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Review

Charting the travels of copper in eukaryotes from yeast to mammals ☆

Tracy Nevitt [1], Helena Öhrvik [1], Dennis J. Thiele *

Department of Pharmacology and Cancer Biology, Duke University Medical School, Durham, NC 27710, USA

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ABSTRACT

Throughout evolution, all organisms have harnessed the redox properties of copper (Cu) and iron (Fe) as a cofactor or structural determinant of proteins that perform critical functions in biology. At its most sobering stance to Earth's biome, Cu biochemistry allows photosynthetic organisms to harness solar energy and convert it into the organic energy that sustains the existence of all nonphotosynthetic life forms. The conversion of organic energy, in the form of nutrients that include carbohydrates, amino acids and fatty acids, is subsequently released during cellular respiration, itself a Cu-dependent process, and stored as ATP that is used to drive a myriad of critical biological processes such as enzyme-catalyzed biosynthetic processes, transport of cargo around cells and across membranes, and protein degradation. The life-supporting properties of Cu incur a significant challenge to cells that must not only exquisitely balance intracellular Cu concentrations, but also chaperone this redox-active metal from its point of cellular entry to its ultimate destination so as to avert the potential for inappropriate biochemical interactions or generation of damaging reactive oxidative species (ROS). In this review we chart the travels of Cu from the extracellular milieu of fungal and mammalian cells, its path within the cytosol as inferred by the proteins and ligands that escort and deliver Cu to intracellular organelles and protein targets, and its journey throughout the body of mammals. This article is part of a Special Issue entitled: Cell Biology of Metals.

1. Copper awareness at a cellular level: yeast as a model system

All organisms have evolved mechanisms to respond to extracellular stimuli that allow for the homeostatic acquisition of nutrients to ensure cellular function, proliferation or differentiation, for evasion or adaptation to environmental stresses that compromise viability or for defense against competing organisms that represent an obstacle to sustained survival. These response mechanisms rely on the precise integration of extracellular cues with signal transduction cascades that initiate regulated responses to the perceived stimulus. The strict requirement to maintain cellular Cu levels within the threshold of adequacy to meet metabolic demand, and below those that exceed the capacity of the cell to appropriately bind and store Cu to avert cytotoxicity, demands a sensitive awareness of the dynamic fluctuations in extracellular and intracellular Cu.

The model eukaryote, *Saccharomyces cerevisiae*, has proven an invaluable tool in understanding fundamental aspects of cell biology that are conserved from yeast to mammals (Fig. 1). In this respect, advances in the identification of proteins and ligands involved in yeast Cu metabolism have provided critical insights into the identification and characterization of orthologous players that similarly operate in mammalian cells, many of which, when mutated, lead to human disease. The striking functional conservation shared by yeast and mammalian Cu handling, Cu-dependent and Cu-storage proteins has permitted the characterization of mammalian orthologues and disease-associated mutations in the facile and genetically-tractable yeast *S. cerevisiae*, underscoring the continued importance of this model in advancing knowledge on Cu metabolism as it relates to human health and disease.

Awareness of cellular Cu status in the yeast *S. cerevisiae* is, in part, accomplished by the reciprocal activities of two nuclear Cu-binding transcription factors, Ace1 and Mac1 [1–4]. As cells undergo a shift from Cu-deficient (or Cu-adequate) to high extracellular Cu bioavailability (>1 μM), nuclear inactive, apo-Ace1 undergoes a conformational change elicited as a result of the formation of a tetra-copper-thiolate cluster within its amino-terminal DNA binding domain. Activated Ace1 is then competent to bind to a specific *cis*-element (TX_3GCTG) within the promoter of target genes that include *CUP1* and *CRS5*, encoding the Cu-binding cysteine-rich metallothioneins (MTs) critical for buffering intracellular Cu, as well as *SOD1* encoding Cu, Zn superoxide dismutase, that, in addition to its role in ROS detoxification, endows cells with enhanced ability to maintain a safe reservoir of intracellular ligand-bound Cu [5]. In addition to enhanced expression of Cu detoxification genes, *FET3* and *FTR1* encoding the ferroxidase and ferrous iron (Fe) permease, respectively, involved in elemental Fe uptake, are similarly indirectly induced by the Fe-responsive transcription factor Aft1,

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* Corresponding author.
E-mail address: Dennis.Thiele@duke.edu (D.J. Thiele).

[1] Co-first authors.

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suggesting an as yet poorly understood impaired cellular Fe homeostasis under high Cu conditions [6]. Given the role of Fe-dependent catalase in decomposing H_2O_2, one possibility is an enhanced requirement for this co-factor in mounting a response to increased oxidative burden to match ROS disproportionation to H_2O_2 catalyzed by Cu,Zn Sod1.

Cellular Cu satiety maintains Mac1 in an inactive state, potentially through the coordination of up to 8 Cu ions by 2 cysteine-rich domains within the transactivation domain at the carboxyl-terminus of the protein, promoting an intra-molecular interaction with the amino-terminal DNA-binding domain [7,8]. As a result of Cu binding to Mac1, DNA binding to Cu-responsive *cis*-elements (CuREs; TTTGC(T/G)C(A/G)) is mitigated, leading to diminished expression of target genes that include the high affinity Cu transporters *CTR1* and *CTR3*, as well as the cell surface ferroreductases encoded by *FRE1* and *FRE7* [9–12]. Deletion of Mac1 abrogates growth on non-fermentable carbon sources, due to the absolute requirement for Cu as a co-factor in the mitochondrial electron transport chain, impairs Sod1 activity and compromises growth under Fe deficiency due to the inability to metallate the Fet3 ferroxidase essential for high affinity reductive Fe^{2+} assimilation.

The strikingly antagonistic role of bound Cu on Ace1 and Mac1 activities, in addition to the estimated intracellular free [Cu^+] of approximately 10^{-18} M [13], suggests a complementary affinity of each transcription factor for Cu binding such that the range of activation of one shares little or no overlap with that of the other. This notion is supported by recent studies that determined the Cu binding constants for Ace1 and Mac1 using a FRET-based reporter system that exploits the intra-molecular conformational changes that ensue upon Cu binding to the cysteine-rich domains of the proteins [14]. The calculated dissociation constants revealed a higher affinity of Mac1 for Cu^+ (K_d of approximately 9.7×10^{-20} M) when compared to that of Ace1 (K_d of approximately 4.7×10^{-18} M) consistent not only with apo-Mac1 binding to the promoters of genes involved in high affinity Cu uptake under conditions of Cu deficiency but also at cellular Cu concentrations' orders of magnitude below the binding affinity of Ace1 for Cu^+.

The constitutive nuclear localization of the Cu-sensing transcriptional regulators Ace1 and Mac1 predicts the existence of a kinetically labile Cu pool within the nucleus yet, in contrast to the existing body of knowledge on the routing of Cu by cytosolic chaperones and transporters between the secretory compartment, vacuole and mitochondria, the nature or mechanism of Cu transport into the nucleus is not well understood. Although a reactive pool of Cu^+–GSH is known to exist in the nucleus [15], over-expression of the Crs5 metallothionein in this subcellular compartment does not compromise Mac1 Cu-responsive regulation [1], indicative of the inability of this labile nuclear Cu pool to promote Mac1 metallation. Furthermore, Cu delivery to the nucleus does not require *de novo* protein synthesis as evidenced by efficient Cu-responsive Ace1 and Mac1 activities in cycloheximide-treated cells [1]. Consistent with this observation and perhaps providing further insights into the mechanism of Cu accumulation into the nucleus, recent studies suggest a role of Sod1 in Mac1 activation under Cu deficiency [16]. Similar to previous observations in mammalian cells [17], these studies revealed that yeast Cu chaperone for Sod1 (Ccs1) and Sod1 are partially localized to the nucleus. Moreover, both Cu-binding proteins are required for robust activation of Mac1-dependent gene expression. Furthermore, the requirement for Ccs1 appears to be limited to its role in delivering Cu to Sod1 since the expression of *Caenorhabditis elegans* Sod1, that acquires Cu independently of Ccs1 (*CCS1* is not encoded in the genome of *C. elegans*), is fully capable of activating Mac1 in a *ccs1Δ* strain. Although the mechanism of Sod1 function in Mac1 activation is unclear, both Sod1 catalytic activity as well as its nuclear localization are required for this process which is unaffected by the levels of intracellular superoxide. It is possible that the disproportionation of superoxide into H_2O_2 by Sod1 generates a signal or is required as a reactant for Mac1 activation. It would be interesting to over-express catalase in the nucleus to evaluate the effects of enzymatic decomposition of H_2O_2 on Sod1-mediated Mac1 activation under Cu deficiency.

