Combination Control of Yeast FET4 Gene Expression by Iron, Zinc, and Oxygen

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Combinatorial Control of Yeast FET4 Gene Expression by Iron, Zinc, and Oxygen

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Abstract

Acquisition of metals such as iron, copper, and zinc by the yeast Saccharomyces cerevisiae is tightly regulated. High affinity uptake systems are induced under metal-limiting conditions to maintain an adequate supply of these essential nutrients. Low affinity uptake systems function when their substrates are in greater supply. The FET4 gene encodes a low affinity iron and copper uptake transporter. FET4 expression is regulated by several environmental factors. In this report, we describe the molecular mechanisms underlying this regulation. First, we found that FET4 expression is induced in iron-limited cells by the Aft1 iron-responsive transcriptional activator. Second, FET4 is regulated by zinc status via the Zap1 transcription factor. We present evidence that FET4 is a physiologically relevant zinc transporter and this provides a rationale for its regulation by Zap1. Finally, FET4 expression is regulated in response to oxygen by the Rox1 repressor. Rox1 attenuates activation by Aft1 and Zap1 in aerobic cells. Derepression of FET4 may allow the Fet4 transporter to play an even greater role in metal acquisition under anaerobic conditions. Thus, Fet4 is a multisubstrate metal ion transporter under combinatorial control by iron, zinc, and oxygen.

Combinatorial control of gene expression occurs when a single gene is regulated in response to different signals (1). This control is often mediated by the combination of different transcription factor-binding sites in a promoter of the gene and action on those elements by multiple transcriptional activators and/or repressors. Combinatorial control allows expression of a gene to be modulated by multiple signals and this can provide several advantages to a cell or an organism. For example, activity of a particular gene product may be beneficial under a variety of conditions. Alternatively, a gene product may play different roles under different conditions and combinatorial control can allow this to occur as well. Combinatorial control also allows modulation of expression by more than
one signal to optimize the expression of a gene to match a particular combination of factors. In this paper, we describe the combinatorial transcriptional regulation of the yeast \textit{FET4} gene. \textit{FET4} encodes a metal ion transporter and its regulation by multiple signals allows modulation of expression in response to iron, zinc, and oxygen.

Metals ions such as iron and zinc are essential nutrients for cells. However, if accumulated to excessive levels, these same elements can be toxic. Therefore, genes encoding the proteins responsible for uptake of metal ions are highly regulated. Metal uptake systems have been well characterized in the yeast \textit{Saccharomyces cerevisiae} (2). For example, iron uptake is mediated by several different pathways in this organism. First, yeast can accumulate iron bound to microbial siderophores (3). Although \textit{S. cerevisiae} does not produce its own siderophores, a number of different transporters can accumulate iron bound by siderophores secreted by other microbes. \textit{S. cerevisiae} also uses a reductive mechanism for iron uptake. Extracellular Fe(III) is reduced to Fe(II) by the Fre1 and Fre2 reductases located in the plasma membrane (4, 5). The Fe(II) product is then the substrate of a high affinity transport system composed of the Fet3 multicopper oxidase and the Ftr1 permease (6, 7). Fet3 uses O\textsubscript{2} as a substrate to oxidize Fe(II) back to Fe(III) for subsequent transport by Ftr1 (8). High affinity copper uptake is also mediated by a reductive mechanism. Extracellular Cu(II) is reduced to Cu(I) by Fre1 reductase prior to transport across the plasma membrane by the Ctr1 and Ctr3 transporters (9–12). Finally, high affinity zinc transport is mediated by the Zrt1 and Zrt2 proteins (13, 14).

These high affinity uptake systems are tightly regulated in response to the cellular status of their respective metal substrates. Iron-limiting growth conditions trigger increased expression of the genes encoding Fre1, Fre2, Fet3, Ftr1, and many other genes involved in iron acquisition (2). This induction is mediated by two related transcription factors, Aft1 and Aft2, that share overlapping but non-identical sets of target genes (15, 16). In a similar fashion, expression of the \textit{ZRT1} and \textit{ZRT2} genes is induced under zinc deficiency by the Zap1 transcription factor (17) and the copper uptake transporters are regulated by the Mac1 copper-responsive activator protein (18). Each of these factors controls their respective target genes by binding to specific sequences in their promoters.

