Iron transport across the plasma membrane appears to be a unidirectional process whereby iron uptake is essentially irreversible. One of the major sequestration sites for iron is the vacuole that stores a variety of metals, either as a mechanism to detoxify the cell or as a reservoir of metal to enable the cell to grow when challenged by a low iron environment. Exactly how the vacuole contributes to the overall iron metabolism of the cell is unclear because mutations that affect vacuolar function also perturb the assembly of the plasma membrane high affinity transport system composed of a copper-containing iron oxidase, Fet3p, and an Fe^{3+}-specific iron transporter, Ftr1p. Here, we characterize the iron transporter homologue Fth1p, which is similar to the high affinity plasma membrane iron transporter Ftr1p. We found that Fth1p was localized to the vacuolar surface and, like other proteins that function on the vacuole, did not undergo Pep4-dependent degradation. Co-immunoprecipitation experiments showed that Fth1p also associates with the Fet3p oxidase homologue, Fet5p; and disruption of the FET3 gene results in the accumulation of Fth1p in the endoplasmic reticulum. We also found that loss of this protein complex leads to elevated transcriptional activity of the FET3 gene and compromises the ability of the cell to switch from fermentative metabolism to respiratory metabolism. Because the Fet5 protein is oriented such that the oxidase domain of Fet5p is luminal, this complex may be responsible for mobilizing intravacuolar stores of iron.

The mechanism by which iron is transported across the plasma membrane in yeast has been intensively studied (1, 2). High affinity transport is catalyzed by a complex of proteins that comprise the gene products of FET3 and FTR1 (3, 4). FET3 encodes a multicopper iron oxidase, whereas FTR1 encodes a polytopic membrane protein that is thought to be a Fe^{3+}-specific transporter. Together, Fet3p and Ftr1p are responsible for the transport of Fe^{2+} across the plasma membrane; the observation that a functional Fth1p in the endoplasmic reticulum is essential for mobilizing intravacuolar stores of iron.

We also found that loss of this protein complex leads to elevated transcriptional activity of the FET3 gene and compromises the ability of the cell to switch from fermentative metabolism to respiratory metabolism. Because the Fet5 protein is oriented such that the oxidase domain of Fet5p is luminal, this complex may be responsible for mobilizing intravacuolar stores of iron.
lyses. Finally, we find that disruption of the Fth1p-Fet5p complex alters iron homeostasis and can inhibit the transition from fermentative to respiratory metabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glutathione-agarose beads were purchased from Amer sham Pharmacia Biotech. Yeast nitrogen base was purchased from DIFCO. Amino acid supplements were purchased from Bio101 (La Jolla, CA). Chemiluminescent developer was purchased from Pierce. Bathophenanthroline disulfonic acid (BPS) was purchased from Sigma. Bathocuproinedisulfonic salt was purchased from Aldrich. All other chemicals were of high grade and were purchased from commercial sources. Oxytetracycline was purchased from Enogenetics (Corvallis, OR). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Protease inhibitor mixture (Complete™) was purchased from Roche Molecular Biochemicals.

**Yeast Strains and Culture Conditions**—The yeast strains used in this study were SEY6210 (MATa ura3-52 leu2-3,112 his3-d200 trp1-901 lys2-801 suc2-D9) (19), PLY1313 (MATa fet5Δ:His3 ura3-52 leu2-3-112 his3-d200 trp1-901 lys2-801 suc2-D9) (19), PLY1546 (MATa fet5Δ::His3 fth1Δ::NEO ura3-52 leu2-3-112 his3-d200 trp1-901 lys2-801 suc2-D9) (19), SFS38-9Da (MATa pep4-3 ura3-52 leu2-3-112;111 his3-d200 trp1-901 lys2-801 suc2-D9). PLY1546 and SFS38-9Da were transformed with plasmid expressing plasmid, pRS316, containing a 400-bp fragment was transformed into SEY6210. Genomic DNA was prepared from Research Genetics (Huntsville, AL).

