Table of contents

Zinc in yeast: mechanisms involved in homeostasis

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Abstract

The first eukaryotic zinc uptake transporter was discovered in the yeast, *Saccharomyces cerevisiae*. Since then, this organism has been an invaluable tool for the discovery of genes involved in zinc homeostasis. Genomic and proteomic studies have revealed an abundance of Zn^{2+}-regulated genes and Zn^{2+}-binding proteins. The large number of essential functions of Zn^{2+} necessitates a complex homeostatic mechanism involving the transport and storage of Zn^{2+} as well as its allocation to essential sites. Studies in yeast have elucidated the opposing roles of the ZIP and CDF Zn^{2+} transporter families and uncovered additional transport systems. The transcription factor, Zap1p, functions as the central Zn^{2+} sensor by regulating genes involved in Zn^{2+} uptake and adaptation to Zn^{2+}-deficiency. The investigation of the role of Zn^{2+} in the regulation of signaling pathways is becoming a primary research direction, and yeast will undoubtedly play a major role in any discoveries in this field as well.

1 Introduction

Cellular organisms are constrained by an absolute requirement for ionic Zn^{2+} (Vallee and Falchuk 1993). The relatively high bioavailability and useful chemical properties of Zn^{2+} allow its extensive use in three general biochemical capacities. Zn^{2+} is primarily used as a structural component of proteins, serving to stabilize a wide variety of architectures. The Lewis acidity of Zn^{2+} also makes it an excellent cofactor for catalysis and many enzymes require Zn^{2+} for full catalytic potential. Finally, Zn^{2+}, like Ca^{2+}, is highly labile and capable of forming transient, yet robust, associations with proteins (Bertini and Luchinat 1994). It is this property that allows zinc to function as a signaling molecule.

The versatility and abundance of Zn^{2+} have made it indispensable. As a consequence, cells must maintain optimal levels of cellular Zn^{2+}, regardless of supply, via a complex process known as homeostasis (Eide 2003). Under conditions of low nutritional Zn^{2+}, cells must ensure that adequate quantities are acquired from the environment. This entails the activation of specific transporters that scavenge Zn^{2+} from the surroundings and transport it across the plasma membrane. Furthermore, the various intracellular uses of Zn^{2+} must be prioritized so that growth

can be optimized during periods of limitation. When cells encounter nutritional surplus, one general strategy is to exclude excess Zn^{2+} from the interior of the cell by downregulating the plasma membrane transporters. Another strategy involves the continuous acquisition of Zn^{2+} so that it can be stockpiled for leaner times. In the latter case, cells require both a means of storing large quantities of zinc in a manner that does not upset homeostasis and a controlled way to release these stores at the appropriate times.

A proper understanding of Zn^{2+} homeostasis requires the identification of all the players in the game. It is, therefore, beneficial to study an organism for which the most information is known. The yeast, *Saccharomyces cerevisiae*, has proven to be an invaluable model system for this purpose. The genome sequence is complete and decades of research have allowed an in-depth analysis of almost every biochemical system. More importantly, the proteins involved in metal metabolism are remarkably conserved from *S. cerevisiae* to humans. This review will summarize what is known about zinc metabolism in *S. cerevisiae* and the mechanism by which homeostasis is sustained. Appropriate consideration will be given to the discussion of zinc metabolism in other yeast species.

2 Zap1p: The zinc sensor

Any discussion of Zn^{2+} in *S. cerevisiae* should begin with Zap1p (Zinc-regulated Activator Protein). Zap1p is an 880 amino acid transcription factor that functions as the central sensor and regulator of zinc homeostasis (Bird et al. 2003). In response to Zn^{2+}-deficiency, Zap1p becomes active and binds to Zinc Response Elements (ZREs) in the promoters of genes involved in Zn^{2+} uptake. The ZRE is an 11 base pair palindrome that has the consensus sequence ACCTTNAAGGT. Zap1p is comprised of a C-terminal DNA binding domain and two distinct activation domains (AD1 and AD2) that recruit RNA polymerase II to the promoter (Fig. 1). Close homologues of Zap1p are found in fungi alone and only the DNA binding domain is fully conserved. [PSI-BLAST and homology searches were performed on the NCBI website (Altschul et al. 1997) or the Saccharomyces Genome Database (Christie et al. 2004).]

2.1 Regulation of Zap1p activity

Zap1p is constitutively located in the nucleus; therefore, its translocation from the cytosol to the nucleus does not seem to be a primary determinant of its transcriptional activity. In addition, there is no evidence to suggest that Zap1p activity is regulated by any type of posttranslational modification. The current state of understanding is that nuclear localized Zap1p generally binds to ZREs during Zn^{2+}-deficiency, but not during Zn^{2+}-repletion and that a direct interaction with Zn^{2+} is responsible for this phenomenon (Bird et al. 2000).

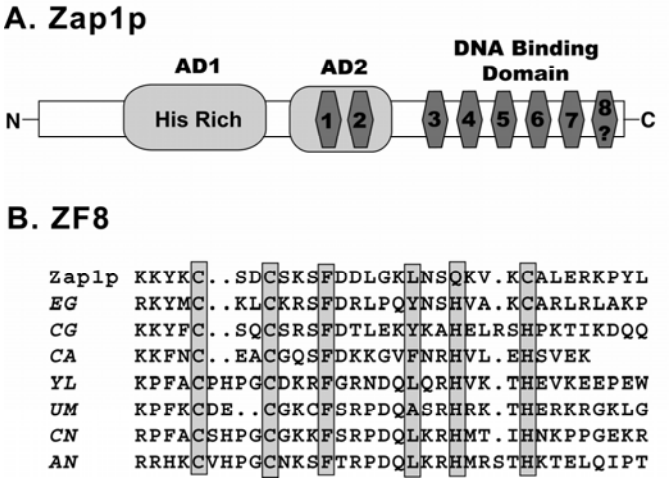

Fig. 1. Structural characteristics of Zap1p. A. The major structural domains of Zap1p. AD = activation domain. Zinc fingers (ZF) are numbered beginning with the most N-terminal finger. B. A multiple sequence alignment of ZF8, including fungal Zap1p homologues in which all eight zinc fingers are conserved. Shaded boxes show conserved residues. *EG, Eremothecium gossypii*; *CG, Candida glabrata*; *CA, Candida albicans*; *YL, Yarrowia lipolytica*; *UM, Ustilago maydis*; *CN, Cryptococcus neoformans*; *AN, Aspergillus nidulans*.

