

Saccharomyces cerevisiae Mutants Altered in Vacuole Function Are Defective in Copper Detoxification and Iron-Responsive Gene Transcription

MARK S. SZCZYPKA, ZHIWU ZHU, PHILIPPE SILAR† AND DENNIS J. THIELE*

Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606, U.S.A.

Received 18 February 1997; accepted 3 May 1997

The metal ions, $\text{Cu}^{2+/+}$ and $\text{Fe}^{3+/2+}$, are essential co-factors for a wide variety of enzymatic reactions. However, both metal ions are toxic when hyper-accumulated or maldistributed within cells due to their ability to generate damaging free radicals or through the displacement of other physiological metal ions from metalloproteins. Although copper transport into yeast cells is apparently independent of iron, the known dependence on Cu^{2+} for high affinity transport of Fe^{2+} into yeast cells has established a physiological link between these two trace metal ions. In this study we demonstrate that proteins encoded by genes previously demonstrated to play critical roles in vacuole assembly or acidification, *PEP3*, *PEP5* and *VMA3*, are also required for normal copper and iron metal ion homeostasis. Yeast cells lacking a functional *PEP3* or *PEP5* gene are hypersensitive to copper and render the normally iron-repressible *FET3* gene, encoding a multi-copper Fe(II) oxidase involved in Fe^{2+} transport, also repressible by exogenous copper ions. The inability of these same vacuolar mutant strains to repress *FET3* mRNA levels in the presence of an iron-unresponsive allele of the *AFT1* regulatory gene are consistent with alterations in the intracellular distribution or redox states of $\text{Fe}^{3+/2+}$ in the presence of elevated extracellular concentrations of copper ions. Therefore, the yeast vacuole is an important organelle for maintaining the homeostatic convergence of the essential yet toxic copper and iron ions. © 1997 John Wiley & Sons, Ltd.

Yeast 13: 1423–1435, 1997.

No. of Figures: 6. No. of Tables: 1. No. of References: 37.

KEY WORDS — *Saccharomyces cerevisiae*; metal ion toxicity; vacuole; protein sorting; gene regulation

INTRODUCTION

The redox active metal ions, $\text{Cu}^{2+/+}$ and $\text{Fe}^{3+/2+}$, play essential roles in the biochemistry of life processes as co-factors for a wide variety of enzymes such as Cu, Zn superoxide dismutase, cytochrome oxidase and ribonucleotide reductase (Karlin, 1993; Lippard and Berg, 1994; Linder and Hazegh-Azam, 1996). This same redox active

nature of copper and iron also renders these metal ions highly toxic when present in abnormally high intracellular concentrations or when maldistributed within organisms. Both Cu^{+} and Fe^{2+} readily engage in Fenton chemistry to generate the highly toxic superoxide radical, which leads to damage to proteins, membranes and nucleic acids (Halliwell and Gutteridge, 1988). The intrinsically cytotoxic yet essential nature of both copper and iron dictates that all organisms must regulate metal ion reduction, uptake, distribution, sequestration and removal for normal homeostatic control.

It is thought that the fundamental mechanisms for metal ion homeostasis are conserved throughout nature. The most detailed picture of copper

*Correspondence to: D. J. Thiele, Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606, U.S.A. Phone: (313)763-5717; fax: (313)763-4581; e-mail: DTHIELE@UMICH.EDU.

†Present address: Centre de Genetique Moleculaire, Centre National de la Recherche Scientifique, 91198 Gif sur Yvette cedex, France.

Contract grant sponsor: National Institutes of Health

and iron control mechanisms has emerged from studies in prokaryotes and yeast (reviewed in Askwith *et al.*, 1995; Vulpe and Packman, 1995). High affinity copper transport into yeast cells involves a plasma membrane-associated Cu^{2+} - Fe^{3+} reductase encoded by the *FRE1* and *FRE2* genes and two integral membrane proteins, Ctr1p and Ctr3p (Dancis *et al.*, 1992; Askwith *et al.*, 1994; Hassett and Kosman, 1995; De Silva *et al.*, 1995; Knight *et al.*, 1996). Yeast cells transport reduced Fe^{2+} ions with high affinity via the plasma membrane-associated Ftr1 protein and a multi-copper ferroxidase activity associated with the Fet3 protein (Askwith *et al.*, 1994; Stearman *et al.*, 1996). Since Fet3p activity is Cu^{2+} -dependent, depletion of copper ions through chelation, mutations in both the *CTR1* and *CTR3* copper transport genes, or inactivation of a putative copper transporting ATPase encoded by the *CCC2* gene, render cells partially or completely defective in high affinity Fe^{2+} uptake (Dancis *et al.*, 1994; Yuan *et al.*, 1995). Therefore, although there is currently no evidence for an essential role of iron in copper transport in yeast, copper is inextricably linked to the high affinity transport of iron.

An additional level of iron and copper control in yeast is achieved through the transcriptional regulation of genes encoding proteins required for copper and iron homeostasis. Yeast cells respond to high environmental copper ion levels by activating transcription of the *CUP1* and *CRS5* genes, encoding metal-sequestering metallothionein proteins, and the *SOD1* gene, encoding Cu, Zn superoxide dismutase, via the Ace1 Cu^+ -metalloregulatory transcription factor (reviewed in Zhu *et al.*, 1995). In response to copper ion starvation, expression of the *FRE1*, *CTR1* and *CTR3* genes is induced, whereas the expression of these genes is down-regulated as a consequence of copper ion repletion via an incompletely characterized mechanism (Dancis *et al.*, 1994; Hassett and Kosman, 1995; Knight *et al.*, 1996). Expression of the *FRE1*, *FRE2*, *FET3*, *FTR1* and *CCC2* genes is elevated by iron ion starvation and repressed by iron ion repletion via the iron-responsive Aft1 protein (Yamaguchi-Iwai *et al.*, 1996). Therefore, copper and iron ions specifically regulate the expression of genes which play critical roles in their homeostasis.

The yeast vacuole is an acidic compartment which contains hydrolytic enzymes and functions in metabolite storage and in ion and pH homeostasis (reviewed in Jones, 1984; Klionsky *et al.*,

1990). Studies investigating the process of vacuole assembly and vacuolar protein sorting have identified six distinct classes of mutations which give rise to partially functional or non-functional vacuoles (classes A-F), distinguishable by a number of criteria, including unique morphological features, defects in vacuolar segregation to developing buds, proper vacuole acidification and mis-sorting of the soluble vacuolar protease carboxypeptidase Y (CPY) (Jones, 1984; Klionsky *et al.*, 1990). The four known complementation groups which comprise the class C mutants, *pep3*, *pep5*, *vps16* and *vps33*, exhibit a variety of phenotypes. These include defects in sorting and transport of vacuolar enzymes, most are temperature sensitive for growth, and all exhibit gross defects in vacuolar biogenesis (Banta *et al.*, 1988; Preston *et al.*, 1991; Woolford *et al.*, 1990; Raymond *et al.*, 1992). Strains harboring an inactivated *VMA3* gene, which encodes the 16 kDa proteolipid subunit of the vacuolar H^+ -ATPase, display characteristics of the class D type of vacuolar protein sorting mutants (Raymond *et al.*, 1992). Previous studies of the vacuolar H^+ -ATPase have shown that assembly of this multi-subunit protein complex is essential to maintain the acidic environment of the vacuole; therefore, *vma3Δ* mutants possess morphologically normal vacuoles but are deficient in vacuolar acidification (Nelson and Nelson, 1990). In addition to this defect, *vma3Δ* mutants are defective in respiration, display an increased sensitivity to environmental pH, and exhibit a slow growth phenotype on media containing high copper ion concentrations (Eide *et al.*, 1993).