In addition to the high affinity systems, low affinity uptake systems for these substrates are also present in yeast. This is evident because mutational inactivation of the high affinity systems does not result in nonviable cells. Previous work identified a low affinity iron transporter encoded by the \textit{FET4} gene (19, 20). The Fet4 protein has six predicted membrane spanning regions and is localized to the plasma membrane. In addition to its role in iron transport, Fet4 has also been shown to be a physiologically relevant copper transporter (21, 22). Other studies suggested that Fet4 may be capable of transporting cobalt, manganese, and zinc as well (23). These observations lead us to the hypothesis that Fet4 plays a central role in the accumulation of a number of metal ions in yeast.
Various studies have indicated that \textit{FET4} expression and/or activity is influenced by a number of environmental factors. First, levels of Fet4 were \textasciitilde 4-fold higher in iron-deficient cells than in iron-replete cells (20). A recent microarray study (24) from our laboratory revealed that \textit{FET4} transcription was increased under zinc deficiency and that \textit{FET4} may be a Zap1 target gene. Finally, another microarray study comparing yeast cells grown in aerobic and anaerobic conditions indicated that \textit{FET4} mRNA levels were greatly elevated under anaerobiosis (25). These results suggested that \textit{FET4} transcription is under combinatorial control and regulated by multiple environmental factors. However, the underlying mechanisms for this regulation were unknown. In this report, we provide an understanding of \textit{FET4} regulation at a molecular level. \textit{FET4} transcription is subject to regulation by iron status via the Aft1 transcription factor and zinc status via Zap1. We present evidence that Fet4 is a physiologically relevant zinc transporter in yeast and this is likely the reason for its regulation by zinc status. Finally, repression of \textit{FET4} expression in aerobic cells is mediated by the Rox1 repressor in response to O\textsubscript{2} levels. These different regulatory proteins integrate \textit{FET4} gene expression with several environmental factors.

\textbf{Experimental Procedures}

\textbf{Yeast Strains and Culture Methods}

Yeast strains used are described in table 1. Cells were grown in metal-replete synthetic defined medium (SD) with necessary auxotrophic supplements at 30°C overnight in a shaking incubator. Cells were also cultured in Chelex-treated synthetic defined medium (CSD)\textsuperscript{1} to control metal availability. CSD was prepared by dissolving 20 g of glucose, 5.1 g of yeast nitrogen base without divalent cations or potassium phosphate (Bio 101, Inc., Vista, California), and 0.1 g each of adenine, histidine, tryptophan, and leucine, in a final volume of 1 liter of distilled water. Twenty-five g of Chelex-100 ion exchange resin (Sigma) was added and the mixture was stirred overnight at 4°C. After removal of the resin by filtration, the pH was adjusted to 4.0 with HCl, and the following were added to the indicated final concentrations: 0.4 mg/liter MnSO\textsubscript{4}, 0.04 mg/liter CuSO\textsubscript{4}, 100 mg/liter CaCl\textsubscript{2}, 500 mg/liter MgSO\textsubscript{4}, and 100 g/liter KPO\textsubscript{4}. The medium was then filter sterilized into polycarbonate flasks that had been washed with Citranox detergent (Alconox, White Plains, New York). Zinc was added as ZnCl\textsubscript{2} and iron was added as FeCl\textsubscript{3} to the indicated concentrations. In some experiments, the copper chelator bathocuproine disulfonic acid (Sigma) or the iron chelator bathophenanthroline disulfonic acid (BPS, Sigma) were added as indicated. Cells were inoculated at an initial level that allowed each strain to reach mid-log phase (\textit{A}\textsubscript{600} \textasciitilde 0.5) in \textasciitilde 18 h for aerated cultures and 24 h for anaerobic cultures. Cultures were made anaerobic by cultivation in a CO\textsubscript{2}-enriched, O\textsubscript{2}-depleted environment (BBL GasPak system, BD PharMingen).
DNA Manipulations

Reporter gene plasmids were constructed by fusion of promoter regions to lacZ (26). Reporter pFET4-lacZ contains 990 bp of DNA sequence from the upstream region of the FET4 open reading frame (24). Control reporters were FET3-lacZ (pFET3-lacZ, gift of A. Dancis), HIS4-lacZ (pHYC3) (27), ZRE-lacZ (pDg2-1) (28), CTR1- lacZ (pCTR1-lacZ) (a gift from A. Dancis), and ANB1-lacZ (YCP(33)AZ) (29). Mutation of specific sites within the FET4 promoter was carried out by introducing transversion mutations into the designated sites by PCR, followed by co-transformation of overlapping fragments and cloning into YEp353 (30) by gap repair (31). All mutations were confirmed by DNA sequencing. Strain BMW1 (zrt1::LEU2 zrt2::HIS3 fet4::TRP1) was constructed by γ-deletion of the FET4 locus (19) in strain CM34 (32). The FET4 overexpression plasmid was pCB1 (19) and the corresponding empty vector was pRS316-GAL1 (33).

β-Galactosidase Assays

In all experiments, cells were grown to mid-log phase (A600 ~0.5), harvested, and assayed for β-galactosidase activity. β-galactosidase activity was measured as described previously (26) and activity units were calculated as follows: (ΔA420 × 1,000)/(min × ml of culture used × culture A600). The data were plotted with error bars indicating ±1 S.D. Many values had small standard deviations such that the error bars are not apparent on the graphs.

