Molecular Mechanisms of Cellular Copper Homeostasis in Mammals

Sylwia HERMAN, Paweł LIPIŃSKI, Rafał STARZYŃSKI, Aleksandra BEDNARZ, Paweł GRZMIL, and Małgorzata LENARTOWICZ

Copper (Cu) is a trace element necessary for the growth and development of all living organisms, and is the third most abundant trace metal in the body after iron and zinc. Copper is essential for maintaining the life processes in all living cells, because several copper-dependent enzymes play an important role in key physiological processes like cellular respiration, oxygen radical scavenging, the transport of iron and neurotransmitter synthesis. Maintaining copper homeostasis implies maintaining the constancy of copper levels in the cells and fluids throughout the body, in order to support the enzymes and other factors that underlie normal life processes. Therefore, living organisms have developed complex mechanisms for maintaining their physiological copper level, because an excess copper level can be toxic for the cells. In the cell, copper homeostasis is controlled by a network of copper-binding proteins and transporters. Furthermore, copper uptake is mediated by the membrane transporter CTR1 and CTR2 proteins. In the cytoplasm, it is bound to a unique group of metallochaperones (ATOX1, CCS, COX17) and transported to different cell compartments, where it is linked to the recipient proteins. The Cu-transporting ATPases (ATP7A and ATP7B) are responsible for transferring copper into the Golgi apparatus, where the copper is added to the active sites of enzymes, and it is also directed onto the path of excess cellular copper removal to prevent the occurrence of toxicity.

Key words: SOD1, copper metabolism, CCS, Cu-transporting ATPases, CTR1, CTR2, ATOX1.

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Copper (Cu) is an essential micronutrient, which plays a role in several fundamental processes that are critical for the normal growth and development of all living organisms. In humans (Homo sapiens), copper is the third most abundant trace metal in the body after iron and zinc (TÜMER & MØLLER 2010); on average, the organism of an adult human contains 110 mg of Cu (LINDER et al. 1998). Due to its ability to accept and donate electrons, this reactive element can exist in two oxidation states: as a reduced, cuprous (Cu+) ion; and as an oxidized, cupric (Cu2+) ion (VELDHUIS et al. 2009). The extensive range of potential redox reactions by copper, and its capacity to participate in one-electron transfer reactions, determine the biological activity and function of this microelement. Owing to its redox properties, copper is an integral part of enzymes, where the metal works as a prosthetic group to facilitate the transfer of electrons from one molecule to another. Copper can coordinate with several electron donor ligands, and in the proteins, it is often ligated to amino acids like cysteine, methionine and histidine. The cyclic amino acids tyrosine, phenylalanine and tryptophan also have significant copper-binding capacities (VELDHUIS et al. 2009). Copper is a cofactor for more than 30 enzymes, many of which catalyse the reactions used in fundamental metabolic processes. A copper deficiency leads to the impaired function of these enzymes and, in consequence, disturbances in an organism’s homeostasis. The Cu-containing enzyme cytochrome c oxidase,
which is the terminal enzyme in the mitochondrial respiratory chain, catalyses the reduction of dioxygen to water in the process of cellular respiration (KADENBACH & HÜTTEMANN 2015). Superoxide dismutase SOD1 and SOD3, containing copper and zinc as cofactors, catalyses the reaction of superoxide to hydrogen peroxide and plays a critical role in the detoxication of oxygen free radicals (MONDOLA et al. 2016). The synthesis of neurotransmitters is catalysed by dopamine β-hydroxylase and peptidylglycine alpha-amidating monoxygenase (PAM). Meanwhile, pigment and connective tissue synthesis are catalysed by tyrosinase and lysyl oxidase, respectively. The Cu-dependent ferroxidases ceruloplasmin and hephaestin regulate the cellular and systemic iron metabolism (VASHCHENKO & MACGILLIVRAY 2013; GUPTA & LUTSENKO 2009; ÖHRVIK et al. 2017; LUTSENKO et al. 2007). However, excess copper can be toxic to organisms; thus, to avoid copper toxicity, organisms have developed special proteins and enzymes to prevent any free copper ions from floating about that might result in a Fenton reaction (LUTSENKO et al. 2007; VAN DEN BERGHE & KLOMP 2010). To ensure the constancy of copper availability at both the cellular and systemic levels, organisms have also developed a complex network of proteins involved in the regulation of copper uptake, transport, utilisation, storage and excretion. The cellular copper uptake, transport and utilisation are coordinated by a closely integrated network of three groups of proteins. The first are copper transporters belonging to the CTR/SLC31 (copper transporter) family, which are proteins that are involved in the copper uptake and intracellular distribution. The second group is the metallochaperones ATOX1, COX17 and CCS, cytosolic copper transporters, which bind the Cu ions and deliver them to specific cellular organelles. Third are the transmembrane P-type Cu-transporting ATPases (ATP7A and ATP7B), which are shuttled between the trans-Golgi network (TGN) and the plasma membrane as proteins anchored in the membrane of the Golgi vesicles. In the Golgi apparatus, the ATP7A and ATP7B proteins transfer copper into the lumen of the secretory pathway, where this metal is incorporated into the active sites of the Cu-dependent enzymes. Both ATP7A and ATP7B are also involved in the ATP-dependent transport of Cu ions across the plasma or intracellular membranes (ÖHRVIK et al. 2017; LUTSENKO et al. 2007; LIN et al. 2015).

