

## Molecular Mechanisms of Cellular Copper Homeostasis in Mammals

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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<sup>+</sup>) ion; and as an oxidized, cupric (Cu<sup>2+</sup>) 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 co-factors, catalyses the reaction of superoxide to hydrogen peroxide and plays a critical role in the detoxification of oxygen free radicals (MONDOLA *et al.* 2016). The synthesis of neurotransmitters is catalysed by dopamine  $\beta$ -hydroxylase and peptidylglycine alpha-amidating monooxygenase (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 & KLUMP 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 (GAM-

BLING *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 (MERCER & 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<sup>+</sup> 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; OGÓREK *et al.* 2017). CTR1 is composed of three transmembrane domains: an extracellular N-terminal domain with a characteristic Mets 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<sup>+</sup> 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<sup>+</sup> 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 that are believed to provide an initial coordinating site for the permeating Cu. The intracellular C-terminus then provides a putative Cu-binding site at the exit of the pore in the cytosol. Numerous studies have confirmed that the intracellular localisation of the CTR1 protein is dependent on the copper status. Under a basal copper concentration, CTR1 is found in both locations, i.e. in the plasma membrane and in the cytoplasm in the intracellular vesicles. However, under low copper conditions, CTR1 is mainly localised in the plasma membrane. High copper levels induce CTR1 endocytosis and translocation, in the vesicles, to the cytoplasm. Such a mechanism probably prevents the excessive import of copper into the cells, and thus counteracts an intracellular copper overload and toxicity (NEVITT *et al.* 2012; WEE *et al.* 2013; GUPTA & LUTSENKO 2009; ÖHRVIK & THIELE 2015; LUTSENKO 2021; MANDAL *et al.* 2020). Results of the studies performed on mice have shown that the expression of the Ctr1 protein, encoded by the *Slc31a1* gene (gene ID: 20529), is required for normal embryonic development. The expression of the *Slc31a1* gene in mouse embryos starts on Day 7 (E7) (LEE *et al.* 2002; KUO *et al.* 2006). The analysis of *Slc31a1*<sup>-/-</sup> embryos between E7 and E8 embryonic days exhibited a dramatic size reduction compared to the control foetus. A morphological analysis of *Slc31a1*<sup>-/-</sup> embryos revealed severe developmental

impairments; for example, their neural ectoderm and mesoderm cell layers were poorly developed. The heterozygous *Slc31a1*<sup>+/-</sup> mice developed normally, but in those mice, the copper concentration in the brain and spleen was reduced by around 50%. Similarly, the activity of the copper-dependent enzyme Sod1 was also reduced (LEE *et al.* 2002). The *Slc31a1*<sup>-/-</sup> knockout mice died in mid-gestation on Day 9 (E9) (LEE *et al.* 2002; KUO *et al.* 2006).

In contrast to CTR1, CTR2, a second cellular copper transporter, which is encoded in humans by the *SLC31A2* (*solute carrier family 31 member 2*) gene (gene ID: 1318) and in mice by the *Slc31a2* gene (gene ID: 20530) as the Ctr2 protein (Table 1), exhibits an exclusively intracellular localisation. CTR2 is localised in the membranes of the vacuoles, vesicles, endosomes and lysosomes of mammalian cells (ÖHRVIK *et al.* 2017; NEVITT *et al.* 2012; WEE *et al.* 2013; MANDAL *et al.* 2020). In contrast to CTR1, CTR2 is characterised by a low affinity for Cu ions, probably because this protein lacks the Met and His-rich motif in the N-terminus domain. The major role of CTR2 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; OGÓREK *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 (MØLLER *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<sup>+</sup> 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.*

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>

Organism	Gene name	Accession number (NCBI)	Gene length (bp)	Exon count	Gene localisation	mRNA length (bp)	Protein length	Gene product (protein)
<i>Homo sapiens</i>	<i>CTR1 (SLC31A1)</i>	NP_001850	42,965	5	9q32	4797	190 aa	CTR1 (SLC31A1)
<i>Mus musculus</i>	<i>Ctr1 (Slc31a1)</i>	NP_780299	31,188	6	4 C1-C2	3764	196 aa	Ctr1 (Slc31a1)
<i>Homo sapiens</i>	<i>CTR2 (SLC31A2)</i>	NP_001851	13,185	5	9q32	1791	143 aa	CTR2 (SLC31A2)
<i>Mus musculus</i>	<i>Ctr2 (Slc31a2)</i>	NP_001277447	18,020	6	4 B3	1803	141 aa	Ctr2 (Slc31a2)
<i>Homo sapiens</i>	<i>ATOX1</i>	NP_004036	15,828	4	5q32	498	68 aa	ATOX1
<i>Mus musculus</i>	<i>Atox1</i>	NP_033850	14,496	4	11 B1.3	501	68 aa	Atox1
<i>Homo sapiens</i>	<i>COX17</i>	NP_005685	17,467	6	3q13.33	423	63 aa	COX17
<i>Mus musculus</i>	<i>Cox17</i>	NP_001017429	5,780	4	16 B3	436	63 aa	Cox17
<i>Homo sapiens</i>	<i>CCS</i>	NP_005116	12,858	8	11q13	1068	274 aa	CCS
<i>Mus musculus</i>	<i>Ccs</i>	NP_058588	13,962	8	19 A	1057	274 aa	Ccs
<i>Homo sapiens</i>	<i>ATP7A</i>	NP_000043	139,740	23	Xq21.1	8540	1500 aa	ATP7A
<i>Mus musculus</i>	<i>Atp7a</i>	NP_001103227	100,937	23	X D	8198	1492 aa	Atp7a
<i>Homo sapiens</i>	<i>ATP7B</i>	NP_000044	79,462	30	13q14.3	6655	1465 aa	ATP7B
<i>Mus musculus</i>	<i>Atp7b</i>	NP_031537	67,292	24	8 A2	4711	1462 aa	Atp7b

