Review

Maria Matson Dzebo[a], Candan Ariöz[a] and Pernilla Wittung-Stafshede*

Extended functional repertoire for human copper chaperones

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Abstract: Copper (Cu) ions are cofactors in many essential enzymes. As free Cu ions are toxic, most organisms have highly specialized Cu transport systems involving dedicated proteins. The human cytoplasmic Cu chaperone Atox1 delivers Cu to P_{1B}-type ATPases in the Golgi network, for incorporation into Cu-dependent enzymes following the secretory path. Atox1 homologs are found in most organisms; it is a 68-residue ferredoxin-fold protein that binds Cu in a conserved surface-exposed CXXC motif. In addition to Atox1, the human cytoplasm also contains Cu chaperones for loading of superoxide dismutase 1 (i.e. CCS) and cytochrome c oxidase in mitochondria (i.e. Cox17). Many mechanistic aspects have been resolved with respect to how Cu ions are moved between these proteins. In addition to the primary cytoplasmic Cu chaperone function, all three cytoplasmic chaperones have been reported to have other interaction partners that are involved in signaling pathways that modulate cell growth and development. These new discoveries imply that humans have evolved a highly sophisticated network of control mechanisms that connect Cu transport with cell regulatory processes. This knowledge may eventually be exploited for future drug developments towards diseases such as cancer and neurodegenerative disorders.

Keywords: Atox1; cell signaling; copper transport proteins; growth and development; transcription factor.

[a]**Maria Matson Dzebo and Candan Ariöz:** These authors contributed equally to this article.
***Corresponding author: Pernilla Wittung-Stafshede,** Department of Biology and Biological Engineering, Chalmers University of Technology, S-41296 Gothenburg, Sweden,
e-mail: pernilla.wittung@chalmers.se
Maria Matson Dzebo and Candan Ariöz: Department of Biology and Biological Engineering, Chalmers University of Technology, S-41296 Gothenburg, Sweden

Introduction: copper transport in the human cytoplasm

Cu is found in the active sites of essential proteins that participate in cellular reactions such as respiration, antioxidant defense, neurotransmitter biosynthesis, connective-tissue biosynthesis and pigment formation (1–3). The redox ability (switch between Cu^{+} and Cu^{2+}) of Cu allows Cu-containing proteins to play vital roles as electron carriers and redox catalysts in living systems. To avoid toxicity of free Cu ions, the intracellular concentration of Cu is regulated *via* dedicated proteins that facilitate its uptake, efflux, as well as distribution to target Cu-dependent proteins and enzymes (4–6). In humans, Cu^{2+} is reduced to Cu^{+} prior to cell uptake. Cu^{+} ions then enter the cell via the membrane-bound Cu importer Ctr1 (Figure 1A). The cytoplasm is a highly reducing environment so the Cu ions remain in the 1+ form throughout cytoplasmic transport. After release from the C-terminal cytoplasmic tail of Ctr1, Cu is transported by the 68-residue Cu chaperone Atox1 in the cytoplasm for delivery to cytoplasmic metal-binding domains of ATP7A and ATP7B (also called Menke's and Wilson disease proteins, respectively), two homologous multi-domain P_{1B}-type ATPases located in the trans-Golgi network (4–6). These ATPases use ATP hydrolysis and conformational changes to transfer Cu ions through the membrane to the lumen of the Golgi where loading of target proteins occurs. Many human copper-dependent enzymes such as the blood clotting factors, tyrosinase, lysyl oxidase and ceruloplasmin acquire Cu from these ATPases before reaching their final destination in the body (4–6).

In addition to the Atox1 pathway, there are at least two other distinct paths for Cu transport in the cytoplasm (Figure 1A). In one, the Cu chaperone for superoxide dismutase 1 (CCS) directs Cu to cytoplasmic Cu/Zn superoxide dismutase 1 (SOD1) and, in the other, Cu is transported to the mitochondria by the Cu chaperone for cytochrome *c* oxidase (Cox17) and, with help from a number of additional proteins, Cu is incorporated into cytochrome c oxidase (COX) (4–6). It is not clear how the cytoplasmic

