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Copper trafficking to the secretory pathway

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Abstract

Copper (Cu) is indispensible for growth and development of human organisms. It is required for such fundamental and ubiquitous processes as respiration and protection against reactive oxygen species. Cu also enables catalytic activity of enzymes that critically contribute to the functional identity of many cells and tissues. Pigmentation, production of norepinephrine by the adrenal gland, the key steps in the formation of connective tissue, neuroendocrine signaling, wound healing – all these processes require Cu and depend on Cu entering the secretory pathway. To reach the Cu-dependent enzymes in a lumen of the trans-Golgi network and various vesicular compartments, Cu undertakes a complex journey crossing the extracellular and intracellular membranes and staying firmly on course while traveling in a cytosol. The proteins that assist Cu in this journey by mediating its entry, distribution, and export, have been identified. The accumulating data also indicate that the current model of cellular Cu homeostasis is still a "skeleton" that has to be fleshed out with many new details. This review summarizes recent data on the mechanisms responsible for Cu transfer to the secretory pathway. The emerging new concepts and gaps in our knowledge are discussed.

1. Cu entry

1.1. CTR1 is a major Cu uptake system, but other pathways for Cu entry also exist

The widely-accepted model of cellular Cu homeostasis indicates that Cu entry into a cell is mediated by the high affinity Cu transporter CTR1 (Fig. 1). This model is supported by studies in the HEK293 cells, which demonstrated that in these cells CTR1 is responsible for the majority (~70%) of Cu intake.[1] Physiological importance of CTR1-mediated Cu uptake is further illustrated by embryonic lethality of the CTR1 knockout mice[2] and marked pathologic changes in a cardiac tissue of mice with a targeted deletion of CTR1 in heart.[3] In these latter animals, the loss of CTR1 function is associated with a severe copper deficiency. Other tissues show more complex responses to CTR1 inactivation, which indicate existence of additional mechanisms of Cu uptake. Inactivation of CTR1 in intestine does not prevent Cu entry into enterocytes from the lumen of the gut. Instead, the loss of CTR1 decreases Cu availability for the Cu-dependent enzymes and Cu-export systems within the intestinal cells. Retention of Cu in enterocytes and an impaired Cu efflux lead to a profound Cu deficiency in the rest of the body despite intestinal Cu overload.[4] By contrast, targeted inactivation of CTR1 in the liver decreases the hepatic Cu content by ~ 50% without having apparent pathologic consequences on either liver morphology or function.[5] In other words, some tissues have mechanisms to significantly supplement the CTR1- mediated Cu uptake.

The form(s), in which Cu enters the intestine and other tissues, are not necessarily the same. The environment of a gut lumen, especially pH, is very different from that of a bloodstream and thus likely to require engagement of a different type of transporters than those operating at the basolateral membranes of internal tissues. It is believed that the dietary Cu exists in an oxidized Cu(II) form and, therefore, it has to be either reduced or transported across the apical membrane as a Cu^{2+} ion or Cu(II) complex with small molecules or short peptides. It is worth noting that the lumen of the gut is inhabited by various species of commensal bacteria (fewer in a small intestine and many more in a colon) that secrete numerous metabolites. These metabolites may influence/modulate dietary Cu absorption directly by binding Cu or through complex signaling mechanisms, as was shown for other nutrients.[6] Identification of a Cu-binding siderophore yersiniabactin, a polypeptide-polyketide produced by infectious Yersinia species and uropathogenic *E. coli*[7] raises many interesting questions. For example, do members of the normal gut microbiota produce similar Cu chelating molecule(s) for their own Cu acquisition or protection? What is the role of a commensal flora in modulating the host's Cu uptake/reabsorption by the gastrointestinal tract, kidneys, or epithelia of airways? Does the composition/abundance of Cu binding metabolites change during infection, when the composition of gut microbiome changes? These questions have practical implications for treatment of infections and in the food industry, where antibiotics to control microbiota are often included into the poultry/cattle diets.

1.2. Small Cu carrier (SCC) could be an important source of Cu for CTR1

It is currently unclear in which form Cu exits the enterocytes to be transferred to tissues. As it will be discussed below, the Cu efflux form enterocytes is mediated, primarily, by the Cu-transporting ATPase ATP7A,[8] which transports a reduced Cu(I) (see ref. 9 for one recent review). Cu(I) is unstable in aqueous solutions and has to be either stabilized by binding to Cu(I) carriers or oxidized to a more stable Cu(II). Existence of oxidoreductase(s) that facilitate Cu exit and/or uptake has been postulated, but molecular identity has not been established. The members of STEAP family were suggested as candidates,[10] however their direct involvement with Cu fluxes remains to be demonstrated. Recent biochemical studies of the N-terminal region of CTR1 suggest that dedicated oxidoreductases may not be required for Cu uptake. Specifically, the extra-cellular N-terminal domain of CTR1 is highly enriched in His and Met residues (Fig. 2 left) and is likely act as a "sponge" for the exchangeable Cu in a serum. Experiments with the model peptides have found that the very N-terminal 13 aminoacids segment of CTR1 can bind both Cu(I) and Cu(II). Furthermore, Cu(II) can be reduced in the presence of reducing equivalents and in the absence of other proteins.[11] It was also found that the HisHis motif within the N-terminus of CTR1 binds Cu(I) in a pseudotetrahedral coordination environment. In addition to stabilizing Cu(I), this coordination environment renders Cu available for ligand exchange and further transfer to the CTR1 ion-translocation pathway.[11] The proposed "guiding" role for the HisHis motif is supported the structural model of CTR1, that is based on the available EM data.[12] This model shows a close proximity of the HisHis motif to the Met-rich entry of the Cu translocation pathway (Fig. 2, right).

