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The multi-layered regulation of copper translocating P-type ATPases

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Abstract The copper-translocating Menkes (ATP7A, MNK protein) and Wilson (ATP7B, WND protein) P-type ATPases are pivotal for copper (Cu) homeostasis, functioning in the biosynthetic incorporation of Cu into copper-dependent enzymes of the secretory pathway, Cu detoxification via Cu efflux, and specialized roles such as systemic Cu absorption (MNK) and Cu excretion (WND). Essential to these functions is their Cu and hormone-responsive distribution between the *trans*-Golgi network (TGN) and exocytic vesicles located at or proximal to the apical (WND) or basolateral (MNK) cell surface. Intriguingly, MNK and WND Cu-ATPases expressed in the same tissues perform distinct yet complementary roles. While intramolecular differences may specify their distinct roles, cellular signaling components are predicted to be critical for both differences and synergy between these enzymes. This review focuses on these mechanisms, including the cell signaling pathways that influence trafficking and bi-functionality of Cu-ATPases. Phosphorylation events are hypothesized to play a central role in Cu homeostasis, promoting multi-layered regulation and cross-talk between cuproenzymes and Cu-independent mechanisms.

Keywords Copper · P-type ATPase · Protein trafficking · Cell signaling

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Mammalian copper-ATPases are essential copper homeostatic enzymes

The ability of copper (Cu) to adopt distinct redox states by cycling between the oxidized Cu^{2+} and reduced Cu^{+} forms makes it a desirable cofactor for a diverse range of metal-binding enzymes. It is distributed throughout the body and participates in a range of physiological processes including central nervous system (CNS) function, connective tissue and blood vessel development, pigmentation, reactive oxygen species (ROS) detoxification, synaptogenesis and mitochondrial function (Danks 1988; El Meskini et al. 2007; Horn and Barrientos 2008; Niciu et al. 2007; Ohta et al. 1982; Tumer and Horn 1996). Paradoxically, the same redox properties also lead to cellular oxidative damage when Cu is found in excessive concentrations. Cu readily participates in reactions that lead to production of highly reactive oxidative species (such as hydroxyl radicals) that can have devastating effects including DNA damage and oxidation of proteins and lipids (Gutteridge et al. 1984; Pena et al. 1999). Thus, Cu intake, distribution,

utilization and excretion must be tightly regulated and Cu ions must be bound rather than "free" throughout this process (O'Halloran and Culotta 2000; Thiele 2003). This ensures correct delivery to Cu-dependent enzymes and subsequent removal from tissues and the body to avoid toxicity.

Both Menkes and Wilson Disease are severe inherited human diseases involving dysfunctional Cu homeostasis, caused by mutations in the ATP7A (Menkes or MNK protein) and ATP7B (Wilson or WND protein) genes, respectively. These genes encode Cu-translocating transmembrane P-type ATPases that function in vectorial Cu transport across biological membranes. The central role of these Cu-ATPases in Cu homeostasis is demonstrated by the severe phenotypes of the two diseases. The malabsorption of Cu in Menkes disease results in Cu accumulation in intestinal cells, placenta, mammary tissue and the kidneys and deficiency in the brain, liver and serum. This leads to disrupted neurological and connective tissue development, causing mental retardation and neurodegeneration and usually results in early childhood death (Danks 1995; Kaler 1998; Moore and Cox 2002). Non-functional WND leads to reduced hepatic Cu excretion into bile, reduced ceruloplasmin in the serum and subsequent hepatic and brain Cu accumulation in Wilson disease patients. Cu accumulation in the kidney and cornea also occur as a result of mutant WND expression in these tissues (Das and Ray 2006; Tumer and Horn 1996). Disturbances in Cu homeostasis are associated with neurodegenerative disorders such as Parkinson's and Alzheimer's disease, age-related macular degeneration and prion-related disease (Barnham and Bush 2008; Bush and Tanzi 2008; Erie et al. 2008). The extreme phenotypes of these diseases reflect, in part, the role of Cu in synaptogenesis, neurotransmission, axonal extension, angiogenesis, wound healing and cell cycle regulation (El Meskini et al. 2005; El Meskini et al. 2007; Hwang et al. 2007; Mandinov et al. 2003; Soldi et al. 2007).

