Molecular mechanisms of copper homeostasis in yeast

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Abstract

Copper ions play critical roles as electron transfer intermediates in various redox reactions. The yeast *Saccharomyces cerevisiae* has served as a valuable model to study copper metabolism in eukaryotic cells. The systems for copper homeostasis; including the uptake, cytoplasmic trafficking, and metabolism in intracellular organelles, detoxification, and regulation of these systems have been characterized. Most of the molecular components for copper metabolism identified in yeast are functionally and structurally conserved in mammals. These findings have underscored the importance of evolving delicate mechanisms to utilize copper. Studies on copper metabolism in yeast certainly have opened up interesting and important research avenues that have shed light on the molecular details of copper metabolism and the physiological roles of copper.

1 Introduction

Copper (Cu) is a metal-ion abundantly found in the earth's crust. It easily accepts and donates electrons through redox reactions. Aerobic organisms have taken advantage of the chemical properties of Cu by incorporating it in various biological processes. Thus, organisms have developed mechanisms for acquiring Cu from the environment. Mechanisms for homeostatic Cu metabolism have been uncovered in prokaryotes, fungi, plants, and mammals. Among these organisms, the yeast *Saccharomyces cerevisiae* has served as a model organism to study Cu metabolism in eukaryotes. A number of experimental tools are available to understand the molecular mechanisms of Cu homeostasis. The sequencing of the yeast genome has provided an extremely valuable source of information. Deletion or expression control of yeast genes is much easier than in higher eukaryotes. Growth environments of yeast can be easily manipulated. Furthermore, most of the mechanisms and components in physiological and biochemical processes identified in yeast are conserved in higher eukaryotes.

Cu is required for at least three biological processes in yeast, (i) mitochondrial oxidative phosphorylation, (ii) superoxide anion detoxification, and (iii) iron metabolism. In the mitochondria cytochrome c oxidase subunits 1 and 2 contain Cu as an electron transport intermediate in oxidative phosphorylation (Tsukihara et al. 1995; Iwata et al. 1995). Thus, Cu is an essential micronutrient for yeast under

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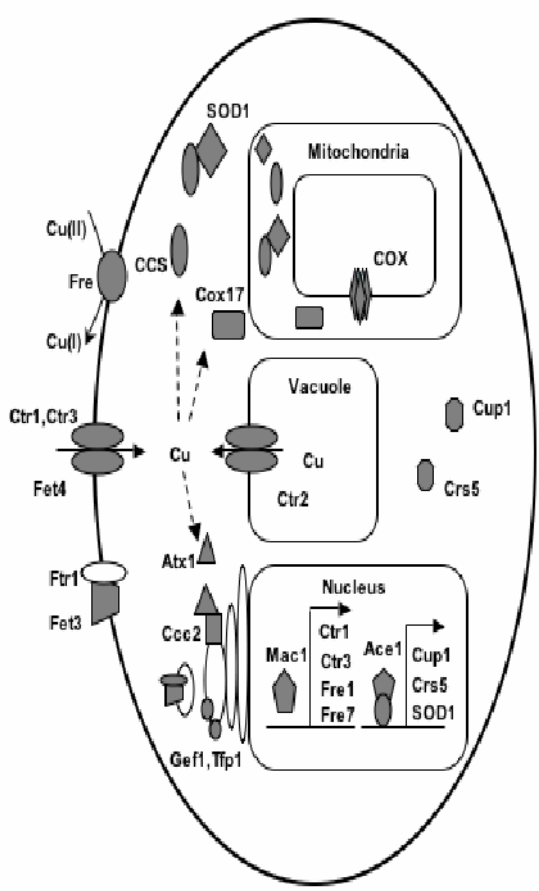

Fig. 1. Copper (Cu) homeostasis in yeast *S. cerevisiae.* Molecular mechanisms of Cu transport, distribution and detoxification have been characterized in yeast. Cu is reduced by cell surface reductases (Fre) prior to uptake by Ctr1 and Ctr3 Cu transporters. Fet4 serves as a low affinity Cu transporter. Ctr2 transports Cu form the vacuole. Cytosolic Cu chaperones Atx1, Cox17 and CCS deliver Cu to the secretory pathway, mitochondria and Cu, Zn superoxide dismutase (SOD1), respectively. At the post-Golgi vesicles Ccc2 accepts Cu from Atx1, followed by incorporation of Cu to Fet3, a multicopper ferroxidase. Gef1 and Tfp1 facilitate the transport and incorporation of Cu into Fet3. Fet3 forms a complex with the iron permease Ftr1 and both proteins are responsible for high affinity iron uptake at the plasma membrane. In mitochondria Cox17 plays essential roles in Cu incorporation into cytochrome c oxidase (COX) subunit 1 (Cox1) and subunit 2 (Cox2). CCS delivers Cu specifically to SOD1 in the cytosol. SOD1 and CCS also localize at the mitochondrial intermembrane space. Two metallothioneins, Cup1 and Crs5, are critical for Cu detoxification. Mac1 and Ace1, Cu-responsive transcription factors, regulate expression of genes involved in Cu metabolism. Mac1 and Ace1 directly bind to the *cis*-acting element of their target genes.

aerobic conditions. Yeast cells that are defective in Cu metabolism are not able to grow on media containing ethanol and glycerol as sole carbon source. These non-fermentable carbons need mitochondrial oxidative phosphorylation to generate energy and Cu serves as an essential cofactor (Keyhani and Keyhani 1975). Cu metabolism is linked to iron (Fe) metabolism, since Cu is a cofactor of Fet3 feroxidase, which plays an essential role in Fe transport at the plasma membrane (Dancis et al. 1994a; Askwith et al. 1994). Cu is also a critical cofactor for Cu,Zn superoxide dismutase (Cu/Zn SOD, SOD1), which detoxifies superoxide anions generated by aerobic biological processes. Thus, yeast cells defective in Cu uptake exhibit sensitivity to superoxide anion stress (Greco et al. 1990). The counterparts of these Cu-containing proteins in higher eukaryotes play the same roles as those in yeast cells (Peña et al. 1999).

Cu is able to catalyze reactions generating reactive oxygen intermediates that are highly toxic to all cellular components (Halliwell and Gutteridge 1984, 1990). The beneficial and detrimental roles of Cu in biological systems demand that cells maintain delicate control of intracellular Cu metabolism. Cu-chelating metallothionein is known as a primary defense system against Cu toxicity (Hamer 1986). Molecular characterization of the metallothionein gene and its transcription regulation has provided an initial picture that reflects the significance of homeostatic Cu metabolism. Further understanding of Cu-metabolism has been taken by cloning the genes encoding components involved in Cu uptake and intracellular distribution in yeast (Fig.1). Phenotypic analyses of yeast cells defective in any of these components led to the identification and characterization of their counterparts in higher eukaryotes.

This chapter will address molecular mechanisms of Cu metabolism in yeast, including baker's yeast, fission yeast and pathogenic yeast, by which uptake, distribution and detoxification of Cu are precisely controlled. The major focus will be in the baker's yeast *S. cerevisiae*. Although this chapter describes the most updated information regarding yeast Cu metabolism in a comprehensive manner, there are excellent review articles focusing on specific topics in Cu metabolism such as transporters (Labbé and Thiele 1999; Puig and Thiele 2002), intracellular distribution (O'Halloran and Culotta 2000; Rosenzweig 2001; Huffman and O'Halloran 2001), mitochondrial Cu metabolism (Carr and Winge 2003), and transcriptional regulation (Thiele 1992; Winge 1998).

2 Cu uptake at the plasma membrane

2.1 High affinity Cu transporters

Initial studies on Cu uptake in yeast demonstrated that Cu transport at the plasma membrane is a saturable and carrier-mediated process (Lin and Kosman 1990). An elegant genetic approach identified the Copper Transporter 1 (Ctr1), which plays critical roles in Cu metabolism (Dancis et al. 1994a). Ctr1 was actually identified from yeast mutants that are unable to transport iron. Subsequent analysis demon-

strated that the Fe-deficiency phenotypes of the Ctr1 mutants were the consequence of Cu deficiency and that Ctr1 is a Cu transporter. Indeed, Cu transported through Ctr1 serves as a cofactor of the Fet3 ferroxidase required for Fe uptake (Askwith et al. 1994). The mammalian Cu-containing ferroxidases, ceruloplasmin and hephaestin, also play an important role in Fe metabolism in mammals (Osaki and Johnson 1969; Vulpe et al. 1999).

Ctr1 appears to be specific for Cu, since Ctr1-mediated Cu uptake is not competed by other metal-ions such as Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, or Zn^{2+} (Dancis et al. 1994a). Expression of Ctr1 is a limiting factor in Cu uptake, since overexpression of Ctr1 increases Cu uptake. Non-functional mutations of the Ctr1 gene result in altered cellular responses to extracellular Cu(I), demonstrating a physiological role for Ctr1 in delivering bio-available Cu. Ctr1-defective cells exhibit deficiency in Cu/Zn SOD activities resulting in growth arrest in Cu-deficient media (Dancis et al. 1994b).