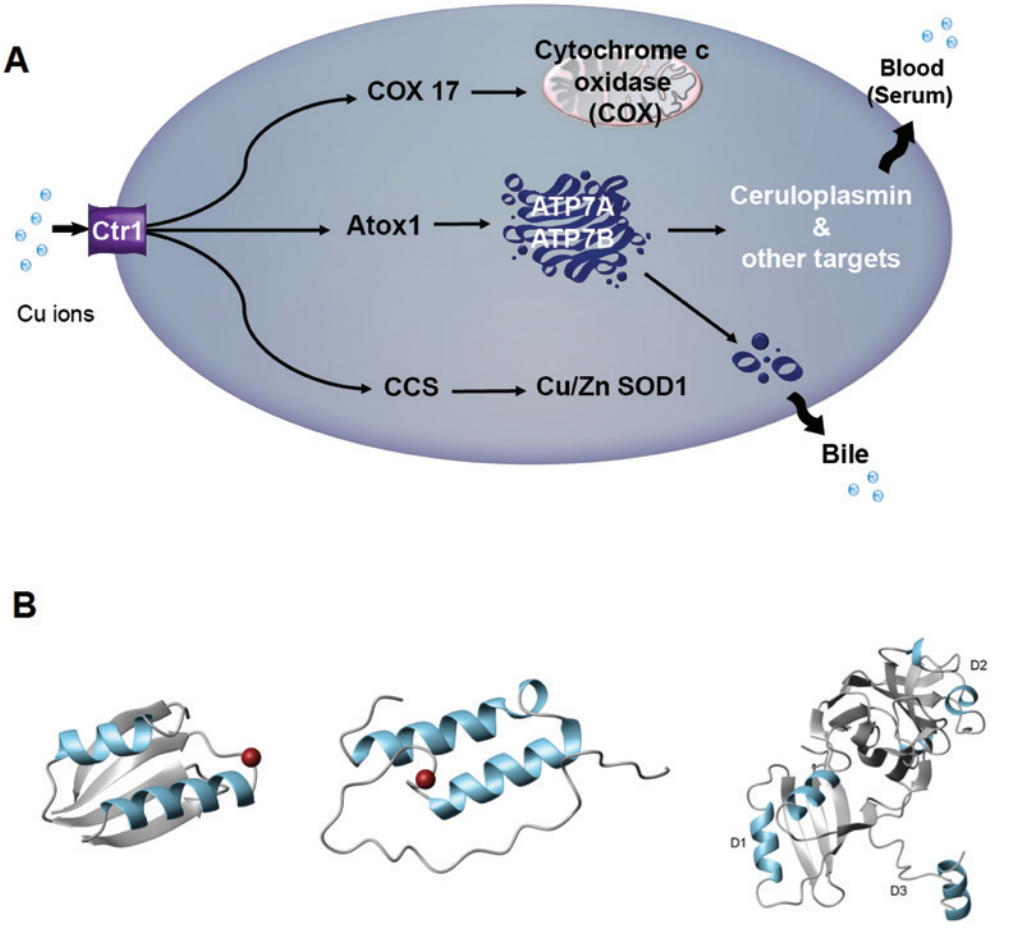

Figure 1: Cu transport in human cytoplasm.
(A) A simplified overview of intracellular pathways for Cu transport in humans. Cu ions enter the cytoplasm through the membrane-bound copper transporter Ctr1 and then follow three different pathways mediated by three different Cu chaperones in the cytoplasm: COX17, Atox1 and CCS. COX17 delivers Cu ions to COX in mitochondria with the help of various additional proteins. The membrane-bound P_{1B}-type ATPases, ATP7A and ATP7B, receive Cu from Atox1, and then delivers the metals to Cu-dependent enzymes in the secretory pathway (i.e. ceruloplasmin). At elevated cytoplasmic Cu levels, ATP7A and ATP7B can translocate into vesicles and export excess Cu from the cell at the plasma membrane. The third Cu chaperone, CCS, transfers Cu to cytoplasmic Cu/Zn SOD1. (B) Schematic structures of Atox1 (PDB code: 1FEE), COX17 (PDB code: 2RNB) and CCS (PDB code: 1JK9). Cu is shown as red sphere in Atox1 and COX17; for CCS, the three domains are labeled as D1, D2 and D3.

Cu chaperones receive the Cu from Ctr1, although direct protein-protein interactions with the C-terminal tail of Ctr1 appears likely. Since CCS possesses the capacity to interact with lipid bilayers, membrane scaffolding may be a mechanism that facilitates interactions between Ctr1 and cytoplasmic chaperones (7). As an alternative to direct Ctr1-chaperone interactions, it has also been proposed that glutathione may act as an intermediate Cu-binding molecule bridging between Ctr1 and the cytoplasmic chaperones (8). We recently reported that, at least *in vitro*, in addition to ATP7A/B interactions, Atox1 can cross-react and exchange copper with CCS (9). Therefore, cross talk between the cytoplasmic chaperones may be a yet unexplored mechanism that allows for more efficient usage and distribution of Cu ions that have entered the cytoplasm.

