

Molecular mechanisms of copper uptake and distribution

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In the past few years, exciting advances have been made toward understanding how copper is transported into and distributed to cupro-proteins within cells. Recent work has identified high-affinity copper transporters at the plasma membrane in a number of organisms. The elucidation of the three-dimensional structure of copper chaperones and target cupro-proteins has shown that highly specific interactions between homologous domains foster copper transfer between conserved copper ligands, and facilitate a detailed understanding of vectorial copper-transfer reactions. Furthermore, the recent generation of mouse-knockout models, deficient in a high-affinity copper transporter, or in copper chaperones, has demonstrated the importance of copper uptake and targeted distribution in both predicted and fascinating unanticipated ways in growth and development.

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Abbreviations

CCS	copper chaperone for SOD
COX	cytochrome oxidase
Ct	carboxy-terminal
Ctr	copper transporter
FALS	familial amyotrophic lateral sclerosis
IMS	intermembrane space
MBD	metal-binding domain
Nt	amino terminal
SOD	superoxide dismutase

Introduction

Copper is a transition metal able to cycle between two redox states, oxidized Cu(II) and reduced Cu(I). Virtually all organisms require copper as a catalytic cofactor for biological processes such as respiration, iron transport, oxidative stress protection, peptide hormone production, pigmentation, blood clotting and normal cell growth and development. However, copper also participates in redox reactions that generate the hydroxyl radical, which causes catastrophic damage to lipids, proteins and DNA [1]. Copper imbalances in humans lead to serious diseases such as Menkes syndrome or Wilson disease, characterized by the inability to appropriately distribute copper to all cells and tissues [2,3]. Additionally, copper has been strongly implicated in neurodegenerative diseases such as familial amyotrophic lateral sclerosis (FALS), Alzheimer's disease, and prion diseases of neuronal spongiform encephalopathy [4,5] (see also review by Lehmann, this issue).

Genetic and biochemical studies, largely conducted with microbial model systems including bacteria and yeast, have firmly established that cells have developed dedicated components and sophisticated homeostatic mechanisms to acquire and maintain adequate intracellular copper concentrations. Indeed, the strong conservation of these copper-balancing proteins and their function in mammals have been recently confirmed through studies involving targeted gene inactivation in the mouse genome. In this review, we focus on the most recent advances in copper transport and intracellular distribution to specific targets, processes that, in their fundamental features, are conserved from microbes to humans.

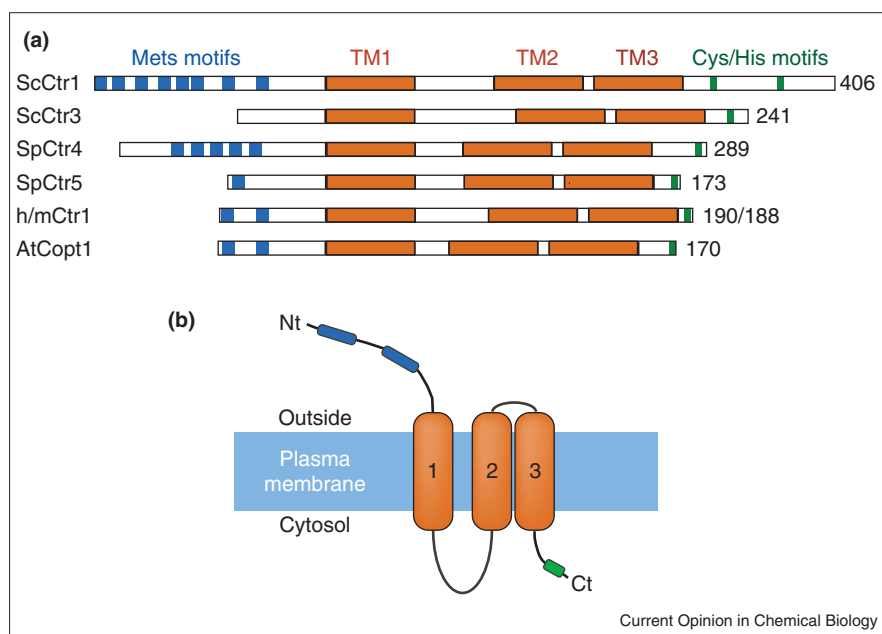
Copper uptake

High-affinity copper transport proteins

Physiological and biochemical studies many years ago suggested the presence of at least two independent activities responsible for copper uptake in eukaryotic cells, of distinct affinities and capacities for copper [6]. The identity and mechanism of action of proteins that transport copper into cells was largely unknown until recently, although a number of distinct candidates were postulated. Indeed, ceruloplasmin, a serum glycoprotein that harbors over 95% of the plasma copper and serves as an important ferroxidase in iron distribution, was long speculated as an important part of the copper-uptake machinery. However, recent definitive studies of a mouse ceruloplasmin gene knockout have firmly established that aceruloplasminemic mice have no defect in intestinal copper absorption, uptake in the liver or biliary excretion, though these mice do exhibit expected defects in iron mobilization [7,8].