2016, ÖHRVIK *et al.* 2017). 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<sup>+</sup> 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

Antioxidant 1 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

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  $\text{Cu}^+$  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 MetxCysxxxCys 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 & KLUMP 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  $\text{O}_2^-$  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*<sup>-/-</sup> 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 endothelium 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice was also very close to that observed in copper-deprived animals. These *Atox1*<sup>-/-</sup> 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*<sup>+/-</sup> 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  $\text{Cu}^+$  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

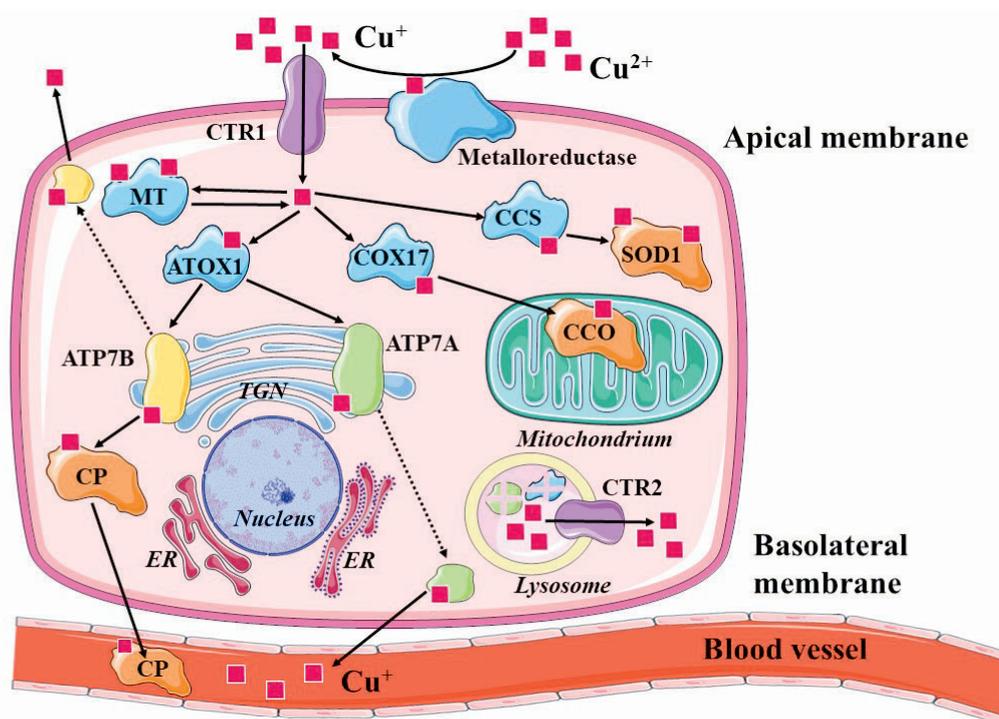


Fig. 1. Copper transport in human cells.

After a reduction by metalloreductase, copper ions can be transported into the cell by the transmembrane importer CTR1. In the cytoplasm, the reduced copper ions can be reversibly bound by metallothionein (MT) and stored in the cell. They can also be bound by metallochaperones: copper chaperone for superoxide dismutase 1 (CCS), the cytochrome c oxidase copper chaperone (COX17), and the antioxidant 1 copper chaperone protein (ATOX1), which deliver copper to diverse proteins such as superoxide dismutase 1 (SOD1), cytochrome c oxidase (CCO), ATP7A and ATP7B in different compartments of the cell including the endoplasmic reticulum (ER), mitochondria and the Golgi apparatus. The ATP7A and ATP7B proteins, while they are located in the trans-Golgi network (TGN), bind the reduced copper ions and incorporate them into the apoenzymes. As an example, ATP7B can transfer copper to apo-ceruloplasmin (CP) to form holo-ceruloplasmin. The copper in the complex with ceruloplasmin can then be transported to the blood vessels. When there is an excess of copper in the cell, the above ATPases can remove the excess copper outside the cell: ATP7B across the apical membrane and ATP7A across the basolateral membrane of the polarised cells. Thereafter, they return to the TGN membrane, where they can be used again. When copper-dependent enzymes are degraded in the lysosomes, CTR2 allows the transport of the released copper ions back into the cytosol for their reutilisation. This figure was made using the freely available illustrations from <https://smart.servier.com/> under the Creative Commons Attribution 3.0 Unported Licence.