While non-functional or mutated Cu-transporters such as mammalian Cu-ATPases can lead to severe disturbances in Cu homeostasis, there is also evidence for Cu-independent mechanisms contributing to these outcomes. For example, iron (Fe) and Cu homeostasis are closely linked (Garrick et al. 2003; Ramirez-Cardenas et al. 2005). Estrogen and insulin regulate Cu-ATPase expression and function and conversely, abnormal metabolism of Cu and other metals cause complications in diabetes (Hardman et al. 2007; Zheng et al. 2008). Identifying regulatory influences on Cu homeostasis, particularly on Cu-ATPases, will enhance development of diagnostic and therapeutic approaches for disorders resulting from disturbances in Cu homeostasis. We will discuss several of these issues with particular reference to the regulation of Cu-ATPase function and the key cell signaling pathways through which these systems communicate.

The localization of a copper P-type ATPase determines its function

MNK and WND Cu-ATPases translocate Cu across biological membranes. This is accomplished through a complex series of binding and ATP hydrolysis steps, a common feature of all members of the ion-transporting P-type ATPase family (Moller et al. 1996). They share ~60% amino acid identity and the ability to perform two key functions: delivery of Cu to Cu-dependent enzymes in the secretory pathway (a physiological function), and Cu export via active efflux (a homeostatic function). The dual function is a result of a regulated trafficking process where MNK or WND can be expressed and localized to: (1) the *trans* Golgi network (TGN) for Cu transfer to enzymes in the secretory pathway such as tyrosinase and peptidylglycine α-amidating monooxygenase (by MNK) and ceruloplasmin (by WND) under basal conditions; or (2) endosomes or rapidly recycling vesicles in the vicinity of the cell surface to facilitate Cu efflux or secretion when intracellular Cu concentrations become elevated (Hellman and Gitlin 2002; Linz et al. 2008; Nyasae et al. 2007; Pase et al. 2004; Petris et al. 2000; Setty et al. 2008; Steveson et al. 2003).

The Cu-responsive Cu-ATPase trafficking process was first demonstrated in non-polarized Chinese Hamster Ovary (CHO) cells (Petris et al. 1996). The same exocytic trafficking was also observed in cultured Menkes patient ($Atp7a^{-/-}$) fibroblasts, demonstrating rescue and correction of the Cu accumulation defect by expression of wild type MNK, triggering significant interest in understanding regulation of the intracellular localization of MNK and WND. In the last decade, the expression profile and localization of Cu-ATPases has

been examined in a range of tissues. This includes hepatocytes (Huster et al. 2003; Schaefer et al. 1999), cultured kidney epithelial cells (Greenough et al. 2004), mouse cerebellum and kidney (Linz et al. 2008; Linz and Lutsenko 2007), hippocampal neurons (Schlief et al. 2005), retina (Krajacic et al. 2006) and intestinal epithelia (Monty et al. 2005; Nyasae et al. 2007). Initial CHO cell expression studies revealed Cu-stimulated WND translocation to cytoplasmic vesicles (La Fontaine et al. 1998). These vesicles have since been more accurately associated with the membrane of bile ducts in the liver, and studies in non-hepatic cells indicate that the vesicles are large, apical/sub-apical-localized Cu-storage compartments destined for secretion (Hardman et al. 2004; Huster et al. 2003; La Fontaine et al. 2001; Linz et al. 2008). In contrast, Cu stimulation results in MNK localization to vesicles throughout the secretory pathway, with particular enrichment within basolateral regions of polarized endothelial and epithelial cells for Cu efflux (Greenough et al. 2004; Nyasae et al. 2007; Pascale et al. 2003; Petris et al. 1996).

Mechanisms for Copper-ATPase trafficking

The trafficking of Cu-ATPases is a complex process requiring a number of intracellular players to act in a concerted fashion in order to maintain physiological Cu levels in the body. This complexity is also observed for other proteins that transport metals and other factors across membranes. The trafficking of the GLUT4 glucose transporter, aquaporin water channel AQP2, CFTR chloride channel and many other ion channels is regulated by cytoskeletal components, Rab GTPases, PDZ adaptor proteins and a range of protein kinases (Hou and Pessin 2007; Ishikura et al. 2008; Lamprecht and Seidler 2006; Saxena and Kaur 2006; Vossenkamper et al. 2007). To fully appreciate how such components affect Cu-ATPase localization and trafficking, it is important to firstly understand what is known about the trafficking mechanisms. These processes have been described in detail elsewhere (La Fontaine and Mercer 2007; Lutsenko et al. 2007; Lutsenko et al. 2008) and are summarized here. Briefly, Cu-ATPase trafficking is regulated by Cu and is closely linked to catalytic activity (La Fontaine et al. 2001; Petris et al. 2002; Voskoboinik et al. 2001a). The catalytic cycle and its relationship with trafficking has been described (Barnes et al. 2005; Hung et al. 2007; Voskoboinik et al. 2001b, 1999). Cu-ATPases such as MNK remain largely in the TGN region for delivery of Cu to the lumen of secretory compartments (TGN, melanosomes) for cuproenzymes (Petris et al. 1998; Setty et al. 2008). This is predicted to be dependent on the cytosolic Cu chaperone Atox1 transferring Cu directly to at least one of the six conserved cytosolic metal binding domains (MBDs) in the N-terminus (Hamza et al. 2003; Walker et al. 2002). When intracellular Cu concentrations increase, ATP-binding and hydrolysis and Cu-translocating activity into the secretory pathway is up-regulated and Cu loading in the secretory lumen rapidly results in Cu saturation in these compartments. Importantly, the Cu-ATPase remains in a conformationally active state which is favorable for post-Golgi protein trafficking (Lutsenko et al. 2007). Catalytic activity of metal ion dependent P-type ATPases involves transfer of the γ-phosphate from the ATP molecule to an invariant aspartate residue (transient acyl-phosphorylation) and is then followed by rapid dephosphorylation coupled to Cu transport across the membrane (Valverde et al. 2008; Voskoboinik et al. 2001b).

