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CELLULAR MULTITASKING: THE DUAL ROLE OF HUMAN CU-ATPASES IN COFACTOR DELIVERY AND INTRACELLULAR COPPER BALANCE

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Summary

The human copper-transporting ATPases (Cu-ATPases) are essential for dietary copper uptake, normal development and function of the CNS, and regulation of copper homeostasis in the body. In a cell, Cu-ATPases maintain the intracellular concentration of copper by transporting copper into intracellular exocytic vesicles. In addition, these P-type ATPases mediate delivery of copper to copper-dependent enzymes in the secretory pathway and in specialized cell compartments such as secretory granules or melanosomes. The multiple functions of human Cu-ATPase necessitate complex regulation of these transporters that is mediated through the presence of regulatory domains in their structure, posttranslational modification and intracellular trafficking, as well as interactions with the copper chaperone Atox1 and other regulatory molecules. In this review, we summarize the current information on the function and regulatory mechanisms acting on human Cu-ATPases ATP7A and ATP7B. Brief comparison with the Cu-ATPase orthologues from other species is included.

Keywords

ATP7A; ATP7B; P-type ATPase; copper; transport; trafficking

1. Introduction

Diverse P-type ATPases play numerous roles in human cell biology. The ion gradients generated and maintained by these transporters are utilized for nutrient uptake, propagation of electrical signals, and muscle contraction. The subfamily of the P-type ATPases involved in transport of copper have additional and unique functions, including absorption of dietary copper, transfer of copper to CNS, regulation of overall copper status in the body, and many other essential processes [1]. In the past decade, there has been a growing appreciation for the role of copper and copper transporters in human metabolism [2,3]. Copper is a micronutrient required by all organisms to maintain life [4]. It serves as a co-factor for enzymes that catalyze a diverse array of essential biochemical reactions. In mammalian cells, the biosynthesis of

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neuroendocrine peptides and neurotransmitters, detoxification of radicals, formation of connective tissues and blood vessels, myelination of neurons and many other important physiological processes rely on proper supply of cells with copper. Cells have developed an intricate network of copper-binding and transporting proteins to maintain copper homeostasis [2]. It is thought that copper enters the cell in a highly reactive Cu(I) form. In this form, it can activate molecular oxygen, oxidize hydrogen peroxide producing free radicals, or disproportionate to Cu(II) and Cu(0). To avoid these wasteful and potentially harmful reactions, Cu(I) is sequestered by a set of specific proteins, which carry and transfer copper to various cell destinations [5]. The work of many research groups over the last decade has led to a greater understanding of the copper distribution in cells (for recent reviews see [2–4]), however the entire array of proteins responsible for the uptake, intracellular compartmentalization of copper and copper efflux is yet to be fully characterized.

It is now apparent that the copper-transporting ATPases (Cu-ATPases) are central to the maintenance of copper homeostasis in mammalian cells. Human cells express two homologous Cu-ATPases, ATP7A and ATP7B. Both of these transporters couple ATP hydrolysis to the transport of copper from the cytosol across cellular membranes, thus decreasing cytosolic copper concentration. The transported copper is either released into the bloodstream for further distribution to tissues or it is exported into the bile for eventual removal from the body. In both cases, copper export across the plasma membrane is thought to be a result of exocytosis of vesicles that have been filled with copper as a result of Cu-ATPase transport activity (see section 5 for details).

In addition, the Cu-ATPases are essential for transporting copper from the cytosol into the lumen of such intracellular compartments as trans-Golgi network, secretory granules, or melanosomes, where copper is utilized for biosynthetic incorporation into secreted copper-requiring enzymes [6–8]. ATP7A is known to deliver copper to peptidyl- α -monooxygenase [9,10], tyrosinase [7], and lysyl oxidase [9,10], while the primary acceptor of copper released from ATP7B is ceruloplasmin [11,12]. The dual role of Cu-ATPase in cofactor delivery to copper-dependent enzymes and in copper export from a cell requires trafficking of Cu-ATPases between intracellular compartments (see section 5 for details).

The essential physiological roles of human Cu-ATPases are evident from the existence of disease phenotypes in humans caused by inactivation of these transporters. Mutations or deletions in the gene encoding ATP7A are associated with Menkes disease, while inactivation of ATP7B leads to Wilson disease syndrome. Abnormalities in copper metabolism due to defects in Cu-ATPases affect multiple organs with the major impact on the CNS (Menkes disease, MNK) and/or liver (Wilson disease, WD) [13,14]. Most Menkes disease patients display severe neurological symptoms, mental retardation, and other developmental delays that are invariably followed by death in early childhood [15]. These physiological abnormalities are caused by impaired absorption of dietary copper and disrupted delivery of copper to the copper-dependent proteins in the secretory pathway in other tissues. Normally, ATP7A is expressed in intestine and is required to transport copper across the basolateral membrane of enterocytes into the circulation [16–18]. In Menkes disease patients or in the animal models of Menkes disease, copper export from enterocytes is greatly diminished [19,20]. ATP7A is also highly expressed in the choroid plexus and is critical for the proper supply of copper to the brain [21–23]. Normally, the levels of ATP7A expression peak during axon extension and synapse formation in several neuronal subpopulations [22]. The up-regulation suggests the involvement of ATP7A in synaptogenesis. The specific role of ATP7A in synaptogenesis is currently unknown; the disruption of this important function of ATP7A during development could be the origin of the neurodegeneration seen in Menkes disease [22]. ATP7A is also abundant in vascular endothelial tissue, cerebrovascular endothelial and smooth muscles [24]. Thus, vascular abnormalities, lung vasculature defects, and poor muscle tone are

commonly observed in Menkes disease patients. The lack of copper delivery to tyrosinase explains the loss of pigmentation, while the lack of copper delivery to the lysyl oxidase accounts for connective tissue abnormalities.

WD symptoms are caused by the abnormal accumulation of copper. The vast majority of WD patients present with either hepatic or neuro-psychiatric symptoms, reflecting the protein's primary expression in the liver and the brain [25]. The major function of ATP7B in the brain is likely at the basal ganglia, since this and surrounding regions are most affected in WD patients with neurological symptoms [26]. About 20% of patients with WD have symptoms attributable to the involvement of other organs [25]. The liver is particularly affected by copper accumulation due to disrupted export of excess copper into the bile. In WD, copper delivery to the secretory pathway is also abolished, resulting in the excretion of apo-ceruloplasmin. In humans, the apo-ceruloplasmin is unstable and rapidly degrades when released into the serum [27]. The low levels of ceruloplasmin in a serum along with high hepatic copper content serve as diagnostic markers for WD. The presence of Kayser-Fleischer rings caused by the deposition of copper in the cornea is also a common diagnostic feature in neurological cases of WD.

In the last decade, significant progress has been made in understanding the biochemistry and cellular regulation of Cu-ATPases [1,28]. The initial discovery of human transporters has been followed by highly successful studies on identification and characterization of their bacterial and plant homologues (for reviews see [29,30]). Recent characterization of thermophilic Cu-ATPases yielded detailed biochemical information about the catalytic activity of Cu-ATPases, the structure of several functional domains [31–35], the conformational transitions [36], and the first low resolution structure of the full-size protein [37]. The human Cu-ATPases are more complex, both in their structure and regulation, compared to their bacterial counterparts. In this review, we will summarize what we know today about the structure, mechanism, and regulation of human Cu-ATPases ATP7A and ATP7B. We will refer to the key studies of Cu-ATPase in other phyla that enhance our understanding of human transporters.