Ctr1 is a 406 amino acid integral membrane protein with three putative transmembrane domains. Ctr1 is heavily glycosylated with *O*-linkages (Dancis et al. 1994b) but it is not known whether glycosylation of Ctr1 is essential for its membrane localization and function. The hydrophilic amino terminal contains eight MXXM or MXM sequence repeats that have been identified as Cu-binding motifs in other Cu-transporting proteins from prokaryotes (Cha and Cooksey 1991; Odermatt et al. 1993). The amino-terminus of Ctr1 localizes at the extra-cellular surface of the plasma membrane (Puig et al. 2002), and those methionine residues appear to play a role in capturing Cu from the environment. Sequence alignment of Ctr family proteins has revealed that a methionine at the extracellular domain and two methionines (MXXXM) at the second transmembrane domain are conserved among these transporters. Site-directed mutagenesis experiments have demonstrated that the conserved methionines are critical for Ctr1 function (Puig et al. 2002). The methionines may serve as Cu ligands in Ctr1-mediated Cu transport. Given that most of the membrane transport proteins possess more than 6 trans-membrane domains, it is reasonable to predict that Ctr1 assembles in a homo or hetero multimer to make a Cu channel at the membrane. This is supported by *in vitro* cross-linking experiments (Dancis et al. 1994b). Interactions of glutamate residues at the third transmembrane domain of each monomer have been implicated in multimer formation (Aller et al. 2004).

Ctr3 was identified in mutant yeast cells defective in Ctr1 (Knight at al. 1996). Ctr3 is a 241 amino acid protein that has 11 cysteine residues of which three pairs are arranged in a potential CXC or CXXC metal binding motif. Ctr3 expression in many laboratory yeast strains is blocked by Ty2, a transposable DNA element. In strains that do not possess a Ty2 transposon, Ctr3 expression is regulated by Cu as is Ctr1. Ctr3 is able to replace Ctr1 function and has the same basic structural features as Ctr1, including three transmembrane domains and a functionally important MXXM motif at the second transmembrane domain. Despite these similarities, Ctr3 bears little sequence identity to Ctr1. The expression of both proteins provides enhanced proficiency in Cu uptake under Cu-limiting conditions. However, it is not clear whether Ctr1 and Ctr3 have any specificity in their roles.

Cu transporters have been identified from other yeast species as well as plants, fruit fly, and mammals due to their functional and structural similarity to yeast Ctr1 or Ctr3 Cu transporters (Puig and Thiele 2002). The structural features observed in yeast Ctr1 are common to the Ctr family of Cu transporters, which suggests that Ctr1 proteins in different organisms function with the same mode of action. However, the actual mechanisms of Ctr1 and Ctr3-mediated Cu transport are poorly understood. In mammalian cells, Fe-containing transferrins bind to their plasma membrane receptor, and Fe is released from transferrin at the endosomes to be transported by a transporter. Similarly, Ctr proteins may simply serve as Cu receptors at the cell membrane. Ctr1 endocytosis may be one mechanism for Cu transport. However, since endocytosis of Ctr3 has not been observed (Peña et al. 2000) as has been for yeast and human Ctr1, endocytosis-mediated intracellular compartmentalization of Cu transporters does not appear to be an absolutely required process in Cu acquisition in yeast. A Cu transport assay of purified Ctr1 protein in a lipid vesicle will be an approach for direct demonstration of Cu transport by Ctr1.

Given that Ctr1 does not possess an obvious ATPase domain, the driving force for Ctr1-mediated Cu transport is not known. Initial characterization of Cu transporting activities in yeast cells suggested a temperature and ATP-dependent high affinity Cu transport process (Lin and Kosman 1990). It is possible that another subunit binding to Ctr1 is an ATPase. Electrochemical measurements have shown that Cu-ion uptake is coupled with K^+ efflux in a 1:2 stoichiometry (De Rome and Gadd 1987), suggesting that Cu transport may take place via a $Cu^+/2K^+$ antiport mechanism. Since elevated extra-cellular K^+ levels enhance Cu uptake in mammalian cells (Lee et al. 2002), there may be a connection between the transport of K^+ and Cu.

An interesting observation is that the Ctr1 Cu transporter determines intracellular accumulation of cisplatin (Ishida et al. 2002). Cisplatin, a platinum-based anticancer drug, is highly active against a wide variety of tumors; however, resistance to this drug upon treatment limits its effectiveness (Loehrer and Einhorn 1984; Giaccone 2000). A genetic screen of yeast loss-of-function mutants for cisplatin resistance has uncovered that deletion of the CTR1 gene results in increased cisplatin resistance and reduced intracellular accumulation of cisplatin. Furthermore, cisplatin regulates Ctr1 stability and trafficking in similar ways as Cu in yeast cells. Cu pre-treatment reduces toxicity of cisplatin. These results suggest that Ctr1 transports cisplatin as well. This has been observed for both yeast and mammalian Cu transporters (Ishida et al. 2002; Lin et al. 2002). The link between Cu transporters and cisplatin may explain some cases of resistance in humans and suggest ways of modulating sensitivity and toxicity to this important anticancer drug.

2.2 Cu transporters identified from other yeast

The fission yeast *S. pombe* is particularly interesting due to its somewhat unique system of Cu uptake. A high affinity Cu uptake protein from *S. pombe*, Ctr4, re-

sembles a chimera between the *S. cerevisiae* Ctr1 and Ctr3 proteins (Labbé et al. 1999; Zhou and Thiele 2001). Ctr4 harbors five MXXMXM repeats in the predicted amino-terminal extracellular region similar to Ctr1, yet transmembrane domains are homologous to Ctr3. A Cu transport assay of Ctr4 in *S. cerevisiae* revealed that Ctr4 fails to complement baker's yeast cells defective in high affinity Cu transport and is not able to localize to the plasma membrane. Selection for *S. pombe* genes, which, when co-expressed with Ctr4, confer high affinity Cu transport to *S. cerevisiae* cells resulted in the identification of Ctr5. Ctr4 forms a complex with Ctr5 to function as a Cu transporter (Zhou and Thiele 2001). Both Ctr4 and Ctr5 are integral membrane proteins, and the physical association between them is interdependent for their secretion to the plasma membrane and for high affinity Cu transport. The specific roles of each subunit in the secretion to plasma membrane and Cu transport have not been defined. It is also interesting to ascertain whether there are homologous subunits in high affinity transport complexes in other eukaryotes.

A Ctr1 Cu transporter has been identified from the pathogenic yeast *C. albicans*. A *C. albicans* Ctr1-null mutant displays phenotypes consistent with the lack of Cu uptake, and its Ctr1 can complement Cu deficiency in *S. cerevisiae* (Marvin et al. 2003). Consequently, *C. albicans* does not appear to have a redundant high affinity Cu transporter similar to Ctr3.

2.3 Low affinity Cu transporters: Fet4, Smf1, and Pho84

The saturable and low-affinity Cu transport activities in Ctr1 and Ctr3 knockout yeast cells are attributed to the Fet4 plasma membrane protein. The Cu transported by Fet4 is available for intracellular Cu-requiring proteins and Cu-responsive transcription factors as is the Cu uptake by Ctr1 and Ctr3 (Hassett et al. 2000). Fet4 has been known as a transporter of divalent metals including Fe, Co, and Cd (Dix et al. 1994), as well as reduced monovalent Cu (Hassett et al. 2000). Mutant Fet4 alleles that are non-functional in Fe transport are also defective in Cu transport. However, since Cu inhibits Fet4-mediated Fe uptake in a non-competitive manner, the Cu and Fe transport by Fet4 is not necessarily through the same mechanism (Hassett et al. 2000).

In addition to Fet4, the Smf1 and Pho84 metal transporter appears to contribute to Cu accumulation in yeast. Smf1 is a plasma membrane transporter for manganese, and transports other metal-ions including Cu (Supek et al. 1996; Liu et al. 1997). Overexpression of Smf1 has been associated with increased accumulation of Cu. Smf1 degradation is controlled by Bsd2-mediated ubiquitination (Liu and Culotta 1999; Hettema et al. 2004). Consequently, a non-functional mutation in Bsd2 upregulates Smf1 expression, resulting in hyper-accumulation of Cu, Cd and Mn (Liu et al. 1997). A phosphate transporter encoded by the PHO84 gene functions as a low affinity transporter of metals including Mn, Zn, Co and Cu (Jensen et al. 2003). This transporter is implicated in metal accumulation when extracellular metals are in excess. However, Smf1 and PHO84-mediated Cu uptake most likely play minimal roles in Cu metabolism. Ctr1, Ctr3, and Fet4 triple knockout

yeast cells do not exhibit saturable Cu transport activities at a wide range of Cu concentration, even though the cells are expressing Smf1 and Pho84 (Hassett et al. 2000). Additionally, Cu transported by Smf1 and Pho84 does not appear to be bioavailable, since cells defective in all other Cu transporters, Ctr1, Ctr2, Ctr3, and Fet4, are not able to acquire Cu for Cu/Zn SOD (Portnoy et al. 2001).

The biological significance of these low affinity Cu transporters is not well understood. The high affinity Cu transport machinery is either down regulated or not expressed to prevent Cu toxicity when yeast are exposed to excess Cu. Under these conditions, the low affinity Cu transporters may play roles in Cu acquisition.

2.4 Cu reductases

Oxygen in the environment oxidizes Cu(I) to Cu(II). Reduction of Cu(II) appears to be an important step for Cu transport into yeast cells. Fre1 and Fre2 metalloreductases initially identified as components of Fe transport also play roles in Cu metabolism. Both FRE1 and FRE2 knockouts have impaired Fe uptake but also Cu transport (Hassett and Kosman 1995; Georgatsou et al. 1997). They are plasma membrane electron transport proteins that mobilize cytoplasmic electrons through the membrane. Five other homologous proteins have been identified from the yeast genome (Martins et al. 1998). Regulation patterns of FRE genes by Cu and Fe further demonstrates that these metalloreductases play roles in Fe and Cu metabolism. FRE1, FRE6, and FRE7 genes are induced when cells are cultured under Cu deficiency. FRE1 to 6 are induced in cells cultured in Fe chelator-treated media as well. The functional specificities among these reductases and subcellular localization have not been fully characterized.

