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INVITED REVIEW

The molecular mechanisms of copper metabolism and its roles in human diseases

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Abstract

Copper is an essential element in cells; it can act as either a recipient or a donor of electrons, participating in various reactions. However, an excess of copper ions in cells is detrimental as these copper ions can generate free radicals and increase oxidative stress. In multicellular organisms, copper metabolism involves uptake, distribution, sequestration, and excretion, at both the cellular and systemic levels. Mammalian enterocytes take in bioavailable copper ions from the diet in a Ctr1-dependent manner. After incorporation, cuprous ions are delivered to ATP7A, which pumps Cu^{+} from enterocytes into the blood. Copper ions arrive at the liver through the portal vein and are incorporated into hepatocytes by Ctr1. Then, Cu^{+} can be secreted into the bile or the blood via the Atox1/ATP7B/ceruloplasmin route. In the bloodstream, this micronutrient can reach peripheral tissues and is again incorporated by Ctr1. In peripheral tissue cells, cuprous ions are either sequestrated by molecules such as metallothioneins or targeted to utilization pathways by chaperons such as Atox1, Cox17, and CCS. Copper metabolism must be tightly controlled in order to achieve homeostasis and avoid disorders. A hereditary or acquired copper unbalance, including deficiency, overload, or misdistribution, may cause or aggravate certain diseases such as Menkes disease, Wilson disease, neurodegenerative diseases, anemia, metabolic syndrome, cardiovascular diseases, and cancer. A full understanding of copper metabolism and its roles in diseases underlies the identification of novel effective therapies for such diseases.

Keywords Copper · Metabolism · Homeostasis · Chaperon · Transporter

Introduction

As a transition metal, copper (Cu) plays several key roles in the human body. These various roles are due to its two oxidation states, Cu(I)/Cu^{+} (cuprous ion) and Cu(II)/Cu^{2+} (cupric ion), which endow it with the ability to act as either a recipient or a donor of electrons. Cu^{+} prefers to bind to the thiol group in cysteine or the thioether group in methionine, while Cu^{2+} exhibits a high affinity for the secondary carboxyl group in aspartic/glutamic acid or the imidazole nitrogen group in histidine [37]. As a result, copper ions readily form complexes with biomolecules containing these amino acid residues. It has been reported that Cu atoms are involved in a wide spectrum of proteins, such as copper/zinc superoxide dismutase (Cu/Zn SOD or SOD1), cytochrome *c* oxidase (C*c*O), mitogen-activated protein kinase MEK1 [10, 149], and cAMP-degrading phosphodiesterase PDE3B [79]. In these proteins, copper ions participate in diverse biochemical reactions (especially redox reactions) via donation or accepting of electrons [30], and maintain specific protein structures by coordinating with the abovementioned groups. Thus, although copper is much less abundant in organisms compared with iron, it is indispensable in cell

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metabolism, especially in the mitochondrial respiratory chain.

However, excess intracellular free copper ions are potentially toxic [30]. During the switch between Cu(I) and Cu(II) states, electron transfer results in the generation of reactive oxygen species (ROS), including superoxide anion (O_2^-), nitric oxide (NO^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2), through Fenton reactions [151]. ROS can damage organic molecules, including proteins, nucleic acids, and lipids. ROS can also interfere with the synthesis of iron-sulfur clusters, which have pivotal roles in a myriad of essential enzymes. Additionally, superfluous copper ions may displace other metals from their cognate ligands in metalloproteins, resulting in improper protein conformations or impairment of enzymatic activities [37].

Given that both an excess and a lack of copper ions are pernicious, copper homeostasis must be maintained in living organisms. Otherwise, a copper imbalance may lead to various kinds of disorders. The following sections aim to provide an updated summary of the literature on copper metabolism and diseases related to copper dyshomeostasis.

Copper metabolism in mammals

In multicellular animals, copper ions undergo uptake, distribution, and efflux. Each event takes place at both the cellular and systemic levels. Over the last several decades, the mechanisms underlying these pathways have been intensively studied and several molecules involved in these processes have been verified in mouse models (shown in Table 1). A schematic diagram of copper metabolism in mammals at the cellular and molecular levels is shown in Fig. 1.

Uptake of copper ions

Humans and other mammals obtain copper from their diet. This micronutrient is present in high concentrations in foods such as liver, crustaceans, red meat, milk, chocolate, mushrooms, nuts, and beans [108]. According to the US Food and Nutrition Board, a human adult should consume 0.9 mg of copper per day [15]. It has been reported that the median intake of dietary copper in adults falls between 1.0 and 1.6 mg daily [9]. Thus, most adults obtain adequate copper from their daily diet. Pregnant women, infants, and children should consume more copper through their diet [108].

In the mammalian digestive tract, copper in consumed food is taken up by enterocytes. The extracellular copper ions are in the form of Cu(II), which can be directly incorporated by DMT1 (divalent metal transporter 1). However, these cupric ions cannot be used directly by cells [131]. It was conventionally assumed that all types of cells, including enterocytes, share the same copper incorporation pathway: 4 reductases; DCYTB; and Steap 2, 3, and 4 are responsible for reducing Cu^{2+} into Cu^+ on the surface of mammalian cells [112, 160]; then, the cuprous ions are incorporated by Ctr1 (copper

Table 1 Phenotypes of knockout (KO) or randomly mutated (RM) mice lacking normal genes involved in copper metabolism