Systemic Copper Metabolism in Mammals

In mammals, during gestation, copper is transported from the mother’s circulation to the foetus throughout the placenta, and copper uptake is enhanced during the last part of gestation. Mammalian foetuses accumulate stores of copper in the liver, and this indispensable element is then utilised by the rapidly-growing young organism during the time of lactation (GAMBILING et al. 2011; WADWA et al. 2014). In adult mammals, copper is predominantly absorbed from the diet through the duodenum and small intestine and is transported to the blood from the enterocytes. In adult humans, the average daily intake of copper ranges from 0.8 to 3 mg (TURNLUND 1998; LUTSENKO et al. 2007). In the blood, the absorbed copper is bound to the albumin or macroglobulin and transcuprein, and is then transported via the portal vein into the liver, a central organ that maintains copper homeostasis (LENARTOWICZ et al. 2010a; GUPTA & LUTSENKO 2009; LINDER et al. 1998; FOCARELLI et al. 2022). The liver is the site of copper storage in the form of the Cu-metallothionein complex (DAVIS & COUSINS 2000). In the hepatocytes, copper is also bound to the apo-ceruloplasmin and, in the form of a holo-ceruloplasmin complex, it is again secreted into the circulatory system to be redistributed to other organs and tissues (BARTEE & LUTSENKO 2007; LENARTOWICZ et al. 2010a). Ceruloplasmin is a main copper carrier in the blood, responsible for transporting this microelement to various organs and tissues. Approximately 65% of serum copper occurs in the complex with CP, while the remaining serum copper is bound to the albumin or transcuprein (FOCARELLI et al. 2022). The liver also takes part in removing copper from the body, because excess cytosolic copper in the hepatocytes is expelled into the bile and removed from the organism in the faeces, which is the main route of copper excretion (MERCR & LLANOS 2003; BARTEE & LUTSENKO 2007). Only 2% of copper is removed from the body by the kidneys via the urine (LINZ et al. 2008; LENARTOWICZ et al. 2010b). Copper, once delivered to various organs, is utilised in the metabolic processes by Cu-dependent enzymes. Although various tissues differ in their copper requirements, the set of proteins regulating copper distribution within the cells is thought to be the same in all tissues.

Orchestration of cellular copper metabolism by a closely integrated network of proteins

The protein-mediated distribution of copper from the uptake system (CTR1) to the export system (Cu-ATPases) is a unique feature of copper transport, when compared with the transport of other ions such as sodium or calcium (ÖHRVIK et al. 2017; LUTSENKO et al. 2007). The high binding affinities of the cellular machinery help in the delivery of copper to the various cellular compartments. In consequence, the cellular and systemic Cu⁺ movement appears to be mediated via ligand exchanges upon protein-protein interactions (LUTSENKO et al. 2007). Currently, we know that cellular copper homeostasis requires a series of copper importers, carriers, chaperones, recipient proteins and exporters, to achieve the essential level of this biometal and to prevent toxicity (summarised in Fig. 1 and Table 1).
Copper transport to the cells