The Fre proteins are homologous to the gp91-*phox* subunit of the human NADPH phagocyte oxidoreductase. These proteins possess two heme-binding motifs, a flavin adenine dinucleotide (FAD) binding site, and two NADPH binding sequences (Roman et al. 1993; Finegold et al. 1996; Lesuisse et al. 1996; Shatwell et al. 1996). Given that gp91-*phox* is a subunit of the NADPH oxidase system (Rotrosen et al. 1992; Chanock et al. 1994), it is feasible to postulate that Cu reductases may need other components for their activities. Secondly, since reduced Cu is easily oxidized in the extracellular aerobic environment, there may be a mechanism that protects reduced Cu during the Cu transport process. Reduced Cu may bind to another molecule, and this complex may be a substrate of Ctr1 or Ctr3-mediated Cu transport. Alternatively, it is possible that Fre proteins may form a complex with Cu transporters passing the reduced Cu to them. Third, given that most other proteins involved in Cu metabolism are conserved between yeast and mammalian cells, yeast Cu reductases may provide critical information for identifying their mammalian counterparts.

3 Intracellular Cu distribution

Once Cu is transported into the cell, a mechanism must be in place to safely distribute Cu to specific intracellular targets. It is known that a class of small proteins referred to as chaperones or metallochaperones are required for this role (Fig.1). There are three known pathways in which Cu is directed in yeast. The Cu chaperone Atx1 directs Cu into the secretory compartments. CCS (copper chaperone for SOD1) incorporates Cu into Cu/Zn SOD (SOD1). The putative Cu chaperone Cox17 distributes Cu to the mitochondria. Chaperones are specific for their targets, since CCS, a Cu chaperon for SOD1, is not required for Cu trafficking to the secretory pathway or the mitochondria. Similarly, overexpression of other Cu chaperones such as Atx1 cannot restore SOD1 activity in cells lacking CCS (Culotta et al. 1997). The physiological necessity of metallochaperones was made apparent by the discovery that the ambient free Cu concentration in yeast cells is exceedingly low (Pufahl et al. 1997). With such a low availability of free Cu in the cell, there must be a mechanism to deliver Cu to the appropriate Cu-dependent enzyme while minimizing its toxic effects.

In addition to the chaperones there are many other downstream components required for intracellular distribution of Cu. These include the Atx1 target, Ccc2, which plays an obligatory role for Cu incorporation into the secretory pathway. There are accessory proteins or co-chaperones, which aid in Cu insertion into specific active sites. Furthermore, a member of the Ctr family has been implicated in mobilizing Cu stores from the vacuole. All together these components act in concert for the precise distribution of intracellular Cu.

3.1 Atx1-mediated Cu delivery to the secretory compartment

The Cu chaperone Atx1 was first identified as a high-copy suppressor of oxidative damage in yeast cells lacking SOD1 (Lin et al. 1995). Atx1 appears to have dual roles as both a scavenger of superoxide anions and a Cu chaperone. The first clue for its role as a Cu chaperone was provided by sequence homology with other metal binding proteins. Another clue came from an observed increase in cellular Cu levels upon overexpression (Lin et al. 1995). Atx1 was found to have conserved homologues in other eukaryotes, including the Hah1(Atox1) in humans (Klomp et al. 1997).

Once Cu is transported into the cytosol, it is captured by Atx1 and shuttled to the trans-golgi network (TGN) for delivery into the secretory pathway. Here, Atx1 transfers its cargo to the Cu translocating ATPase, Ccc2. In an ATP dependent manner, Cu is transported across a vesicular membrane for incorporation into the Fet3 multi-Cu oxidase (Lin et al. 1997). The mature Fet3 with its Cu cofactor forms a complex with the Ftr1 iron permease, which is responsible for iron uptake at the plasma membrane (Steaman et al. 1996). This explains the inextricable link previously observed between iron and Cu metabolism. Consistent with its role in iron metabolism, Atx1 is regulated by the iron sensing transcription factor Aft1 (Lin et al. 1997). However, unlike other Aft1 targets, Atx1 expression is unaf-

fected by a null mutation in AFT1, suggesting regulation by an additional transcription factor. The oxygen sensing transcription factor, Yap1 has been implicated in the regulation of Atx1 as its expression is strongly induced by oxygen (Harshman et al. 1988). The regulation of Atx1 by two different transcription factors is consistent with its dual functionality, Cu and Fe metabolism and oxidative stress defense.

Atx1 is a small cytosolic 8.2 kD polypeptide composed of 73 amino acid residues. The N-terminus of Atx1 contains a single MTCXXC metal binding motif. Electron paramagnetic resonance (EPR) and X-ray absorption spectroscopy (XAS) of Atx1 have supported the binding of a single Cu (I) ion per polypeptide (Pufahl et al. 1997). Extended X-ray absorption fine structure (EXAFS) measurements of Atx1 indicated an all sulfur coordination environment through either a two or three liganded complex. The NMR solution structure indicates that the Atx1 protein has an αβαββαβ fold and places a single Cu ion between Cys15 and Cys18 (Arnesano et al. 2001). Atx1 is comprised of multiple Lys residues generating a positively charged surface (Portnoy et al. 1999). Mutations of conserved lysines crippled Atx1 function *in vivo*. In particular, a mutation of Lys65 to Glu, which introduces a negative charge adjacent to the metal-binding motif, severely abrogates Atx1 function. Interestingly, a basic, neutral or hydrophobic residue at this position was tolerated (Portnoy et al. 1999). The importance of Lys65 has been proposed to partially neutralize the net negative charge resulting from the coordination of Cu(I) by two or more cysteines.

3.2 Ccc2, and other factors necessary for Cu incorporation into Fet3

Several genetic and biochemical experiments support the pathway of Cu(I) delivery from Atx1 to Ccc2 for incorporation into Fet3. Yeast ATX1 null mutants are unable to grow in iron limiting conditions, which can be rescued by Cu supplementation (Lin et al. 1997). *In vivo* ^{64}Cu labeling experiments have confirmed that Cu incorporation into Fet3 is defective in Δatx1 yeast strains (Klomp et al. 1997). Furthermore, overexpression of CCC2 can correct the defect of poor growth on limited iron in Δatx1 mutants corroborating the model in which Atx1 delivers Cu(I) to Ccc2 prior to incorporation into Fet3.

Ccc2 is 110kD transmembrane protein consisting of 1004 amino acid residues which has been localized to the late or post Golgi network (Yuan et al. 1997). It is a P-type ATPase belonging to the P1-type subfamily, which is specific for metal ion transport (Lutsenko and Kaplan 1995). P1-type ATPases contain 8 putative transmembrane domains with a conserved CPC/H motif located in the sixth transmembrane domain which is believed to be critical for metal ion translocation. P1-type ATPases contain 1 to 6 CXXC metal-binding domains at their N-terminus. The N-terminus of Ccc2 contains two of these metal-binding domains. The cytoplasmic domain of Ccc2 contains two loops with the largest containing the catalytic ATP-binding and phosphorylation sites. Ccc2 is a functional and structural homolog of the mammalian WND and MNK, P1-type ATPase Cu transporters, which are impaired in Wilson and Menkes disease, respectively. Both

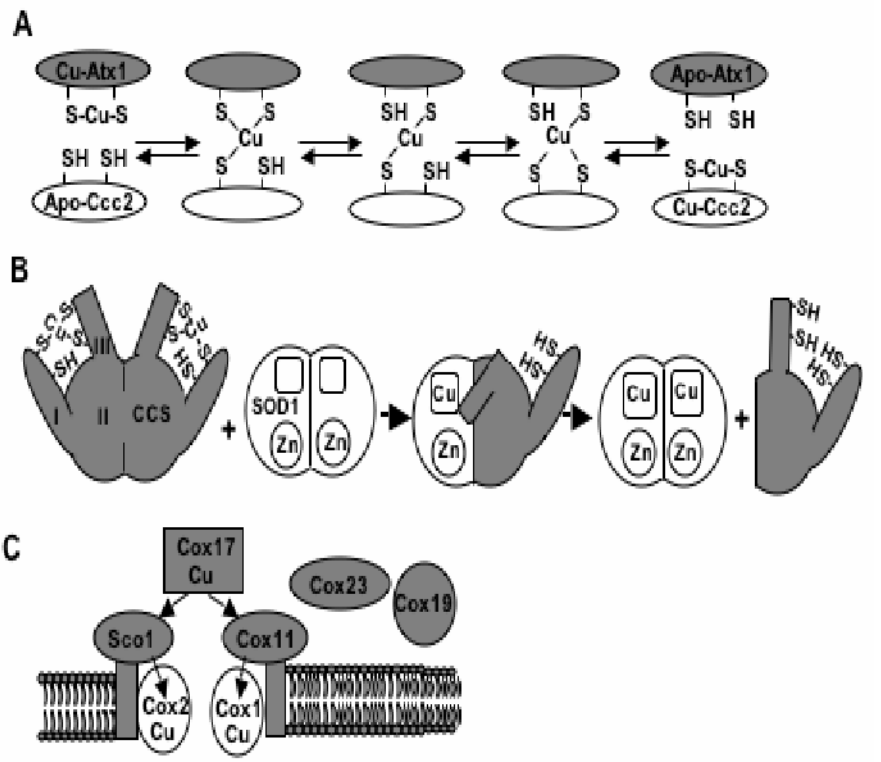

Fig. 2. Schematic illustrating the mechanisms of copper (Cu) transfer between metallochaperones and targets. (A) Atx1 and Ccc2 form a transiently docked complex bringing each metal binding domain into proximity. Cu equilibrates between both metal binding domains involving two and three liganded intermediates for Cu transfer. (B) Dimerization and Cu transfer between CCS and Cu,Zn superoxide dismutase (SOD1). Domain II forms docking interface with SOD1. At low Cu concentrations domain I potentially provides Cu ions to domain III. Domain III swings into position for Cu transfer into the active site of SOD1 (C) Cu incorporation into mitochondrial cytochrome c oxidase (COX) subunits 1 and 2. Cox17 transfers Cu to Sco1 and Cox11 prior to incorporation into the Cox2 Cu_A site and Cox1 Cu_B site, respectively. Cox19 and Cox23 both partially localize to the mitochondrial intermembrane space and are also important for COX assembly. Figures are modified from previous publications, (Fig. 2A: Pufahl et al. 1997; Fig. 2B: Lamb et al. 2001; Fig. 2C: Carr and Winge 2003).

