δ* strains. As noted above, Stearman *et al.* (3) demonstrate that deletion of *FTR1* resulted also in a loss of Fet3p oxidase activity. That is, the *ftr1Δ* strain, like the *fet3Δ* mutant, is iron-deficient and devoid of Fet3p oxidase activity (3). Investigating the copper sensitivity in the *ftr1Δ* strain gave us the means to assess the relative contributions to this sensitivity of iron deficiency *versus* the absence of plasma membrane Fet3p activity.

To distinguish between these two mechanisms we produced in the *ftr1Δ* strain, strain 42C, either the wild type Ftr1p or an uptake-negative Ftr1p mutant, Ftr1p(RAGLA), using plasmids YEp351FTR1 and YEp351FTR1(RAGLA) (3). Stearman *et al.* (3) demonstrate that high affinity iron uptake was virtually absent in strain 42C when it produced the Ftr1p(RAGLA) mutant as its sole Ftr1p protein, whereas Fet3p oxidase activity was unaffected. This defect in iron uptake is shown here in Fig. 2, panel A, as judged by the extremely slow growth of strain 42C carrying mutant Ftr1p(RAGLA) on the non-fermentable carbon source (ethanol). This respiratory defect was suppressed by iron supplementation as expected (Fig. 2, panel B). In contrast to this failure of Ftr1p(RAGLA) to rescue the iron uptake deficiency in the *ftr1Δ* strain, this iron uptake-negative Ftr1p mutant did suppress the copper sensitivity of this strain (Fig. 2, compare panel A with panel D). This growth

¹ The abbreviations used are: SC medium, synthetic complete medium; MES, 4-morpholineethanesulfonic acid; GFP, green fluorescent protein.

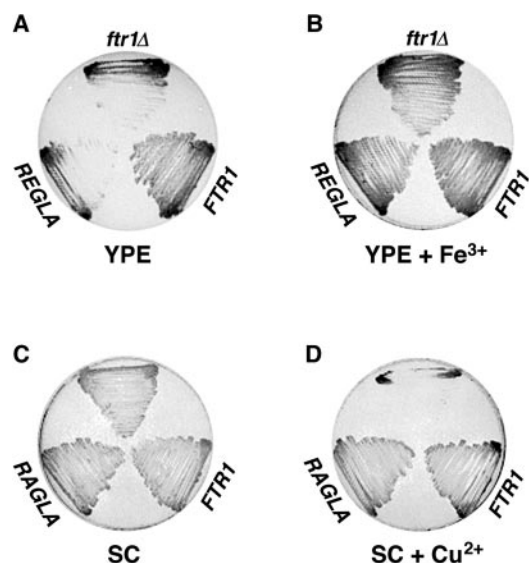


FIG. 2. Loss of Fet3p activity, not iron-deficiency, leads to copper sensitivity. Panels A and B, the *ftr1Δ* strain 42C bearing plasmid YEp351, YEp351FTR1, or YEp351RAGLA was streaked onto yeast growth medium containing a non-fermentable carbon source ethanol (YPE) without (panel A) or with FeCl_3 ($50 \mu\text{M}$, panel B). Panels C and D, the same set of transformants were streaked onto agar containing SC medium in the absence (panel C) or presence (panel D) of exogenous CuSO_4 ($800 \mu\text{M}$). These cells were incubated at 30°C for 3 days and photographed.

pattern indicates that iron deficiency *per se* does not correlate with the copper sensitivity exhibited by the *ftr1Δ* mutant. This pattern is consistent, however, with the inference that the copper sensitivity is linked to the lack of active Fet3p in the yeast plasma membrane in this *ftr1Δ* mutant strain.

Stearman *et al.* (3) demonstrate that the Fet3p produced in a strain producing Ftr1p(RAGLA) was enzymatically active. That this Fet3p was trafficked normally to the plasma membrane was demonstrated directly by *in situ* confocal fluorescence microscopy. In this experiment, wild type Ftr1p or the Ftr1p(RAGLA) mutant was co-produced with Fet3p:GFP. As the image in Fig. 3, panel C demonstrates, this mutant Ftr1p supports the plasma membrane localization of Fet3p comparable with that of wild type Ftr1p (panel B); in the absence of any Ftr1p, Fet3p:GFP fails to localize to the plasma membrane (panel A). These results indicate that the copper sensitivity of *fet3Δ* and *ftr1Δ* mutants correlates most strongly with the absence of Fet3p in the yeast plasma membrane and not with the iron deficiency of these strains.