The CTR1 and CTR2 proteins belonging to the CTR/SLC31 (copper transporter) family are involved in the copper uptake and intracellular distribution. Studies conducted on embryonic fibroblast cell lines have shown that in mammalian cells, the copper import is primarily mediated by the high-affinity copper membrane transporter CTR1 (NEVITT et al. 2012; WEE et al. 2013). In humans, the SLC31A1 protein, widely known as CTR1, is encoded by the SLC31A1 (solute carrier family 31 member 1) gene; while in mice (Mus musculus), the Slc31a1 protein (Ctr1) is a product of the Slc31a1 gene (Table 1). The human CTR1 is ubiquitously expressed with the highest expression in the liver, kidneys, small intestine, ovaries, testes and heart (GUPTA & LUTSENKO 2009; ÖHRVIK & THIELE 2015; LUTSENKO 2021; MANDAL et al. 2020; ÖGÖREK et al. 2017). CTR1 is composed of three transmembrane domains: an extracellular N-terminal domain with a characteristic M et motif that is rich in methionine and histidine; a large intracellular loop; and a short intracellular C-terminus domain. CTR1 transports copper in the form of Cu+ ions in an ATP-independent manner, using conserved methionine residues located in the N-terminal domain. Two-dimensional electron microscopy studies have demonstrated that CTR1 forms a pore for the transport of Cu+ ions across membranes, by the formation of a homotrimer within the cell membrane. The pore is lined on its extracellular side by a ring of six methionine residues located in the N-terminal domain. The major role of CTR1 in this mechanism is to facilitate the release of copper from degraded cuproenzymes in the lysosomes and to transport it back into the cytosol for re-utilisation (ÖHRVIK et al. 2017; WEE et al. 2013; MANDAL et al. 2020). Similarly to SLC31A1/Slc31a1, the expression of SLC31A2/Slc31a2 is found in all tissues and organs, with a high abundance in the liver, kidneys and testis (ÖHRVIK & THIELE 2015; WEE et al. 2013; ÖGÖREK et al. 2017). Transfection of human fibroblasts with the hCTR1 (human CTR1) cDNA sequence resulted in an increased capacity for copper uptake by these cells. Meanwhile, no effect on the copper uptake was obtained in human fibroblasts transfected with the hCTR2 (human CTR2) cDNA sequence. CTR1 is the only identified major Cu uptake transporter in the plasma membrane and it plays a pivotal role in the copper absorption in human cells (MOELLER et al. 2000). On the other hand, the results obtained by ÖHRVIK et al. in 2013 indicated that CTR2 plays a crucial role in regulating the function of the CTR1 protein, by the cleavage of the CTR1 methionine-rich ectodomain to generate a form that is more active for mobilising endosomal copper stores than the full-length mammalian CTR1, which is the more active form for Cu+ imports across the plasma membrane. How the process of CTR1 cleavage occurs in endosomes was shown during in vitro studies (ÖHRVIK et al. 2013). However, it has not been fully elucidated how CTR2 participates in the CTR1 cleavage, although it has been suggested that CTR1 and CTR2 interact as a complex, and CTR2 enhances the abundance of truncated Ctr1 either by the recruitment of a protease, or by stabilising the truncated Ctr1 against degradation (ÖHRVIK et al. 2013). A subclass of lysosomal cysteine proteases, cathepsin B and cathepsin L, were shown to be responsible for the cleavage of the ectodomain of CTR1 (ÖHRVIK et al.
The cleavage of mouse Ctr1 resulted in the removal of all 11 His residues and 10-13 Met residues in the Ctr1 ectodomain. However, the majority of Ctr1 truncations retained one of two Met residues of the M-X-M sequence, both of which were previously shown to be essential for the Ctr1 Cu⁺ transport activity (ÖHRVIK et al. 2013).

However, CTR1 is not the only mechanism by which copper enters the cells. Divalent metal transporter 1 (DMT1) also imports copper ions into some types of cells under certain circumstances (LIN et al. 2015; JIANG et al. 2013). Conversely to the CTR1 protein, the DMT1 levels increase in response to a rise in copper concentrations, and therefore, it is inversely correlated to a decrease in CTR1. Studies have demonstrated that a deficiency in both CTR1 and DMT1 will completely inhibit the uptake of copper ions. The conclusion can be made that the DMT1 protein imports copper ions in the case of a CTR1 deficit, and vice versa. Therefore, a compensation mechanism occurs here. (LIN et al. 2015). However, the results of another study demonstrated that in the cells of intestine, DMT1 is not required for the transport of copper (SHAWKI et al. 2015). Therefore, the role of DMT1 in copper transport is controversial and should be further investigated.

Intracellular copper transport and distribution

The second group is comprised of metallochaperones, cytosolic copper transporters that bind the Cu ions and deliver them to the cellular organelles, to reduce possibility of Cu ions participating in the Fenton reaction (WERNIMONT et al. 2000; LUTSENKO et al. 2007).