Early studies identified an orthologue of Ace1 in *Candida glabrata*, a phylogenetically close relative of the *Saccharomycetes* and an increasingly relevant human fungal pathogen. Amt1 (Activator of MT transcription 1) was originally isolated as a genomic DNA fragment that conferred robust reporter activity and a Cu-resistance growth phenotype when episomally-expressed in a *S. cerevisiae ace1Δcup1Δ* strain harboring a genomic copy of the *C. glabrata* MT1 gene and an MT1 promoter-driven reporter. [18]. Sequencing of the *AMT1* gene revealed an encoded polypeptide with striking similarities to the amino terminus of Ace1 encompassing the Cu-activated DBD, including 11 cysteine residues the first 7 being exactly conserved between the 2 proteins, as well as a corresponding enrichment of acidic residues that comprise the Ace1 trans-activation domain at the carboxyl terminus. Deletion of *AMT1* abolishes the growth of *C. glabrata* under high exogenous Cu conditions due to the inability of cells to activate MT gene expression [19]. Despite the structural and functional similarities between Ace1 and Amt1, their regulation at the level of transcription is distinct, with Ace1 showing Cu-independent constitutive gene expression while Amt1 auto-activates its own transcription in response to elevated Cu, correlating with an accumulation in mRNA and protein levels required for resistance to Cu toxicity [20,21]. At present, it is not known whether *C. glabrata* encodes an orthologue for Mac1 but the high degree of phylogenetic and syntenic similarity between these two species would certainly support the notion that this may be the case. Indeed, perusal of the *C. glabrata* genome (Genolevures) for a Mac1 orthologue reveals an ORF (*CAGL0M07590g*) with similarity to and in gene synteny with *S. cerevisiae MAC1* (Gene Order Browser). Given the relevance of *C. glabrata* in human infectious disease as well as recent evidence for the use of Cu as a microbiocidal mechanism employed by mammalian immune cells [22], it would be exciting to begin to characterize Mac1 in this pathogenic fungus.

Despite *S. cerevisiae* serving as one of the most valuable model eukaryotes to date, the existence of two Cu-sensing transcriptional regulators that, together, sense extremes in Cu availability is not a widespread feature among fungal species. Of the phylogenetically more distant fungi to *S. cerevisiae* studied, most also encode a Cu-sensing transcription factor with functional similarity to Mac1 but appear to lack a distinct Ace1 counterpart. Curiously, both *Schizosaccharomyces pombe* and the opportunistic pathogen *Cryptococcus neoformans* seem to have evolved a single regulator with striking similarities to both Mac1 and Ace1. In the fission yeast, *S. pombe*, the nuclear transcription factor Cuf1 (Cu factor 1) activates the expression of genes involved in high affinity Cu uptake under Cu deficiency [23–25], akin to Mac1, as well as that of the vacuolar Cu exporter *CTR6*, an orthologue of *CTR2* in *S. cerevisiae* that is not regulated by Cu but rather by Fe availability [26]. The amino-terminal 63 amino acids of Cuf1 exhibit 51% identity to the corresponding region of *S. cerevisiae* Ace1 (and 45% identity to Amt1), a region that includes the Zn coordination domain responsible for DNA binding in Ace1 and Mac1, as well as extended homology to the Cu-binding domain responsible for Ace1 Cu-inducible DNA binding to target gene promoters. Heterologous Cuf1 expression in an *ace1Δ* strain can partially complement growth in high Cu but does not substitute for the activity of Mac1 in *S. cerevisiae* despite displaying high identity to the Cu-sensing domain of Mac1 in the carboxyl terminus, indicating a critical role in Cu uptake but not in its detoxification [24]. Deletion of *cuf1*$^+$ generates many of the phenotypes observed in the *mac1Δ* mutant, including impaired respiratory growth, diminished Sod1 activity and inability to grow under low Fe bioavailability. Interestingly, and in contrast to Mac1, under low Cu availability Cuf1 represses several genes encoding components of high affinity reductive Fe uptake, including *frp1*$^+$, which encodes a metalloreductase, and the *fip1*$^+$/*fio*$^+$

Fe^{2+} permease/oxidase-encoding genes that mobilize extracellular Fe via tandemly repeated *cis*-elements similar in sequence to the CuREs found in the promoters of *CTR1*, *CTR3*, *FRE1*, *FRE7* and *FET3 S. cerevisiae* genes. Given the critical role of Cu in high affinity Fe uptake as well as in Sod1-mediated ROS detoxification, it is perhaps not surprising that cells undergoing Cu starvation actively down-regulate Fe transport mechanisms that would be compromised in activity and would furthermore promote the influx of a redox active metal under conditions of compromised antioxidant function.

C. neoformans Cuf1, while sharing significant similarity to its *S. pombe* counterpart, both at the level of protein structure and its activity in orchestrating the cellular response to Cu deficiency, has the added functional attributes of *S. cerevisiae* Ace1 in mounting a cytoprotective response to Cu toxicity [27–29]. As expected, the *cuf1Δ* strain is unable to grow under Cu deficient conditions even in the presence of glucose unless supplemented with exogenous Cu, underscoring the preference of this fungal pathogen for respiratory over fermentative growth. This growth phenotype results from the inability of the *cuf1Δ* mutant to activate the expression of the high affinity Cu transporters *CTR4* and *CTR1*, that in addition to catering for the demand of Cu-dependent enzymes involved in mitochondrial respiration and ROS detoxification, supply this essential co-factor to the polyphenolic oxidase, laccase, involved in melanin synthesis and a critical virulence determinant in this organism [27,28]. Analogous to that observed in *S. cerevisiae* Mac1-deleted strains, *cuf1Δ* cells are hyper-sensitive to oxidative stress due to decreased Cu availability for incorporation into Sod1 as well as diminished activity of the Fe-dependent enzyme catalase, due to secondary Fe deficiency [29]. In contrast to *S. pombe* Cuf1, the *C. neoformans* orthologue is critically required for resistance to Cu toxicity and the *cuf1Δ* strain displays severe growth impairment under elevated exogenous Cu, likely due to failure to induce the expression of MT-encoding genes and perhaps other genes [28,29].

The existence of two dedicated regulators of Cu homeostasis in *S. cerevisiae* and close phylogenetic relatives, which are functionally replaced by a single regulator in more distantly-related fungal species, begs the question whether this results from neo-functionalization of an ancestral gene in species that underwent whole genome duplication (WGD) [30]. Alternatively, both genes may have been present in a common ancestor and have been subsequently lost or a chimeric protein generated through recombination events. Inspection of synteny at the *S. cerevisiae MAC1* and *ACE1* loci across several yeast genomes reveals orthologues of both genes in pre-WGD genomes [31], arguing for genetic loss in species harboring a single Cu-sensing regulator rather than neo-functionalization of a duplicated ancestral gene.

1.1. Enter copper

As an essential yet toxic trace element, cellular Cu acquisition, handling and storage must be rigorously governed. In the aerobic environment, Cu is predominantly present in its oxidized cupric form (Cu^{2+}). The dynamic bioavailability of Cu, as a function of extracellular pH, oxygenation, changes in water saturation or as a result of human-imposed interventions such as the use of copper-based fungicides, poses a challenge in articulating a graded response to cellular demand. This is in large part accomplished by the integration of Cu sensing mechanisms with the regulation of high affinity Cu transporters, intracellular buffering and sequestration mechanisms. The common theme, therefore, is that mechanisms of high affinity Cu acquisition are activated only under conditions where environmental levels of Cu availability fall below the binding affinity of low affinity metal transporters constitutively expressed at the cell surface. In *S. cerevisiae* growing under conditions of Cu adequacy, the activities of the Fe^{2+} transporter Fet4 and the Smf (Suppressor of mitochondrial function) family of divalent metal transporters orthologous to the NRAMP (Natural Resistance-Associated Macrophage Protein) family of mammalian metal permeases, fulfill the cellular demand for Fe, Cu, Mn and Zn [32,33] Liu, 1999). Smf transporters are similarly present in several yeast species including *C. glabrata*, *S. pombe* (Pdt1), *C. neoformans* and *C. albicans*. However, when bioavailable Cu concentrations fall below the K_m for transport by Smf transporters (approximately 25–50 μM Cu), the expression of cell surface metalloreductases, required to reduce Cu^{2+}, and high affinity Cu^{+} transporters (K_m for Cu approximately 1–5 μM) is activated by the Mac1 and Cuf1 transcription factors leading to the accumulation of Ctr (Cu transporter) and Fre (ferro-reductase) proteins at the plasma and vacuolar membranes, that together drive the concerted mobilization of this metal from environmental sources as well as intracellular stores. Copper uptake by the fungal Ctr proteins requires its prior reduction from Cu^{2+} to Cu^{+}. This is accomplished by cell surface metalloreductases encoded by the *FRE1* and *FRE7* genes in *S. cerevisiae* whose expression, under Cu deficiency, is regulated by Mac1 [9,10,34]. Analyses of genome-wide transcriptional responses to altered Cu availability in *S. pombe* and *C. neoformans* reveal conserved regulation of core Cu-responsive genes despite significant evolutionary divergence between these three yeast species [28,35].