**Immunofluorescence microscopy**—Whole cell lysates from growing cultures of yeast cells were made by pelleting yeast, washing the pellet once with water, and resuspended and incubated in 4% fresh paraformaldehyde containing 50 mM KPO4, pH 7.5. Cells were then pelleted and resuspended in 10 ml of 1.2 M sorbitol, 50 mM Tris-HCl, pH 8.0, 5% SDS, 10% β-mercaptoethanol, and then spheroplasted in 1.2 M sorbitol, 50 mM Tris-HCl, pH 8.0, 5% SDS, 10% β-mercaptoethanol, and then spheroplasted in 1.2 M sorbitol. Cells were then adhered to polylysine-coated glass slides prior to immunolabeling. For labeling with FM4-64 (Molecular Probes, Eugene, OR), cells were labeled for 30 min at 30 °C in 500 mM FM4-64 in SD medium containing 50 mM KPO4, pH 7.5. Cells were then washed in water and cultured in unbuffered SD medium or yeast extract-peptone-dextrose for 30 min. For labeling with plasmid expressing GFP, cells were grown in SD medium to a density of 0.8–1.2. An 0.5 volume of GFP-KILL buffer (1 x Tris, pH 8.0, 0.5% sodium azide) was added to ensure that membrane traffic had ceased and that the GFP protein was kept at the optimal pH level for fluorescence detection. For some experiments, 10 μM DAPI was included in the GFP-KILL buffer. GFP fusion proteins were imaged using a 470-nm excitation filter, 485-nm dichroic mirror, and 525-nm emission filter. Images were captured with a Hamamatsu ORCA CCD camera mounted on an Olympus BX-60 microscope equipped with a 100× oil objective. Image sets were processed and overlaid using Adobe Photoshop™.

**Affinity purification**—A yeast strain (RGYS3347) carrying the HIS3 gene deletion was transformed with plasmids containing the oligonucleotides ATGGTTCTTCTCCCGTGTTGGTCTGATCGCGCATGTTCTTCTCAGCAGAGAT- TCTTCCTAATTGTTTTGATCACCGG. The resulting PCR fragment was transformed into SEY6210. Genomic DNA was prepared from HIS+ transformants by pelleting 1–2 optical density units of cells and resuspending in 300 μl of 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Complete™, Roche Molecular Biochemicals). Cell lysates were then adhered to polylysine-coated glass slides prior to immunolabeling.

**Immunoblotting**—Whole cell lysates from growing cultures of yeast cells were made by pelleting yeast, washing the pellet once with water, and resuspending in 50 mM Tris, pH 6.5, 8% urea, 2% SDS. Glass beads (150–200 μm) were added, and the sample was vortexed for 2 min. Samples were then adjusted to 1× Laemmli sample buffer (20). For GST-Fet5p, the samples were run in a 4% stacking and a 10% separating gel. Proteins were transferred to a nitrocellulose membrane, blocked in 3% nonfat dry milk, and probed with affinity-purified antibodies, followed by the mouse anti-VMA2p (13D11) and anti-ALP (Pho8p; 1D3) monoclonal antibodies were purchased from Molecular Probes. The polyclonal anti-HA antibody was a kind gift of Kathy Hill and Tom Stevens (University of Oregon, Eugene, OR). The monoclonal anti-HA antibody 12D12 and the monoclonal anti-6×His antibody, which recognizes an epitope composed of six tandem histidine residues, was purchased from Babco (Berkeley, CA). Rabbit polyclonal anti-Kar2p was a kind gift of Mark Rose (Princeton University). Texas Red-labeled secondary antibodies were purchased from Molecular Probes. Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech. DIGoxin labeling antibodies was performed according to the manufacturer’s instructions using a digoxin labeling kit (Roche Molecular Biochemicals).

**Bathocuproinedisulfonic salt**—Bathocuproinedisulfonic salt was purchased from Aldrich. All other chemicals were of high grade and were purchased from commercial sources.