**ATOX1 – metallochaperone, transcription factor, and antioxidant protein**

An antioxidant copper chaperone protein (ATOX1) is a cytosolic protein which transports Cu from the membrane-bound CTR1 to the Cu-ATPases - ATP7A and ATP7B - while they are located in the membrane of the trans-Golgi network (TGN) and stimulates their catalytic activity. Human ATOX1 is a small (consisting of 68 amino acids) and soluble protein that is ubiquitously expressed. It is encoded by the ATOX1 gene (STRAUSAK et al. 2003; HATORI & LUTSENKO 2013), which is localised on chromosome 5, while in mice, the Atox1 gene is localised on chromosome 11 (Table 1). A large-scale analysis of the human transcriptome showed the highest expression of ATOX1 in the adult kidney, liver and spleen. The

| Table 1 |

Characteristics of human (*Homo sapiens*) and murine (*Mus musculus*) genes encoding the proteins involved in the cellular copper uptake (CTR1 and CTR2), copper metallochaperones (ATOX1, COX17 and CCS) and the copper transporting ATPases (ATP7A and ATP7B). Source: http://www.ncbi.nlm.nih.gov

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene name</th>
<th>Accession number (NCBI)</th>
<th>Gene length (bp)</th>
<th>Exon count</th>
<th>Gene localisation</th>
<th>mRNA length (bp)</th>
<th>Protein length</th>
<th>Gene product (protein)</th>
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<td>42,965</td>
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<td>31,188</td>
<td>6</td>
<td>4 C1-C2</td>
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<td>196 aa</td>
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ATOX1 protein contains a single metal-binding site (MBS) with a characteristic MetxCysxxCys motif. In the MBS, a copper ion is bound by sulphur atoms from two cysteines; thus, one molecule of ATOX1 can bind and transfer one Cu^+ ion (HATORI & LUTSENKO 2013). Among the different roles played by ATOX1, the most important is the transfer of copper to the Cu-transporting ATPases, and there is data which indicates that ATOX1 also delivers Cu to metal-binding domains (MBD) of ATP7A and ATP7B. The MetxCysxxCys copper-binding sequence of ATOX1 has a significant homology and a similar folding to the N-terminal metal-binding domain of the Cu-ATPases, and interacts with them in a copper-dependent manner (LUTSENKO et al. 2008; HATORI & LUTSENKO 2013). Thus, the Cu delivered to the TGN is transferred into the lumen, via the ATP7A and ATP7B action, where it is incorporated into the Cu-dependent enzymes. The inactivation of ATOX1 inhibits the maturation of cuproenzymes, as well as copper exports from the cells (BARRY et al. 2010). It is known that ATOX1 regulates the catalytic activity of ATP7B, because the ATOX1-Cu transfer stimulates the phosphorylation of this protein (BARRY et al. 2010; HATORI & LUTSENKO 2013). ATOX1 can also play a role as a Cu-ATPase inhibitor, because at a low cellular copper concentration the apo-form of ATOX1 can remove copper from 4-5 MBDs of the Cu-ATPases and therefore down-regulate the enzyme activity to about 50% (LUTSENKO et al. 2007). The cellular functions of ATOX1 are not limited to its copper-trafficking role, and may include the storage of labile copper, modulation of the gene transcription and antioxidant defence (MULLER & KLOMP 2009). The ATOX1 protein contains a nuclear localisation signal (NLS) at the C-terminal domain, and in the nucleus it plays the role of a transcription factor (TF) regulating the expression of genes (ITOH et al. 2008; ITOH et al. 2009). As an example, direct copper-dependent interactions of Atox1 with the promoter region of the Ccnd1 gene encoding cyclin D1 have been demonstrated in mice. Under a high concentration of copper ions inside the cell, Atox1 also stimulates the expression of the SOD3 (superoxide dismutase 3, extracellular) gene. The SOD3 protein is the major extracellular antioxidant enzyme protecting against O_2^- by catalysing the dismutation of two superoxide radicals into hydrogen peroxide and oxygen. The results obtained by ITOH et al. (2009) revealed that copper induces the binding of Atox1 to the Atox1-responsive element of the mouse Sod3 promoter, which is indispensable for the copper-dependent Sod3 transcription (ITOH et al. 2009). ATOX1 also plays an essential role in inflammatory neovascularisation and wound healing. This is possible because ATOX1, as a metallochaperone, delivers copper ions through the CTR1-ATOX1-ATP7A pathway to the active centre of lysyl oxidase (LOX), the enzyme which is involved in the process of angiogenesis (SUDHAHAR et al. 2019; CHEN et al. 2015; DAS et al. 2016).