The Atox1 pathway

Atox1 binds Cu via two conserved Cys residues in a surface-exposed copper-binding motif CXXC (1, 10) (Figure 1B). Intriguingly, Atox1 contains an apparent nuclear localization sequence (NLS) KKTGK within its C-terminal part and, although not discussed, in the initial discovery paper of Atox1 from 1999 (11), immunofluorescence of HeLa cells indicated that Atox1 was distributed throughout the cell, including the nucleus. Nonetheless, Atox1 is monomeric in solution, it forms Cu-dependent hetero-dimers with target metal-binding domains in ATP7A/B that are structurally similar to the homo-dimer of Atox1 found in the X-ray structure (12–18). In these dimers, a Cu ion bridges two proteins

via interactions with cysteine sulfurs from both metal-binding sites (19).

ATP7A and ATP7B are homologous multi-domain copper pumps with eight membrane-spanning helices, an actuator (A) domain, as well as nucleotide- (N) and phosphorylation- (P) domains, with nucleotide-binding site and an invariant Asp (that is transiently phosphorylated during the catalytic cycle), respectively, protruding into the cytoplasm. In addition, ATP7A and ATP7B both have six cytoplasmic metal-binding domains in the N-terminus connected by peptide linkers of various lengths (20). Notably, much of our current knowledge of ATP7A/B comes from studies of individual domains and from work on yeast and bacterial homologs (21, 22).

Each metal-binding domain in ATP7A/B, as well as Atox1, has a ferredoxin-like α/β fold and a surface-exposed invariant MXCXXC motif (X=any residue) in which a single Cu can bind via the two cysteine sulfurs. In contrast to humans, bacterial and yeast P_{1B}-type ATPases have only one or two metal-binding domains. The purpose for as many as six metal-binding domains in ATP7A/B is unknown, albeit regulation of Cu transfer activity has been proposed. The MXCXXC motif does not confer intrinsic specificity to Cu ions, although soft metals are favored by sulfur ligands, as both Atox1 and individual ATP7B metal-binding domains can bind other metals, such as Zn, strongly *in vitro* (23). Under normal conditions in cells, however, metal-binding degeneracy is not a problem since metal transport is strictly governed by protein-protein interactions (24, 25).

It was originally assumed that Atox1 delivers Cu to one of the metal-binding domains of ATP7A/B and the metal then is shuttled within the protein to Cu binding sites in the membrane channel. *In vitro* (12–17) and in silico (18) work has shown that Cu transfer from Atox1 to metal-binding domains of ATP7A/B proceeds via copper-bridged hetero-dimer complexes where the metal is shared between the two metal-binding sites. Cu is thought to move from one protein to the other via ligand-exchange reactions involving tri-coordinated Cu-sulfur intermediates (15). All six domains of ATP7A/B can be loaded with Cu by Atox1 but only in some cases have Cu-dependent protein-protein complexes been detected by NMR via slower tumbling times (12, 13, 26, 27). Based on affinity and NMR studies, Cu binding to a ATP7B metal-binding domain is favored over binding to Atox1 by a factor of 3–5 providing a shallow directional thermodynamic driving force. We found that upon mixing of Cu-Atox1 and the fourth metal-binding domain of ATP7B (WD4), a stable ternary complex assembled that was in equilibrium with substrates and products (19). In contrast, when mixing a two-domain construct of domains 5 and 6 in ATP7B (WD56) and Cu-Atox1, the protein-protein interaction was transient such that it did not survive size exclusion chromatography (SEC) but Cu transfer still took place (28).

For the Atox1 and WD4 pair, we identified that Atox1-Cu-WD4 hetero-protein complex formation is driven by favorable enthalpy and entropy changes, whereas the overall reaction, from Atox1 to WD4, relies on only enthalpy (19). In additional studies, we revealed that the first cysteine in each protein's copper binding motif is essential for complex formation and the copper site in the hetero-protein complex appears to be dynamic (29). It remains unknown if the same principles apply to Atox1 interactions with the other five domains in ATP7B and when the target domain is surrounded by neighboring domains within the full-length protein. Information on these (apparent) straightforward issues has been hampered by the difficulty of purifying large membrane proteins.

The finding that the Cu chaperone in bacteria, CopZ, (that is homologous to Atox1) could bypass the single metal-binding domain of the bacterial P_{1B}-type ATPase and instead deliver Cu directly to the membrane entry site (30) reinforced the idea that the human metal-binding domains played regulatory roles. Nonetheless, yeast complementation studies have shown that the presence of the human and yeast metal-binding domains, at least some domains in the case of the human protein, is essential for Cu transfer activity (31, 32). In 2011, the crystal structure of the bacterial ATP7A/B homolog *Legionella pneumophila* CopA was reported (22). Although the CopA structure was a breakthrough, there was no electron density resolved for its metal-binding domain (22). The CopA structure revealed a putative docking site for a chaperone, or an internal metal-binding domain, at the membrane entry site for Cu in the form of a kinked helix. Subsequent modeling studies indicated that this kinked helix could be a docking site for Atox1 (33) as well as for the 6th metal-binding domain (34) leaving the question of where Atox1 delivers the copper ion still unresolved. Regardless, the importance of the metal-binding domains *in vivo* is clear: at least three disease-causing point mutations are found in the metal-binding domains of ATP7B (11).

