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REVIEW

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Copper metabolism in cell death and autophagy

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ABSTRACT

Copper is an essential trace element in biological systems, maintaining the activity of enzymes and the function of transcription factors. However, at high concentrations, copper ions show increased toxicity by inducing regulated cell death, such as apoptosis, paraptosis, pyroptosis, ferroptosis, and cuproptosis. Furthermore, copper ions can trigger macroautophagy/autophagy, a lysosome-dependent degradation pathway that plays a dual role in regulating the survival or death fate of cells under various stress conditions. Pathologically, impaired copper metabolism due to environmental or genetic causes is implicated in a variety of human diseases, such as rare Wilson disease and common cancers. Therapeutically, copper-based compounds are potential chemotherapeutic agents that can be used alone or in combination with other drugs or approaches to treat cancer. Here, we review the progress made in understanding copper metabolic processes and their impact on the regulation of cell death and autophagy. This knowledge may help in the design of future clinical tools to improve cancer diagnosis and treatment.

Abbreviations: ACSL4, acyl-CoA synthetase long chain family member 4; AIFM1/AIF, apoptosis inducing factor mitochondria associated 1; AIFM2, apoptosis inducing factor mitochondria associated 2; ALDH, aldehyde dehydrogenase; ALOX, arachidonate lipoxygenase; AMPK, AMP-activated protein kinase; APAF1, apoptotic peptidase activating factor 1; ATF4, activating transcription factor 4; ATG, autophagy related; ATG13, autophagy related 13; ATG5, autophagy related 5; ATOX1, antioxidant 1 copper chaperone; ATP, adenosine triphosphate; ATP7A, ATPase copper transporting alpha; ATP7B, ATPase copper transporting beta; BAK1, BCL2 antagonist/killer 1; BAX, BCL2 associated X apoptosis regulator; BBC3/PUMA, BCL2 binding component 3; BCS, bathocuproinedisulfonic acid; BECN1, beclin 1; BID, BH3 interacting domain death agonist; BRCA1, BRCA1 DNA repair associated; BSO, buthionine sulphoximine; CASP1, caspase 1; CASP3, caspase 3; CASP4/CASP11, caspase 4; CASP5, caspase 5; CASP8, caspase 8; CASP9, caspase 9; CCS, copper chaperone for superoxide dismutase; CD274/PD-L1, CD274 molecule; CDH2, cadherin 2; CDKN1A/p21, cyclin dependent kinase inhibitor 1A; CDKN1B/p27, cyclin-dependent kinase inhibitor 1B; COMMD10, COMM domain containing 10; CoQ10, coenzyme Q 10; CoQ10H2, reduced coenzyme Q 10; COX11, cytochrome c oxidase copper chaperone COX11; COX17, cytochrome c oxidase copper chaperone COX17; CP, ceruloplasmin; CYCS, cytochrome c, somatic; DBH, dopamine beta-hydroxylase; DDIT3/CHOP, DNA damage inducible transcript 3; DLAT, dihydrolipoamide S-acetyltransferase; DTC, diethyldithiocarbamate; EIF2A, eukaryotic translation initiation factor 2A; EIF2AK3/PERK, eukaryotic translation initiation factor 2 alpha kinase 3; ER, endoplasmic reticulum; ESCRT-III, endosomal sorting complex required for transport-III; ETC, electron transport chain; FABP3, fatty acid binding protein 3; FABP7, fatty acid binding protein 7; FADD, Fas associated via death domain; FAS, Fas cell surface death receptor; FASL, Fas ligand; FDX1, ferredoxin 1; GNAQ/11, G protein subunit alpha q/11; GPX4, glutathione peroxidase 4; GSDMD, gasdermin D; GSH, glutathione; HDAC, histone deacetylase; HIF1, hypoxia inducible factor 1; HIF1A, hypoxia inducible factor 1 subunit alpha; HMGB1, high mobility group box 1; IL1B, interleukin 1 beta; IL17, interleukin 17; KRAS, KRAS proto-oncogene, GTPase; LOX, lysyl oxidase; LPCAT3, lysophosphatidylcholine acyltransferase 3; MAP1LC3, microtubule associated protein 1 light chain 3; MAP2K1, mitogen-activated protein kinase kinase 1; MAP2K2, mitogen-activated protein kinase kinase 2; MAPK, mitogen-activated protein kinases; MAPK14/p38, mitogen-activated protein kinase 14; MEMO1, mediator of cell motility 1; MT-CO1/COX1, mitochondrially encoded cytochrome c oxidase I; MT-CO2/COX2, mitochondrially encoded cytochrome c oxidase II; MTOR, mechanistic target of rapamycin kinase; MTs, metallothioneins; NAC, N-acetylcysteine; NFKB/NF-Kb, nuclear factor kappa B; NLRP3, NLR family pyrin domain containing 3; NPLOC4/NPL4, NPL4 homolog ubiquitin recognition factor; PDE3B, phosphodiesterase 3B; PDK1, phosphoinositide dependent protein kinase 1; PHD, prolyl-4-hydroxylase domain; PIK3C3/VPS34, phosphatidylinositol 3-kinase catalytic subunit type 3;