Results

FET4 Is Regulated by Metal Availability

A FET4-lacZ reporter fusion was used to assess the regulation of FET4 gene expression in response to various growth conditions. To examine expression of FET4 under low iron and zinc conditions, we compared the FET4-lacZ reporter expression to that of control reporters in wild-type yeast. Expression of the FET4-lacZ reporter was induced ~2-fold by iron deficiency. This induction, while small, was very reproducible and was further increased by the addition of an iron chelator, BPS, to the growth medium (fig. 1A). The iron-deficient properties of these growth conditions were confirmed using an iron-responsive FET3-lacZ reporter (fig. 1B). The FET4-lacZ reporter was induced to an even greater extent, ~6-fold, in response to zinc deficiency (fig. 1A). The FET3-lacZ reporter was unaffected by zinc deficiency, whereas the zinc-responsive ZRE-lacZ reporter was highly induced by low zinc (fig. 1C) indicating the metal specificity of these responses. Finally, a HIS4-lacZ reporter (fig. 1D) revealed that the observed metal responses were not because of general alterations in lacZ expression resulting from either
iron or zinc deficiency. The reduced expression of HIS4-lacZ in low zinc has been observed previously (13) and is likely a consequence of the zinc deficiency impairing overall protein synthesis. Microarray experiments indicated that expression of many genes involved in protein synthesis (e.g., those encoding ribosomal subunits S5, S9A, L4A, and L10) and amino acid biosynthesis (e.g., THR1 and MET6) is repressed in zinc-deficient cells.

These data suggested that FET4 expression is regulated specifically by iron and zinc status. If so, we reasoned that FET4-lacZ expression would be altered in yeast strains defective for iron or zinc accumulation. The FET4-lacZ reporter and control reporters were transformed into mutant strains defective in high affinity iron uptake (fet3Δ) or high affinity zinc uptake (zrt1Δzrt2Δ). FET4-lacZ reporter activity was induced to a higher level in a fet3Δ mutant than wild-type cells and required more iron added to the growth medium to reduce expression to basal levels (fig. 2A). A similar effect was observed using the FET3-lacZ reporter (fig. 2B). The FET4-lacZ reporter was also induced to higher levels in the zrt1Δzrt2Δ strain and required higher levels of zinc to reduce expression (fig. 2C). A similar profile of zinc responsiveness was observed with the ZRE-lacZ reporter (fig. 2D). Additional assays of ZRE-lacZ and FET3-lacZ reporter expression also demonstrated that the zinc status was not altered under the variable iron conditions in figure 2, (A) and (B), and the iron status was not altered by changing the zinc status in figure 2, (C) and (D) (data not shown). These results are consistent with both iron and zinc status acting as signals for FET4 regulation.

**FET4 Is Not Regulated by Copper Status**

Previous studies indicated that Fet4 is a physiologically relevant copper uptake transporter (21, 22). Therefore, we tested whether FET4 transcription was regulated by the copper-responsive transcription factor Mac1. First, we compared expression of the FET4-lacZ reporter with a Mac1-regulated reporter (CTR1-lacZ) over a range of decreasing copper availability (fig. 3A). FET4-lacZ reporter activity was unchanged in medium supplemented with the copper chelator bathocuproine disulfonic acid up to 50 µM. CTR1-lacZ reporter expression increased up to 4.5-fold under these conditions. Higher concentrations of bathocuproine disulfonic acid did induce FET4-lacZ expression (data not shown) but this effect was likely an indirect effect of severe copper limitation on iron status (34). Furthermore, as shown in figure 3B, FET4-lacZ reporter activity in a mac1Δ strain was similar to that seen in a wild-type strain in iron- and zinc-limiting conditions. These results indicate that FET4 is not regulated by copper status despite its known role in copper uptake. This conclusion is also in agreement with a recent microarray study that failed to identify FET4 as a Mac1 target (35).
**Aft1 Is Required for Iron Responsiveness, whereas Zap1 Is Required for Zinc-responsive FET4 Expression**

To assess which factors regulate FET4 in response to iron and zinc, we examined regulation of the FET4-lacZ reporter in strains defective for three metal-responsive transcription factors. To determine whether Aft1 and/or Aft2 are required for FET4 activation under iron-deficient conditions, FET4-lacZ reporter activity was examined in aft1Δ and aft2Δ mutant strains. In the aft1Δ mutant, iron responsiveness was abolished (fig. 4A), but zinc-responsive expression was unaffected. In an aft2Δ mutant, FET4-lacZ expression was unchanged compared with wild type (data not shown). This result is in agreement with a recent microarray study that did not identify FET4 as an Aft2 target gene (16). Results of Lyons et al. (24) indicated that FET4 may be a Zap1 target. To test whether Zap1 was required for increased FET4 transcription under zinc deficiency, FET4-lacZ reporter activity was examined in a zap1Δ mutant. β-Galactosidase activity showed a strong response to iron deficiency but most of the increase resulting from zinc deficiency was abolished (fig. 4B). These data suggest that Aft1 and Zap1 are responsible for the iron and zinc responsiveness of FET4 expression, respectively. The zap1Δ cells grown without added iron and with BPS grew much more slowly than the cells grown without iron (data not shown) perhaps because of the dual problem of zinc deficiency (caused by the loss of Zap1) and severe iron deficiency (because of BPS addition). This slowed growth may explain the absence of additional FET4-lacZ expression when BPS was added to the medium as observed in figure 1A.