The CCS and Cox17 pathways

SOD1 is mostly found in the cytoplasm and requires incorporation of zinc and copper ions, as well as the

formation of a disulfide bond, before reaching its active dimeric form (35). The activation of human SOD1 occurs via its specific chaperone CCS (36), although the molecular mechanisms of CCS-dependent maturation of SOD1 is not fully resolved. CCS is a protein with three distinct domains (37) (Figure 1B). Domain 1 of human CCS has a ferredoxin-like fold and MTCXXC motif as Atox1 and is thought to be the initial Cu receiver domain (35, 38). Domain 2 of CCS binds Zn and is structurally similar to SOD1; this domain appears critical for CCS-SOD1 protein recognition (37). The 3rd domain of CCS is a short polypeptide segment that lacks secondary structure but contains a CXC motif that is essential for CCS-dependent activation of SOD1 (37). A minor fraction of CCS is found in the inner membrane space of mitochondria where it activates mitochondrial SOD1. How Cu and Zn loading are coordinated has been the focus of several studies and it is believed that Zn binding may couple to early folding events of the protein (39).

The mitochondrial assembly of the copper sites in COX involves a series of accessory proteins, including (but not limited to) Cox11, Cox17 and Sco1. The two mitochondrial inner membrane proteins Cox11 and Sco1 are thought to be copper donors to the Cu(B) and Cu(A) sites of COX, respectively, whereas Cox17 is believed to be the copper donor to Sco1 within the inter membrane space (IMS) (40). Cox17 is a 69-residue cysteine-rich (three pairs of cysteines) protein that shuttles between the cytoplasm and mitochondria (41, 42) (Figure 1B). How Cox17 obtains Cu in the cytoplasm is unclear, but likely this involves Ctr1. Cox17 is imported to the IMS of mitochondria by the MIA pathway, a specific translocation pathway of metal-binding IMS proteins that couples disulfide exchange to facilitate transport (43, 44). It has been demonstrated that Cox17 is a specific copper donor to both Sco1 and Cox11 in the mitochondrial IMS and the interactions likely involve complementary electrostatic surfaces (45). Moreover, in contrast to the cytoplasmic reactions, it appears that reversible changes in the redox state of cysteines in these proteins represent important steps in the pathways ultimately providing Cu to COX (46, 47). The IMS is a more oxidizing environment than the cytosol (48) and, in accord, Cox17 exists in a partially oxidized form with two S-S bonds and two reduced cysteines. It was shown that Cu-loaded Cox17 can transfer simultaneously a reduced Cu ion and two electrons to Sco1, when the latter is in the oxidized state, i.e. with metal-binding cysteines forming a disulfide in Sco1 (47). Electron transfer-coupled metalation is thus a mechanism that provides efficient Cu transfer when metal-binding thiols are oxidized in the target protein.

Atox1 as a transcription factor?

That Atox1 is essential already in embryonic development is clear from studies of Atox1$^{-/-}$ mice that generally die after birth, and those that survive have severe malfunctions and malformations (49). This agrees with the established role of Atox1 in Cu loading of essential cuproenzymes but also suggests additional Atox1 functions at early embryonic stages. Moreover, fibroblast cells lacking Atox1 displayed a diffusive distribution of Cu throughout the entire cell according to quantitative synchrotron X-ray fluorescence (SXRF), also implying additional Atox1 functions, here as a player in maintaining proper organization of Cu ions at the cellular level (50).

In 2008, it was reported that Atox1 had an additional activity acting as a Cu-dependent transcription factor (TF) that drives the expression of *Ccnd1* (cyclin D1), a protein involved in cell proliferation. Increased levels of cyclin D1 together with other factors promote the transition from G1 to S phase in the cell cycle. To induce and maintain increased expression of cyclin D1, the growth factor signal ERK (extracellular-signal-regulated kinase; also called classical MAP kinase) has to be activated (51). A direct Cu-dependent interaction of GST-tagged Atox1 with a GAAAGA sequence in the promotor region of the *Ccnd1* gene was demonstrated by an electrophoretic mobility shift assay (EMSA) (52). The GAAAGA sequence was also identified by the same authors in the promoter region of SOD3, which is the major extracellular antioxidant protein in humans and this protein also protects against angiotensin II-induced hypertension. It was shown by EMSA that GST-tagged Atox1 associated directly with the SOD3 promotor segment *in vitro* (53) and that angiotensin II promoted Atox1 translocation to the nucleus followed by stimulation of SOD3 expression in mice (54). It was subsequently proposed that, in similarity to many other TFs, Atox1 acted as a TF in the homo-dimer form and the same GAAAGA sequence was found in additional gene promotors (55). Most recently, it was reported that Atox1 promoted inflammatory neovascularization by acting as a Cu-dependent transcription factor for NADPH oxidase organizer p47phox (containing GAAAGA motifs), leading to increased ROS-NFκB-VCAM-1/ICAM-1 expression and monocyte adhesion in endothelial cells (56).