**Fluorescence Microscopy**—Immunofluorescence microscopy using anti-HA, anti-Vma2p, and anti-Kar2p was performed essentially as previously described (19). Briefly, cells were grown in selective SD medium and adjusted to 3.7% formaldehyde. After 30 min, cells were then resuspended and incubated in 4% fresh paraformaldehyde containing 50 mM KPO4, for 12 h. Cells were washed in 200 mM Tris, pH 8.0, + 20 mM EDTA + 1% β-mercaptoethanol, and then spheroplasted in 1.2 M sorbitol, 50 mM Tris, pH 8.0, containing 10 μg/ml oxytetracycline for 30 min. Cells were then washed in 1.2 M sorbitol, permeabilized for 1 min in 1% SDS, 1.2 M sorbitol, and washed once more in water. Cells were then adhered to polylysine-coated glass slides prior to immunolabeling. For labeling with FM4-64 (Molecular Probes, Eugene, OR), cells were labeled for 30 min at 30 °C in 500 mM FM4-64 in SD medium containing 50 mM KPO4, pH 7.5. Cells were then washed in water and cultured in unbuffered SD medium or yeast extract-peptone-dextrose for 30 min. For labeling with plasmid expressing GFP, cells were grown in SD medium to a density of 0.8–1.2. An 0.5 volume of GFP-KILL buffer (1 x Tris, pH 8.0, 0.5% sodium azide) was added to ensure that membrane traffic had ceased and that the GFP protein was kept at the optimal pH level for fluorescence detection. For some experiments, 10 μM DAPI was included in the GFP-KILL buffer. GFP fusion proteins were imaged using a 470-nm excitation filter, 485-nm dichroic mirror, and 525-nm emission filter. Images were captured with a Hamamatsu ORCA CCD camera mounted on an Olympus BX-60 microscope equipped with a 100× oil objective. Image sets were processed and overlaid using Adobe Photoshop™.

**Antibodies**—Plasmid pJL54 was transformed into MCI1061 Escherichia coli, which were then induced with isopropyl-1-thio-b-D-galacto pyranoside for 20 h. The GST-Fet5p-GFP fusion protein was purified using glutathione-agarose, dia lyzed against phosphate-buffered saline, and used to immunize rabbits. Anti-GFP antibodies were affinity purified over an Affi-Gel 10/15 column to which was attached the GST-GFP fusion protein according to manufacturer’s directions (Bio-Rad). Polyclonal anti-GST antibodies were purified from serum from rabbits immunized with irrelevant GST fusion proteins over an Affi-Gel 10/15 column to which was attached the GST protein alone.

The mouse anti-Vma2p (13D11) and anti-ALP (Pho8p; 1D3) monoclonal antibodies were purchased from Molecular Probes. The polyclonal anti-HA antibody was a kind gift of Katharyn Hill and Tom Stevens (University of Oregon, Eugene, OR). The monoclonal anti-HA antibody 12D12 and the monoclonal anti-6×His antibody, which recognizes an epitope composed of six tandem histidine residues, was purchased from Babco (Berkeley, CA). Rabbit polyclonal anti-Kar2p was a kind gift of Mark Rose (Princeton University). Texas Red-labeled secondary antibodies were purchased from Molecular Probes. Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech. DIGoxin labeling antibodies was performed according to the manufacturer’s instructions using a digoxin labeling kit (Roche Molecular Biochemicals).

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**Bathocuproinedisulfonic salt**—Bathocuproinedisulfonic salt was purchased from Aldrich. All other chemicals were of high grade and were purchased from commercial sources.
washed three times in phosphate-buffered saline, 1 mM EDTA, 0.5% Triton X-100. Samples were then solubilized in Laemmli sample buffer and analyzed by immunoblotting using a mouse monoclonal anti-HA antibody or digoxin-labeled rabbit anti-GFP antibody to assess the amount of HA- and GFP-tagged protein immunoprecipitated by the rabbit anti-GFP antibody. As a negative control, PAP was analyzed independently. Several changes from the original sequence were noted and submitted to GenBank™ (accession number AF177330).