2. Structural features of human copper-transporting ATPases

Human Cu-ATPase are large polytopic membrane proteins (Figure 1). Cu-ATPase ATP7A consists of 1500 residues (163,334 kDa; pI 5.94) and is glycosylated, which brings the apparent molecular weight of this protein to 175–180 kDa when it is analyzed on denaturing SDS gels. Human Cu-ATPase ATP7B is slightly smaller, consisting of 1465 residues (157,339 kDa, pI 6.29). ATP7B is not glycosylated and runs with the mobility corresponding to a protein of 165 kDa. At the protein level, ATP7A and ATP7B are 50–60% identical. Both Cu-ATPases are phosphorylated at Ser residue(s) by a yet to be characterized kinase; the extent of a kinase-mediated phosphorylation is regulated by copper ([38,39], see section 5 for details).

The membrane portion of ATP7A and ATP7B has 8 transmembrane segments (TMS) with the N- and C-termini of the protein both oriented towards the cytosol [40]. The topology of human Cu-ATPases has not been analyzed in detail, however recent epitope insertion studies using ATP7B indicate that most of the luminal loops connecting TMS are short and the insertion of the HA-epitope in the most predicted loops interferes with the ATP7B folding and/or activity, with the loop connecting the TMS5 and 6 being the exception [40]. The only data available for ATP7A indicate that the HA epitope can be incorporated in to the first luminal loop without an apparent negative effect on either activity or trafficking [41].

The TMS form the intra-membrane copper-binding sites (Figure 1) to which copper is delivered from the cytosol and from which it is subsequently exported into the lumen of intracellular compartments. The packing of TMS in the membrane has been predicted by recent electron microscopy structure of bacterial Cu²⁺-transporting ATPase CtrA3 [37], however mutual orientation and function of TMS in either bacterial or human transporters remain largely

uncharacterized. The analysis of the distribution of WD-causing mutations in ATP7B [42] illustrates that all TMS in ATP7B are necessary for the Cu-ATPase folding or function. Furthermore, several disease mutations cause the substitutions of Gly or Pro at the predicted membrane interface. This observation suggests that flexible connections allowing conformational changes are essential for the Cu-ATPase transport activity. The nature of the copper coordinating ligands in the membrane can be glimpsed from the site-directed mutagenesis in human Cu-ATPases and in several model systems, most notably the Cu-ATPase of *Archaeoglobus fulgidus* [16,32,34,43–48]. Altogether these studies suggest that the CPC motif in TMS6, NY motif in the TMS7 and MxxS motif in TMS8 are likely to contribute to copper coordination during transport (Figure 1).

The cytosolic portion of the Cu-ATPases contains all other functional domains: the N-terminal copper binding domain, the ATP-binding domain (which includes the nucleotide-binding, or N-domain, and the phosphorylation, or P-domain), the actuator (A-domain) and the C-terminus (Figure 1). The N-terminal copper binding domain is composed of 6 homologous sub-domains (Figure 1). The structures of all individual sub-domains of ATP7A and the 5th and 6th sub-domains of ATP7B together have been determined by the NMR [16,43–45]. Each of the sub-domains is 72 amino-acid residues long, has a ferredoxin-like fold $\beta\alpha\beta\beta\alpha\beta$ -fold [49], and houses a copper-binding site GMxCxxC, in which two invariant cysteines of the CxxC motif coordinate Cu(I) [50–52]. The sub-domains 5 and 6 are connected by a short linker and their metal-binding sites are spatially far apart [16]. However, other linkers connecting the sub-domains are longer and in a fully folded N-terminus of Cu-ATPases, the subdomains appear to form pairs in which metal-binding sites are in sufficiently close proximity for the Cu-Cu distance to be detected by the X-ray absorption spectroscopy [50].

While the structures of the N-terminal metal-binding sub-domains have been studied in significant detail, the overall fold of the N-terminus remains unexplored and little is known about regions that are thought of as “flexible linkers”. However, these regions appear to play a key role in regulating the human Cu-ATPases. For example, the N-terminal segment prior to the first copper-binding subdomain of ATP7B (the first ~ 50 amino acids) is much shorter in ATP7A (~5 amino acids). The first 63 amino acids in ATP7B including this extension have been implicated in the apical targeting of ATP7B ([53], see below for details). In addition, alternative splicing of exon 1, which encodes the N-terminal extension in ATP7B, further increases diversity of this region. In sheep (*Ovis*), two forms of ATP7B generated by alternate splicing of exon 1 have been identified [54,55]. The exon1 of a shorter form, sATP7B, encodes an 18-amino-acid sequence, while in the longer form lATP7B, it is replaced by a 79-amino-acid sequence [55]. It has been hypothesized that the longer isoform might be associated with the additional role for ATP7B copper storage in cells to combat copper deficiency, which is common in sheep.

The ATP-binding domain (ATP-BD) is located between TMS6 and TMS7 and includes the conserved motifs DKTGT, TGDN, and GDGxxD, which are essential for the catalytic activity of these transporters. The ATP-BD is composed of two interacting sub-domains (Figure 1), the P-domain (that houses the Asp residue that undergoes catalytic phosphorylation during ATP hydrolysis, see section 3 for details) and the N-domain (responsible for nucleotide binding). The sequence and the structure of the P-domain was recently determined for the archae Cu-ATPase CopA [31]. This domain, containing signature motifs for P-type ATPases (DKTG and GDGxxD), is conserved between Cu-ATPases and the more distant members of the P-type ATPase family such as Ca-ATPase or KdpB. This structural conservation reflects the common enzymatic mechanism through which all P-type ATPase, including copper pumps, operate.

The sequence of the N-domain is unique for the Cu-ATPases. The residues forming the ATP binding pocket are highly conserved in all Cu-ATPases (in ATP7B, these residues are H1069, G1099, G1101, I1102, G1149, and N1150, [56]), but they differ from the ATP-coordinating residues of other P-type pumps. Such difference in coordination environment may explain a tighter binding of nucleotide by the isolated N-domain of ATP7B [33] compared to the N-domains of other P-type pumps [57,58] and may contribute to a slow turnover rates of Cu-ATPases compared to other eukaryotic P-type pumps (see section 3 for details). Despite a very low sequence similarity and unique ATP-coordination environment, the three-dimensional fold of the N-domain of ATP7B (and of *A.fugidus* Cu-ATPase CopA [31]) is similar to those of other P-type ATPases; i.e. the core of the domain consists of a six-stranded beta-sheet with two adjacent alpha-helical hairpins [56]. The similarity of 3 dimensional structures of key functional domains strongly suggests that the overall transport mechanism, which requires cooperation of different domains, is preserved among all members of the P-type ATPase family, including Cu-ATPases. Recent analysis of conformational changes in Cu-ATPase from hyper-thermophilic bacterium *Thermotoga maritima* provides direct evidence that this Cu-ATPase undergoes domain rearrangements very similar to those of SERCA1 [36]. This latter study also revealed an association between the N-terminal copper-binding domain and the A-domain, an arrangement that could be essential for copper-dependent conformational transitions in Cu-ATPases.