There are at least three direct mechanisms by which Zn^{2+} binding affects the activity of Zap1p. First, the DNA binding domain is Zn^{2+}-regulated (Bird et al. 2003). This domain contains five classical TFIIIA-type zinc fingers (ZF3-ZF7 in Fig. 1A). Each contains one Zn^{2+} ion that facilitates domain folding by coordinating two cysteines and two histidines via their side chain sulfur and nitrogen atoms, respectively. Conserved phenylalanine and leucine residues help form the hydrophobic core of the fingers. Although not strictly conserved in all species, the unequivocal presence of an additional non-canonical zinc finger (ZF8) at the extreme C-terminus of Zap1p is elucidated by alignment with other fungal Zap1p homologues (Fig. 1B). ZF4, ZF5, ZF6, and ZF7 are believed to make direct contact with bases in the ZRE (Evans-Galea et al. 2003). Enigmatically, the structural integrity of these fingers is essential for DNA binding, yet the domain shows decreased DNA binding activity at high Zn^{2+} concentrations. The means by which excess Zn^{2+} decreases the affinity for the ZRE is unknown, however, ZF3 and/or perhaps ZF8 may be involved in this process.

The two other mechanisms of Zap1p regulation involve the repression of activation domain function by Zn^{2+} binding. AD1 is a very large region of the protein rich in histidine residues. This domain is the least conserved across species and almost nothing is known about how Zn^{2+} affects its activity. The working hypothesis is that Zn^{2+} binding to histidine residues alters the conformation of the domain and abrogates its interaction with RNA polymerase. AD2 contains two atypical TFIIIA-like fingers (ZF1 and ZF2) that lack the consensus phenylalanine and leucine residues. In vitro, these fingers have a decreased affinity for Zn^{2+}

when compared with fingers from the DNA binding domain, perhaps due to the loss of the hydrophobic core residues. At higher Zn^{2+} concentrations, the folding of these fingers is postulated to induce a conformational change that results in decreased AD2 activity (Bird et al. 2003).

It is important to note that, on a few promoters, Zap1p remains active even under Zn^{2+}-replete conditions. Two notable examples are the *ZRT2* (Bird et al. 2004) and *ZPS1* (Lamb et al. 2001) genes that encode a low affinity zinc transporter and a metalloprotease-like protein, respectively. Unlike classical Zap1p target genes, *ZRT2* has high Zap1p-dependent expression in Zn^{2+}-replete cells. This elevated expression can be repressed by the addition of excess Zn^{2+}. In the case of *ZPS1*, expression in Zn^{2+}-replete conditions is induced by alkaline pH in a manner that is dependent upon both the Zap1p protein and the pH-responsive transcription factor Rim101p. Zap1p and Rim101p interact in a yeast two-hybrid screen (Uetz et al. 2000), suggesting they may collaborate during the regulation of *ZPS1*. The induction profiles of *ZRT2* and *ZPS1* suggest that the inactivation of Zap1p can be prevented or perhaps shifted to higher Zn^{2+} concentrations by other proteins in the nucleus.

2.2 The Zap1p regulon

Zap1p was first discovered as a positive regulator of both *ZRT1* and *ZRT2,* the genes encoding the high- and low-affinity Zn^{2+} uptake transporters, respectively (Zhao and Eide 1997). Three distinct ZREs can be found in the promoter regions of both of these genes. A ZRE was subsequently identified in the promoter of the *ZAP1* gene as well (Zhao et al. 1998). The autoregulation of *ZAP1* by Zap1p represents a fourth, indirect mechanism by which Zn^{2+} regulates the activity of Zap1p.

DNA microarrays were used to identify all Zap1p-target genes in the yeast genome (Lyons et al. 2000). Global expression changes in response to Zn^{2+}-depletion were monitored in wild type and *zap1*Δ cells. This screen yielded over forty genes whose expression suggested Zap1p-dependent regulation and whose promoter regions contained sequences that resemble the consensus ZRE. There is no reason to believe that the Zap1p regulon defined in these experiments is complete. The inherent errors of DNA microarray analysis notwithstanding, many yeast genes are constitutively repressed under the conditions used for these experiments (i.e. glucose as a carbon source, aerobic culture, etc.) (Courey and Jia 2001). It is possible that, if different culture conditions were used, new Zap1p-target genes would be found. Many of the genes belonging to the Zap1p regulon were either known or expected to be Zap1p targets due to their predicted roles in Zn^{2+} metabolism. The majority of Zap1p target genes, however, encode proteins not directly involved in Zn^{2+} metabolism (Lyons et al. 2000). Some of these genes will be discussed in later sections.

3 Zinc transporters

The main line of defense against ion loss or overaccumulation are membranes. The lipid bilayer presents a formidable barrier to the diffusion of charged molecules. This necessitates the existence of specific transporters that can selectively allow the passage of ions in response to environmental conditions. Several major families of Zn^{2+} transporters have been characterized and extensively reviewed.

3.1 Import into the cytoplasm

The ZIP (Zrt-like, Irt-like Proteins) family of proteins is ubiquitous in biology, indicating a very early origin. ZIP proteins are responsible for transporting Zn^{2+} into the cytoplasm from either outside of the cell or from various internal organelles. The ZIP family is defined by a characteristic topology. Although most members have eight transmembrane domains (TM), some have as few as five. TMs 4 and 5 contain conserved histidines that are predicted to line a channel involved in metal binding and transport. Another conserved region of unknown importance is the cytoplasmic loop between TM3 and TM4 that contains an $(HX)_n$ motif (Fig. 2) (Eide 2004).

S. cerevisiae possess five genes encoding ZIP proteins (Fig. 2). Indeed, the aforementioned Zrt1p and Zrt2p high- and low-affinity plasma membrane Zn^{2+} transporters are the flagship members of this family. The third ZIP protein, Zrt3p, is closely related to the Zn^{2+} uptake transporter, ZupT, from *Escherichia coli* (Grass et al. 2002). Zrt3p is involved in the liberation of Zn^{2+} from vacuolar stores (Section 6) (MacDiarmid et al. 2000). Of the remaining ZIP proteins, only Atx2p has been characterized. Atx2p is thought to reside in the Golgi complex where it may function in the transport of Mn^{2+} from the lumen to the cytoplasm, but its involvement in zinc homeostasis has not been investigated (Lin and Culotta 1996). The last ZIP protein, Yil023cp, although completely uncharacterized, is closely related to the human ZIP4 protein mutated in congenital zinc deficiency (acrodermatitis enteropathica).