We validated the authenticity of the various GFP constructs by complementation: Vph1-GFP could restore vacuolar acidification to a vph1Δ mutant; Ste3-GFP could restore mating efficiency to a ste3Δ mutant; and GFP-PHOS was found to traffic appropriately to the vacuole in an APM3-dependent manner.²

RESULTS

Fth1p and Fet5p Localize to the Vacuole Surface—We tagged Fth1p with the green fluorescent protein as part of a separate study to find membrane protein markers of different subcellular compartments. Surprisingly, we found Fth1p localized to the vacuole and went on to characterize this protein further. Using a set of GFP-tagged constructs, we found that Fth1p tagged with GFP at the C terminus was clearly localized to the limiting membrane of the vacuole, where it colocalized with the endocytic tracer FM4-64 that was allowed to transit to the vacuole for 1 h (Fig. 1). Despite localization to the vacuole, these data did not necessarily indicate that Fth1p functions at the vacuole. Indeed, overexpression of many membrane proteins that function, such as the Golgi apparatus or plasma membrane, can accumulate within the vacuole upon overexpression or upon deletion of Golgi-targeting motifs. This phenomenon is absent in cells lacking the vacuolar proteases that allow these proteins to accumulate without degradation. Recently, it has been found that membrane proteins destined for degradation in the vacuole actually localize within the lumen of the vacuole, whereas other proteins that function at the vacuole are localized to the vacuole surface (24). Other examples of this type of intravacuolar localization of proteins destined for degradation have been found previously (25–30).

Thus, to further examine the subcellular distribution of Fth1p, we compared the labeling pattern of Fth1p-GFP to a panel of GFP fusion proteins made with either proteins known to function at the vacuole or proteins known to undergo PEP4-dependent degradation in the vacuole. This analysis was performed in a pep4Δ mutant strain that lacked vacuolar proteases to ensure that proteins localized to the interior of the vacuole could be visualized. In the case of Fth1p, Fth1p-GFP could clearly be seen on the vacuolar surface in Pep⁻ and pep4 Δ mutant cells, indicating that Fth1p does not localize within the interior of the vacuole. This same labeling pattern was also observed for Vph1p and Pho8p GFP fusion proteins. Vph1p is a component of the vacuolar-ATPase known to function at the vacuole, whereas PHOS encodes the type II membrane protein alkaline phosphatase, which has been localized to the vacuole surface in previous studies (24, 28). In contrast, proteins such as Ste3p, which function at the plasma membrane and then undergo PEP4-dependent degradation, were clearly seen within the vacuole and did not overlap with the FM4-64 label that was restricted to the limiting membrane of the vacuole characteristic of FM4-64 labeling (31).

As a further analysis, we examined Fth1p-GFP from wild-type and pep4 Δ mutant cells by immunoblot analysis (Fig. 2). We found that with anti-GFP antibodies we could label a single 75-kDa band from both wild-type and pep4 Δ cells. This is the predicted molecular mass of the fusion protein; 51502 kDa for the FTH1 open reading frame that also had ends that overlapped the insertion site of the HA epitope. Therefore, the predicted molecular mass of the fusion protein; 51502 kDa for the FTH1 open reading frame and 50.5 kDa for the GFP open reading frame. When equal amounts of whole cell extract were

² J. Urbanowski, D. Swartz, and R. C. Piper, unpublished data.
loaded, the levels of Fth1p were the same. In contrast, this same analysis performed with Ste3p showed that the pep4 mutation dramatically increased the level of Ste3p, consistent with previous studies showing that Ste3p is rapidly degraded by vacuolar proteases after endocytosis from the plasma membrane (27). Taken together, these data indicate that Fth1p is a resident protein of the vacuole and thus likely functions at this compartment.