The A-domain of Cu-ATPases is formed by a cytosolic loop located between TM4 and TM5 (Figure 1). The structure of this domain has been solved for Cu-ATPase from *A. fulgidus* [31] and is very similar to the structure of the A-domain of other P-type ATPases. The A-domain contains the highly conserved TGE motif, which is required for dephosphorylation step during catalysis (see section 3). By analogy with Ca^{2+} -ATPase SERCA, the A-domain of Cu-ATPases is thought to interact with the ATP binding domain and play an important role in conformational transitions associated with the catalytic activity of the transporter [59]. The triple mutation TGE>AAA disrupts the ability of protein to proceed through the catalytic cycle and stabilizes the phosphorylated intermediate [60].

The C-terminal domains of the human Cu-ATPases are approximately 90 amino acids long and about 56% identical. No structural information is available for the C-terminal region of the human Cu-ATPases, however some interesting sequence motifs in this region have been noticed and characterized. The C-termini of ATP7A and ATP7B contain dileucine and tri-leucine motifs (Figure 1), respectively. In ATP7A, the L¹⁴⁸⁷L¹⁴⁸⁸ sequence is important for the retrieval of the protein from the plasma membrane [41,61,62], as evident from a trapping of the L¹⁴⁸⁷A-L¹⁴⁸⁸A mutant at the plasma membrane. Surprisingly, in polarized MDCK cells the L¹⁴⁸⁷A-L¹⁴⁸⁸A mutant of ATP7A is trapped at the apical, and not basolateral membrane [63]. This result seems to suggest a more complex role for the ATP7A C-terminus than was initially thought. It could be that the dileucine motif of ATP7A participates in the targeting of ATP7A to the appropriate (basolateral membrane) in polarized epithelial cells [63]. Alternatively, and perhaps more likely, the polarized delivery of Cu-ATPases to appropriate membranes is coordinated through a fine-tuned interaction of several domains, which became hidden or exposed as protein exits certain compartments.

The role of a tri-leucine motif LLL1454–1456 in the C-terminus of human ATP7B is also unclear. The mutation of two leucines to alanines within this sequence results in ATP7B being trapped in the vesicles, and not at the plasma membrane [35], as would be expected for a “regular” endocytic signal. It is interesting that the C-terminus of ATP7B also has a cluster of negative residues D¹⁴⁴⁶DDGD¹⁴⁵⁰ preceding the tri-leucine motif. Previous studies of the insulin-regulated aminopeptidase demonstrated that a combination of an acidic cluster and two Leu residues may not only determine the targeting of membrane proteins to distinct membrane compartments, but also regulate the rate of their trafficking from the endosomal compartments

[64]. It could be that in ATP7B the hydrophobic LLL and the negative cluster work together to retain ATP7B in recycling vesicles (see below in section 5 of Cu-ATPase trafficking) or to regulate the rate of ATP7B trafficking between intracellular compartments.

The extreme C-terminus of ATP7A contains a PDZ binding motif, DTAL [63,65], which interacts with AIPPI (ATPase-interacting PDZ) protein [65]. This interaction is likely to be important for the targeting and localization of ATP7A at the basolateral surface of polarized cells, however the functional consequences of this interaction have not yet been demonstrated. Deletion of DTAL is associated with the relocalization of ATP7A, in polarized renal cells, to the vesicles and the apical membrane [63]. In ATP7B, the deletions in the C-terminal domain reduce the protein steady-state levels [47]. Whether this latter effect is due to reduced RNA stability or enhanced proteolysis of the protein remains to be determined. *In-silico* analysis using web-based modeling program SOSUI suggested that the absence of the C-terminal disrupts the TM8 domain stability, which in-turn affects the global protein structure [47].

The presence of the HxxM motif (¹³⁸⁹HGHM in ATP7B and ⁶⁹⁶HSSM in ATP7A) that is conserved in Cu-ATPases has been also noticed, and the role for this motif in metal coordination was proposed [66]. It is interesting that in some plant and bacterial Cu-ATPases, the C-terminus contains distinct metal binding domains, suggesting that the C-terminus may play a more important regulatory role in the Cu-ATPase transport mechanism than is currently understood. Detailed functional characterization and kinetic measurements of Cu-ATPases trafficking and recycling are necessary to understand the precise roles of various motifs in their C-termini.

3. Transport mechanism and regulation of catalytic activity of human Cu-ATPases

ATP7A and ATP7B belong to the large family of P-type ATPases. Similarly to other members of this family, Cu-ATPase use the energy of ATP hydrolysis to transport ions across membranes. Unlike many other members of the P-type ATPase family, Cu-ATPases do not generate ion gradients, at least not in a direct sense, since copper stays bound to proteins both in the cytosol and outside of the cell. The major steps of the catalytic cycle through which Cu-ATPases proceed while transporting copper have been identified [33,67–70] and can be described briefly as follows. The Cu-ATPase binds ATP and copper from the cytosolic side; ATP is then hydrolyzed, and during this process the γ -phosphate of ATP (Pi) is transferred to the invariant Asp residue in the DKTG sequence motif, forming a transient phosphorylated intermediate, E1P-Cu. A Cu-ATPase then undergoes conformational change into the so-called E2P-Cu form. Copper is released at the opposite side of the membrane, Asp becomes dephosphorylated, and the Cu-ATPase returns to the initial state for re-initiation of the cycle. The formation of a transient phosphorylated intermediate has been demonstrated for both ATP7A and ATP7B [33,46,69], the existence of conformational changes coupled with ligand binding has recently been shown for thermophilic Cu-ATPase [36].

The analysis of kinetic of phosphorylation and dephosphorylation under identical conditions revealed that both partial reactions are faster for ATP7A compared to ATP7B [67], suggesting that ATP7A may have a faster turnover rate. This property may explain why ATP7A appears to serve as a housekeeping Cu-ATPase, while the role of ATP7B in copper balance in tissues could be more specialized [71]. The studies of the ATP dependency and Cu-dependency of catalytic phosphorylation yielded very similar values for both Cu-ATPases [67]. This is not surprising given high conservation of the ligand binding sites for ATP7A and ATP7B. However, the interactions of the full-size ATP7A and ATP7B with the intracellular copper donor, the copper chaperone Atox1 (see section 4 for details), have not been directly compared. The interaction with the chaperone is mediated by the N-terminal domain of Cu-ATPases,

which is structurally less conserved between two ATPases compared to other domains. Consequently, the possibility remains that the interaction of the N-domain with Atox1 may differ significantly for the Atox1-ATP7A and Atox1-ATP7B pairs. Therefore, in cells in which both Cu-ATPases are expressed there could be a preference as to which Cu-ATPase predominantly receives copper from Atox1, particularly under conditions of copper deprivation. Consistent with this idea, recent NMR experiments using individual metal-binding subdomains provide *in vitro* evidence that there is a difference in the interactions between ATP7A and ATP7B copper-binding sub-domains and Atox1 [16,44].

So far, very few studies reported direct measurements of copper transport by human Cu-ATPase [31,46,68–70,72]. The transport activity of ATP7A and ATP7B is often evaluated indirectly using either yeast complementation assay (the assay is based on the ability of human overexpressed Cu-ATPase to compensate for the lack of yeast Cu-ATPase CCC2, see for example [28,73,74]) or using the tyrosinase activity assay in cells (tyrosinase activity depends on copper, which is delivered to tyrosinase by the Cu-ATPase, see for example [7,53]). The first attempts to measure copper transport for purified ATP7A and ATP7B directly using liposome preparations revealed very slow rates of either ATP hydrolysis or copper transport, at least *in vitro* [68,72]. These observations may reflect the fact that the important function of Cu-ATPases - to provide copper for the biosynthetic incorporation of a cofactor into apo-enzymes-may not require fast rates of copper transport. It is also possible that the optimal conditions for measuring Cu-ATPase turnover, particularly following protein reconstitution into liposomes are yet to be determined, as was recently discussed [68].

