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PERSPECTIVE

Unresolved questions in human copper pump mechanisms

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Abstract. Copper (Cu) is an essential transition metal providing activity to key enzymes in the human body. To regulate the levels and avoid toxicity, cells have developed elaborate systems for loading these enzymes with Cu. Most Cu-dependent enzymes obtain the metal from the membrane-bound Cu pumps ATP7A/B in the Golgi network. ATP7A/B receives Cu from the cytoplasmic Cu chaperone Atox1 that acts as the cytoplasmic shuttle between the cell membrane Cu importer, Ctr1 and ATP7A/B. Biological, genetic and structural efforts have provided a tremendous amount of information for how the proteins in this pathway work. Nonetheless, basic mechanistic-biophysical questions (such as how and where ATP7A/B receives Cu, how ATP7A/B conformational changes and domain–domain interactions facilitate Cu movement through the membrane, and, finally, how target polypeptides are loaded with Cu in the Golgi) remain elusive. In this perspective, unresolved inquiries regarding ATP7A/B mechanism will be highlighted. The answers are important from a fundamental view, since mechanistic aspects may be common to other metal transport systems, and for medical purposes, since many diseases appear related to Cu transport dysregulation.

Key words: copper transport, copperchaperone, Wilson disease protein, Menke's disease protein, ceruloplasmin, biophysical methods.

Copper (Cu) pumps of the human secretory pathway

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 (Harris, 2003; Huffman & O'Halloran, 2001; Puig & Thiele, 2002). The ability of Cu to oxidize/reduce (switch between Cu^{+} and Cu^{2+}) allows Cu-containing proteins to play important roles as electron carriers and redox catalysts in living systems. To avoid Cu toxicity, the intracellular concentration of Cu is regulated *via* dedicated proteins that facilitate uptake, efflux as well as distribution to target Cu-dependent proteins and enzymes (Festa & Thiele, 2011; O'Halloran & Culotta, 2000; Robinson & Winge, 2010). In the human cytoplasm, after Cu has entered the cell via the Cu importer Ctr1 (Ohrvik & Thiele, 2014), there are at least three independent Cu transport pathways.

In the general pathway, conserved in most organisms, the 68-residue Cu chaperone Atox1 transports the metal to cytoplasmic metal-binding domains in 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 (Fig. 1). Most Cu-dependent enzymes acquire Cu from ATP7A/B in the Golgi before reaching their final destination (e.g. blood clotting factors, tyrosinase, lysyl oxidase and ceruloplasmin) (Festa & Thiele, 2011; Koch *et al.* 1997; O'Halloran & Culotta, 2000; Robinson & Winge, 2010). Once transferred to ATP7A/B, the Cu ion is channeled to the lumen of the Golgi and loaded onto target Cu-dependent proteins. We recently reported that at least *in vitro*, in addition to ATP7A/B interactions, Atox1 can cross-react and exchange Cu with another cytoplasmic Cu chaperone, the Cu chaperone for superoxide dismutase, CCS, which delivers Cu specifically to SOD1 (Petzoldt *et al.* 2015). Thus, cross talk between cytoplasmic chaperones may be an unexplored mechanism that allows for efficient usage of cytoplasmic Cu ions.

ATP7A/B are elaborate multi-domain Cu pumps with eight membrane-spanning helices, an actuator (A) domain, as well as nucleotide-(N) and phosphorylation-(P) domains,