Fet3p Catalyzes Oxidation of Cu^{1+} to Cu^{2+} Ions—Currently, Fet3p is known only for its role in the oxidative conversion of Fe^{2+} to Fe^{3+} for iron uptake (1, 4, 5, 7). The results above suggested that we attempt to determine if Fet3p was catalytically active with Cu^{1+} as substrate as well. Oxidation of Cu^{1+} would naturally diminish copper toxicity because Cu^{1+} is known to be more toxic than Cu^{2+} (20). Indeed, wild type Fet3p is an active “cuprous” oxidase as demonstrated by the Fet3p-catalyzed O_2 uptake supported by Cu^{1+} as substrate (Fig. 4, panel A). In a standard velocity *versus* $[\text{Cu}^{1+}]$ analysis employing an O_2 electrode to measure oxygen consumption (6, 19), the kinetic constants for Cu^{1+} turnover have been determined: $K_m = 37.9 \pm 3.6 \mu\text{M}$, and $k_{\text{cat}} = 79.2 \pm 2.7 \text{ min}^{-1}$.² These values can be compared with those for Fe^{2+} oxidation under the same conditions: $K_m = 5.4 \mu\text{M}$, and $k_{\text{cat}} = 63.9 \text{ min}^{-1}$.² Importantly, a ferroxidase-negative Fet3p mutant, Fet3p(T1D), failed to catalyze this oxidation of Cu^{1+} (Fig. 4, panel A). This mutant

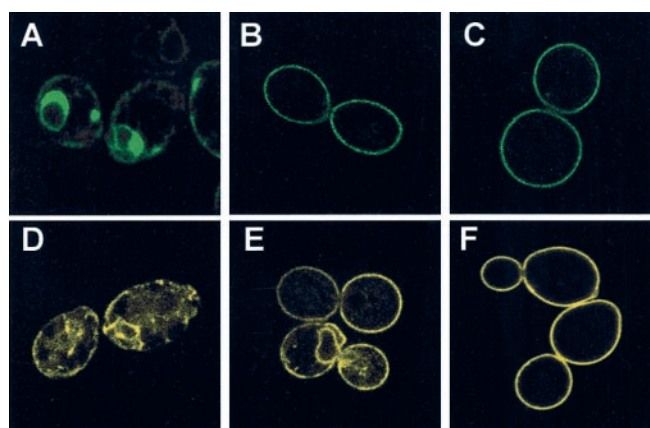


FIG. 3. Fluorescent fusion proteins localize Ftr1p and Fet3p alleles to the plasma membrane. Strain AMR76(*fet3Δftr1Δ*) was transformed with plasmid pDY133FET3:GFP (panels A–C) or with p703FTR1:YFP (panels D–F). Live cells were imaged with a $60\times$ (oil) as described under “Experimental Procedures.” The three cell samples transformed with pDY133FET3:GFP were co-transformed with empty vector (A) YEp351FTR1 (B), and YEp351RAGLA (C). The three cell samples transformed with p703FTR1:YFP were co-transformed with empty vector (D), pDY133FET3 (E), and pDYFET3(T1D) (F). Note that in the absence of the partner protein, the fluorescent fusion remained intracellular (panels A and D); with co-production of the partner protein, wild type or mutant, the fusion was trafficked to the plasma membrane (panels B and E and C and F, respectively).

contains a C484S substitution and so lacks the type 1 copper required for the initial electron transfer from the reducing substrate; it retains the remaining three copper atoms found in wild type Fet3p, however (18). These results suggest that Fet3p most reasonably could suppress copper toxicity by catalyzing the conversion of Cu^{1+} to the less toxic Cu^{2+} rather than by serving as a “sink” for excess cell copper.

A Cuprous Oxidase-negative Fet3p Does Not Protect against Copper Sensitivity—This conclusion was tested by evaluating the ability of the cuprous oxidase-negative Fet3p(T1D) to suppress the copper sensitivity of a *fet3Δ* strain. We first demonstrated that this mutant Fet3p failed to support iron uptake in this background (Fig. 4, panel B); whereas wild type Fet3p conferred ^{59}Fe uptake to this strain (closed circles), the Fet3p(T1D) mutant did not (open circles). Uptake by this transformant was not different from the vector-only control (\times 's). On the other hand, this protein did support the trafficking of the Ftr1p-Fet3p(T1D) complex to the plasma membrane. This was determined by co-expression of either wild type Fet3p or Fet3p(T1D) and Ftr1p:YFP (Fig. 3, panels D–F). In the absence of any Fet3p protein, the Ftr1p:YFP fusion remained intracellular (panel D); in contrast, in the presence of either wild type Fet3p or Fet3p(T1D), this fusion protein was strongly localized to the plasma membrane (panels E and F, respectively). We can conclude from these results that a strain producing Fet3p(T1D) has a structurally intact but functionally inactive Fet3p-Ftr1p complex in the plasma membrane.