**FET4 Transcription Is Regulated by Oxygen**

Previous microarray studies revealed that FET4 expression is increased in anaerobic cells (25). One of the important components of O₂ regulation in yeast is the Rox1 repressor (36). Rox1 represses target gene expression in aerobic cells and this repression is relieved in low O₂ conditions. Therefore, we tested the effects of O₂ on FET4 transcription in wild-type and rox1Δ strains. We also assayed an ANB1-lacZ reporter, a known target of Rox1 repression, as a control (37). Consistent with the microarray studies, FET4-lacZ expression increased ~10-fold in wild-type cells grown anaerobically (fig. 5A). In the rox1Δ mutant, FET4-lacZ expression was greatly increased in aerobic cells supporting a role of Rox1 in repressing FET4 expression in the presence of O₂. In support of this hypothesis, similar results were obtained with the control ANB1-lacZ reporter.

Oxygen sensing by Rox1 occurs in response to heme; heme synthesis requires oxygen and, therefore, anaerobic cells are heme-deficient. Heme status is communicated to Rox1 indirectly through the Hap1 transcription factor. In low heme, Hap1 represses expression of the ROX1 gene thereby allowing derepression of Rox1 target genes (36). Although Hap1 also activates expression of ROX1 in the
presence of heme, this induction is not required for repression of at least some Rox1 target genes (e.g., ANB1). Thus, Hap1-independent expression of ROX1 is sufficient for full repression. To further assess the role of Rox1 in FET4 regulation, we examined the effects of heme status and the HAP1 genotype on FET4-lacZ expression under aerobic conditions. To control heme status, cells mutated in the HEM1 gene were used. HEM1 encodes δ-aminolevulinic acid (ALA) synthase and heme levels can be controlled in this mutant by increasing the amount of ALA supplemented into the medium. Increasing ALA levels had no effect on FET4-lacZ expression in either wild-type or hap1∆ mutant cells (fig. 5B). Consistent with a role of Rox1 repressing FET4 expression in response to heme, aerobically grown hem1∆ mutants had high FET4-lacZ expression when supplemented with low ALA levels (i.e., low heme conditions). This expression was reduced with increasing ALA supplementation. Mutation of the HAP1 gene greatly impaired the increased expression observed in low ALA grown cells. These data are consistent with oxygen controlling FET4 expression via heme-responsive Hap1 regulation of the Rox1 repressor.

**Rox1 Repression Attenuates Activation by Aft1 and Zap1**

Rox1 represses gene expression by recruiting the Tup1 and Ssn6 general transcription repressors to its target promoters where they block activation by positive factors also bound to these promoters (38). To determine whether Rox1 attenuates Aft1- and Zap1-mediated FET4 expression, we examined FET4-lacZ expression in wild-type and rox1∆ cells over a range of iron (fig. 6A) and zinc (fig. 6B) concentrations. Regulation of FET4-lacZ expression by iron and zinc was still observed in the rox1∆ strain. However, expression was elevated ~10-fold relative to wild-type cells at all concentrations of either metal. These data indicate that Rox1 repression modulates both Aft1 and Zap1 in regulating the FET4 promoter in response to metals.

**Mapping Regulatory Sites in the FET4 Promoter**

Potential binding sites for Rox1, Aft1, and Zap1 were identified in the 990-bp region upstream of the FET4 start codon based on the sequences of known binding sites in other promoters (fig. 7A). Six potential Rox1-binding sites (39), two potential Aft1 sites (40), and one Zap1 site (ZRE) (24) were identified in this region. A diagram depicting the locations of these sites is also shown. A deletion mutation removing Aft1 site 1 and Rox1 site 1 had no effect on FET4-lacZ expression indicating that these sites were not involved in FET4 regulation (data not shown). To assess the role of the remaining sites, mutations were constructed in the full-length promoter in which the nucleotides in each site were altered by transversion mutations. Mutation of Aft1 site 2 resulted in complete loss of iron regulation of the FET4-lacZ reporter while zinc-responsive expression was
unaffected (fig. 7B). Similarly, mutation of the potential ZRE eliminated almost all zinc responsiveness while iron regulation was unaffected. A residual 2-fold induction under zinc limitation was observed in the ZRE mutant suggesting that a Zap1-independent mechanism of zinc regulation is also present.

Mutagenesis of potential Rox1 sites in the promoter indicated that site 2 and/or 3 play the most important roles in mediating repression. Mutation of both Rox1 sites 2 and 3 (RoxM2,3) resulted in almost full derepression of the FET4-lacZ reporter in wild-type cells when compared with rox1Δ mutants (fig. 7C). In contrast, mutations in either sites 4 (RoxM4) or 5 (RoxM5) had no effect on Rox1-dependent repression. However, site 4 may contribute a small amount of repression given that FET4-lacZ expression in wild-type cells was routinely higher in RoxM2,3,4 (and RoxM2,3,4,5) than that observed with the RoxM2,3 allele. The role of the potential Rox1-binding site 6 was not examined in this study because deletion mutations indicated that this site was not required for repression (data not shown).