The Ctr proteins are ubiquitous among all eukaryotes evaluated and typically harbor three transmembrane domains (TM), multiple potential Cu-binding ligands (methionine and histidine) along the extracellular amino terminus and at the extracellular boundary of TM2, as well as in the cytosolic carboxyl terminus. The Cu^{+} transport function requires Ctr multimerization that results in the formation of a Cu-permeable pore through which Cu^{+} is ferried in a series of putative exchange reactions between Cu-binding amino acid residues of TM2 that line the interface of the trimer pore [36,37]. Unlike Cu^{+} transport by the P-type ATPase Ccc2 discussed below, Ctr activity is not dependent on ATP hydrolysis, thus the mechanistic understanding of how Cu^{+} traverses through the Ctr pore remains to be fully deciphered. However, recent insights into the 3D structure of human Ctr1 revealed a homo-trimeric complex arranged in a tail-to-tail orientation in which the carboxy-termini face each other within the cytosol, generating a cone-like pore that is narrow at the extracellular face (approximately 8 Å across) and wide at the intracellular aqueous exit (approximately 22 Å across) [37]. Additionally, the position of the conserved M–X_3–M Cu-binding motif on TM2 along the pore interface of the symmetric Ctr1 trimer, relative to the regional intra- and inter-monomeric packing of the membrane-spanning domains, suggests that Cu^{+} exchange reactions between neighboring methionines induce conformational changes responsible for controlled conduction of the metal ion from the extracellular entry point to the HCH-motif near the carboxyl terminus of the protein. The higher stability of Cu^{+}–cysteine interactions when compared to those between Cu^{+}–methionine, would thus favor cation enrichment (in the form of a thermodynamic sink) at the wider aqueous intracellular exit, thereby obviating the energetic requirement for ATP hydrolysis to drive Cu transport.

Although the Ctr proteins exhibit functional and topological conservation from yeast to mammals, sequence homology along the protein length is unimpressive, particularly outside of the highly conserved methionine-rich (Mets) Cu-coordinating motifs. Notwithstanding, human Ctr1 can functionally complement for the loss of Ctr1 and Ctr3 in *S. cerevisiae* [38] and recent studies evidence an overall common membrane organization shared by the two most distantly-related orthologues, *S. cerevisiae* Ctr3 and human Ctr1. Differences in non-homologous regions between these proteins could be accommodated by an adaptor function of TM1 that allows evolutionarily distant Ctr proteins to adopt largely analogous higher-order protein structures [39]. Some eukaryotic cells express multiple Ctr proteins. In *S. cerevisiae*, Ctr1 and Ctr3 localize to the plasma membrane whereas Ctr2 is present on the vacuolar membrane and transports lumenal Cu stores into the cytosol. *S. pombe* also expresses two cell surface Ctr proteins, Ctr4 and Ctr5, but in contrast to that observed in *S. cerevisiae* and *C. neoformans*, functional Cu transporter

activity requires hetero-multimerization of Ctr4 and Ctr5 with a ratio of 2 Ctr4 molecules to 1 Ctr5 molecule [40]. The contribution of each protein to the function of the heterotrimer was recently addressed where it was found that a single Ctr4- or Ctr5-derived Mets-motif-containing amino-terminal domain is required for Cu transport activity [41]. Furthermore, Ctr4–Ctr5 assembly and trafficking to the plasma membrane is dependent on heterotrimer formation, since in the absence of either counterpart Ctr4 and Ctr5 fail to accumulate at the cell surface. Recently, a second *C. neoformans* cell surface Ctr protein, in addition to Ctr4, was identified, with high phylogenetic relation to *S. cerevisiae* Ctr1 [28]. Unlike the seemingly redundant functions of Ctr1 and Ctr3 in bakers yeast, deletion of *C. neoformans* Ctr1 leads to a dramatic elevation in *CTR4* mRNA expression, indicative of cellular Cu deficiency in the mutant, whereas an analogous effect on *C. neoformans CTR1* mRNA levels was not observed in the absence of *CTR4*. This would suggest that in *C. neoformans* Ctr1 plays a primary role in high affinity Cu transport under standard laboratory growth conditions and that Ctr4 may function predominantly under conditions of more severe Cu deficiency.

While much is known regarding the acquisition and intracellular distribution of Cu in mitotically-dividing cells, Cu metabolism during developmental processes such as meiosis has not been investigated in detail. It is widely accepted that conditions of nutrient starvation, notably that of nitrogen sources, induce meiotic differentiation among fungal species. In an elegant study by Beaudoin et al. [42], Cu deficiency was shown to induce *S. pombe* meiotic arrest and meiosis-specific elevation of *mfc1*$^+$ expression, encoding a novel Cu transporter belonging to the major facilitator superfamily. Mfc1 harbors 4 Mets motifs and is enriched for methionine and cysteine residues along the protein length. Strikingly, while *ctr4*$^+$ expression is induced in a Cuf1-dependent manner under meiosis, albeit at significantly lower levels when compared to its induction in cells undergoing mitotic cell division, *mfc1*$^+$ expression during mitosis is undetectable and its expression is Cuf1-independent during meiosis. Curiously, *zym1*$^+$, encoding a putative MT or cytosolic metallocarrier in *S. pombe*, and not induced under high Cu conditions but rather by elevated Zn or Cd concentrations, shows an almost 3-fold elevation in mRNA levels in Cu-deficient meiotic cells (Borrelly [42,43]. It would be interesting to evaluate whether this represents impaired Zn homeostasis in meiotically-dividing Cu deficient cells or a meiosis-specific Cu homeostasis activity. Functional studies in an *S. pombe ctr4Δctr5Δ* double mutant in which *mfc1*$^+$ expression was placed under the control of the *cuf1*$^+$ promoter demonstrate that Mfc1 rescues the respiratory growth defect of this mutant strain on non-fermentable carbon sources, with a predicted affinity for Cu in the very low micromolar range but below that of the Ctr4–Ctr5 transporter complex. During meiosis, Mfc1 localizes to the forespore membrane where it presumably delivers Cu to the forespore where it may be used as a cofactor for Cu-dependent enzymes critical for spore formation, maturation and release. Given the conservation between *S. pombe* mfc1 protein and proteins found in higher eukaryotes including mammals, the discovery of Mcf1 sets the stage for the identification of novel Cu transporters, and Cu-dependent proteins, that may operate in the germ line and in stem cell differentiation.

1.2. Intracellular copper relay

The recruitment of Cu into cells demands the concomitant implementation of active measures for relaying this redox-active ion to its target proteins or subcellular compartments in a controlled fashion. The accepted observation, based on several independent studies, that cells harbor virtually no free intracellular Cu underscores the existence of mechanisms for routing this essential metal while maintaining minimal cytotoxicity. Two cytosolic Cu chaperones, Ccs1 and Atx1 deliver Cu to Sod1 and the Ccc2 transporter localized to the membrane of the secretory compartment, respectively. While we know much about how Cu is loaded onto mitochondrial cytochrome oxidase, the mechanism by which Cu is routed from the site of import at the plasma membrane to the mitochondria is still largely unknown.

1.3. Ccs1–Sod1 copper relay

Ccs is widely distributed amongst eukaryotes where it is required for delivery and insertion of Cu into Sod1. Ccs proteins harbor 3 domains, two of which (domains II and III) are required for interaction with and activation of Sod1. Within domain II lies a region of striking homology to Sod1 that is responsible for Ccs1 docking onto Sod1. This is followed by insertion of the Cu cofactor from Ccs1 into the active site of Sod1 as well as the oxidation of an intramolecular disulfide in Sod1, both required for its activation and mediated by Ccs1 Domain III that contains a CXC Cu-binding motif. Comparatively less is understood about Domain I, which, in addition to 2 cysteine residues at positions 27 and 64, also contains a CX_2C motif shared by Atx1 and the Ccc2 P-type ATPase Cu transporter. However, mutation of this motif in *S. cerevisiae* does not appear to strongly impair Ccs1 activity *in vivo* and, furthermore, is completely absent in *S. pombe* Ccs. Given that Domain I is indispensible to Ccs function, it is thought that this region may be required for interaction with Cu-ligands upstream of Cu delivery to Sod1. Although there is a Ccs1-independent mechanism of Sod1 activation, and several species lack Ccs1 altogether, the Ccs1-Sod1 partnership in eukaryotes that express both proteins is underscored by their matched intracellular localization that has been detected in the cytosol, the mitochondrial IMS and in the nucleus. Ccs1 is imported into mitochondria via the Mia40/Erv1 disulfide relay system that promotes the transport of cysteine-rich proteins into the IMS. Although Ccs1 does not contain the twin CX_3C or CX_9C domains that are required for substrate intermediate disulfide-mediated interaction with Mia40, Domain I cysteine 64 interacts with Mia40 to generate the disulfide intermediate [44]. Subsequent disulfide bond transfer to Ccs1 occurs between cysteines 27 and 64 to form a stable IMS-localized Ccs1 protein that is competent in promoting apo-Sod1 retention to this subcellular compartment by introducing Cu and the disulfide bond into TOM-translocated apo-Sod1.