In a serum, Cu is bound to both small molecules (<10 kDa) as well as high molecular weight carriers. Fractionation of human serum demonstrated that adults have approximately 600 ng ml^{-1} of Cu in fractions containing ceruloplasmin; 120 ng ml^{-1} of Cu in factions with transcuprein (β-macroglobulin), 150 ng ml^{-1} of Cu in fractions with albumin; and at 90 ng ml^{-1} Cu in low molecular weight fractions.[13] Which of these pools is the primary source of Cu for cellular uptake is unclear, and all of them may contribute under certain circumstances. Albumin represents 50% of the total serum proteome[14] and has a well characterized Cu(II) site, the so called ATCUN motif,[15] which is also found in other proteins such as hemoglobin.[16] However, physiologic significance of Cu binding to albumin or globulins is uncertain. Mice with genetic inactivation of albumin or a double knockout of alpha-macroglobulin and murinoglobulin-1 are healthy[17,18] and show no apparent signs of Cu misbalance, although careful measurements of Cu uptake in these mice have not yet been done. Ceruloplasmin, an abundant Cu-containing enzyme in the serum, which accounts for 70–90% of total serum Cu, was also discussed as a potential Cu carrier. However, inactivation of ceruloplasmin in mice alters iron fluxes and does not cause notable changes in the gastrointestinal absorption, hepatic uptake, or biliary excretion of Cu.[19] These results argue against an obligatory role of ceruloplasmin in Cu delivery to tissues.[19] Compensatory changes in Cu-containing protein pools may also explain the lack of effects on Cu homeostasis following inactivation of individual Cu-binding components.

Recent data suggest that a small Cu carrier(s), SCC, plays a significant role in regulation of Cu distribution between tissues. Although the low molecular weight Cu-containing components of a serum have been repeatedly observed,[13,20,21] identification of their molecular nature has been challenging. This is largely due to serum complexity, the lack of prominent spectral features in the SCC-Cu complexes and a relatively low binding affinity of SCC for Cu, which also makes SCC-Cu good candidates for an “exchangeable” Cu pool.

SCC was also identified in the urine of animals with Cu-overloaded livers.[22] In this latter study, the levels of SCC in the urine increased when Cu uptake by the liver decreased, suggesting that Cu-SCC was present in a serum and was, normally, taken in by the liver.[22] Size-exclusion chromatography showed that SCC was larger than a single amino acid or the Gly-His-Lys tripeptide,[22] which was previously isolated from the serum in a Cu-bound form and subsequently shown to act as a growth factor.[23,24] Mass-spectrometry of the SCC containing fractions confirmed that SCC co-elutes with molecules that had molecular mass of 1–2 kDa (our unpublished data). When added to HEK293 cells, SCC competed with the radioactive Cu for the uptake by Ctr1, further indicating that SCC could be a source of Cu for cells.[22] Finally, experiments with cultured cells first loaded with Cu and then washed and placed in a serum-free medium revealed that cells secrete Cu in a complex with SCC or the SCC-like molecule. This result is interesting, as it suggests that SCC interacts and forms complex with Cu within the cell, probably within the secretory pathway. It is tempting to speculate that the transporters, which transport various organic molecules and drugs may transfer SCC to the lumen of the ATP7A-containing vesicle allowing for the SCC-Cu complex assembly and subsequent secretion. From this point of view, a recent discovery of the role of ABCA12 transporter in Cu homeostasis in a dog liver is extremely interesting.[25] ABCA12 is regulated by glucosylceramides[26] and inactivation Minireview of ABCA12 in mice alters lipid metabolism in skin.[27] In dogs, loss of ABCA12 activity is associated with a

massive accumulation of Cu in hepatocytes pointing to a direct or indirect association between Cu and lipid efflux.

1.3. Candidate proteins for the low affinity uptake systems

The “low affinity” pathways, supplementary to CTR1, are likely to mediate transport of Cu(II) or Cu-complexes since this transport route is not inhibited by silver (an effective inhibitor of CTR1 and a Cu(I) competitor). The identity of the low affinity Cu transporters could be tissue-specific (*i.e.* different tissues may utilize different transporters) and their molecular identity in different tissues remains to be established. The Cu-transporter 2, CTR2, was initially thought to play a role in the low affinity Cu uptake across the plasma membrane, but accumulating data strongly point to its role in Cu transport in intracellular compartments[28] and in regulation of CTR1, to which it can bind.[29] The divalent metal transporter DMT1 has been proposed as a candidate for the low affinity Cu uptake system in intestine and other tissues, but its precise role is still uncertain. Expression in oocytes directly showed the ability of DMT1 to transport Cu(II),[30] and a stable expression of DMT1 in HEK293 cells was shown to increase Cu uptake and intracellular Cu accumulation.[31] In human umbilical vein endothelial cells down-regulation of CTR1 upregulates DMT1 and inactivation of both transporters completely blocked Cu uptake.[32] These data strongly suggest a compensatory role of DMT1 in maintaining a total cellular Cu content. At the same time, mice with global inactivation of DMT1 show no defects in their intestinal absorption of Cu when compared to the wild-type animals;[33] these results argue against a significant role for DMT1 in Cu homeostasis under regular circumstances. Studies in embryonic fibroblasts, human embryonic kidney cells, and Cos-7 cells also ruled out DMT as a major route of apical Cu uptake and, instead, identified chloride-dependent anion exchange systems as a potential mechanism for the low affinity Cu uptake.[34]

Recent genome-wide screening of HeLa cells illustrates challenges associated with identification of the low affinity Cu transporters. This study used the siRNA library to inactivate individual genes and then characterize changes in a cellular metal content by ICP-MS. Down-regulation of CTR1 (Cu import) and ATP7A (Cu efflux) was associated with the expected metal-specific decrease and increase of Cu content, respectively, and were taken as controls.[35] Down-regulation of several other transporters also caused changes in the cellular Cu levels, but these changes were often accompanied by changes in other metal ions raising questions about specificity of the transporters and/or the nature of their primary substrate (see example in Fig. 3).

Inactivation of chloride channel CLC7 was also associated with a lower cellular Cu content; however CLC7 is an intracellular Cu transporter and its direct role in Cu uptake seems unlikely. In yeast, the CLC system (Gef1) operates intracellularly to counteract vesicular acidification and is important for Cu binding to a Cu-dependent ferroxidase Fet3.[36] Interestingly, inactivation of Gef1 is associated with an apparent redox misbalance manifested as a poorer recovery of cells from oxidation despite their overall higher glutathione content.[36] Since glutathione facilitates Cu uptake,[37] the lower Cu content in human cells lacking CLC7 could be due to glutathione misbalance cased by CLC7 inactivation. These studies provide an excellent example of interconnection between the Cu

homeostasis and other cellular pathways as well as a cautionary note to interpretation of potentially indirect effects of genes' down-regulation.