WND and MNK can complement a Ccc2 null mutation in heterologous yeast systems (Hung et al. 1997; Payne and Gitlin 1998).

The Tfp1 subunit of the yeast vacuolar H^+ - ATPase and the yeast chloride channel Gef1 have been implicated in aiding Ccc2 mediated Cu transfer to Fet3 (Gaxiola et al. 1998). Yeast cells lacking Tfp1 or Gef1 are unable to grow on non-fermentable carbon sources; however, they can be rescued by Cu supplementation.

Additionally, Gef1 was also shown to co-localize with Ccc2 (Gaxiola et al. 1998). These observations led to the following model: Tfp1 ensures the maintenance of an acidic environment of the lumen in the late- or post-golgi vesicles required for Cu loading onto Fet3. Acidification and concomitant import of Cu ions lead to an increase of membrane potential further impeding transport of metal cations. The potential can be counter balanced by the influx of Cl^- anions mediated through Gef1 restoring Ccc2 mediated delivery of Cu into the lumen. In addition to the stabilization of the electrochemical potential, Cl^- anions have been proposed to act as an allosteric effector required for the incorporation of Cu into Fet3 (Davis-Kaplan et al. 1998).

3.3 Interaction between Atx1 and Ccc2 for Cu transfer

Structural studies of Atx1 and Ccc2 have shed light onto possible mechanisms of Cu transfer. Yeast two-hybrid experiments have demonstrated a Cu dependent interaction between Atx1 and the N-terminus of Ccc2 (Pufahl et al. 1997). NMR solution structures available for Atx1 (Arnesano et al. 2001) and the soluble N-terminal domain of Ccc2 (Banci et al. 2001) indicated that both have a similar secondary structure. The metal-binding domain of Atx1 is located at a surface-exposed loop which is buried in the metal bound form (Arnesano et al. 2001). Conversely, the Ccc2 N-terminal structure remains relatively invariant upon metal binding (Banci et al. 2001).

It has been demonstrated that a Cu(I) loaded Atx1 can directly transfer Cu to Ccc2 in a reversible manner (Huffman and O'Halloran 2000). Cu(I) rapidly equilibrates between the two proteins suggesting that the thermodynamic gradient is quite shallow. Thus, the vectoral delivery of Cu(I) from Atx1 is not dependent on higher Cu affinity of the Ccc2 target domain. Rather, Atx1 acts as an enzyme by lowering the energy required for transfer specifically for the Ccc2 target site while protecting it from adventitious reactions with non-partner proteins (Huffman and O'Halloran 2000).

A model using current structural information has been proposed for Cu transfer between Atx1 and Ccc2. According to this model, the low activation barrier for metal transfer is achieved from complementary electrostatic forces which orientate the metal binding domains of Cu(I) loaded Atx1 and apo-Ccc2 (Huffman and O'Halloran 2000). The conformational changes observed in the apo-Atx1 to Cu-Atx1 transition are thought to poise Atx1 for direct interaction with Ccc2. Atx1 possesses many positively charged residues on its surface while Ccc2 possesses multiple negatively charged residues at its N-terminus serving as a docking interface (Portnoy et al. 1999). Computer modeling, using the available structural information, shows stable electrostatic interactions amongst specific Atx1 Lys residues and N-terminal Ccc2 Glu and Asp residues (Arnesano et al. 2004). Additionally, an intermolecular hydrogen bond could be placed between the critical Lys65 of Cu-Atx1 and Ccc2. Thus, computer aided modeling has given a structural basis to explain previous mutational data.

It has been proposed that after protein-protein recognition, Cu undergoes a series of rapid associative exchange reactions involving two- and three-coordinate intermediates between the Atx1 and Ccc2 metal-binding motifs (Fig. 2a) (Pufahl et al. 1997). Once Cu is bound to Ccc2, it is believed to be transferred to the intermembrane CXC motif. The binding of Cu to the CXC motif is believed to trigger ATP hydrolysis which drives a conformational change, releasing Cu into a vesicle that is thermodynamically distinct from the cytosol (Huffman and O'Halloran 2000). However, the details of this mechanism still remain poorly understood.

3.4 CCS, a Cu chaperone for Cu,Zn superoxide dismutase (SOD1)

The chaperone which delivers Cu to SOD1 in yeast is CCS (copper chaperone for SOD1). CCS was first identified by mutations in the Lys7 gene, which displayed a phenotype of a non-functional SOD1 (Culotta et al. 1997). A Lys7 null mutation results in the loss of SOD1 function despite normal levels of SOD1 protein. SOD1 function is restored by the supplementation of Cu supporting that its inactivity is the result of inadequate Cu incorporation.

CCS is a 249-amino acid protein, which is much larger in comparison to Atx1. Similar to SOD1, CCS exists as a homodimer (Fig. 2B). There are three functionally distinct domains in CCS (Lamb et. al 1999). The N-terminal domain, domain I, resembles Atx1 and contains the same MXCXXC Cu binding motif; however, is only necessary for function under Cu limited conditions. The central domain, domain II, is homologous to SOD1 and is critical for recognition, but lacks residues important for SOD1 function. Domain III, a short peptide of the carboxyl terminus, bears an invariant CXC Cu binding motif conserved in all members of the CCS family and is crucial for function (Schmidt et. al 1999).

CCS activates SOD1 by directly inserting the Cu cofactor while protecting it from intracellular Cu scavengers. This function was first made apparent as a Cu loaded CCS could activate apo-SOD1 in the presence of Cu chelating agents *in vitro* (Rae et al. 1999). The transfer mechanism must be direct because any Cu ions released into solution would be immediately sequestered by the Cu chelators. Yeast two hybrid assays showed *in vivo* protein-protein interactions between SOD1 and domains II and III of CCS, however, no interaction was observed with the Atx1-like domain I. Instead, this domain most likely captures Cu under limited conditions and transfers it to the CXC motif of Domain III.

It would appear that a similar mechanism of Cu transfer exists for CCS and SOD1 as does for Atx1 and Ccc2. In this case the CCS and SOD1 homodimers would have to disassociate prior to docking with one another. As with Atx1, Cu binding to CCS elicits a conformation suitable for docking with its partner. The Cu transfer is directed by specific protein-protein interactions which guide the Cu binding domains of donor and acceptor within proximity for Cu transfer.

The crystal structure of CCS complexed with SOD1 has provided a structural model for CCS Cu insertion into SOD1 (Lamb et al. 2001). The "frozen" complex was obtained by replacing SOD1 His48 with Phe so that it was unable to bind Cu

yet retain Zn in the active site. The crystal structure shows a heterodimer consisting of a single CCS monomer and a single SOD1 monomer linked together by an intermolecular disulfide bond. Previous biochemical studies have also supported the formation of a heterodimer between CCS and SOD1 which is facilitated by Zn (Lamb et al. 2000). Most contacts are made between SOD1 and the SOD1-like domain II of CCS. Many conformational changes occurred upon the reorganization of CCS and SOD1 homodimers to form a heterodimeric duplex. These conformational changes have given mechanistic clues into how Cu is loaded into the SOD1 active site. CCS has two potential Cu binding sites, one in the MHCXXC sequence motif of the Atx1-like domain I and the other CXC sequence motif in domain III. The structure showed that the MHCXXC motif of domain I is too far away for Cu transfer, however, the cysteines of the CXC motif of domain III lie adjacent to the SOD1 active site. Interestingly, one of these cysteines forms an intermolecular disulfide bond with SOD1 disrupting a previous intramolecular disulfide within SOD1. This displacement appears to open up the active site of SOD1 for Cu incorporation (Lamb et al. 2001).

Recently, the presence of O_2 has been shown to be necessary for the post-translational activation of SOD1 in a CCS dependent manner (Brown et al. 2004). Mutagenesis and biochemical studies have shown that the activity of SOD1 was dependent on an intramolecular disulfide bond which is catalyzed by Cu-CCS in the presence of O_2 (Furukawa et al. 2004). It has been proposed that the oxygen affords an oxidizing environment catalyzing the disulfide-linked heterodimer of CCS and SOD1 which was observed in the crystal structure. Following isomerization the intermolecular disulfide would exchange to form an intramolecular disulfide bond activating SOD1. This would explain how the SOD1 intramolecular disulfide bond is achieved in the reducing environment of the cytosol. This provides for a means of post-translational activation in which a pool of apo-SOD1 could be activated in the presence of oxygen when most needed (Furukawa et al. 2004).