This mutant Fet3p did not suppress the copper sensitivity observed for the *fet3Δ* strain (Fig. 5). Whereas transforming this deletion strain with pDY133FET3(WT) suppressed the copper sensitivity, transformation with pDY133FET3(T1D) did not (Fig. 5, panel B). This result is consistent with the hypothesis that the catalytic activity of Fet3p correlates with this protein's link to copper resistance in *S. cerevisiae*, most reasonably as a result of the cuprous oxidase activity demonstrated above.

Deletion of the Cu^{2+} Reductase FRE1 Suppresses the Copper-sensitive Growth Phenotype of *fet3Δ* Cells—The cell surface metalloreductase Fre1p catalyzes reduction of Cu^{2+} to Cu^{1+} for

² C. Stoj and D. Kosman, manuscript submitted for publication.

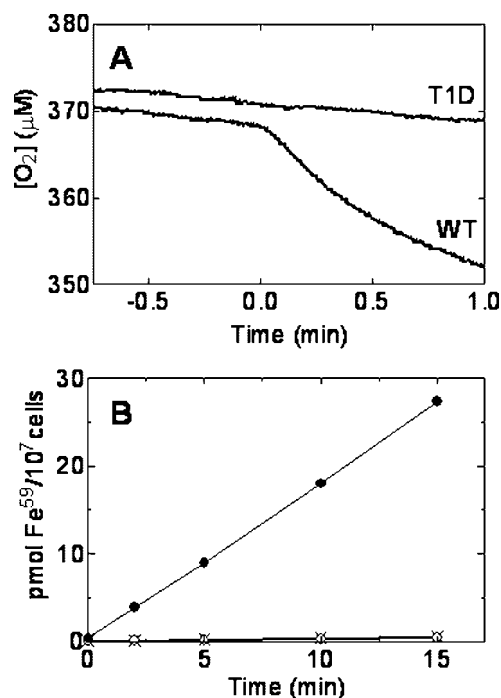


FIG. 4. *In vitro* cuprous oxidase activity of Fet3p and its correlation with ⁵⁹Fe uptake. O₂ uptake data (panel A) were obtained using an Oxygraph oxygen electrode system and the associated OXYGRAPH software (Hansatech). The buffer used for O₂ uptake measurements was air-saturated, pH 5.0, 0.1 M MES containing 5% (v/v) acetonitrile (final volume percent). The [protein] = 0.75 μM and was added at time 0 in the figure; the [Cu¹⁺] = 300 μM, essentially V_{max} conditions for wild type (WT) Fet3p (see “Results”). The O₂ uptake traces for wild type Fet3p and the ferroxidase-negative Fet3p(T1D) (8) mutant are as indicated. This mutant represented a control demonstrating that the cuprous oxidase activity was truly a characteristic of catalytically active Fet3p. Panel B, the lack of ⁵⁹Fe uptake activity in DEY1394 (*fet3Δ*) producing ferroxidase-negative Fet3p(T1D) (open circles) is compared with the same strain producing wild type Fet3p (closed circles). The (lack of) ⁵⁹Fe uptake in the vector-only negative control is given by the ×’s.

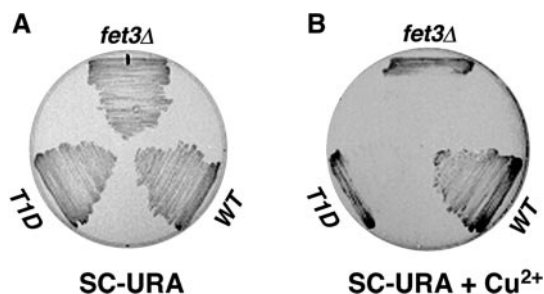


FIG. 5. Copper sensitivity in *fet3Δ* is not suppressed by a cuprous oxidase-negative Fet3p mutant. Strain DEY1394 (*fet3Δ*) was transformed with the empty vector pRS416 (transformant indicated as *fet3Δ*), pDY133FET3 (WT), and pDY133FET3(T1D) (T1D) plasmid, respectively. Cells were streaked onto agar containing SC-URA medium alone (panel A) or SC-URA medium containing Cu²⁺ (800 μM, panel B). Cells were incubated at 30 °C for 3 days and photographed.