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.* 2017; WONG *et al.* 2000; PROHASKA *et al.* 2003a). The CCS protein expression is very sensitive to the copper level, where even a marginal decrease in the copper level causes an increased CCS protein production; thus, CCS has been proposed as a marker of copper deficiency. Conversely, the concentration of the CCS protein is reduced in conditions of excess cellular copper, presumably due to an increased proteasomal degradation of CCS (BERTINATO *et al.* 2003; 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*<sup>-/-</sup> mice copper ions were not delivered to the Sod1 enzyme. In the *Ccs*<sup>-/-</sup> 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*<sup>-/-</sup> females also had problems with fertility and the structure of their ovaries was disturbed. A histological analysis of the *Ccs*<sup>-/-</sup> female ovaries revealed abnormally formed follicles instead of *corpus luteum* (WONG *et al.* 2000).

#### COX17 – 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<sup>+</sup> ions from the CTR1 membrane protein and delivers copper to CCO through other metallochaperones directly associated with the metalation of CCO (PALUMAA 2013; NEVITT *et al.* 2012). 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 & GYBINA 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*<sup>-/-</sup> mice died between embryonic days E8.5 and E10 (PROHASKA & GYBINA 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 suffi-

cient 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 (P1<sub>B</sub>-ATPases), which has distinct structural and mechanistic characteristics (VELDHUIS *et al.* 2009). ATP7A and ATP7B share a significant (50-60%) primary sequence homology, have a similar architecture and are composed of several functional domains. An N-terminal cytosolic portion of these proteins contains six cysteine-rich copper-binding domains (MBD1-MBD6), with characteristic MetxCys<sub>3</sub>xxCys motifs that bind the reduced form of copper (Cu<sup>+</sup>) (LUTSENKO *et al.* 2007; GUPTA & LUTSENKO 2009; LUTSENKO 2021). Eight transmembrane domains (TM1-TM8) stabilise the protein in the membrane and form the intramembrane channel, with a highly-conserved CysProCys sequence in the TM6 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<sup>+</sup> ions transport (LUTSENKO *et al.* 2007; VELDHUIS *et al.* 2009; BANCI *et al.* 2010; PIERSON *et al.* 2019). During the catalytic cycle, the ATP7ases are autophosphorylated by a conserved aspartic acid in the ATP-binding domain to form the cytoplasmic loop between TM6 and TM7, 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 TM4 and TM5. The C-terminus of the ATP7A contains dileucine (LeuLeu), and this motif play an important role in the retrograde transport of ATP7A, because is essential and sufficient for the retrieval of ATP7A from the plasma membrane back to the TGN. In the ATP7B, trileucine (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 (BANCI *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 & MERCER 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 & MERCER 2007). Another important role of ATP7A involves delivering Cu<sup>+</sup> ions to the secretory pathway, where they are incorporated into the Cu-dependent enzymes such as lysyl oxidase, tyrosinase, dopamine- $\beta$ -hydroxylase, peptidylglycine- $\alpha$ -amidating monooxygenase 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* (*Atp7a* in rodents) gene (Table 1) (MERCER *et al.* 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; OGÓREK *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; OGÓREK *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 & MØLLER 2010; MAUNG *et al.* 2021). The phenotypic features of Menkes disease can be divided in 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 (MØLLER *et al.* 2009). Mice with mutations in the *Atp7a* 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 *Atp7a* protein (SKJØRRINGE *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 *Atp7a* 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. Mutants from the second class, i.e. *brindled* (*Atp7a*<sup>mo-br</sup>), *macular* (*Atp7a*<sup>mo-ml</sup>) and *mosaic* (*Atp7a*<sup>mo-ms</sup>) 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 males 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 (SKJØRRINGE *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; NICIU *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 Menkes 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 mammary 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; MAUNG *et al.* 2021). Mice with a mutation in the *Atp7b* gene (*tx* – toxic milk mice) and *Atp7b*<sup>-/-</sup> knockout mice are the animal model of Wilson disease (CORONADO *et al.* 2001; BUIAKOVA *et al.* 1999; HADRIAN & PRZYBYŁKOWSKI 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 & PRZYBYŁKOWSKI 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. Adult *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 (JOŃCZY *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-

lochaperones 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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