A number of factors may contribute to a faster rate of copper transport *in vivo*. For example, it is interesting that the rate of copper transport by ATP7B appears significantly increased by lowering the pH below the neutral range [46]. This result suggests that a relatively low pH, common for the intracellular compartments in which the Cu-ATPases operate, may facilitate copper dissociation from the transporter and hence stimulate metal transport. Perhaps the lack of appropriate pH balance in current *in vitro* systems could be responsible for strong retention of copper by the Cu-ATPase and slow transport rates *in vitro*. Alternatively, the copper-binding proteins that *in vivo* accept copper released from the Cu-ATPases may play a significant role in copper transport by “buffering” copper in the lumen of the secretory pathway.

It is also possible that the rate of copper transport is regulated and may increase upon demand. The deletion of the first 4 copper-binding sub-domains in ATP7A increases the rate of catalytic phosphorylation, and presumably the copper transport rate, due to the disruption of autoinhibitory interactions between the N-terminal domain and ATP-BD [75]. Such domain-domain interaction is also decreased when the N-terminal domain binds copper [33]. Thus, it is possible that the rate of copper transport by Cu-ATPase may differ greatly depending on the copper occupancy of the regulatory N-terminal domain; the occupancy may in turn be determined by copper availability in a cell. In addition, the trafficking of Cu-ATPases between various cellular compartments (see section 5 for details) may also have an effect on the rate of copper transport. As we describe below, the human Cu-ATPases traffic in response to changes in the intracellular copper concentration or other signals from their basal localization in the *trans*-Golgi network to various vesicles. The luminal pH in these various compartments differs and may have a significant effect on the release of copper from the transporters, as was shown *in vitro* by Safaei and colleagues (see above, [46]).

4. Copper transfer by Atox1 and the role of Cu-ATPase individual metal-binding subdomains in this process

It is thought that human Cu-ATPases receive copper from a small cytosolic protein called Atox1. Deletion of the Atox1 gene in mice is associated with growth failure, skin laxity, and

hypopigmentation [76], a phenotype resembling the copper deficiency due to ATP7A inactivation. The metabolic studies confirmed that *Atox1*^{-/-} cells have impaired cellular copper efflux consistent with the disrupted copper delivery to Cu-ATPases. It is interesting that the copper-dependent trafficking of ATP7A from the TGN is slower but not completely abolished in the *Atox1*^{-/-} cells. This observation suggests that other cytosolic copper carrier(s) may substitute for Atox1 at least in regulation of ATP7A trafficking. Alternatively, copper dependent trafficking may depend less on the copper-transport activity of Cu-ATPases (a process that requires functional Atox1) but instead require a posttranslational modification of Cu-ATPase, which could be an Atox1-independent event (see more on trafficking in section 5).

In vitro, Atox1 transfers copper to the N-terminus of Cu-ATPase by directly docking to this domain [16,44,45,50,77,78]. Atox1 is a small 68 amino-acid residue soluble protein that is ubiquitously expressed [79]. Atox1 contains a MxxCxxC metal-binding motif that binds copper in a linear coordination by two Cys residues [80]. Structurally, Atox1 is similar to the individual copper-binding domains of Cu-ATPase. In solution, Cu induces homodimerization of Atox1 [81], and the crystallographic structure was determined for Atox1 dimer held together by a metal coordinated via CxxC motif in each monomer [82]. Whether such Atox1 dimerization is physiologically significant or it simply reflects the ability of the Atox1 metal-binding domain to form adducts with a similar CxxC site on another protein is currently unknown.

It is clear that in solution, Atox1 interacts with several individual metal-binding sites of Cu-ATPases with some discrimination. In the case of the isolated metal-binding subdomains of ATP7B, Atox1 interacts most strongly with the subdomain #4, less with the subdomain #2 and does not interact with the subdomains #5 and #6 [16]. In the case of ATP7A, the preferences are different. The NMR experiments using the entire N-terminal domain of ATP7A suggest that Atox1 in a copper-bound form interacts with the subdomains 1 and 4 [45]. Mutation of the metal binding cysteines to alanine in either of these two domains abrogated its interaction with each other but left the other domain capable of forming the intermolecular adduct [45]. This difference in the ATP7A and ATP7B behavior with respect to Atox1 may be essential in a cell, where one Cu-ATPase can be preferred over the other as a copper acceptor, particularly under conditions of copper deprivation (see above).

It is also possible that the reported differences in interaction of Atox1 with different metal-binding subdomains have limited physiological relevance and simply reflect the properties of individual recombinant subdomains in solution. These properties (a surface charge distribution, the exposure of the metal binding sites, and the affinity for copper) could be altered significantly in a fully folded structure of the entire N-terminal domain of Cu-ATPase composed of 6 subdomains. For example, the studies of the full-length ATP7B demonstrate that although in solution the recombinant subdomain 2 by itself interacts less efficiently with Atox1 than subdomain 4 (see above), in the folded full-length protein the subdomain 2 and not domain 4 is a preferential site for copper-transfer by Atox1 [83]. In fact, the CxxC to AxxA mutation of the metal-binding subdomain 2 completely abolishes the Atox1-dependent activation of ATP7B (without affecting activation by free copper) even though the subdomain 4 is intact [83]. In the murine ATP7B orthologues, the subdomain 4 lacks the copper-coordinating residues [84]. This observation further argues against the role of this sub-domain as a primary site for Atox1 docking and copper transfer. Clearly further studies are needed to better understand the sequence of events during copper transfer and the role of the individual domains in this process.

There is current agreement in the literature that for the copper-transport activity of human Cu-ATPases only one metal-binding sub-domain in the vicinity to the membrane (sub-domain 5 or 6) is sufficient [85]. More N-terminally located subdomains (1 to 4) cannot sustain copper

transport in the absence of domains 5 and 6 [85]. These results indicate that the spatial location of the metal-binding subdomains and, possibly, correct interactions with the other functional domains of the Cu-ATPase are essential for the overall transport activity. Mutational analysis of the N-terminal subdomains suggest that the domains 5 and 6 are likely to regulate the affinity of the intramembrane sites for copper [75], while the first four subdomains serve as a docking station for Atox1 (see above) and may control the Cu-ATPase turnover [75]. It is interesting that in ATP7B Atox1 does not interact with metal-binding sites 5 and 6 [16,86]. This observation suggest that either the sites 5 and 6 receive copper from other metal-binding subdomains [16] or, in solution the properties of recombinant subdomains differ from their characteristics in the full-size protein (see [45] and also above). When all six metal-binding subdomains in the N-terminus are mutated no transport is observed [28], although earlier studies suggested that some activity might have been retained [70].