1.4. Copper and the mitochondria

The small, hydrophilic protein, Cox17, was originally identified from an *S. cerevisiae* cytochrome oxidase (CCO) assembly mutant that was unable to carry out respiratory growth despite expression of CCO components [45]. The observation that growth on non-fermentable carbon sources could be rescued by Cu supplementation indicated a role of Cox17 in delivering this metal to CCO or other target mitochondrial proteins. It is now known that Cox17 delivers Cu to Sco1 and Cox11, two Cu-binding IM-associated proteins required for the metallation and assembly of CCO. The localization of Cox17 both to the cytoplasm and mitochondrial IMS, mediated by Mia40 intermediate formation via twin CX_9C motifs on Cox17, initially suggested that Cox17 delivers Cu from the cytosol to the mitochondria. However, elegant experiments tethering Cox17 to the IM did not compromise CCO biogenesis [46], indicating that Cox17 is competent to access mitochondrial Cu stores for metallation of Sco1 and Cox11 and suggests that Cox17 does not ferry cytosolic Cu to the mitochondria. At present, it remains unclear how Cu is delivered from its site of entry across the plasma membrane to the mitochondria. It is possible that an as yet unidentified Cu protein/ligand or transporter mediates Cu shuttling to this organelle or perhaps, as an unexplored alternative possibility, inter-organellar fusion between the secretory compartment and mitochondrial tubules, that have been recently described [47], allows for the passage of Cu into the mitochondria. It would be interesting to evaluate mitochondrial Cu stores as well as respiratory activity in *S. cerevisiae* strains lacking either Atx1 or Ccc2, both required for mobilization of Cu into the secretory compartment. In

this experiment iron could be provided to CCO by siderophore supplementation that would bypass the Ccc2-dependent Fet3 mediated iron uptake pathway.

1.5. Atx1–Ccc2 and the secretory compartment

A recent review on transition metal homeostasis in eukaryotes made the striking observation that the vast majority of the thirty or so known Cu-binding proteins are predicted to be extracellular, underscoring the critical importance for Cu mobilization into the secretory compartment [48]. This notion was firmly established in *S. cerevisiae atx1Δ* strains that are compromised in high affinity Fe uptake due to the inability to metallate the multi-Cu ferroxidase Fet3 in the Golgi, leading to an inactive Fet3–Ftr1 Fe oxidase-permease complex at the cell surface. Atx1 is a small, cytosolic Cu chaperone with a ferredoxin-like fold and a Cu-coordinating motif within its first loop. This region shows high similarity to the amino terminal metal-binding domains (MBD) of the Cu-transporting P-type ATPase Ccc2, with which Atx1 transiently interacts to deliver Cu for translocation into the secretory compartment. Copper exchange between Atx1 and Ccc2 is dependent on the presence of at least one of the two amino-terminal Ccc2 MBDs that not only receives the cation for transport, but also mitigates intra-molecular inhibitory interactions between the amino-terminal domains and the active site of the ATPase that allow subsequent delivery of Cu to the transport site. Despite the *atx1Δ* mutant exhibiting a severe growth defect under Fe-limiting conditions, the *ccc2Δ* strain is completely abrogated in ferrous Fe uptake [49] evidencing the existence of an Atx1-independent mechanism of Cu delivery to Ccc2. Although the details of this mechanism remain elusive, it is likely not mediated by Sod1 or MTs, although the role of Ccs1 in this process has not been addressed. Instead, studies revealing the requirement for endocytosis as well as Ctr1 activity in Atx1-independent Ccc2 Cu delivery raise the possibility that internalization of Ctr1 into intracellular vesicles may contribute towards direct Ccc2 Cu delivery. Given the high degree of identity shared between the MBD of Atx1 that receives Ctr1-internalized Cu and that of Ccc2, it is possible that a similar mechanism of Cu relay to the Ccc2 MBD occurs upon endocytic relocalization of plasma membrane Ctr1 to intracellular vesicles. Alternatively, perhaps glutathione–Cu^{+} coordination complexes deliver Cu to Ccc2 in a manner similar to the CCS-independent pathway for Sod1 [50].

1.6. The intracellular exchangeable copper pool and its storage

The dynamic changes in the cellular demand for Cu pre-empt the existence of an exchangeable pool of intracellular Cu to allow for critical biological processes in the face of a dramatic decrease in extracellular Cu bioavailability. This reactive Cu pool is in part maintained by the ubiquitous and abundant cysteine containing tri-peptide, glutathione (GSH) that also plays a critical role in cellular ROS detoxification [51]. Upon entry of Cu into the reducing environment of the cell, Cu^{+} spontaneously reacts with the internal cysteine-SH of GSH and the Cu-GSH complex is thought to be available to mediate Cu transfer to Cu-binding proteins within the cytosol, the nucleus and the mitochondria, including the detoxifying MTs, Atx1 and Sod1 [52,53]. In addition to cytoprotection against Cu overload, yeast MTs, known to bind up to 8 Cu ions, have also been proposed to mediate Cu transfer through direct interaction with apo-metallo-chaperones, fulfilling both a role as a Cu sink when Cu concentrations reach above the levels of cellular demand as well as in its mobilization and delivery under conditions of Cu scarcity [52].

Yeast cells utilize the vacuole as a dedicated organelle that, in addition to playing a critical role in maintaining intracellular pH, also serves as a storage and sequestration compartment for amino acids, metals and other metabolites. Evidence supporting the role of the vacuole in Cu homeostasis arises from the observation that mutants defective in the biogenesis of this organelle display sensitivity to high levels of exogenous Cu [54–56]. Although the mechanism of Cu delivery to the vacuole is as yet unknown, mobilization of vacuolar Cu stores into the cytosol is mediated by Ctr proteins, such as Ctr2 in *S. cerevisiae* and Ctr6 in *S. pombe*, the activities of which make Cu available for Cu-chaperone-mediated intracellular distribution [23,26,57]. In *S. cerevisiae*, the Fre6 metalloreductase acts in concert with Ctr2 in vacuolar Cu mobilization [26], suggesting that vacuolar Cu is mobilized to the cytosol as Cu^{+}.

2. Copper in mammals: a fantastic voyage

The powerful genetics of yeast model systems has, to a large extent, led to the discovery of analogous mammalian genes and proteins that carry out Cu metabolism. Despite the high degree of structural and functional conservation of Cu metabolism proteins between yeast and mammals (Fig. 1), mammalian orthologues are primarily regulated via multiple post-transcriptional mechanisms, rather than at the level of metal-responsive gene expression. A major challenge faced by mammals in maintaining Cu homeostasis is the integration of Cu sensing and mechanisms for ensuring satiety beyond the level of the individual cell, within an organismal context comprised not only of multiple cell types with unique cellular demands for Cu, but also separated from each other by distance. This multi-dimensional regulation of Cu must efficiently and correctly escort, deliver, and store the metal to cater for its demand at the cellular, organ, and systemic levels. Furthermore, while Cu is essential for the entirety of the mammalian life span and is distributed throughout the body, specific developmental stages and organs exhibit distinct demands for Cu that reflect their precise physiological function or role in Cu storage. Indeed, while the liver contains the highest steady state levels of Cu, followed by the brain, heart and kidneys, muscle and bone display the lowest concentration of this metal. However, when taking into account the abundance of these two tissue types in relation to the liver, Cu levels in muscle and bone collectively account for approximately 50% of the total amount of Cu in the body whereas the liver contains a mere 10% [58]. Here, we will review current knowledge for how dietary Cu is acquired from the intestinal lumen, mobilized into the portal circulation to the liver and subsequently systemically distributed to the peripheral organs.

2.1. Copper import into cells and tissues

Mammalian Cu acquisition, be it at the apical surface of intestinal epithelial cells or by cells located in the periphery, is mediated in large part by the Ctr1 plasma membrane high affinity Cu importer, a structural and functional homologue of the yeast Ctr family of Cu importers. Consistent with its functional activity in yeast, mammalian Ctr1-mediated Cu import requires its prior reduction to Cu^{+} and, despite the lack of formal identification of the cell surface metalloreductase that fulfills this role, studies have demonstrated that members of the Steap family of metalloreductases (Steap2, Steap3 and Steap4) can function as cupric reductases in cultured cells, suggesting a physiological role in both Fe and Cu reduction [59]. Mice systemically deleted for Ctr1 exhibit severe growth and developmental defects and die *in utero* during mid-gestation, evidencing the essentiality of this Cu importer, and for Cu, in normal mammalian growth and development [60,61].

The regulation of Ctr1 activity occurs predominantly at the level of transporter localization and abundance. Ctr1 is present at the plasma membrane at times of cellular demand for Cu and on the membrane of intracellular vesicles as a result of Ctr1 endocytosis in response to elevated exogenous Cu [62,63]. Whether Ctr1 that is localized to these intracellular vesicles functions to mobilize lumenal Cu to the cytosol, akin to the role we suggested for Ctr1 in Atx1-indendent Cu delivery to Ccc2 in *S. cerevisiae*, is not currently

known. Moreover, elevated Cu levels have been shown in cultured cell models, and in mouse intestinal epithelium, to increase Ctr1 protein degradation [62,64].