Zn-transporters are attractive candidates for the role in the low affinity Cu(II) uptake. The ability of Cu(II) to inhibit zinc uptake across the basolateral membrane was demonstrated. Cu overload in human livers of patients with Wilson disease (WD) or in the animal model of WD alters expression of Zn transporters (Table 1) suggesting a potential involvement of Zn transport system in maintaining Cu balance. In the genome-wide metallomics study, down-regulation of the basolateral transporter SCL39A5 (Zip5) in HeLa cells was shown to decrease significantly both zinc and Cu content (Fig. 3), although the detailed follow up studies are needed to verify the results of the screen. Previous biochemical studies of the recombinant Zip5 showed inhibition of Zn uptake by "free" Cu(II) suggesting possible completion, although Zn^{2+} was clearly a preferred substrate for Zip5.[38] Altogether, these data suggest that Zn^{2+} transporters may be involved in transporting Cu under certain physiologic conditions. Systematic studies of the low affinity Cu transport and a better understanding of the nature of Cu carriers would help to identify molecules and pathways that supply 30-50% of cellular Cu. Table 1 lists the transporters that change their expression under conditions of Cu overload; the results of the genome-wide screen and effects of down-regulation of various transporters can be found at http://gladyshevlab.bwh.harvard.edu/ICPMS_Human/.

2. Cu trafficking within the cell

2.1. How does Cu exit CTR1?

CTR1 was discovered 15 years ago, and since then a great deal of information about its structure and function has been accumulated (for recent review see ref. 39). Yet, the mechanism of Cu transport by CTR1, especially Cu release into a cytosol, is far from being fully understood. The 3D structure of trimeric CTR1 closely resembles that of a gated channel with the His- Met-enriched extracellular domain employed for Cu binding, a narrow Met-rich "selectivity filter" – for guiding Cu(I) to the entry into the otherwise wide Cu-translocation pathway and a Cys/His ring at the narrow cytosolic portion of the pathway – to regulate Cu exit[12,40] (Fig. 2). The Cys-His ring is likely to have a higher affinity for Cu than the Met-rich "entrance" and may facilitate the vectorial movement of Cu within CTR1. How does the Cis/His ring open to let Cu out is unclear; one likely mechanism involves conformational changes triggered by Cu binding to the extracellular portion of the transporter.

The amount of Cu entering cells through CTR1 is low, because the transporter is non-abundant (HEK293 cells contain about 1000 copies per cell) and the transport rates are slow (10 ions per trimer per s).[41] Such slow rates of Cu transport are inconsistent with a mechanism involving formation on an open pore through which Cu ions can diffuse. The typical ion-channels have permeation rates close to free diffusion, *i.e.* orders of magnitude higher than the rates seen for CTR1. Thus, Cu either does not diffuse out of CTR1 on its own and needs to be retrieved (see below) or, as soon as Cu is out of the CTR1, the exit closes and has to be opened again for the next round of Cu transport. In either case the available structural model (Fig. 2) is likely to represent CTR1 is its closed, inactive state.

HEK293 cells have a radius of 7.9 micrometer,[42] which translates into a cell volume of approximately 2 pL. With CTR1 working at its full capacity (and no Cu export occurring) the intracellular Cu concentration would increase by approximately 8 nM s^{-1} and within a minute would increase by only 0.5 μM. Given that cells contain 1–10 mM glutathione, which binds Cu with a 10^{-11} M affinity,[43] all Cu entering cells is effectively buffered. In fact, the glutathione-mediated binding of incoming Cu may be one of the reasons for its ability to facilitate Cu uptake.[37] The current model (Fig. 1) suggests that Cu is retrieved from the initial entering pool by soluble cytosolic Cu shuttles or metallochaperones, which then transfer Cu to various destinations (Fig. 1). Cu chaperones CCS and Atox1 facilitate Cu delivery to the cytosolic Cu^{+}/Zn^{2+}-dependent superoxide dismutase SOD1 and the ATP-driven Cu transporters in the secretory pathway, respectively.[44] Recent studies demonstrated that the chaperones can bind to the plasma membrane and also directly interact with the Cu-bound CTR1.[45,46] It was also found that the synthetic 13 aminoacid residues peptide corresponding to the cytosolic C-terminal tail of CTR1 can bind Cu and transfer it to Atox1 *in vitro*.[47] These observations seem to support the model of Cu release from CTR1 mediated by protein–protein interactions between the transporter and the chaperones. However, inactivation of CCS or Atox1 is not detrimental to the cellular Cu uptake and down-regulation of both Cu chaperones does not noticeably alters the rates of CTR1-mediated Cu transport.[37] Thus, it is not clear at which point of Cu entry the chaperones become engaged. It cannot be ruled out that interactions between the chaperone(s) and CTR1 serve important regulatory function and control Cu occupancy of the intracellular Cu binding sites. The existence of the regulatory sites is suggested by the Cu-dependent trafficking of CTR1. Specifically, elevated Cu triggers CTR1 endocytosis from the plasma membrane,[41,48,49] and, when Cu levels decrease, CTR1 returns to the plasma membrane. The endocytic step depends on the CTR1 ability to transport Cu,[41,48,49] and therefore endocytosis is caused by Cu accumulation inside the cell. It seems possible that intracellular sites in CTR1 may "sense" high cytosolic Cu and respond by changing protein conformation, as was shown for yeast CTR1,[50] allowing engagement of trafficking machinery. The CTR1 residues M117, His120, and M126, located in the vicinity to each other in the flexible cytosolic loop (Fig. 2), are interesting candidates for the role of the intracellular Cu sensor sites.