Gene	Model type	Phenotypes and etiologies
Ctr1	Systemic KO	Embryonic lethality due to systemic deficiency of Cu [80, 85]
	Enterocyte-specific KO	Severe growth and viability defects due to peripheral Cu deficiency, which was rescued by postnatal intraperitoneal Cu injection [109, 110]
	Cardiac-specific KO	Cardiac hypertrophy due to cardiac Cu deficiency [77]
CCS	Systemic KO	Reduced female fertility and increased sensitivity to paraquat due to a reduction in SOD1 activity [159]
SOD1	Systemic KO	Vulnerable to motor neuron loss after axonal injury [118] and subfertile [101], due to increased oxidative burdens.
Atox1	Systemic KO	Death before weaning or growth failure, hypopigmentation, skin laxity, and seizures due to perinatal copper deficiency [58]
ATP7A	Motor neuron-specific KO	Progressive deterioration of gait, age-dependent muscle atrophy, denervation of neuromuscular junctions, and a loss of motor neuron cell bodies, likely due to increased Cu in motor neurons and decreased Cu in the spinal cord [64]
	RM (systemic)	Coat color defects and death before or after birth due to excess Cu in the small intestine and kidney, as well as low Cu in the brain, liver, and heart [50, 57, 88, 119]
ATP7B	Systemic KO	Cirrhotic liver disease caused by intracellular accumulation of toxic Cu, as well as increased Cu in other organs such as the kidneys and brain due to defects in Cu excretion [11, 65]
	RM (systemic)	Production of milk with low copper content (toxic milk) and hepatic Cu accumulation due to abnormal Cu efflux [24, 81, 145]
CP	Systemic KO	Anemia due to progressive iron accumulation in the liver and spleen [18, 61]

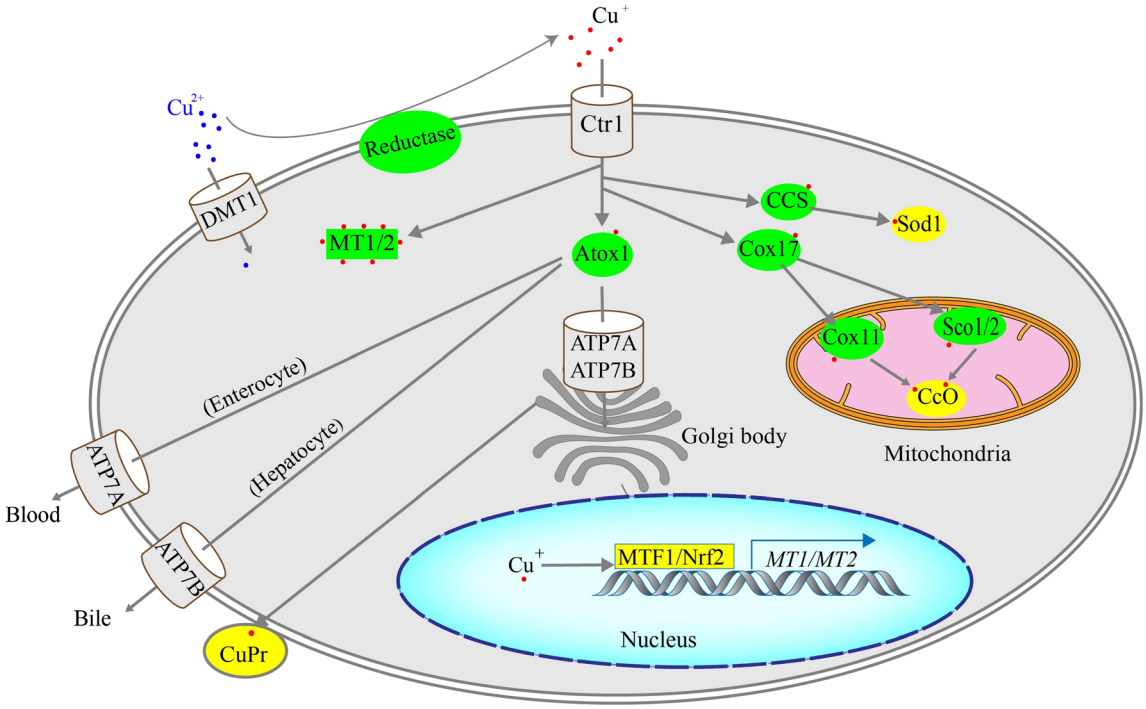

Fig. 1 Schematic diagram of copper metabolism at the cellular and molecular levels in mammals. Bioavailable copper ions are primarily transported by Ctr1. Then, they are reduced by reductases such as Steap 2, 3, 4, and DCYTB. Cu^{2+} can be incorporated by DMT1. After incorporation, cuprous ions undergo utilization and detoxification pathways: the chaperons CCS, Cox17, and Atox1 target Cu^{+} to SOD1, C*c*O, and Golgi bodies, while superfluous cuprous ions are sequestrated by metallothioneins (MT1 and MT2). Cuproproteins (CuPrs) assembled in the Golgi compartment are secreted out of the cell or sorted to specific organelles. In enterocytes or hepatocytes, ATP7A and ATP7B relocate to the plasma membrane to pump Cu^{+} into the blood or bile, respectively. Elevated copper levels stimulate expression of MT1 and MT2, mediated by the transcription factors MTF1 and Nrf2, to scavenge the excessive Cu^{+}

transporter 1) in a high-affinity manner [34]. It has been widely accepted that Ctr1-dependent copper incorporation is the primary means of incorporation of cupric ions in peripheral tissues. However, the process for enterocytes is more complicated. It has been reported that Ctr1 is located at the apical membrane of enterocytes [109, 110]. This is consistent with the assumption that Ctr1 might mediate uptake of dietary copper from intestinal lumen. Confusingly, other studies have shown that Ctr1 is located at both the apical and blastolateral membranes, as well as inside enterocytes [166]. A further investigation revealed that copper absorption from the intestinal lumen is largely independent of Ctr1, because inactivation of intestinal Ctr1 barely impaired accumulation of copper in enterocytes [110]. This indicates that copper transportation across the apical membrane is mainly an uncharacterized low-affinity process, which might be mediated by transporters such as DMT1 or driven by endocytosis [96]. It is likely that Ctr1 is required to release dietary copper from subapical vesicles for further utilization; however, the underlying mechanism remains unclear. Another possibility is that only cuprous ions transported by Ctr1 from intestinal lumen are bioavailable [54, 109]. Thus, in the absence of Ctr1, copper ions absorbed via other pathways are unavailable to the organism because they are sequestrated in subapical vesicles [116]. This has been confirmed by the observation that enterocyte-specific Ctr1 knockout mice exhibit severe copper deficiency in peripheral tissues and die about 3 weeks post-birth; these effects can be rescued by intraperitoneal copper injection [109, 110]. On the other hand, systemic knockout of Ctr1 leads to embryonic lethality [80, 85], while cardiac-specific Ctr1 knockout produces cardiac hypertrophy and a decrease in copper accumulation in cardiac tissue [77]. These genetic models confirm the importance of Ctr1 in both central and peripheral copper uptake (Table 1).