An alternative mechanism for the uptake of Cu into mammalian cells has been proposed that involves the transport of Cu–chloride complexes by an anion exchanger [65]. However, confirmation of this observation awaits both the identification of the anion exchanger and demonstration that its genetic ablation in cells or animals results in physiological Cu deficiency *in vivo*. In addition to Ctr1, Lee et al. [66] reported that cells also possess another biochemically distinct functional Cu transport activity with a lower affinity that Ctr1 by approximately 10-fold. While this has yet to be identified, there is a possibility that the human counterpart to the recently identified Cu importer Mfc1 in *S. pombe*, is responsible for this activity. Furthermore, new findings demonstrate that the human zinc importer Zip4 (Zrt- and Irt-like protein 4) expressed in *Xenopus laevis* oocytes transports Cu across a wide concentration range, raising the exciting possibility for a role of Zip4 in mammalian Cu transport [67].

A second Ctr protein, structurally related to Ctr1, has also been identified in mammals and designated Copper transporter 2 (Ctr2). Ctr2 harbors three putative transmembrane domains, is thought to homo-multimerize and has conserved the Met–X_3–Met motif in the second transmembrane domain that is essential for Cu^+ transport by Ctr1. In contrast to the wealth of information regarding the structure, physiological function and mechanism of action of mammalian Ctr1, comparatively little is known about that of Ctr2. Studies thus far have suggested a role for Ctr2 in mammalian cells analogous to the yeast vacuolar Ctr2/Ctr6 proteins in mobilizing Cu from intracellular vesicles into the cytosol [68] or that Ctr2 functions as a low affinity Cu importer on the plasma membrane [69]. Recently studies have shown that Ctr2 mRNA and protein levels decrease in the liver and heart of Cu deficient rats, which, *a priori*, would be inconsistent with a role for Ctr2 as a Cu importer [70]. Furthermore, cells in which *CTR2* mRNA has been knocked down by RNAi exhibit an overall increased rate of macropinocytosis, suggesting that Ctr2 might also function as a regulator of macropinocytosis [71]. Notwithstanding, studies on the role of Ctr2 in mammalian Cu homeostasis have not been reported in animal models and it remains unclear whether Ctr1 and Ctr2 collaborate to regulate mammalian Cu acquisition. Definitive evidence regarding Ctr2 function *in vivo* currently awaits the generation of systemic or tissue-specific knockout mice.

2.2. Copper chaperones in the cytoplasm

The intracellular relay of internalized Cu ions to the Cu-dependent proteins and compartments for which it is destined follow the same theme as we have outlined for yeast, with the transfer of Cu to specific Cu chaperones and other ligands such as GSH or MT. CCS is similarly required for Cu delivery to Cu,Zn SOD1 but unlike yeast, cellular Cu status regulates CCS at a post translational level involving the 26S proteasome in mammals, resulting in high protein levels during Cu deficiency and low CCS levels during times of elevated intracellular Cu [72–74]. Although alterations in CCS expression do not affect SOD1 protein levels, the activity of the enzyme changes as a function of CCS (and thus Cu) levels [75,76]. Deletion of the CCS gene in mice is not lethal, but impacts female fertility and reduces SOD1 activity by 70–90% as well as protein levels in some reports, [75–77]. This indicates that while CCS is critically important for SOD1 activation, a CCS-independent pathway is in place to deliver Cu to SOD1. Fascinating studies in *C. elegans*, which lacks CCS, have demonstrated that this CCS-independent mechanism involves Cu–GSH complexes [15,78].

Analogous to the Atx1-Ccc2 Cu relay and transport into the secretory compartment, the orthologous mammalian chaperone Atox1 transfers Cu to the two Cu-transporting ATPases, Atp7a and Atp7b, themselves orthologues of Ccc2. In mice lacking Atox1, reduced placental Cu transport causes deficiency in both the brain and liver of newborn pups, paralleled by reduced activity of the Cu-dependent cytochrome oxidase and tyrosinase enzymes [79]. The *Atox1*$^{-/-}$ newborns also exhibit severely impaired growth and perinatal viability, underscoring the critical role of Atox1 in Atp7a/b-mediated Cu transport across the placenta and in systemic distribution. Interestingly, in addition to its Cu-chaperone role in the cytosol, it has been suggested that Atox1 also functions as a transcription factor, translocating into the nucleus under conditions of high Cu and stimulating the expression of the gene encoding extracellular SOD3 as well as other genes involved in cell proliferation such as cyclin D1 [80,81]. Follow-up studies will be important to decipher the mechanisms by which Atox1 regulation allows for these seemingly mutually exclusive functions in delivering Cu to Atp7a/b for transport into the secretory compartment or out of cells, and its accumulation in the nucleus for transcriptional activation of target genes, under high Cu conditions.

2.3. Mitochondrial copper escort

In mammals, the enzyme cytochrome c oxidase (CCO) (Complex IV in the mitochondrial respiratory chain) is composed of 13 subunits, ten of which are encoded by nuclear genes and three by mitochondrial genes. As described for yeast, Cu insertion into two of the mitochondrially-encoded subunits, Cox1 and Cox2, is mediated by the concerted action of several Cu-binding proteins, Cox17, Cox 11, Sco1 and Sco2. Mice lacking Cox17 show impaired CCO activity and die early *in utero* [82], demonstrating the critical importance of this mitochondrial Cu chaperone. Supporting the importance of the Cu binding subunits in CCO assembly, silencing of Cox17 in cell culture reduces the steady state levels of Cox1 and Cox2 proteins as well as CCO activity [83]. The two Sco proteins are responsible for receiving Cu from Cox17 and loading two Cu atoms into the Cu_A site in Cox2. Mutations in human Sco1 or Sco2 correlate with decreased tissue Cu levels, decreased CCO activity and cause neonatal hepatic failure and fatal hypertrophic cardiomyopathy [84–86]. In keeping with the multiple proposed roles for Atox1, both Sco1 and Sco2 have been demonstrated not only to be essential assembly factors for CCO, but also to have regulatory roles in Cu homeostasis, particularly in the regulation of cellular Cu export [87]. While these mitochondrial-associated proteins clearly play a critical role in the assembly of CCO and the insertion of Cu into specific subunits, it is currently unclear how Cu travels from the site of import at the plasma membrane via Ctr1 or other transporters, to the mitochondria and into the mitochondrial lumen.

2.4. Cellular copper detoxification and storage

As Cu is an essential component in life that is neither created nor destroyed, the ability to store excess Cu and to mobilize cellular Cu stores during times of need is likely to be critical to the survival of organisms. While as we discuss below, the liver is thought to be a major Cu storage organ in mammals, little attention has been paid to the precise biochemical mechanisms by which metazoans store and mobilize Cu, and the regulation of these processes. Perhaps we can glean some preliminary insights into these processes from understanding Cu detoxification pathways, as these Cu-sequestering mechanisms that function during times of Cu excess, may also have a physiological role in Cu storage.

Mammalian metallothioneins (MTs) are small cysteine-rich polypeptides that tightly bind up to 12 Cu^+ atoms through thiolate bonds such that the Cu atoms are largely shielded from solvent. MT expression is regulated at the transcription level by MTF1 (metal transcription factor 1), which in response to elevated Cu levels accumulates in the nucleus and binds the metal responsive elements (MREs) in the promoter regions of its target genes [88]. MTs have

been extensively studied for over half a century, primarily from a biochemical and toxicological perspective due to the rapid induction of MT gene transcription in response to exposure to Cu and other metals. While mice deleted for the MTI and MTII genes exhibit sensitivity to Cd, Cu and other metals [89] it has also been proposed that MTs contribute towards homeostasis under conditions of Cu limitation [90]. In support of this, mouse embryonic fibroblasts lacking MT have reduced viability under Cu deficiency [91], suggesting a role for MT as a storage protein for cellular Cu that could be mobilized during times of Cu deficit. Consistent with this notion, RNAi-mediated knockdown of CCS induces the expression of *MT-I* and *MT-II* genes, as well as that of *ATOX1*, *COX17* and *ATP7A* [92], suggesting that perhaps the loss of abundant Cu binding proteins induces expression of other Cu homeostasis proteins to protect cells against excessive amounts of the metal.

How might Cu be mobilized if it is so tenaciously bound by MTs? MT–Cu complexes are localized to lysosomes [93] and in rat liver homogenates, MTs are partially degraded by lysosome-associated enzymes, leading to the release of bound metals [94]. The acidic pH of the lysosome would favor displacement of metal ions from MT and it has been proposed that an initial displacement of metals is necessary to initiate the degradation of MT. Klaassen et al. [94] also demonstrated that apo-MT degradation occurs much faster than that of metal-bound MT, indicating higher holo-MT stability under conditions of metal excess. Lysosomal MT degradation and Cu release raises the possibility that autophagy may play an active role in Cu homeostasis. In line with this hypothesis, Nose et al. [95] found that a single systemic injection of Cu into mice carrying an intestinal-specific excision of Ctr1 not only rescued the perinatal lethality of these mice, but also endowed these mutant mice with a lifespan that is indistinguishable from wild type. These observations suggest that a Cu bolus may be stored and efficiently recycled with minimal loss or excretion.