The use of genetic studies in yeast, the complementation of yeast copper-uptake mutants with mammalian cDNAs, and protein database searches have recently resulted in the identification of proteins from several species that serve as high-affinity ($K_M \sim 1\text{--}5 \mu\text{M}$) copper transporters of the Ctr family [9–15]. An alignment of known Ctr1 family members from yeast, plants and mammals highlights domains that are structurally conserved, and probably functionally important, during the evolution of these proteins (Figure 1a). All Ctr family members contain three putative transmembrane regions, within which most of the amino acid similarity lies in a largely hydrophobic stretch (Figure 1a, TM1–3 shown in orange). The amino-terminal (Nt) region is rich in methionines that are arranged as MxxM and MxM motifs (Figure 1a, Mets motifs shown in blue). On the basis of homology with motifs present in bacterial copper homeostasis proteins [16,17], it has been proposed that the Ctr1 Mets motifs could play a role in copper binding [9]. Finally, the carboxy-terminal (Ct) portion of Ctr1 proteins is rich in charged amino acids and contains quite well conserved cysteines and histidines at or

Figure 1



Model for the structure of Ctr family high-affinity copper transport proteins. **(a)** Copper transport proteins from *S. cerevisiae* (ScCtr1, ScCtr3), *S. pombe* (SpCtr4, SpCtr5), human and mouse (h/mCtr1) and *A. thaliana* (AtCopt1) were aligned. Conserved features are represented from Nt (left) to Ct (right). Most proteins contain: three transmembrane domains (TM1–3, shown in orange); methionine-rich motifs or Mets motifs (blue boxes) consisting of three to five methionines arranged as MxxM and/or MxM; and Cys/His motifs (green boxes) composed of three amino acids with two cysteines or histidines. The length of each protein in amino acids is shown on the right. **(b)** Proposed topological structure of the Ctr family of high-affinity copper transporters. Mets motifs (Nt) would be located outside the cell, whereas Cys/His motifs (Ct) would be cytosolic.

close to the carboxyl terminus (Figure 1a, Cys/His motifs shown in green). The proposed topological structure of Ctr family members, on the basis of computer algorithm analyses, would locate Mets motifs outside the cell where they may function in copper capture, and Cys/His motifs in the cytosol (Figure 1b). Although experimental evidence supporting this topology is currently lacking, this does provide a working model for probing structure–function relationships in Ctr copper transporters.

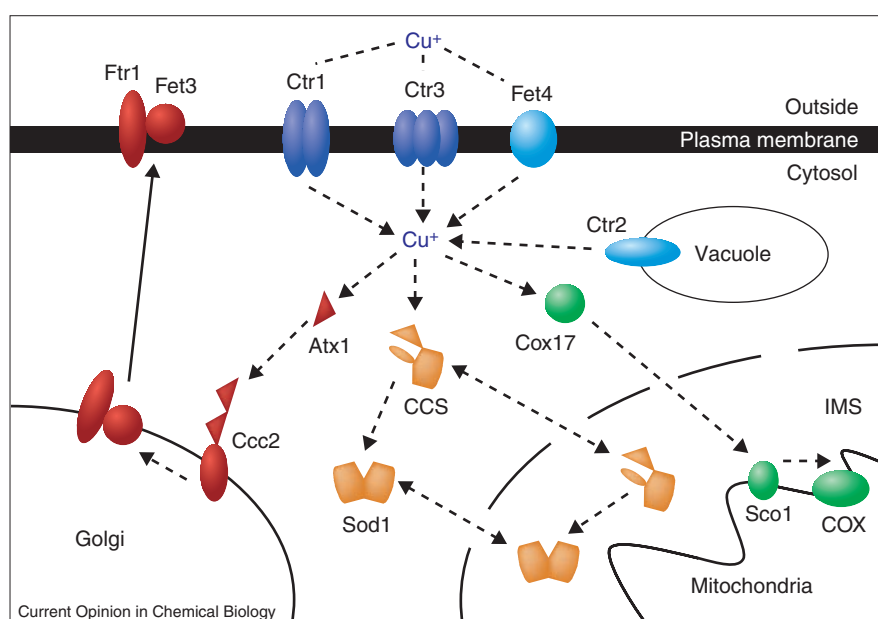
What is the subunit composition of the yeast Ctr family members? While the budding yeast *Saccharomyces cerevisiae* contains two independent, functionally redundant high-affinity copper transporters, Ctr1 [9] and Ctr3 [10], it is curious that each protein is quite distinct in primary sequence [18]. Ctr1 displays low similarity to Ctr3 at its carboxyl terminus and the cytosolic tail (Figure 1a). On the other hand, Ctr3 is devoid of Mets motifs in the amino terminus. Just how these two proteins transport copper into yeast cells is likely to depend on those residues in transmembrane domains, regions likely to form copper channels, that are conserved. Although protein cross-linking and co-immunoprecipitation studies suggest that both Ctr1 and Ctr3 from *S. cerevisiae* are homo-multimeric [19,20], the fact that these cells can dispense with one or the other protein and still carry out high-affinity copper uptake eliminates requisite hetero-oligomerization. In contrast, however, high-affinity copper uptake in the fission yeast *Schizosaccharomyces pombe* is carried out by a heteromeric complex formed by at least two proteins, Ctr4 [11] and Ctr5 [12], that are both structurally related to each other and to the Ctr family of copper transporters. Both proteins physically associate *in vivo*, are interdependent for