Although the conformational shifts and domain interplay in the Cu-ATPases during Cu translocation is not entirely clear, trafficking studies on mutants possessing truncations of N-terminal Cu-binding domains indicate that metallation (Cu-binding to conserved CxxC motifs) within all six MBDs aids Cu sensing and may promote the switch to an active conformation. An important feature of this process is the minimal requirement for MBD5 or MBD6, the two domains also conserved in homologous invertebrate Cu-ATPases (Cater et al. 2004; Guo et al. 2005; Strausak et al. 1999; Voskoboinik et al. 2002). A potential mechanism may involve interactions between metallated MBD5/6 with the phosphatase or "A" domain (TGEA motif) when intracellular Cu is elevated. This allows the catalytic cycle to proceed and the Cu-ATPase to remain in a transiently acyl-phosphorylated state (Lutsenko et al. 2008). This is contradictory to earlier models where MBDs were predicted to shuttle Cu into the central channel. The current model is supported by structural studies in the homologous bacterial CopA Cu-ATPase (Wu et al. 2008) and evidence for a direct transfer from the bacterial Atox1-like chaperone CopZ to CopA in the

absence of metal binding sites (Gonzalez-Guerrero and Arguello 2008). Logically, metallation of MBDs may also lead to exposure of other regions of the protein harboring signal sequences for interactions with trafficking machinery and therefore subsequent post-Golgi trafficking of the ATPase to the cell surface.

Two major targeting motifs have so far been identified in the C-terminal cytoplasmic tail of MNK; a PDZ protein–protein interaction motif required for basolateral targeting and retention, and a dileucine motif which is required for endocytic retrieval and basolateral targeting (Greenough et al. 2004; Petris et al. 1998). The mechanism of dileucine regulated cell surface retrieval is unclear, yet the apical/sub-apical MNK distribution of the MNK L1487-1488A mutant raises the possibility that MNK may be sorted through apically localized recycling endosomes (Greenough et al. 2004). Our data suggests that the majority of MNK does not traffic via the Rab11 positive recycling endosomes *en route* to the basolateral membrane in the presence of Cu (A. Gaeth and P. Jamieson, unpublished data). Therefore the dileucine signal may regulate sorting into other endosomal compartments. This motif is recognized by trafficking machinery such as adaptor complexes (Bonifacino and Traub 2003). The WND protein also contains a dileucine motif (within a tri-leucine sequence) and there is an important N-terminal 63 residue domain which is absent in MNK (Cater et al. 2006; Guo et al. 2005). This domain is essential for the apical distribution and TGN retention of WND, as deletion studies (N-terminal truncations up to MBD5) show that the WND protein is constitutively localized at basolateral regions of the cell (Guo et al. 2005). Since wild type Cu-ATPases are also known to undergo low-level constitutive trafficking in basal Cu conditions (Cobbold et al. 2002; Petris and Mercer 1999) the function of the N-terminal apical-targeting domain may be to facilitate a role in protein sorting under both basal and elevated cellular Cu conditions. Thus, increased apical trafficking would occur under elevated Cu due to increased exposure of the domain following MBD-Cu interactions.

The functionality of these key targeting motifs can be further considered in evolutionary terms. For example, the *Drosophila melanogaster* ATP7 homolog (DmATP7) lacks the WND N-terminal apical-targeting domain, but contains a putative dileucine and presumptive PDZ target motif and is similar in function to the mammalian MNK protein e.g., role in CNS development and pigmentation (Norgate et al. 2006). When DmATP7 is expressed in mammalian cells it localizes to the TGN and shows rapid Cu-responsive trafficking to the basolateral membrane in polarized cultured cells (A. Southon, unpublished data). This exciting finding demonstrates that key trafficking and localization signals for basolateral MNK trafficking are conserved. This provides a powerful system for Cu-ATPase structure/function analysis.