2.2. Cu transfer to SOD1 and COXIV involves protein–protein interaction and redox reactions

While the precise functional consequence of interactions between the Cu chaperones and CTR1 are still being examined, accumulating data leave little doubt that the cellular roles and the mechanism of Cu chaperones are more complex than initially thought. Transfer of Cu from CCS to SOD1 has been explored *in vitro* and in cells in great detail[51-53] and is best understood (for review see ref. 54). After forming specific complex with SOD1, CCS facilitates not only the insertion of a Cu cofactor, but also formation of an important disulfide bond. This latter function depends on availability of oxygen which serves as a source of oxidizing equivalents[52,53] and is performed by Cu-CCS (and not by apo-CCS). Cu transfer to cytochrome *c* oxidase located in the mitochondria inner membrane is even more complex, as it requires moving Cu across the outer membrane of mitochondria and a subsequent incorporation of Cu into two different sites of cytochrome *c* oxidase formed by its membrane-embedded subunits COX1 and COX2.[55] The fascinatingly intricate process

involves both soluble (Cox17 and Cox11) and membrane bound proteins (Sco1, Sco2) with distinct and essential roles in activation of cytochrome c oxidase.[56-59] Recent studies identified additional factors: CoA6, which works together with Sco1,[60,61] and Cox19 that interact with Cox 11 and Sco1.[61] It has also become apparent that Cu incorporation into cytochrome c oxidase involves redox reactions coupled with the transfer of Cu between the chaperones.[56,62]

2.3. The Atox1-mediated Cu transfer to the Cu-transporters ATP7A/7B is well studied but many questions persist

The delivery of Cu to the secretory pathway is mediated by the small cytosolic protein Atox1, which binds Cu with $K_D = 2.1(2) \times 10^{-18}$–$6.3 \times 10^{-19}$ M depending on pH.[63] At the first glance, the Atox1-mediated Cu transfer is a simple process compared to Cu delivery to SOD1 or COXIV. The experiments on Atox1 inactivation in cells confirmed its role in activation of the Cu transporters ATP7A and ATP7B, which reside and operate in the secretory pathway.[64,65] Numerous studies have demonstrated the ability of Atox1 to exchange Cu with the structurally similar metal binding domains (MBDs) of ATP7A and ATP7B (for recent reviews see ref. 66 and 67). However, it remains completely unclear how Cu migrates from the cytosolic MBDs to the sites within the membrane portion of the transporters. In fact, it is still unknown whether Cu, which is bound to the cytosolic MBDs of ATP7A and ATP7B, is transported at all or it only regulates the transporters activity without being translocated across the membrane. Studies using the archeal Cu ATPase CopA and the Cu-binding domain of the presumed metallochaperone CopZ (structurally similar to Atox1) led to the suggestion that the chaperone docks at a highly conserved membrane-embedded region of the Cu-ATPases (called “a platform”) and directly transfer Cu to the membrane site(s). This hypothesis is supported by the mutational studies[68] and is appealing, as it explains how Cu enters the Cu translocation pathway. Further experimental evidence using the full-sized LCopA and CopZ (instead of the artificially truncated proteins) and similar experiments/findings in the mammalian systems would further strengthen the model. If these findings are confirmed for the human Atox1/Cu-ATPase relay, the current model of how Atox1 operates would have to be significantly updated to explain how Atox1 detects structurally dissimilar regions in the protein (“the platform” and MBDs), in what order the metal-binding sites are being filled and, importantly, how “the platform” region of the Cu-ATPases discriminates between the apo- and Cu bound chaperone.

2.4. Atox1 is a redox sensitive molecule

Recent data add further intriguing complexity to the Atox1- driven Cu delivery to the secretory pathway. It was shown that glutaredoxin Grx1 is required for the transport activity and intracellular trafficking of ATP7A and ATP7B[69] and that Grx1 binds Cu with high affinity, at least *in vitro*.[63] It was originally suggested that Grx1 removes glutathione from MBDs of the transporters priming them for Cu binding. More recent studies suggest that Grx1 is involved in regulation of the oxidation state of Atax1.[63,70] The Cu-binding site of Atox1, Cys-Gly-Gly-Cys, is sensitive to changes in the ratio of the reduced and oxidized glutathione (GSH : GSSG). The metal-coordinating cysteines of Atox1 undergo reversible oxidation forming a disulfide bond within the physiological range of redox potentials set by the GSH: GSSG pair.[70] Grx1 influences the redox status of Atox1 by increasing the rate of

Atox1 reduction by glutathione, although the precise mechanism needs further study. In one report, Grx1 was shown to facilitate reduction of Atox1 by glutathione,[70] whereas another study reported a more complex behavior.[63] Specifically, Grx1 was show to catalyze oxidation of Atox1 with GSSG in the absence of Cu and also reduction of oxidized Atox1 by GSH, but only if Cu was present.[63] Altogether, the redox sensitivity of Atox1 and the involvement of Grx1 in Cu homeostasis raise questions about the nature of physiological processes employing redox regulation of Cu transport to the secretory pathway. Recent studies of neuronal differentiation and spinal cord development provide first insights.[71]

2.5. Atox1 is a redox-sensitive Cu router in differentiating neurons

Dynamic changes in the amount of glutathione and glutathione oxidation (GSH: GSSG) are among numerous metabolic changes that occur upon cell differentiation and organogenesis.[72,73] Reductive and oxidative shifts in the status of the GSH/GSSG pair are cell-type specific and contribute to development of unique cell functionalities.[72] Upon differentiation, utilization of Cu by different cellular compartments also changes to accommodate increased levels of Cu-binding proteins and/or an altered number of organelles, such as mitochondria.[71,74,75] Similarly to redox changes, changes in the demand for Cu by different compartments are cell-type specific. In differentiating neuronal cells, the largest use for Cu is within the secretory pathway, where several Cu-dependent enzymes are significantly upregulated.[71] How do neuronal cells shuttle Cu where it is needed most? Recent evidence points to the central role of Atox1 in this process. In proliferating cells, the GSH:GSSG ratio is lower compared to differentiated cells and this is associated with a partial oxidation of Atox1. increasing the GSH :GSSG ratio upon differentiation causes complete reduction of the Cys-Gly-Gly-Cys site in Atox1, increasing the Cu binding capacity of this chaperone and thus making more Cu available for the secretory pathway.[71] Whether normal physiological fluctuations in a cellular redox balance have similar influence on the Cu-binding/delivery properties of CCS or modulate activity of the redox-sensitive Cu chaperones in mitochondria is a fascinating question that remains to be explored.

