Minireview

Regulation of Transition Metal Transport across the Yeast Plasma Membrane*

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Transition metals are essential for the function of many proteins, either by facilitating redox reactions or by stabilizing protein structure. To satisfy requirements for these metals, cells have numerous mechanisms for the solubilization and uptake of metals from the extracellular environment. Cells must, however, simultaneously protect themselves from the hazards inherent in the versatility of these metals, as the facile redox reactions can produce toxic free radicals if cytosolic metal concentrations are not carefully regulated. Numerous human disorders of metal homeostasis have been identified. Excess iron uptake has been implicated in the pathologies of hereditary hemochromatosis, Parkinson’s disease, and the neurological disease Friedrich ataxia. Menkes’ syndrome and Wilson’s disease result from aberrant copper homeostasis. Excess manganese supplements have been implicated in pediatric neurological disorders. The yeast Saccharomyces cerevisiae has proven a powerful model for investigation of the metal uptake machinery. Extensive investigations using this single cell organism have demonstrated parallels between the mechanisms by which this simple eucaryote obtains different transition metals from the extracellular environment. Metal transport systems have been found to consist of both low and high affinity transporters (Fig. 1). High affinity transporters are selective for their target metals and are tightly regulated according to metal need. Low affinity transporters are less responsive to metal need and are somewhat less selective for metals transported. This system of dual uptake allows the maintenance of metal homeostasis in conditions of either metal limitation or excess.

Iron

Iron is the most versatile transition metal in biological redox reactions. Variation in the environment of the iron-binding site can effect a 1000-mV difference in redox potential (1), and iron is a component of numerous cellular redox reactions. The high affinity of iron for oxygen has also made iron the active site in heme, which is commonly involved in oxygen binding and oxygen-based enzymatic reactions. The same properties of facile electron transport, however, make iron potentially toxic, as free iron generates toxic superoxide anion and hydroxyl radicals in the presence of oxygen. Cells face the conundrum of accumulating an essential but relatively toxic metal by tightly regulating the concentration of free cytosolic iron. Higher eucaryotes regulate cytosolic free iron by controlling both the amount of iron uptake mediated by the transferrin receptor and the amount of iron storage in ferritin. These processes are inversely regulated by the iron regulatory proteins (cf. Ref. 2 for review). To date, there is no compelling data that yeast cells contain a ferritin homologue. Some experiments have suggested that iron is stored in the vacuole (3, 4), although alternative explanations for these experiments have also been proposed (5). Regulation of iron status in yeast, therefore, is mediated primarily by regulating plasma membrane iron transport.

Yeast have multiple iron transport systems, all of which appear to require ferrous iron as a substrate. This form of iron, however, is not usually present under aerobic conditions, as ferrous iron is rapidly oxidized to ferric iron, which is essentially insoluble at physiological pH. The first step in iron transport is the reduction of ferric iron mediated by a transmembrane electron transporter system encoded by the FRE1 and FRE2 genes (6, 7). Although this step is essential for iron transport, Ftr1p and Ftr2p can also reduce copper; thus, the term ferrireductase is a misnomer as they are metalloreductases (8, 9). Although both FRE1 and FRE2 are highly homologous and mediate the same reaction they are differentially regulated. FRE1 is regulated by both iron deprivation through the action of the iron transcription factor Aft1p and copper deprivation through the action of the copper transcription factor Mac1p. FRE2 is solely regulated by iron deprivation through Aft1p (8, 10). In addition to these two cell surface ferrireductases, a family of putative metalloreductases has also been identified whose function is unknown (10).

High affinity iron transport is mediated by a bipartite system composed of a ferroxidase and a transmembrane permease. The ferroxidase Fet3p is a multicopper oxidase that catalyzes the oxidation of 4 mol of Fe(II) with the concomitant reduction of 1 mol of molecular oxygen (11). The S. cerevisiae ferroxidase has been biochemically characterized both as the native protein (12) and as a secreted protein lacking the transmembrane domain (13). The protein contains four copper atoms and shows the spectroscopic properties of a multicopper oxidase having copper in all three spectroscopic forms. Fet3p oxidizes iron, with a $K_m$ of 0.15 μM, which is close to the $K_m$ for cellular iron transport. Genetic experiments suggest that oxidized Fe(III) is then transported by a multitiopic membrane protein encoded by the FTR1 gene (14). Ftr1p has six potential transmembrane domains as well as a potential iron-binding motif. This motif, composed of the amino acids REGLE, is similar to a ferrireductase motif. Co-expression of either the FET3 and FTR1 homologues fio1” and fip1” is sufficient to confer high affinity iron transport in yeast strains with defective chromosomal FET3 (15). These results suggest that the permease and the oxidase are the only plasma membrane proteins required for high affinity iron transport. Because Fet3p has ferroxidase activity in vitro, a plausible hypothesis is that it oxidizes Fe(II) to Fe(III), which is then transported across the membrane by the permease Ftr1p. An alternative hypothesis that Fet3p oxidizes Ftr1p has not been formally excluded. The motive force behind transport is unclear, as is the mechanism by which iron is released from the transporter. An attractive hypothesis is that cytosolic re-

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ductants might provide a driving force for both iron transport and release.