Potential for kinase phosphorylation to regulate Cu-ATPase trafficking

Current evidence suggests that the N-terminal MBDs do not act alone to promote the exposure and action of trafficking/sorting signals. Indeed, both mammalian Cu-ATPases undergo Cu-dependent kinase-mediated phosphorylation (distinct from the acyl-phosphorylation in the catalytic cycle of P-type ATPases) that is predicted to regulate catalytic activity and trafficking responses, given that levels of ^{32}P radioactive label incorporation into MNK correlate strongly with the Cu concentration mediated change in localization (Vanderwerf et al. 2001; Voskoboinik et al. 2003). This is not entirely unexpected since the introduction of a negatively charged phosphate moiety to an amino acid is known to alter solvent accessibility of amino acid or peptide domains and subsequently affect protein function (Hoffert et al. 2006; Nowicki et al. 1996). Hyper-phosphorylation of ion channels and other transmembrane proteins can also have profound effects on catalytic activity and protein localization (Gadsby and Nairn 1999; Guggino and Stanton 2006; Kato et al. 2002). The paradigm has already been investigated and we know that the insulin-dependent packaging of GLUT4 into rapidly trafficking glucose storage vesicles is stimulated by PI3 Kinase-dependent phosphorylation, but it is important to note that phosphorylation of the GLUT4 protein is not essential for its translocator activity (Bai et al. 2007; Hou and Pessin 2007; Peck et al. 2006). This trafficking response is analogous to that recently described for Cu-stimulated packaging of MNK into rapid recycling Cu vesicles (Pase et al. 2004). Thus, kinases and

lipid signaling may act on MNK function via multiple layers of regulation, potentially via phosphorylation of MNK, as well as stimulating the trafficking machinery required to localize MNK into rapid recycling vesicles.

Both MNK and WND are phosphorylated on serine residues, indicating a key role for one or more serine/threonine kinases for regulation of their trafficking and possibly function (Vanderwerf and Lutsenko 2002; Voskoboinik et al. 2003). In the most detailed analysis of Cu-ATPase phosphorylation, comparisons were made between radioactive phosphopeptides isolated from wild type and MNK mutant cells, where the conserved CxxC metal binding sites of the N-terminal domain of MNK were substituted for serine residues (MBSm1-6 mutant)(Strausak et al. 1999). This study strongly suggests the occurrence of metal-binding site-dependent and independent phosphorylation, and suggests that MNK phosphorylation occurs at the TGN in conditions of low Cu (MBSm1-6 cannot exit the TGN) (Voskoboinik et al. 2003). In addition, studies of the yeast Cu-ATPase ortholog Ccc2 indicate that N-terminal phosphorylation may be coupled to the ATPase domain catalytic cycle (Valverde et al. 2008). Relative to mammalian Cu-ATPases, the Protein Kinase A (PKA) phosphorylation site at Ser-258 is located between MBD6 and the first transmembrane domain. Furthermore, Cu is predicted to promote Ser-258 phosphorylation by increasing solvent accessibility of the region as a consequence of Cu-MBD interactions (Valverde et al. 2008). This is consistent with reduced phosphorylation in the mammalian MNK mMBS1-6 mutant (Voskoboinik et al. 2003). Thus kinases may play a key role in mammalian Cu-ATPase regulation (Lutsenko et al. 2008). In addition, specific kinase inhibitor studies have demonstrated a role for PKA in MNK exocytic trafficking (Cobbold et al. 2002).

Such an example indicates that Cu interactions are not solely responsible for the regulation of Cu-ATPase function. We have recently identified regions in both C- and N-terminal cytosolic domains in polarized epithelial cells which display Cu-responsive phosphorylation (Veldhuis et al., manuscript in preparation). This indicates that the function of MNK may be regulated by interaction with signal transduction pathways and suggests roles for both of these cytosolic domains in regulation of trafficking and/or activity. Other recent studies are also providing compelling evidence for the involvement of a range of proteins and other Cu-independent pathways to regulate Cu homeostasis, either indirectly or in conjunction with Cu and characterized cuproenzymes.