Interestingly, a strain lacking both Zrt1p and Zrt2p (*zrt1*Δ*zrt2*Δ) is still viable, indicating that these transporters are not the sole vehicles for Zn^{2+} transport from outside of the cell. Another protein, Fet4p, was found to function as a low affinity Fe^{2+}, Cu^{2+}, and Zn^{2+} uptake transporter (Waters and Eide 2002). Fet4p has an interesting evolutionary history. It is yeast-specific and no closely related proteins can be found in homology searches. More extensive BLAST searches, however, revealed that Fet4p is distantly related to a widely dispersed family of bacterial proteins (COG5478) that have two transmembrane domains. Fet4p is a fusion protein made up of four tandem repeats of the COG5478 motif. The most highly conserved amino acids are two tryptophan residues found within the transmembrane domains (Fig. 2).

Since a *zrt1*Δ*zrt2*Δ*fet4*Δ triple mutant strain is still viable when grown in high Zn^{2+}, other Zn^{2+} uptake mechanisms must exist (Waters and Eide 2002). The current assessment of the phosphate transporter, Pho84p, suggests it too functions as

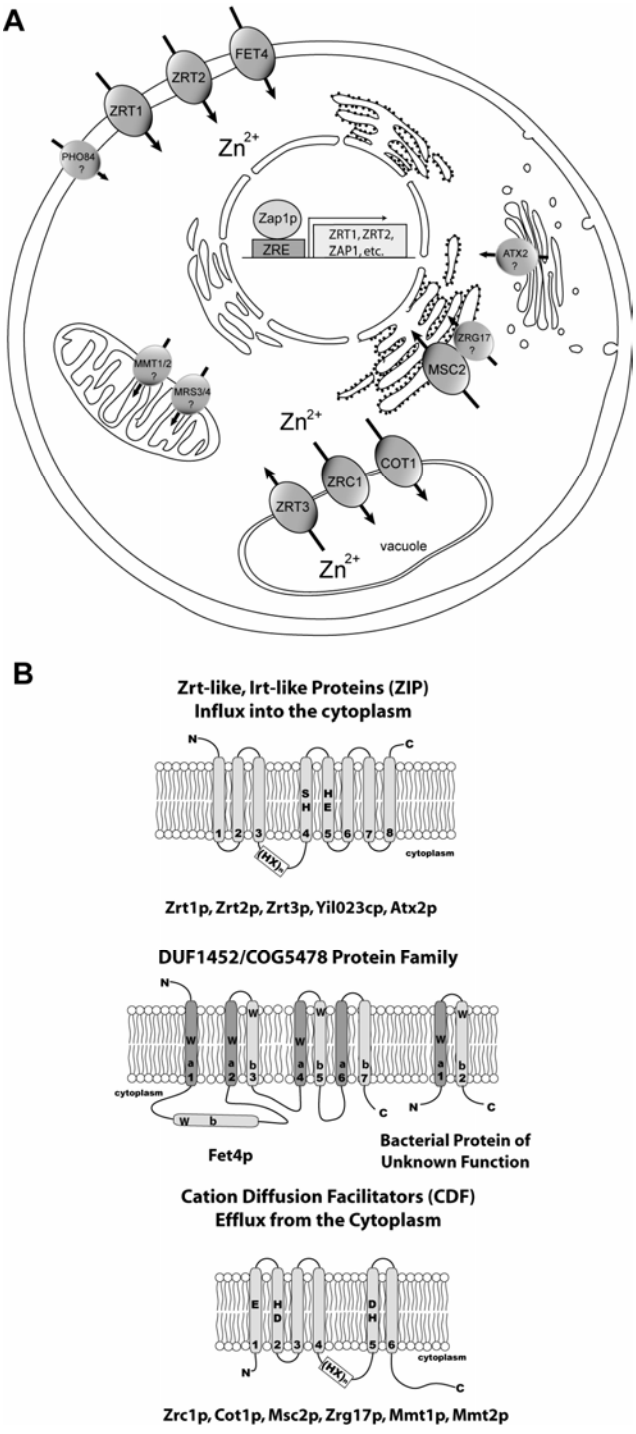
A
FET4
ZRT2
ZRT1
PHO84
?
Zn2+
Zap1p
ZRE
ZRT1, ZRT2,
ZAP1, etc.
ATX2
?
ZRG17
?
MSC2
MMT1/2
?
MRS3/4
?
Zn2+
COT1
ZRC1
ZRT3
vacuole
Zn2+
B
Zrt-like, Irt-like Proteins (ZIP)
Influx into the cytoplasm
N
C
S
H
H
E
1 2 3 4 5 6 7 8
cytoplasm
(HX)n
Zrt1p, Zrt2p, Zrt3p, Yil023cp, Atx2p
DUF1452/COG5478 Protein Family
N
W
a 1
a 2
b 3
a 4
b 5
a 6
b 7
b 1
b 2
cytoplasm
w b
C
N
C
Fet4p
Bacterial Protein of
Unknown Function
Cation Diffusion Facilitators (CDF)
Efflux from the Cytoplasm
E
H
D
D
H
1 2 3 4 5 6
cytoplasm
N
(HX)n
C
Zrc1p, Cot1p, Msc2p, Zrg17p, Mmt1p, Mmt2p

Fig. 2 (overleaf). Zinc transporters in *Saccharomyces cerevisiae*. A. A model of a yeast cell with the predicted locations and directionality of Zn^{2+} transport for known and putative transporters. B. Predicted topology and important structural features of the ZIP, Fet4p, and CDF proteins.

a low affinity transporter of Zn^{2+} and other divalent cations, presumably via metal-phosphate complexes (Jensen et al. 2003).

3.2 Export out of the cytoplasm

It is clear that yeast possess many systems for the transport of Zn^{2+} into the cytoplasm. Once Zn^{2+} builds up in the cytoplasm, however, it must be trafficked across the membranes of various internal organelles to maintain homeostasis. The CDF (Cation Diffusion Facilitator) family of proteins assumes this responsibility. Like the ZIP family, the CDF proteins are ubiquitous. Their predicted topology generally consists of proteins with six transmembrane domains, although some CDF proteins have twelve. As with the ZIP proteins, a long loop region between TM4 and TM5 contains an $(HX)_n$ motif. The highly amphipathic nature of TM1, TM2, TM5, and TM6, along with a preserved intermembrane aspartate residue, suggest a cation transporting channel (Fig. 2) (Palmiter and Huang 2004).

In *S. cerevisiae*, Zrc1p and Cot1p are the best characterized members of the CDF family. Both confer resistance to metals when overexpressed and sensitivity when deleted (Kamizono et al. 1989; Conklin et al. 1992; MacDiarmid et al. 2000). Zrc1p and Cot1p are found on the vacuolar membrane and function to transport Zn^{2+} into this compartment (Section 6) (MacDiarmid et al. 2000). Msc2p is believed to transport Zn^{2+} into the lumen of the endoplasmic reticulum and perhaps an additional organelle involved in the secretory pathway. Protein folding in the ER is impaired in an *msc2*Δ strain, a phenotype which can be rescued by addition of excess Zn^{2+} (Ellis et al. 2004). Msc2p has been shown to physically interact with a fourth CDF protein, Zrg17p. These two proteins function as a complex to transport Zn^{2+} into the secretory pathway (Ellis 2005). The final two members of the CDF family in *S. cerevisiae*, Mmt1p and Mmt2p, are thought to participate in the transport of iron into the mitochondrion (Li and Kaplan 1997). No evidence to date links them to the metabolism of Zn^{2+}.