Once translated, Fet3p and Ftr1p must be synthesized concomitantly for localization to the plasma membrane (14). If either protein is synthesized by itself, it is restricted to an intracellular compartment. The requirement that both proteins must be simultaneously synthesized for proper processing suggests that these proteins might function as a complex on the cell surface. The sorting process that requires both proteins to be processed together, however, does not require an active Fet3p. Mutated Fet3p, either due to a defect in copper loading or to a mutation in a copper-binding domain, is capable of being properly glycosylated and of accompanying Ftr1p to the cell surface. Overexpression of soluble Fet3p lacking the transmembrane sequence in a fet3Δ strain does not produce surface localization of Ftr1p, suggesting that the cytosolic or transmembrane domain may be required. The interaction domains of Fet3p and Ftr1p that are required for interaction and transport to the surface are not known.

The high affinity iron transport system is primarily regulated transcriptionally through the action of the DNA-binding protein Aft1p (16). Deletion of AFT1 blocks transcription of both FTR1 and FET3, whereas a mutant protein Aft1pα results in constitutive expression of these high affinity transport genes. Aft1p has been shown to bind DNA in an iron-dependent manner, in which iron precludes binding (17), whereas the mutant Aft1pα binds to DNA even in the presence of iron. Thus, both biochemical and genetic studies demonstrate that AFT1 encodes an iron-sensing transcriptional activator that regulates the expression of FRE2, FET3, and FTR1. Aft1p also regulates the expression of CCC2, a gene required for the incorporation of copper into apo-Fet3p.

The Fet3p/Ftr1p transport system mediates iron transport only under aerobic conditions because the transport system requires oxygen to catalyze the oxidation of Fe(II) (18). An alternative iron transport system, mediated by the FET4 gene product, effects iron uptake in high iron conditions and is required for iron transport under anaerobic conditions (19).

Fet4p is a multitopic plasma membrane protein capable of transporting a number of transition metals, and the Fet4p transporter is sufficient to maintain growth in fet3Δ cells. Although there are increased amounts of FET4 mRNA as a result of iron deprivation, the gene is not regulated by Aft1p (20). The mechanism by which iron regulates the expression of FET4 is yet to be elucidated.

A third potential yeast iron transporter has been defined through studies on mammalian cells. Two independent studies identified homologous genes that function in intestinal and endosomal Fe(II) transport (21, 22). The genes (Nrramp2, DCT1) encode multtopic proteins that are H+-transition metal transporters. When defective, these genes are responsible for the phenotypes of anemia and defective transport of iron from the gut seen in the mk mouse and Belgrade rat, respectively. When expressed in oocytes, DCT1 results in increased uptake of iron and other metals (22). Expression of Nramp2 in yeast also shows an effect of iron accumulation (23). The mammalian genes have two yeast homologues, SMF1 and SMF2, genes previously identified as encoding manganese transporters (24). SMF1/SMF2 may constitute a third cell surface iron transport system, as deletion of SMF2 results in an exacerbation of iron deprivation in yeast with deletions in FET3 and FET4 (5).

Copper

Copper, like iron, is a redox-active metal, and although essential, it is potentially toxic. Unlike iron, for which no storage mechanism has yet been defined in yeast, the cellular concentration of copper is regulated both at the level of sequestration as well as the level of uptake. Once taken up by cells, copper can be sequestered by binding to metallothioneins, small proteins that bind copper and other potentially toxic metals. Induction of metallothionein synthesis is regulated transcriptionally by the product of the ACE1 gene, which encodes a copper-dependent transcription factor (25).

Transport of copper is similar to iron in that it is highly regulated. Copper is found in the environment as the oxidized, cupric (Cu2+) form but is transported as the reduced, cuprous (Cu+) form. Reduction of extracellular copper results from the action of Fre1p and Fre2p (8, 9). The first yeast copper transporter gene CTR1 was identified through its effects on iron metabolism. Klausner and colleagues (26) had developed a clever genetic scheme to identify yeast mutants defective in iron metabolism. The basis of this screen was to have the prototrophic gene HIS3 placed under the control of the FRE1 promoter. Mutations that resulted in decreased cytosolic iron produced increased transcription of the HIS3 gene, which overcame His auxotrophy (26). Through this approach a mutant was discovered that had reduced transport of both copper and iron. The primary defect in this mutant was found to be in plasma membrane copper transporter. Subsequent analysis revealed that defective copper transport resulted in decreased iron transport through a deficit in the copper loading of Fet3p.