Copper-independent trafficking

A) Lessons from co-expressing Cu-ATPases

Studies have previously focused on the role of distinct ATP7A and ATP7B expression patterns in dictating the clinical manifestations of Menkes or Wilson disease. ATP7B (WND) expression is highest in liver tissue, with lower expression patterns in the brain and kidney (Bull et al. 1993; Tanzi et al. 1993; Yamaguchi et al. 1994). ATP7A (MNK) expression is observed in most extra-hepatic adult tissues, with high expression in the intestinal mucosa, kidney and neuronal cells (Abe et al. 1994; Monty et al. 2005). Recent evidence has changed our understanding of expression patterns and the interplay of MNK and WND expressed in the same cells (Linz and Lutsenko 2007).

Hormone signaling is a key regulator of ion channels in many tissues (Cheng et al. 1997; Feraille and Doucet 2001; Gimenez and Forbush 2003; Golestaneh et al. 2001; Mirshahi et al. 1999). In mammary cells the importance of Cu delivery to infants in milk was first identified in the Toxic milk (*Tx*) mouse mutants. The *Tx* mutation prevents WND Cu-translocating activity and WND exocytosis from the TGN, causing Cu accumulation within the tissue and reduced Cu secretion into milk (Michalczyk et al. 2000; Rouch 1983; Voskoboinik et al. 2001a). It is now clear that WND plays a physiological secretory role by delivering Cu to infants in milk and ceruloplasmin (Ackland et al. 1999; Ackland et al. 1997; Kelleher and Lonnerdal 2006). MNK is responsible for direct Cu export in response to lactation, where hormonal induction by estrogen, progesterone, insulin and prolactin stimulate the expression and basolateral localization of MNK and the Cu uptake protein Ctr1 to perform a homeostatic function in the mother's tissue (Ackland et al. 1997; Llanos et al. 2008). Studies in cultured placental cells support this finding, where insulin and estrogen treatment of

cultured placental JEG-3 cells promotes MNK trafficking toward the basolateral surface for enhanced Cu export, in the absence of added Cu. Interestingly, estrogen and insulin have an opposing effect on WND, causing reduced WND expression and restricting localization to perinuclear regions, leading to reduced Cu transport across the apical membrane (Hardman et al. 2007) and these effects occur independently of additional intracellular Cu. Thus, in contrast to the case in mammary tissue, MNK is performing a Cu secretion function in delivering Cu to the fetal circulation whilst WND is performing a homeostatic function in placental epithelium (Fig. 1). This reinforces the concept that signaling pathways can result in differential outcomes for Cu-ATPases. The described differences in localization can be partially explained by Cu-binding and the key trafficking motifs such as the WND apical-targeting domain or MNK dileucine and PDZ motifs. As we learn more about the recently discovered Cu-ATPase complementary function and differential localization, it is likely to emerge that similar to the examples described above, MNK and WND are subject to Cu-independent regulation in many tissues. For example, investigations into renal Cu distribution demonstrate a role for WND in apical Cu excretion and storage in Cu-loaded vesicles, whereas Cu-stimulated MNK trafficking from internal secretory compartments to the basolateral membrane aids Cu re-absorption (Linz et al. 2008). Enterocytes are an important site for Cu uptake in the body. Recently, gastric and intestinal tissues were shown to express WND mRNA and protein, where Cu-responsive WND re-localization from the TGN to vesicles was consistent with WND localization in other tissues (Weiss et al. 2008). The precise function remains unclear yet supports a physiological role in fine-tuning Cu homeostasis, potentially by sequestering Cu into endosomal/vesicular pools or returning Cu into the apical lumen, while MNK participates in vectorial Cu transport across the basolateral membrane and into the bloodstream (Weiss et al. 2008). Krajacic et al. (2006) have demonstrated the presence of both MNK and WND in the retina. Preliminary results (Rodriguez-Boulan, Deora and Gaeth, unpublished data) utilizing cultured retinal pigment epithelial cells (RPE) reveal, in the presence of Cu, a basolateral distribution for MNK and a sub-apical localization for WND, suggesting a "homeostatic" role for MNK by pumping Cu into the choroid, whereas WND may be performing a physiological role in delivering Cu to photoreceptor cells. Reduced levels of Cu and zinc (Zn) in retinal pigment epithelium from patients suffering from age-related macular degeneration suggest that the localization and function of the Cu-ATPases is a pivotal process in retinal health (Erie et al. 2008).