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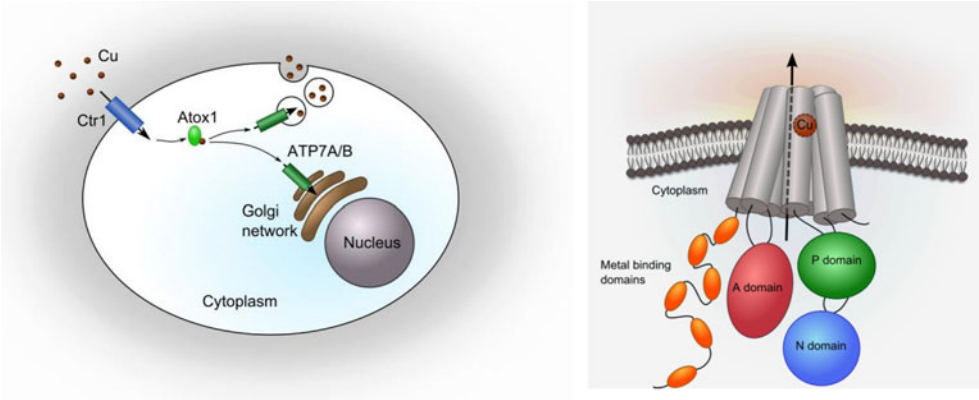

Fig. 1. Left: Illustration of the Cu transport pathway to the Golgi for Cu loading of proteins in the secretory path. Uptake of Cu takes place via Ctr1, then cytoplasmic transport is facilitated by the Cu chaperone Atox1 to membrane-bound ATP7A/B for loading of Cu-dependent enzymes. If there is too much Cu in the cell, ATP7A/B can move to vesicles and facilitate Cu export out of the cell. Right: Schematic structure of the domain arrangement of ATP7A/B (six metal-binding domains, an actuator (A) domain, N- and P-domains that bind ATP and become phosphorylated, respectively, and membrane-spanning helices (gray).

with nucleotide-binding site and an invariant Asp (that is transiently phosphorylated during the catalytic cycle), respectively, protruding into the cytoplasm (Fig. 1). In addition, ATP7A and ATP7B both have six cytoplasmic metal-binding domains in the N-terminus connected by peptide linkers of various lengths (Lutsenko *et al.* 2007). Notably, much of our current knowledge of ATP7A/B comes from studies of individual domains (as it is difficult to prepare the full length proteins) and from work on yeast and bacterial homologs (Culotta *et al.* 2005; Gourdon *et al.* 2011).

Each metal-binding domain in ATP7A/B, as well as Atox1, has a ferredoxin-like $\alpha\beta$-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 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* (Niemiec *et al.* 2014). At normal cell conditions, however, metal-binding degeneracy is not a problem since metal transport is strictly governed by protein–protein interactions (Tottey *et al.* 2005, 2008).

Moving Cu from chaperone to ATP7A/B

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* (Achila *et al.* 2006; Banci, 2006; Banci *et al.* 2008, 2009*a*, *b*; Pufahl *et al.* 1997; Wernimont *et al.* 2000) and *in silico* (Rodriguez-Granillo *et al.* 2010) work has shown that Cu transfer from Atox1 to metal-binding domains of ATP7A/B proceeds via Cu-bridged hetero-dimer complexes where the metal is shared between the two metal-binding sites (Fig. 2). Cu is thought to move from one protein to the other via ligand-exchange reactions involving tri-coordinated Cu–sulfur intermediates (Pufahl *et al.* 1997). 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 (Achila *et al.* 2006; Banci *et al.* 2005, 2008, 2009*a*, *b*). Based on affinity and NMR studies, Cu binding to an 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 (Niemiec *et al.* 2012). 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 (Nilsson *et al.* 2013).

For the Atox1 and WD4 pair, SEC in combination with titration calorimetry made possible thermodynamic analysis of the reaction in Fig. 2 and 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 (Niemiec *et al.* 2012). In additional studies, involving protein engineering, we revealed that the first cysteine in each protein's Cu binding motif was essential for hetero-protein complex formation but one of the second cysteines was not required. Thermodynamic analysis disclosed that the wild-type Cu site in the hetero-protein complex was dynamic (in agreement with positive entropy change, see above), involving entropy–enthalpy compensation (Niemiec *et al.* 2015). It remains unknown if the same mechanism and thermodynamic principles