Mrs3p and Mrs4p comprise another pair of homologous proteins, unrelated to the CDF family, that are thought to transport iron into the mitochondrion. Two lines of evidence suggest these two proteins also play a role in mitochondrial Zn^{2+} uptake. First, *MRS3* expression seems to be regulated by Zap1p, either directly or indirectly (Lyons et al. 2000). Second, mitochondrial Zn^{2+} concentrations in iron-deficient yeast are highest in strains overexpressing Mrs3p or Mrs4p and lowest in strains that lack these transporters (Muhlenhoff et al. 2003).

3.3 Zinc regulation of transporter function

Due to their role in scavenging extracellular or stored Zn^{2+}, it is not surprising that the primary transcriptional regulator of *ZRT1, ZRT2, ZRT3,* and *FET4* is Zap1p (Lyons et al. 2000). The *ZRT2* gene, however, presents an interesting case. Not only does *ZRT2* retain elevated expression in high Zn^{2+} (Section 2.1), at very low Zn^{2+} concentrations, its expression is repressed by Zap1p. This phenomenon is due to the binding of Zap1p to a weak ZRE adjacent to the TATA box, thereby, preventing the recruitment of RNA polymerase. These findings reflect the function of Zrt2p as a low affinity transporter. Since Zrt2p does not function at extremely low Zn^{2+} concentrations, its expression is not needed. During Zn^{2+}-repletion, it may play a role in constitutive zinc uptake (Bird et al. 2004).

In mammalian cells, excess Zn^{2+} is sensed by the MTF-1 transcription factor that induces the expression of proteins which expel Zn^{2+} from the cytoplasm (Andrews 2001). This mechanism is absent in *S. cerevisiae*. The only CDF gene induced by Zn^{2+}-excess is *COT1* and this effect is not direct (Section 7) (Lyons et al. 2004). On the contrary, *ZRC1* and *ZRG17* are induced by Zn^{2+}-deficiency via Zap1p (Lyons et al. 2000). It is possible that essential proteins in the vacuole and ER require Zn^{2+} for function and the upregulation of *ZRC1* and *ZRG17* indicates an increased need for Zn^{2+} transport to these sites. While this may be the case for *ZRG17*, the induction of *ZRC1* during Zn^{2+}-deficiency is more complex and will be discussed in Section 6.

The shift from a nutrient-limiting to nutrient-replete environment is problematic because many nutrients are toxic at high concentrations. Zn^{2+} is no exception (Dineley et al. 2003). Transcriptional changes are unlikely to occur quickly enough for cells to adapt to rapid environmental changes. Therefore, yeast respond to these extreme changes via posttranslational control of Zn^{2+} transporters, particularly Zrt1p. Under conditions of Zn^{2+} limitation, Zrt1p is a stable protein. Upon exposure to high levels of Zn^{2+}, Zrt1p is internalized via ubiquitin-dependent endocytosis. Although, the exact trigger for ubiquitination is poorly understood, the modification is known to occur on lysine 195. After endocytosis, Zrt1p traffics to the vacuole where it is degraded, thereby preventing additional Zn^{2+} uptake (Gitan et al. 1998, 2003). To date, this is the only known posttranslational regulatory mechanism of Zn^{2+} transporters in yeast.

4 The zinc proteome

To gain a complete understanding of zinc homeostasis in yeast, one must first identify all of the genes and gene products involved in the process. Several papers have attempted to define the "zinc proteome" in both *E. coli* and yeast using 2D gel electrophoresis and mass spectrometry (Obata et al. 1996; Zhu et al. 2002). Many new Zn^{2+}-containing proteins have since been identified. There is also a wealth of genomic data that can be mined to identify putative Zn^{2+} proteins using known Zn^{2+}-binding motifs.

4.1 Structural zinc

Since the characterization of the classical TFIIIA-type zinc finger motif, a plethora of distinct structural Zn^{2+}-binding motifs have been discovered and characterized by x-ray crystallographic and nuclear magnetic resonance techniques. In general, structural Zn^{2+} sites have only four tetrahedrally coordinated protein side chain atoms. With rare exceptions, these coordinating atoms are cysteine sulfurs and histidine nitrogens. The most common ligand sets are four cysteines (C_4), three cysteines and one histidine (C_3H) and two cysteines and two histidines (C_2H_2). The characteristics of zinc finger and related domains have been extensively reviewed elsewhere (Grishin 2001; Laity et al. 2001; Matthews and Sunde 2002; Krishna et al. 2003).

The entire yeast proteome can be scanned for proteins that contain known Zn^{2+} binding motifs. A summary of such a search, including Zn^{2+}-binding proteins discovered by other means, is shown in Table 1. Proteins are listed by structural motif or functional classification. As can be seen, there are hundreds of proteins with known Zn^{2+}-binding motifs. Due to the prevalence of CxxC motifs (where x = any amino acid) in structural Zn^{2+} sites, proteins that have closely spaced CxxC pairs (leading to a C_4 ligand set) are also predicted to bind Zn^{2+}. If these putative Zn^{2+}-binding proteins were added to Table 1, they would place the number of proteins that require structural Zn^{2+} at approximately four hundred. Since glutamate and aspartate can occasionally replace cysteines and histidines in Zn^{2+}-binding motifs, it is likely that the proteome search performed for this review missed many bona fide Zn^{2+}-binding proteins. Moreover, novel Zn^{2+}-binding motifs are being discovered at a regular pace and it is likely that Table 1 is far from complete. Approximately 6-7% of the yeast proteome (depending on varying estimates for its size) require zinc for structural integrity. Based on this fact, zinc can be thought of as an essential building block for proteins.