These differences in localization and function of Cu-ATPases co-expressed in various tissues are

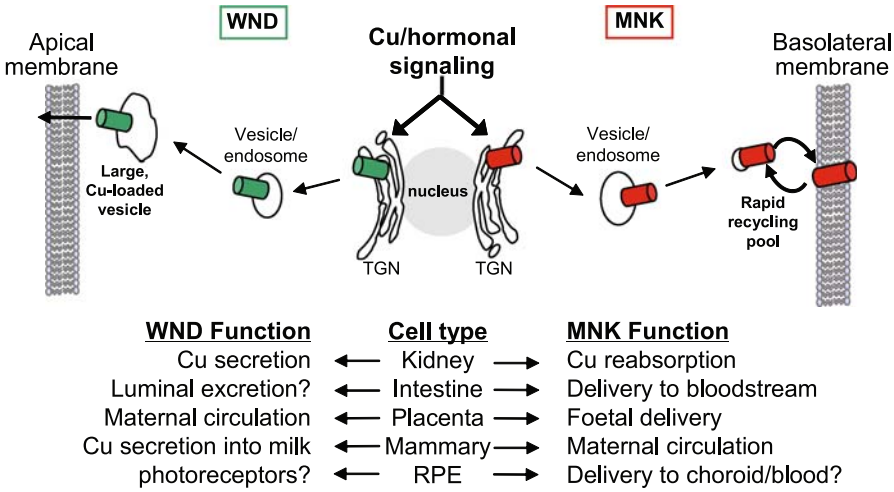

Fig. 1 Complementary function of Cu-ATPases expressed in the same tissues. The identification of tissues expressing both Cu-ATPases has shown that each Cu-ATPase undergoes Cu-stimulated trafficking from the TGN to different membrane domains in polarized cells for specialized functions. This demonstrates that Cu-ATPase function is tissue specific and varies in nature. Functions followed by "?" have not been confirmed in the literature

summarized in Fig. 1, consistent with complementary physiological and homeostatic functions. Linz and Lutsenko (2007) have also provided evidence for distinct functions of the two ATPases in the same tissues (Linz and Lutsenko 2007). There are multiple indicators suggesting that cell signaling components regulate Cu-ATPase function. For example, estrogen and insulin not only stimulate MNK trafficking but also increase MNK expression while reducing expression of WND (Hardman et al. 2007). This suggests an additional layer of transcriptional control affects Cu homeostasis, a concept that is well supported in previous studies (Itoh et al. 2008; Mattie et al. 2008). Additionally, if Ctr1 is responsible for the Cu uptake and stimulation of Cu-ATPases in these tissues, there is potential for hormonal signaling to indirectly regulate Cu-ATPase function by inducing expression and targeting of the Ctr1 Cu uptake protein.

B) Copper release from hippocampal neurons

Another interesting model for Cu-ATPase trafficking is observed in hippocampal neurons. Activation of the N-methyl-D-aspartic (NMDA) receptor by glycine–glutamate stimulates synaptic release of intracellular Cu, thus linking Cu homeostasis to neuronal activation. MNK function is linked to this response where upon NMDA activation MNK undergoes rapid, reversible relocalization from the TGN to post-Golgi neuronal processes for Cu release (Schlief et al. 2005). This neuroprotective effect occurs independently of intracellular Cu concentrations. Furthermore, in the absence of large neuronal intracellular Cu stores, NMDA-mediated efflux is associated with lower MNK catalytic activity, suggesting neuronal MNK trafficking may occur independently of its catalytic cycle (Lutsenko and Petris 2003; Rae et al. 1999; Schlief et al. 2005). The NMDA receptor also controls Ca^{++} permeability and modulates Ca^{++}-dependent signal cascades leading to neurotransmission for various cellular responses (Liao et al. 2001; Lu et al. 2001). These processes suggest that MNK trafficking is under control of several pathways that are likely to involve cell signaling. The major signaling pathways downstream of NMDA activation involve mitogen-activated protein kinase (MAPK) family members (Hardingham and Bading 2003). However, studies to determine physiological substrates of the NMDA-induced extracellular signal-regulated kinases ERK1 and ERK2 (mitogen-activated (MAP) kinases) showed no MNK phosphorylation when ERK1/2 was stimulated (Cuthbert et al. 2007). This implicates ERK-independent pathways in MNK neuronal trafficking, despite known links between ERK-dependent signaling and at least the Wilson copper P-type ATPase (Ko et al. 2006).