In this work, we have initiated a genetic screen for genes which play important roles in metal detoxification and homeostasis in *Saccharomyces cerevisiae*. We have isolated two genes that have been previously isolated and identified as *PEP3* and *PEP5* and are known to play critical roles in yeast vacuolar biogenesis. Studies of these two class C mutants, *pep3Δ* and *pep5Δ*, and cells harboring a *vma3Δ* mutation, demonstrate that all three strains display copper ion sensitive growth phenotypes and, surprisingly, exhibit copper-dependent repression of a normally iron-responsive gene. Furthermore, we have identified distinct differences in metal ion homeostasis defects between these two classes of vacuolar mutants. The data described here demonstrate that vacuolar function and integrity are important in normal copper and iron metal ion homeostasis in *S. cerevisiae*.

Table 1.

Strain	Genotype	Reference or Source
DTY1	<i>MATa gal trp1-1 his4 ade8 CUP1R-10</i>	Thiele, 1988
DTY7	<i>MATa ura3-52 his6 leu2-3,-112 CUP1R-3</i>	Thiele, 1988
DTY186	<i>MATa pep3::LEU2 ura3-52 his6 leu2-3,-112 CUP1R-3</i>	This work
DTY190	<i>MATa vma3::LEU2 ura3-52 his6 leu2-3,-112 CUP1R-3</i>	This work
SEY6210	<i>MATa ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 CUP1R</i>	Banta <i>et al.</i> , 1988
DTY213	<i>MATa pep3::LEU2 ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 CUP1R</i>	This work
DTY214	<i>MATa pep5::LEU2 ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 CUP1R</i>	This work
DTY212	<i>MATa fet3::HIS3 ura3-52 leu2-3,-112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 CUP1R</i>	This work

MATERIALS AND METHODS

Strains and media

Escherichia coli strains XL1 blue, DH11S, and GM119 were used in this study. The genotypes of *S. cerevisiae* strains used in these studies are described in Table 1. Strains DTY186, DTY187 and DTY190, isogenic to DTY7, were created by transformation and homologous recombination to disrupt either the *PEP3*, *PEP5*, or *VMA3* genes (Sherman *et al.*, 1986). Disruption of the *PEP3* gene was accomplished by transforming DTY7 with an 8 kb *SacI/SphI* DNA fragment from plasmid p1A3 (described below), which contains the disrupted *PEP3* gene, and selecting for leucine prototrophy. Similarly, *PEP5* was disrupted in the DTY7 background using a 4.8 kb DNA fragment from plasmid pMMM4A-L (described below), which contains the disrupted *PEP5* gene, and selecting for leucine prototrophy. Yeast strains were grown on standard rich (YPD) or synthetic complete (SC) medium lacking selected amino acids as described in the figure legends. Solid media used to test metal resistance phenotypes were prepared as described (Sherman *et al.*, 1986). Appropriate concentrations of metal ions or metal ion chelators were added to liquid or solid media from stock solutions using water as solvent. Spotting of yeast cells to metal containing plates was performed by dilution of logarithmic phase cultures grown to OD₆₅₀=1.0–2.0, to a final OD₆₅₀ of 0.3 into SC medium. Cell number was estimated using standard calculations (Sherman *et al.*, 1986), serial dilution performed, and 10 µl of cell suspension were spotted onto plates.

Plasmids

Standard techniques for plasmid construction, maintenance, and single-stranded DNA production were used in this study (Ausubel *et al.*, 1987). Plasmids used for disruption of the *PEP3* gene were constructed as follows. A 5 kb *SacI/SspI* DNA fragment containing the entire *PEP3* open reading frame (ORF) and flanking sequence was subcloned into the *SacI/SmaI* sites of pUC19 creating intermediate plasmid pMMM3N. pMMM3N was linearized at a unique *BamHI* site located within the *PEP3* ORF and a 2.8 kb *BglII* fragment containing the *LEU2* gene was ligated into this site, disrupting the *PEP3* gene and resulting in plasmid p1A3.

Plasmids for *PEP5* disruption were created as follows. Intermediate plasmid pMMM4A was constructed by subcloning a 4.3 kb *KpnI/HindIII* fragment encompassing the *PEP5* ORF and flanking sequences into the same sites of pBluescript SK+. This plasmid was cleaved with *BclI* after passage through the *dam* and *dcm* methylation-deficient *E. coli* strain GM119 and the 2.8 kb *BglII* *LEU2* fragment was used to replace a 2.5 kb *BclI* DNA fragment which contains 85% of the *PEP5* ORF. This plasmid was designated pMMM4A-L. pT14, a centromere-based plasmid containing the *AFT1-1^{up}* mutant allele was obtained from Andrew Dancis (Univ. of Pennsylvania) and was previously described (Yamaguchi-Iwai *et al.*, 1996). The YIpFET3 plasmid used to create the *fet3Δ* strain was obtained from David Eide (Univ. of Missouri) and was previously described (Askwith *et al.*, 1994).

Mutant isolation

A 100 ml culture of exponentially growing wild-type cells (DTY1) was mutagenized with ethyl methane sulfonate (EMS) to a point at which 28% of the cells survived (Ausubel *et al.*, 1987). Approximately 5000 viable colonies from this mutagenesis were grown on YPD agar medium for 3 days at 30°C and then replica plated to SC agar plates in the absence or presence of 300 µM-CuSO₄. Colonies which grew on SC but not SC containing 300 µM-copper sulfate were identified and colony purified for further analysis.

Vacuolar staining

Cultures were grown to log phase and 5 µl of a 10 mM solution of 5- (and-6)-carboxyfluorescein diacetate was added (CFDA, Molecular Probes). Cells were incubated at 30°C, 300 rpm for 30 min to allow for the uptake of CFDA into vacuoles (Roberts *et al.*, 1991) and then pelleted and washed with phosphate-buffered saline (pH=7.3) containing 0.05% aniline blue, a dye which specifically stains the cell wall. Vacuolar fluorescence was visualized under ultraviolet light fluorescence and photographed with a Zeiss Axioskop microscope at 1000 × magnification.

RNA blot analysis

Total RNA was prepared from exponentially growing cells (OD₆₅₀=1.0–2.0) under conditions indicated in the appropriate figure legends. RNA was loaded into each well of a 1% formaldehyde-agarose gel and subjected to electrophoresis blotted to Nytran membranes (Schleicher & Schuell, Keene, NH) and probed as recommended by the manufacturer with either *CUP1*, *FET3*, *CTR1* or *ACT1* specific DNA fragments labeled with [³²P]dATP by nick translation (Ausubel *et al.*, 1987). *CUP1*, *FET3*, and *CTR1* mRNA species were visualized by autoradiography of RNA blots using Kodak Biomax X-ray film and phosphorimaging using a Molecular Dynamics Model SP Phosphorimager. Blots were normalized for RNA loading by washing and re-probing with an actin mRNA specific probe followed by phosphorimaging and quantitation using ImageQuaNT software (Molecular Dynamics).