3. Cu transport through the secretory pathway

3.1. Cu transport to the secretory pathway enables Cu homeostasis along with activation of enzymes critically involved in metabolism and signaling

The Cu-transporting ATPases ATP7A and ATP7B are indispensible for human Cu metabolism. ATP7A is expressed in the majority of tissues and plays a central role in the dietary Cu uptake,[8] transfer of Cu from the choroid plexus into the brain,[76] and activation of Cu dependent enzymes within the secretory pathway. ATP7B activity is most important in the liver (Fig. 1) where it maintains hepatic (and systemic) Cu levels by exporting excess Cu into the bile. In hepatocytes, ATP7B also delivers Cu to the Cu-dependent feroxidase ceruloplasmin that undergoes its functional maturation within the trans-Golgi network.[77] This latter function is not unique for ATP7B; in macrophages, Cu delivery to ceruloplasmin is mediated by ATP7A.[78] The physiological roles of ATP7A and ATP7B are often discussed in the context of Cu export out of the cell, which is necessary to avoid toxicity. This function is essential and was been explored in detail, whereas an equally important role of Cu-ATPases in activation of various Cu dependent enzymes is much less studied and is poorly

understood. Strong evidence exists for the role of ATP7A in activation of peptidyl-alpha-monooxygenase,[79,80] tyrosinase,[81] and superoxide dismutase 3.[82,83] The role of ATP7B in Cu transfer to any Cu-dependent enzymes, except ceruloplasmin, is unclear. In the intestine, ATP7B may deliver Cu to ceruloplasmin homologue, hephaestin, because inactivation of ATP7A does not impair iron uptake, in which hephaestin plays an important role.[8] In other words, in the absence of ATP7A, hephaestin is likely to receive its Cu-cofactor from ATP7B, although the role of ATP7B in hephaestin activation under normal physiologic conditions remains to be formally demonstrated. In many cells and tissues, where both ATP7A and ATP7B are co-expressed (see an example below), their individual roles in activation of Cu enzymes remain unclear. Do the Cu ATPases transfer Cu to the enzymes in the distinct cellular compartments or, rather, one Cu-ATPase plays a primary role in the activation of Cu-dependent enzymes, whereas another Cu-ATPase regulates Cu levels in a cell and thus has indirect influence? Further studies are needed to answer this question.

3.2. Localization of ATP7A and ATP7B in a cell is regulated by various signals and involves a kinase-mediated phosphorylation

Cu regulates the activity of the secretory pathway (Cu secretion *versus* Cu utilization by enzymes) by modulating the intracellular localization of the Cu-transporting ATPases (for reviews see ref. 84 and 85). In the vast majority of cells, characterized so far, ATP7A and ATP7B are targeted to the trans-Golgi network (TGN). Upon Cu elevation, ATP7A and ATP7B traffic to vesicles and then to the basolateral and apical plasma membrane, respectively. Cu depletion results in the redistribution of ATP7A and ATP7B from the vesicles back to the TGN. Intracellular localization may change during organ development, as was shown for ATP7A in kidneys.[86] In the non-epithelial cells and in tissues, where Cu fluxes are low, additional signals for trafficking of Cu-ATPases exist. Hormonal signaling was shown to trigger movement of ATP7A from the TGN to the plasma membrane;[87,88] another example is trafficking of ATP7A to presynaptic membrane in response to activation of NMDA receptor and resulting calcium signaling.[89] In macrophages, hypoxia alters both the expression of the Cu transport proteins as well as localization.[78]

The mechanism of Cu dependent trafficking of either ATP7A or ATP7B is best characterized. Both ATP7A and ATP7B have numerous sites for kinase-mediated phosphorylation at their N-terminal and C-terminal tails. Phosphorylation of different residues may serve distinct purposes, and be involved in regulation of trafficking or/and modulation of transport activity in response to various stimuli.[90] Elevation of cytosolic Cu was shown to increase the kinase-mediated phosphorylation of the transporters.[90-93] For ATP7B, Cu-dependent kinase-mediated phosphorylation occurs prior to ATP7B exit from the TGN and requires a long-range communication between the transmembrane portion of ATP7B and its very N-terminal segment.[94] The N-terminal domain is a target of Cu-dependent phosphorylation, which is accompanied by structural changes within the domain that in turn influence the inter-domain interactions.[90,92] Changes in protein conformation are likely to expose the regions in Cu-ATPase that are involved in interactions with trafficking machinery. Communication between the N-terminal Cu-binding domain and the C-terminal region that contains the invariant dileucine motif important for trafficking has been demonstrated.[90] Recent studies also indicate that both ATP7A and ATP7B interact with the

heterotetrameric adaptor protein AP1 (an important regulator of protein trafficking within the secretory pathway) *via* their C-terminal dileucince motifs.[95,96]

Although largely uncharacterized, the molecular mechanisms underlying regulation of ATP7A and ATP7B by hormones and metabolites deserve particular attention. This is because the Cu-independent regulation is likely to represent a very significant component of Cu homeostasis. With the exception of intestine, liver, placenta, and mammary gland, most tissues do not experience significant fluctuations in Cu levels. By contrast, many tissues produced Cu-dependent enzymes, the activity of which have significant local and systemic effects and, therefore, must be tightly regulated. For example, the enzymatic activity of peptidyl-alpha-monooxygenase, dopamine beta hydroxylase, superoxide dismutase 3, amino oxidase 3 yields products with important physiological functions: neurotransmitters, neuroendocrine peptides, H_2O_2. Currently, little, if any information, is available on the feed-back regulation of Cu transporters by such metabolites; this area is an exciting and important frontier for future research.

3.3. Tyrosinase assay

Tyrosinase is a Cu-dependent enzymes that is involved in melanogenesis and chatecholamine synthesis pathways.[97] Tyrosinase is a glycosylated membrane protein with one transmembrane domain and two Cu ions in the active site. Human tyrosinase uses dioxygen to hydroxylate tyrosine with a formation of dihydroxyphenylalanine (DOPA) and then oxidative DOPA to form DOPA quinone; the product has dark color and can be easily detected in cells. Mutations in the TYR gene cause oculocutaneous albinism type 1.[98] Petris and co-workers were first to demonstrate that tyrosinase was inactive in skin cells lacking functional ATP7A (Menkes disease fibroblasts) and that co-expression of ATP7A and tyrosinase results in activation of tyrosinase and production of the pigment, L-DOPA.[81] This experimental system is now commonly used to evaluate the Cu-transport activity of ATP7A and ATP7A mutants. The tyrosinase assay was also used to measure activity of ATP7B in fibborblasts derived from patients with Menkes disease, and the results indicate that ATP7B can activate tyrosinase. In skin, ATP7A is thought to be the primary Cu transporter that delivers Cu to tyrosinase. This model is consistent with hypopigmentation observed in Menkes disease patients and animals lacking ATP7A. By contrast, Wilson disease patients have darkening skin,[99] indicative of increased melanin production. The mechanism of this phenomenon is currently unknown. In retinal pigment epithelium both ATP7A and ATP7B are expressed.[100,101] Studies of macular mice (a murine model of Menkes disease) revealed a complex picture: these animals have altered functional integrity of retina, which was partially reversed by Cu-chelation therapy.[102] Thus, specific roles of ATP7A and ATP7B in activation/regulation of tyrosinase activation *in vivo* need to be further explored.