Copper-dependent activation of cell signaling pathways

Metals and oxidative stress are linked to the pathogenesis of many disorders including cancer, diabetes and cardiovascular disease (Barthel and Klotz 2005; Gupte and Mumper (2008); Li et al. 2007). The importance of understanding the role of Cu in these systems is now becoming clear and may provide new drug targets (Qin et al. 2008). At the transcriptional level, exposure of cells to Cu induces several signaling pathways (Mattie and Freedman 2004; Mattie et al. 2008; Ostrakhovitch and Klotz 2007). The MAP kinases p38 and ERK, CKII and PKC for example, are all reported to regulate metallothionein (MT) expression. Transcription is activated via regulation of the metal transcription factor MTF-1 in response to Cu and ROS (Mattie and Freedman 2004). Up-regulation of PKC expression (alpha, delta and zeta isoforms) initiating colonic tumor growth has also been demonstrated in rats fed on a high Cu diet (Davis and Johnson 2002).

Copper increase is correlated with phosphorylation and activation of the c-Jun N-terminal kinase/stress-activated protein kinase and p38/MAPK pathways, having potential proto-oncogenic effects (Mattie et al. 2008). Similarly, phosphoinositide 3-kinase (PI3K) and PI3K-dependent activation of the Ser/Thr kinase Akt (PKB) is stimulated by Cu and Zn ions. This occurs in part via increased levels of Cu-induced reactive oxygen species (ROS), causing phosphorylation and nuclear exclusion of the FoxO family of transcription factors. This in turn affects gene expression relating to cell cycle, apoptosis and glucose metabolism (Barthel et al. 2007; Walter et al. 2006). This insulin mimetic effect occurs via Glucose 6-Phosphatase promoter activity, being dependent on both PI3K and Akt-dependent and independent pathways (Barthel et al.

2007; Ni et al. 2007). The link between Cu and glucose metabolism is an intriguing one that was noticed many years ago (described in detail by Walter et al. 2006). The protective effect of metallothionein in diabetes and diabetic complications is also well characterized (Li et al. 2007). Analogous effects have also been observed in the protist *Chlamydomonas reinhardtii*, where Cu and hypoxia share the same signaling pathways for expression of oxygen-dependent glycolysis enzymes (Quinn et al. 2002). The similarities suggest that as a redox metal, Cu (and metallothionein) initiates signaling responses as an indirect measure of intracellular oxygen levels, thus regulating processes associated with energy and oxidative metabolism.

Reactive Cu ions and Cu-independent ROS induce oxidative damage due to the generation of hydroxyl radicals and these can also function at the post-translational level, due to interaction with exposed thiol groups of multiple proteins. Oxidative stress contributes to the onset and development of age-related diseases such as atherosclerosis, diabetes, Parkinson's and Alzheimer's diseases as well as various diseases of autoimmunity and inflammation (Cherny et al. 2001; Donnelly et al. 2007; Smith et al. 1994; Stockel et al. 1998). Alzheimer's Precursor Protein (APP) and Prion Protein (PrP) are susceptible to misfolding leading to protein aggregations or plaques (e.g., when APP is cleaved into the amyloidogenic Aβ peptide). However, brain Aβ accumulation is minimized through activation of matrix metalloproteases (MMPs) and current evidence suggests that Cu participates as a co-factor in the activation of signaling pathways to up-regulate MMP activity. Clioquinol (CQ) is a compound which complexes zinc and Cu leading to dissolution of Aβ aggregates (White et al. 2006). The CQ-Cu^{2+} mechanism of action occurs at least in part via PI3K/Akt activation and downstream phosphorylation of glycogen synthase kinase (GSK-3) and MAP kinases such as ERK1/2 (Donnelly et al. 2008; Price et al. 2007). However, similar effects were not observed by Cu treatment alone, suggesting Cu ions were first delivered to a sub-cellular location before being released for its effect to take place (White et al. 2006).

Copper pathways as therapeutic targets

There are many pathways activated by the presence of Cu or ROS and these are potential targets for regulation of Cu-ATPase function, at least in part due to the function of cell signaling pathways. It is also becoming clear that understanding the complex signaling pathways potentially regulating Cu-ATPase function may also lead to new therapeutic targets. MNK itself modulates angiotensin-II induced hypertension via SOD3 activity, and is considered a major therapeutic target for ROS-dependent cardiovascular disease (Qin et al. 2008). Copper and Cu-signaling is also implicated in angiogenesis (blood vessel development) and tumor progression, where Cu stimulates the phospholipid kinase SGK1 as well as angiogenic factors like fibroblast growth factors FGF-1 and FGF-2, being stimulators of Akt signaling. Hence, Cu chelators are being tested to reduce progression of melanomas (Di Serio et al. 2008; Soldi et al. 2007). As outlined in this review, key components of the signaling and trafficking pathways used by MNK and WND to maintain Cu homeostasis are likely to be universal and important for other functions rendering them difficult targets for therapeutic compounds. Thus a better strategy to control copper levels may be to directly target proteins required for the regulation of homeostatic enzymes participating in systemic Cu distribution, such as MNK and WND. Solving the structure of these proteins will greatly enhance the chances of designing novel drug targets in the future. If successful, such drugs could be used to treat Cu-associated neurodegenerative disorders such Alzheimer's and Prion disease by modulating the absorption of Cu across the blood/brain barrier.