secretion to the plasma membrane and for high-affinity copper transport, and are co-regulated at the level of gene transcription by the copper-sensing transcription factor Cuf1 [11,12].

Recent genetic and bioinformatic approaches have led to the identification of high-affinity copper transporters in mice and humans that are members of the Ctr1 family [13,14] (Figure 1a). While both mouse and human *Ctr1* expression can rescue phenotypes in yeast associated with defects in copper transport (mitochondrial respiration defects caused by cytochrome oxidase (COX) deficiency, oxidative stress sensitivity due to Cu,Zn superoxide dismutase (SOD) defects and inability to transport iron as a result of defects in the Fet3 multi-copper ferroxidase) [13,14], transfection studies demonstrated that both mouse and human Ctr1 proteins are able to stimulate copper uptake and accumulation in mammalian cells [21,22^{••}]. Recently, the physiological role played by Ctr1 in mammals has been investigated by creating a targeted deletion of the mouse *Ctr1* gene [22^{••},23^{••}]. These studies demonstrated that Ctr1 is essential for embryonic development, because mice with a homozygous deletion of *Ctr1* die approximately mid-way through gestation [22^{••},23^{••}]. Although the critical targets for Ctr1-mediated copper delivery during development are currently unknown, future studies of mice in which *Ctr1* has been deleted or silenced in specific tissues, or at specific times during development, should be informative. It is interesting to note that in both mice and humans, *Ctr1* is strongly expressed in the liver and kidney, important organs for copper metabolism and reabsorption, respectively, but poorly expressed in brain in spite of the importance of

Figure 2

Copper transport and distribution in baker's yeast. Depending on extracellular copper concentrations, copper is transported inside the cell by two high-affinity copper transporters Ctr1 and Ctr3, or by a low-affinity Cu/Fe-transporter Fet4. Ctr2 could be involved in copper efflux from the vacuole. Copper is distributed by an unknown mechanism to three different metallochaperones: Atx1, CCS and Cox17. Delivery of copper to the secretory compartment (pathway shown in red) involves the metallochaperone Atx1 that shuttles copper to Ccc2, a P-type ATPase located in the trans-Golgi network, and is responsible for copper translocation. Once copper is in the lumen of the secretory pathway, it is loaded onto Fet3, a multicopper-ferroxidase essential for high-affinity iron uptake that partners with the Ftr1 subunit. The delivery of copper to Sod1 is mediated by the copper chaperone for SOD, CCS (pathway shown in orange). This protein contains three different domains: an Nt domain I similar to Atx1, a central domain II with homology to Sod1 and an essential Ct domain III. CCS directly interacts with Sod1 through the central domain and contacts from domain III to form a



heterodimer. The pathway for delivery of copper to the mitochondria is shown in green. The metallochaperone Cox17 delivers copper

to Sco1, and other proteins, located in the inner mitochondrial membrane. Then copper is transferred to specific subunits of COX.

copper in neonatal brain development [13,14,24]. Consistent with this observation, mouse *Ctr1* heterozygotes display a 50% reduction in total brain copper levels, and reductions in Cu,Zn SOD activity as compared with in wild-type mice, but no such defects in liver [22^{••},23^{••}]. Perhaps liver tissue utilizes or recycles copper efficiently, or there are alternative copper transporters that are more active in some tissues than in others. The identification of high-affinity copper transporters from microbes and mammals serves as a crucial launching point for understanding several important aspects of their mechanism of action, including the nature of the copper, or copper–ligand complex that is transported, the energetics of this process, the structure of the Ctr1 copper transporters and potential forms of regulation.