Atomic absorption spectroscopy

Cultures of isogenic wild-type, *pep3Δ*, *pep5Δ*, and *vma3Δ* strains were grown to log phase

(OD₆₅₀=1.0–2.0) in SC medium. A 50 ml aliquot from each culture was then incubated in the absence or presence of 100 µM-CuSO₄ at 30°C for 1.5 h. Five ml of each culture was removed for protein determination using a modification of the method of Lowry (Herbert *et al.*, 1971) and cells from 40 ml of the remaining culture were pelleted at room temperature, 3000 rpm. Pellets were washed two times with ice-cold 10 mM-EDTA and resuspended in 1 ml concentrated nitric acid (Baker, Analytical Grade). Cell suspensions were boiled until the liquid had evaporated and 1 ml of 0.5% nitric acid was added to each sample. The resulting solution was analysed for copper and iron content by flameless atomic absorption spectroscopy using a Perkin Elmer PEC 3000 spectrophotometer. All experiments were conducted at least five times.

RESULTS

Isolation of *S. cerevisiae* mutants defective in copper ion homeostasis

To isolate mutants defective in copper ion homeostasis in *S. cerevisiae*, the wild-type strain DTY1 was subjected to mutagenesis with EMS and surviving colonies were replica-plated from YPD to SC agar containing a range of CuSO₄ concentrations. Of approximately 5000 colonies screened, six were identified which grew on YPD or SC at 30°C, but which failed to grow on SC agar containing 300 µM-CuSO₄. The parental wild-type strain is capable of robust growth on CuSO₄ concentrations exceeding 2 mM-CuSO₄. In addition to copper ion sensitivity, these strains exhibited comparable sensitivity to Fe³⁺, Zn²⁺ and Cd²⁺ (data not shown). Genetic analysis of these isolates revealed the presence of two complementation groups with single recessive nuclear mutations unlinked to either the *CUP1* or *ACE1* genes. One strain representing each of the two complementation groups was used as a recipient for transformation by a centromere-based yeast genomic library and two copper ion resistant transformants were identified for each mutant. The analysis of plasmids rescued from each copper ion resistant transformant demonstrated that the clones which complemented each mutant were unique, but that for each mutant the clones contained overlapping restriction fragments. Sub-cloning and complementation analysis for copper ion resistance, followed by sequence analysis,

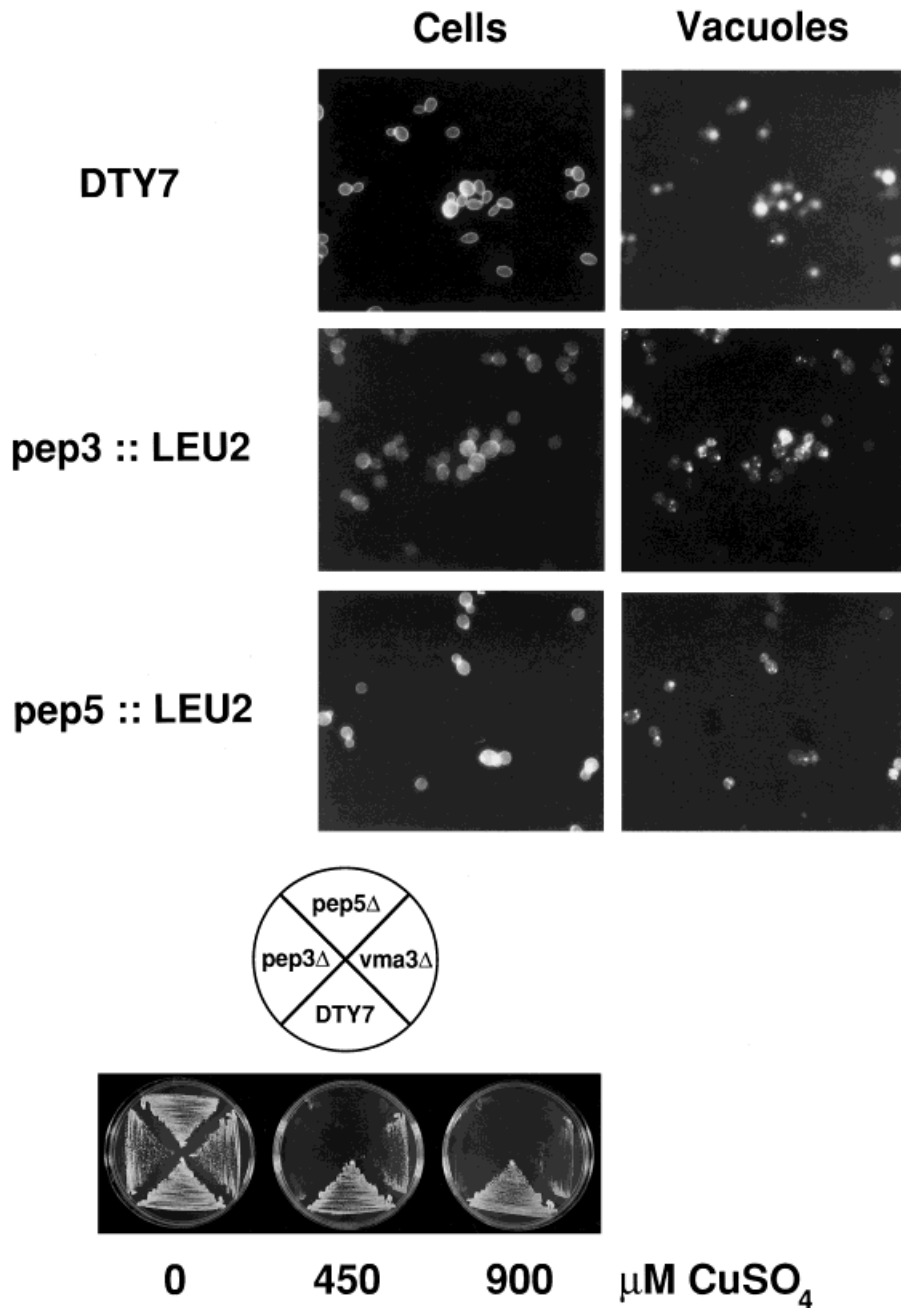
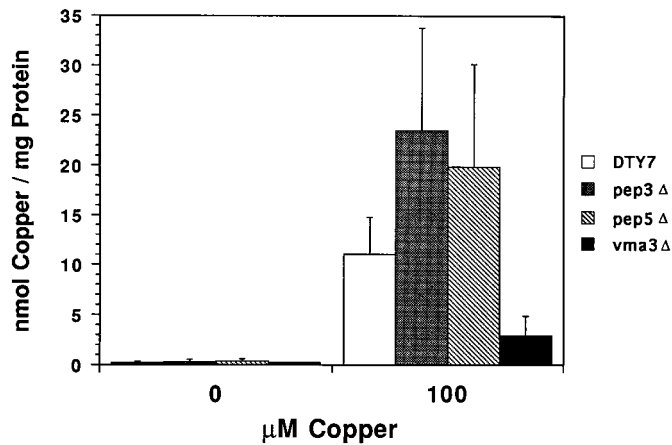
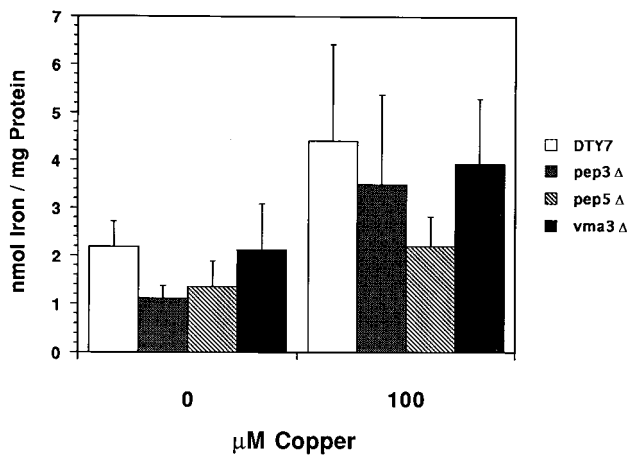