It is currently thought that the N-terminal domain is involved in a series of complex copper-dependent interactions. The transfer of copper by Atox1 affects other subdomains, as evidenced by chemical shifts in the NMR spectra [45] and induces conformational changes in the connecting loops that can be revealed by limited proteolysis [77]. Further conformational changes upon increased copper-binding stoichiometry were reported by CD-spectroscopy [52]. As described above, these changes in the N-terminal domain structure are associated with the changes in the interdomain interactions, which affect the Cu-ATPase activity and may open sites for interaction with cytosolic protein regulators. It is interesting that Atox1 in its apo form can remove several but not all copper atoms from the N-terminal sub-domains and down-regulate the catalytic activity of Cu-ATPase [77]. It is tempting to speculate that fine-tuned interactions between Atox1 and various N-terminal subdomains are at heart of the mechanism that controls transport activity and the trafficking of human Cu-ATPases.

5. Localization and trafficking of human Cu-ATPases

Consequences of genetic inactivation of human Cu-ATPases indicate that they perform at least two major functions in a cell. Specifically, the Cu-ATPases deliver copper to the copper-dependent enzymes in the secretory pathway and maintain the intracellular concentration of copper in a cell by exporting excess of copper either into circulation for further distribution between tissues or into the bile for removal with feces. To mediate these functions Cu-ATPases traffic between cell compartments (Figure 2). Co-localization studies with compartment-specific markers and electron microscopy studies [75] have aided understanding of the localization and trafficking behavior of Cu-ATPases throughout the secretory pathway (see for example [87]).

It is now well established that under basal (low copper) conditions both ATP7A and ATP7B reside in the terminal portion of the Golgi apparatus - the *trans*-Golgi network (TGN), where they display a characteristic perinuclear staining pattern. It is also well established that both ATP7A and ATP7B respond to an increase in copper by trafficking from the TGN towards the plasma membrane, although their intracellular itineraries differ (Figure 2). ATP7A relocates towards the basolateral membrane to facilitate copper export into blood [63], while in hepatic cells ATP7B traffics towards the apical canalicular membrane to transport copper into the bile [88]. A very recent study has suggested that ATP7B may also participate in the copper export via distinct mechanism involving the paracellular transport throughout tight junctions [89]. This very interesting hypothesis awaits further verification (see [90]).

Immunofluorescence studies of cultured Chinese Hamster Ovary (CHO) cells made to over-express ATP7A were the first to illustrate a change in the subcellular localization of the transporter from the TGN to vesicles and then the plasma membrane in response to copper elevation [91]. To determine whether the relocation of ATP7A to the cell surface is due to

a copper-stimulated forward traffic of ATP7A from the TGN to the cell surface (exocytosis) or due to the decreased retrieval at the plasma membrane (endocytosis), Petris and Mercer introduced an epitope tag for the antibody (myc-tag) to the first luminal/extracellular loop of ATP7A [41]. Immunofluorescence imaging of this ATP7A variant in non-permeabilized cells showed that the internalization of the myc-tagged ATP7A occurred under both basal and elevated copper conditions, supporting the model that copper stimulates anterograde trafficking from the TGN [41].

The presence of ATP7A at the plasma membrane was confirmed by cell-surface protein biotinylation [17,43], suggesting that ATP7A may function at this location and export copper directly across the plasma membrane. However, these studies also revealed the presence of a recycling pool of ATP7A [43]. In addition, recent quantitative analysis of ATP7A distribution in intestinal cells [17] as well as immunocytochemistry of ATP7A in tissues [18,92] convincingly demonstrates that the plasma membrane represents a minor and transient location for Cu-ATPases. The vast majority of protein in high copper condition resides in vesicles in close vicinity to the plasma membrane. Altogether these observations lead to a current model of constitutive cycling of ATP7A between the TGN, vesicles, and the plasma membrane under basal conditions. When copper is added, the steady state distribution of ATP7A is shifted towards the vesicles and cell surface (see [28,53]).

According to this model, ATP7A mediates export of copper from the cell in at least two steps (Figure 2). In response to copper elevation, ATP7A traffics to the vesicles in the proximity to the plasma membrane, where it sequesters excess copper into the lumen of the vesicles. The vesicles then fuse with the plasma membrane allowing for the appearance of the Cu-ATPase at the cell surface, and copper is released into the extracellular milieu. ATP7A is then endocytosed and returns to a recycling vesicular compartment. Whether it then continues to transport copper into the exocytic vesicles or returns to the TGN appears to depend on the intracellular copper levels. How these intracellular levels are “sensed” by the Cu-ATPase is currently unknown. Given the ability of copper-bound Atox1 to transfer copper to the Cu-ATPases and ability of the apo-Atox1 to remove copper from the N-terminal domain of the Cu-ATPase (plus the conformational changes that accompany copper binding and release, see above), it is tempting to speculate that Atox-1 and the Cu-ATPase N-terminus both contribute to this copper sensing mechanism.

ATP7B trafficking was investigated in hepatic cells where it is primarily expressed and where ATP7A is absent. Both, *in vitro* and *in vivo* experiments have illustrated copper dependent trafficking of ATP7B in hepatocytes [1,53,88,93]. Current data suggest a mechanism of the ATP7B trafficking that is very similar to the one described for ATP7A. An interesting difference between ATP7A and ATP7B is observed when putative endocytic di/tri-leucine motif, which is present in their C-terminus (see section 2), is mutagenized. In ATP7A the LL>AA replacement traps the transporter at the plasma membrane consistent with the role of the motif in endocytosis. In contrast, similar mutation in ATP7B traps protein in vesicles. Such difference could be either do to the fact that the trafficking experiments were performed in non-polarized cells. In such cells, the apical delivery pathway may not be fully functional precluding the appearance of ATP7B at the cell surface. Alternatively, the phenotype of this mutant may reflect the role of the di-leucine motif in ATP7B in the redistribution between different vesicular populations and the TGN (see also section 2).

Despite it being repeatedly shown that the addition of copper to cell culture medium results in trafficking of ATP7A and ATP7B, the underlying mechanism for the exit of Cu-ATPases from the TGN is not well understood. A scenario where the Cu-ATPase trafficking to vesicles occurs due to increased intracellular copper is the most probable and currently accepted. However, the model that involves receptor/kinase mediated signaling from cell surface cannot be fully

discounted. The trafficking of Cu-ATPase in response to signaling at the plasma membrane is supported by the recent demonstration of the ATP7A relocalization in response to changes in Ca concentration (induced by the opening of the NMDA receptors [94]) or in response to hormonal signaling [95]; both these trafficking events occur without extracellular additions of copper. Elevated copper, in turn, was shown to activate such kinases as Akt [96] and induce kinase-mediated phosphorylation of ATP7A [39] and ATP7B [38] although the identity of kinase(s) acting on Cu-ATPases remains unknown. The change in the phosphorylation of Cu-ATPase is linked to the change in subcellular localization. Specifically, the re-localization of Cu-ATPases from the TGN to vesicles is associated with a hyper-phosphorylated state of Cu-ATPases, while recycling back to the TGN with dephosphorylation to the basal level. A kinase-mediated phosphorylation may provide a link between various signaling events and Cu-ATPase trafficking. It is possible that the kinase-mediated phosphorylation serves as a signal for the exit of the transporter from the TGN by recruiting the components of trafficking machinery to the phosphorylated protein. Alternatively, kinase-mediated phosphorylation may shift the equilibrium in the intracellular distribution of the transporter by providing a better retention of Cu-ATPase in the vesicles. The precise mechanism and the role of kinase-mediated phosphorylation in regulating the Cu-ATPase trafficking awaits further exploration.