The Fth1p homologue, Ftr1p, is a Fe\(^{3+}\)-specific transporter that forms a complex with the iron oxidase Fet3p. Fet3p is a type I membrane protein that is a multi-copper-dependent oxidase and bears homology to the iron oxidase ceruloplasmin. The Ftr1p-Fet3p complex has been localized to the plasma membrane and is responsible for high affinity uptake of iron into the cell (4). Another protein that bears close homology with the Fet3 iron oxidase is Fet5p (32). FET5 was originally identified as a high copy suppressor of iron-limited growth of a fet3 fet4 double mutant in which both the high affinity iron transport system (FET3-dependent) and the low affinity transport system (FET4-dependent) were disrupted. FET5 itself is responsible for ~85% of the total cellular iron oxidase activity, although disruption of FET5 does not lead to a decrease in the apparent transport of iron into the cell (32). Although the suppresser activity of high copy FET5 was dependent on the plasma membrane transporter Ftr1p, the observation that disruption of FET5 did not result in a decrease in iron uptake indicates that Fet5p might normally function elsewhere. Thus, given the functional interaction between Ftr1p and Fet3p, we were interested in the potential interaction between Fth1p and the oxidase Fet5p. Examining this possibility, we localized Fet5p using indirect immunofluorescence in pep4 mutant cells to determine whether Fet5p was also localized to the vacuole surface. To facilitate localization, we inserted an epitope tag encoding 6 tandem epitopes derived from the hemagglutinin spike glycoprotein (HA epitope). Immunoblot analysis showed that the Fet5-HA protein migrates as an ~100-kDa band con-
sistent with what has been observed previously as glycosyla-
tion of the predicted 71-kDa core protein (32). Fig. 3 shows
double immunofluorescence localization of Fet5-HA with the
vacuolar protein Vac8p as well as with the ER protein Kar2p in
pep4 mutant cells. Fet5p could clearly be seen on the vacuolar
membrane in a pattern very similar to Vac8p, a cytosolic pro-
tein associated with the vacuole (34). In contrast, Fet5p did not
colocalize with the ER marker Kar2p. Importantly, we did not
detect any fluorescence labeling within the vacuole but rather
only on the limiting membrane of the vacuole, indicating that,
like Fth1p, Fet5p is a resident protein of the vacuole.

Interaction between Fth1p and Fet5p—Given the observation
that Fet5p colocalizes with Fth1p on the vacuole, we sought to
determine whether these two proteins indeed formed a complex
as do Ftr1p and Fet3p. The model that Fet3p forms a complex
with Ftr1p is based on the observation that disruption of FET3
resulted in the accumulation of Ftr1p in the endoplasmic reticu-
ulum (4). Thus, we reasoned that if Fet5p and Fth1p also
formed a complex before exiting the ER, we might observe a
similar mislocalization of one putative subunit upon deletion of
the other partner subunit. Fig. 4 shows the localization of
Fth1p to the ER in a strain lacking the FET5 gene. In fet5Δ
cells, Fth1-GFP was not localized to the vacuole as denoted by
FM-64 labeling. Rather, Fth1p was localized to structures sim-
ilar to the ER, located underneath the plasma membrane and
around the nucleus identified by DAPI labeling (Fig. 4A). To
confirm this localization, we also performed double labeling
experiments with Kar2p and Vma2p (Fig. 4, B and C). Here we
observed good colocalization of Fth1p with the ER marker
Kar2p and very little, if any, colocalization with the vacuolar
ATPase subunit, Vma2p. In this experiment we chose to in-
crease the levels of Fth1p as much as possible to enable better
detection by growing cells in an iron-limited medium. Growth
of cells in SD containing the Fe²⁺ chelator BPS at 100 μM was
shown to significantly increase the levels of Fth1 protein (see
below). To control for any effects of higher levels of Fth1p
expression on localization, we also localized Fth1p in wild-type
cells grown in 100 μM BPS (Fig. 4D). Under these conditions,
the higher levels of Fth1p were localized to the vacuole with no
Fth1p apparent in the ER in wild-type cells. Also, the localiza-
tion of Fth1-GFP to the ER was observed in fet5Δ cells grown in
the absence of BPS (data not shown).

The observation that Fth1p was dependent on the presence

**FIG. 2.** Fth1p does not undergo PEP4-dependent degradation. SF838-9Δa pep4Δ mutant cells (lanes 1 and 3) or congenic RPY10 wild-type Pep⁺ cells (lanes 2 and 4) were transformed with the Fth1-GFP-expressing plasmid pJLU47 (lanes 1 and 2) or the Ste3-GFP-expressing plasmid pJLU34 (lanes 3 and 4). Cell lysates were immuno-
blotted with anti-GFP antibodies or anti-ALP antibodies (loading control).