Figure 1. Copper ion-sensitive mutants are defective for vacuolar biogenesis. (A) Wild-type, *pep3Δ* and *pep5Δ* strains were incubated for 30 min in the presence of a pH-sensitive dye, CFDA, and visualized at 1000 × magnification by fluorescence microscopy. Cell walls were stained with 0.05% aniline blue in phosphate-buffered saline. (B) Isogenic strains of the relevant indicated genotype and the parental wild-type strain DTY7, were streaked to SC plates containing a range of copper ion concentrations. Agar plates containing 0, 450 and 900 μM-CuSO₄ are shown. Plates were incubated at 30°C for 3 days and photographed.

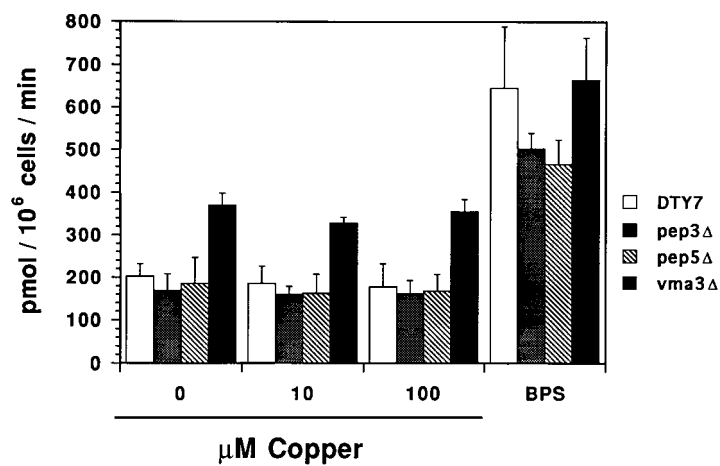
COPPER ACCUMULATION



IRON ACCUMULATION



Ferrireductase Activity



indicated that the complementing genes for the two independent copper ion-sensitive mutants correspond to *PEP3* and *PEP5*, which encode proteins previously demonstrated to play critical roles in vacuolar biogenesis (Woolford *et al.*, 1990; Preston *et al.*, 1991; Robinson *et al.*, 1991).

For further investigations, isogenic *pep3Δ* and *pep5Δ* deletion strains were constructed by homologous recombination and the structure of the inactivated chromosomal alleles verified. Fluorescence microscopy using CFDA, a pH-sensitive dye which fluoresces in acidified vacuoles, demonstrated that the *pep3Δ* and *pep5Δ* deletion strains exhibit the punctate vacuolar staining characteristics of class C vacuolar mutants (Banta *et al.*, 1988; Raymond *et al.*, 1992), while the wild-type strain exhibited normal vacuolar morphology (Figure 1A). Furthermore, the *pep3Δ* and *pep5Δ* strains exhibited the same degree of copper sensitivity as the original mutant isolates (Figure 1B). Previous work by Eide and colleagues has demonstrated that a mutation in the *VMA3* gene, encoding the 16 kDa subunit of the vacuolar H⁺-ATPase, is important for copper ion detoxification and respiration (Eide *et al.*, 1993). Therefore, to directly compare the role of the *PEP3*, *PEP5* and *VMA3* gene products in copper homeostasis, a *vma3Δ* mutant was also constructed in the DTY7 background. Although, as previously observed, the *vma3Δ* mutant grew poorly on medium containing CuSO₄ after a 3-day incubation, this strain was able to grow on CuSO₄ concentrations identical to those of the wild-type parental strain after a 6- to 10-day incubation at 30°C (data not shown). Taken together, these data demonstrate that vacuolar function is essential for normal copper ion detoxification in *S. cerevisiae* and that mutants with defects in vacuole assembly display greater copper ion sensitivity as compared to a mutant with structurally normal vacuoles that are defective in acidification.

Metal ion accumulation and reduction in vacuolar mutants

To investigate the nature of the copper ion homeostatic defects in the isogenic vacuolar mutant strains, total cell-associated copper ions were quantitated by atomic absorption spectroscopy. No significant differences were observed in copper ion accumulation between the wild-type strain, DTY7 and either of the three mutant strains grown in SC medium in the absence of exogenous copper ions (Figure 2A). Incubation of each of the strains in the presence of 100 μM-CuSO₄ for 1.5 h (a sublethal copper ion concentration for all of the vacuolar mutant strains) showed that the *pep3Δ* and *pep5Δ* mutant strains had no significant difference in copper ion accumulation as compared to the wild-type parental strain. In contrast, the *vma3Δ* strain reproducibly accumulated three-fold less copper ions than the wild-type strain (Figure 2A).

Copper influences iron transport in yeast by activating Fet3p multi-copper Fe(II) oxidase activity and via the de-repression of the *FRE1/2* genes, encoding the Cu²⁺/Fe³⁺ reductase required for high affinity Fe²⁺ transport, as a consequence of copper ion starvation (Askwith *et al.*, 1994; Dancis *et al.*, 1994; Hassett and Kosman, 1995). Therefore, to ascertain if the vacuolar mutants have alterations in these aspects of iron regulation, we measured total cell-associated iron and Fe³⁺ reductase activity in untreated or copper ion-treated wild-type and vacuolar mutant strains. The data in Figure 2B show that there was a modest stimulation of iron accumulation by exogenous copper ions in all four strains, presumably due to the activation of Fet3p or other copper-dependent Fe(II) oxidase activities. However, there were no significant differences in total iron accumulation in the vacuolar mutant strains as compared with the wild-type strain. Furthermore, although the *pep3Δ* and *pep5Δ* strains exhibited ferrireductase activity