4.2 Catalytic zinc

Several excellent reviews have considered the role of Zn^{2+} as a catalytic cofactor (Coleman 1992, 1998; Parkin 2004). While hundreds of enzymes are known to utilize Zn^{2+}, most can be categorized into two basic groups, both of which use the positive charge of zinc to stabilize negative charges on substrates. The first class of enzymes uses Zn^{2+} to coordinate the oxygen or sulfur atoms in water molecules, alcohols or thiols. Coordination to Zn^{2+} polarizes the O-H or S-H bond, making the proton more acidic and allowing its abstraction by a basic amino acid side chain. The hydroxide, alkoxide or thiolate generated can then act as a nucleophile in catalysis. The second class of enzymes utilizes Zn^{2+} as an electron-withdrawing group to polarize carbonyls. This makes the carbon atom more electrophilic, thereby, stabilizing enolates or making carbonyls more amenable to nucleophilic attack. Still other enzymes, such as α-1,2-mannosidase, are thought to use zinc for substrate recognition (van den Elsen et al. 2001). Table 2 lists all of the proteins in yeast that are known or suspected to contain tightly bound catalytic Zn^{2+}.

Table 1. Compendium of yeast proteins with structural zinc sites

Structural Class/Function	Proteins
Single zinc binding domains	
Classical TFIIIA tandem zinc fingers (C_2H_2)	Ace2, Adr1, Azf1, Crz1, Fzf1, Gis1, Lpz12, Lpz14, Nrg1, Nrg2, Map1, Met31, Met32, Mig1, Mig2, Mig3, Mot3, Msn2, Msn4, Mub1, Pzf1, Rgm1, Rim101, Rme1, Rph1, Rpn4, Set5, Sfp1, Stp1, Stp2, Stp3, Stp4, Swi5, Usv1, Zap1, Zms1, Yer130c, Ygr067c, Yml081c, Ypr022c
U1-like zinc fingers (C_2H_2)	Bud20, Dbf4, Jjj1, Luc7, Prp9, Prp6, Prp11, Reh1, Rei1, Rts2, Sad1, Snu23, Spt10, Yhc1, Yod1, Ydr049w
TFIIIA-like (C_2H_2)	Abf1, Eco1, Luc7, Pcf11, Sas2, Sas3
GATA-type zinc finger (C_4)	Ash1, Dal80, Gat1, Gat2, Gat3, Gat4, Gln3, Gzf3, Rad16, Srd1, Srd2
ACE1 structural zinc (C_3H)	Ace1, Haa1, Mac1
Viral-type zinc knuckle (C_2HC)	Air1, Air2, Atg14, Bik1, Gis2, Itt1, Mpe1, Msl5, Slu7, Ykr017c, Yol029c
TIS11 RNA binding finger (C_3H)	Cth1, Dus3, Lee1, Nab2, Tis11, Yth1, Yor091w
Rad50 zinc hook (C_4)	Rad50
PKC1-like fold/ARF GAP (C_4)	Age1, Age2, Gcs1, Glo3, Gts1, Sps18
MOB1 four helix bundle (C_2H_2)	Mob1
NEW1/DHHC (predicted)	Akr1, Akr2, Erf2, Swf1, Ydr459c, Ynl155w, Ynl326c, Yol003c
Class II histone deacetylase(C_4)	Hst1, Hst2, Hst3, Hst4, Sir2
DnaJ/CSL zinc finger (C_4)	Apj1, Dph3, Hua1, Jjj1, Mdj1, Nob1, Scj1, Xdj1, Ydj1
Ubiquitin interacting zinc fingers	
HIT znf-UBP (C_4)	Bcd1, Hit1, Plb1, Plb2, Plb3, Spo1, Ubp8, Ubp14, Vps71, Yhl010c
RBZ/NFZ (C_4)	Npl4, Nrp1, Ubp14, Vps36
E1 protein zinc finger (C_4)	Atg7, Uba2, Uba3, Uba4
Deubiquitinase finger(C_4)	Ubp1, Ubp4, Ubp7, Ubp8, Ubp9, Ubp10, Ubp11, Ubp13, Ubp14, Ubp16
Sec23/24 zinc finger (C_4)	Sec23, Sec24, Sfb2, Sfb3, Yhr035w
ZPR1 finger	Zpr1
tRNA binding proteins (C_4)	Ism1p, Mes1p, Nam2p, Trm1
DNA replication machinery (C_4)	Mcm2, Mcm6, Mcm7, Mcm10, Pol1, Pol2, Pol3, Rev3, Rfa1
RNA polymerase complex (at least 8 zinc/complex)	Brf1, Dst1, Rpa9, Rpa135, Rpa190, Rpb1, Rpb2, Rpb3, Rpb9, Rpb10, Rpc2, Rpc10, Rpc11, Rpo31, Spt4, Sua7, Tfa1, Tfb4
Ribosome associated proteins	Mrpl32, Rps26, Rps27, Rps29, Rpl34, Rpl37, Rpl43, Tif5, Sui3
TIM22 complex	Tim8, Tim9, Tim10, Tim12, Tim13

Multinuclear zinc binding domains	
PKC1	Pkc1
FYVE	Fab1, Pep7, Pib1, Pib2, Vps27
ZZ domain	Ada2, Rsc8
RING finger and related motifs	Asr1, Apc11, Asi1, Asi3, Asr1, Bre1, Cst9, Cwc24, Dma1, Dma2, Far1, Hex3, Hrd1, Hrt1, Hul4, Itt1, Mag2, Nfi1, Pep3, Pep5, Pex2, Pex10, Pex12, Pib1, Psh1, Rad5, Rad16, Rad18, Ris1, San1, Sig1, Siz1, Slx1, Slx8, Ssm4, Ste5, Tfb3, Tul1, Ubr1, Ubr2, Vps8, Ybr062c, Ydr128w, Ydr266c, Yhl010c, Ykr017c, Ylr247c, Ymr187c, Ymr247c, Yol138c
PHD	Asr1, Bye1, Cti6, Ecm5, Hop1, Ioc2, Nse1, Nto1, Pho23, Rco1, Rds3, Set2, Set3, Set4, Snt2, Spp1, Yng1, Yng2, Yer051w, Yjr119c
LIM domain/RHO GAP	Lrg1, Pxl1, Rga1, Rga2
Binuclear zinc clusters (Zn_2Cys_6)	Arg80, Aro81, Cat8, Cep3, Cha4, Dal81, Ecm22, Eds1, Gal4, Hal9, Hap1, Leu3, Lys14, Mal13/2/23/33/83, Oaf1, Pdr1, Pdr3, Pdr8, Pip2, Ppr1, Put3, Rdr1, Rds1, Rds2, Rgt1, Rsc3, Rsc30, Sef1, Sip4, Stb4, Stb5, Sut1, Sut2, Tbs1, Tea1, Thi2, Uga3, Ume6, Upc2, War1, Yrm1, Yrr1, Ybr239c, Ydr520c, Yer184c, Yfl052w, Yil130w, Yjl103c, Yjl206c, Ykl222c, Ykr064w, Yll054c, Ylr278c, Ynr063w
Multinuclear metalloenzymes with known and potential structural zinc sites	
PPP family phosphatases	Cmp2, Cna1, Glc7, Ppg1, Pph3, Pph21, Pph22, Ppq1, Ppt1, Ppz1, Ppz2, Sit4
Pseudouridine synthase	Deg1, Pus1, Pus2
Alcohol dehydrogenases	Adh1, Adh2, Adh3, Adh5, Adh6, Adh7, Bdh1, Sfa1, Yalo61w
Miscellaneous	Car1, Cox4, Sod1