In addition, the ATOX1 protein participates in placental copper transport. Experiments on mice with a knockout of the Atox1 gene (Atox1^-/- mice) showed that most of the copper is trapped in the placenta in the case of Atox1 deficient mice. In the embryonic cells, such as the syncytiotrophoblasts and capillary endothelial cells, in the absence of a functional Atox1 chaperone, copper is not delivered to the Atp7a and Atp7b proteins, which are responsible for the copper transfer between mother and foetus (HAMZA et al. 2001). Most of the homozygous Atox1^-/- pups died just after birth, and only around 67% survived the critical period; however, some of them also died subsequently, before weaning. The overall perinatal mortality of these mice was 43%. The phenotype of the homozygous Atox1^-/- mice was also very close to that observed in copper-deprived animals. These Atox1^-/- mice exhibited growth retardation, central nervous system deformations, skin laxity, hypothermia and hypopigmentation, and they also suffered from peripartum haemorrhaging. Some of the pups had severe congenital eye defects (microphthalmia). On postnatal day 2 (day P2), the tested knockout mice showed lower copper concentrations in the liver and in the brain (around 50% compared to their wild-type littermates). The activity of the copper-dependent enzymes, such as cytochrome c oxidase in the brain and tyrosinase in the skin cells, was also lower. However, the heterozygous mice Atox1^-/- were indistinguishable from their wild-type littermates (HAMZA et al. 2001).

**CCS – metallochaperone mediated SOD1 maturation**

The copper chaperone for superoxide dismutase 1 (CCS) is a ubiquitously expressed protein with a molecular weight of 35 kDa, which forms a 70 kDa homodimer. The CCS (copper chaperone for superoxide dismutase) gene is localised on chromosome 11 in humans and the mouse Ccs is located on chromosome 19 (Table 1). In the cells, CCS is localised in the cytosol and intermembrane space of the mitochondria (ANTINONE et al. 2017; CULOTTA et al. 1997; PROHASKA et al. 2003a; PALUMAA 2013). As a metallochaperone, CCS binds Cu^2+ and is responsible for delivering copper to the Cu,Zn-superoxide dismutase (SOD1), ensuring the conversion of apo-SOD1 to holo-SOD1. The CCS protein consists of three domains that carry out the separate functions of copper-binding, trafficking and docking to the adaptor SOD1 protein. The N-terminal domain I is an ATOX1-like domain, and it includes the MetxCysxxCys motif in the loop responsible for binding copper (ANTINONE et al. 2017; PROHASKA et al. 2003b). Domains II and III are required for the interaction with, and activation of, SOD1. The central and largest domain II contains a region of close homology with SOD1 (in humans it is close
to 50%), and it is responsible for the formation of heterodimeric CCS-SOD1 intermediate and docking CCS to the SOD1 during the protein metalation. On the C-terminus, there is the smallest domain (domain III) that contains the most conserved motif CysxCys across the whole CCS protein. It is responsible for binding copper to apo-SOD1 by catalysing the formation of disulphide bonds between the copper and protein, and any mutation in this region will cause a CCS dysfunction. CCS not only plays a crucial role in SOD1 metalation, but is also necessary for SOD1 activation and maturation (ALLEN et al. 2012; ANTINONE et al. 2017). The SOD1 maturation from an inactive monomer to a functional dimeric stable form of the enzyme requires zinc-binding, copper acquisition, the formation of an intramolecular disulphide bond between cysteine (Cys) 57 and Cys 146, and homodimerisation (BOYD et al. 2020; ANTINONE et al. 2017). In the absence of metal cofactors, and upon a reduction of the disulphide bonds, the SOD1 dimer is destabilised and exists as an inactive monomer (ANTINONE et al. 2003a; PROHASKA et al. 2003a; SUAZO et al. 2008). Ccs<sup>−/−</sup> knockout mice with the deletion of exons 1 and 2 were viable and exhibited normal amounts of Sod1, but there was an abnormal activity of this protein in comparison to the wild-type littermates. The Ccs<sup>−/−</sup> mice demonstrated a similar
phenotype to Sod1 knockout mice, because in the Ccs−/− mice copper ions were not delivered to the Sod1 enzyme. In the Ccs−/− mice, the activity of the Sod1 protein was lowered by 15% in the brain, kidneys and spinal cord. Moreover, it was lowered by 30% in the liver, even when the copper amount delivered to hepatocytes was normal. The Ccs−/− females also had problems with fertility and the structure of their ovaries was disturbed. A histological analysis of the Ccs−/− female ovaries revealed abnormally formed follicles instead of corpus luteum (Wong et al. 2000).

COX17 – a metallochaperone essential for the Cu-mitochondrial pathway

COX17 is a small (8 kDa) hydrophilic protein located both in the cytoplasm and in the mitochondrial intermembrane space (IMS). This small metallochaperone contains 6 conserved cysteine residues; thus, it binds and transports Cu+ ions from the CTR1 membrane protein and delivers copper to CCO through other metallochaperones directly associated with the metalation of CCO (Palumaa et al. 2612). In mammals, 4 copper chaperones are required to transfer copper from the incoming CTR1 to CCO: COX17, SCO1, SCO2 and COX11 (Cobine et al. 2006; Prohaska & Gymba 2004).