Figure 2. Copper and iron ion accumulation and Fe³⁺ reductase activity in *Saccharomyces cerevisiae* wild-type and isogenic *pep3Δ*, *pep5Δ* and *vma3Δ* strains. Wild-type (DTY7), *pep3Δ*, *pep5Δ* and *vma3Δ* strains were grown to log phase (OD₆₅₀ = 1.0–2.0) in SC media and cultures were incubated at 30°C, 300 rpm for 1.5 h in the absence or presence of 100 μM-CuSO₄, or Fe²⁺ chelator bathophenanthroline sulfonate (BPS). An aliquot was removed for protein quantitation before the cultures were harvested. Cell pellets were prepared as described in Materials and Methods and flameless atomic absorption spectrophotometry was used to determine the copper ion concentrations of these cells. The copper ion concentration for each sample is presented as the mean and standard deviation (indicated by bars) from at least three independent experiments. (B) Accumulation of iron ions in *S. cerevisiae* wild-type and isogenic *pep3Δ*, *pep5Δ* and *vma3Δ* strains. (C) Ferrireductase activity was measured in wild-type, *pep3Δ*, *pep5Δ* and *vma3Δ* mutants grown in the absence or presence of the indicated copper ion concentrations as described in Materials and Methods.

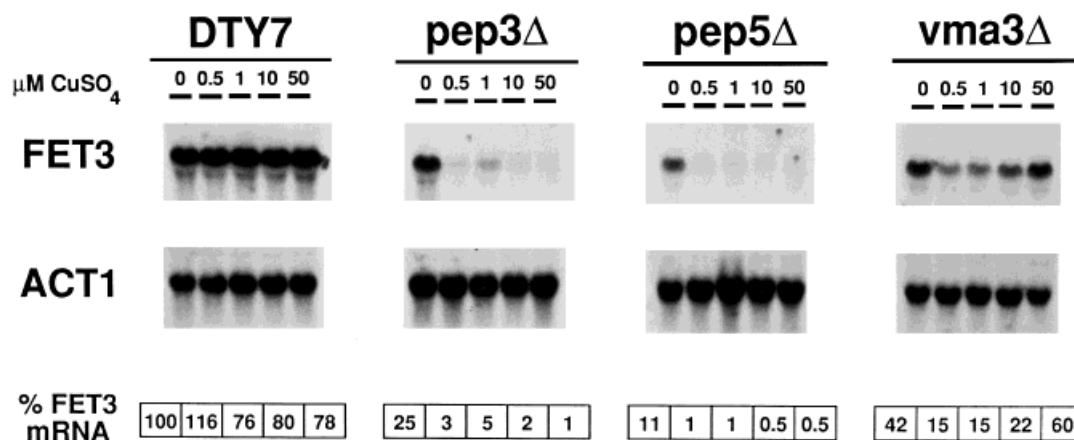


Figure 3. Aberrant regulation of *FET3* in vacuole biogenesis mutants. RNA was isolated and prepared from the indicated wild-type (DTY7) and vacuolar mutant strains (*pep3Δ*, *pep5Δ* and *vma3Δ*). Blots were probed with a *FET3* mRNA specific probe and exposed to x-ray film and quantitated using a Molecular Dynamics phosphorimager. After exposure and quantitation, blots were stripped using standard procedures and then re-probed with an actin specific mRNA probe to normalize for total RNA loaded. Copper ion concentrations added to each culture (in μM) are indicated at the top. The mRNAs of *FET3* and *ACT1* are indicated as FET3 and ACT1, respectively. The percentage of *FET3* mRNA in each lane, as compared to the wild-type strain with zero exogenous copper ions, is shown below each lane of the autoradiogram.

levels that were similar to the wild-type strain both in the absence and presence of CuSO_4 . *vma3Δ* cells reproducibly displayed Fe^{3+} reductase activity that was two-fold higher than the wild-type, *pep3Δ* and *pep5Δ* strains (Figure 2C). This is consistent with the decrease in total copper ion accumulation observed in the *vma3Δ* strain and suggests that the *vma3Δ* mutation may render these cells partially starved for copper. Furthermore, the iron starvation phenotype previously found to be associated with *vma3Δ* mutants (Eide *et al.*, 1993) is also likely to contribute significantly to the increased reductase activity. Under iron starvation, generated by addition of an impermeable Fe^{2+} chelator bathophenanthroline sulfonate (BPS) in the growth medium, the Fe^{3+} reductase activities in all four strains as expected are significantly increased.

Aberrant expression of *FET3* in vacuole mutants

The data described above demonstrate that *pep3Δ*, *pep5Δ* and *vma3Δ* mutants have altered copper ion sensitivity but no significant increase in iron or copper ion accumulation. To examine the status of intracellular iron or copper ion distribution in the wild-type parental strain and vacuolar mutants, we assessed copper- and iron-dependent expression of the *CUP1* and *FET3* genes. *CUP1* gene activation occurs via Cu^+ -activation of the Ace1p metalloregulatory transcription factor (Zhu

et al., 1995). *FET3* gene repression occurs in response to iron via the binding of the Aft1p to a regulatory sequence in the *FET3* gene promoter (Yamaguchi-Iwai *et al.*, 1996). These genes are transcriptionally regulated by iron or copper ions, respectively, in a highly metal ion specific manner. Therefore, *CUP1* gene activation and *FET3* gene repression are sensitive indicators of intracellular copper and iron availability, respectively. We observed no significant difference in the responses of the wild-type, *pep3Δ*, *pep5Δ* or *vma3Δ* strains to increasing concentrations of copper ions for the induction of *CUP1* mRNA levels (data not shown). The analysis of *FET3* mRNA levels in

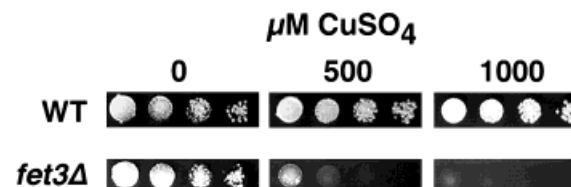


Figure 4. The *fet3Δ* strain is sensitive to elevated copper ion concentrations. Isogenic wild-type (SEY6210, WT) and *fet3Δ* strains were grown to logarithmic phase and cells were diluted to an $\text{OD}_{650}=0.3$. Serial dilutions were performed in SC medium and 10 μl suspension containing either 30,000, 3000, 300 or 30 cells were spotted on agar plates containing the indicated copper ion concentrations. The plates were incubated at 30°C for 3 days and photographed.

these strains, however, revealed a striking metal ion-dependent regulatory defect. First, *FET3* mRNA levels in the *pep3Δ*, *pep5Δ* and *vma3Δ* strains were significantly lower than the isogenic wild-type strain when cells were grown in SC medium (Figure 3). Moreover, *FET3* mRNA levels were dramatically reduced upon incubation of the cells in the presence of as little as 0.5 μM-CuSO₄, resulting in only 3, 1 and 15% of the wild-type basal levels in the *pep3Δ*, *pep5Δ* and *vma3Δ* strains respectively. The severity of this response was much more dramatic for the *pep3Δ* and *pep5Δ* mutants than for *vma3Δ*. Therefore, strains harboring defects in vacuolar function render the normally iron-responsive *FET3* gene repressible by very low concentrations of exogenous copper ions. In contrast, *FET3* mRNA levels in the wild-type strain were only modestly reduced even in the presence of 50 μM-CuSO₄. To ascertain if *FET3* gene function confers protection from copper ion toxicity, isogenic wild-type and *fet3Δ* strains were compared for their ability to grow in the presence of increasing concentrations of CuSO₄. As shown in Figure 4, *fet3Δ* mutants are more sensitive to copper ions than the isogenic parental wild-type strain, strongly suggesting that the copper ion-dependent decrease in *FET3* mRNA is, at least in part, responsible for the copper ion sensitivity of *pep3Δ*, *pep5Δ* and *vma3Δ* mutants.