Intracellular Cu has also been shown to be bound to GSH, albeit with lower affinity than to MT. In support of Cu-GSH representing a potential pool of exchangeable Cu, experimentally depleted hepatic GSH levels in Cu deficient rats results in decreased Cu in the bile and blood but increased Cu in the liver, suggesting that decreased GSH levels may give rise to an oxidative environment in the liver that renders Cu less bioavailable [96]. An additional explanation for the high hepatic Cu levels while hepatic GSH levels are low might be that GSH plays a role in mobilizing hepatic Cu stores. Decreased GSH levels have also been shown to inhibit the Atp7a/b Cu pumps, resulting in cellular Cu accumulation [97]. Perhaps, as GSH functions in a CCS-independent manner in loading Cu onto Sod1, GSH may also deliver Cu to the cytosolic metal binding domains of the Atp7a/b Cu exporters independently of Atox1. Alternatively, cellular redox state, influenced by GSH levels, may be important for the function of Atp7a/b in Cu export.

2.5. Copper export mechanisms

The two mammalian Cu^{+}-transporting ATPases, Atp7a and Atp7b, exhibit high sequence homology and, like the yeast Ccc2 protein, are responsible for the compartmentalization of Cu into the lumen of the secretory compartment for its loading onto Cu-dependent enzymes such as ceruloplasmin, tyrosinase, hephaestin and other enzymes. Moreover, Atp7a and Atp7b play key roles in exporting Cu from specific cells and in specific tissues, as a means of driving vectorial Cu movement. For both the Cu export and Cu compartmentalization functions, these Cu pumps are thought to predominantly receive their Cu cargo directly from the cytosolic Cu chaperone Atox1. Although Atp7a and Atp7b pump Cu across membranes via similar biochemical mechanisms, they are clearly not biologically redundant. Mutations in the ATP7A gene result in Menkes Disease, characterized by an intestinal Cu block, peripheral Cu deficiency and lethality typically by 2 or 3 years of age. Physiologically, Menkes disease manifestations are due to the inability to pump Cu across the basolateral membrane of intestinal epithelial cells to the periphery, or across the blood brain barrier, resulting in a build up of intestinal Cu and inadequate supplies for Cu-dependent enzymes in the periphery. Mutations in the ATP7B gene cause Wilson's disease, characterized by excessive accumulation of Cu in hepatic and neuronal tissues. Both Cu homeostasis disorders present with severe neuromuscular, neuropsychiatric, and cognitive defects, that, while arising from distinct etiologies, result from specific cell and tissue types where Atp7a and Atp7b function to move Cu across membranes.

2.6. Extracellular and circulating copper

In the bloodstream approximately 70–95% of total circulating Cu is bound to the acute phase protein ceruloplasmin (Cp). Cp is a Cu-dependent ferroxidase whose primary function is in loading Fe^{3+} onto transferrin for Fe distribution to the periphery. Given its abundance and a Cu-binding stoichiometry of 6 Cu atoms per Cp monomer, this protein was initially proposed to function in systemic Cu transport, much akin to Fe-transferrin delivery to peripheral tissues. However, ablation of the Cp gene in mice demonstrated that while Fe homeostasis was severely impaired, Cu was delivered to peripheral tissues in a manner essentially indistinguishable from that of wild type mice [98,99]. The half-life of holo-Cp is much longer than the apo-enzyme, indicating that both Cp catalytic function and protein stability are Cu-dependent [100]. Furthermore, recent findings in Cu deficient animals show reduced expression also of the membrane bound splicing variant of Cp named GPI-Cp (glycosylphosphatidylinositol- anchored Cp) [101]. GPI-Cp is a membrane bound multicopper ferroxidase that is expressed in several cell types and tissues [101,102].

In addition to GPI-Cp, two other membrane-bound multicopper ferroxidases have been identified that include hephaestin and the recently identified zyklopen [103]. Hephaestin is strongly expressed in enterocytes where it is important for the export of Fe across the basolateral membrane and into the portal circulation. Hephaestin protein abundance is regulated by Cu status, with low Cu causing decreased steady state levels of the protein [104,105]. Zyklopen has been suggested to mediate placental Fe transport [103] but studies addressing the full spectrum of the physiological roles of zyklopen have not yet been reported. The critical role of the Cu-dependent ferroxidases in Fe uptake and homeostasis imparts an intimate relationship between Cu and Fe, whereby Cu deficiency leads to a secondary Fe deficiency. Once in the circulation, Cu distribution to the periphery likely occurs in a ligand-bound manner, as free Cu is not well tolerated in biological systems. Several low affinity carriers have been suggested to transport Cu in the blood, including macroglobulins and albumin [106,107]. However, analbumenic mice show no critical effects on Cu transport or homeostasis [108], suggesting that albumin is not an essential Cu carrier under standard laboratory conditions. The generation of different macroglobulin knockout animal models will provide opportunities for further insights on the role of macroglobulins, in systemic distribution of Cu.

3. Copper homeostasis from an organ-specific perspective

Mammalian organs have distinct physiological functions and cell types, reflecting diverse requirements for Cu and distinct contributions towards Cu homeostasis. To be able to fulfill these tasks, the localization and distribution of Cu transporters is distinct in different cell types, as summarized in Fig. 2. Here we briefly review key aspects of Cu homeostasis in major organs.

3.1. Intestinal copper uptake and mobilization into portal circulation

The journey of Cu through mammals begins at the apical membrane of enterocytes within the lumen of the intestine. Here, dietary Cu^{2+} is reduced and subsequently transported into enterocytes primarily via Ctr1 localized on the apical membrane. Once inside, Cu binds to ligands and proteins including GSH and intracellular chaperones, and is routed to intracellular targets or pumped across the basolateral membrane by Atp7a for peripheral distribution. During Cu satiety, Atp7a localizes primarily to the membrane of the TGN, but relocalizes to vesicles proximal to the apical and basolateral membranes of enterocytes when Cu bioavailability increases, leading to the transport of Cu into portal circulation [109,110].

Dietary Cu uptake depends on several factors including age, sex and the amount of Cu in the diet. Mice carrying an intestinal-specific Ctr1 excision display peripheral Cu deficiency, growth retardation, hepatic Fe-overload, cardiac hypertrophy and die before weaning [95]. Biotin labeling of Ctr1 in intact mouse intestine demonstrated that in response to a Cu adequate diet, Ctr1 is found on the apical membrane of intestinal epithelial cells and in intracellular vesicles [64]. However, the majority of Ctr1 is localized to the apical membrane in intestinal samples from mice reared on a Cu deficient diet, evidencing changes in Ctr1 localization as a function of dietary Cu availability. The apical localization of mouse Ctr1 has also been verified in other animal species, including rat and pig [64]. The observation that suckling mice exhibit higher Ctr1 expression at the apical membrane of enterocytes when compared to older animals supports the notion of age-specific differences in the systemic demand for Cu [111], that is likely higher in younger animals due to the presumably lower stores of this metal and to ongoing organ maturation.

While multiple studies support the presence of Ctr1 at the plasma membrane in cultured cells and the apical membrane in intestinal epithelial cell sections [64,111–113], another report suggests that Ctr1 does not localize to the apical membrane of enterocytes, but rather to the basolateral membrane of cultured CaCo-2 cells and mouse intestinal sections [114]. This observation, and other studies, remain to be reconciled. In addition to the role of Ctr1 in Cu transport from the intestinal lumen, *in vitro* studies have also suggested that DMT1 (Divalent Metal Transporter 1) may contribute towards dietary Cu uptake [115,116]. However the contribution of DMT1 to mammalian Cu acquisition has not yet been fully validated *in vivo*, even though in the Belgrade rat, a genetic model of Fe deficiency, expressing high levels of an inactive form of DMT1, there is no evidence for altered Cu transport or accumulation [117].

3.2. Hepatic copper storage, excretion and role in systemic copper distribution

The liver is the main storage organ for Cu and other trace metals, and consequently, is a central player in the regulation of systemic Cu homeostasis. Primarily, the liver has four distinct responsibilities when it comes to Cu homeostasis. It must store Cu, mobilize Cu to peripheral tissues, incorporate Cu into Cp and other Cu-dependent proteins, and excrete Cu, when in excess of its storage capacity, into the feces via the bile. Hepatic Ctr1 is localized to the basolateral membrane of the hepatocyte and mediates Cu transport from the blood into cells [111,118]. Mice with a liver-specific reduction in Ctr1 have reduced activity of Cu-dependent enzymes, decreased Cu levels in liver and kidney, and diminished Cu excretion through the bile [119]. Cu levels in other organs were not reduced, suggesting the existence of a compensatory Ctr1-independent mechanism that facilitates uptake and/or enhanced systemic delivery of Cu from the liver into systemic circulation, despite diminished Cu stores in this organ. However, the molecular mechanisms by which this occurs remain to be elucidated.