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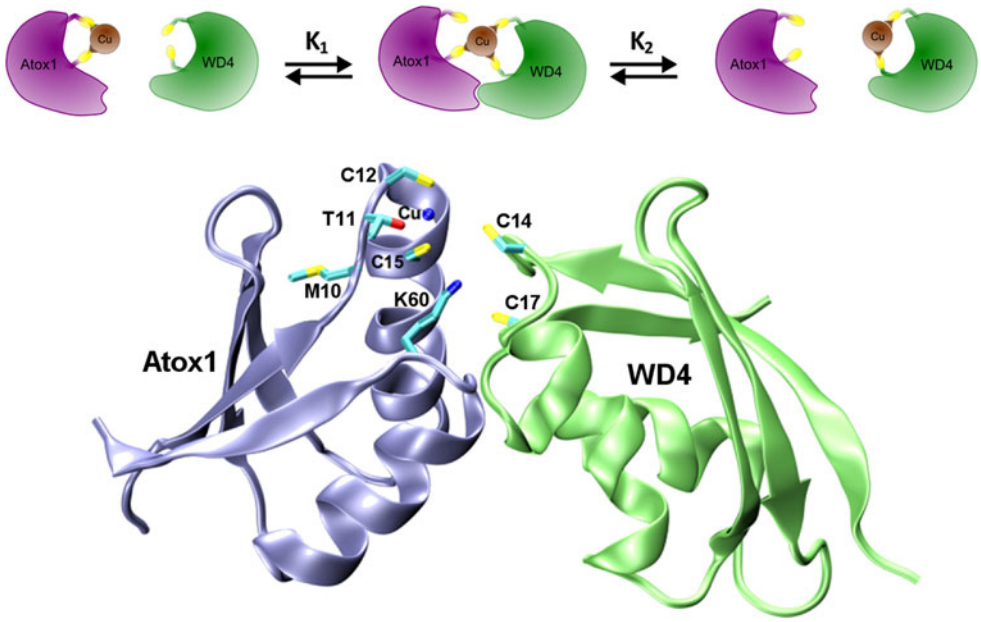

Fig. 2. Top: Scheme of Cu transfer mechanism from Atox1 to the 4th metal-binding domain of ATP7B (WD4) indicating an intermediate hetero-protein complex in which the Cu ion is coordinated by cysteines in both proteins' metal-binding loops. Bottom: Structural model of the Cu-dependent Atox1–WD4 hetero-protein complex.

apply to Atox1 interactions with the other five domains in ATP7B and when the target domain is surrounded by its natural domains within the full-length protein. Information on these (apparent) straightforward issues has been hampered by the difficulty to prepare large membrane proteins for biophysical studies.

The finding that the Cu chaperone in bacteria (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 (Gonzalez-Guerrero & Arguello, 2008) 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 (Forbes *et al.* 1999; Morin *et al.* 2009). In 2011, the crystal structure of the bacterial ATP7A/B homolog *Legionella pneumophila* CopA was reported (Gourdon *et al.* 2011). Although the CopA structure was a breakthrough, there was no electron density resolved for its metal-binding domain (Gourdon *et al.* 2011). 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 (Gourdon *et al.* 2012) as well as for the 6th metal-binding domain (Arumugam & Crouzy, 2012) making the question of where Atox1 delivers the Cu 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 (Hamza *et al.* 1999).

Internal interactions that modulate Cu movement

During the catalytic cycle, that requires ATP hydrolysis and ultimately results in Cu transfer to the lumen side of the membrane, ATP7A/B are likely to undergo significant conformational changes driven by domain–domain interactions (Lutsenko *et al.* 2007). Available predictions for how ATP7A/B works catalytically come from analogy with the calcium pump SERCA, for which structures of different enzymatic stages have been resolved (Bublitz *et al.* 2013). Since there are no structures of the arrangement of the six metal-binding domains within full-length ATP7A/B, it is unclear how these domains participate in the catalytic cycle. Interactions among the metal-binding domains of ATP7A/B are proposed to transmit signals long-range (Gourdon *et al.* 2012). In support, we found that even in a small construct of only domains 5 and 6 of ATP7B (WD56), minute variations in salt and pH conditions perturbed domain–domain relative fluctuations such that the efficiency of Atox1-mediated Cu delivery to these domains was modulated (Nilsson *et al.* 2013). This implies that local (temporal and spatial) fluctuations in the cellular environment may tune overall Cu pump activity via changes in domain–domain interactions.