3.4. Inactivation of ATP7A causes intracellular Cu redistribution and changes in glutathione balance in mitochondria

Consequences of inactivation of either ATP7A or ATP7B at the organism level are well known. Loss of ATP7A function in Menkes disease patients is associated with Cu deficiency in most tissues due to diminished Cu efflux from intestine and loss of activity of Cu-dependent enzymes. Kidneys represent an interesting exception – they accumulate Cu

despite low systemic Cu levels. The reason behind this phenomenon is not clear; one possible explanation could be that kidneys are the primary acceptors of small amounts of Cu that exit the intestine through the non-ATP7A related mechanisms. Cu accumulation is also seen in a mouse model of X-linked spinal muscular atrophy,[103] where ATP7A is selectively inactivated in motor neurons. This genetic intervention causes Cu accumulation in neurons without significant changes in Cu levels else-where. In Wilson disease (ATP7B inactivation), Cu accumulates initially in the liver and then later in the brain and some other tissues, yet some tissues (for example, adrenal gland) show functional Cu deficiency.[104] Altogether, these observations suggest that inactivation of either ATP7A or ATP7B is associated in a spectrum of cellular and systemic effects and changes in Cu levels are tissue and cell-specific.

At the molecular levels, the best characterized consequences of ATP7A/7B inactivation are changes in the intracellular Cu distribution. In skin fibroblasts isolated from patients with Menkes disease, Cu accumulates in the cytosol, nuclei, and mitochondria. This observation illustrates the primary role of ATP7A in maintaining the cellular Cu balance and also suggests that the transport of Cu to the secretory pathway restricts Cu entry to other compartments.[105] One may conclude that CCS in the cytosol and the Cu chaperones for mitochondria have to compete with Atox1 for their quota of Cu. This conclusion is somewhat problematic because the mitochondria respiratory function is paramount for cell survival, whereas cells with a disrupted Cu flow to secretory pathway can survive and proliferate. More likely, mitochondria have a high affinity but low capacity Cu-delivery/uptake system, which is saturated at low Cu levels and is sufficient to provide Cu to the only one Cu-dependent enzyme in this compartment – COXIV. The rest of the Cu in cytosol would bind to Atox1 and CCS, which might have lower affinity than the mitochondria Cu-shuttles, but be more abundant. This hypothesis awaits further testing.

In fibroblasts, the loss of Cu transport to the secretory pathway causes significant changes in the redox environment of several cell compartments; these change do not seem to directly correlate with changes in the Cu levels in these compartments. An increase in Cu concentration is comparable in the nucleus, cytosol, and mitochondria of ATP7A$^{-/-}$ cells, but mitochondria shows more dramatic changes in their redox environment, as indicated by the diminished ratio of the reduced to oxidized glutathione and increased levels of peroxide.[105] Although mitochondria sensitivity to high Cu is a well-known phenomenon, it is not obvious why mitochondria are especially sensitive. Cu can significantly alter the formation of Fe-S clusters, which is an important mitochondria function, and/or inhibit the characteristic redox-dependent Mia40-mediated protein translocation pathway. The human ATP7A$^{-/-}$ skin fibroblasts as well as Atox1$^{-/-}$ mouse embryonic fibroblasts are extremely sensitive to glutathione depletion[70,105] and diminished levels of glutathione and a lower GSH : GSSG ratio would negatively affect both Fe-S cluster formation as well as Mia 40 transport. Understanding of the precise functional consequences of Cu overload in mitochondria is an important subject of future research because structural and functional abnormalities in mitochondria may significantly contribute to pathogenesis of human disorders of Cu metabolism.

4. Conclusions

Cu transfer to the secretory pathway is a sophisticated process orchestrated by both soluble and membrane proteins. Emerging data suggest existence of additional important players and regulatory pathways that may utilize metabolic feedback loops. Discovery and characterization of the new players and pathways is necessary for understanding of both normal physiological and pathologic processes associated with Cu homeostasis in human cells and tissues.

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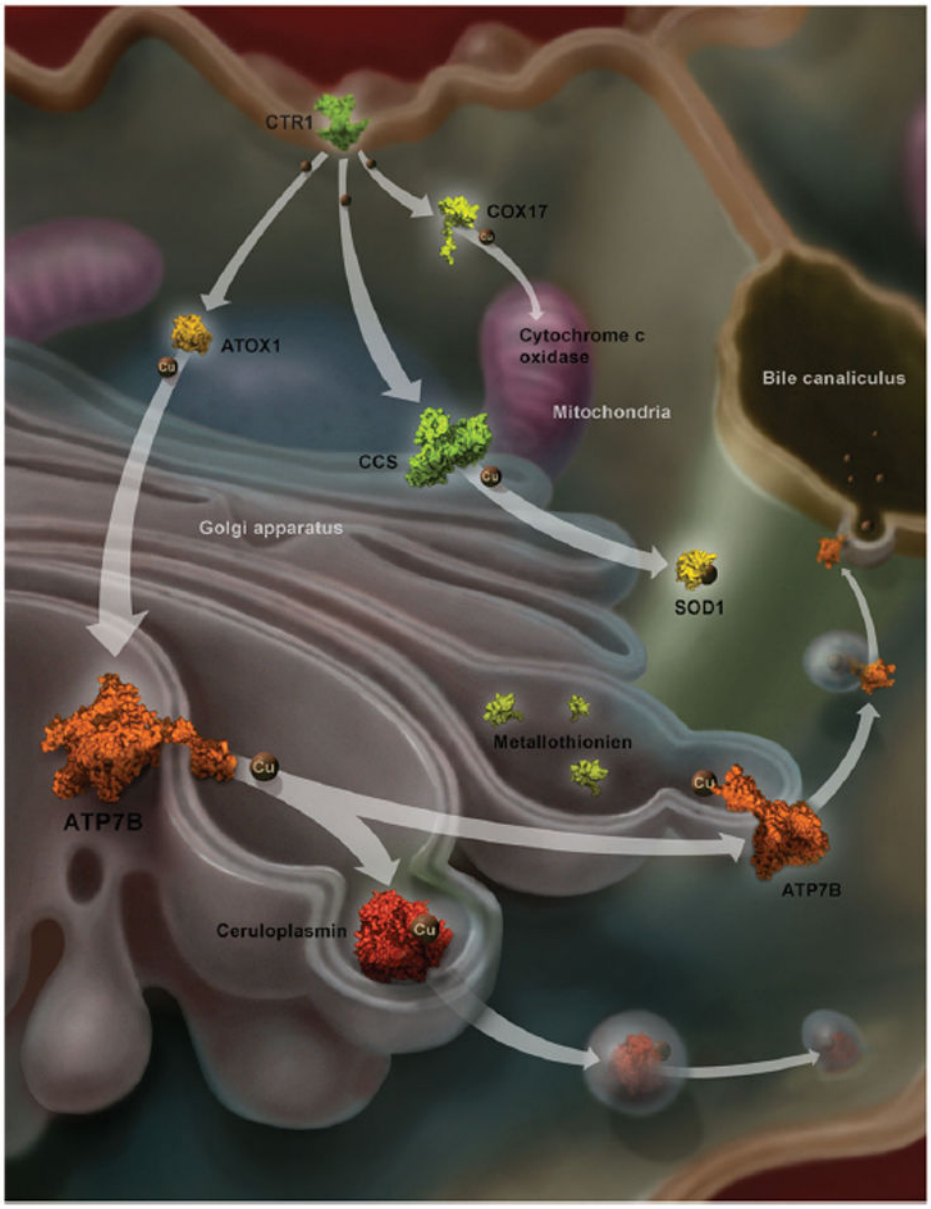