**FET4 Is a Physiologically Relevant Zinc Uptake System**

One possible explanation for the regulation of FET4 expression by Zap1 is that Fet4 also serves as a relevant pathway of zinc uptake for yeast cells. Several observations support this hypothesis. Consistent with an ability of Fet4 to transport zinc, overexpressed Fet4 could suppress the growth defect of a zrt1Δzrt2Δ mutant on medium without added zinc (fig. 8A). Wild-type cells grew well on this medium while zrt1Δzrt2Δ mutants transformed with the pRS316-GAL1 vector did not. FET4 overexpressed from the GAL1 promoter greatly improved growth of the mutant. Second, FET4 overexpression caused zinc sensitivity (fig. 8B). Relative to wild-type cells or zrt1Δzrt2Δ mutant cells transformed with the vector, zrt1Δzrt2Δ cells overexpressing FET4 grew more slowly on a medium containing 1 mM zinc. Third, mutation of FET4 in a zrt1Δzrt2Δ mutant resulted in slow growth of the strain on low zinc liquid medium when compared with the zrt1Δzrt2Δ strain (fig. 8C). This growth defect was largely suppressed by supplementing the medium with 1 mM zinc (fig. 8D). These results indicate that Fet4 is important for zinc uptake in mutants where the primary uptake pathways are inactive. A fet4Δ single mutant had no effect on growth in low zinc relative to a wild-type strain (data not shown) suggesting that wild-type cells could compensate for loss of Fet4 activity by up-regulating ZRT1 and ZRT2 expression. This conclusion was supported using a ZRE-lacZ reporter in wild-type and fet4Δ mutant strains. Both wild-type and fet4Δ mutant cells showed similar levels of ZRE-lacZ expression when severely zinc limited or zinc replete (fig. 8E). However, under conditions of moderate zinc availability, mutation of FET4 resulted in a 2–3-fold induction of ZRE-lacZ expression. These results suggest that in wild-type cells, Fet4 is contributing to zinc uptake and affecting Zap1 activity.
Discussion

During the preparation of this manuscript, Jensen and Culotta (41) reported a related analysis of \textit{FET4} gene expression. Our studies confirm and extend on their work. We demonstrate here that the \textit{FET4} gene is subject to transcriptional regulation in response to iron and zinc status and the presence or absence of O$_2$. These studies also provide an understanding of this regulation at a molecular level. In response to iron- or zinc-limiting growth conditions, \textit{FET4} expression is activated by Aft1 or Zap1, respectively. The role of Rox1 is to attenuate iron and zinc responsive expression in aerobic cells and allow derepression in anaerobic conditions. We have not provided evidence here that these proteins directly interact with the \textit{FET4} promoter. However, the effects of mutations in the genes encoding these transcription factors on \textit{FET4} expression plus the presence and importance of promoter elements similar to their consensus binding sites strongly support a direct role of these factors in regulating \textit{FET4} transcription.

Regulation of \textit{FET4} by iron status is easily understood given the importance of the Fet4 protein in iron accumulation (19, 20). Both our results and those of Jensen and Culotta (41) indicate that \textit{FET4} is regulated in response to iron status by Aft1. This conclusion is also supported by the work of Li and Kaplan (23) who showed that \textit{FET4} expression is up-regulated in a \textit{fet3A} mutant. Our results indicate that this increase is because of iron limitation imposed by loss of the high affinity iron uptake system. These observations were surprising given that two previous studies addressing \textit{FET4} regulation had discounted a role of Aft1. Casas et al. (42) observed that \textit{FET4} mRNA levels were not altered in cells treated with the iron chelator ferrozine, whereas other Aft1 target genes were induced. One possible explanation is that this treatment regimen was not sufficiently iron limiting to elicit a detectable response from \textit{FET4}. Also of note in their study (42) was that \textit{FET4} mRNA was detected in an \textit{aft1A} mutant, indicating that other factors were involved in its expression. We now know that at least one other positive factor—that is, Zap1—activates \textit{FET4} expression. Intriguingly, Dix et al. (20) noted that the Fet4 protein level and activity were induced ~4-fold in response to iron limitation. However, a constitutive allele of \textit{AFT1}, \textit{AFT1–1\textsuperscript{op}}, did not cause increased Fet4 uptake activity in iron-replete cells. Given that the \textit{AFT1–1\textsuperscript{op}} allele greatly increased \textit{FET4} mRNA levels in iron-replete cells (41), it now seems likely that some form of post-transcriptional regulation of Fet4 activity may occur in response to iron status. Post-translational control of copper (43), manganese (44), and zinc (45, 46) uptake transporters has already been observed in yeast so this seems a likely prospect.

Previous microarray analyses from our laboratory suggested the zinc-responsive control of \textit{FET4} transcription by Zap1 (24). Here we confirm that \textit{FET4} expression is increased under zinc-deficient conditions. Most of the increase in \textit{FET4} expression under zinc limitation was lost in the \textit{zap1A} mutant or when the ZRE in the \textit{FET4} promoter was mutated. However, there was a residual 2-fold
response to zinc deficiency observed in a zap1Δ mutant strain and with a \textit{FET4} reporter where the ZRE was mutated. This residual zinc responsiveness may be because of loss of some Rox1 repression in zinc-deficient cells resulting from a possible decrease in heme levels. The second enzyme in heme biosynthesis, ALA dehydratase, is zinc-dependent (47), and in zinc-deficient conditions, this enzyme could conceivably become limiting for heme biosynthesis. It is also attractive to speculate that the iron status of aerobic cells may be communicated to \textit{FET4} transcription through effects of iron depletion on heme levels with a resultant decrease in Rox1 repression. However, this does not appear to occur; all iron responsiveness we observed in aerobic cells required Aft1. Iron-deficient \textit{aft1Δ} mutants did not show residual iron responsiveness as we would expect if heme levels were also used as an indicator of iron status.