For ATP7B, an interaction between the ATP-binding domain (N-domain) and a construct of the six metal-binding

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domains of ATP7B was reported based on pull-down data (Tsivkovskii *et al.* 2001); this interaction was found to depend on the metal-loading status as well as on phosphorylation events and it was suggested to be an auto-inhibitory interaction (Hasan *et al.* 2012). A similar intra-protein interaction was reported for domains of a homologous bacterial Cu pump, also using pull down experiments with tagged proteins (Gonzalez-Guerrero *et al.* 2009). Since the bacterial homolog has only one metal-binding domain, one may speculate that the 6th metal-binding domain in ATP7B plays a key role in the interaction within the human protein, perhaps with the additional five metal-binding domains and the unstructured loop, unique to the human N-domain, fine-tuning the binding. However, our biophysical studies using ^{15}N-labeled purified domains (N-domain mixed with four-domain construct, WD1-4 or with WD56) did not reveal any interaction for any protein pair (Åden and Wittung-Stafshede, unpublished), although we used high μM protein concentrations (Fig. 3). This suggests that the interaction identified by pull-down experiments depends sensitively on the environment, such as pH, salt and inter-domain interactions, or possibly on additional components present in the lysate. In a general sense, this result highlights the elusive nature of regulatory interactions; one must clearly test a range of conditions and constructs before making conclusions.

Fate of Cu after reaching the lumen

After Cu has passed the ATP7A/B channel to the lumen it is unclear how it is added onto target polypeptides. In ATP7A, a luminal loop has been identified that has Cu-binding capacity (Barry *et al.* 2011). In ATP7B, this loop is shorter but still has Cu-binding residues. Thus, this loop may be a transient site for the Cu ion before it is loaded on a target polypeptide. The mechanism of Cu loading onto target polypeptides is unknown; one possibility is that Cu becomes free in solution after leaving ATP7A/B and binding is simply driven by Cu–protein affinity.

Ceruloplasmin is a large six-domain ferroxidase, requiring six Cu ions for activity, which is Cu-loaded by ATP7B in the secretory pathway. Our *in vitro* studies of ceruloplasmin unfolding imply that metal binding must take place early on during protein folding in order for the polypeptide to fold into its functional form. If the polypeptide is allowed to fold without metals, then it misfolds into a dead end species that cannot bind Cu (Sedlak & Wittung-Stafsheden, 2007; Sedlak *et al.* 2008). Thus, appropriate timing of folding and binding events in the final step of the Cu transport cascade appears crucial. In the case of ceruloplasmin, this becomes important in the treatment of aceruloplasminemia (a condition where mutated ceruloplasmin it not loaded with Cu) since Cu supplementation will not rescue already misfolded apo-forms of ceruloplasmin.

Interactions with other proteins and new functions

In addition to internal domain–domain interactions, other proteins have also been proposed to regulate ATP7A/B activity. For example, the protein COMMD1 (Copper Metabolism Murr1 Domain) was recently shown to interact with the metal-binding domains of ATP7B and this appeared to regulate overall ATP7B stability via the ER degradation pathway (de Bie *et al.* 2007; Materia *et al.* 2012). The binding sites on neither protein nor the physical mechanism resulting in ER degradation (interaction causing protein destabilization or triggering of a cellular signal) are known. Surprisingly, COMMD1 was found to specifically bind Cu^{2+} *in vitro* and, therefore, it was speculated that the COMMD1–ATP7B interaction may be a way to eliminate oxidized (toxic) Cu from cells (Sarkar & Roberts, 2011).