Fig. 1.

The major pathways of Cu distribution in human cells. The cartoon illustrates the main Cu distribution pathways in hepatocytes. The high affinity Cu transporter CTR1 at the basolateral membrane is responsible for at least 50% of Cu entering the cell. The low affinity transporters are unknown. Upon entry, Cu may bind transiently to glutathione and be retrieved from glutathione or directly from CTR1 by cytosolic Cu chaperones. Cytosolic Cu chaperone for SOD1 in a Cu-bound form facilitates Cu incorporation into SOD1 and the formation of an important disulfide bond. Atox1 binds Cu within the Cys-Gly-Gly-Cys metal binding site, which is sensitive to changes in redox environment. Increase in the GSH : GSSG ratio increases the reduction of Atox metal-binding site, enabling more Cu binding to Atox1 and thus increasing the delivery of Cu to the secretory pathway. In the TGN, Cu-

transporting ATPase ATP7B transfers Cu to ceruloplasmin, which is then secreted across the basolateral membrane. Cu elevation triggers trafficking of ATP7B from the trans-Golgi network toward the apical membrane (ATP7A in other cells traffics to basolateral membrane in response to high Cu), thus facilitating export of excess Cu. Complex set of soluble and membrane-bound chaperones facilitates Cu transfer to mitochondria.

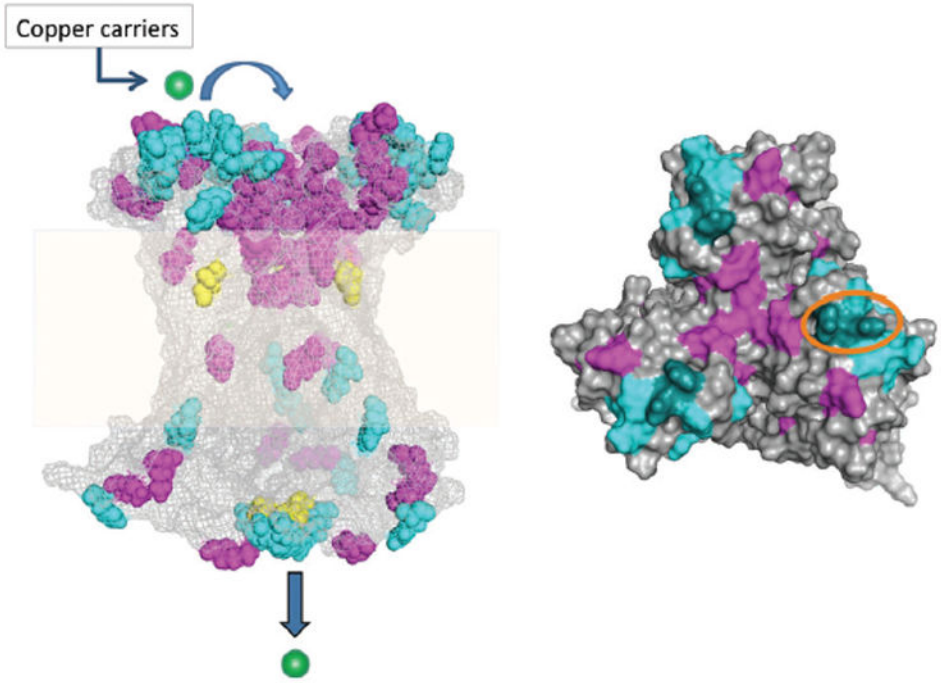

Fig. 2.

Structural model of CTR1. Left: A side view of the CTR trimer with the Met residues shown in purple, Cys in yellow, and His in cyan. Extracellular Cu (green ball) enters the transporter through the Met-rich selectivity filter (either directly or *via* intermediate binding to the HisHiscontaining site in the N-terminus of CTR1) and is directed through the wide vestibule of the transporter towards the narrow Cys/His-based ring at the cytoplasmic side. The release of Cu from CTR1 is driven either by conformational changes that disrupt the Cis/His ring or by interaction with the cytosolic molecule(s). Right: The top view of the Ctr1 trimer. The HisHis pair located in the vicinity of Met-rich entry is indicated by dark cyan and is circled. The images are generated using CTR1 pdb coordinates generated by Tsigelny and co-workers.[12]