Regulation of \textit{FET4} by Zap1 is now understandable given our new appreciation of the role of Fet4 in zinc uptake. Several of our observations indicate that Fet4 is a relevant pathway for zinc uptake in wild-type cells. Most zinc uptake in aerobically grown yeast is mediated by the Zrt1 and Zrt2 transporters (14). However, a \textit{zrt1Δzrt2Δ} double mutant is viable indicating that additional systems of zinc uptake are present and our results suggest that Fet4 is one of those systems. A \textit{zrt1Δzrt2Δfet4Δ} mutant, whereas requiring more zinc to grow than the double mutant, is also viable indicating that still other mechanisms of zinc uptake are present. One possible pathway for zinc accumulation is by fluid-phase endocytosis to the vacuole where the Zrt3 zinc transporter could mediate transport of zinc into the cytoplasm (32).

Microarray analysis indicated that \textit{FET4} expression was greatly increased in anaerobic cells when compared with cells grown aerobically (25). Our results are in agreement with this previous study and we further showed that Rox1 is responsible for repressing \textit{FET4} expression in aerobic conditions. Loss of Rox1 repression under anaerobiosis results in increased expression of \textit{FET4}. In aerobic cells, Rox1 serves to attenuate activation by both of the metal-responsive activators, Aft1 and Zap1, that control \textit{FET4} expression. Rox1 functions by recruiting the Ssn6 and Tup1 repressors to promoters where they block activation by positive factors (38). It is interesting to note that in \textit{Candida albicans}, Tup1 regulates iron uptake genes in response to iron status (48). Thus, ours is not the first example of these multifunctional transcription repressors acting to control metal ion uptake in fungi.

Given that \textit{FET4} can contribute to the accumulation of iron and zinc in aerobic cells, the question arises as to why \textit{FET4} is repressed under these conditions. One possible explanation for this repression is that, because Fet4 is relatively non-selective in its metal substrates, metal toxicity may occur by expressing \textit{FET4} at high levels in aerobic conditions. Indeed, such an effect was observed in experiments with \textit{fet3Δ} mutants where \textit{FET4} is more highly expressed; \textit{fet3Δ} mutants have increased sensitivity to cobalt, copper, manganese, and zinc (23). For cobalt, this increased sensitivity was shown to be dependent on \textit{FET4} expression.
Moreover, Jensen and Culotta (41) showed that cells overexpressing \textit{FET4} aerobically because of mutation of \textit{ROX1} are hypersensitive to cadmium. Thus, repression of the relatively nonspecific Fet4 transporter allows aerobic cells to rely on the more specific high affinity metal uptake systems.

If high level Fet4 activity is so dangerous to cells, why then is \textit{FET4} expression derepressed in anaerobic cells? One possible explanation is that Fet4 is required under anaerobic conditions to compensate for the loss of Fet3 activity. The high affinity iron uptake system is composed of the Ftr1 transporter and the Fet3 multicopper oxidase. Fet3 activity is oxygen-dependent and the high affinity iron uptake system does not function under anaerobic conditions (8). Up-regulation of Fet4 in anaerobic cells may be a necessary risk taken by cells to maintain adequate iron accumulation and predicts that Fet4 will play a predominant role in iron accumulation under anaerobic conditions. In support of this hypothesis, Jensen and Culotta (41) observed that anaerobically grown \textit{fet4}\textDelta mutants accumulated much less iron than wild-type cells. Furthermore, expression of many iron uptake genes including \textit{FET3}, \textit{FTR1}, \textit{SIT1}, and \textit{FIT2} was found to be repressed in anaerobic conditions (25, 49). This repression, which is mediated through Aft1 (49), may result from changes in iron status because of \textit{FET4} derepression. An additional reason underlying the increased expression of \textit{FET4} under anaerobic conditions may be related to changes in metal toxicity. Under anaerobic conditions, metal ions such as iron and copper are less likely to participate in metal-catalyzed Fenton chemistry that contributes to their toxicity (50). Thus, higher levels of some metals may be more tolerable under conditions of low O\textsubscript{2}. One scenario that illustrates this point is that zinc-limited anaerobic cells may accumulate more iron and/or copper through Fet4 than would be tolerable under aerobic conditions.