Many more enzymes can be activated by Zn^{2+} in vitro, however, the functional cation in vivo is not known. Such enzymes, known as Zn^{2+}-activated enzymes, may be far more numerous than is currently recognized. One example of this type of enzyme is enolase, which is highly active in the presence of Zn^{2+} in vitro. The pI of enolase has been shown to change when cells are grown under Zn^{2+}-deficient conditions, suggesting that Zn^{2+} is the functional cofactor in vivo as well. Based on Table 2, it is estimated that at least 100 enzymes, or 1-2% of the yeast proteome, require or can utilize Zn^{2+} for catalysis.

5 Prioritizing zinc

Estimates for the concentration of Zn^{2+} inside eukaryotic cells are remarkably consistent from species to species. In yeast, this value is approximately 180 μM (Lyons and Eide, unpublished data). Back-of-the-envelope calculations based on several different studies suggest that a yeast cell grown in normal media (Zn^{2+}-

Table 2. Compendium of yeast proteins that use zinc in a catalytic capacity

Functional Class	Proteins
Nucleophile stabilization by -X-H bond polarization	**Zn^{2+} + X-H ------> Zn^{2+}-X^- + H^+**
Alcohol dehydrogenases (R-O-H)	Adh1, Adh2, Adh3, Adh5, Adh6, Adh7, Bdh1, Sfa1, Sor1, Sor2, Xyl2, Yal061w
Prokaryotic-type carbonic anhydrase (H-O-H)	Nce103
Hydrolases (H-O-H)	
AlkP (alkaline phosphatase) superfamily: binuclear metallohydrolase	Gpi13, Las21, Mcd4, Pho8, Ycr026c, Yel016c
Type I cyclic nucleotide phosphodiesterase	Pde2
Trinuclear zinc phosphodiesterase	Apn1
HIT family diadenosine polyphosphatase hydrolases	Apa1, Apa2, Hnt1, Hnt2
β-lactamase fold: binuclear zinc site	
Phosphodiesterases	Pde1, Pso2, Trz1, Ysh1
Glyoxalases I and II	Glo2, Glo4
Class I histone deacetylases	Hda1, Hos1, Hos2, Hos3, Rpd3
Cytosine deaminase fold	
Nucleic acid/riboflavin deaminase	Amd1, Cdd1, Dcd1, Fcy1, Gud1, Rib2, Tad1, Tad2, Tad3
Cyclic imidohydrolases/dihydropyrimidase family	Dal1, Ura4, Yjl213w
Jab1/MPN proteasomal metalloprotease	Ron8, Rpn11, Rri1
MH clan binuclear zinc metalloproteases ($HxDx_nDx_nEEx_nDx_nH$)	Ape3, Cps1, Lap4, Vps70, Ybr074w, Ybr281c, Ydr415c, Yfr044c, Yhr113w, Yol153c
MA clan zinc metalloproteases ($HExxHx_nE$)	Aap1, Afg3, Ape2, Lta1, Oct1, Prd1, Rca1, Yme1, Zps1, Yil137c, Ynr020c
MC clan metalloprotease ($HxxEx_nHx_nE$)	Ecm14
ME clan metalloprotease ($HxxEHx_nEx_nE$)	Axl1, Cym1, Mas1, Mas2, Ste23, Yol098c
MG clan binuclear metalloprotease ($Dx_nDx_nHx_nEx_nE$)	Map1, Map2
Integral membrane proteases (HExxH)	Oma1, Ste24
Thiol Activation (R-S-H)	
Methionine synthases	Met6, Mht1, Sam4
Prenyltransferases	Bet4, Ram2
Methionine sulfoxide reductase (potential)	MsrB
Disulfide isomerases (potential)	Eug1, Mpd1, Mpd2, Pdi1
Electrophile stabilization by R=O bond polarization	**Zn^{2+} + O=C-R ------> Zn^{2+}-$^{\delta-}$O-$C^{\delta+}$-R**
Alcohol dehydrogenase (reverse rxn, carbonyl activation)	See above
Aldol cleavage/condensation (enolate stabilization)	
Type II aldolase	Fba1
DAHP synthase	Aro3, Aro4
5-aminolevulinate dehydratase/PBGS	Hem2

HMGL fold	
Homocitrate synthase	Lys20, Lys21
Isopropylmalate synthase	Leu4, Yor108w
Pyruvate carboxylase	Pyc1, Pyc2
Phosphomannose isomerase (enediolate stabilization)	Pmi40
Substrate recognition	
Alpha-1,2-mannosidase	Ams1

replete) contains roughly 6-8 million Zn^{2+} atoms (Korhola and Edelmann 1986; Obata et al. 1996; Lyons and Eide, unpublished data). When grown in normal media, however, yeast stop growing due to glucose-depletion before Zn^{2+} becomes limiting. Therefore, much of the Zn^{2+} content may result from the continuous uptake and storage of Zn^{2+} by cells that have entered stationary phase. In support of this hypothesis, the Zn^{2+} content in yeast significantly drops when Zn^{2+} is the limiting nutrient. Under these conditions, estimates for intracellular Zn^{2+} range from 600,000 to 3 million atoms per cell (Obata et al. 1996; Lyons and Eide, unpublished data). This value, albeit crude, can be thought of as the minimum cellular Zn^{2+} requirement for cells that have undergone growth arrest due to lack of Zn^{2+}.

Although Zn^{2+} is clearly abundant inside of cells, Table 1 and 2 show that the proteins requiring this metal for proper function are as well. It is therefore important for yeast to prioritize the uses of Zn^{2+} so the most important functions are retained during Zn^{2+}-limitation. To complicate matters, most intracellular Zn^{2+} seems to be tightly bound to a variety of intracellular ligands. 'Free Zn^{2+}', or the amount of Zn^{2+} that remains unchelated inside of a cell is predicted to be quite low (Finney and O'Halloran 2003). Work done in *E. coli* estimates the amount of 'free Zn^{2+}' to be less than one atom per cell (Outten and O'Halloran 2001).

How then does the cell distribute the infinitesimal amount of 'free Zn^{2+}' to the appropriate sites? The answer may lie in the lability of Zn^{2+}. The relatively fast ligand exchange rate of Zn^{2+} makes it likely to associate and dissociate quickly from solvent accessible sites. Thus, when an ample supply exists, Zn^{2+} may diffuse rapidly throughout the cell without ever being 'free' for very long. Small molecules such as glutathione may also mediate the fast exchange of Zn^{2+} from site to site (Mason et al. 2004).