It is generally assumed that the increase in extracellular copper results in increased copper uptake. ^{64}Cu provides a means of measuring uptake and intracellular accumulation, and it has been shown that a maximum amount of ATP7A at the plasma membrane coincided with the accumulation of 0.1 pmol of $^{64}\text{Cu}/\mu\text{g}$ of cellular protein [43] and that as little as 1 μM in the medium is sufficient to trigger the trafficking [17]. Once copper has entered cells, it may stimulate Cu-ATPase trafficking through several mechanisms. For example, the binding of copper to the regulatory N-terminal domain may induce conformational changes in this domain triggering the Cu-ATPase re-localization. However, deletion experiments revealed that only one of the N-terminal metal-binding sites is required for copper-dependent trafficking. In fact, if appropriate conformation of protein is stabilized through site-directed mutagenesis (for example, the triple mutation TGE>AAA in the A-domain, see below), the N-terminal metal-binding sites are not required at all [97]. Therefore other factors/domains must play a more important role. Recent studies identified at least two such factors.

It has been shown that the copper-transport activity is necessary for the ability of Cu-ATPases to leave the TGN. Mutations that prevent copper binding within the transmembrane portion or those that disrupt the formation of phosphorylated intermediate also prevent trafficking of Cu-ATPase from the TGN (for review see [28]). The mutation of the phosphatase motif (TGE>AAA) in ATP7A, which inhibits copper transport but stabilizes the phosphorylated intermediate resulted in vesicular localization under both basal and high copper conditions [60,97]. These experiments provided convincing evidence for the existence of a conformational state of Cu-ATPase that is preferentially recognized by cellular trafficking machinery. Further evidence for the importance of Cu-ATPase conformation came from the studies on ATP7B, in which the deletion of the part of the N-terminal domain including the first 63 residues also led to relocalization from the TGN even in the basal copper [53]).

In a wild-type protein, a series of events, such as copper binding to the regulatory sub-domains, changes in the inter-domain interactions and transport on copper into the lumen of the TGN are likely to precede the stabilization of such a “trafficking-compatible” conformational state. It could be that in low copper, the copper that is released in the lumen of the TGN is buffered away from the Cu-ATPases by the luminal apo-copper binding proteins. Saturation of the acceptor proteins with copper may slow down the release of copper leading to “substrate inhibition” of Cu-ATPases. Stabilization of a copper-bound form of Cu-ATPase may serve as a signal for leaving the TGN. Other mechanisms are also possible and further studies are needed.

Another set of interesting observations was revealed by the experiments trying to determine the molecular basis of Cu-ATPase trafficking to different membranes in polarized cells. As described above, a series of studies (both in cultured cells and tissues) revealed that ATP7A traffics towards the basolateral membrane, while ATP7B moves to the apical membrane. Subsequent studies demonstrated that the very N-terminal 63 amino acids of ATP7B that are absent in ATP7A not only important for the copper-dependence of ATP7B trafficking, but also are essential for the apical delivery of the transporter in polarized cells [53]. If this segment is kept intact, then the deletion of the N-terminal copper-binding subdomains 1–4 does not affect its trafficking in polarized hepatic (WIF-B) cells [53]. This is in agreement with a previous study in CHO cells, where ATP7B exhibited wild type trafficking behavior despite having a deletion of MBSs 1–5 (amino acids 64–540) [98]. However, the deletion of MBSs 1–4 together with the most N-terminal 63 amino acids results in a functional transporter that traffics towards the basolateral membrane and vesicles even when copper is low [53]. This observation suggests that copper sensing and subsequent trafficking are intrinsic properties of the transporter (rather than being determined by copper-binding proteins in the cytosol). Furthermore, it seems clear that the cell's trafficking machinery recognizes specific signal sequences within ATP7B to direct it towards the appropriate membrane. Whether these signal sequences are located within the 1–63 region or this segment of the protein acts as an important regulator of conformational changes remains to be determined. The summary of possible movements and rearrangement in ATP7B domains during copper binding, transport, and trafficking is shown in Figure 3.

6. Cu-ATPases in various phyla

The presence of Cu-ATPases in all phyla and the dichotomization of P1B (heavy metal transporting) ATPase and other P-type ATPases before the division into eukaryotic and prokaryotic cells illustrate an early evolution origin of Cu-ATPases. The evidence of Cu metabolism is already seen in thermophiles, such as primitive sulphur-metabolizing archaea *Archaeoglobus fulgidus* and hyper-thermophilic bacterium *Thermotoga maritima*, a representative of a very deep branch of eubacteria. The exact functional role of Cu ATPases in the physiology of the respective microorganisms is not yet known, however the thermophilic Cu-ATPases have been extremely useful as model systems to study structure-function relationship in Cu-ATPases [33–36].

The role of Cu-ATPases in the physiology of lactobacterium *Enterococcus hirae* is much better characterized. This organism possesses a *cop* operon that is required for copper homeostasis and which consists of the four genes *copY*, *copZ*, *copA*, and *copB*. *copY* encodes a copper-responsive repressor, *copZ* a copper chaperone, and *copA* and *copB* code for Cu-ATPases [99]. Current evidence suggests that CopA might be required for copper uptake under copper-limiting conditions, while CopB may mediate copper export [100]. Interestingly, a *Staphylococcus* gene *ivi44*, encoding a homologue to CopA, is upregulated during *Staphylococcus* infection in a renal abscess model [101], suggesting a potentially important role of copper transporters in virulence. Specific role of Cu-ATPase in bacterial pathogenesis was shown for CtpA, a Cu-ATPase from pathogenic *Listeria monocytogenes* [102]. Mutants in CtpA had dramatically reduced growth in tissue of infected mice and a significantly impaired *in vivo* persistence. The roles of *ivi44* and CtpA in pathogenesis are not clear but may involve acquisition of copper during bacterial growth or copper delivery to surface expressed copper-dependent enzymes, required for bacterial infection [103].

In many bacteria (e.g. *Haemophilus influenza*, *Deinococcus radiodurans*, *Bacillus subtilis*) the genes for the metallochaperones and the Cu-ATPases form a part of the same operon as seen in Cop system of *E.hirae* [104]. The difference in orientation of the coding regions as well as the lack of sequence similarity of each copper chaperone to the adjacent metal-binding domains of Cu-ATPases, suggests that the copper chaperones evolved independently from the Cu-

ATPases [105]. The *E.coli* genome does not encode an apparent copper chaperone, however this bacterium possess the Cu-ATPase YbaR with a longer N-terminal fragment. It has been hypothesized that a proteolytic product of the N-terminal end of this Cu-ATPase acts as a copper chaperone, although direct experimental evidence for this interesting hypothesis is still lacking [104,106]. It is known that operons are relatively unstable. They represent evolutionary intermediates with low half-lives, and few operons are conserved in phylogenetically distant organisms [107]. Thus, the consistent presence of copper operon system in a diverse set of microorganisms suggests strong selective pressure to maintain tight levels of intracellular copper, taken care by chaperones in the initial phase of entry and Cu-ATPases in the later stages including excretion.