Interestingly, the heart-specific excision of *CTR1* that demonstrably leads to low cardiac Cu levels and lethal hypertrophy, is accompanied by an increase in the abundance of Cu in the serum. The export of Cu from the liver into the blood is suggested by elevated serum Cu levels concomitant with an increase in ATP7A expression at the level of its mRNA and protein accumulation at cell surface of hepatocytes [120]. Moreover, Atp7a protein levels in intestinal epithelial cells are elevated in parallel with hepatic Atp7a expression. While this finding suggests a Cu-sensing mechanism by which the liver and enterocytes respond to peripheral Cu deficiency by elevating the levels of Atp7a in order to mobilize Cu stores, the mechanism by which this signal is generated and interpreted are not yet understood.

Similar to the age-dependent localization observed for Ctr1 in intestinal epithelial cells [111], hepatic expression of mammalian Atp7a diminishes during the life span of animals reared under Cu adequacy during development from birth into adulthood [120,121]. This is consistent with measurements of hepatic Cu stores, which reach a maximum around birth and decline progressively thereafter [120,122,123]. The positive association between Atp7a expression and Cu levels in the neonate liver may relate to the suboptimal transport capacities of the immature liver, resulting in limited export of the metal to the periphery in young animals, or limiting hepatic stores to meet higher developmental demands for Cu. Presently, it is not known whether changes in hepatic Cu levels are solely correlated to Atp7a expression or whether other as yet unidentified proteins are also involved in sensing, mobilizing and sustaining the systemic demand for Cu during different developmental stages and altered Cu availability. Although, the observation that Cp levels in the serum start off low at birth and thereafter rapidly increase at the same time as hepatic Cu levels decrease [124], suggests that the rapid reduction in hepatic Cu concentrations after birth probably is caused by several proteins.

In contrast to the perinatal expression of mammalian Atp7a, developmental maturity correlates with a predominance of Atp7b expression in the liver, responsible for pumping Cu into the secretory compartment for metallation of Cp and other proteins and for the mobilization of Cu into bile for excretion of excess Cu stores. In hepatocytes Atp7b is partially regulated by COMMD1 (Cu metabolism Murr1 domain 1), which was first discovered in Bedlington terriers harboring a mutation in this gene that presented with hepatic Cu toxicosis [125]. Recent work shows that COMMD1 protein, in addition, regulates SOD1 maturation and activity by reducing the level of SOD1 homodimers [126]. The finding of COMMD1 also led to the discovery of another hepatic protein involved in Cu homeostasis in the liver, X-linked inhibitor of apoptosis (XIAP) [127]. Under conditions of Cu excess, XIAP regulates its own protein stability through a negative feedback loop, the mechanism of which involves CCS-mediated Cu delivery to XIAP, that primes it for proteasomal degradation via auto-ubiquitination, and, in turn, the degradation of CCS through XIAP-dependent poly-ubiquitination [128]. In contrast, when Cu levels are low, apo-XIAP-mediated ubiquitination of CCS instead increases CCS-mediated Cu delivery to SOD1, concomitant with elevated activity of this enzyme in the cell [129]. The precise mechanism for the interaction between Cu and XIAP requires further elucidation, but this discovery raises the possibility that aspects of the pathophysiology observed during liver Cu toxicosis may result from reduced XIAP expression and an increased sensitivity to apoptosis. Furthermore, these findings underscore the possible role of ubiquitin/SUMO post-translational modifications in modulating the abundance or activity of other Cu dependent enzymes and proteins involved in cellular and systemic Cu homeostasis or other processes in mammals.

3.3. Copper in the kidney

Given that circulating Cu is predominantly bound to Cp or sequestered within blood cells, the relative amounts of this trace metal that

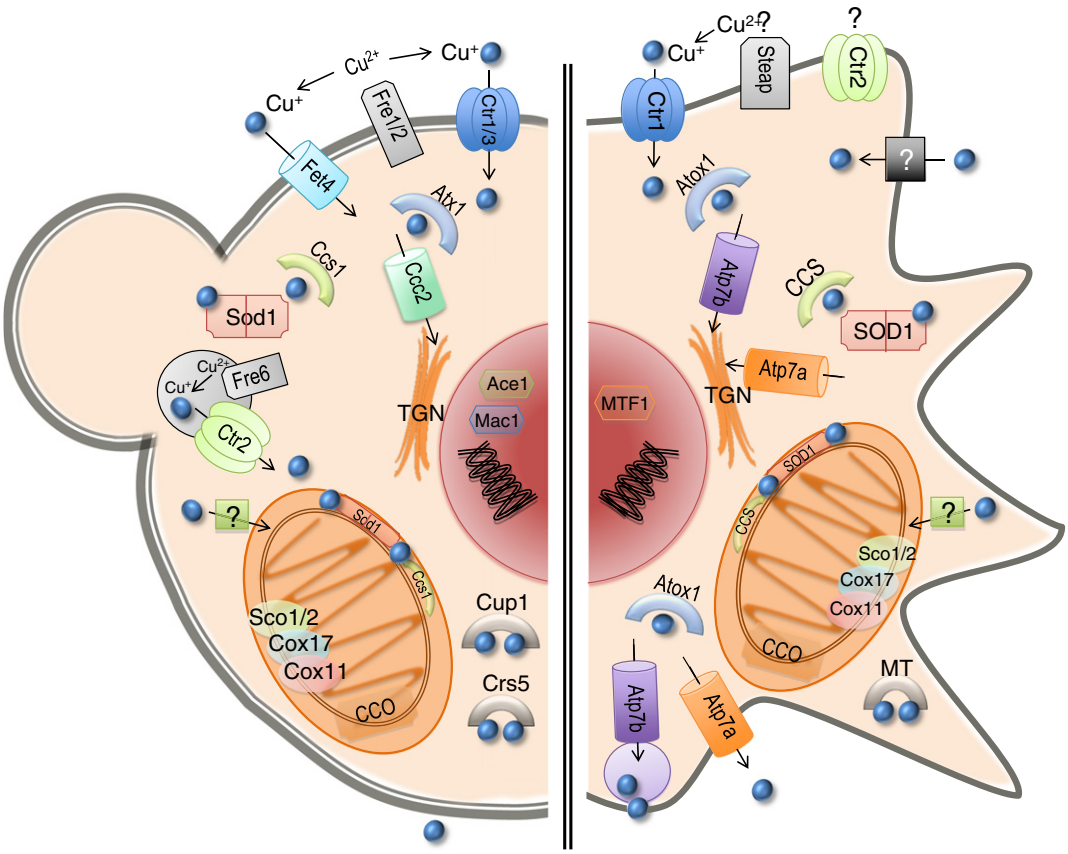

Fig. 1. Eukaryotic Cu homeostasis. A summary of the eukaryotic proteins involved in the sensing, acquisition, intracellular distribution and mobilization of copper (Cu). Each protein, and its function and mode of action, are discussed in the text. The model to the left depicts the copper homeostasis machinery in *S. cerevisiae*, whereas the model on the right depicts these components in a generic mammalian cell.

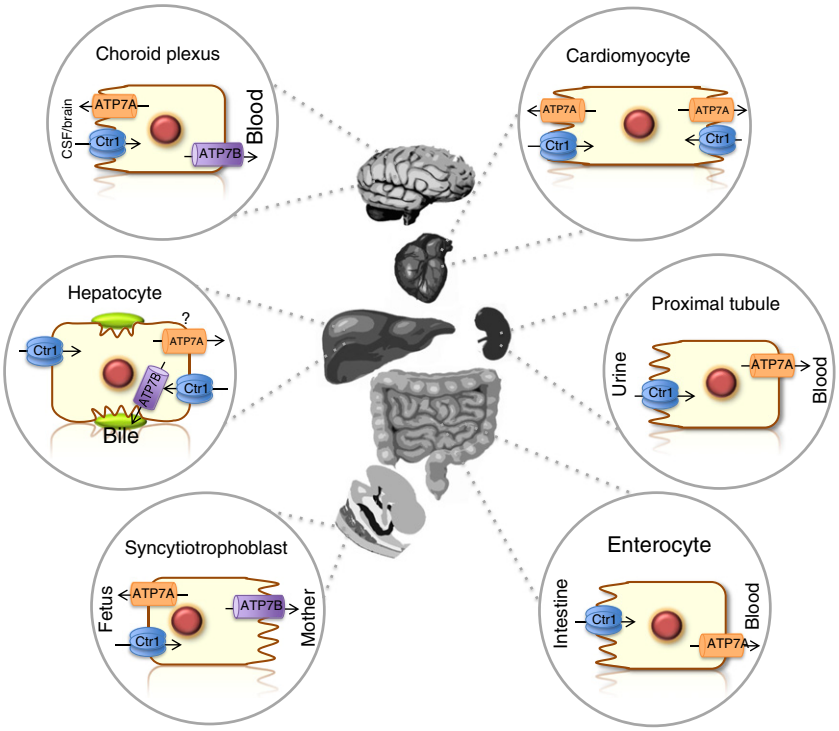

Fig. 2. Mammalian Cu homeostasis in select organs and cell types. Several organs where Cu homeostasis has been investigated are shown, including blood brain barrier, liver, placenta, intestinal epithelial cells, kidney and heart. In the indicated cell types the membrane localization of Cu transporters and directionality are indicated. CSF, Cerebral Spinal Fluid.

are filtered through the glomerular capillaries during Cu satiety are low, suggesting that urinary Cu excretion plays a minor role in systemic Cu homeostasis under normal conditions. Ctr1 is expressed in both proximal and distal tubular cells in the kidney and may import glomerular-filtered Cu from primary urine back into the blood [111]. In support of this observation, Ctr1 levels in the kidney become elevated under systemic Cu deficiency [111], which may serve to increase the reabsorption of Cu from the urine. Curiously, the renal Cu levels are refractory to Cu deficiency [95], suggesting that the kidney at a higher degree than other tissues possesses mechanisms that makes it more resistant to low Cu status. Atp7a and Atp7b colocalize in both the proximal and distal cells of the cortical tubules, however only Atp7a has been reported to relocalize to the basolateral membrane during high Cu, possibly to protect renal cells from Cu toxicity by increasing the cellular export of Cu into the blood for storage or excretion by the liver [130]. The generation of mouse models specifically deleted for Ctr1 or Atp7a/b in the kidneys would be a valuable tool with which to dissect the role of this organ and its resistance to Cu lowering, as well as that of the Cu transporters, in systemic Cu homeostasis.