3.5 Cu transport to the mitochondria

Cu is an essential electron carrier for cytochrome c oxidase (COX). COX is the terminal enzyme of the electron transport chain within the mitochondrial inner membrane (IM). COX is composed of many different subunits, three of which (Cox1, Cox2, and Cox3) are encoded and assembled within the mitochondria (Carr and Winge 2003). Two of these subunits, Cox1 and Cox2, utilize Cu as their cofactor. Cox2 contains two Cu ions in the binuclear Cu_A site which functions as the initial electron acceptor. Cox1 contains a single Cu and a heme cofactor forming the heterobimetallic Cu_B site. These mitochondrial encoded subunits are assembled within the mitochondria and are localized to the IM. A mechanism must be in place to deliver Cu ions to the mitochondria, traverse the outer membrane (OM) and incorporate the metal cofactors into the appropriate COX subunits. Since it has already been demonstrated that metallochaperones are responsible for the delivery of Cu to the secretory pathway and to Cu/Zn SOD1, it is likely that Cu delivery to the mitochondria requires metallochaperones as well.

Cox17 has been implicated as the main Cu shuttle, which delivers cytosolic Cu to the mitochondria. Cox17 was first identified by genetic screening of yeast for respiratory deficient mutants (Glerum et al. 1996a). Cells harboring a COX17-1 mutation are respiratory deficient and lack COX activity; however, the phenotypes were reversed by the addition of exogenous Cu suggesting a role of Cox17 in the metalation of COX. Currently, in addition to Cox17, four other proteins (Cox11, Sco1, Cox19, and Cox23) have been implicated in Cu loading of COX (Fig. 2C).

Cox17 is a hydrophilic, cysteine rich polypeptide composed of 69 amino acid residues. It resides partially in the cytosol (40%) and the mitochondrial intermembrane space (IMS) (60%) (Beers et al.1997). This dual localization is consistent for a chaperone that ferries Cu across the mitochondrial OM. Cox17 exists as a dimer or a tetramer where the apo form is predominantly monomeric, suggesting that Cu binding is important for oligimerization and correlated to function (Heaton et al. 2001). Of the seven total cysteines, C23, C24, and C26 are part of a CCXC sequence motif and important for function (Heaton et al. 2000). Mutation of any one cysteine results in loss of function despite retaining the ability to bind Cu and localize to the mitochondria. However, two Cys->Ser mutations of the Cox17 CCXC motif results in the loss of Cu binding yet localization to the mitochondria is retained. Thus, a Cu(I) conformer of Cox17 is not necessary for mitochondrial import as was once previously thought. Recently, the Cox17 NMR solution structure places a single Cu(I) ion coordinated by C23 and C26 of the conserved CCXC motif, which is consistent with previous mutagenesis studies (Abajian et al. 2004). Interestingly, the original COX17-1 mutation turned out to be a C57Y substitution first believed to abrogate Cu binding. It was later found that this mutant retains the ability to bind Cu(I) but no longer localizes to the mitochondria (Heaton et al. 2000). Conversely, a C57S mutant is still functional but only low levels accumulate in the mitochondria suggesting that minimal quantities of Cox17 are required for function (Heaton et al. 2000).

Direct transfer of Cu from Cox17 to both Sco1 and Cox11 has been demonstrated (Horng Y-C et al. 2004). Thus, Cox17 seems to have the unique ability to mediate Cu transfer to two different proteins. Sco1 has been proposed to play a role in Cu delivery to the Cu_A site of Cox2 subunit. Previously, it was shown that overexpression of Sco1 or its close homolog Sco2 could suppress the respiratory deficient phenotype of a Cox17-1 strain (Glerum et al. 1996b). Overexpression of Sco2 only partially restored Cox17-1 respiratory deficiency and only when exogenous Cu was added to the growth medium. In yeast, Δsco1 null mutants are respiratory deficient while Δsco2 null mutants lack an obvious phenotype. Overexpression of Sco2 fails to suppress the respiratory deficiency of Δsco1 null mutants. However, Sco2 overexpression does show partial allele-specific suppression for a Sco1 point mutant. One explanation for the allele-specific suppression observed is that Sco1 and Sco2 physically interact; however, no such interaction was detected. Instead Sco2 may be providing one of the functions lost in the Sco1 mutant suggesting that some redundancy exists between these two proteins (Glerum et al. 1996b). Contrary to human Sco2 which is essential, the functional role of Sco2 in yeast still remains unclear (Lode et al. 2002).

Both Sco1 and Sco2 possess a single transmembrane N-terminal helix and are associated with the mitochondrial IM (Glerum et al. 1996b). Sco1 has been demonstrated to bind Cu through a conserved CXXC sequence, which is critical for *in vivo* function (Nittis et al. 2001; Rentzsch et al. 1999). X- Ray absorption spectroscopy suggested that Cu(I) is coordinated by three ligands provided by the two cysteines of the CXXC motif and a conserved histidine (Nittis et al. 2001). A mutation of any one of these conserved residues of Sco1 abolished function and resulted in a non-functional COX (Rentzsch et al. 1999). These findings established that the function of Sco1 is dependent on Cu(I) binding. Additionally, Sco1 has been demonstrated to interact specifically with the Cox2 subunit, which has further supported its role for the incorporation of Cu(I) into the Cu_A site (Lode et al. 2000).

Cox11 has been proposed to insert Cu into the Cu_B site of the Cox1 subunit. Like the Sco1 and Sco2 proteins, Cox11 contains a single N-terminal transmembrane helix and is localized to the mitochondrial IM (Tzagoloff et al. 1990). Similarly, Cox11 is a Cu(I) binding protein, coordinating Cu(I) by three conserved cysteines (Carr et al. 2003). As with Sco1 any mutation of the Cu(I) coordinating residues resulted in respiratory deficiency due to reduced COX activities (Carr et al. 2003). These findings also correlate Cox11 function with Cu(I) binding. Evidence for specific incorporation of Cu into the Cox1 subunit Cu_B site has been provided by genetic and biochemical experiments. Yeast Δcox11 mutants have lower levels of Cox1 subunit (Tzagoloff et al. 1990). Additionally, COX isolated from *R. sphaeroides* Δcox11 cells lacked Mg^{2+} and a Cu_B center, yet retained all other cofactors including the Cu_A site in Cox2 subunit (Hiser et al. 2000). The combined evidence suggests that Cox11 has a functionally specific role in Cu(I) loading of the Cu_B center in the Cox1 subunit.

Cox19 (Nobrega et al. 2002) and Cox23 (Barros et al. 2004) are important for COX assembly. Like Cox17, Cox19, and Cox23 show a dual localization between the cytosol and the mitochondrial IMS. Cox19 lacks a CCXC Cu (I) binding motif and Δcox19 mutants cannot be rescued by the addition of Cu (Nobrega et al. 2002). It is more likely that Cox19 plays another role in COX assembly other than Cu metalation of COX. However, Cox23 does seem to play a role in mitochondrial Cu homeostasis. Δcox23 unlike Δcox19 mutants can be rescued by exogenous Cu, however, only when transformed with a Cox17 high copy plasmid. Conversely, overexpression of Cox23 does not suppress the Δcox17 respiratory deficient phenotype suggesting that Cox17 functions downstream of Cox23 in the same pathway possibly involving Cox19 (Barros et al. 2004).

One thing that is apparent now is that Cu homeostasis in the mitochondria is much more complicated than once previously thought. Recently, the role of Cox17 as the main mitochondrial Cu shuttle has come into doubt. Cox17, tethered to the mitochondrial IM by fusion with the N-terminal transmembrane domain of Sco2, was still able to compliment the respiratory deficiency of Δcox17 cells (Maxfield et al. 2004). Evidence also exists for a non-proteinaceous pool of Cu in the mitochondrial matrix (Cobine et al. 2004). If Cox17 is not supplying Cu to the mitochondria, then how is it getting there? One possibility is that a Cu transporter exists within the mitochondrial OM; however, such a transporter has remained

elusive. The role of Cox17 seems to be confined to the mitochondrial IMS where it passes Cu(I) to the IM proteins Sco1 and Cox11 for incorporation into the Cu_A and Cu_B sites, respectively (Fig. 2C). The transfer process is likely to be similar to the docking mechanism described between Atx1 and Ccc2 and CCS and Cu/Zn SOD1.

3.6 How do the cytoplasmic Cu chaperones acquire Cu?

Both Cu transporters at the plasma membrane and cytoplasmic Cu chaperones play critical roles in Cu delivery to subcellular targets. However, the mechanisms of Cu acquisition by Cu chaperones are not known. Since Cu transported by Ctr1, Ctr3, Fet4, and Ctr2 is available for intracellular Cu metabolism (Portnoy et al. 2001), it is unlikely that Cu chaperones that are distinct in their structural features interact directly with all these heterogeneous Cu transporters. Furthermore, any common sequence among these Cu transporters that may serve as an interacting motif with cytoplasmic Cu chaperones does not exist either.

A report has addressed the potential direct interaction between yeast Ctr1 and the Atx1 Cu chaperone (Xiao and Wedd 2002). C-terminal sequences (amino acid 280-406) of Ctr1 are hydrophilic and include two Cys-X-Cys motifs. The Ctr1 C-terminus exchanges Cu(I) rapidly with the Atx1 protein *in vitro*. This domain of Ctr1 binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster (Xiao et al. 2004). However, it has not been demonstrated whether other Cu chaperones acquire Cu in the same experimental conditions. Although the metal-binding motifs are not conserved among Cu transporters, three-dimensional structures of the transporters will be much more informative in the investigation of their direct interaction with Cu chaperones. Additionally, since their interactions may be transient, conventional biochemical approaches may not be the best methods for determining their interactions.