The general idea has been that mammalian Cu transport protein levels are primarily regulated post-transcriptionally, such as *via* degradation pathways (Hasan & Lutsenko, 2012). To control for elevated Cu levels, ATP7A/B can redistribute reversibly from the Golgi to the plasma membrane to expel Cu and thereby protect against Cu toxicity (La Fontaine & Mercer, 2007). However, in 2008 it was reported that Atox1 had dual functionality and also acted as a Cu-dependent transcription factor (TF) that drives the expression of *Ccd1* (cyclin D1), a protein involved in cell proliferation. A direct Cu-dependent interaction of GST-tagged Atox1 with a GAAAGA sequence in the promotor region of *Ccnd1* was demonstrated by an electrophoretic mobility shift assay (EMSA) (Itoh *et al.* 2008). Subsequently, this motif was found in the promotor regions of several other genes (Muller & Klomp, 2009). In support of playing a role in the nucleus, the sequence of Atox1 contains an apparent nuclear localization signal KKTGK within its C-terminal part and, although not discussed, in the initial discovery paper of Atox1 from 1999 (Hamza *et al.* 1999), immunofluorescence of HeLa cells indicated that Atox1 was distributed throughout the cell, including the nucleus. In our work, we have also detected Atox1 in the nuclei of HeLa cells (Fig. 4) although we find no binding to DNA duplexes harboring the target sequence *in vitro* (Kahra *et al.* 2015). The answer to this apparent paradox may be that Atox1 is an indirect TF working via interaction with another protein that contains the DNA-binding ability.

To search for new Atox1 partners, we performed a yeast two-hybrid screen (Hybrigenics; similar to (Rain *et al.* 2001)) using Atox1 as a LexA fusion bait toward a human placenta library of protein fragments (Wittung-Stafshede, unpublished); among 98 million possible interactions, we found 25 confident target proteins that interacted with Atox1 (Table 1). Of these interactions partners, at least six are known DNA/RNA-binding proteins. The results of this screen demonstrate that the Cu transport network is greater than what is currently known and, moreover, suggest that

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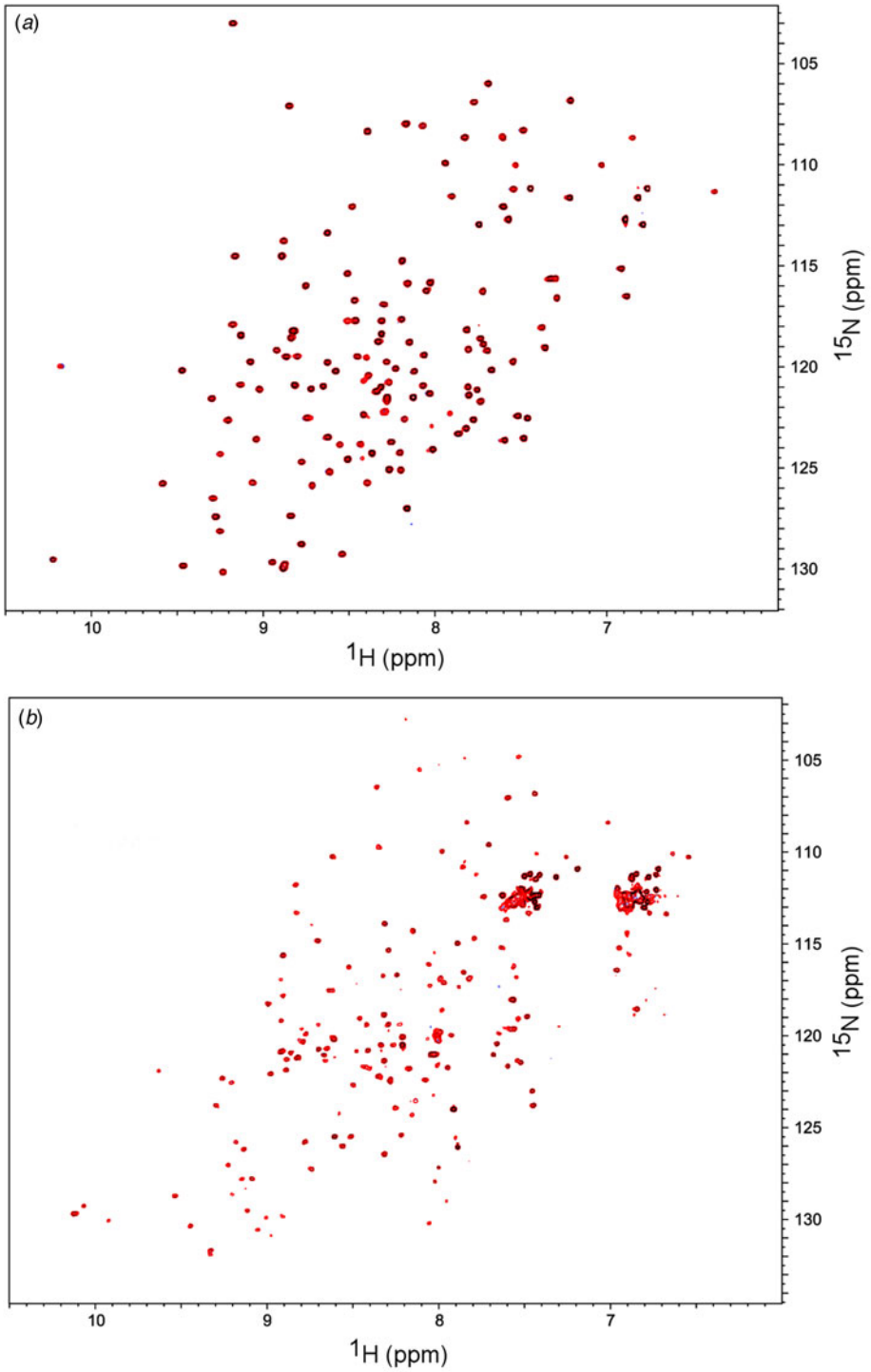