Copper-repression of FET3 expression occurs through the Aft1p iron sensor

The observations that *pep3Δ* and *pep5Δ* mutants exhibit copper-dependent repression of *FET3* mRNA levels, but no significant defect in iron or copper accumulation, suggest that copper might influence the intracellular distribution of iron in cells defective for vacuolar function. To test this model, we ascertained if the dramatic copper-dependent repression of *FET3* mRNA in the *pep3Δ* and *pep5Δ* mutants depended upon a functional gene encoding Aft1p, the iron-sensing transcription factor important for the regulation of *FET3*, *FRE1*, *FRE2*, *CCC2* and other iron homeostatic genes.

The data in Figure 5A show, as previously demonstrated, that in a wild-type strain (*AFT1*) *FET3* mRNA levels are repressed by iron but not copper ions. Under these growth conditions *FET3* mRNA levels in the *pep3Δ* and *pep5Δ* mutants were induced by the presence of the Fe²⁺ chelator BPS, consistent with a partial iron starvation when

cells are grown on YPD. As expected, the copper ion chelator bathocuproinedisulfonic acid (BCS) did not alter *FET3* mRNA levels. However, in the *pep3Δ* and *pep5Δ* strains, basal *FET3* mRNA levels were much lower than wild-type levels and were repressed by either exogenous iron or copper ions. Furthermore, *FET3* mRNA levels are induced in these mutant strains after treatment with BPS but not BCS. In contrast, the same strains transformed with a dominant allele of the *AFT1* gene, *AFT1-1^{up}*, in which a single amino acid substitution renders Aft1p non-responsive to iron (Yamaguchi-Iwai *et al.*, 1996), did not repress *FET3* mRNA levels in the presence of copper or iron ions (Figure 5B).

These observations strongly suggest that the copper ion sensitivity and copper ion-mediated repression of *FET3* mRNA levels in the *pep3Δ* and *pep5Δ* mutants occur via copper exacerbation of intracellular iron distribution defects. Cells harboring the *AFT1-1^{up}* allele display two phenotypes consistent with this hypothesis. First, both wild-type and the *pep3Δ* and *pep5Δ* mutant cells displayed increased copper ion sensitivity when the *AFT1-1^{up}* gene was present on a centromeric plasmid, with the sensitivity being more severe in the *pepΔ* mutants (Figure 6A). This may represent a sensitivity to abnormal iron distribution that is augmented as a consequence of added copper ions. Second, in both the *pep3Δ* and *pep5Δ* mutants (but not the wild-type strain) containing either the wild-type *AFT1* gene or the *AFT1-1^{up}* allele, growth was severely inhibited when cells were starved for iron, but growth was only modestly inhibited when cells were starved for copper (Figure 6B, BPS and BCS, respectively). This was observed in response to a range of BPS or BCS concentrations (data not shown).

DISCUSSION

Previous studies have clearly demonstrated that yeast vacuoles play pivotal roles in a number of cellular processes including protein processing, amino acid and ion homeostasis, the storage of metals, polyphosphates and other metabolites and in cellular pH and osmoregulation (Jones, 1984; Klionsky *et al.*, 1990). The data in this manuscript demonstrate that yeast cells harboring mutations in genes important for either vacuolar structure or acidification have clear defects in copper ion detoxification and metal ion-dependent gene expression. Furthermore, the consequences of mutations

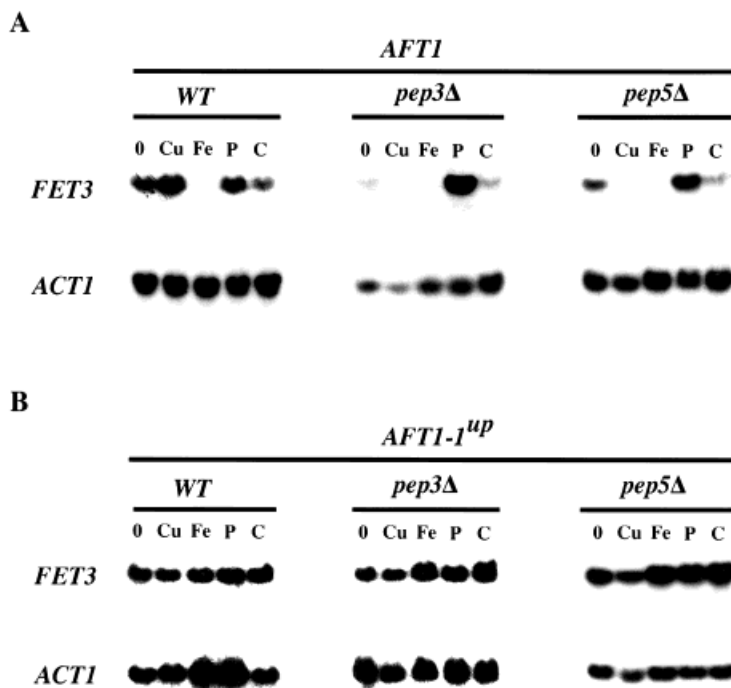


Figure 5. Copper ion regulation of *FET3* mRNA requires a functional *AFT1* gene. RNA was isolated from the wild-type (WT) *pep3Δ* and *pep5Δ* strains transformed with the control plasmid YCp50 (A) or the dominant *AFT1-1^{up}* mutant allele (B) grown under control conditions (0) or in the presence of CuSO₄ (Cu), FeCl₃ (Fe), BPS (P) or BCS (C). Blots were probed with a *FET3* mRNA-specific probe and after exposure and quantitation, blots were stripped using standard procedures and then re-probed with an actin-specific mRNA probe to normalize for total RNA loaded. The mRNAs of *FET3* and *ACT1* are indicated as *FET3* and *ACT1*, respectively.