3.7 Ctr2-mediated mobilization of intracellular Cu stores

Recent evidence has shown that the yeast *S. cerevisiae* has the ability to mobilize Cu stores from the vacuole and that this mobilization is mediated through the Cu transporter Ctr2 (Portnoy et al. 2001; Rees et al. 2004). Ctr2 was identified along with the *Arabidopsis* COPT1 by sequence homology in an attempt to identify Cu transporters in plants (Kampfenkel et al. 1995). Both proteins were characterized as belonging to the Ctr1 family of integral membrane proteins.

Ctr2 was previously thought to be low affinity plasma membrane Cu transporter. Several early observations led to this misconception. Due to the homology to other Ctr transporters, it seemed logical that Ctr2 would also be a plasma membrane Cu transporter. High levels of Cu could suppress Fe uptake in Ctr1 mutants, which was indicative of the presence of a low affinity Cu transporter at the plasma membrane (Dancis et al.1994). However, disruption of the CTR2 gene did not show any respiratory deficiency when grown on a non-fermentable carbon source

or under iron limiting conditions (Kampfenkel et al. 1995). Overexpression of Ctr2 led to increased sensitivity to high Cu levels while conversely its disruption led to greater resistance. Furthermore, overexpression of Ctr2 did not complement a Ctr1 mutant on a non-fermentable carbon source or under iron limiting conditions suggesting that its contribution for Cu uptake was minimal (Kampfenkel et al. 1995). These findings were all consistent for a low affinity plasma membrane Cu transporter. This role was largely dismissed after Ctr2 was localized to the vacuole (Portnoy et al. 2001; Rees et al. 2004). It is now apparent that Ctr2 plays a role in mobilizing vacuolar Cu stores to readily make Cu available for cellular needs. Evidence for this role was provided by Cu measurements of purified vacuoles. Cu levels in vacuoles from Δctr2 cells were fourfold higher than cells that were constitutively expressing Ctr2 (Rees et al. 2004). Ctr2 has been shown to make Cu available for all known Cu-requiring processes (Portnoy et al. 2001; Rees et al. 2004). Interestingly, Ctr2 does not appear to be regulated as are the high affinity Cu transporters, Ctr1 and Ctr3 (Portnoy et al. 2001).

Sequence homology and predicted topology suggest that Ctr2 utilizes a similar mechanism of transport as Ctr1. This is further supported by genetic and biochemical evidence. It was illustrated that Ctr2 assembles as a homomultimer as does Ctr1 (Rees et al. 2004). Both Ctr1 and Ctr2 possess conserved methionine residues at the N-terminus and possess an MXXXM sequence motif within the second transmembrane domain. Mutations of conserved methionines displayed similar defects in both Ctr1 and Ctr2, which is consistent for a similar mechanism of Cu transport.

If Ctr2 is in fact a Cu transporter which mobilizes Cu from the vacuole, then how does Cu get there in the first place? Since yeast contain an iron and Mn^{2}+ transporter, which stores these metals within the vacuole (Chen et al. 2001), it is quite possible that a similar unidentified Cu transporter is responsible for Cu uptake within the vacuole. Cu transfer mediated through endocytosis or fusion of vesicles loaded with Cu cannot be excluded either.

3.8 Pca1, a P-type ATPase

A second P-type ATPase in *S. cerevisiae* in addition to Ccc2 has been designated as Pca1 (putative P-type cation-transporting ATPase) (Rad et al. 1994). Pca1's role in Cu homeostasis remains obscure. Pca1 is a large 132kD transmembrane protein consisting of 1216 amino acid residues. Pca1 has one N-terminal MTCXXC metal binding motif compared to the two found in Ccc2. Mammals possess two P-type ATPases, which are WND and MNK, and another yeast species *C. albicans* expresses two P-type ATPases that play independent roles, Cu incorporation at the secretory pathway and Cu export to reduce intracellular Cu accumulation. It is curious that *S. cerevisiae* would possess two P-type ATPases that may have distinct roles exampled in mammals and other yeast. Pca1 is believed to play a role in Cu homeostasis and defense against Cu toxicity since Δpca1 strains show sensitivity to high concentrations of Cu (Rad et al. 1994). Interestingly, the single amino acid substitution R970G confers cadmium resistance in yeast

(Shirashi et al. 2000). Microarray data confirmed by quantitative PCR shows that Cu or Fe deficiency regulates Pca1 expression (De Freitas et al. 2004). A respiratory deficient phenotype has been described for Δpca1 null mutants (De Freitas et al. 2004). Thus far, the available evidence suggests some role in Cu and/or Fe homeostasis, however, the details still remain to be studied.

4 Defense systems to Cu toxicity

Cu transporters and cytoplasmic chaperones deliver Cu to Cu-requiring proteins. However, Cu accumulated in excess, like other transition metal ions, is toxic. Consistent to the potential toxicity arising from accumulation or releasing of Cu to its free forms, yeast has equipped defense mechanisms for Cu toxicity. Systems for chelation, sequestration and export of Cu, and scavenging oxygen free radicals generated by Cu-mediated reactions have been characterized in yeast.

4.1 Metallothioneins

Metallothionein (MT) is a 61 amino acid polypeptide, which coordinates seven to eight Cu(I)-ions as a Cu-S polynuclear cluster utilizing 10 of its 12 cysteines (Hamer 1986). Each of the eight Cu(I) ions are bridged trigonally by cysteine residues through Cu(I)-thiolate bonds (Winge et al. 1985; Thrower et al. 1988). Yeast *S. cerevisiae* possesses up to 15 tandem copies of the Cup1 gene encoding metallothionein (Fogel and Welch 1982). The gene copy number of Cup1 is directly correlated to the resistance level to external Cu. Yeast cells lacking Cup1 are hypersensitive to high Cu concentrations (Hamer et al. 1985), which is consistent to the role of MT in Cu detoxification. In addition to Cu chelation, MT partially suppresses the oxygen sensitivity of SOD1-defective yeast cells, since MT carries superoxide dismutase activities (Tamai et al. 1993).

Crs5 encodes a small molecular weight cysteine-rich protein with an amino acid sequence bearing all the features of a eukaryotic metallothionein, yet shares little sequence similarity with Cup1 (Culotta et al. 1994). Given that the Cu(I) ions bound to Crs5 are kinetically more labile, MT plays a dominant role in Cu detoxification (Jensen et al. 1996). The modest effects of Crs5 may imply it may have additional roles that are distinct from Cu chelation.

C. glabrata contains three MT-encoding genes, MTI, MTIIA, and MTIIB, which are highly induced by Cu (Mehra et al. 1989, 1990, 1992). The MTIIA locus contains a tandem array of a gene like Cup1 in *S. cerevisiae*, and Cu tolerance is correlated to the gene copy number (Mehra et al. 1990). The MTIIA and MTIIB genes encode the same polypeptide, but their 5' and 3' non-coding sequences are not identical (Mehra et al. 1992).

4.2 Cu,Zn superoxide dismutase (SOD1)

Cu readily catalyzes reactions that result in the production of hydroxyl radicals through the Fenton and Haber-Weiss reactions ($Cu^{+} + H_2O_2 \rightarrow HO^{\cdot} + HO^{-} + Cu^{2+}$, $O_2^{\cdot -} + Cu^{2+} \rightarrow Cu^{+} + O_2$) (Halliwell and Gutteridge 1984, 1990). Superoxide anion ($O_2^{\cdot -}$) is a critical factor that propagates the reaction generating highly reactive hydroxyl radicals ($HO^{\cdot}$). Thus, superoxide dismutase is obviously linked to a defense mechanism for Cu toxicity. Consistently, excess Cu induces Cu/Zn superoxide dismutase (Cu/Zn SOD, SOD1) expression (Gralla et al. 1991).

SOD1 is primarily located in the cytoplasm, but SOD1 has also been identified in other organelles including mitochondria (Weisiger and Fridovich 1973). It is interesting that a fraction of SOD1 and its Cu chaperone CCS localize to the mitochondrial IMS (Sturtz et al. 2001). Only a very immature form of the SOD1 polypeptide that lacks both Zn and Cu cofactors in its reduced disulfide form efficiently enters the mitochondria (Field et al. 2003), and SOD1 retention in the mitochondria is largely dependent on CCS. When CCS synthesis is repressed, SOD1 levels in the mitochondria are low, and conversely SOD1 levels in the mitochondria are high when CCS is abundant in this organelle. Yeast cells with elevated levels of SOD1 in the mitochondria exhibit pro-longed survival in the stationary phase (Sturtz et al. 2001). Given that death of yeast cells in the stationary phase is linked with mitochondrial reactive oxygen production (Jakubowski et al. 2000; Longo et al. 1999; Ashrafi et al. 1999), SOD1 accumulated in the mitochondria may play a significant role in protection against oxidative stress when cells are in the stationary phase. This study may have an important implication with familial amyotropic lateral sclerosis (ALS), a fatal neurodegenerative disease resulting from gain-of function mutations of SOD1 (Rosen et al. 1993; Gurney et al. 1994). It would be interesting to test whether the SOD1 mutants causing ALS behave abnormally in their retention and mode of actions in the mitochondria.

4.3 CuS biomineralization

The SLF1 gene was identified as a multicopy suppressor of a Cu sensitive mutant (Yu et al. 1996). This protein is important for the physiological Cu sulfide (CuS) mineralization on the cell surface, resulting in a brownish coloration when cells are grown in $CuSO_4$-containing media. Since overexpression of the SLF1 gene confers Cu resistance and disruption of the gene increases Cu sensitivity, CuS biomineralization should be an important mechanism to prevent Cu toxicity.