Cu-ATPases in plants

Plants harbor a complex system of copper transporters and chaperones to orchestrate both copper delivery and detoxification in response to environmental and developmental demands. Though copper is essential for all living organisms, plants (and cyanobacteria) have additional requirements for copper due to their photosynthetic machinery. Plants must be able to mobilize copper in a large-scale and organized manner, as in biosynthesis of the photosynthetic apparatus. Critical copper-containing proteins in plants are found in several intracellular compartments including the cytosol, mitochondria, endo-membrane system, chloroplasts and peroxisomes. Table 1 provides examples of copper-containing enzymes/proteins and their locations. Further, copper enzymes in the apoplast include ascorbate oxidase, diamine oxidase and polyphenol oxidase. Several copper-transporting ATPases have been identified and functionally characterized to move intracellular copper into specific compartments. In addition, copper homeostasis in plants is controlled via copper-responsive microRNA that regulate levels of copper-binding proteins [108].

RAN1 (HMA7, Heavy Metal ATPase7) transports copper from the cytosol to the endomembrane system and was initially identified in plants deficient in the ethylene receptor. This transporter functionally complements yeast $\Delta Ccc2$ [109] and is characterized by the canonical CPC motif in transmembrane helix 6 and two cytosolic N-terminal CxxC metal binding motifs. This protein is also thought to export copper from the cell at the plasma membrane, though loss-of-function mutation in RAN1 does not increase copper sensitivity. Rather, excess copper in growth media suppresses the phenotype [109].

HMA5 is a close homologue of RAN1 that is expressed predominantly in roots and flowers [110]. Mutants in HMA5 show increased copper sensitivity, indicating that the protein transports copper out of cells. These mutants do not show defects in ethylene sensitivity, unlike mutants in RAN1. Curiously, defects in HMA5 as well as RAN1 affect cell expansion [110, 111]. In a recent review, Pilon and colleagues suggest loss of function in these transporters may affect apoplastic oxidase activity and cell expansion [30].

The critical departure in plant copper homeostasis from other organisms is the presence of chloroplasts and photosynthetic machinery. Copper is a critical cofactor in the photosynthetic machinery, particularly with respect to balancing the benefits of energy acquisition from photosynthesis with the increased oxidative stress due to generation of reactive oxygen species by the light-harvesting complex and electron transport. Study in both photosynthetic cyanobacteria (representing the evolutionary precursor to chloroplasts) and vascular plants has provided insight into copper delivery to chloroplasts.

In the model cyanobacterium *Synechocystis* PC 6883, the P-type ATPase CtaA transports copper into the cell across the plasma membrane, while the P-type ATPase PacS transports copper into the thylakoid lumen for incorporation into plastocyanin [112]. Key targets of copper delivery in chloroplasts are the copper-zinc superoxide dismutase (CSD2) in the stroma and

the electron carrier plastocyanin in the thylakoid lumen. Two P-type ATPases localized to chloroplasts were identified by defects in photosynthesis [113]. PAA1/HMA6 shows similarity to CtaA, while PAA2/HMA8 is similar to PacS. PAA1 is localized to the chloroplast envelope and loss-of-function mutation in this transporter shows a decrease in CSD2 activity and holoplastocyanin levels, consistent with envelope location [114]. PAA2 defects show increased CSD2 activity and decreased holoplastocyanin [113], consistent with the localization of PAA2 in the thylakoid membrane. Copper supplementation can overcome these phenotypes, while PAA1/PAA2 double mutant is seedling lethal and cannot be overcome with copper supplementation [113]. Curiously, copper is preferentially delivered to plastocyanin over CSD2, indicating the physiological necessity of copper delivery to this protein [113].

A third chloroplast copper ATPase, HMA1 was identified due to an increase in light sensitivity of the corresponding mutant. Like PAA1, HMA1 is localized to the chloroplast envelope; however, the N-terminal (presumably cytosolic) domain contains a histidine-rich region in place of the typical N-terminal CxxC-containing metal-binding domain. Like PAA1 mutants, plants deficient in HMA1 show reduced CSD2 activity as well as decreased ATPase activity in isolated envelope membranes [115]. HMA1 ATPase activity is specifically stimulated by copper, again indicating that this transporter is a route of entry for copper into chloroplasts. It has been proposed that HMA1 primarily supplies copper for CSD2, while PAA1 predominantly transports copper destined for the photosynthetic apparatus (i.e. plastocyanin).

Similarly to human system, the copper is thought to be delivered to the N-terminal domains of plant copper-transporting ATPases by copper chaperones. The interaction has been demonstrated for ScAtx1 with both CtaA and PacS. Structural data on heterodimers of these transporter-chaperone complexes provides strong indication of a defined shuttle role for chaperone interactions [16]. Although a chloroplast-targeted chaperone has been identified to supply CSD2 (AtCCS) in *Arabidopsis*, no stromal copper chaperone has been identified to date that transfers copper from PAA1 or HMA1 to PAA2.

Utilization of yeast for analysis of copper homeostasis

Saccharomyces cerevisiae has been an invaluable model system for identifying the major components of cellular copper transport machinery. The high affinity copper transporter, Ctr1 was first discovered in yeast. The identification of Cu-ATPase Ccc2p and of copper chaperones has followed outlining the network of key proteins involved in regulating the intracellular copper homeostasis. The yeast strains, in which individual components of copper transporting machinery are deleted serve as convenient background strains for complementation studies in which the functional activity of mammalian transporters can be evaluated. Overall, it is difficult to overestimate the important contribution of yeast system to understanding of copper biology in eukaryotic cells, yet there are important differences in the regulation of copper homeostasis in yeast and human cells, particularly with respect to Cu-ATPases.

The Ccc2 protein, a structural and functional homologue of ATP7A and ATP7B, is located in the Golgi and transports copper to the lumen of the secretory pathway where copper is incorporated into the extracellular domain of Fet3, a copper-dependent ferroxidase. Disruption of *ccc2* prevents copper incorporation into Fet3 resulting in iron deficiency [116]. Interestingly, unlike Cu-ATPases in bacteria and mammals, Ccc2 plays the role of copper transporter only in the secretory pathway. No significant effect on copper resistance or accumulation is observed in yeast cells with disrupted *ccc2*. Instead, the regulation of copper concentration in the cell is primarily controlled by Ctr1 and its homologues, which are endocytosed and degraded in response to copper elevation [117,118] preventing further copper accumulation.

Significant variations in copper metabolism pathways seem to exist among yeast strains. The pathogenic yeast *Candida albicans* has a higher resistance to elevated copper compared to

Saccharomyces cerevisiae. Weissman and colleagues reported the presence of a *Candida*-specific copper-transporting P-type ATPase, *CaCRP1* that plays an important role in the excretion of copper excess and hence imparting high copper resistance [119]. *CaCRP1* was also shown to be required for cell resistance to silver [120]. In contrast to mammalian Cu-ATPases, the yeast Cu-ATPases do not traffic and different gene products seem to mediate copper delivery to the secretory pathway and copper export across the plasma membrane. For example, the *Candida* Cu-ATPase *CaCrp1* is localized at the plasma membrane and does not rescue the $\Delta Ccc2$ phenotype. *Candida albicans* has also a Cu-ATPase, which is a functional homologue of *Saccharomyces* *Ccc2*, *CaCcc2*, and is functionally distinct from the copper exporter *CaCrp1*. Similarly to *S. cerevisiae* *Ccc2*, the *Candida* *CaCcc2* is localized at the Golgi network. Here, it mediates copper delivery to *CaFET3*, which is required for high affinity iron uptake and thus important for the survival of the pathogen when iron in the environment is limiting. Further studies of two Cu-ATPases in *Candida* may supply important insight into the complementary roles of the two mammalian Cu-ATPases ATP7A and ATP7B, especially in the tissues in which two Cu-ATPases are co-expressed.