in the *PEP3* or *PEP5* genes are distinct from those observed for the *VMA3* gene. With respect to copper resistance, the *pep3Δ* and *pep5Δ* mutants displayed *CUP1* mRNA and protein levels that are essentially indistinguishable in these strains as compared to the isogenic wild-type parental strain. Therefore, the copper ion-sensitive phenotype displayed by the *pep3Δ* and *pep5Δ* mutants is not a consequence of a defect in *CUP1* biosynthesis. Although the intracellular location of the *CUP1*-encoded metallothionein (MT) is thought to be cytosolic in wild-type cells (Wright *et al.*, 1987), we cannot at this time rule out the possibility that the intracellular localization of MT in the vacuolar mutant strains is altered and contributes to the copper-sensitive phenotype displayed by these strains. Therefore, differences in the accumulation of copper ions and the expression of the Cup1 metallothionein are unlikely to represent mechanisms for copper ion sensitivity. Since the Fet3

protein is predicted to bind four copper ions per polypeptide and has Cu²⁺-dependent oxidase activity (Askwith *et al.*, 1994; Yuan *et al.*, 1995), we tested whether Fet3p may also contribute to copper ion resistance by disruption of the chromosomal *FET3* gene. Indeed, cells bearing a *fet3Δ* allele exhibited a shift in copper sensitivity from wild-type levels that is comparable to that exhibited by the *pep3Δ* and *pep5Δ* mutants. Consistent with this observation, *pep3Δ* and *pep5Δ* cells respond to submicromolar copper ion concentrations by almost completely repressing *FET3* mRNA levels. Taken together, these two observations strongly suggest that the Fet3 multi-copper oxidase provides one line of defence against copper ion toxicity. It is interesting that in higher eukaryotes, the Fet3p homologue ceruloplasmin has been suggested to scavenge free radicals generated through Fenton chemistry (Samokyszyn *et al.*, 1989; De Silva and Aust, 1992). Therefore, Fet3

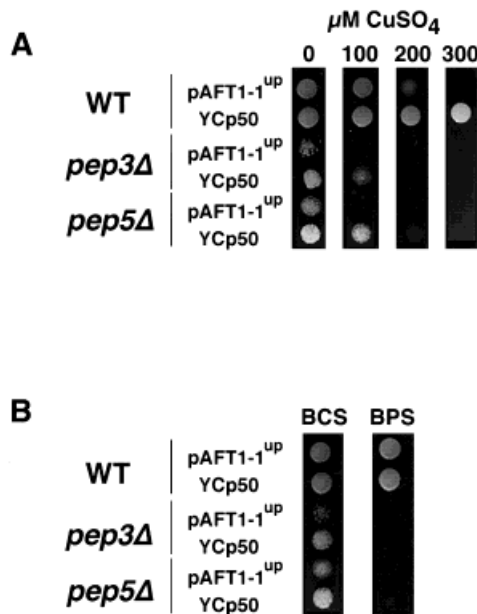


Figure 6. Expression of the *AFT1-1^{up}* allele in vacuolar mutants generates a copper ion-sensitive phenotype and induces Fe starvation. (A) Wild-type (WT), *pep3Δ* and *pep5Δ* cells transformed with a control plasmid (YCp50) or a plasmid containing the *AFT1-1^{up}* allele were grown to logarithmic phase, diluted and 10 μl containing approximately 3000 cells was spotted to plates containing 0, 100, 200 and 300 μM -CuSO₄. (B) 10 μl of the same cell suspension used in (A) was spotted to plates containing either 100 μM -BCS (copper ion-limiting conditions) or 100 μM -BPS (iron and copper ion-limiting conditions). Plates were incubated at 30°C for 3 days and photographed.

might play a role in the protection from copper ion toxicity both by binding and sequestering copper ions and through serving as an anti-oxidant.

What might be the mechanisms by which *pep3Δ* and *pep5Δ* mutants dramatically repress *FET3* mRNA levels in response to copper? We observed that *pep3Δ* or *pep5Δ* cells expressing a mutated form of the *FET3* regulatory protein Aft1, which renders it non-responsive to copper, are also insensitive to copper for *FET3* repression. These results suggest that in *pep3Δ* and *pep5Δ* strains, vacuolar defects in iron homeostasis, either at the level of intracellular distribution or iron redox states, are worsened by elevations in copper ion concentrations. Alternatively, it is possible that in the *pep3Δ* and *pep5Δ* mutants, a maldistribution of copper ions might provide a mechanism for the direct interference of iron sensing or transcriptional activation by Aft1p by copper. A number of investigations have suggested that yeast vacuoles

serve to store intracellular iron (Kitamoto *et al.*, 1988; Raguzzi *et al.*, 1988; Bode *et al.*, 1995). Furthermore, Eide and colleagues clearly demonstrated that the *gef2* mutant, allelic to *vma3* and *cup5*, is copper ion sensitive and defective in the utilization of non-fermentable carbon sources and oxygen consumption (Eide *et al.*, 1993). This respiratory defect could be suppressed by the addition of iron to the growth medium, suggesting that the primary defect in vacuolar H⁺-ATPase mutants is in iron homeostatic control mechanisms. Consistent with this idea, it has recently been demonstrated that *pep5Δ* mutants exhibit altered intracellular iron distribution (Bode *et al.*, 1995). One possibility is that iron is mobilized from the vacuole by a mechanism involving a Cu²⁺-dependent ferroxidase similar to Fet3p and that in the context of the abnormal vacuoles found in *pep3Δ* and *pep5Δ* mutants, this machinery is hyper-sensitive to added copper ions, resulting in increased levels of Fe (III). Interestingly, a search of the yeast genome data base indicates the presence of an ORF on chromosome VI (YFL0141W) encoding a protein with significant homology to the Fet3p. Indeed, recent studies have demonstrated that this ORF encodes a vacuolar protein which participates in iron homeostasis (D. Eide, personal communication).

The data presented in this work has demonstrated that *pep3Δ* and *pep5Δ* mutants exhibit defects in copper sensitivity and iron-responsive gene regulation. Since *pep3Δ* and *pep5Δ* mutants lack morphologically normal vacuoles and have a number of other defects, but *vma3Δ* mutants have normal vacuolar structure, vacuolar integrity may be important for copper detoxification and only a subset of copper homeostatic functions carried out by the vacuole are defective in mutants with altered vacuolar acidification. A number of studies have also demonstrated that yeast vacuoles are important for resistance to Ca²⁺, Cd²⁺, Zn²⁺ and other metal ions (Li *et al.*, 1997; Klionsky *et al.*, 1990). Therefore, vacuolar function plays an important role in the protection and homeostatic control of both toxic and essential metal ions.

ACKNOWLEDGEMENTS

We are grateful to Drs Kevin Morano, Marj Peña, Simon Labbé, Jason Brickner and David Eide for critical comments on this manuscript. We thank Drs Andrew Dancis for the *AFT1-1^{up}* plasmid, N. Nelson for the *VMA3* disruption plasmid, David

Eide for the *FET3* disruption plasmid, Scott Emr and Randy Schekman for yeast strains and John Szczypka for technical assistance. This work was supported by a Postdoctoral Fellowship–National Research Service Award from the National Institutes of Health (F32 GM17067) to Z.Z. and by a grant from the National Institutes of Health (GM41840) to D.J.T. D.J.T. is a Burroughs Wellcome Toxicology Scholar.