copper uptake as well as reducing Fe³⁺ to Fe²⁺ for iron uptake (6, 7). If the role of Fet3p in copper sensitivity is linked to its activity toward Cu¹⁺, then as the source of this Cu¹⁺, Fre1p should be epistatic to Fet3p in this copper sensitivity. We tested this inference by comparing the copper sensitivity of wild type (WT), *fet3Δ*, *fre1Δ*, and *fet3Δ fre1Δ* cells. These isogenic strains grew equally well on SC plates (Fig. 6, panel A). In the presence of exogenous copper, the *fet3Δ* mutant exhibited, as expected, a severe growth defect; in contrast, under the same conditions, the *fet3Δ fre1Δ* double deletion mutant was able to

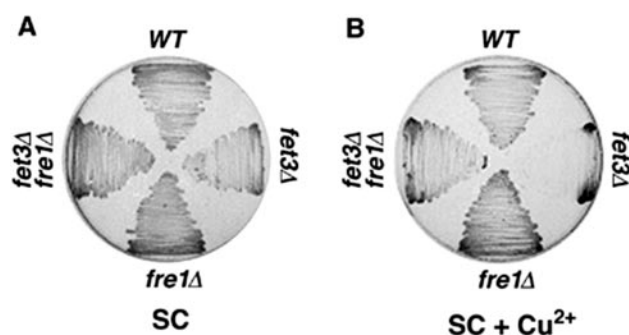


FIG. 6. Copper sensitivity in *fet3Δ* is suppressed by deletion of FRE1. Strains carrying targeted gene deletions as indicated were streaked onto agar containing SC growth medium without (panel A) or with CuSO₄ (700 μM, panel B). The cells were incubated at 30 °C for 3 days and photographed. WT, wild type.

grow as well as the wild type and *fre1Δ* strains, exhibiting little if any growth defect in this assay (Fig. 6, panel B). This growth difference between the *fet3Δ* and *fet3Δ fre1Δ* strains demonstrated that deletion of *FRE1* significantly suppressed the copper sensitivity of the *fet3Δ* cells. This result supports the model that Fet3p suppresses copper toxicity by catalyzing the conversion of Fre1p-produced Cu¹⁺ to Cu²⁺.

Overproduction of Fre1p Induces Copper Sensitivity; Deletion of Genes Encoding Copper Uptake Proteins Does Not—The distinguishing aspect of this model is that the copper sensitivity in the *fet3Δ* strain results from a defect in the handling of copper on the extracellular surface of the plasma membrane rather than as a consequence of aberrant copper accumulation. This model predicts that overproduction of Fre1p, and, therefore, of Cu¹⁺ at this surface would exaggerate copper sensitivity, whereas deletion of genes encoding copper transport proteins would not. We tested these two interrelated predictions by taking advantage of the *MAC1^{up1}* allele (21, 22). Mac1p is a transcriptional activator of the *CTR1* and *FRE1* genes that is repressed by copper in the growth medium (21, 23–25); Mac1p, therefore, is the key player in control of copper homeostasis in yeast. The *Mac1^{up1}* protein carries a single point mutation that confers to the protein a copper-independent, constitutive transcriptional activity. Thus, *FRE1* is strongly expressed in a strain carrying this allele (21, 22); this strain exhibits a strongly elevated copper (and iron) reductase activity (22) and copper sensitivity (21). This latter phenotype is illustrated in Fig. 7, panel B, and is consistent with the prediction our model affords. The model is further supported by the fact that deletion of *FRE1* in the *MAC1^{up1}* background suppressed this strain’s copper sensitivity (Fig. 7, panel B).

However, this result does not exclude the possibility that the copper toxicity observed in either *fet3Δ* or *fre1Δ* cells or in the *MAC1^{up1}* strain was linked also to an increase in copper accumulation. Increased copper accumulation has been reported in *fet3Δ* cells (11) and in yeast carrying the *MAC1^{up1}* allele (22). As noted above, *CTR1* is strongly up-regulated in the *MAC1^{up1}* background. To assess the contribution that increased copper uptake might make to the copper-sensitive phenotypes documented above, we carried out two experiments. First, we assessed whether deletion of *CTR1* suppressed the copper sensitivity linked to the constitutively active *Mac1^{up1}*. Our results show clearly that, unlike the suppressive effect of *FRE1* deletion (Fig. 7, panels A and B), deletion of *CTR1* had little or no impact on the copper sensitivity associated with the gain-of-function Mac1 protein (Fig. 7, panels C and D); this result has been noted previously (27). Second, we determined whether deletion of *CTR1* or of the low affinity copper transporter, *FET4* (26), could suppress the copper sensitivity in the *fet3Δ*