**FIG. 3.** Fet5p is localized to the vacuole. Pep⁻ yeast cells (LW5541: pep4) carrying the Fet5-HA-expressing plasmid pJLU61 were fixed,
permeabilized, and labeled with monoclonal anti-HA and polyclonal antibodies to either Kar2p (panels in row A) or Vac8p (panels in row B). Shown
are matched images of GFP, Texas-Red immunofluorescence, DAPI, RGB merged images, and dichroic (DIC) images, respectively, left to right. C,
anti-HA immunoblot of LW5541 cells carrying the pJLU61 or pRS316 vector only.
of Fet5p for exiting the ER strongly indicated that Fth1p forms a complex with Fet5p. As a more direct test, we performed coimmunoprecipitation experiments from cells harboring the Fth1-GFP-producing plasmid (pJLU73) and the Fet5-HA-producing plasmid (pJLU61). Detergent-solubilized spheroplasts were subjected to immunoprecipitation with anti-HA or anti-GFP antibodies, and the immunoprecipitates were analyzed by Western blot for GFP- and HA-tagged proteins. As a control for nonspecific precipitation, we also performed reactions with a polyclonal anti-GST antibody and a monoclonal antibody that recognizes a 6-histidine tag. Fig. 5 shows the anti-GFP antibody immunoprecipitated the Fth1-GFP protein and the Fet5-HA protein. In contrast, neither the anti-GST nor the anti-6xHis antibody immunoprecipitated the Fth1-GFP or the Fet5-HA protein. Comparable results were obtained when detergent extracts were made with CHAPS (data not shown).

Orientation of the Fth1p-Fet5p Complex—Given the similarities in the primary structure of Fet5p and Fet3p, one would expect the orientation of the Fet5p-Fth1p complex to be similar to the orientation of the Fet3p-Ftr1p complex. The fact that the Fet5p glycoprotein has a predicted short N-terminal hydrophobic signal sequence as well as having all of the predicted N-linked glycosylation sites on the N-terminal side is strong evidence that Fet5p shares the same topology as Fet3p in that the copper-binding oxidase domain is luminal (32). To confirm this orientation as well as probe the orientation of Fth1p, we subjected membrane fractions prepared from cells producing Fet5p-HA and Fth1-GFP to limited proteolysis and then immunoblotted with anti-GFP antibodies. As a control, we also
Fth1p Forms a Complex with Fet5

The transcription of the copper chelator bathocuproinedisulfonic acid. Thus, as shown in previous studies, the transcription of Fet5 is activated by low iron conditions (1, 35). Importantly, we found that the addition of 500 μM FeSO₄ further decreased the level of Fet3 and FTH1 promoter-dependent lacZ production, indicating that our SD medium had intermediate levels of iron such that the level of intracellular iron was low enough to induce some activation of the Fet3 gene.

In contrast to FTH1, the level of Fet3 mRNA is augmented in strains carrying an activated allele of AFT1 (35). As confirmation of the iron responsiveness of the FTH1 gene, we found that the levels of Fet3 protein are significantly elevated in cells grown in low iron medium (SD medium containing the iron chelator BPS at 100 μM) (Fig. 7A). Likewise, we found that activation of the FTH1 promoter, as assessed by β-galactosidase activity produced from the appropriate FTH1 promoter-lacZ fusion plasmid, was greatly elevated in response to 100 μM BPS (Fig. 7B), whereas no effect was observed upon the addition of the copper chelator bathocuproinedisulfonic acid. Thus, as shown in previous studies, the transcription of FTH1 and Fet5 is activated by low iron conditions (1, 35). Importantly, we found that the addition of 500 μM FeSO₄ further decreased the level of Fet3 and FTH1 promoter-dependent lacZ production, indicating that our SD medium had intermediate levels of iron such that the level of intracellular iron was low enough to induce some activation of the Fet3 gene.