4.4 P-type ATPase-mediated Cu export

The pathogenic yeast *C. albicans* has a higher resistance to Cu toxicity than baker's yeast. Interestingly, this Cu resistance is mediated by a P-type ATPase (CaCRP1/CRD1) and MT (CaCUP1/CRD2) that have homology to their human counterparts (Weissman et al. 2000; Riggle and Kumamoto 2000). Cu induces

transcription of the CaCRP1/CRD1 gene, while CaCUP1/CRD2 expression is constant. These gene disruptions indicate that the CaCRP1/CRD1 is the major component for Cu resistance. Furthermore, under acidic and anaerobic growth conditions, CaCRP1/CRD1 function becomes essential for survival in the presence of even very low Cu concentration. Cu export mechanisms have been characterized in pathogenic enteric bacteria. Since CaCRP1/CRD1 primarily localizes at the plasma membrane and a knockout of this gene does not affect Fe metabolism, this Cu export pump is a unique example in eukaryotes. The *C. albicans* genome contains another P-type ATPase that may function at the secretory pathway (Weissman et al. 2000). This Cu export mechanism by a P-type ATPase is conserved in mammals, but the P-type ATPases in mammals play dual roles in Cu metabolism, Cu transport at the secretory compartment and Cu export at the plasma membrane (DiDonato and Sarkar 1997; Cox 1999; Schaefer and Gitlin 1999; Llanos and Merser 2002). Given that the baker's yeast *S. cerevisiae* has two P-type ATPases as well, Ccc2 and Pca1 (Yuan et al. 1995; Rad et al. 1994), Pca1 may play the same role as the CaCRP1/CRD1 of *C. albicans*.

4.5 Multi-drug resistance protein

It has been known that yeast cells defective in Pdr13 are more sensitive to Cu toxicity (Kim et al. 2001). Pdr13 activates Pdr1, a transcription factor that upregulates the expression of Pdr5 and Yor1 genes encoding ATP-binding cassette transporters involved in drug efflux (Moye-Rowley 2003). Consistently, a gain of function mutation of Pdr1 transcription factor shows increased resistance to Cu, Fe, and Mn (Tuttle et al. 2003). These results suggest that expression of multidrug resistance genes play a role in Cu resistance by altering their efflux and/or sequestration.

4.6 Vacuole and Cu sequestration

Isolation of yeast mutants sensitive to Cu ion toxicity revealed that genes that play critical roles in vacuolar assembly or acidification, including Pep3, Pep5, and Vma3, are required for normal Cu resistance (Szczypka et al. 1997). Yeast vacuoles serve as a storage organelle for metal-ions including Cu, Fe, and Zn (Bode et al. 1995; Ramsay and Gadd 1997; Paidhungat and Garrett 1998; MacDiarmid et al. 2000). Since baker's yeast Ctr2 protein and Ctr6 of *S. pombe* mobilize stored Cu from the vacuole (Bellemare et al. 2002; Rees et al. 2004), there should be mechanism(s) for Cu sequestration to the lumen of the vacuole.

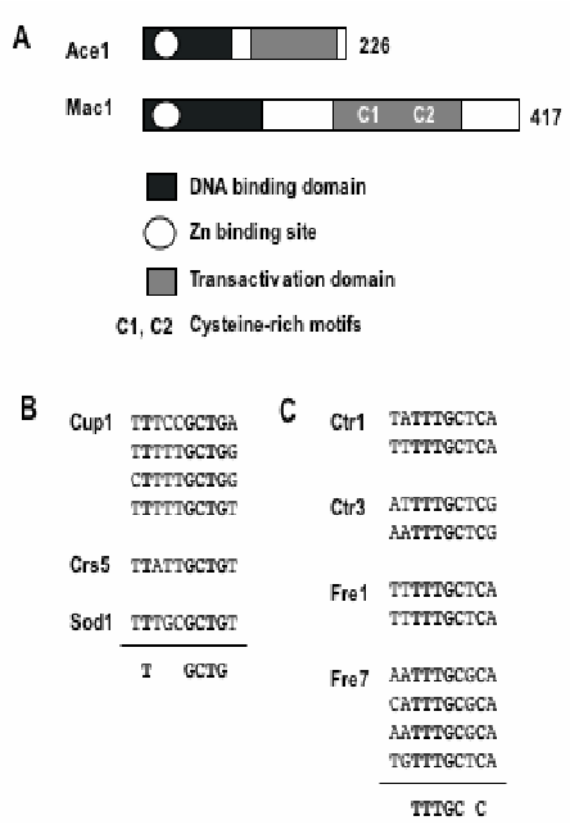

Fig. 3. (A) Ace1 and Mac1 are two Cu-responsive transcription factors in yeast *S. cerevisiae*. They share high sequence similarity in the DNA-binding domain, Cu-binding site and transactivation domain. Carboxyl-terminal cysteine-rich C1 and C2 motifs ($CXCX_4CXCX_2CX_2H$) of the Mac1 are important for its Cu sensing and transcription regulation of its target genes. (B) Sequences of the *cis*-acting elements of Ace1. Ace1 induces expression of *CUP1*, *CRS5* and *SOD1* genes upon binding to the promoter region of theses genes. The bold characters indicate the conserved sequences among the Ace1 DNA-binding sites. (C) Mac1-binding sequences identified from the promoter of *CTR1*, *CTR3*, *FRE1*, and *FRE7* genes.

5 Regulation of Cu metabolism

Copper ion homeostasis in yeast is maintained by different modes of regulation both at the transcriptional and post-translational levels. Cu uptake at the plasma membrane, sequestration of Cu in the cytoplasm, and defense systems against Cu toxicity are all regulated by Cu-activated and Cu-repressed transcription factors.

Turnover of Cu transporters and Cu-responsive transcription factors are also regulated by Cu levels.

5.1 Ace1/Amt1

Studies of the transcriptional regulation of Cup1 metallothionein (MT) gene by the Cu-regulatory transcription factors, Ace1 in *S. cerevisiae* (Thiele 1988; Welch et al. 1989) and Amt1 from *Candida glabrata* (Zhu and Thiele 1991), have provided insights into the mechanisms of Cu sensing and signal transduction. When the extracellular Cu concentration is in excess (10 μM), Ace1 is activated to induce expression of genes including Cup1, Crs5 and Sod1 (Hamer et al. 1988; Grallar et al. 1991; Culotta et al. 1994). Cu(I)-activated gene expression occurs through direct Cu binding to the regulatory domain of the Ace1 transcription factor (Dameron et al. 1991). Cu(I) cluster formation stabilizes a specific conformation of Ace1 that leads to binding with a response element (TXXXGCTG) of its target gene (Fig. 3B) (Buchman et al. 1989; Dobi et al. 1995). Chromosomal footprinting demonstrated that Ace1 binds, as a monomer, to three different regions of the Cup1 promoter comprising four different binding sites (Huibregtse et al. 1989; Evans et al. 1990). The DNA-binding domain of Ace1 was shown to map to the amino-terminal (Fig. 3A) in which 11 cysteine residues (CXXC or CXC) out of 12 are critical for Cu-induced gene expression (Hu et al. 1990). The Cu-regulatory domain binds 4 Cu(I) ions through 8 cysteinyl thiolates forming a polycopper cluster (Furst and Hamer 1989). Ace1 DNA binding is dependent on not only the Cu coordination but also by Ag(I) ions (Dameron et al. 1991; Furst el al. 1988). In addition to a Cu-responsive DNA-binding domain, Ace1 carries two other functional domains. The N-terminal of Ace1 has a Zn(II)-binding module (Farrell et al. 1996). Transcription assays using the fusion protein between a heterologous yeast DNA binding domain and the carboxy-terminal half of Ace1 indicates that this C-terminal region of Ace1 contains a transcription activation domain (Hu et al. 1990).

The trans-acting factor that mediates Cu-induced expression of MT genes in *C. glabrata* is Amt1, which is similar to Ace1 in its structure and mode of action (Zhou and Thiele 1991). Interestingly, unlike Ace1, expression of Amt1 is auto-regulated by Cu (Zhou and Thiele 1993). A homopolymeric (dA·dT) stretch located adjacent to the metal-response element of the Atm1 promoter plays a critical role in rapid transcriptional auto-regulation of the Atm1 gene (Zhu and Thiele1996). This structure fosters binding of Cu-activated Atm1 to the promoter resulting in rapid regulation of Atm1 expression.

The *S. cerevisiae* genome contains Haa1, which is homologous to Ace1. The N-terminal Zn-binding domain and Cu regulatory domain of Haa1 is similar to those of Ace1. Haa1 is indeed a transcription factor, but Cu and other metal-ions do not regulate expression of its target (Keller et al. 2001). The functions of Haa1-regulated genes do not appear to be directly related to Cu metabolism since over-expression of Haa1 does not suppress the Cu sensitivity of Ace1-defective cells (Keller et al. 2001).

5.2 Mac1

Cu-mediated repression of genes involved in Cu uptake in *S. cerevisiae* occurs through Mac1. Mac1 was identified by its similarity with the cysteine-rich copper-binding domain of Ace1 and Amt1 (Jungmann et al. 1993). Cu(I) binding to Mac1 represses an array of genes involved in Cu uptake, including CTR1, CTR3, FRE1, and FRE7, but Cu ion starvation de-represses these genes (Georgatsou et al. 1997; Yamaguchi-Iwai et al. 1997; Labbé et al. 1997; Peña et al. 1998). Deletion of the MAC1 gene results in phenotypes similar to those in cells lacking high affinity Cu transport that can be rescued by the addition of Cu ions.