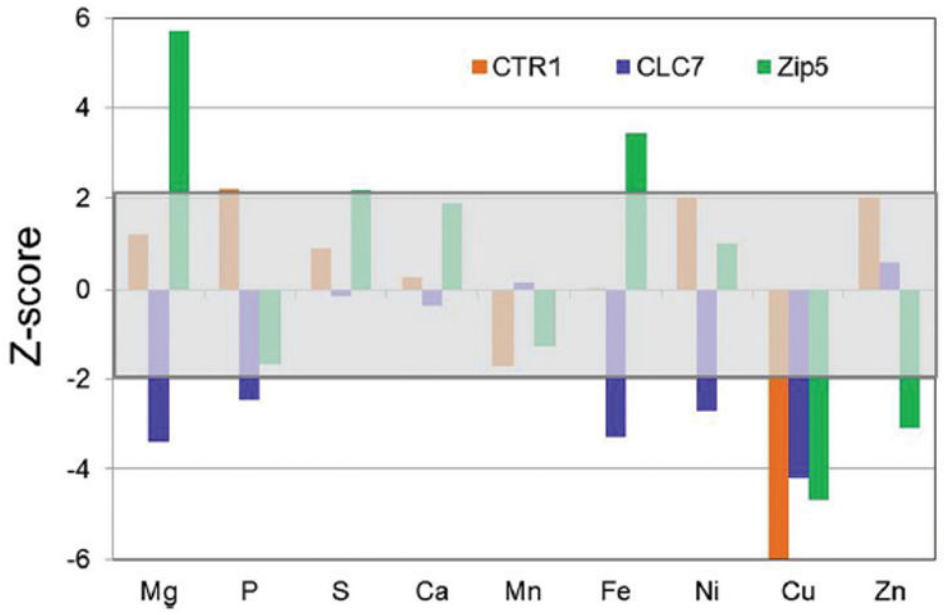

Fig. 3.

Changes in the intracellular levels of metals in response to down regulation of specific transporters. The *Z*-score higher than plus 2 or lower than minus 2 indicates a significant change; a variation within this range (marked by a gray block) is insignificant. Inactivation of CTR1 (positive control) is associated with a selective decrease of Cu levels in cells. Inactivation of CLC7 (the intracellular chloride transporter) lowers Mg^{2+}, iron, and Cu levels; inactivation of Zip5 (Zn^{2+}-uptake transporter) significantly decreases the cellular Cu content and zinc levels, but also results in increases of iron and Mg^{2+} levels. The data illustrate importance of determining the status of entire metallome following inactivation of transporters with an unknown specificity.

Table 1

Transporters with higher (A) and lower (B) expression in human Wilson' disease liver compared to control livers

Gene ID	Protein name	Transported solute
(A)		
AL518391	Aquaporin 1 (Colton blood group)	H_2O
AK025062	SLC12, member 2	Na, K/Cl
NM_000492	CFTR	Cl
AI638295	Chloride intracellular channel 6, CLC6	Cl
BF247552	SLC38A1	Glutamine
U51478	Na^+/K^+ ATPase, beta 3	Na/K
NM_018658	KCNJ16 channel	K
NM_000165	Gap junction protein, alpha 1, 43 kDa	Solutes
NM_006931	SLC2A 3	Glucose, sugars
AF034607	Chloride intracellular channel 1, CLC1	Chloride
NM_000701	Na^+/K^+ ATPase, alpha 1	Na/K
NM_004731	SLC16A7 transporter 2	Pyruvate, lactate
AW068936	ATP11A	Phospholipid?
BG150485	SLC 6A6 (2-aminoethanesulfonic acid)	Taurine and b-alanine
BC000006	Na^+/K^+ ATPase, beta 1 polypeptide	Na^+/K^+
NM_004207	SLC16A 3	Monocarboxylic acid
NM_01421	GABRP (GABA receptor)	Cl
NM_002560	Purinergic receptor P2RX4	Ca^{2+}
AI539710	ABCC1	As-triglutathion
(B)		
BF514158	Potassium channel, subfamily J, member 8	K^+
N80922	SLC 35 (dual transporter), member D1	UDP-glucuronic acid and UDP-N-acetylgalactosamine
NM_000443	SLC22A 7	Organic acid
N30257	SLC16A 10	Aromatic amino acid
NM_003051	SLC16A 1	Monocarboxylic acid
AW268880	SLC25A13	Aspartate, glutamate
AL136828	SLC41A 2	Magnesium
BF433180	Na^+/H^+ exchanger domain containing 2	Na^+/H^+
NM_030777	SLC 2, member 10	Glucose
NM_173596	SLC 39, member 5	divalent metal ions
NM_018043	Anoctamin 1, Ca-activated chloride channel	Cl
NM_000166	Gap junction protein, beta 1, 32 kDa	Solutes, metabolites
NM_006672	SLC 22A 7	Organic anion
NM_001859	SLC 31A1	Cu
AA834576	Inositol 1,4,5-triphosphate receptor, type 2	Ca^{2+}
AB040120	SLC 39, member 8	Zn^{2+}
BC001689	SLC 25A20	Carnitine/acylcarnitine

Gene ID	Protein name	Transported solute
NM_020980	AQP9	Glycerol
NM_006841	SLC 38A 3	Glutamate
AA876372	SLC 7A2	Cationic amino acid
AL157452	SLC 1, member 2	Glutamate
NM_014252	SLC25, member 15	Ornitine
R52647	Ca++ATPase, plasma membrane 2	Ca^{2+}
AW235061	SLC 1 member 1	Glutamate
AY008285	SLC25A18	Glutamate
AF153330	SLC 19, member 2	Thiamine
AI074459	ABCC6	Lipid, bile acid
AF098951	ATP-binding cassette, sub-family G (WHITE), member 2	Retinal
NM_006517	SLC16A2	Thyroid hormone
	SLC21A6	Taurochlate, steroid conjugates prostaglandin E2
BF475862	ATPase, class VI, type 11C	Lipids (?)
BC020618	ABCB4/MRP3	Lipid, drugs
AI272941	TRPM8 channel	Cations
NM_007168	ABCA8	Drugs
NM_019844	SLC1B3	Organic anion
AA777852	SLC 22, member 7	Organic anion
AI797218	SLC 13, member 5	Na citrate
AI003579	SLC 6, member 1	GABA
NM_003049	SLC 10, member 1	Bile acid
NM_021614	K^+ channel, subfamily N, member 2	K^+
NM_000340	SLC 2, member 2	Glucose
AF193836	SLC38, member 4	Amino acid
M_005835	SLC17, member 2	Phosphate
NM_018242	SLC 47A1	Flavonoid
NM_003057	SLC22A1	Choline, histamine, epinephrine, adrenaline, noradrenaline, dopamine