Distribution and sequestration of copper ions

After incorporation, some copper ions will be targeted to different cuproproteins (CuPrs) via three utilization pathways: cytosolic, mitochondrial, and Golgi routes [37]. In cytosol, CCS (copper chaperone for Sod1) mediates Cu^{+} loading and activation of superoxide dismutase 1 (Sod1). This role of CCS has been verified in *CCS* knockout (KO) mice (Table 1) [101, 159]. On the other hand, KO mouse models have demonstrated the key roles of CCS and Sod1 in ROS scavenging in germline cells and neurons (Table 1) [101, 118, 159]. A recent study suggested that CCS acquires copper from Ctr1 and passes it to Sod1 via formation of a Ctr1-CCS-Sod1 complex, which can be broken with complete activation of Sod1 [135]. Besides CCS, the chaperons Cox17 and Atox1 can also escort

Cu^+ from Ctr1 [37]. Cox17 shuttles between the cytoplasm and mitochondrial lumen, delivering cytosolic cuprous ions to Sco1 or Sco2, both of which are located on the inner membrane of the mitochondrion. They pass copper atoms to the copper A (Cu_A) site of Cox2 [20]. Cox17 also delivers copper from the cytoplasm to Cox11, which acts as a copper donor to the copper B (Cu_B) site of Cox1 [20]. Cox1 and Cox2 are two subunits of C*c*O, which is an essential copper-dependent enzyme in the oxidative phosphorylation process [20]. It has been reported that Cox17-mediated chaperoning of copper is also crucial to C*c*O biogenesis [20, 152]. Atox1 passes copper to P1B-type ATPases, including ATP7A (ATPase copper-transporting α) and ATP7B (ATPase copper-transporting β) [58]. These ATPases are located on the *trans*-Golgi network (TGN) or plasma membrane in different circumstances, and pump Cu^+ from Atox1 to the other side of the membrane [27]. ATP7A and ATP7B transporters on the plasma membrane are pivotal in the systemic distribution of copper ions (detailed below), while those on the TGN membrane contribute to loading of copper to various CuPrs, such as Sod3 (superoxide dismutase 3) and ceruloplasmin (CP). Then, these CuPrs assembled with Cu^+ are sorted to specific organelles or secreted out of the cell [27].

In enterocytes, a large proportion of absorbed cuprous ions are secreted into circulation to reach other tissues. This process is dependent on the abovementioned Atox1/ATPase routes. ATP7A is widely expressed and is located on the TGN membrane in most cases [27]. Unlike in other cell types, in enterocytes, a large amount of ATP7A molecules relocate to the basolateral membrane. These P-type ATPases on the plasma membrane also receive cuprous ions from Atox1 like they do on the TGN membrane, and mediate the exodus of copper from enterocytes into the bloodstream [73]. Genetic mouse models have demonstrated that deficiency of the Atox1/ATP7A route causes defects in Cu distribution, leading to pathological changes in several organs (Table 1) [50, 57, 64, 88, 119]. Albumins and free amino acids in the blood associate to the copper ions secreted from enterocytes, and these can be absorbed by Ctr1 upon arrival at peripheral tissues/organs [143].

Under physiological conditions, a healthy adult body contains about 80 mg of copper in total, with the concentrations being highest in the eyes, heart, liver, and brain [51]. Among these organs, the liver is an important organ that stores copper from the portal vein. Surplus cuprous ions in hepatocytes bind to metallothioneins (MTs) in the cytosol or are stored within the lysosome as a reservoir [8]. ATP7A is normally expressed in many tissues, except for the liver, where it is replaced by its paralogue, ATP7B [37]. One study reported that cardiac-specific KO of Ctr1 leads to a dramatic increase in expression of ATP7A in the mouse liver [77].

A large proportion of copper in hepatocytes is targeted to CP via the Atox1/ATP7B/Golgi route. Then, CP is secreted into the blood and oxidizes Fe(II) into Fe(III) to facilitate its absorption and mobilization [18, 32, 61]. It has been reported that the normal serum copper concentration is approximately 109 μg/100 mL, with 90% bound to CP and the remaining cuprous ions associated with albumins or free amino acids [51].

Since free copper ions have the potential to generate ROS in cells, the excess intracellular Cu^+ must be sequestered by molecules such as MTs and glutathione (GSH). MTs are a family of low molecular weight proteins encoded by four genes (MT1, MT2, MT3, and MT4), whose expression can be induced by increased metal ions, such as copper and zinc. MTs contain 61–68 amino acid residues, among which, 20 cysteine residues are distributed in two globular domains, α and β clusters [70]. Thiol groups in cysteins endow MTs, especially MT1 and MT2, with the capacity to chelate a large portion of Cu^+ incorporated by Ctr1 to avoid cellular toxicity [13].

GSH is a tripeptide containing glutamate, cysteine, and glycine residues. It is synthesized by two enzymes, γ-glutamylcysteine sythetase and GSH synthase, instead of by ribosomes [7, 95]. Like MTs, GSH can buffer excess copper via its association with this ion. This tripeptide is also crucial to intracellular distribution of copper, as GSH is likely the first acceptor of copper after its entry into the cell, following which, Cu^+ is either delivered to the chaperones or sequestered by MTs [42, 99, 135]. On the other hand, GSH plays a key role in maintaining the redox condition of cysteines in the N-terminus of ATP7A and ATP7B, which is important for the exportation of copper ions by ATPases [134].