In the presence of low levels of Cu, Mac1 constantly surveys the intracellular Cu status, and regulates the expression of the Cu transport genes depending on cellular needs. This regulation requires the interaction of Mac1 with the Cu-responsive *cis*-acting elements [CUREs, 5'-TTTGC(T/G)C(A/G)-3'] (Fig. 3C). This sequence is found as tandem or inverted repeats in the promoters of the high affinity Cu transport genes and a reductase encoded by the FRE1 gene (Yamaguchi-Iwai et al. 1997; Labbé et al. 1997; Peña et al. 1998). Consistently, *in vivo* footprinting experiments have shown that the CUREs within the CTR3 promoter are occupied during Cu ion starvation to induce target gene expression (Labbé et al. 1997; Peña et al. 1998). Mac1 appears to bind to these elements as a $(Mac1)_2$/DNA ternary complex with two molecules of Mac1 bound to each CURE element (Joshi et al. 1999). A module in the DNA binding motif interacts with the TTT sequence at the 5' end of the CURE site, and another DNA binding module interacts with the adjacent major groove in the GCTCA sequence (Jamison McDaniels et al. 1999).

Mac1 has several structural features that are consistent with its role as a regulatory protein. Its amino terminal has ~50% similarity to Ace1 and Amt1 (Jungmann et al. 1993). This domain is thought to bind to the minor groove of DNA and to bind Zn(II) (Jensen et al. 1998). The carboxyl terminal of Mac1 has two clusters of Cys-His repeats arranged as Cys-X-Cys-X_4-Cys-X-Cys-X_2-Cys-X_2-His (Fig. 3A). These two Cys-rich motifs bind a total of 8 Cu(I) ions (Jensen and Winge 1998). The first cluster appears to play a role in Cu sensing and the second cluster may be involved in transcriptional activation. Cu ions stimulate an intramolecular interaction between the amino terminal DNA binding domain and the cysteine-rich carboxyl terminus of Mac1, resulting in a Cu-dependent stabilization of Mac1 (Jensen and Winge 1998). Such interaction is believed to mask the activation domain of Mac1 resulting in repression of transcription of the Cu transport genes under high Cu conditions. Yeast strains that harbor the dominant allele $MAC1^{up1}$, are hypersensitive to Cu and exhibit high mRNA levels of Cu transport genes where expression is no longer regulated by Cu (Jensen et al. 1998; Hassett and Kosman 1995). The mechanisms of Cu incorporation to Mac1 are not well characterized. Given that intracellular trafficking of Cu to all known targets is mediated by Cu chaperones and the negligible amount of free Cu-ions in cells, there may be a shuttle that transfers Cu to Mac1 when Cu is in excess.

Mac1 has additional modes of regulation. As the intracellular Cu concentration approaches toxic levels (10 μM), Mac1 undergoes proteolytic degradation (Zhu et

al. 1998). This mode of regulation is specific for Cu and does not require the synthesis of new proteins. Conformational changes of Mac1 induced by Cu-binding may expose protease-binding domains. Alternatively, Cu may activate a specific protease that leads to degradation of Mac1. Another level of Mac1 regulation is phosphorylation. Unphosphorylated Mac1 is unable to bind to the Ctr1 promoter (Heredia et al. 2001). The kinase responsible for the phosphorylation of Mac1 is yet to be identified.

Microarray studies have identified two new Mac1-regulated genes in addition to other well-characterized targets (Gross et al. 2000). YFR055W shares homology with a family of trans-sulfuration enzymes involved in cysteine biosynthesis. The other target is YJL217W, which does not share significant sequence similarity to any known proteins. The functions of these genes in Cu metabolism are unclear. Mac1-deleted yeast cells exhibit alterations in expression of a number of genes (De Freitas et al. 2004). Since Cu is a critical cofactor for Fe transport, the responses in Mac1-defective yeast partially reflect Fe-deficiency. Defects in mitochondrial oxidative phosphorylation resulting from reduced activities of mitochondrial Cu-containing cytochrome c oxidase may lead to metabolic reorganization. Genes involved in the metabolism of amino acids, carbohydrates, and lipids are differentially expressed in the Mac1-deficient cells. Other genes that play roles in cell cycle regulation and stress response are also differentially expressed in Mac1-defective yeast. These observations further attest the critical roles of Cu in cellular metabolism.

5.3 Cuf1

The fission yeast *S. pombe* has a Cu-regulated transcription factor Cuf1 that carries homology with Ace1/Atm1 and Mac1 (Labbé et al. 1999). The Cuf1 amino-terminal is more similar to that found in Ace1 and Atm1 than Mac1. The Cys-rich domain near its carboxyl-terminal shares similarity to the domain of Mac1 that is known to play a critical role in Cu ion sensing. Cuf1 activates expression of Cu transporter genes under Cu starvation conditions by binding to a Cu-responsive *cis*-acting element of the Ctr4 Cu transporter (Beaudoin and Labbé 2001). Consistent to sequence similarity of the DNA binding domain between Cuf1 and Ace1, the Cuf1 recognition region bears strong sequence similarity to that of Ace1. A chimeric Cuf1 protein bearing the amino-terminal 63-residue segment of Ace1 functions like Cuf1 (Beaudoin et al. 2003).

Interestingly, Cuf1 activated by Cu starvation represses expression of genes encoding components of the Fe transport machinery (Labbé et al. 1999). This result suggests that, in the absence of sufficient levels of Cu cofactor, *S. pombe* prevents futile synthesis of Fe transporters. It appears that Fet3 in *S. cerevisiae*, which contains Cu as a cofactor is also regulated in a Mac1 activity-dependent manner, but the pattern of regulation is distinct from that of Cuf1. Cu supplementation in wild type cells that leads to Mac1 inactivation induces Fet3 expression, and a hyperactive allele of Mac1 represses Fet3 expression. It would be interesting to test

whether there is a regulatory mechanism of Cu metabolism under Fe starvation to supply sufficient Cu required for Fe metabolism.

5.4 Post-translational regulation of Cu transporters

Cu also triggers the degradation of the *S. cerevisiae* Ctr1 high affinity Cu transporter. Ctr1 is rapidly and specifically degraded at the plasma membrane in the presence of excess extracellular Cu (Ooi et al. 1996). This mechanism of Cu transporter regulation is conserved in its mammalian counterpart (Petris et al. 2003). Regulation of Cu transporters is likely a critical mechanism for preventing excess accumulation of Cu. Studies using mutant yeast strains that have defects in the endocytic pathway, and vacuolar degradation suggest that this process in yeast does not require internalization of Ctr1 or its delivery to the vacuole for proteolytic degradation. A cytosolic metal-binding motif (CX_5CXCX_2H) of Ctr1 appears to play an important role in Ctr1 degradation. Interestingly, the Mac1 transcription factor is also required for Cu-dependent Ctr1 degradation (Yonkovich et al. 2002). Ctr1 is much more stable in the Mac1-deleted cells. It is interesting that a transcription factor controls its target at both transcriptional and post-transcriptional levels. The exact mechanisms and implications of Mac1 in Ctr1 turnover have not been resolved. A simple explanation of this observation is that one or multiple Mac1 target gene(s) play a role in Cu-dependent Ctr1 degradation. Whereas the Ctr3 high affinity Cu transporter has the same functions and similar structural features to those of Ctr1, post-transcriptional regulation of these proteins is distinct. Unlike Ctr1, the Ctr3 transporter is neither regulated at the level of protein degradation nor endocytosis as a function of elevated Cu levels (Peña et al. 2000).

5.5 Regulation of Cu metabolism by stress and other nutritional factors

The Cu transport machinery is not only regulated by extracellular Cu levels but also by growth conditions such as pH and nutrient limitations. First, many other genes relevant to Cu and Fe metabolism such as Fre1, Ctr1, Lys7, and Ccc2 are induced when cultured in an alkaline media (Serrano et al. 2002, 2004). Consistently, yeast cells with deletions in any of these genes are sensitive to alkaline pH, and overexpression of the Ctr1 or Fet4 Cu transporters confers resistance to alkaline pH (Serrano et al. 2004). These results suggest that Cu and Cu-requiring physiological processes are important for yeast growth under such conditions. A possible explanation of this regulation is that Cu uptake is slow under alkaline conditions. Second, it has been shown that Ctr3 is upregulated by carbon-limitations, and Ctr1 and Ctr3 are downregulated under sulfur limitations (Boer et al. 2003). Carbon limitation may force yeast cells to generate energy through mitochondrial oxidative phosphorylation in which Cu and Fe play an essential role in the electron transfer. This may explain why cells induce Cu transport. Downregu-

lation of Ctr1 and Ctr3 in yeast suffering sulfur limitation could be important for preventing Cu toxicity. The underlying mechanisms and biological significance of these regulations remain to be studied.

6 Conclusions

The power of techniques of genetics, molecular biology and chemistry has led to discovery of delicate and fascinating mechanisms of Cu homeostasis. Interestingly, most of the molecular mechanisms of Cu homeostasis are well conserved between yeast and human. Further progress in understanding Cu metabolism will lead to the determination of the roles for Cu homeostasis in biochemical processes and prevention and treatment of Cu-related diseases in human. Advances in understanding Cu metabolism will also lead in to further studies on other physiologically important metal ions.

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Abbreviations

ATP: adenosine triphosphate
COX: cytochrome c oxidase
FAD: flavin adenine dinucleotide
IM: inner membrane
IMS: intermembrane space
kD: kilodalton
OM: outer membrane
MT: metallothionine
MNK: Menkes disease protein
NMR: nuclear magnetic resonance
NADPH: nicotinamide adenine dinucleotide phosphate
Cu,Zn SOD: Cu, Zn Superoxide Dismutase
SOD1: Cu, Zn Superoxide Dismutase
WND: Wilson's disease protein

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