Early studies of metal ion transport suggested the existence of a general divalent metal ion uptake system that was responsible for accumulation of many different substrates (51). With the subsequent characterization of the high affinity systems responsible for the specific uptake of copper (Ctr1 and Ctr3), iron (Fet3/Ftr1), manganese (Smp1), and zinc (Zrt1 and Zrt2), this hypothesis fell from favor. However, with our growing appreciation of the function of Fet4 in the uptake of multiple metal ion substrates, this model gains renewed credibility. We now know that Fet4 is a relevant transporter of iron, copper, and zinc in aerobic cells. Given the even higher levels of \textit{FET4} expression in anaerobic cells, our data and those of others (41) argue that Fet4 plays an even greater role in the uptake of multiple metal ions under these conditions. Thus, combinatorial control of \textit{FET4} expression allows this protein to function in iron and/or zinc uptake depending on the metal status of the cell. Combinatorial control also allows modulation of activity of the Fet4 in aerobic cells to prevent overaccumulation of toxic metal ions and compensate for lost high affinity transporter activity under these conditions.
| Strain      | Genotype                      | Source          |
|------------|-------------------------------|-----------------|
| BY4743     | MATa/α his3/his3 leu2/leu2 met15/MET15 lys2/LYS2 ura3/ura3 | Invitrogen Corp. |
| 36192      | BY4743 fet3::KanMX           | Invitrogen Corp. |
| 30596      | BY4743 mac1::KanMX           | Invitrogen Corp. |
| 34438      | BY4743 aft1::KanMX           | Invitrogen Corp. |
| 31090      | BY4743 aft2::KanMX           | Invitrogen Corp. |
| 31367      | BY4743 zap1::KanMX           | Invitrogen Corp. |
| 35484      | BY4743 rox1::KanMX           | Invitrogen Corp. |
| DY1457     | MATα ade6 can1 his3 leu2 trp1 ura3 | D. Stillman     |
| CM34       | DY1457 zrt1::LEU2 zrt2::HIS3 | Ref. 32         |
| BMW1       | DY1457 zrt1::LEU2 zrt2::HIS3 fet4::TRP1 | This work     |
| DEY1422    | MATα can1 his3 leu2 trp1 ura3 fet4::LEU2 | Ref. 20       |
| BWG1–7a    | MATα leu2ade1 his4 ura3      | L. Guarente    |
| MH1–4c     | BWG1–7a hem1::URA3           | M. Haldi        |
| BWG1–7a hap1 | BWG1–7a hap1::LEU2          | T. Keng         |
| DEY1       | BWG1–7a hem1::URA3 hap1::LEU2 | L. Guarente    |
Figure 1. *FET4* expression is induced by both iron and zinc limitation. Wild-type (BY4743) cells bearing the indicated lacZ reporter were grown in aerated CSD medium with (+) or without (−) added iron or zinc (50 µM). BPS was added where indicated at 50 µM. Cells were grown to mid-log phase (*A*₆₀₀ = ~0.5), harvested, and assayed for β-galactosidase activity. (A) *FET4*-lacZ reporter activity. (B) *FET3*-lacZ reporter activity. (C) ZRE-lacZ reporter activity. (D) *HIS4*-lacZ reporter activity. Results of a representative experiment are shown and the error bars represent ±1 S.D. of three replicates.
Figure 2. Mutant cells lacking high affinity uptake systems have altered \textit{FET4} expression. Wild-type cells (BY4743 in panels A and B, DY1457 in panels C and D) and the corresponding isogenic mutants lacking the high affinity iron uptake system (\textit{fet3\Delta}, 36192) or high affinity zinc uptake systems (\textit{zrt1\Delta zrt2\Delta}, CM34) were transformed with the indicated \textit{lacZ} reporter. These cells were grown in aerated CSD medium with iron or zinc added over a range of concentrations. Where the concentration of one metal was varied, the other was added at replete levels (50 µM). Cells were grown to mid-log phase (\textit{A}_{600} \approx 0.5), harvested, and assayed for \textit{β}-galactosidase activity. Results of a representative experiment are shown and the \textit{error bars} represent ±1 S.D. of three replicates.
Figure 3. FET4 expression is not regulated by copper status. (A) wild-type cells (BY4743) bearing the FET4-lacZ reporter (open bars) or the CTR1-lacZ reporter (filled bars) were grown in aerated SD medium with the copper chelator bathocuproine disulfonic acid (BCS) added at 0, 15, 30, or 50 µM. (B) mutant cells lacking the copper-responsive transcription factor Mac1 (mac1Δ, 30596) bearing the FET4-lacZ reporter were grown in aerated iron- or zinc-deficient medium as described in the legend to figure 1. Cells were grown to mid-log phase ($A_{600} \sim 0.5$), harvested, and assayed for β-galactosidase activity. Results of representative experiments are shown and the error bars represent ±1 S.D. of three replicates.
Figure 4. Aft1 and Zap1 are necessary for FET4 metal responsiveness. \textit{aft1Δ} (34438) and \textit{zap1Δ} (31367) mutants bearing the \textit{FET4-lacZ} reporter were grown in metal-deficient or replete conditions as described in the legend to figure 1. (A) \textit{FET4-lacZ} reporter activity in the \textit{aft1Δ} mutant. (B) \textit{FET4-lacZ} reporter activity in the \textit{zap1Δ} mutant. Cells were grown to mid-log phase (A$_{600}$ \textasciitilde 0.5) with aeration, harvested, and assayed for β-galactosidase activity. Results of a representative experiment are shown and the error bars represent ±1 S.D. of three replicates.