3.4. Copper in the central nervous system

Much clinical and basic research data support the critical requirement for the tight regulation of Cu homeostasis in neurological development and function [131,132]. The pathophysiology resulting from both extremes of Cu availability notoriously manifests itself at the level of diverse neurological impairments, which if left untreated, lead to neuronal degeneration and death. There are several Cu dependent enzymes that are crucial for appropriate neurological development and function, including peptidylglycine-alpha-amidating monooxygenase (PAM) that is important for the synthesis of amidated neuropeptides and dopamine-beta-hydroxylase (DBH), that is involved in production of norepinephrine, a critical neurotransmitter. Ctr1 is highly expressed at the apical membrane of cells in the choroid plexus that line the ventricular lumen as well as in endothelial cells of the capillaries [111]. Under Cu deficiency, Ctr1 expression increases in the choroid plexus, perhaps to facilitate the uptake of Cu from the cerebrospinal fluid required to meet the demands for this metal ion in the brain [111,133]. In addition, CCS levels in the cerebellum increase in Cu deficient rat pups but those of CCS in the choroid plexus do not, suggesting that there are distinct differences in Cu sensing, regulatory responses or Cu-dependent activities in the choroid plexus versus other brain regions [133]. Together, these findings stress the importance of the choroid plexus in the third and fourth ventricles for maintaining Cu homeostasis in the brain.

Similar to findings in other tissues, the expression of Atp7a and Atp7b in the brain changes during development, from embryonic stages into adulthood. In the neonate, Atp7a can be readily detected throughout the brain but decreases during development except in cells of the choroid plexus and Purkinje neurons [134,135]. Atp7b is similarly expressed in many regions of the brain, including the cerebellum and Purkinje neurons [136,137]. The ontogenic expression of Ctr1, the P-type ATPase Cu transporters and perhaps other proteins involved in Cu homeostasis in the central nervous system is an area that is currently poorly understood. However given the striking neurological pathologies associated with perturbations in Cu homeostasis, this certainly represents an exciting and medically-relevant area for future investigation.

3.5. The heart-felt requirement for copper

The demand for Cu in cardiac function is very high due to, among other things, requirements for mitochondrial Cu-dependent ATP production that drives cardiomyocyte contractility and the concomitant elevated SOD1 activity needed to cope with the respiration-dependent generation of superoxide. The effects of Cu deficiency in experimental animals have been recognized to cause cardiac hypertrophy, a pathological condition characterized by enlargement of cardiomyocytes, which if left untreated leads to hypertension and congestive heart failure. This pathophysiological manifestation of Cu deficiency has been very clearly shown in several laboratories in different mammalian animal models [120,138,139].

In cardiomyocytes, Ctr1 localizes to the intercalated discs [111], complex structures that connect single cardiomyocytes to an electrochemical syncytium that allows for the coordinated contraction of myocytes along their entire length. In support of the high demand for Cu by this organ, recent studies in a cardiac-specific Ctr1 knockout mouse revealed severe Cu deficiency in the heart, growth retardation and hypercardiomyopathy, leading to perinatal death [120]. This recapitulated the pathophysiology described in dietary Cu deficient humans and in animal models, but furthermore highlights the intrinsic requirement for Cu in the heart in the absence of effects resulting from systemic deficiency. Interestingly, systemic ATP7A overexpression in mice results in significant depletion of Cu, particularly in the heart [140], despite its fundamental role in dietary Cu uptake and mobilization into the portal circulation. These findings underscore the importance and differences in tissue-specific expression of Cu-homeostasis proteins and the elaborate mechanisms at play in achieving regulated systemic Cu metabolism.

The critical demands imposed by cardiac function require efficient ROS detoxification mechanisms. Furthermore, access to reactive cellular Cu stores to accommodate for fluctuations in the demand for and storage of excess Cu likely represent important strategies for maintenance of cardiomyocyte function. Indeed, cardiac-specific overexpression of MT *in vivo* has been reported to increase cardiac Cu and Zn concentrations in mice and also protect against Cu deficiency-induced cardiac hypertrophy [141]. These findings propose that in the heart, MT proteins serve a dual role in the safe sequestration of redox-active Cu as well as in maintaining a store of this metal for use in times of Cu scarcity. How Cu might be mobilized from MT during times of deficiency is an area for further investigation.

3.6. Placental copper exchange during pregnancy

During pregnancy the systemic demand for Cu increases to meet the needs of the developing fetus and this correlates with an elevation of serum Cu levels. In the placenta Ctr1 has been localized at the basal membrane, where it presumably serves to transport Cu into syncytiotrophoblasts [142]. The localization of Ctr1 at the basal membrane is somewhat unexpected as it suggests that Ctr1 transports Cu from the fetus into the placenta. However, circulating maternal Cu levels are significantly higher (approximately 5 times) than those in cord blood [143], supporting a basal localization of Ctr1 to favor transport of excessive Cu from the fetus to the mother rather than in the opposite direction. In line with this, one may speculate that the Cu levels in the fetus need to be extremely well regulated due to immaturity of Cu binding enzymes, transporters, protection against oxidative stress and shifting demands of Cu during different periods of the embryogenesis. Atp7a and Atp7b are both expressed in the human placenta tissue. However, as observed in other tissues, these transporters perform distinct functional roles as inferred by differences in their subcellular localization [144,145]. Specifically, while Atp7a is responsible for the transport of Cu to the fetus, consistent with its localization at the basal membrane, Atp7b localizes to the apical membrane and that of the secretory compartment where it may function in cellular Cu export and mobilization of the metal into the Golgi for insertion into Cu-requiring proteins [145,146]. Further studies on Cu homeostasis during pregnancy, and the influence of hormonal responses, represent a promising field for future

research, given the sub-optimal levels of Cu present in the typical American diet [147].

3.7. Sensing peripheral changes in copper availability

Given the differences in Cu requirements as a function of development, cell or organ type and dietary status, mechanisms must be in place to sense and respond to overall body Cu status. Currently, very little is known about systemic Cu signaling mechanisms. However, in addressing the intrinsic role of Cu acquisition in cardiac function, an interesting observation was made implicating the liver and intestinal epithelium in responding to cardiac-specific Cu deficiency. This study revealed that, while Cu levels remained unchanged in the vast majority of organs and tissue types tested, there was a significant reduction in liver-associated Cu accompanied by an elevation in the circulating levels of the metal in the serum as well as elevated Atp7a expression in intestinal epithelial cells and in the liver [120]. This was interpreted as the possible mobilization of dietary and hepatic Cu into circulation as a means to elevate its supply to distal organs, such as the heart, experiencing a Cu deficit. Although the mechanism by which the liver or intestine sense inadequate Cu supply in a specific peripheral organ remains to be elucidated, it argues for the existence of a signal, perhaps synthesized or activated and secreted by the afflicted tissue and sensed by the Cu acquisition and storage organs. In support of this notion, human epithelial cells treated *in vitro* with serum harvested from cardiac-specific Ctr1 knockout mice strongly elevated Atp7a protein expression. The existence of a metal-responsive regulator of systemic metal homeostasis is not a new concept. Hepcidin is a small peptide hormone, synthesized primarily by the liver under conditions of elevated Fe levels, that effectively blocks Fe mobilization from the diet and hepatic stores under conditions of Fe excess [148–150]. It is interesting to speculate that a mechanism operates in mediating Cu homeostasis whereby a secreted factor serves as a signal to integrate the requirements of distal organs and tissue types for this essential trace metal.

4. Concluding remarks

The last decades have witnessed phenomenal progress in our understanding of how cells transport and utilize Cu. Recent findings revealing new aspects of how Cu homeostasis is regulated at the multicellular organismal level have now paved the way for decades of further investigations to elucidate the mechanisms by which cells and organs communicate and coordinately cater for the changing developmental and temporal demands for Cu over the life-time of the organism.

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