The observation that in some yeast strains inactivation of CTR1 does not lead to copper deprivation led to the identification of a second copper transporter encoded by the CTR3 gene (27). Although both Ctr1p and Ctr3p are membrane proteins, they have low sequence homology. The proteins encoded by these genes, however, appear to have redundant function. Each gene by itself can maintain copper homeostasis and supply copper to all intracellular targets. The origin of two high affinity copper transporters with little homology is curious. A possibility is that the gene products may transport other metals in addition to copper, although a complete characterization of the metal specificity of Ctr1p and Ctr3p has not yet been accomplished.

Deletion of CTR1 and CTR3 results in a dramatic reduction of...
in growth because of copper deprivation. The double deletion mutant provides a phenotype that has been used in expression cloning to identify other eucaryotic copper transporters. Using this approach, Zhou and Gitschier (28) identified a putative human copper transporter, hCTR1, which was homologous with CTR1 and could functionally replace CTR1 in maintaining cellular copper homeostasis in yeast. A similar approach was used to identify an Arabidopsis copper transporter (29). The plant transporter showed a high degree of homology to a then unknown yeast gene. That gene, CTR2, appears to be a low affinity copper transporter as overexpression of CTR2 can complement copper deficiency of a ctr1Δ ctr3Δ strain. CTR2 has higher homology with CTR3 than with CTR1. The metal specificity of CTR2 has also not been defined.

As transcription of FET3 and FTR1 is regulated by iron, transcription of CTR1 and CTR3 is regulated by copper through the action of the DNA-binding, metal-sensitive transcription factor, Mac1p (25, 30). Similar to Aft1p, Mac1p recognizes specific DNA sequences and binds to these sequences in the absence of copper. Null mutations of both transcription factors prevent transcription of target genes, and up-regulation mutants of both transcription factors show constitutive metal independent activity. Copper inhibition of Mac1p activity has been shown to be the direct result of metal association with the protein (31). Low or intermediate copper conditions stabilize Mac1p (31). High copper conditions, however, produce rapid degradation of Mac1p (32). The degradation of Mac1p protects the cell from copper toxicity by reducing transcription of the copper transporter genes.

Ctr1p is regulated transcriptionally by Mac1p and post-translationally by a copper-dependent proteolysis (33) (Fig. 2). Degradation appears to result from activation of a surface-bound protease, as degradation occurs even in the absence of Ctr1p endocytosis. This copper-activated protease is unknown, but its action must be specific, as other surface membrane proteins are not affected by high concentrations of copper.

**Zinc**

The same themes observed in the physiology of copper and iron transport have also been found in zinc transport, in which multiple zinc transporters are regulated by zinc at both transcriptional and translational levels. The gene encoding the high affinity zinc transporter, ZRT1, was identified on the basis of homology to a family of iron-regulated plant transporters (34). The plant transporters were initially identified by complementation of the low iron growth defect of a fet3Δ/fet4Δ mutant (35). The protein product of the homologous yeast gene, ZRT1, was found to transport not iron, but zinc (34). Transcriptional analysis showed that ZRT1 was highly regulated by zinc. Expression studies revealed that Zrt1p effected high affinity zinc transport. Zrt1p showed a marked preference for zinc, as only Cu⁺ and Fe²⁺ showed competition with zinc and then only at superphysiological concentrations (36).

Examination of the concentration dependence of zinc transport in wild type and zrt1Δ cells revealed both an inducible high affinity transport system with a \( K_m \) of 1.0 \( \mu \)M and a low affinity system with a \( K_m \) of 10 \( \mu \)M. The gene responsible for low affinity zinc transport, ZRT2, was identified on the basis of sequence homology with ZRT1. Sequence analysis showed both proteins to be highly homologous (44% sequence identity) with eight potential transmembrane domains. The zrt2Δ deletion strain was shown to be viable in both high and low zinc medium, though a strain deleted for both ZRT1 and ZRT2 showed a growth limitation for zinc. Analysis of both the zrt2Δ deletion strain and strains in which ZRT2 was overexpressed demonstrated that Zrt2p was responsible for low affinity zinc uptake (36). Zinc transport by Zrt2p showed a similar inhibition spectrum by other transition metals to Zrt1p. It is interesting that both Zrt1p and Zrt2p have the same metal specificity, yet at a 10-fold difference in affinity. Both transporters show a cluster of histidine residues in a variable loop that is predicted to be cytosolic. Histidine residues are capable of binding transition metals, and similar clusters are found in a wide variety of transition metal transporters. That the clusters are found in what may be a cytosolic loop suggests that they are not involved in the initial recognition of transport of metals from the extracellular surface but rather may be involved in a feedback regulation system.