Given the ability of the cell to rapidly and markedly up-regulate transcription of Fet3 to compensate for iron-poor conditions, we decided to use the transcriptional activity of the Fet3 gene as a functional indicator of the overall level of intracellular iron. We reasoned that if the Fet5p-Fth1p complex was important for providing iron to the cell from intracellular stores, the absence of this complex might lead to activation of Fet3 under conditions of intermediate levels of iron. Thus, we assessed the transcriptional activity of the Fet3 promoter by measuring the β-galactosidase activity produced from the Fet3-lacZ fusion plasmid in wild-type and fet5Δ cells grown in SD medium containing 10 μM BPS, 100 μM BPS, or 500 μM FeSO₄. At 100 μM BPS, the level of β-galactosidase activity was the same for both the wild-type strain and the fet5Δ mutant, showing that the maximal response of the Fet3 promoter to low levels of iron was similar between wild-type and fet5Δ mutants (data not shown). Likewise, the Fet3 promoter activity in high levels of iron was the same between both

Role of the Fth1p-Fet5p Complex in Iron Metabolism—One of the key responses to growth in low iron conditions is the transcriptional up-regulation of the high affinity plasma membrane iron transport system (1). Consistent with a role in iron metabolism, Fet5 message levels have been shown to increase as the levels of Fet3 protein are augmented under conditions of intermediate levels of iron such that the level of intracellular iron was low enough to induce some activation of the Fet3 gene.

Given the ability of the cell to rapidly and markedly up-regulate transcription of Fet3 to compensate for iron-poor conditions, we decided to use the transcriptional activity of the Fet3 gene as a functional indicator of the overall level of intracellular iron. We reasoned that if the Fet5p-Fth1p complex was important for providing iron to the cell from intracellular stores, the absence of this complex might lead to activation of Fet3 under conditions of intermediate levels of iron. Thus, we assessed the transcriptional activity of the Fet3 promoter by measuring the β-galactosidase activity produced from the Fet3-lacZ fusion plasmid in wild-type and fet5Δ cells grown in SD medium containing 10 μM BPS, 100 μM BPS, or 500 μM FeSO₄. At 100 μM BPS, the level of β-galactosidase activity was the same for both the wild-type strain and the fet5Δ mutant, showing that the maximal response of the Fet3 promoter to low levels of iron was similar between wild-type and fet5Δ mutants (data not shown). Likewise, the Fet3 promoter activity in high levels of iron was the same between both

![Fig. 6. Protease susceptibility of the Fth1p C terminus.](Image)

The protease susceptibility of the Fth1p C terminus (Fig. 6A). As an internal control, we immunoblotted Pho8p; note that upon protease treatment, Pho8p migrates slightly faster indicating that the short N-terminal portion of Pho8p was cleaved by proteases. In the case of Fet5-HA, the HA epitope was susceptible to limited protease treatment, confirming that the orientation of the Fet5 protein is similar to that of a type I transmembrane protein such that the short C-terminal portion is oriented toward the cytosol. Likewise, the C-terminal GFP domain within the Fth1p-GFP protein was also completely accessible to protease treatment. These data not only confirm the localization of Fth1p to the vacuole surface, but they also indicate that the C terminus Fth1p is oriented toward the cytosol.

![Fig. 7. Iron homeostasis and regulation of Fth1p levels.](Image)

A, SYE6210 cells harboring the Fth1-GFP-expressing plasmid pJLU47 were cultured in SD medium or SD containing 100 μM BPS for 12 h as indicated. Equivalent extracts were prepared and immunoblotted with anti-GFP, anti-HA, or anti-ALP antibodies. B, SYE6210 cells were transformed with the FTH1-lacZ construct pJLU65, the Fet3-lacZ construct pJLU62, or the Fet4-lacZ construct pJLU68 and grown in SD medium. Cells were then shifted to SD medium containing 500 μM FeSO₄, 100 μM BPS, 100 μM BPS or no additions for 6 h prior to assaying for β-galactosidase activity. Enzyme activity was normalized to cell number and expressed relative to the mean ± S.D.
wild-type and fet5Δ cells (Fig. 7C). In contrast, fet5Δ cells grown in SD medium or SD medium containing only 10 μM BPS showed a 2-fold increase in the level of β-galactosidase activity, indicating that iron levels were compromised enough in fet5Δ mutants to result in activation of the FET3 gene.