Figure 5. FET4 expression in response to oxygen is mediated by the heme/Hap1/Rox1 pathway. (A) wild-type (BY4743) and rox1∆ (35484) cells bearing the FET4-lacZ or ANB1-lacZ reporters were grown in metal-replete CSD medium with aeration (+O₂) or under anaerobic conditions (–O₂). (B) FET4-lacZ reporter activity in wild-type (BWG1–7A), hem1∆ (MH1–4c), hap1∆ (BWG1–7a hap1::LEU2), or hem1∆hap1∆ (DEY1) cells grown in aerated metal-replete SD medium supplemented with ALA at the indicated concentrations. Cells were grown to mid-log phase (A₆₀₀ ~ 0.5), harvested, and assayed for β-galactosidase activity. Results of representative experiments are shown and the error bars represent ±1 S.D. of three replicates.
Figure 6. Rox1 repression attenuates *FET4* metal responsiveness. Wild-type (BY4743) and *rox1Δ* (35484) cells were grown over a range of iron or zinc concentrations. (A) *FET4-lacZ* reporter in CSD medium supplemented with a range of iron concentrations. The zinc concentration was 50 µM. (B) *FET4-lacZ* reporter in CSD medium supplemented with a range of zinc concentrations. The iron concentration was 50 µM. Cells were grown with aeration to mid-log phase (*A*$_{600}$ ~0.5), harvested, and assayed for β-galactosidase activity. Results of a representative experiment are shown and the error bars represent ± 1 S.D. of three replicates.
Figure 7. Mapping regulatory elements in the FET4 promoter. (A) alignment of FET4 promoter sequences with consensus binding sites for Rox1, Zap1, and Aft1/Aft2 (Y = C or T, and R = A or G). Bases matching the consensus sequences are shown in bold. The locations of these elements relative to the FET4 start codon are also indicated. The asterisks denote elements on the noncoding strand. Below the table is a diagram of the FET4 promoter depicting the linear arrangement of these sites. The potential Rox1 sites are numbered 1–6. (B) wild-type (BY4743) cells bearing the FET4-lacZ reporter (FET4), the FET4-lacZ reporter with Aft1 binding site 2 mutated (AFTM), and the FET4-lacZ reporter with the Zap1 binding site mutated (ZREM) were grown in metal-replete CSD medium (50 µM iron, 50 µM zinc, open bars), or CSD without added iron (gray bars) or zinc (black bars). Cells were grown to mid-log phase (A600 ~0.5) with aeration, harvested, and assayed for β-galactosidase activity. Results of representative experiments are shown and the error bars represent ±1 S.D. of three replicates. (C) wild-type (BY4743, open bars) or rox1∆ cells (35484, gray bars) bearing the FET4-lacZ reporter (FET4), the FET4-lacZ reporter with potential Rox1 sites 2 and 3 mutated (ROXM2,3), the FET4-lacZ reporter with potential Rox1 site 4 mutated (ROXM4), the FET4-lacZ reporter with potential Rox1 site 5 mutated (ROXM5), and FET4-lacZ reporters with combinations of these mutations (ROXM2,3,4, ROXM2,3,5, ROXM4,5, and ROXM2,3,4,5) were grown in metal-replete CSD medium (50 µM iron, 50 µM zinc). Cells were grown to mid-log phase (A600 ~0.5) with aeration, harvested, and assayed for β-galactosidase activity. Results shown are from three combined experiments and the error bars represent ±1 S.D. (n = 9).
Figure 8. Evidence for a role of Fet4 in zinc uptake. Panels (A) and (B), wild-type (DY1457) and zrt1Δzrt2Δ (CM34) cells were transformed with the vector pRS316-GAL1 or the FET4 overexpression plasmid pCB1. Five µl of a cell suspension (1.5 × 10⁷ cells/ml) and three 10-fold serial dilutions (left to right) were plated onto SD-galactose agar plates (panel A) and SD-galactose plates were supplemented with 1 mM zinc (panel B). These plates were incubated for 3 days at 30°C. Panels (C) and (D), growth of zrt1Δzrt2Δ (CM34) (open circles) and zrt1Δzrt2Δfet4Δ (BMW1) (filled circles) cells in iron-replete (50 µM) CSD medium supplemented with 0.25 µM zinc (panel C) or 1 mM zinc (panel D). Cells were inoculated at an initial A₆₀₀ of 0.01 and the A₆₀₀ was measured over time. Results of a representative experiment are shown. (E) wild-type (DY1457) and fet4Δ (DEY1422) cells transformed with the ZRE-lacZ reporter were grown in iron-replete (50 µM iron) CSD medium supplemented with the indicated concentrations of zinc. Cells were grown with aeration to mid-log phase (A₆₀₀ ~ 0.5), harvested, and assayed for β-galactosidase activity. Results of a representative experiment are shown and the error bars represent ±1 S.D. of three replicates.
Notes

1. The abbreviations used are CSD, Chelex-treated synthetic defined medium; ALA, δ-aminolevulinic acid; BPS, bathophenanthroline disulfonic acid.
2. T. Lyons, A. Gasch, P. Brown, D. Botstein, and D. Eide, manuscript in preparation.
3. D. Kosman, personal communication.

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