Excretion of copper ions

Copper ions can be secreted out of cells via several pathways (shown in Fig. 1). In all cell types, various CuPrs are loaded with Cu^+ by ATP7A or ATP7B at the TGN; they are then secreted out of cells. In enterocytes, incorporated Cu^+ can be directly pumped into the blood by ATP7A residing at the blastolateral membrane; these copper ions can be delivered to the liver and absorbed by hepatocytes in a Ctr1-dependent manner [73]. In hepatocytes, CP is synthesized and assembled with Cu^+ at the TGN; these holo-CPs can then be released into the blood along with the copper ions bound on them, which can, in turn, be recycled by other tissues via blood circulation [90]. In response to elevated copper levels, ATP7B in hepatocytes also translocates from the Golgi apparatus to the lysosome and imports copper into its lumen. Then, lysosomal ATP7B collaborates with p62 to trigger exocytosis, releasing excess copper into the bile; this is then excreted out of the body [117]. Hepatic ATP7B on the apical membrane can also directly pump copper ions into bile to be recycled or excreted via the digestive tract [5]. The importance of ATP7B in copper excretion has been verified in mouse models generated by

either homologous recombination or random mutation [11, 24, 65, 81, 145].

Copper homeostasis in mammals

In mammals, homeostasis of a nutrient consists of two aspects: cellular homeostasis and systemic distribution. Copper homeostasis in mammalian cells is achieved through the regulation of genes involved in copper influx and detoxification. The abundance of the mammalian Ctr1 protein at the plasma membrane is negatively regulated by the cellular copper concentration according to a feedback mechanism: Ctr1 is removed from the cell surface and undergoes internalization or endocytosis-dependent degradation in response to elevated copper levels [106, 115]; the internalized Ctr1 can also be recycled back to the plasma membrane when the extracellular copper concentration is reduced [43, 126]. This regulation may explain the observation that varying dosages of dietary copper affect the copper incorporation efficiency of enterocytes: copper depletion increases the fraction of copper that is absorbed, with little drained out of the digestive tract, while chronic dietary consumption of copper significantly impairs the absorption efficiency, regardless of the high level of overall copper in the intestine [116]. On the other hand, it has been reported that high concentrations of cellular copper ions can enhance transcription of metallothionein genes (*MT1* and *MT2*) to scavenge the excess and toxic copper ions. This transactivation is mediated by the transcription factor MTF1, which is indirectly regulated by copper. Furthermore, data from the Tohru Fukai group revealed that mammalian Atox1 not only acts as a copper chaperon but also acts as a copper-dependent transcription factor. The researchers found that copper stimulates nuclear translocation of Atox1, which then binds to promotors of genes including cyclin D1, NADPH oxidase organizer p47phox, and SOD3 [16, 67, 75, 113]. Whether this transcription factor function of Atox1 is involved in copper homeostasis in mammals remains unclear. There also is evidence suggesting that the *MT1* gene contains four functional metal response elements (MREs) and that the transcription factor Nrf2 is crucial to MRE-mediated transcription of *MT1* in response to increased copper [136]. Under low cellular concentrations of copper and other metal ions, the metal-free MTs, termed apo-MTs, are highly vulnerable to proteolytic processes, resulting in a low level of these chelators [137].

A large body of studies demonstrates that mammals have evolved an exquisite ability to sense and retain copper in the organs; however, the underlying mechanisms are still not clearly understood. In early studies, Levenson CW measured copper turnover by monitoring two naturally occurring stable isotopes of this metal (^{63}Cu and ^{65}Cu) [86, 87]. They found that copper conservation in organs could be induced in response to dietary copper restriction. This conservation was highly organ specific: copper conservation in organs such as the brain and heart was very efficient, with little loss of copper, while conservation in the liver was induced only after the loss of a significant amount of copper [86, 87].

It is well-established that liver and hepatic ATP7B are crucial to systemic homeostasis of copper. As mentioned above, copper ions incorporated by enterocytes are delivered to the liver via the blood, and are then secreted out of hepatocytes through different ATP7B-dependent routes: at basal copper concentrations, hepatic ATP7B resides at the TGN and loads Cu^+ to CP, which mediates copper recycling through the circulation system [90, 163]; high concentrations of hepatic copper induce ATP7B translocation to either the apical surface or lysosome, where copper excretion occurs via bile [5, 117]. In the cardiac-specific *Ctr1* knockout mouse model mentioned above, expression of ATP7A was strongly increased in the liver and intestine [77]. These data suggest that demand for copper in peripheral tissues can be sensed via an as-yet unknown mechanism, which then upregulates expression of ATP7A in both the liver and intestine to promote copper distribution.

Roles of copper in human diseases

Due to the dual role of copper ions, either insufficiency or superfluity of this metal may cause various diseases (listed in Table 2). As discussed above, ATP7A and ATP7B play key roles in copper distribution in the human body. Genetic inactivation of *ATP7A* leads to a syndrome characterized by severe systemic copper deficiency, known as Menkes disease (MD), while mutations of *ATP7B* impair the ability of the body to excrete copper into bile, causing accumulation of this metal in the body and a disease known as Wilson disease (WD). Perturbations in copper homeostasis due to ATP7A/B mutations manifest as a variety of disorders, including neuropathy, anemia, and cardiovascular diseases. These pathological manifestations of MD and WD offer clues to understanding the physiological consequences of copper deficiency, overload, and misdistribution.