In humans, the COX17 (cytochrome c oxidase copper chaperone COX17) gene is localised on chromosome 3 (3q13.33), but its pseudogene is also localised on chromosome 13 (13q14-21). In mice, Cox17 is localised on chromosome 16 (Table 1) (Horvath et al. 2000; Cobine et al. 2006). The expression of Cox17 was found in all tissues, but the Cox17 mRNA level was highest in the heart, kidneys, brain and also in some endocrine cell types, as was shown in rodents. A low expression was observed in the liver, small intestine and some fibroblast cell lines (Kako et al. 2000). A lack of activity of Cox17 in mice leads to early prenatal lethality. For example, Cox17−/− mice died between embryonic days E8.5 and E10 (Prohaska & Gymba 2004) because the proper activation of Cco during embryonic development is essential (Horvath et al. 2000; Cobine et al. 2006; Kako et al. 2000). Heterozygous mice with a disabling mutation in this gene, which spans from the middle of exon 1 until the end of exon 2, appeared healthy, were fertile and were of a normal size. However, a closer analysis showed that in their brains, hearts, kidneys and skeletal muscles, the expression of the Cox17 gene was around 50% lower compared to wild-type mice. Interestingly, the expression of Cox17 in the other tissues was not changed in the mutants. Furthermore, in the brains of those mice, the Cco activity was lower by 20%, but there were no changes in Cco activity in the kidneys or skeletal muscles (Horvath et al. 2000; Cobine et al. 2006; Kako et al. 2000). This may indicate that even with a 50% decrease in the Cox17 gene expression, a sufficient amount of the Cox17 protein is being produced. Moreover, this process is organ dependent, because a lower expression of Cox17 in the brain, heart, kidneys and skeletal muscles allows for their normal functioning.

The copper-transporting ATPases, ATP7A and ATP7B, are critical components of copper metabolism

ATP7A and ATP7B belong to the large family of P-type ATPases and form a separate subgroup within the P-type ATPase family (P1A-ATPases), which has distinct structural and mechanistic characteristics (Veldhuis et al. 2009). ATP7A and ATP7B share a primary sequence homology, have a similar architecture and are composed of several functional domains. A N-terminal cytosolic portion of these proteins contains six cysteine-rich copper-binding domains (MBD1-MBD6), with characteristic M etxCysxxxCys motifs that bind the reduced form of copper (Cu+) (Lutsenko et al. 2007; Gupta & Lutsenko 2009; Lutsenko 2021). Eight transmembrane domains (TM 1-TM 8) stabilise the protein in the membrane and form the intramembrane channel, with a highly-conserved CysProCys sequence in the TM 6 for the transport of metal ions. The ATP7A and ATP7B proteins play a crucial role in the maintenance of cellular Cu homeostasis by delivering copper ions to various copper-dependent enzymes and, in the case of a copper overload in the cell, by transporting the excess copper out of the cell. Both ATPases use energy from the ATP hydrolysis for the Cu+ ions transport (Lutsenko et al. 2007; Veldhuis et al. 2009; Banchi et al. 2010; Pierson et al. 2019). During the catalytic cycle, the ATP7Aases are autophosphorylated by a conserved aspartic acid in the ATP-binding domain to form the cytoplasmic loop between TM 6 and TM 7, and subsequently to bind Cu to the transmembrane domains (Lutsenko et al. 2007). After the release of Cu, the ATPases are dephosphorylated by the A-domain which is located between TM 4 and TM 5. The C-terminus of the ATP7A contains dileucine (LeuLeu) and this motif play an important role in the retrograde transport of ATP7A, because it is essential and sufficient for the retrieval of ATP7A from the plasma membrane back to the TGN. In the ATP7B, tri-leucine (LeuLeuLeu) motif, has also been predicted to act as an endocytic retrieval signal and could be involved in returning ATP7B from the vesicles to the TGN (Banchi et al. 2010; Gupta & Lutsenko 2009; Lutsenko et al. 2007).