Independent of the work by the Fukai group, it was demonstrated that Atox1 expression and nuclear accumulation were stimulated by cell treatment with the cancer drug cisplatin (57). We recently confirmed the presence of Atox1 in the nucleus of HeLa cells but no DNA binding of Atox1 to the proposed GAAAGA promotor sequence *in vitro* was detected (58). Despite our negative result for DNA

binding which does not support a direct TF role, Atox1 may nonetheless regulate gene transcription via additional proteins that in turn bind DNA.

New interaction partners for Atox1

Yeast two-hybrid (Y2H) screens using the protein of interest as bait is becoming a powerful tool to identify known and unknown protein-protein interactions and, thus, assign new functions of proteins (59). To identify new partners that may facilitate Atox1-mediated signals to DNA we recently applied Y2H screening, using Atox1 as the bait, on 98 million human protein fragments and 25 unique proteins of which 16 were characterized proteins (two of those were ATP7A and ATP7B) were identified as confident hits (60) (Table 1). Among the 14 new proteins, six were reported as detected in the nucleus: ICE1, DNMT1, ZHFX3, TRIM26, USP48, and ZNF521 with all but USP48 described as DNA/RNA-binding proteins (Table 1). DNA/RNA binding has been reported also for the identified hit proteins CPEB4, LMCD1 and PPM1A (60). Although biochemical validations of these putative interactions are required, the new data triggers a number of speculations regarding functional consequences that are briefly described below.

The DNA methyltransferase DNMT1 is responsible for the propagation of methylation patterns to the next generation via its preferential methylation of hemi-methylated CpG sites in the genome (61). Thus, like cyclin D1 (whose expression was proposed to be driven by Atox1, as mentioned above), DNMT1 expression is cell cycle dependent and, in fact, the expression patterns of DNMT1 and cyclin D1 during the cell cycle are known to correlate with each other (62). In DNMT1, Atox1 interacted with the first BAH (bromo-adjacent homology) domain (60) which has β-structure with many surface exposed regions serving as a platform for protein-protein interactions. Since DNMT1 is thought to be in an auto-inhibitory state in which a linker stretches over the first BAH domain and blocks DNA from entering the catalytic site (63), it may be speculated that Atox1 binding to the first BAH domain may turn on activity.

Table 1: The interaction partners of Atox1 identified in a Y2H (yeast two hybrid) screen (60), described with full name and reported/proposed function.[a]

Protein	Full name	Function
ATP7A	ATPase, Cu transporting, beta polypeptide (Menkes disease protein)	Cu transport
ATP7B	ATPase, Cu transporting, beta polypeptide (Wilson disease protein)	Cu transport
ARMC6	Armadillo repeat-containing protein 6 isoform 2	Hematopoietic progenitor cell differentiation
CPEB4	Cytoplasmic polyadenylation element-binding protein 4	Nucleic acid binding, cytoplasmic polyadenylation element-binding
CRELD2	Cysteine-rich with EGF-like domain protein 2 isoform b precursor	Protein binding; calcium binding
DNMT1	DNA (cytosine-5)-methyltransferase 1 isoform a	DNA methylation on cytosine; DNA-methyltranferase activity; regulation of gene expression; epigenetic zinc binding
ICE1	Little elongation complex subunit 1	transcriptionally active chromatin; transcription elongation factor complex; positive regulation of protein complex assembly, intracellular protein transport
LMCD1	LIM and cysteine-rich domains protein 1 isoform 1	Negative regulation of transcription; zinc binding
PPM1A	Protein phosphatase 1A isoform 1	Transcription initiation; insulin receptor signaling; growth factor pathway; protein de-phosporylation; phosphatase; manganese and magnesium binding
PTPRF	Receptor-type tyrosine-protein phosphatase F isoform 2 precursor	Cell migration and adhesion; integral component of plasma membrane; receptor regulation activity; peptidyl-tyrosine de-phosphorylation
TRIM26	Tripartite motif-containing protein 26	DNA binding, transcription factor; regulation of viral release from host cell; innate immune response; zinc binding
USP19	Ubiquitin carboxyl-terminal hydrolase 19 isoform	Cysteine-type endopeptidase activity protein de-ubiquitination
USP33	Ubiquitin carboxyl-terminal hydrolase 33 isoform	Cysteine-type endopeptidase activity protein de-ubiquitination
USP48	Ubiquitin carboxyl-terminal hydrolase 48 isoform a	Cysteine-type endopeptidase activity protein de-ubiquitination
ZFHX3	Zinc finger homeobox protein 3 isoform B	DNA binding transcription factor activity; protein binding; zinc binding
ZNF521	Zinc finger protein 521	Nucleic acid binding; metal ion binding

[a]Bold entries represent nuclear and/or DNA/RNA binding proteins.