Menkes disease and copper deficiency disorders

ATP7A is vital for pumping copper from enterocytes to the blood. Inactivation of this transporter results in disturbed copper acquisition and systemic copper deficiency, giving rise to Menkes disease (MD). The *ATP7A* gene, located at *Xq13.3*, encodes a transmembrane peptide with 1500 amino acid residues. To date, more than 370 different mutations of *ATP7A* have been identified as implicated in MD [53, 147]. Classical MD and its milder allelic variant, occipital horn syndrome (OHS), are characterized by progressive cerebral and cerebellar neurodegeneration, fair skin with kinky hair, and

Table 2 Copper-related diseases and mechanisms

Diseases	Symptoms	Cu status	Molecular mechanisms	Pathogenic CuPrs
Menkes disease	Neurodegeneration; abnormal connective tissue, defects in collagen and elastin, vasculopathy, kinky hair	Deficiency	Primary: *ATP7A* mutations cause systemic deficiency of Cu. Secondary: decreased activities of various enzymes, prolonged and deleterious NMDAR activation, reduced mRNA levels of LOX and its substrate tropoelastin	ATP7A [53, 73, 147], DBH [47, 74, 147], PAM [33, 59], C*c*O[73], SOD1 [73], tyrosinase [71, 73], NMDAR [128], LOX [25, 44], etc.
Wilson disease	Heptic injury and neurodegeneration	Overload	*ATP7B* mutations cause accumulation of Cu in the liver and brain	ATP7B [29, 62, 103, 105]
Fatal infantile cardioencephalomyopathy	Neonatal death	Deficiency	*SCO2* mutations cause Cu deficiency for C*c*O and lead to neonatal death	Sco2 and C*c*O [114, 144]
Metabolic syndrome	Non-alcoholic fatty liver disease and obesity	Deficiency	Derepression of PDE3B causes a decrease in cAMP-dependent lipolysis, and affects plasma cholesterol and lipoprotein levels	PDE3B [79]
Anemia	Hemolytic anemia	Overload	High levels of free copper ions in the blood cause hemolysis	ATP7B [139]
	Iron deficiency anemia	Deficiency	Low activities of hephestin and CP impair iron transportation	Hephestin and CP [108]
Cardiovascular diseases	Hypertension and atherosclerosis	Overload	Cu activates LOX, IL-1α , FGF1, etc., promoting atherogenesis and hypertension	LOX [43, 167], IL-1α [97] , FGF1 [97], etc.
		Deficiency	Impaired SOD3 and increased O_2^-	SOD3 [92, 93]
Cancer	Malignant proliferation and metastasis	Overload	Cu stimulates proliferative immortality, angiogenesis, and metastasis	MEK1 [10, 149], Atox1 [67], LOX [60, 94, 150], etc.
Neurodegenerative diseases	Impairment of cognition, memory, or movement	Misdistribution	Cu accumulates with Aβ, α-synuclein, etc., to increase ROS; DBH, PAM, etc., are deficient in Cu for neuronal activity	Aβ [4, 56], Tau [49], α-Syn [21, 45], SOD1 [156], Prion [157], ATP7A [76], DBH [153], etc.
Diabetes	Insulin deficiency or resistance, hyperglycemia	Misdistribution	Cu increases in the liver and kidneys but is insufficient for vascular SOD3 to scavenge detrimental O_2^-	ATP7A [140, 141], Atox1 [43], SOD3 [140, 141]

connective tissue disturbances [73]. In some individuals with MD, intervention with supplemental copper can attenuate disease progression and relieve most of the symptoms, highlighting the essential role of copper in humans [147].

Copper deficiency results in decreased activities of multiple enzymes, and this is believed to underlie the neuropathic manifestations of MD. Dopamine-β-hydroxylase (DBH) is a copper-dependent enzyme responsible for converting dopamine to norepinephrine; it is a crucial neurotransmitter in norepinephrinergic neurons. Reduced DBH activity contributes to the abnormal neurochemical patterns observed in the plasma and cerebrospinal fluid (CSF) of MD patients [47, 73, 74, 164]. Peptidyl-α-amidating monooxygenase (PAM) is another copper-requiring enzyme. Like DBH, PAM is metallated at the TGN. PAM is essential in neurogenesis and neuronal survival via mediation of the maturation of a variety of neuroendocrine peptides such as corticotropin-releasing hormone, neuropeptide Y, and pituitary adenylyl cyclase–activating polypeptide [33, 59]. In addition, copper deficiency in MD may also result in decreased activities of other cuproenzymes, including C*c*O, SOD1, and tyrosinase, which might also contribute to the neuropathology observed in this disorder [71, 73]. It also has been reported that synaptic release of copper might competitively inhibit *N*-methyl-D-aspartate receptors (NMDAR) in a neuroprotective fashion, while dysfunction of ATP7A and copper deficiency might lead to prolonged, potentially deleterious NMDAR activation, inducing degenerative loss of neurons in MD patients [73, 128].

OHS is a less severe form of MD, and manifests as abnormal connective tissues. These connective tissue defects are likely due to impairment in lysyl oxidase (LOX) activity. LOX is a copper-requiring enzyme that normally deaminates lysine and hydroxylysine at the first step of collagen crosslink formation. In MD patients, reduced plasma copper levels and defective transportation seem to directly decrease LOX

enzyme activity, and indirectly downregulate mRNA levels of both LOX and its substrate tropoelastin [25, 44]. As a result, defects in collagen and elastin may lead to connective tissue abnormalities, kinky hair, and abnormal vasculopathy such as vascular tortuosity and peripheral aneurysms, which are observed in MD and OHS [25, 46, 107].