ATP7A and ATP7B are encoded by different genes localised in separate chromosomes, and both ATP7A and ATP7B prevent a toxic accumulation of copper by expelling Cu ions from the cells. It has been proposed that this Cu-induced trafficking of both ATPases is fundamental for maintaining cellular copper homeostasis (Lutsenko et al. 2007). When the
cells are exposed to increased copper concentrations, ATP7A binds the copper ions and is transported from the TGN via vesicles that bud off from the trans-Golgi network, travelling to the cytoplasm and plasma membrane (in polarised cells to the basolateral membrane) and releasing copper into the blood fluid (Gupta & Lutsenko 2009; Linz & Lutsenko 2007; La Fontaine & Merc 2007). Under the same conditions, ATP7B also carries copper ions and is transported in vesicles to the plasma membrane (in polarised cells to the apical membrane) (Veldhuis et al. 2009; Barry et al. 2010; La Fontaine & Merc 2007). A further important role of ATP7A involves delivering Cu⁺ ions to the secretory pathway, where they are incorporated into the Cu-dependent enzymes such as lysi oxidase, tyrosinase, dopamine-β-hydroxylase, peptidylglycine-α-amidating mono-oxygenase and extracellular superoxide dismutase (SOD3) (Lutsenko et al. 2007; Petris et al. 2000; Qin et al. 2006). In humans and laboratory rodents, the ATP7A protein is encoded by the X-linked ATP7A (Ap7a in rodents) gene (Table 1) (Merc 1993), and its expression has been reported in nearly all the cells in the body. For this reason, ATP7A is considered to be a housekeeping gene. Nevertheless, its expression differs among the cells and tissues, and is age-dependent (Lutsenko et al. 2007; Lenartowicz et al. 2010a; Lenartowicz et al. 2015a; Ogorek et al. 2017). The highest expression levels have been found in the brain, kidneys, small intestine, testis and the heart (Lutsenko et al. 2007; Linz et al. 2008; Lenartowicz et al. 2011; Lutsenko et al. 2008; Ogorek et al. 2017; Pierson et al. 2019). In humans, a lack of ATP7A activity caused by a mutation in the ATP7A gene leads to a severe metabolic syndrome called Menkes disease (Tümer & Mossler 2010; Maung et al. 2021). The phenotypic features of M enkes disease can be divided into at least three categories: Classical Menkes disease, with death in early childhood; Mild Menkes disease with long-term survival; and the mildest form, called occipital horn syndrome (OHS, previously known as X-linked cutis laxa or Ehlers-Danlos syndrome (Moller et al. 2009). Mice with mutations in the Ap7a gene are called mottled mutants. Many mottled mutants have arisen spontaneously in different laboratories, or have been induced by chemical or radiation mutagenesis. Currently, about 44 different mutations in the mottled locus have been described, and 15 of them have been characterised at the molecular level (Lenartowicz et al. 2015b). Several disease-causing variants affect the copper-induced trafficking of the Ap7a protein (Skjöringle et al. 2017). Mottled mutants exhibit defects in copper metabolism, and hemizygous mottled males exhibit a severe and often lethal phenotype. As in humans, the severity of the phenotype in Ap7a mutant mice is dependent on the mottled allele and varies between mottled mutations (Lenartowicz et al. 2015b; Lenartowicz et al. 2012). In general, the affected males belong to one of three classes of phenotypic severity: (1) mutant males that die in utero; (2) mutant males that die in the 3rd week of postnatal life; and (3) mutant males that die within a few postnatal months. M utants from the second class, i.e. brindled (Atp7a molt−br), macular (Atp7a molt−mac) and mosaic (Atp7a molt−mos) forms, have been extensively studied and are widely-accepted as good animal models for the severe form of Menkes disease (La Fontaine et al. 1999; Kodama et al. 1993; Lenartowicz et al. 2012). A lack of activity of the Atp7a protein in mottled mutants leads to a defect in the transport and absorption of copper. Analyses of the copper content in the organs of these mutants have indicated that copper is accumulated in the small intestine and kidneys, while the brain, liver and heart suffer from a copper deficiency. Macular, mosaic and brindled mutant mice exhibit many clinical features characteristic of defective copper metabolism, including defects in their pigmentation and hair structure, a decrease in body weight and poor viability. During the second week of life, they develop neurological symptoms like tremors, seizures, ataxia and progressive paresis of the hind limbs, and they die in the third week postpartum (Kodama et al. 1993; Lenartowicz et al. 2012; Phillips et al. 1986; La Fontaine et al. 1999; Lenartowicz et al. 2017). These pathological symptoms are connected to the specific function of ATP7A in several cell types, which consists of expelling Cu from the cells to the extracellular environment. Consequently, dysfunctional ATP7A in the absorptive enterocytes and epithelial cells of the renal tubules not only leads to the accumulation of Cu in these cells, but also limits the presence of this metal in other cells of the body (Lenartowicz et al. 2010b; Phillips et al. 1986; Kodama et al. 1993). Some studies on fibroblasts from patients with Menkes Disease caused by different missense mutations in the ATP7A gene were conducted. They showed that under the conditions of high copper levels in the cell, the ATP7A is still localised in the TGN and does not migrate to the plasma membrane as it should. Thus, several disease-causing mutations of the ATP7A protein appearing separately affect the copper-induced migration of ATP7A from the TGN, and thereby indirectly affect the transport of excess copper out of the cell (Skjöringle et al. 2017). Furthermore, because ATP7A plays an important role in the delivery of Cu to the Cu-containing enzymes, a large number of essential enzymes in the mottled mutants are dysfunctional or exhibit decreased activity (Lenartowicz et al. 2017; Nicu et al. 2007; Donsante et al. 2013; Kodama et al. 2005). The phenotypic diversity of the mottled alleles is a valuable source of knowledge, not only about the molecular basis of M enkes disease, but also about the role of copper in metabolism and development.