Low-affinity copper transport proteins

The existence of a low-affinity copper transport activity in budding yeast was first suggested based on the ability to suppress growth phenotypes associated with an *S. cerevisiae* *ctr1ctr3* strain by increasing the concentration of copper in the growth medium [9,10]. Several proteins have been implicated in low-affinity copper uptake in baker's yeast, including Fet4 and Ctr2, for which the most compelling evidence exists. Fet4 was first described as a low-affinity iron transporter with no significant homology to the Ctr family of high affinity copper transporters [25]. However, biochemical and genetic evidence suggests that Fet4 is able to transport both iron and copper with a K_M of $\sim 35 \mu M$ [26]. Recent data suggest that yeast Ctr2, which bears homology to the Ctr family of high-affinity copper transporters [15], may be localized in the vacuolar membrane,

and may play a role in the mobilization of intracellular pools of copper [27]. Copper transported by both Fet4 and Ctr2 is normally distributed to the three known delivery pathways in yeast cells [26,27] (Figure 2, see below). Curiously, a human protein encoded by a gene adjacent to *hCtr1*, *hCtr2*, has also been described [13]. Like yeast Ctr2, *hCtr2* is unable to complement the respiratory deficiency of a *ctr1ctr3* yeast mutant and *hCtr2* shows a tissue distribution of mRNA expression that is different from *hCtr1*, with high levels in placenta and, in contrast to *hCtr1*, reduced expression in liver [13,21]. The precise role for either yeast or mammalian Ctr2 in copper homeostasis is currently unknown, but will be of great interest.

Copper distribution

The essentiality and toxicity of copper requires the presence of precise mechanisms for acquisition that are intimately linked to controlled distribution. Over the past few years, genetic studies in yeast demonstrated the existence of three independent copper-delivery pathways: delivery to the secretory pathway, to Cu,Zn SOD, and to COX in mitochondria (Figure 2) [28–31]. More recently, biochemical and structural studies have shed light on the distribution pathway specificity, and the chemical mechanisms for copper shunting, while gene knockout experiments in mice have revealed fascinating aspects of the role of copper chaperones in mammalian physiology and development.

Delivery of copper to the secretory pathway

Many cupro-proteins, from yeast to humans, traverse the secretory pathway to reach their destination at the plasma membrane, or *en route* for secretion to the extracellular

environment. These include such proteins as blood clotting factors V and VIII, tyrosinase, lysyl oxidase and ceruloplasmin in mammals, or the Fet3 multicopper ferroxidase that is essential for high-affinity iron uptake in yeast [3,32]. Two types of proteins have been identified in the delivery of copper to the secretory pathway: the yeast Atx1 (or Atox1 in mammals) copper chaperone and the copper-transporting ATPase Ccc2 (or ATP7A, ATP7B in mammals) (Figure 2). Atx1/Atox1 is a small soluble protein with an overall $\beta\alpha\beta\beta\alpha\beta$ fold structure, which coordinates one atom of copper to the cysteines of a conserved MxCxxC motif (where x represents other amino acids) [33–36,37[•]]. The NMR solution structure of Atx1 suggests that both cysteines move from a buried to a solvent-exposed conformation after copper release [38]. In this pathway, Atx1/Atox1 delivers copper to the Ccc2/ATP7A/B copper-transporting P-type ATPases, multi-membrane-spanning proteins localized in the trans-Golgi network [39–41] (Figure 2). The Ccc2/ATP7A/B copper pumps also contain between two and six of the MxCxxC metal-binding domains (MBDs) at their amino termini, each with an overall tertiary structure similar to that of Atx1/Atox1, where each MBD binds a single copper atom *in vitro* [42–45]. The Atx1/Atox1 and Ccc2/ATP7A/B proteins directly interact, in a copper-stimulated manner [33,39,40]. According to structural predictions, the metal is transferred via a series of ligand-exchange reactions involving two- and three-coordinate intermediates between cysteine ligands in the CxxC motifs on Atx1/Atox1 and the recipient copper-transporting ATPases [33,36,37[•]]. A positively charged surface on Atx1/Atox1 is thought to engage in ionic interactions with negatively charged residues on the MBDs of copper pumps, facilitating the appropriate alignment and specificity of the interaction [36]. Once transferred, copper is then translocated into the lumen of the Golgi, with ATP consumption, in an as-yet incompletely defined mechanism [46]. Once inside the lumen of the secretory compartment, it is not known how copper is loaded onto proteins, or whether there is a luminal copper chaperone required for this process, but these are important questions to address.