The tandem RNA recognition motif (RRM) that Atox1 interacts with in CPEB4, based on the Y2H results (60), is one of the most abundant protein domains in eukaryotes (64). CPEB proteins bind cytoplasmic polyadenylation elements of mRNA (elements involved in regulation of cell proliferation, chromosome segregation and cell differentiation) via its RRM and can act as either translational repressors or activators. CPEB4 was observed to support tumor growth in pancreatic tumors via translational activation of mRNAs that are silenced in normal tissue (65). Notably, cyclin D1 expression is inversely linked to the expression of CPEB4 through the activation of ERK and down-regulation of CPEB4 promoted G1 to S phase transition (51). Thus, an inhibitory Atox1 interaction with CPEB4 may result in increased levels of cyclin D1 and promotion of cell proliferation, as reported previously (52).

The Atox1 Y2H interaction partners TRIM26 and PPM1A (60) are both involved in viral defense signaling and stress response (66). In analogy, Atox1 was originally discovered as an antioxidant protein expressed in response to oxidative stress (11). PPM1A is an essential manganese/magnesium ion-dependent Ser/Thr phosphatase (in the family protein phosphatase 2C, PP2C) that regulates cellular stress response in eukaryotes (67). Reversible phosphorylation of proteins acts as activation/inactivation mechanisms in cellular processes such as cell cycle progression. Based on the two-hybrid screen result, Atox1 interacted with the C-terminal helices in PPM1A, which are unique to mammalian PP2C isoforms (68) and are thought to contribute to substrate specificity. Notably, Atox1 is predicted to be phosphorylated at Ser44 and Ser47 and if so, in order to be recycled, there may be a requirement for a phosphatase such as PPM1A.

Because many of the Y2H identified Atox1 partners are eukaryotic-specific (60) (Table 1), it is conceivable that additional functionalities for Atox1 have evolved during evolution in order to adapt to increased organism complexity. One may speculate that these interactions are regulated by Cu in several different ways: 1. Cu-loaded Atox1 may have a different affinity for a partner as compared to the apo form, 2. Cu-loaded Atox1 may promote interaction with a partner due to physical relocation to the nucleus, or 3. Cu may be transferred from Atox1 to the partner promoting downstream effects.

Role of CCS in apoptosis

In addition to the established target SOD1, the X-linked inhibitor of apoptosis (XIAP) was recently reported as a CCS target (69). XIAP is a Cu-binding protein, overexpressed in many cancers, that is involved in regulation of apoptosis, such that it blocks caspases necessary to promote cell death (70). XIAP is also believed to modulate Cu homeostasis through interactions with a protein named COMMD1, which is important for hepatic Cu excretion and interacts with ATP7B (Figure 2). It was demonstrated that CCS delivers Cu to XIAP and XIAP in turn can promote E3 ubiquitination of CCS, which resulted in enhanced Cu delivery to SOD1 (69). XIAP itself is regulated by Cu such that when Cu is bound it adopts a different conformation that is less stable and impaired in ability to inhibit caspases. Thus, when CCS delivers Cu to XIAP, the Cu form of XIAP becomes degraded and apoptosis can take place (71). Supplying Cu to XIAP may thus be a new approach to kill cancer cells, although the same process may contribute to the pathophysiology of Cu toxicosis disorders. In Wilson disease, Cu levels are raised in hepatocytes due to impaired ATP7B activity, and this extra Cu may bind XIAP and promote XIAP degradation; thereby the apoptotic threshold may be lowered, sensitizing the cells to apoptosis.

CCS was recently suggested to mediate Cu transport to the nucleus where the metal regulated formation of the hypoxia-inducible factor 1 (HIF-1) transcriptional complex, which in turn promoted expression of the vascular endothelial growth factor (VEGF), a potent angiogenic factor (72). Typically, expression of VEGF is induced in cells exposed to hypoxia or ischemia and VEGF-stimulated neovascularization occurs in several clinical contexts, including myocardial ischemia, retinal disease, and tumor growth. Although CCS was originally shown to localize in the nucleus in addition to the cytoplasm (73), mechanistic information of CCS-mediated Cu transport to the nucleus is lacking.