Mutations in *ATP7A* can also cause X-linked Spinal Muscular Atrophy type 3 (SMAX3), a distal hereditary motor neuropathy [76]. In SMAX3, missense mutations induce subtle defects in ATP7A intracellular trafficking, resulting in preferential accumulation at the plasma membrane [163]. By specifically deleting the *ATP7A* gene within motor neurons, Hodgkinson VL et al. generated a SMAX3 mouse model, named the $ATP7A^{MN/Y}$ mouse [64]. This model perfectly mimics the symptoms of SMAX3 patients; although no overt signs of systemic copper deficiency were observed in this model, the $ATP7A^{MN/Y}$ mice exhibited an age-dependent overall reduction in copper concentration in the spinal cord and a concomitant increase in copper content in motor neurons, as compared with wild-type mice (Table 1) [64]. These findings further confirm the importance of ATP7A in copper distribution and neuronal functions.

Besides ATP7A, the C*c*O assembly gene *SCO2* has also been identified to cause severe inherited disease in humans. *SCO2* encodes a copper chaperon located on the mitochondrial inner membrane. As mentioned above, Sco2 passes cuprous ions from Cox17 to Cox2 in mitochondrion and facilitates the formation of holoenzymes of C*c*O. *SCO2* mutations cause C*c*O activity deficiency in several tissues, particularly in skeletal and heart muscles, leading to neonatal death in humans [114, 144].

In addition to the abovementioned hereditary causes of copper disturbance, multiple acquisition-related factors may result in copper deficiency and consequential disorders in humans. Poverty, malnutrition, anorexia, vegetarianism, malabsorption, long-term diarrhea, and parenteral hyperalimentation may lead to inadequate intake of copper [39]. Gestation and lactation in women, as well as the infant and child developmental period, increases the demand for copper, making these groups vulnerable to copper deficiency [108]. Acquired copper deficiency in these cases often leads to serum copper and CP concentrations below the normal range, with symptoms including progressive lower extremity paresthesias, dysesthesias, optic neuropathy, other neurological dysfunctions, weakness, fatigue, and pancytopenia [9, 19, 162]. The neurological symptoms can be explained by the abovementioned functions of CuPrs in the neural system, while the hematological manifestations may be due to the crucial role of copper in CP synthesis and hematopoiesis. Since copper-containing enzymes such as hephestin and CP are essential in iron transportation, copper deficiency always produces iron deficiency and anemia [108]. Copper deficiency anemia, as well as decreased activity of C*c*O, may account for weakness and fatigue in these patients. On the other hand, it has been reported that dietary copper can affect lipid storage [35], and inadequate copper intake can lead to lipid accumulation, causing non-alcoholic fatty liver disease (NAFLD) [2]. This is likely due to copper-mediated inhibition of the cAMP-degrading phosphodiesterase PDE3B: cuprous ion directly binds to PDE3B and inhibits its enzymatic activity; copper deficiency leads to derepression of PDE3B and a reduction in cellular cAMP level, causing a decrease in cAMP-dependent lipolysis in adipocytes [79]. Copper is also thought to be involved in atherosclerosis via its impact on the metabolism of low-density lipoprotein (LDL) and cholesterol [83, 142]. However, whether these pathways are involved in metabolic syndrome remains unknown. Moreover, it has been reported that deletion of extracellular SOD3, whose activity is dependent on copper loading at the TGN via the ATP7A/Atox1 axis, increases O_2^- production and induces hypertension in response to angiotensin II stimuli [92, 93]. Based on data from animal models and clinical research, copper deficiency has been proposed as a risk factor for atherosclerosis and coronary heart disease, although the mechanisms are unknown [78, 82]. Taken together, these findings indicate that acquired copper deficiency can result in neurological, hematological, cardiovascular, and metabolic disorders via varying mechanisms.

For both congenital and acquired copper deficiency, the most effective treatment is copper supplementation. For patients with copper deficiency as a result of inadequate intake of copper, a diet containing sufficient copper is required for recovery [9, 162]. However, since patients with *ATP7A* mutations exhibit defective copper delivery from the intestine to peripheral tissues, the only way to reverse this deficiency is to administer intravenous copper. Given that copper-histidine is naturally present in serum and is quantitatively important in copper transport, this compound is currently the most preferred treatment agent. However, administration of copper-histidine for more than 3 years is not desirable due to the expected nephrotoxicity [72, 74, 148]. Recently, Ogata et al. reported a case of a 29-year-old MD patient who tolerated long-term intravenous therapy with copper chloride for more than 20 years without major adverse effects [111]. The relationship between his survival into adulthood and this unconventional treatment regimen remains controversial [111].

Wilson disease and copper overload disorders

ATP7B is a paralogue of ATP7A. It encodes an intracellular copper transporter in hepatocytes that is located on the Golgi apparatus or canalicular membrane, depending on the cellular cuprous level. Under low concentrations of hepatocellular copper, ATP7B loads cuprous ions onto CP at the Golgi complex; when high levels of copper ions are stored in the liver, ATP7B promotes exodus of them via bile [5, 117]. Since first

identified as the gene responsible for WD in 1993, more than 500 mutations of *ATP7B* have been implicated in the disease. In WD, copper accumulates and causes toxicity in the liver due to inactivation of ATP7B and the consequential defect in biliary excretion of copper [62, 103]. As a result, patients may suffer from hepatitis, liver failure, and cirrhosis [105]. Inactivation or deletion of *ATP7B* in mice also produces WD symptoms [81, 145].

Since ATP7B is inactivated in WD patients, excess hepatic copper ions overflow into the blood, rather than into the bile, with secondary pathological copper accumulation in other tissues, particularly the brain. The toxic copper can produce neurodegeneration, manifesting as neurological symptoms and psychiatric disturbances [29, 62, 103]. Dysfunction of ATP7B may also result in hemolytic anemia. In WD patients, increased non-CP-bound copper in the blood can loosely bind to albumin and attack erythrocyte plasma membranes. This causes coombs-negative hemolysis, and finally, a hemolytic crisis, resulting in anemia [139].