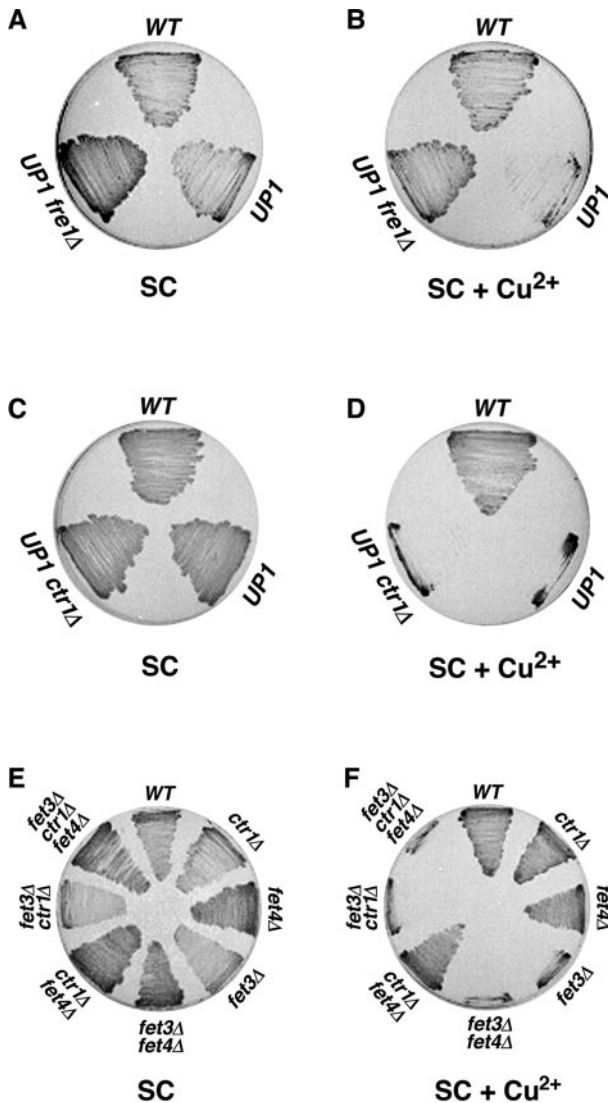


FIG. 7. Copper sensitivity in the $MAC1^{up1}$ and $fet3\Delta$ backgrounds is suppressed by deletion of $FRE1$ but not by deletion of genes encoding copper transport proteins. Strains carrying targeted gene deletions as indicated were streaked onto agar containing SC growth medium without (panels A, C, and E) or with $CuSO_4$ ($700 \mu M$, panels B, D, and F). The cells were incubated at $30^\circ C$ for 3 days and photographed. WT, wild type.

strain. The results of this experiment are shown in Fig. 7, panels E and F. As demonstrated by the plate shown in Fig. 7, panel F, deletion of $FET4$ alone, of $CTR1$ alone, or of both loci was without effect on the copper sensitivity exhibited by the $fet3\Delta$ strain. These two sets of results strongly support our model in two ways. They indicate first that it is the increase in Fre1p metalloredox and not the increase in Ctr1p-dependent copper uptake that leads to the copper sensitivity of the $MAC1^{up1}$ strain. Second, they demonstrate that $FRE1$ and not $CTR1$ (nor $FET4$) interact physiologically with $FET3$ in yeast copper homeostasis. Note that in all of the backgrounds used for the experiments in this work, the $CTR3$ locus was inactivated by a Ty2 insertion found in most laboratory strains of *S. cerevisiae* (17). Ctr3p, when produced, exhibits a copper uptake activity comparable with that supported by Ctr1p (17).

DISCUSSION

High affinity copper uptake through Ctr1p (and Ctr3p) in *S. cerevisiae* is preceded by reduction of Cu^{2+} to Cu^{1+} , a process mediated by the cell surface metalloreductase, Fre1p (7, 22).

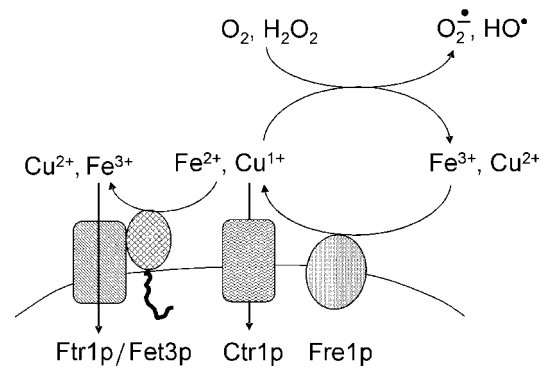


FIG. 8. Model of copper and iron metabolism in *S. cerevisiae*. Note the dual roles of Fre1p and Fet3p. Fre1p generates Fe^{2+} and Cu^{1+} for high affinity uptake of both metal ions; Fet3p targets the Fe^{2+} for iron uptake through ferroxidation and buffers the Cu^{1+}/Cu^{2+} ratio through cuprous oxidation.