The mammalian ATP7A and ATP7B proteins, defective in humans with Menkes syndrome and Wilson disease, respectively, are known to undergo copper-responsive changes in intracellular trafficking (reviewed in [47]). Under low copper conditions, both proteins are predominantly localized in the trans-Golgi network where they deliver copper to secreted cupro-proteins [48–50]. In the presence of elevated copper levels, ATP7A, which is expressed in extra-hepatic tissues, re-distributes to the plasma membrane, where it is thought to efflux copper to the extracellular milieu [51]. ATP7A contains a di-leucine motif near the carboxyl terminus, which mediates its endocytosis back to the Golgi in a reversible process independent of protein synthesis [52–54]. ATP7B, expressed largely in the liver and in neuronal tissue, undergoes copper-induced trafficking from the trans-Golgi network to

intracellular vesicles, where it is likely to function in copper excretion from the liver to the bile [55,56]. Both human ATP7A and ATP7B have six MBDs within the amino-terminal region. However, mutational analysis suggests that these MBDs are not functionally equivalent. MBDs closer to the channel, presumably formed by the transmembrane domains, are more important than Nt MBDs in terms of copper transport function and copper-induced trafficking [57–61]. However, recent results suggest that MBDs are high-affinity copper-binding sites only required because of the low bioavailable copper concentrations in the cell, but dispensable for the copper translocating activity of the ATPase, as shown by the transient and copper-induced acyl-phosphorylation of wild type ATP7A, or forms lacking all MBDs [62]. In *Enterococcus hirae* [17], *Escherichia coli* [63], and *Candida albicans* [64,65], a P-type ATPase similar to Ccc2/ATP7A/B, mediates copper efflux, thereby increasing resistance to elevated copper concentrations.

The generation of a mammalian model for Atox1 deficiency [66^{••}] has given rise to exciting and important insight into the function of this metallochaperone in mammalian copper-trafficking. Atox1^{-/-} mouse pups displayed pleiotropic phenotypes similar to copper-deprived animals, suggesting a crucial role for Atox1 in perinatal copper homeostasis. Phenotypes observed include growth retardation, increased perinatal mortality, hemorrhaging, hypothermia, skin laxity, hypopigmentation and decreased placental copper transport [66^{••}]. Furthermore, Atox1^{-/-} pups showed lower copper levels in liver and brain, and decreased activity of the cupro-enzymes COX and tyrosinase [66^{••}]. Atox1-deficient cells exhibited impaired copper efflux, similarly to Menkes-deficient cells. This suggests that Atox1 might also interact with the MBDs of the ATP7A/B at their sites of efflux, in addition to copper transport into the lumen of the trans-Golgi network. Interestingly, Atox1^{-/-} progeny from Atox1^{-/-} mothers showed a maternal effect consisting of exacerbated phenotypes, and occasionally, congenital eye developmental defects [66^{••}]. Atox1/Atx1 may also play additional roles in cells, such as protection against oxidative damage, as shown in yeast [34,35,67] and in rat neurons [68].

The discovery of the requirement for copper as a co-factor for the *Arabidopsis thaliana* ethylene receptor ETR1 [69[•],70[•]], involved in growth and developmental processes such as germination, cell elongation, sex determination, fruit ripening, pathogenic response or flower and leaf senescence, has recently focused special interest on copper homeostasis in plants. An Atx1/Ccc2-like copper-trafficking pathway composed of CCH (Atx1/Atox1 homologue) [71] and RAN1 (Ccc2/ATP7A/B homologue) [69[•],72] is responsible for copper delivery to ETR1 in the secretory compartment (reviewed in [73]). Transcription of both the CCH and RAN1 genes is induced during senescence and in response to copper scarcity [71,73]. Interestingly, CCH protein has also been localized in plant vascular tissues,

suggesting a potential role in intercellular transport of copper from decaying organs, or protection against oxidative stress that occurs during senescence [74]. CCH contains a unique Ct domain absent in other Atx1/Atox1 homologues identified to date, that could be responsible for protein–protein interactions mediating intercellular transport in plants [75].