Fig. 3. ^{1}H–^{15}N–HSQC spectra recorded at 850 MHz in 50 mM Tris, 50 mM NaCl, 2 mM DTT, 6% D2O (v/v) at pH 8·0 and 25°C. (*a*) 150 μM ^{15}N-labeled N-domain (blue), and together with 150 μM unlabeled WD56 (red). (*b*) 200 μM ^{15}N-labeled WD1-4 (blue), and together with 200 μM unlabeled N-domain (red).

Cu transport proteins (i.e. Atox1) likely have additional (yet unknown) partners and functions. Although only a screen that will require extensive follow up, these types of large-scale experiments that are available today are important in that they may identify new directions for future biophysical work.

Outlook for Cu pump biophysics

Many mutations in the genes that code for Cu transport proteins have been linked to human diseases. Mutations in ATP7A/B constitute the basis of Wilson and Menke's

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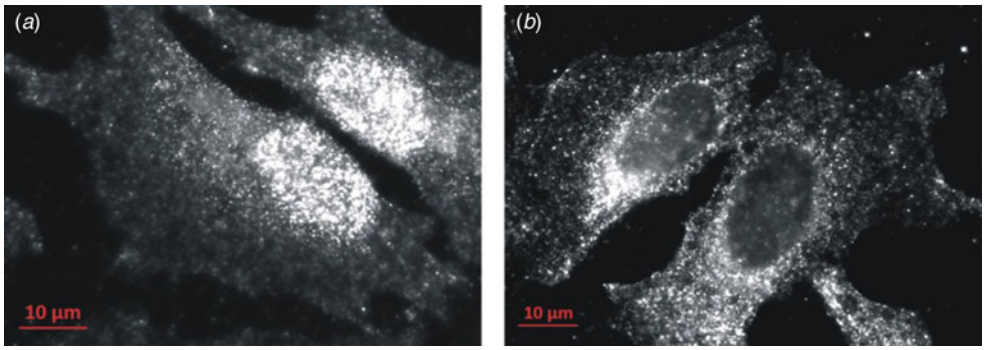

Fig. 4. Localization of Atox1 in HeLa cells detected by wide-field fluorescence microscopy using monoclonal Alexa Fluor488 tagged anti-mouse antibodies specific for Atox1. In most cells Atox1 appears in perinuclear areas and as punctuate structures in close contact with the plasma membrane (*b*), indicative of Golgi compartment and transport vesicle localization, respectively. However, there are also cells with increased Atox1 levels within the nucleus (*a*).