Here, we have described the reverse of that process in which Cu^{1+} is apparently re-oxidized to Cu^{2+} , a process catalyzed by the cell surface ferrous oxidase (ferroxidase) Fet3p (2, 5–7). That is, we suggest that the cuprous oxidase activity of Fet3p we have demonstrated here suppresses the toxicity of copper present in the growth medium. The suppression of the copper-sensitive growth phenotype of the $fet3\Delta$ cells by the simultaneous deletion of $FRE1$ is consistent with this suggestion. In summary, our data suggest the more general conclusion that copper homeostasis is established in part by the physiologic interplay between the cupric reductase, Fre1p, and the cuprous oxidase, Fet3p, as depicted in Fig. 8.

These inferences are supported by the following data. First, we suppressed the copper sensitivity of an $ftr1\Delta$ strain by production in this strain of the Ftr1p(RAGLA) mutant. Stearman *et al.* (3) show that the Ftr1p(RAGLA) mutant was inactive in iron uptake. We confirmed this by demonstrating the respiration deficiency exhibited by the $ftr1\Delta$ strain producing this mutant protein, a growth deficiency corrected by the addition of iron salt to the medium. Stearman *et al.* (3) demonstrate also that this mutant Ftr1p did support the processing and activation of Fet3p. We now have shown that this Fet3p is localized to the plasma membrane. Taken together these data indicate that the loss of $FET3$ function in the plasma membrane, but not the iron deficiency that results from this loss, is causal in the copper sensitivity in the $fet3\Delta$ and $ftr1\Delta$ strains.

We have supported this conclusion by the demonstration that purified Fet3p catalyzes the oxidation of Cu^{1+} to Cu^{2+} ions *in vitro*. Our O_2 uptake data show that Fet3p is an excellent cuprous oxidase with an efficiency with Cu^{1+} as substrate that is only slightly less than that exhibited with Fe^{2+} as substrate. This fact is of particular significance because Cu^{1+} is known to be more toxic to cells than Cu^{2+} (20). Furthermore, the suppression of the copper-sensitive growth phenotype of $fet3\Delta$ cells by $FRE1$ deletion offers additional support to the model that Fet3p protects cells from the deleterious effects of copper by converting Cu^{1+} generated by Fre1p to the less toxic Cu^{2+} redox state of this metal ion. The apparent genetic interaction between $FRE1$ and $FET3$ that we have inferred provides direct evidence that copper homeostasis is based partly on the modulation of the redox balance of this metal ion.

Oxidation of Cu^{1+} by Fet3p would diminish the potential toxic effects of copper ions. In addition, the oxidation could also deprive Ctr1p of its substrate, Cu^{1+} . Therefore, the oxidation of Cu^{1+} might also function as a mechanism for limiting copper uptake by Ctr1p. Consistent with this notion, the Kaplan group found that a $fet3\Delta$ mutant accumulated more copper than the wild type (11). On the other hand our genetic data indicate that

copper uptake *per se* is not responsible for the copper-sensitive phenotype of the *fet3Δ* strain since deletion of the genes encoding the primary copper transporters found in most laboratory strains of *S. cerevisiae*, *CTR1*, and *FET4* had no mitigating effects. Therefore, the data are consistent with the inference that the increased accumulation of copper in the *fet3Δ* background reflects an increase in the steady-state level of Cu^{1+} available to Ctr1p. However, our genetic and biochemical data indicate that, most reasonably, it is the increased extracellular Cu^{1+} that is linked to the copper sensitivity exhibited by this strain, not the increased copper accumulation, since deletion of *CTR1*, in particular, was without protective effect.

Our data cannot fully exclude the possibility that Fet3p also detoxifies copper by scavenging copper ions, as proposed by the Thiele group (10). Note, however, that the cuprous oxidase-negative Fet3p mutant, Fet3p(T1D), did not complement in a *fet3Δ* strain. The Fet3p(T1D) protein retains three of the four copper atoms found in wild type Fet3p (6, 18); there is no evidence to suggest that the type 1 copper that is missing in this protein might have some role in copper metabolism not shared by the type 2 and type 3 copper atoms. Rather, this result is most easily understood in terms of a catalytic rather than copper binding mechanism of Fet3p protection against environmental copper.