As Zn^{2+} is depleted from the cytoplasm, those sites with the lowest binding affinity or that exchange the fastest are likely to lose Zn^{2+} more rapidly. It is possible that natural selection has tuned the K_d and solvent accessibility of the numerous Zn^{2+}-binding sites so that the dispensable functions of Zn^{2+} are lost first. Since structural Zn^{2+} sites have generally high Zn^{2+}-binding affinities (Cox and McLendon 2000) and low solvent accessibilities (Auld 2001), they are probably the last to lose Zn^{2+}. The fact that the DNA binding zinc fingers of Zap1p retain their function even when cells have stopped growing due to Zn^{2+}-limitation supports this conclusion (Lyons et al. 2000). The likelihood is minimal that cells would continue to grow if Zn^{2+}-deficiency had advanced to the point of depleting structural Zn^{2+} sites.

5.1 Zinc chaperones

Another possibility for the distribution of Zn^{2+} involves the existence of specific proteins that escort Zn^{2+} to essential sites, regardless of their physical properties. In the case of copper homeostasis, proteins known as copper chaperones are responsible for directional transcytoplasmic trafficking. For example, the yeast copper chaperone, Ccs1p, is specifically required for the delivery of copper to superoxide dismutase (Elam et al. 2002).

It is important to note that, with the exception of Atx1p, which delivers copper to the entire secretory pathway, all known copper chaperones have specific copper protein targets. This is not surprising since the copper proteome is quite small, consisting of no more than a handful of proteins. It is not a burden for cells to carry genes for both the copper protein and the accessory copper chaperone. On the other hand, the enormity of the zinc proteome makes it unlikely that each zinc protein possesses a cognate zinc chaperone. Although, it is possible that classes of zinc proteins, such as the Zn^{2+}-dependent alcohol dehydrogenases, may have chaperones that serve all the members of the class. To date, however, no zinc chaperone has been identified in eukaryotes.

5.2 Remodeling

The yeast cell is a complex mixture of proteins competing for limited supplies of Zn^{2+}. In the absence of zinc chaperones or some other type of active partitioning, kinetics and thermodynamics determine the fate of Zn^{2+} as nutritional supplies dwindle. Therefore, it may become necessary for the cell to remodel the cellular protein profile to ensure the reallocation of Zn^{2+} to essential sites. If a Zn^{2+}-containing protein is abundant and dispensable, cells may downregulate its expression in response to Zn^{2+}-deficiency, thus, releasing much needed Zn^{2+} for other, more important, uses.

An example of this phenomenon can be seen with the major isoform of Zn^{2+}-containing alcohol dehydrogenase (Adh1p). Based on crude estimates of approximately 250,000 monomers per cell, Adh1p can be considered to be very abundant in yeast cells (Racker 1950). Since each monomer contains two Zn^{2+} ions, Adh1p would consume an enormous percentage of the cellular supply if expressed under conditions of Zn^{2+}-limitation. Part of this problem is solved by thermodynamics, since Adh1p purified from Zn^{2+}-limited yeast is both less active and Zn^{2+}-deficient (Dickenson and Dickinson 1976). Clearly, Adh1p is unable to compete with the myriad of other Zn^{2+}-chelators. Yeast also address this problem by placing the expression of the iron-dependent alcohol dehydrogenase isozyme, Adh4p, under the control of Zap1p, thereby, eliminating the need for Zn^{2+} to perform the dehydrogenase function (Lyons et al. 2000). Lastly, it appears that Zap1p, either directly or indirectly, represses the expression of the *ADH1* gene (Lyons et al. 2000). This remodeling allows yeast to conserve important Zn^{2+}-dependent functions at the expense of Adh1p activity.

Other potential examples of this type of remodeling have recently come to light. In *E. coli* and *B. subtilis*, several non-Zn^{2+}-dependent ribosomal proteins are specifically induced by Zn^{2+}-deficiency, ostensibly to replace Zn^{2+}-binding subunits that can no longer function due to loss of Zn^{2+} (Panina et al. 2003; Nanamiya et al. 2004). An alternative interpretation is that the replacement of ribosomal proteins that require Zn^{2+} with ones that do not is a matter of economy. Ribosomes are numerous. Ergo, ribosomes that require less Zn^{2+} are beneficial during Zn^{2+}-deficiency because they consume less of a limiting resource.

In yeast, a similar situation may exist. Yeast ribosomes are predicted to contain at least six proteins with predicted structural Zn^{2+}-binding sites (Rivlin et al. 1999). Since a vegetative yeast cell is estimated to contain 200,000 ribosomes (Warner 1999), they probably represent the largest pool of Zn^{2+} in the cytoplasm. Zn^{2+}-deficient yeast show repressed expression of over a hundred genes encoding ribosomal proteins. This is not surprising since the repression of ribosomal genes in yeast seems to be a generalized response to stress. Unexpectedly, ribosomal genes showed lower expression in wild type cells than in *zap1*Δ cells when both were grown under Zn^{2+}-deficiency, suggesting a role for Zap1p in the repression of ribosomal gene expression (Lyons et al. 2000).

6 Zinc storage and detoxification

Part of homeostasis is the evolution of mechanisms by which excess nutrients are managed. If accumulated in the wrong location, Zn^{2+} is an effective cellular poison. Although the exact mechanism(s) by which Zn^{2+} exerts its toxic effect(s) are not known, Zn^{2+} may replace other cations in non-Zn^{2+}-dependent enzymes, thereby inactivating them. For example, Zn^{2+} is known to compete with iron for insertion into porphyrin by ferrochelatase (Labbe et al. 1999). Zn^{2+} is also capable of acting as an inhibitor by binding to adventitious sites on enzymes, a mechanism believed to explain its inhibition of mitochondrial function (Link and von Jagow 1995). Whatever the mechanism of toxicity, Zn^{2+} cannot be allowed to hyperaccumulate in the cytoplasm.

6.1 The vacuole

With respect to zinc metabolism, the primary function of the yeast vacuole is storage and detoxification. When Zn^{2+} is plentiful, Zrc1p transports Zn^{2+} from the cytoplasm into the vacuolar compartment. Transport is thought to proceed via secondary active transport driven by the proton gradient (MacDiarmid et al. 2002). Zn^{2+} may also traffic to the vacuole by other indirect pathways, perhaps from the ER or Golgi via secretory vesicles or from the plasma membrane via endosomes. Yeast grown in excess zinc are capable of accumulating large quantities of vacuolar Zn^{2+}, over 80 million atoms/cell (Obata et al. 1996). The speciation of stored Zn^{2+} inside the vacuole is unknown, although it is predicted to form a complex

with polyphosphate. When external supplies diminish, these stores are released primarily by Zrt3p (MacDiarmid et al. 2000).