There is also evidence suggesting the involvement of copper in tumor formation and progression. It is well-known that copper levels are elevated in a variety of malignancies, including both solid tumors and blood cancers. The high levels of copper concentrated in cancer cells are essential in tumorigenesis, angiogenesis, and metastasis [38, 55, 66]. Stephen Howell and his research group revealed that copper acquired from Ctr1 is required to increase phosphorylation of ERK1/2 or AKT via FGF-, PDGF-, and EGF-induced MAPK signaling; importantly, ERK1/2 and AKT play major roles in organismal development and tumorigenesis [146]. Dennis Thiele and Christopher Counter and their research groups reported that copper taken up by Ctr1 can bind to MEK1 kinase, which in turn phosphorylates and activates ERK1/2 kinases, mediating tumorigenesis driven by the V600E mutant of BRAF kinase [10, 149]. In addition, copper directly binds to multiple factors such as FGF1 and its receptor, as well as to angiogenin, to promote angiogenesis via enhancing the release of these factors or the affinity between the ligands and receptors [60, 84, 97, 150]. It has also been reported that copper activates hypoxia-inducible factor-1α (HIF1α) via CCS, and consequentially, induces expression of VEGF, which can mediate angiogenesis [69]. As Atox1 can transactivate cyclin D1 in a copper-dependent manner [67], this likely contributes to cancer cell proliferation and angiogenesis. Moreover, copper may promote tumor progression by elevating diverse cuproenzymes including SOD1, C*c*O, DBH, LOX, and CP [60, 94, 150].

As mentioned above, copper deficiency and the copper-dependent enzyme SOD3 can contribute to cardiovascular diseases. Intriguingly, several researchers have found that excess copper can also induce atherosclerosis and hypertension. It has been reported that hypoxia increases Cu fluxes by up-regulating Ctr1 and ATP7A in pulmonary arteries and pulmonary arterial smooth muscle cells. The increased copper levels elevate LOX activity and cross-linking of extracellular matrix (ECM), eventually leading to an increase in pulmonary arterial stiffness and development of pulmonary hypertension [43, 167]. On the other hand, copper can promote neointimal thickening and vascular remodeling after vascular injury through directly binding to IL-1α and FGF1 to facilitate their release [97]. Transactivation of *p47phox* and *SOD3* mediated by copper and Atox1 may also promote neovascularization and hypertension [16, 113]. Copper can also promote atherogenesis by elevating the expression or activity of proteins involved in LDL uptake and oxidation, as well as those involved in cholesterol biosynthesis [83, 142]. In addition, plasma homocysteine interacts with copper to enhance hydrogen peroxide, leading to peripheral vascular disease and ischemic heart disease [68, 98, 138]. Taken together, these findings indicate that excess copper promotes cardiovascular diseases via multiple mechanisms.

WD can be successfully treated with oral agents, including copper chelators and zinc preparations [29, 91, 124]. Patients with hepatic presentations are more often treated by chelators, including D-penicillamine and trientine or tetrathiomolybdate. Excess Cu ions can bind to these chelators and are excreted from the body via the kidneys. As a result, urinary excretion of non-CP-bound copper is promoted [154, 165]. Unfortunately, chelator therapy, especially D-penicillamine, is associated with numerous side effects. Early sensitivity reactions, characterized by fever and cutaneous eruptions, lymphadenopathy, neutropenia, or thrombocytopenia, and proteinuria, may occur during the first 1–3 weeks. Late reactions include nephrotoxicity, usually heralded by proteinuria or the appearance of other cellular elements in the urine [6, 31, 104, 132, 155]. Liver transplantation is often performed in WD patients with acute liver failure [124]. For pre-symptomatic and neurological patients, zinc therapies such as zinc sulfate and zinc acetate seem to be preferred [158]. With increased zinc incorporated in enterocytes, expression of MTs can be readily induced; MTs have a greater affinity for copper than for zinc. Once bound, the copper is not pumped into the circulation but is lost into the fecal contents [26, 63, 124, 127]. Gastrointestinal reactions and hepatic deterioration are relatively common side effects of zinc therapies [100, 158].

Copper misdistribution and diseases

As discussed above, both MD and WD may present with neurodegeneration, highlighting the complex roles of copper in neuropathies. A considerable body of literature has demonstrated a correlation between neurodegenerative diseases and copper misdistribution in the neural system. As mentioned above, the copper content of motor neurons is increased while that of the spinal cord is decreased in the SMAX3 mouse model [64]. In Parkinson's disease (PD), copper levels are

decreased in the degenerating substantia nigra pars compacta, while Lewy bodies are enriched in this metal because it co-aggregates with α-synuclein (α-Syn) in neurons [21, 45]. In the brains of Alzheimer's disease (AD) patients, copper accumulates in senile plaques composed of amyloid-β (Aβ) peptides as well as in neurofibrillary tangles (NFTs), the latter of which is composed of hyperphosphorylated Tau proteins. However, some important brain regions, such as the neocortex, are deficient of copper [1, 21, 49, 120, 129]. It has also been reported that CCS delivers copper to BACE1, which is a crucial aspartic protease involved in the generation of Aβ; this highlights the involvement of CCS-mediated copper delivery in AD [3]. It is believed that Aβ and α-Syn can sequestrate copper ions and form aggregates, reducing the available copper in some parts of the brain [4, 56]. Copper has also been found to be sequestrated by Huntington, mutant SOD1, and Prion, which are involved in Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and Prion disease (PrD), respectively [41, 125, 153, 156, 157, 161]. In some cases, sequestration of copper ions by these culprit proteins might cause or aggravate these neurodegenerative disorders via synergistic generation of toxic free radicals [12, 133, 151]. However, not all pathogenic characteristics of these diseases can be explained by toxic free radicals. For example, meta-analysis has indicated that copper is significantly reduced in the AD brain [129]. Furthermore, a study in Drosophila showed that ROS do not mediate the copper effects in HD [161]. In these scenarios, a decrease in copper ions available for cuproproteins such as DBH, PAM, C*c*O, NMDAR, and tyrosinase may contribute to the development of these neuropathies, like it does in MD-induced neurodegeneration [33, 47, 59, 71, 73, 74, 128, 153].