REFERENCES

- Askwith, C., Eide, D., Ho, A. V., *et al.* (1994). The *FET3* gene of *S. cerevisiae*: a multicopper oxidase required for ferrous iron uptake. *Cell* **76**, 403–410.
- Ausubel, F. M., Brent, R., Kingston, R. E., *et al.* (Eds) (1987). *Current Protocols in Molecular Biology*. Greene/Wiley, New York.
- Banta, L. M., Robinson, J. S., Klionsky, D. J. and Emr, S. D. (1988). Organelle assembly in yeast: characterization of yeast mutants defective in vacuolar biogenesis and protein sorting. *J. Cell. Biol.* **107**, 1369–1383.
- Bode, H. P., Dumschat, M., Garotti, S. and Fuhrmann, G. F. (1995). Iron sequestration by the yeast vacuole: a study with vacuolar mutants of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **228**, 337–342.
- Dancis, A., Roman, D. G., Anderson, G. J., Hinnebusch, A. G. and Klausner, R. D. (1992). Ferric reductase of *Saccharomyces cerevisiae*: molecular characterization, role in iron uptake, and transcriptional control by iron. *Proc. Natl. Acad. Sci. USA* **89**, 3869–3873.
- Dancis, A., Haile, D., Yuan, D. S. and Klausner, R. D. (1994a). The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). *J. Biol. Chem.* **269**, 25660–25667.
- Dancis, A., Yuan, D. S., Haile, D., *et al.* (1994b). Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. *Cell* **76**, 393–402.
- Dancis, A., Klausner, R. D., Hinnebusch, A. G. and Barriocannal, J. G. (1990). Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**, 2294–2301.
- De Silva, D. M., Askwith, C. C., Eide, D. and Kaplan, J. (1995). The *FET3* gene product required for high affinity iron transport in yeast is a cell surface ferroxidase. *J. Biol. Chem.* **270**, 1098–1101.
- De Silva, D. M. and Aust, S. D. (1992). Ferritin and ceruloplasmin in oxidative damage: review and recent findings. *J. Physiol. Pharmacol.* **71**, 715–720.
- Eide, D. J., Bridgham, J. T., Zhao, Z. and Mattoon, J. (1993). The vacuolar H(+)-ATPase of *Saccharomyces cerevisiae* is required for efficient copper detoxification, mitochondrial function and iron metabolism. *Mol. Gen. Genet.* **241**, 447–456.
- Halliwell, B. and Gutteridge, J. M. C. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219**, 1–14.
- Hassett, R. and Kosman, D. J. (1995). Evidence for Cu(II) reductase as a component of copper uptake by *Saccharomyces cerevisiae*. *J. Biol. Chem.* **270**, 128–134.
- Herbert, D., Phipps, P. J. and Strange, R. E. (1971). Chemical analysis of microbial cells. *Methods Microbiol.* **5B**, 410–452.
- Jones, E. W. (1984). The synthesis and function of proteases in *Saccharomyces*: genetic approaches. *Ann. Rev. Genet.* **18**, 233–270.
- Karlin, K. D. (1993). Metalloenzymes, Structural Motifs, and Inorganic Models. *Science* **261**, 701–708.
- Kitamoto, K., Yoshizawa, K., Ohsumi, Y. and Anraku, Y. (1988). Mutants of *Saccharomyces cerevisiae* with defective vacuolar function. *J. Bacteriol.* **170**, 2687–2691.
- Klionsky, D. J., Herman, P. K. and Emr, S. D. (1990). The fungal vacuole: composition, function and biogenesis. *Microbiol. Rev.* **54**, 266–292.
- Knight, S. A. B., Labbé, S., Kwon, L. F., Kosman, D. J. and Thiele, D. J. (1996). A widespread transposable element masks expression of a yeast copper transport gene. *Genes & Devel.* **10**, 1917–1929.
- Li, Z.-S., Lu, Y.-P., Zhen, R.-G., Szczypka, M., Thiele, D. J. and Rea, P. A. (1997). A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis(glutathionato)cadmium. *Proc. Natl. Acad. Sci. USA* **94**, 42–47.
- Linder, M. C. and Hazegh-Azam, M. (1996). Copper biochemistry and molecular biology. *Am. J. Clin. Nutr.* **63**, 797–811.
- Lippard, S. J. and Berg, J. M. (1994). *Principles of Bioinorganic Chemistry*, University Science Books, Mill Valley, USA.
- Nelson, H. and Nelson, N. (1990). Disruption of genes encoding subunits of yeast vacuolar H(+)-ATPase causes conditional lethality. *Proc. Natl. Acad. Sci. USA* **87**, 3503–3507.
- Preston, R. A., Manolson, M. F., Becherer, K., *et al.* (1991). Isolation and characterization of *PEP3*, a gene required for vacuolar biogenesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**, 5801–5812.
- Raguzzi, F., Lesuisse, E. and Crichton, R. R. (1988). Iron storage in *Saccharomyces cerevisiae*. *FEBS Lett.* **231**, 253–258.
- Raymond, C. K., Howald-Stevenson, I., Vater, C. A. and Stevens, T. H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants: Evidence for a prevacuolar compartment in class I vps mutants. *Mol. Biol. Cell.* **3**, 1389–1402.
- Roberts, C. J., Raymond, C. K., Yamashiro, C. T. and Stevens, T. H. (1991). Methods for studying the yeast vacuole. *Meth. Enzymol.* **194**, 646.
- Robinson, J. S., Graham, T. R. and Emr, S. D. (1991). A putative zinc finger protein, *Saccharomyces cerevisiae* Vps18p, affects late golgi functions required for vacuolar protein sorting and efficient a-factor

- prohormone maturation. *Mol. Cell. Biol.* **12**, 5813–5824.
- Samokyszyn, V. M., Miller, D. M., Reif, D. W., Saito, M. and Aust, S. D. (1989). Inhibition of superoxide and ferritin-dependent lipid peroxidation by ceruloplasmin. *J. Biol. Chem.* **264**, 21–26.
- Sherman, F., Fink, G. R. and Hicks, J. B. (1986). *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Thiele, D. J. (1988). ACE1 regulates expression of the *Saccharomyces cerevisiae* metallothionein gene. *Mol. Cell. Biol.* **8**, 2745–2752.
- Vulpe, C. D. and Packman, S. (1995). Cellular copper transport. *Ann. Rev. Nutr.* **15**, 293–322.
- Woolford, C. A., Dixon, C. K., Manolson, M. F., Wright, R. and Jones, E. W. (1990). Isolation and characterization of *PEP5*, a gene essential for vacuolar biogenesis in *Saccharomyces cerevisiae*. *Genetics* **125**, 739–752.
- Wright, C. F., McKenney, K., Hamer, D. H., Byrd, J. and Winge, D. R. (1987). Structural and functional studies of the amino terminus of yeast metallothionein. *J. Biol. Chem.* **262**, 12912–12919.
- Yamaguchi-Iwai, Y., Dancis, A. and Klausner, R. D. (1995). AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *EMBO J.* **14**, 1231–1239.
- Yuan, D. S., Stearman, R., Dancis, A., Dunn, T., Beeler, T. and Klausner, R. D. (1995). The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proc. Natl. Acad. Sci. USA* **92**, 2632–2636.
- Zhu, Z., Szczyepka, M. S. and Thiele, D. J. (1995). Transcriptional regulation and function of yeast metallothionein genes. In B. Sarkar (Ed.), *Genetic Responses to Metals*. Marcel Dekker Inc., New York.