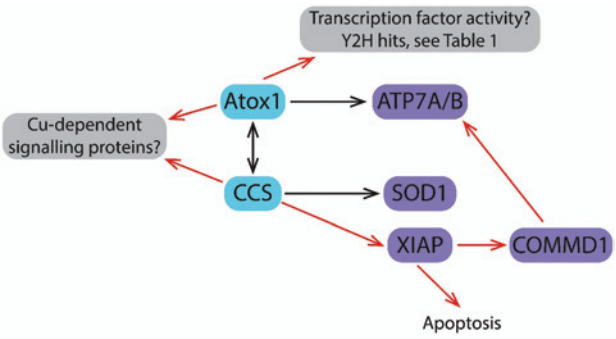

Figure 2: Scheme of interactions of Atox1 and CCS.
The reactions with the Atox1 and CCS partners in the well-established Cu transport pathways ATP7A/B and SOD1, respectively, are indicated with black arrows whereas more recently discovered and putative interactions are indicated with read arrows.

Role of Cox17 in mitochondrial membrane architecture

In addition to the established partner of Cox17, Sco1, it was recently discovered that Cox17 formed an independent complex with a protein named Mic60 (74). Mic60 is one of six subunits in the so-called MICOS (mitochondrial contact site and cristae organizing system) that is essential for establishing and maintaining the proper inner membrane architecture. MICOS is also required for formation of contact sites between the inner and outer membranes to facilitate communication between compartments. Cox17 interaction was Cu dependent such that Cu facilitated the interaction and, upon forming a complex, MICOS integrity was promoted (74). It is yet unclear if the presence of bound Cu affects Cox17 such that MICOS binding is favored or if Cu is transferred from Cox17 to MICOS stabilizing the latter via direct metal binding. Nonetheless, these findings argue for additional roles of Cox17 in mitochondrial membrane organization (74).

Copper ions in signaling – role of Cu chaperones?

Traditionally, metals have been divided into two categories, dynamic signals and static cofactors, where inactive alkali (Na, K) and alkaline (Mg, Ca) earth metals were thought to provide signaling, whereas redox active metals such as iron (Fe) and Cu were considered as static cofactors (75). But, because metals cannot be created or destroyed, their levels have to be regulated by other means and the line between metabolism and signaling must be blurred. The emerging paradigm is that transition metals [Fe, Cu, zinc (Zn), and manganese (Mn)] may also exist in labile pools that are involved in cell signaling (75). In line with this, apart from the established role as cofactor in cuproenzymes, Cu is essential for normal cell growth and development, albeit involved mechanisms are not well understood (76).

Several studies have demonstrated a cellular role of Cu ions in modulation of metabolism, survival, migration, proliferation and differentiation (77, 78). Cu appears involved in many steps of the classical cell signaling cascade, including Cu effects on polypeptides targeting membrane receptors, interaction of Cu with receptors, Cu modulation of kinase and associated phosphatase-signaling processes, Cu as a second messenger, and finally transcriptional control mediated by Cu in the nucleus (for a recent review, see ref. 76). It is clearly a justified question how a metal that is so tightly regulated because of its potential toxicity can provide such diverse and widespread control over cell signaling. Although the roles of Cu chaperones in providing Cu for these cell signaling events are unknown, it has emerged that many of the key signaling proteins utilize structural changes due to Cu binding as a regulatory switch [i.e. XIAP (70) discussed above].

The greatest evidence for Cu participation in the modulation of cell signal transduction comes from studies showing activation of mitogen-activated protein kinase (MAPK) signaling pathways by Cu. For example, it was recently shown that Cu stimulates the MAP kinase kinase Mek1 to phosphorylate ERK (79) and, in order for this to take place, Mek1 had to bind two Cu ions (79). How Mek1 obtains Cu is unclear, but proposed candidates are Atox1, CCS or low molecular weight intracellular ligands.

Another Cu-dependent signaling system is the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) proteins, which are inflammatory transcription factors. In the absence of activating stimulus, NFκB subunits are bound to inhibitor of κB (IκB) proteins in the cytoplasm. But when a cell receives a stimulus, the IκB proteins are phosphorylated, ubiquitinated and degraded, which allows NFκB to form dimers, translocate to the nucleus, and modulate gene expression. Increased Cu levels in the cytoplasm result in potent inhibition of this NFκB signaling and the explanation appears to be a Cu-mediated increase in reactive oxygen species that inhibits IκB phosphorylation (80). One may speculate that Atox1 could play a role in changing the available (labile) pool of Cu ions mediating NFκB inhibition because Atox1 has been proposed to be involved in cellular redox homeostasis due to the particularly low reduction potential of Atox1's cysteine sulfurs (81). Oxidation of cysteine sulfurs will release Cu ions that may catalyze formation of additional reactive oxygen species. This type of redox reactions in a cell can be rescued and it was found that glutaredoxin 1 resolved disulfides in Atox1 created by oxidative stress and reloaded a Cu ion via direct protein-protein interactions *in vitro* (82).