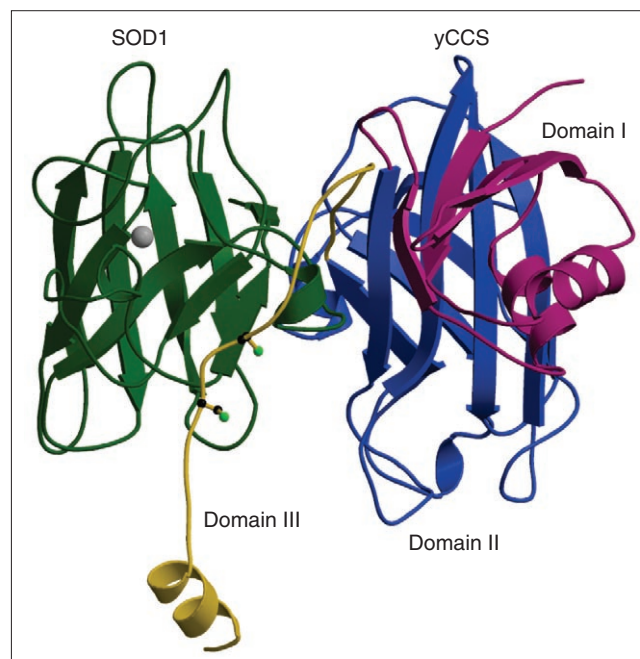
The Gram-positive bacterium *Enterococcus hirae* contains a copper chaperone, CopZ, with an overall tertiary structure and copper-binding features similar to Atx1 [76]. CopZ delivers two Cu⁺ ions to CopY, a copper-responsive DNA-binding repressor, inducing release from the *cop* operon promoter [77]. CopY release allows transcription of an operon encoding CopY, CopZ, and two P-type ATPases, CopA and CopB, involved in copper import and export, respectively [17]. CopZ is post-transcriptionally regulated by copper-induced proteolysis [78[•]], providing an additional level of cellular regulation by copper. Interestingly, recent surface plasmon resonance analysis indicated that CopZ directly interacts in a copper-induced manner with CopA, suggesting a direct transfer of the metal from the plasma membrane transporter to the metallochaperone [79[•]]. Curiously, the mechanism of copper acquisition by metallochaperones, or delivery of copper to the yeast copper-sensing transcription factors Mac1 and Ace1 is completely unknown.

Specific delivery of copper to Cu,Zn SOD

Cu,Zn SOD is a homodimeric enzyme, located in eukaryotes in the cytosol and the intermembrane space (IMS) of mitochondria [80[•],81[•]], which uses the redox properties of copper to catalyze the disproportionation of superoxide (O₂^{•-}) to hydrogen peroxide (H₂O₂) and oxygen, thereby protecting cells against oxidative damage. Recent genetic, biochemical and structural studies have shown that delivery of copper to this enzyme requires a specific copper chaperone for SOD, called CCS (Figure 2) [82,83,84^{••},85,86].

The CCS protein comprises three distinct functional regions, based on initial proteolytic studies, mutagenesis phenotypes and, ultimately, determination of its three-dimensional structure [86,87[•],88–90]. The Nt region of the protein (domain I) is similar in structure to that of the Atx1 metallochaperone, containing a MxCxxC copper-binding motif [88,90]. Despite the similarity between Atx1 and CCS domain I, they are not functionally interchangeable [87[•]] and, surprisingly, domain I is only required under extreme copper scarcity *in vivo* [87[•]]. Domain II is the central portion of the protein, with an overall structure similar to Sod1 [88–90], but lacking crucial residues for superoxide disproportionation [91]. Residues involved in Sod1 homodimerization are conserved in CCS, allowing CCS dimer formation [85,86,87[•],88]. This domain is not involved in copper-binding [86] but is essential for the interaction with Sod1 [83]. Domain III, is a small but essential Ct region [87[•]]. It contains a conserved CxC motif that is required for copper donation, but not for interaction with Sod1 [85].

Figure 3



Structure of the complex between yeast SOD1 and CCS. The SOD1 monomer is shown in green, CCS domain I in magenta, CCS domain II in blue and CCS domain III in gold. The two cysteine residues in CCS domain III are shown in ball-and-stick representation and the zinc ion in the Sod1 monomer is shown as a gray sphere. Reproduced from [93^{••}] with permission.

Recently, Rosenzweig and colleagues have used a mutated version (His48Phe) of yeast Sod1, which cannot accept copper from CCS [92], to trap the formation of the heterodimer complex between Sod1 and CCS. With this strategy, they have determined the three-dimensional structure for the yeast CCS–Sod1 heterodimer [93^{••}] (Figure 3). The structure shows an important interaction between domain II of CCS and Sod1, which is similar to the Cu,Zn SOD homodimer interaction, but with a wider surface of contact because of contributions from CCS domain III [93^{••}]. Furthermore, unanticipated overall changes between the relative position of the CCS domains have been observed by comparing the CCS, Sod1 and CCS–Sod1 structures [93^{••}]. Proteolysis studies have also suggested that copper induces conformational changes in CCS domain III [86,87[•]], and fluorescence anisotropy measurements suggest that copper stabilizes the CCS–Sod1 heterodimer [92]. Recent metal-binding studies suggest that CCS binds one atom of copper with ligands from each of domains I and III, but not II [86]. Formation of the heterodimer was only observed under aerobic conditions [92], suggesting that a potential disulfide bond between CCS and Sod1 could stabilize the complex. Such a bond was also present in the crystal structure between Sod1 and CCS domain III [93^{••}], and domain III, according to two-hybrid assays, is essential for the interaction [85]. Taken together, these results suggest a working model where the CxxC motif in CCS domain I would be

involved in binding and acquisition of copper from the donor [86], while the CxC motif in CCS domain III would translocate the metal from CCS domain I to the active site of the partner protein, Sod1 [86,92,93**].