Northern analysis and reporter constructs suggested that ZTR1 and ZRT2 are both regulated transcriptionally. Using a genetic screen similar to that used to identify AFT1, Zhao and Eide identified ZAP1 as a gene that encodes a zinc-regulated transcription factor (37). When the gene was deleted, severe zinc deficiency arose because of a lack of transcription of both ZRT1 and ZRT2. Promoter analysis revealed that Zap1p binds to specific DNA sequences in the 5′-untranslated regions of ZRT1 and ZRT2. Additionally, the transcription of ZAP1 is itself regulated by zinc. There was increased transcription of ZAP1 in the absence of zinc, as demonstrated by both Northern analysis and the use of Zap1-reporter constructs. Unlike Mac1p, high levels of the zinc did not lead to degradation of the transcription factor, Zap1p. Similar to Ctr1p, however, the transporter Zrt1p shows post-translational regulation. Under high zinc conditions the half-life of Zrt1p is dramatically reduced. A difference between the degradation regulation mechanism of Ctr1p and Zrt1p is that proteolysis of Zrt1p required endocytosis (38). The half-life of Zrt1p is prolonged both under conditions in which endocytosis is inhibited and in mutants defective in vacuolar proteases. These results indicate that high zinc induces the internalization of Zrt1p leading to its degradation.

**Manganese**

As mentioned above, SMF1 and SMF2 have high homology to the mammalian transition metal transporter Nramp2. Although physiological studies show that Nramp2 can transport a number of different transition metals with similar affinity, analysis of the Belgrade rat revealed deficiencies in manganese (39). Yeast strains deleted for SMF1 showed reduced uptake of manganese whereas overexpression of SMF1 resulted in increased levels of cellular manganese. Genetic studies revealed a SMF1 homologue, SMF2, which also affected manga-
nese transport (40). Little is known regarding the normal physiological function or metal preference of Smf1p or Smf2p. SMF2 appears to have much greater preference for cobalt than SMF1, and both are capable of transporting copper (41). Because Nrzp2 is an H+ transition metal symporter, it may be expected that transport by Smf1p/Smf2p would also be pH-dependent. The regulation of SMF1/SMF2 transcription is not known but does not appear to be affected by iron, copper, or manganese. The observation that Smf1p and Smf2p have broad metal specificity and are not transcriptionally regulated by manganese suggests that they may not be the high affinity manganese transporters.

The mediator transport activity, however, is regulated post-translationally by the product of the BSD2 gene (42). This gene encodes an endoplasmic reticulum membrane protein that was shown to be responsive to metal concentrations. In growth medium containing high metal concentrations, BSD2 directs Smf1p to the vacuole for degradation. Cells deleted for BSD2 have increased activity of Smf1p and Smf2p and accumulate high levels of manganese, copper, cadmium, and cobalt.

**Speculation**

Examination of the features of transition metal transport and their regulation reveal consistent themes. The demand for metals is sensed by a transcription factor, which then regulates the rate of transporter transcription. This control of transport activity by transcriptional regulation may extend to genes involved in the assembly of the transporter, the notable example being the vesicular copper transporter Ccc2p that is required for the assembly of the holooFeT3p. Based on the observation that there are metal-sensing transcription factors that regulate the accumulation of iron, copper, and zinc, we speculate that there may well be a similar transcription molecule that may regulate the activity of manganese transport. The only manganese transporters discovered to date have broad metal specificity and do not appear to be transcriptionally regulated by manganese. There may be high affinity manganese transporters that are transcriptionally regulated.

Another consistent theme is that copper, manganese, and zinc transporters show post-transcriptional regulation. When cells starved for a specific metal are then exposed to high concentrations of metals the activity of the transport system is modulated either by degradation or alteration of vesicular traffic. To date no such regulation has been seen for high affinity iron transport in *S. cerevisiae*. Once induced, exposure to iron does not affect a rapid reduction in iron transport activity. Identical experiments suggest that the high affinity iron transport system in *S. pombe* can adapt and reduce transport activity (15). These observations suggest that perhaps there is a regulation mechanism in *S. cerevisiae*, which has been overlooked. An alternative explanation is that *S. cerevisiae* can accommodate excess iron by rapid sequestration. The mechanisms of iron storage in *S. cerevisiae* are unknown, but if *S. cerevisiae* does not rapidly repress iron transport activity, then we predict that there must be a storage vehicle for iron deposition.

![D. Radisky and J. Kaplan, unpublished data.](image-url)