Expert opinion

Copper accumulation (either due to, or causing, copper transport dysregulation) is often found in cancer tumors and upon neurodegeneration. Cu is necessary for angiogenesis which helps facilitate cancer metastasis (83, 84) and Cu can bind to amyloidogenic proteins that are

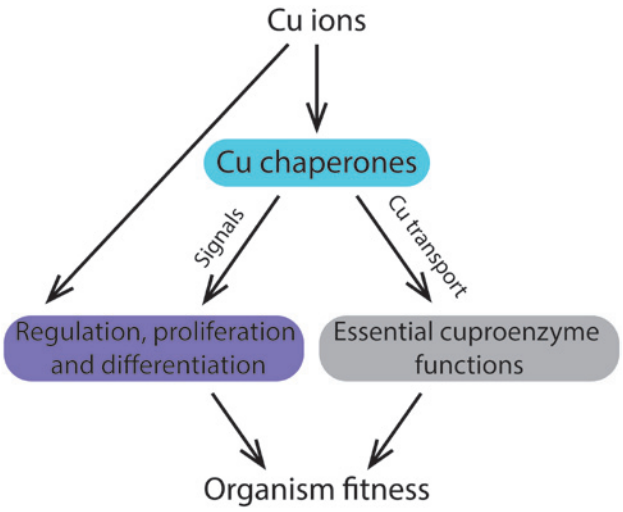

Figure 3: Cu chaperones are essential to many functions in cells and hence aid in organism fitness.
In addition to Cu transport providing essential Cu-dependent enzymes with Cu, Cu chaperones and Cu ions have been found to play roles in cellular growth and development.

involved in Huntington's, Parkinson's and Alzheimer's diseases; upon binding, amyloid formation is promoted *in vitro* (76, 85). Thus, these diseases develop in parallel with failed and/or altered Cu homeostasis. Even if it is not clear what is cause and consequence, the mechanisms for how Cu levels are regulated and how Cu binding to specific target proteins affects cell signals related to growth and development are excellent targets for new drug concepts combating these diseases. Work in this direction is underway in many labs. In summary, it emerges that the Cu chaperones, required for Cu transport to essential Cu-dependent enzymes, have been exploited in humans such that they also aid in the cascade of events that result in cell signals in response to Cu (Figure 3). This may be through new functions gained through evolution taking advantage of existing proteins, or activities acquired in response to a need of tighter control of Cu levels in higher organisms.

Outlook

With better biological and chemical understanding of the emerging network of interactions involving Cu and Cu chaperones; new medical concepts may be discovered and, based on that, new drug leads can be developed. Clearly, there is a lot of rewarding research ahead of us in this field from both fundamental and medical aspects. To be successful, this will require a combination of biophysical, structural, and biological studies on individual protein systems as well as with whole cells and model organisms.

Highlights

- Cu is an essential cofactor in many enzymes but potentially toxic in its free form, thus there are Cu transport systems in the cytoplasm involving Cu chaperones
- In essences, all Cu ions in a cell are thought to be bound to proteins and Cu chaperones deliver Cu to the next target protein via direct protein-protein interactions
- Recent data implies new functions for human Cu chaperones and, for example, Atox1 has been suggested to have TF activities and additional protein partners in the nucleus
- Cu is known to be important for normal growth and development but the mechanisms for this is unclear
- Many proteins in signaling cascades are found to be regulated by Cu, via direct binding of the metal that modulates protein activity
- It is not yet known what role Cu chaperones play in delivering Cu to signaling proteins but an interplay between signaling proteins and Cu chaperones can be proposed
- Further understanding of the extended functional repertoire of the human Cu chaperones may lead to new medical concepts towards metabolic disease, cancer and neurodegenerative disorders

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List of abbreviations

Atox1	antioxidant protein 1
BAH	bromo-adjacent homology
CCS	copper chaperone for superoxide dismutase
COX	cytochrome c oxidase
Cox17	chaperone for cytochrome c oxidase
Cu	copper
EMSA	electrophoretic mobility shift assay
ERK	extracellular-signal-regulated kinase
HIF-1	hypoxia-inducible factor 1
IMS	inter membrane space
MICOS	mitochondrial contact site and cristae organizing system
RRM	RNA recognition motif

SOD1	superoxide dismutase 1
TF	transcription factor
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis
Y2H	yeast-two-hybrid

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