Although its sole purpose is Zn^{2+} detoxification, Zrc1p is highly expressed in Zn^{2+}-limited cells via Zap1p. On the surface, it makes little sense why a protein involved in Zn^{2+}-detoxification would be turned on by low Zn^{2+}-bioavailability. The answer to this mystery lies in the cell's inherent proactive defense against zinc shock, a condition brought about by the induction of Zrt1p during Zn^{2+}-deficiency. If cells expressing Zrt1p are exposed to large quantities of extracellular Zn^{2+}, Zrt1p is endocytosed and inactivated. However, the endocytic process is not fast enough to prevent the rapid influx and temporary cytoplasmic accumulation of Zn^{2+}. Therefore, Zrc1p is induced as a preventative measure, thus, allowing the vacuole to absorb the excess Zn^{2+} before it can exert its toxic effects in the cytoplasm. *zrc1*Δ cells are exceptionally sensitive to a shift from Zn^{2+}-depleted conditions to media containing even small amounts of Zn^{2+}. This phenotype can be rescued by the concomitant deletion of the *ZRT1* gene (MacDiarmid et al. 2003).

6.2 Metallothionein

Many organisms express small cysteine-rich proteins, called metallothioneins, that function as cytoplasmic stores for Zn^{2+}. Mammalian metallothioneins are induced by the MTF-1 transcription factor in response to elevated Zn^{2+} (Andrews 2001). Similar systems can be found in lower eukaryotes and cyanobacteria (Robinson et al. 2001). *Saccharomyces cerevisiae* does have genes encoding metallothioneins, however, their gene products are involved in the detoxification of copper and are not Zn^{2+}-regulated (Pena et al. 1998). The fact that *S. cerevisiae* has co-opted metallothionein function for copper homeostasis may reflect its unique evolutionary history resulting from domestication. The distantly related fission yeast, *Schizosaccharomyces pombe*, does have a Zn^{2+}-inducible system of metal tolerance that includes a Zn^{2+}-binding metallothionein, Zym1 (Borrelly et al. 2002). Taking this into account, *S. pombe* may represent a much better model system for understanding eukaryotic zinc homeostasis than *S. cerevisiae*.

7 Zinc signals and other regulators of zinc homeostasis

There exists the possibility that Zn^{2+} is not the only direct regulator of Zap1p, as is demonstrated by the influence of Rim101p in Zn^{2+}-replete conditions (Lamb et al. 2001). In addition, Zap1p is not the only transcription factor that regulates the expression of Zn^{2+} transporters. For example, *ZRT1* is also regulated by the cell cycle (Cho et al. 1998), nitrogen metabolism (Cox et al. 1999) and the Rpd3p histone deacetylase (Bernstein et al. 2000). It is exciting to speculate that the regulation of proteins and genes involved in zinc metabolism by other biochemical systems points toward a larger role for Zn^{2+} as a signaling molecule.

The prospect that Zn^{2+}, like Ca^{2+}, acts as a second messenger has intrigued researchers in the field for quite some time. Like Ca^{2+}, Zn^{2+} is highly labile, redox inert and fairly promiscuous regarding the ligand sets and geometries it will accept (Vallee and Falchuk 1993; Bertini and Luchinat 1994). These properties allow Zn^{2+} to transmit signals inside cells either by modulating the activity of Zn^{2+}-activated proteins or by binding to structural Zn^{2+} sites. Indeed, Zn^{2+} has many pharmacological effects in eukaryotic cells, including the ability to alter signaling pathways (Korichneva et al. 2002; Min et al. 2003).

Some mammalian tissues accumulate high levels of Zn^{2+} in secretory vesicles (Palmiter et al. 1996). Ample evidence suggests that, in some cases, this pool of Zn^{2+} functions in signaling (Cuajungco and Lees 1997). Localization of Zn^{2+} with fluorescent dyes has also indicated the existence of distinct vesicular compartments, called 'zincosomes', filled with 'labile Zn^{2+}' (Beyersmann and Haase 2001). It remains to be seen whether or not these zincosomes are real and function in zinc homeostasis or are merely artifactual. Zincosomes that transiently accumulate Zn^{2+} in response to influx have been visualized in yeast (Devirgiliis et al. 2004). Since this pool of Zn^{2+} is considered 'labile', it is tempting to postulate a role in zinc signals, although this hypothesis remains to be tested.

Whether or not Zn^{2+} acts as a direct messenger, it is clear that the expression of hundreds of genes show altered expression in response to perturbations in zinc homeostasis. Zn^{2+}-deficiency, in particular, has a profound effect on transcription (Lyons et al. 2000). The majority of transcriptional alterations caused by deficiency are part of a generalized environmental stress response. Other changes, such as the induction of the Unfolded Protein Response, are specifically caused by the loss of Zn^{2+} in critical sites (Ellis et al. 2004). The putative Zap1p regulon, however, includes a variety of proteins that have no apparent role in zinc homeostasis. *NRG2*, for example, encodes a transcriptional repressor involved in the regulation of glucose metabolism. *DPP1* encodes diacylglycerol pyrophosphate (DAGPP) phosphatase, an enzyme involved in the generation of lipid molecules that may act as second messengers (Han et al. 2001).

By comparison, the perturbation of zinc homeostasis by excess Zn^{2+} has far fewer transcriptional consequences (Lyons et al. 2004). DNA microarrays revealed that Zn^{2+}-toxicity resulted in the induction of only two interconnected transcriptional regulons: the Aft1p iron-responsive regulon and the Mga2p hypoxia-responsive regulon. The CDF protein, Cot1p, is slightly induced under these conditions as part of the Aft1p regulon. It is likely that Zn^{2+} affects mitochondrial iron metabolism which, in turn, affects the hypoxia regulon.

8 Conclusions

Saccharomyces cerevisiae affords the unique opportunity to characterize the process of zinc homeostasis in its entirety. Much is known, yet much more remains to be discovered. Several ZIP and CDF proteins remain uncharacterized. In addition, improved structural data is needed to elucidate the chemical mechanisms by which

Zn^{2+} is transported or sensed. New avenues of research will include the complete characterization of the zinc proteome and further studies on the transcriptional effects of imbalances in zinc homeostasis. New Zn^{2+}-specific fluorescent probes will be invaluable in defining the role of Zn^{2+} as a signaling molecule. When appropriate, extrapolation of the lessons learned from yeast will yield a better understanding of zinc metabolism in humans.

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