Because both Fre1p and Fet3p are known to be crucial for iron uptake, the current work, thus, places these two proteins at the interface between copper and iron homeostasis (Fig. 8). In response to changes in iron conditions, *FRE1* and *FET3* are regulated similarly, with their transcription enhanced under iron deficiency and repressed when iron is in excess. *FRE1* expression is also altered when copper concentration changes; similar to *CTR1* expression, *FRE1* transcription is up-regulated under copper deficiency and repressed when copper is replete (22, 28, 29). More recent reports from Thiele and co-workers (30) and Winge and co-workers (31) have describe that *FET3* expression is modulated in response to changes in copper conditions as well, but in a fashion opposite to the response exhibited by the *CTR1* and *FRE1* loci; copper deficiency represses *FET3* transcription, whereas copper excess enhances it (30, 31). This transcriptional behavior suggests that there is regulatory coordination between *FET3* and *FRE1/CTR1* in response to a changing copper environment. In fact, we have found that *CTR1* expression is modulated by changes in iron status.³ These several results may reflect some coordination between copper and iron homeostasis; we are currently investigating the molecular bases of this apparent coordination.

Eukaryotes produce quantities of Cu^{1+} in the course of normal copper metabolism via the action of metalloreductases. In *S. cerevisiae* this reductase is the Fre1p protein (7, 32); in the mammalian epithelial cell, it is the Dcytb protein (33). Obviously, as an enzyme product, this Cu^{1+} has the potential to equilibrate with bulk solvent; that is, it can be considered “free.” This certainly is the case in the yeast Cu^{2+} reduction/copper uptake system where cuprous copper for uptake can come directly from the bulk solvent and not necessarily from Fre1p (22). In short, eukaryotic cells normally produce a fairly aggressive pro-oxidant. In this context, our model predicts generally that an imbalance between Cu^{1+} production and turnover would lead to an exaggerated copper sensitivity. This prediction appears born out by the yeast mutant carrying a gain-of-function mutation in the *MAC1* gene (21–25). Mac1p is a transcriptional activator of expression of *CTR1* and *FRE1*, whose activity is repressed by $<1 \mu\text{M}$ copper in the growth medium. The *MAC1^{up1}* allele encodes a single point mutation

that makes the Mac1^{up1} protein insensitive to copper; therefore, Mac1^{up1} constitutively activates transcription of the *CTR1* and *FRE1* genes, thus, strongly up-regulating plasma membrane reductase activity and copper uptake (22). In the context of our model we have shown here that the copper sensitivity exhibited by strains expressing the *MAC1^{up1}* allele is not, apparently, due to an increase in intracellular copper. If it were, then deletion of *CTR1* would be expected to suppress the copper sensitivity to a significant extent. In fact, such deletion had no such effect. Of note is the fact that in addition to activating transcription from *CTR1* and *FRE1*, the Mac1^{up1} protein appears to repress transcription from the *FET3* locus (30). Thus, the *MAC1^{up1}* carrying strain may be doubly at risk to environmental copper; that is, increased Cu^{1+} production together with decreased Cu^{1+} turnover. Note, however, that the repression by Mac1^{up1} of *FET3* expression has not been consistently observed (31).

Our data indicate that iron deficiency is not the sole or primary cause of the copper sensitivity observed in *fet3Δ* and *ptr1Δ* strains. This inference follows most directly from the fact that the copper sensitivity is suppressed by the trafficking of active Fet3p to the plasma membrane along with the iron uptake-negative Ftr1p(RAGLA) mutant. What then is the explanation for the fact that iron supplementation suppressed the copper sensitivity of the iron uptake-deficient yeast mutants? One explanation of this latter observation is that iron protects from copper toxicity at the level of Fre1p; Fe^{3+} competes with Cu^{2+} for reduction by this cell-surface reductase. In fact, the reactivity of Fe^{3+} with Fre1p is ~ 70 -fold greater than the reactivity of Cu^{2+} when both are present at $40 \mu\text{M}$ (22). To the extent that this difference reflects a greater affinity by Fre1p for Fe^{3+} , a significant inhibition by Fe^{3+} of the concurrent reduction of Cu^{2+} can be predicted. In relation to the relative cytotoxicity of the products of this Fre1p reaction, Cu^{1+} and Fe^{2+} , it is informative that the $\text{Cu}^{2+}/\text{Cu}^{1+}$ cycle is far more reactive under O_2 . As an example of the one-electron oxidative cycle that copper supports more efficiently in comparison to iron, dihydroascorbate is essentially stable in the presence of $10 \mu\text{M}$ Fe^{3+} , whereas this one-electron reductant is completely turned over in the presence of $10 \mu\text{M}$ Cu^{2+} (34). Cytotoxicity derives from this reaction by the production of the one-electron reduction products of O_2 that this redox cycle generates, primarily the superoxide and hydroxyl radicals.