As a more substantial test of Fth1p-Fet5p function, we sought to develop a regimen by which vacuolar iron stores might be necessary for growth. Previous studies have shown that during the switch from growth on glucose to growth on glycerol that vacuolar iron stores are depleted (18). This depletion is observed even when the cells are cultured in iron-replete medium and most likely represents the large amount of iron that is required for the proper synthesis of the heme-containing mitochondrial cytochrome oxidases (18). Thus, we tested whether fet5Δ fth1Δ mutant cells were compromised in making a transition from fermentative to respiratory metabolism. In the course of these experiments we found that prior treatment of cells with the iron chelator BPS accentuated the defects we observed, possibly by limiting the amount of iron stored in the cells with the iron chelator BPS accentuated the defects we observed, possibly by limiting the amount of iron stored in the

As an iron transport complex on the vacuole, Fth1p-Fet5p could serve one of two functions. One possibility is that this complex could be used to detoxify the cell of high intracellular levels of iron. Perhaps the iron oxidase activity of the complex could modify iron to be bio-unavailable as Fe3+, which could be sequestered by binding to polyphosphates or other factors within the vacuole. However, this possibility is counterintuitive given that Fth1p levels increase when cells are challenged by iron starvation. One of the difficulties in assessing the role of these proteins in overall iron-dependent growth of the cell is that there are many mechanisms in place to compensate for the lack of iron. When iron levels are low in the cell, the production of the iron transport systems are elevated by activation of the transcription factor Aft1p to restore intracellular iron levels (35). In the absence of the high affinity Ftr1p-Fet3p system or in low iron medium, Fet4p levels increase and iron uptake across the plasma membrane is maintained by this low affinity/
low specificity transport system (12, 13). Although these mechanisms may not be viable strategies in the wild because they may incur increased risk to other environmental factors such as toxic heavy metals, they do obscure functional analysis of these iron transport proteins (14). We find that when cells are grown in medium containing intermediate levels of iron, disruption of the vacuolar iron transport complex caused the activation of \textit{FET3} transcription, indicating that intracellular iron levels are perturbed enough to activate the \textit{FET3} promoter. Iron can be accumulated to high levels within vacuoles and then mobilized from these stores and incorporated into other cellular proteins, especially during the transition from fermentative to respiratory growth (18). Our analysis indicates that the Fth1p-Fet5p complex plays an important role in the mobilization of vacuolar iron stores during this transition and that absence of the complex results in a significant delay in the synthesis of many iron-containing enzymes required for respiration.

One question that arises from these studies is how iron storage might be regulated. Iron might be stored in the vacuole as Fe\(^{3+}\) and thus not as a substrate for the Fth1p-Fet5p complex. This model would predict a role for a vacuolar localized iron reductase. Currently, there are seven recognized iron reductase-related genes in yeast (38). All but one, \textit{FRE7}, are activated by growth in low iron conditions, advancing the possibility that one of these proteins is localized exclusively to the vacuole. Fet5p has been shown to be a potent iron oxidase, and disruption of \textit{FET5} led to an 85% decrease in total cellular oxidase activity (32). Fet5p was originally characterized as a multicopy suppressor of the iron-limited growth defect of a \textit{fet3 fet4} mutant. Interestingly, this suppression was dependent on Ftr1p, suggesting that the overexpressed Fet5p was helping to assemble a functional Ftr1p complex capable of transporting iron across the plasma membrane. Importantly, in a \textit{fet3 fet4} mutant grown under iron-poor conditions, the expectation would be that Fet5p levels would already be induced because \textit{FET5} transcript levels are increased in iron limited medium, yet this is not enough to suppress the \textit{fet3} mutation. As expressed from a 2-\(\mu\) plasmid, Fet5p levels under these conditions would be expected to be exceptionally high and may allow some association with Ftr1p and exit from the ER. In our experiments, Fth1p-Fet5p was associated only with the vacuole even when levels of this complex were elevated by growing the cells in limited iron medium. Given the similarities between Fet5p and Fet5p and Fth1p and Ftr1p, it will be interesting to determine not only what domains of the iron oxidase are required for recognition of the respective transporter and vice versa but also to determine the mechanisms by which the Fth1p-Fet5p complex is sorted to the vacuole while the Fet3p-Ftr1p complex is sorted to the plasma membrane.
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