Although the soluble nature of Cu,Zn SOD supported the initial notion that this is a cytosolic protein, evidence from the Fridovich laboratory in 1973 suggested that a fraction of Cu,Zn SOD is localized in the mitochondrial IMS [94]. More recent studies, from both the Culotta and Fridovich laboratories, have now clearly demonstrated the accumulation of a significant fraction (1–5%) of Cu,Zn SOD in the IMS in purified mitochondria from both yeast and rat liver, that is otherwise biochemically indistinguishable from cytosolic Cu,Zn SOD [80*,81*]. Yeast cells lacking CCS show reduced mitochondrial SOD levels, while over-expression of CCS, targeted to the IMS, increased IMS Sod1, suggesting that Sod1 metallation and physical interaction with CCS somehow control mitochondrial Cu,Zn SOD accumulation [80*]. According to these results, it has been proposed that apo-SOD would be able to cross the mitochondrial outer membrane but, after interaction and copper transfer from CCS, it would be stabilized within the IMS [80*,81*].

What is the function of this fraction of Cu,Zn SOD localized to the IMS of mitochondria? As noted by Fridovich and colleagues [81*], mitochondria are the major site of superoxide production in cells because of their extraordinary rate of oxygen consumption and the incomplete reduction of oxygen in the respiratory chain, resulting in the production of high levels of superoxide anion. Culotta and colleagues [80*] took advantage of the observations that yeast *sod1Δ* mutants exhibit a number of growth defects including the inability to synthesize lysine and methionine, as well as sensitivity to stationary-phase-induced cell death. Indeed, the localization of Cu,Zn SOD to the IMS using a mitochondrial targeting sequence complemented these phenotypes even more robustly than the wild-type Cu,Zn SOD protein. Taken together, these observations suggest that yeast and mammalian Cu,Zn SOD may represent a proximal line of defense against superoxide anions within the IMS. Because dominant mutations in several Cu,Zn SOD codons are known to cause FALS, a disease of motor neuron degeneration that results in fatal paralysis, it will be interesting to evaluate whether any of the FALS-associated mutations alter IMS targeting or function within the IMS. Furthermore, whether CCS plays a role in the mitochondrial IMS in addition to targeting of Cu,Zn SOD, or Cu,Zn SOD plays an oxidative stress protective role in other subcellular compartments where it has been reported, including the nucleus and microsomal fractions, remain to be determined. Recently, mice with a targeted deletion of the CCS gene were reported [95*]. Similarly to Cu,Zn SOD-deficient mice, CCS^{-/-} mice are viable, but show dramatic reductions in Cu,Zn SOD activity and a parallel sensitivity to the superoxide-generating drug paraquat and, interestingly, reduced female fertility [95*]. It will be

important to ascertain whether the loss of Cu,Zn SOD activity in general underlies this phenotype, or whether specific functions this enzyme or CCS play, for example, within the mitochondrial IMS, are responsible for this phenotype.

Delivery of copper to mitochondria

A series of seminal genetic studies by Tzagoloff and colleagues, describing the isolation and analysis of yeast mutants incapable of assembling the multisubunit complex COX, proved pivotal in breaking open the area of mitochondrial copper delivery [96]. COX is an integral membrane mitochondrial enzyme in which the Cox1 and Cox2 catalytic subunits that require copper as co-factor are expressed from the mitochondrial genome. As such, these subunits must be metallated within the mitochondria. One such COX assembly mutant, *cox17*, also exhibits a respiratory deficiency that can be specifically rescued by addition of copper to growth media containing non-fermentable carbon as the sole carbon source [96]. Subcellular fractionation experiments have demonstrated that Cox17 exists in both the yeast cytosol and the mitochondrial IMS, prompting a model in which copper-metallated cytosolic Cox17 would shuttle into the IMS to deliver copper to COX [97].