Conclusions

Research contributing to understanding the complex network of Cu-regulated biological processes is rapidly expanding and it is evident that multiple layers of transcriptional and post-translational regulation control mammalian Cu-ATPase function. Establishing cause and effect for these studies will be a significant challenge. However, current evidence suggests that Cu-responsive kinase phosphorylation regulation of localization and catalytic activity of the Cu-ATPases would be a key process. Furthermore, phosphorylation of WND and MNK appears to be both constitutive (i.e., operating at basal Cu levels) and Cu-responsive.

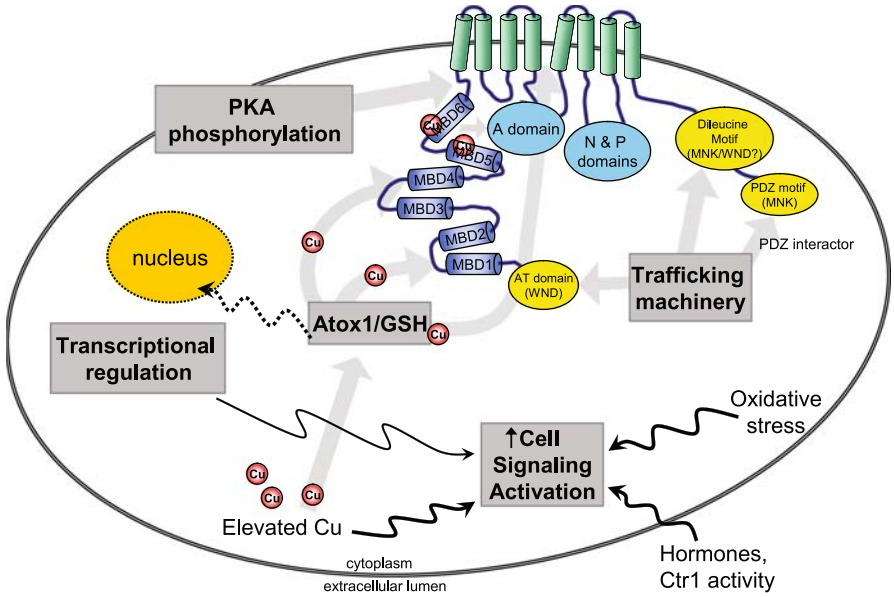

Fig. 2 The multi-layered regulation of Cu-ATPases. MNK and WND (colored) trafficking and activity is regulated by kinase-dependent and independent mechanisms (grey). Signaling components including the kinases PKA, PKC, MAPK pathways (including p38 and ERK1/2) and lipids modulate trafficking machinery and other cuproenzymes in response to intracellular and extra-cellular triggers, including elevated Cu and transcriptional regulation. Elevated intracellular Cu also directly modulates Cu-ATPase activity. The transfer of Cu ions to N-terminal MBDs by an Atox1-GSH complex potentially exposes sorting motifs, including the apical-targeting (AT) domain in WND, and dileucine and PDZ motifs in MNK (in yellow). Metallation of MBD5 and MBD6 is also predicted to expose PKA phosphorylation (in the Ccc2 Cu-ATPase) and initiate interactions with the phosphatase 'A' domain. This promotes catalytic activity, via the ATP-binding 'N' domain and acyl-phosphorylation 'P' domain, and transfer of Cu directly from Atox1/GSH to the Cu-translocating channel

Low-level basal phosphorylation may play an important structural role and is therefore important for steady state Cu-ATPase activity. In contrast, elevated Cu may cause increased phosphorylation due to: (1) direct stimulation of signaling pathways; or (2) exposure of phosphorylation sites due to structural changes as a result of Cu-binding to the transporter. These mechanisms are not mutually exclusive as both are likely to affect Cu-ATPase function and trafficking. In addition, these pathways may contribute to biochemical and localization differences in WND and MNK (summarized in Fig. 2). More research is necessary to elucidate the complexity of these pathways and to understand the relationship between Cu-signaling and Cu-ATPase function. This will have a major impact on understanding upstream signaling events, as well as improving diagnosis and treatment of inherited and acquired diseases of Cu homeostasis. An important step will be determination of phosphorylation sites and the kinases and signal transduction pathways involved and structure/function analysis of WND and MNK, as key mediators of Cu homeostasis.

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