Table 1. *Results from a cDNA yeast two hybrid screen using Atox1 as bait (LexA fusion) and a human placenta RP6 library as prey (Hybrigenics). 98·2 million interactions were analyzed and 310 positive clones were fully processed. Detected interactions with the highest predicted biological scores (PBS) are listed below divided in four categories from A (the highest confidence rank) to D. There were two additional unknown proteins in B, and 12 additional proteins in the D category (three ubiquitin specific peptidases and nine unknown proteins) not listed here. No C scores were found. Several of the detected interaction partners have DNA/RNA binding capacity (bold name)*

PBS	Target	Comment on target protein
A	PTPRF	Protein tyrosine phosphatase, receptor-like; cell signaling, cancer
A	**KIAA0947**	Nuclear protein involved in RNA binding and regulation
A	ATP7B	Known Atox1 partner (positive control)
B	ATP7A	Known Atox1 partner (positive control)
B	**DNMT1**	C-5 cytosine methyl transferase
B	CRELD2	Cysteine-rich with EFG-like Ca-binding domains
B	**ZFHX3**	TF, with zinc fingers
D	**CPEB4**	RNA-binding protein
D	**LMCD1**	Zinc-finger protein with LIM and cysteine-rich domains, regulator of transcription
D	PPM1A	Phosphatase; regulator of stress response, cell cycle control
D	**TRIM26**	Protein with tripartite motif (includes zinc-binding domains), DNA binding

diseases; missense mutations in almost all domains of ATP7B have been linked to Wilson disease with the most common mutation being H1069Q in the N-domain. Using a range of biophysical methods we could explain the underlying mechanism: the mutation did not affect domain stability or ATP-binding affinity; however, it made ATP bind in such a way that hydrolysis was hindered (Rodriguez-Granillo *et al.* 2008). In addition to direct genetic defects, Cu accumulation (either due to, or causing, Cu transport dysregulation) is often found in cancer tumors and upon neurodegeneration. Cu can bind specifically to the amyloidogenic proteins that are involved in Huntington's, Parkinson's and Alzheimer's diseases; upon binding, amyloid formation is promoted *in vitro* (Faller *et al.* 2013), suggesting that Cu may be a causative agent of these diseases *in vivo*.

Another aspect is drug treatment and side effects. For the cancer drug cisplatin it has been reported that the drug hijacks Cu transport proteins as a possible mechanism to become exported out of cells. We showed that Atox1 can bind cisplatin together with Cu creating a di-metal site. Cisplatin binding causes Atox1 unfolding *in vitro* but prior to this, if a partner such as ATP7B is present, the cancer drug can be transferred further (Palm *et al.* 2011; Palm-Espling & Wittung-Stafshede, 2012; Palm-Espling *et al.* 2013, 2014). This may result in drug resistance but, considering the possibility of Atox1 entering the cell nucleus, Atox1 may in fact mediate the delivery of the drug to DNA.

Clearly, biophysical knowledge of mechanisms and regulation of Cu transport proteins may aid the development of new approaches to target disorders involving Cu transport proteins and imbalance in Cu metabolism. Biophysical studies of the mechanisms and proteins that facilitate human Cu transport may also provide predictions for how other metal transport systems work. Like in Cu transport proteins, the ferredoxin-like structural fold appears commonly used by zinc transport proteins (Lu & Fu, 2007; Lu *et al.* 2009), which is a group of metal transporters for which mechanistic and biophysical knowledge is severely lacking. Taken together, the unresolved questions described above emphasize the need for more careful biophysical studies and invite young biophysical scientists to enter this wide-open field.

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Fig. 4. Former graduate student Maria Palm-Espling made Fig. 1. The Swedish Research Council, the Wallenberg foundation, and Umeå University are acknowledged for financial support. This text was written during a sabbatical period at California Institute of Technology.

Conflict of interest

None.

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