ATP7B is encoded by the autosomal ATP7B gene. In humans, the ATP7B (ATPase copper transporting
beta) gene is located on chromosome 13, while in mice Atp7b is found on chromosome 8 (Table 1) (Bull et al. 1993; La Fontaine & Mercer 2007). In mammals, the ATP7B protein is primarily synthesised in the liver in the hepatocytes, but its expression has been also reported in other tissues such as the placenta, mammary glands, eyes, lungs and brain (Lutsenko et al. 2007; La Fontaine & Mercer 2007). The ATP7B protein in the hepatocytes is responsible for the copper binding to apo-ceruloplasmin, resulting in the formation of the redox-active holoceruloplasmin, which is responsible for the transport of copper to the bloodstream (Bartee & Lutsenko 2007; Polishchuk et al. 2014). ATP7B also participates in the excretion of excess copper to the bile (Polishchuk et al. 2014). The ATP7B protein is also responsible for the return transport of copper from the placenta to the maternal compartment, to prevent an excess copper accumulation in the foetus (Hardman et al. 2011). During lactation in the mammalian gland cells, ATP7B participates in the export of copper to the milk (Michalczyk et al. 2000). In human patients with Wilson disease, a mutation in the ATP7B gene results in disturbances in copper binding to the ceruloplasmin (CP) by the ATP7B protein, and leads to a copper accumulation in the liver reaching the toxic level (Lutsenko et al. 2007; Lutsenko 2014; Maun et al. 2021). Mice with a mutation in the Atp7b gene (Tx – toxic milk mice) and Atp7b knockout mice are the animal model of Wilson disease (Coronado et al. 2001; Buiakova et al. 1999; Hadrian & Przybylowski 2021). In Atp7b knockout and mutant mice, a lack of Atp7b activity leads to a progressive copper accumulation in the liver up to the toxic level, resulting in the development of a wide spectrum of hepatic pathological symptoms (Hadrian & Przybylowski 2021; Voskoboinik et al. 2001; Huster et al. 2006). Moreover, in Tx mutants, the copper concentration is increased in the spleen, kidneys and brain, while infant mice are copper deficient and display an increased mortality (Coronado et al. 2001; Buiakova et al. 1999; Huster et al. 2006). Tx mutant mice, during the progress of the disease, also develop some neurological symptoms such as motor and cognitive disturbances (Terwel et al. 2011). A decrease in the ceruloplasmin level and its ferroxidase activity in Tx mutant mice leads to disturbances in the iron metabolism. A dult Tx mutants also develop mild anaemia caused by a functional iron deficiency. The mutant mice showed decreased plasma iron levels with a concomitant iron accumulation in the hepatocytes and liver macrophages (Joncézy et al. 2019).

Summary

Although mammalian organs have distinct physiological functions and consist of different cell types, both of the CTR transporters described above, metal-chaperones and Cu-transporting ATPases, are expressed in all the tissues and organs, and their expression and activity is mainly regulated by the copper level. Cellular copper homeostasis is controlled on several levels: copper uptake by the CTR transporters (CTR1 and CTR2), the intracellular molecules engaged in handling Cu and delivering it to its specific sites by the metallochaperones (ATOX1, CCS and COX17), and copper efflux by the ATP7A and ATP7B proteins. Interactions between all these groups of proteins allow for the transfer of copper from the intramembrane side of the transporter to the binding site of the acceptor proteins, and are also responsible for modulating the intracellular copper levels. A lack of activity by the copper transporters caused by a mutation or a genetic knockout leads to lethality and pathological symptoms in laboratory mice. However, experiments on those mutant and knockout animals give us the possibility to analyse multiple copper-dependent pathways for the regulation of Cu metabolism during physiological and pathological conditions. In humans, mutations in the genes encoding the copper transporting proteins ATP7A and ATP7B results in the severe metabolic disorders called Menkes and Wilson disease.

Author Contributions

Research concept and design: M.L.; Collection and/or assembly of data: A.B.; Writing the article: S.H., M.L.; Critical revision of the article: P.L., R.S., P.G., A.B., M.L.; Final approval of article: S.H, P.L., R.S., P.G., A.B., M.L.

Conflict of Interest

The authors declare no conflict of interest.

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