The suggestion that Fet3p plays a specific physiologic role in preventing this one-electron cycle via its four-electron reduction of O_2 to water using 4Cu^{1+} as substrate is predicated on the assumption that there is a significant steady-state level of reduced metal species at the cell surface due to the Fre1p reaction. This situation would require that the relative velocities of the two processes involved in copper uptake, reduction and transport, and the three involved in iron uptake, reduction, ferrooxidation, and transport, were $V_R > V_F > V_T$, with the ferrooxidation step included for the case of iron. In fact, for iron these relative velocities are ~ 500 (22), 4 (5), and 0.4 (22) nmol of iron/min/mg of cell protein, respectively. The difference between the reduction and uptake velocities for copper is smaller due to the less robust reduction of Cu^{2+} by Fre1p noted above; the reduction and uptake values for copper are 7 and 0.7 nmol of copper/min/mg of cell protein, respectively (22). Thus, the rate of formation of the lower valence metal ion is severalfold greater than the rate of this metal ion uptake (Cu^{1+}) or catalytic re-oxidation (Fe^{2+}). One could infer that the extracellular steady-state level of Cu^{1+} increases in the *MAC1^{up1}* strain based on the copper sensitivity of this strain. In fact, in this background the velocities of Cu^{2+} reduction and Cu^{1+} uptake are each increased ~ 7 -fold to 46.2 and 5.1 nmol of copper/

³ X. Shi and Z. Zhu, manuscript in preparation.

min/mg of protein, respectively (22). Thus, the molar difference between uptake and reduction is increased from ~6 nmol of copper/min/mg of protein in wild type yeast to 40 nmol of copper/min/mg of protein in the *MAC1^{up1}* strain, an increase of nearly 700%. In short, the data available are completely consistent with the notion that there is a steady-state level of both Cu¹⁺ (and Fe²⁺) at the yeast plasma membrane. The results here indicate that the level of this plasma membrane Cu¹⁺ is modulated by the action of Fet3p and that this effect contributes to the copper resistance of *S. cerevisiae*.

The CueO protein, a multicopper oxidase from *Escherichia coli* that is encoded within a copper resistance operon in the genome of this bacterium, is a ferroxidase that exhibits a pattern of substrate reactivity similar to that recorded for ceruloplasmin (12–14). Three groups working with this protein and its encoding cue system (for Cu efflux) have suggested that CueO (previously known as the *yakK* gene product) could contribute to copper resistance by acting as a cuprous oxidase, thus shifting the Cu²⁺/Cu¹⁺ ratio toward Cu²⁺. In this model, the cupric ion would be less readily taken into the cell in addition to its being a weaker pro-oxidant, thus explaining the cytoprotective effect of the CueO protein. However, one of these groups reported that despite a substrate reactivity profile comparable with that of ceruloplasmin and Fet3p, including its reactivity with Fe²⁺, CueO exhibited no oxidase activity with Cu¹⁺ as substrate (14). Therefore, the role of cuprous oxidase activity in copper homeostasis in prokaryotes remains unresolved.

Serum ceruloplasmin plays a critical role in iron release from the reticuloendothelial system by catalyzing the oxidation of Fe²⁺ to Fe³⁺ ions (8). The central nervous system also expresses ceruloplasmin as a glycoposphatidylinositol-anchored protein (35–37). This form of ceruloplasmin most likely plays a critical role in iron metabolism of the central nervous system since patients with aceruloplasminemia, a neurodegenerative disease caused by loss-of-function mutations in the ceruloplasmin gene, accumulate excess iron within the central nervous system (8). Ceruloplasmin shares the cuprous oxidase activity exhibited by Fet3p;² it is likely that this glycoposphatidylinositol-anchored ceruloplasmin also catalyzes the oxidation of Cu¹⁺ ions. As a consequence, mutations in the ceruloplasmin gene might also impair the capacity of the brain to detoxify copper ions. Indeed, copper toxicity might well contribute to the oxidative damage and neurodegeneration associated with aceruloplasminemia. Future studies must now evaluate further what role(s) this family of oxidases plays in both iron and copper homeostasis.

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