What are the data to support this model and how does Cox17 interact with both copper and COX? Both genetic studies and spectroscopic analyses support the idea that Cox17 binds three Cu(I) atoms, via at least three conserved cysteine residues, in a poly-copper cluster [98,99]. Whereas the Cox17 Cu(I) cluster has features consistent with trigonal coordination geometry much like that of the Cup1 metallothionein, the Cox17 Cu(I) cluster is distinct in at least some of the copper atoms being readily exchangeable with ligands in solution [99], a property one would predict for a copper chaperone. Furthermore, recent gel filtration and equilibrium sedimentation analysis strongly suggest that Cox17 exists in equilibrium as a dimer/tetramer with a K_d of approximately 20 μ M [99]. Indeed, calculations suggest that while cytosolic Cox17 levels approximate 0.4 μ M, IMS Cox17 concentrations could be as high as 60 μ M, supporting the potential for an oligomeric state *in vivo* [99]. Although the rationale for Cox17 existing as an oligomer is not yet known, it has been proposed that this may facilitate the ‘protection’ of bound Cu(I) as the protein traverses the pores of the mitochondrial outer membrane, or that the oligomeric state may stabilize interactions with other proteins (see below) *en route* to COX [99]. We suppose that oligomerization could also help temporarily retain Cox17, a rather small protein of ~8 kDa, in the IMS to enhance copper delivery to Cox subunits, or to facilitate its interactions with downstream target proteins.

What are these downstream target proteins? Once again, yeast genetic experiments provided initial evidence for the existence of other components in the pathway for delivery of copper to mitochondrial COX, and their order of function within this pathway. Supra-physiological levels of

expression of *SCO1*, a nuclear gene required for assembly of COX [100], were able to rescue the COX assembly and respiratory defects associated with a null *cox17* mutant [101]. However, overexpression of *Cox17* was not able to complement the respiratory defect of a *sco1* mutant, suggesting that *Sco1* acts downstream of *Cox17* [101]. Interestingly, *Sco2*, a *Sco1* structural homologue, is able to partially complement the respiratory deficiency of a *cox17* mutant in the presence of low levels of copper [101]. *Sco1* [102] and *Sco2* [101] are integral proteins of the mitochondrial inner membrane that each contain a copper-binding signature motif, CxxxC, that is also present, and comprise two of the copper ligands, in the COX2 subunit [103*]. Three key observations support a role in copper delivery to COX subunits by *Sco1*: first, deletion of the yeast *SCO1* gene [100], or mutagenesis of cysteine residues in the *Sco1* CxxxC element [104] also results in COX assembly and respiratory defects; second, a soluble *Sco1* fragment encompassing the CxxxC sequence binds a single Cu(I) atom with trigonal coordination geometry, with the third Cu(I) ligand is likely to be provided by a downstream histidine residue [103*]; and finally, affinity chromatography and co-immunoprecipitation experiments indicate that *Sco1* interacts with a *Cox2* subunit [105].

Two homologues of the *SCO* gene family, *hSco1* and *hSco2*, have been identified in humans [106]. Mutations in human *Sco1* and *Sco2* have recently been related to some diseases showing COX deficiencies. Heterozygous mutations in *hSCO1* cause neonatal ketoacidotic comas and isolated COX deficiency [107]. Additionally, humans heterozygous for the *hSCO2* gene suffer a fatal cardiomyopathy associated with a COX deficiency in smooth muscle [108].

Taken together, these observations suggest that *Cox17* would deliver copper to *Sco1* in the mitochondrial inner membrane, and then copper would be transferred to *Cox2* in the mitochondrial IMS (Figure 2). However, the precise biochemical mechanisms underlying this process are unknown. Other proteins could be also involved in this process, such as *Cox11* required for copper insertion to *Cox1* in *Rhodobacter sphaeroides* [109].

Conclusions

The essential yet toxic nature of copper in biology, strongly suggested the requirement for both specific copper transporters at the plasma membrane and for metallochaperones for copper delivery to subcellular targets. Studies thus far suggest the metal-delivery process is mediated by highly specific protein–protein interactions involving homologous domains between chaperone and target protein. The recent development of genetic models of deficiency or loss of copper uptake or distribution proteins in mice has allowed important glimpses into the role these proteins play at the cellular and at higher levels of biological organization. Additionally, identification of new copper-transport proteins and metallochaperones in different organisms strongly supports an evolutionarily conserved

mechanism of metal exchange. The precise chemical details of these copper-shunting mechanisms, from point of entry to functional targets, will evolve over the next few years from genetic, biochemical, structural and cell biology studies.

Update

The human copper transporter *hCtr1* has recently been characterized in more detail. Data show that *hCtr1* specifically stimulates Cu(I) uptake in human cells with a K_M in the low micromolar range. And, similarly to yeast high-affinity copper transporters, *hCtr1* forms a homo-multimer at the plasma membrane in mammalian cells [110].

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