Cu-ATPases are required for organism development in many species

The Cu-ATPase in *Caenorhabditis elegans* is CUA-1. It is expressed in the intestinal cells of the adult worm and in hypodermal cells of larva. Functionally CUA-1 complements *S. cerevisiae* $\Delta ccc2$ mutants. Similarly, to mammalian Cu-ATPases, CUA-1 receives copper from a copper chaperone. Cuc-1, the *C. elegans* homologue of the mammalian Atox1 transports copper to CUA-1. Precise functional properties of CUA-1 and its intracellular localization remain to be determined.

The copper homeostasis in the *Drosophila melanogaster* S2 cell line was studied in more details. The DmATP7 was identified as the sole Cu-ATPase in *Drosophila*. Inactivation of *DmATP7* significantly increased copper accumulation, demonstrating that DmATP7 was essential for the efflux of excess copper [121]. Recent studies revealed that *DmATP7* is required for the successful completion of embryogenesis, early larval growth and development, as well as adult pigmentation of the insect [122]. It was observed that is independently of dietary copper levels *DmATP7* is required for the early embryonic requirement of flies. Strikingly, the germline clone-derived embryos devoid of maternal *DmATP7*, though alive and fully developed, fail to hatch [122].

DmATP7 mutants show impaired neuronal function, emphasizing the important role of copper in CNS development and the key role that the Cu-ATPases play in this process. Another important role of DmATP7 is the delivery of copper into the secretory pathway for biosynthesis of copper-dependent enzymes. DmATP7 transports copper to the copper-dependent enzyme tyrosinase, which is involved in the pigmentation and sclerotization pathway. Disruption of endogenous *DmATP7* activity bleaches cuticle, indicating that *DmATP7* activity is required for the production of pigments. Clearly, DmATP7A is a multitasking Cu-ATPase, functionally similar to its human counterparts. The *DmATP7* mutant flies provide a phenotypic analogy to poor pigmentation and neuronal disfunction observed in human Menkes disease patients, which are devoid of functional Cu-ATPase ATP7A.

Recently, zebrafish has been utilized as a model to study the functions of *Calamity*, a zebrafish orthologue of ATP7A, in normal development [123]. Studies on *Cal* mutant showed that copper is essential for normal notochord formation and that copper availability is tightly controlled by Cu-ATPase. In mammalian embryonic connective tissue, mesenchyme, that influences notochord development, cuproenzyme lysyl oxidase is heavily expressed. In humans, synthesis of lysyl oxidase is dependent on copper incorporation by ATP7A [60]. On that basis, Mendelsohn and co-authors suggest that the abnormal notochord formation in *cal* arises from impaired lysyl oxidase activity. The notochord is one of the classifying characters of chordates

and has vital roles in vertebrate development as a skeletal element and in the patterning of the surrounding tissues [124]. Some of the vascular and neurologic abnormalities observed in the offspring of copper-deficient animals may result from patterning defects secondary to notochord abnormalities.

7. Conclusion

In recent years, there has been an explosion in identification of new members of the Cu-ATPase subfamily, and the list of these transporters is likely to grow. For most of these proteins, we have only initial data that point to their important functional role in the organism development and/or survival. The studies of structurally simpler members of the Cu-ATPase family, such as CopA, have already provided immensely useful information on the structure and mechanism of copper pumps. The analysis on human Cu-ATPase and other eukaryotic pumps revealed the breadth of physiological processes that require the functional activity of these transporters as well as sophisticated mechanisms that regulate the Cu-ATPases' function. With many new Cu-ATPases identified, some basic functional assays established, and the complex regulation uncovered, the field of Cu-ATPases is facing an exciting task of generating a comprehensive mechanistic picture of Cu-ATPases' function and regulation in the context of entire cell.

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Figure 1. The major functional domains of human Cu-ATPases

The transmembrane portion of Cu-ATPases is composed of 8 transmembrane segments (dark blue) that form intramembrane copper-binding site(s); the CPC, YN and MxxS motifs contribute to these sites. The ATP-binding domain is composed of the P-domain (light blue) and the N-domain (green) and together with the A-domain (turquoise) is responsible for enzymatic cycle (ATP binding, hydrolysis, phosphorylation, and dephosphorylation). The N-terminal domain has six metal (Cu)-binding subdomains (MBD1-6, red) and the very N-terminal 63 residues, that are involved in targeting of ATP7B in polarized hepatocytes. The LL letters indicate the di/tri-leucine motif.

Figure 2. Trafficking of human Cu-ATPases in polarized cells

The localization of Cu-ATPases in low (indicated by a thin arrow) and high (indicated by a thick arrow) copper has been described in polarized epithelial cells and tissues. Caco-2 cells serve as a model for the ATP7A trafficking in enterocytes; WIF cells and HepG2 cells are used to study the localization of ATP7B in polarized hepatocytes. The trafficking from the TGN to a vesicular compartment is observed for both ATP7A and ATP7B, however their destination differs. ATP7A traffics towards the basolateral membrane to facilitate copper export into the blood; in hepatocytes ATP7B traffics towards the apical membrane to export copper into the bile (for copper removal).

Figure 3. The involvement of various domains in the transport activity and trafficking of Cu-ATPases

(A). In the absence of copper, the N-terminal domain of Cu-ATPase interacts with the ATP-binding domain and down-regulates the enzyme activity [33]. (B) Atox1 transfers copper to the metal-binding sites 2; this leads to structural changes and allows further transfer of copper to the metal-binding site 4, which may in turn transfer copper to sites 5 and 6 [16,77]. (C) Recent data suggest that Atox1 may also transfer copper directly to the transmembrane sites, at least in CopA [125]. (D) The binding and hydrolysis of ATP causes the rearrangement of the domains and copper release into the lumen (as suggested in [36]). (E) In elevated copper, the N-terminal dissociates from the ATP-binding domain. This structural change is thought to increase the rate of copper transport into the lumen and expose sites for interaction with cellular trafficking machinery (the example of ATP7B is shown). (F1) The first 63 amino acids may regulate apical trafficking of ATP7B by interaction with specific targeting proteins [53]. (F2) Copper binding also regulates the phosphorylation of Cu-ATPases by kinases [38]. In yeast Cu-ATPase Ccc2, PKA phosphorylation appears to regulate activity [126], the role of the kinase-mediated phosphorylation in either activity or intracellular targeting of human Cu-ATPases is still unknown. The number of copper atoms need to be bound the N-terminal to regulate catalytic activity, kinase-mediated phosphorylation or trafficking remains to be determined

Table 1

Sub-cellular localization of Cu-ATPases and acceptor proteins in plants

Copper protein	Subcellular location	Proposed or defined ATPase required
Ascorbate oxidase, polyphenol oxidase, diamine oxidase	Apoplast (extracellular space)	HMA5, RAN1/HMA1
Ethylene receptor	Endoplasmic reticulum	RAN1/HMA7
Plastocyanin	Thylakoid lumen	PAA2/HMA8
Copper-zinc SOD (CSD2)	Chloroplast stroma	PAA1/HMA6, HMA1
Copper-zinc SOD (CSD1)	Cytosol, peroxisomes	None
Cytochrome C oxidase	Mitochondria	None