Pharmacological intervention for copper distribution can ameliorate the progression of neurodegenerative diseases. Lipophilic chelators like clioquinol and its derivative PBT2 can reduce amyloid burden and improve cognitive performance in AD animal models and patients [17, 52, 122, 130]. Data from us and others have demonstrated that clioquinol and its analogues function as metal-protein attenuating compounds (MPACs), thereby redistributing copper and other metal ions from enriched to depleted brain regions. This redistribution may, at least partially, account for the treatment effects of these MPACs in AD patients and animals [89, 102, 122, 130]. Moreover, copper redistribution or delivery with MPACs can also mitigate cognitive decline and improve memory or movement in mouse models of other neurodegenerative disorders, including ALS and PD [21, 28, 123]. These findings further support the assertion that misdistribution of copper plays crucial roles in the progression of neurodegenerative diseases.

Copper misdistribution also occurs in diabetes. On the one hand, hepatic and renal copper levels are increased in streptozotocin (STZ)-induced diabetes rat models [36]. Furthermore, copper chelation therapy, with drugs such as trientine, has been found to mitigate various pathogenic states of diabetes, suggesting a key role of excess copper in this disorder [14, 22, 23, 48]. However, the underlying mechanism remains to be determined. Furthermore, it was recently reported that metformin, a first-line drug for the treatment of type II diabetes, may act by chelating mitochondrial copper, resulting in alterations to cellular energy metabolism [40, 121]. On the other hand, CuPrs such as SOD3 may lack available Cu in diabetes: insulin deficiency or defects in the insulin/Akt2 pathway cause downregulation of ATP7A in the vessels in multiple diabetes mouse models, including STZ-induced mice; the downregulation of ATP7A impairs metallization/activation of SOD3 with copper at TGN, and in turn, enhances vascular O_2^- production, which induces endothelial dysfunction in diabetes [43, 140, 141]. These findings suggest that an imbalance in copper distribution contributes to diabetes.

Conclusions

Copper levels are tightly controlled through either cellular or systemic homeostasis. The mechanism of copper metabolism in mammals is shown in Fig. 1. First, the influx of extracellular copper ions is primarily mediated by the high-affinity transporter Ctr1. In enterocytes, further detailed understanding of copper influx is still needed, but it is widely accepted that Ctr1 in the apical membrane mediates absorption of most available cuprous ions from the intestinal lumen. After incorporation, most copper ions are sequestrated by MTs or stored within lysosomes, while only a small fraction of them are delivered to different apparatuses. The copper utilization pathways include the CCS-SOD1 route, the Atox1-ATP7A/B-CuPrs pathway, and the Cox17-Sco1/2-C*c*O route. Cells maintain copper homeostasis via regulation of genes involved in incorporation, detoxification, and utilization pathways. In mammalian enterocytes and hepatocytes, ATP7A and ATP7B, respectively, relocate to the basolateral and canalicular membrane to pump copper ions into the blood or bile. Copper ions pumped into the blood by basolateral ATP7A from enterocytes can be transported all over the body, while those released into the bile, mediated by ATP7B, are flushed out of the body. The importance of components of copper transportation has been verified in several mouse models (summarized in Table 1).

Copper deficiency can bring about various diseases (listed in Table 2). In MD, patients generally exhibit copper deficiency due to a loss of function of ATP7A. As a result, CuPrs involved in the production of neurotransmitters/neuropeptides, collagen/elastin, and ATP are impaired, leading to neurodegeneration, connective tissue abnormalities, and other pathologies. Mutation of *SCO2* can lead to neonatal death due to defects in mitochondria. Copper deficiency caused by acquired factors can also bring about metabolic syndrome by disturbing lipolysis, and can lead to cardiovascular diseases by impairing free

radical scavenging. Since copper is required for iron transportation, copper deficiency also leads to anemia.

On the other hand, excess copper is toxic and can also give rise to several disorders. Inactivation of ATP7B can cause WD, with copper accumulation in the liver and brain. Toxicity produced by excess copper in these organs leads to hepatitis, neurodegeneration, and hemolytic anemia. In cancer cells, copper levels are elevated to play a role in proliferation, angiogenesis, and metastasis via modulation of diverse cuproenzymes and signaling pathways. Unlike copper deficiency, excess copper promotes atherosclerosis and hypertension through multiple mechanisms, including by increasing ECM linking and arterial stiffness, neointimal thickening and vascular remodeling, and metabolism of LDL and cholesterol.

In neurodegenerative diseases and diabetes, copper misdistribution seems to occur, and this contributes to the progression of these disorders. Copper ions are sequestrated by several proteins, including Aβ, Tau, α-synuclein, Huntington, mutant SOD1, and Prion, and they tend to accumulate in several inclusion bodies. Binding to copper increases the potency of these proteins to generate ROS, which may play a key role in neurodegeneration. On the other hand, copper deficiency in other CuPrs or regions might exacerbate neuropathies. Although the total level of hepatic and renal copper is increased in animal models of diabetes, vascular SOD3 cannot obtain enough cuprous ions to scavenge free radicals, which aggravates this disease.

Thus, the available evidence indicates that copper is a key nutrient with two opposing functions. Mammals and humans have evolved delicate systems to maintain proper levels of copper ions, both at the cellular and systemic level. Both deficiency and excess of this metal can cause, or produce deterioration in, several kinds of disorders in humans. Copper supplementation or chelation has been widely adopted to treat diseases caused by copper dishomeostasis, especially MD and WD. The use of MPACs like clioquinol and PBT2 is also a promising therapeutic strategy for neurodegenerative diseases; these MPACs act to redistribute copper in the brain. Clarification of the roles and mechanisms of copper under normal physiological conditions and in pathological events will contribute to the identification of novel and effective methods for maintaining copper homeostasis, and these could act as potential therapies for copper-related diseases.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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