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PMAIP1/NOXA, phorbol-12-myristate-13-acetate-induced protein 1; POR, cytochrome P450 oxidoreductase; PUFA-PL, PUFA of phospholipids; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SCO1, synthesis of cytochrome C oxidase 1; SCO2, synthesis of cytochrome C oxidase 2; SLC7A11, solute carrier family 7 member 11; SLC11A2/DMT1, solute carrier family 11 member 2; SLC31A1/CTR1, solute carrier family 31 member 1; SLC47A1, solute carrier family 47 member 1; SOD1, superoxide dismutase; SP1, Sp1 transcription factor; SQSTM1/p62, sequestosome 1; STEAP4, STEAP4 metalloreductase; TAX1BP1, Tax1 binding protein 1; TEPA, tetraethylenepentamine; TFEB, transcription factor EB; TM, tetrathiomolybdate; TP53/p53, tumor protein p53; TXNRD1, thioredoxin reductase 1; UCHL5, ubiquitin C-terminal hydrolase L5; ULK1, Unc-51 like autophagy activating kinase 1; ULK1, unc-51 like autophagy activating kinase 1; ULK2, unc-51 like autophagy activating kinase 2; USP14, ubiquitin specific peptidase 14; VEGF, vascular endothelial gro wth factor; XIAP, X-linked inhibitor of apoptosis

Introduction

Copper is an important trace element in the human body. Its uptake, distribution, utilization, and elimination are tightly regulated at the circulation, tissue, and cellular levels [1]]. Copper has multiple functions at the physiological level, depending on its ability to adopt two distinct redox states – an oxidized state Cu(II) and a reduced state Cu(I). Copper acts as a catalytic cofactor in redox chemistry, involved in regulating the activity of various enzymes such as, but not limited to, electron transport chain cytochrome C oxidase [2], antioxidant SOD (superoxide dismutase) [3], PDE3B (phosphodiesterase 3B) for lipolysis [4], ferroxidase for iron absorption [5], DBH (dopamine beta-hydroxylase) for the synthesis of norepinephrine from dopamine [6], and LOX (lysyl oxidase) for tissue regeneration and rejuvenation [7]. In addition to metabolism-related enzymes, some kinases (e.g., MAP2K1/MEK1 [mitogen-activated protein kinase kinase 1], MAP2K2 [8], ULK1 [unc-51 like autophagy activating kinase 1], ULK2 [9], and PDK1 [phosphoinositide dependent protein kinase 1] [10]) and transcription factors (e.g., TP53/p53 [tumor protein p53] [11]) rely on copper to exert their function or can be activated by copper. Therefore, copper deficiency can lead to the dysfunction of biological systems and cause human diseases, such as cardiovascular disease [12], anemia [13], and osteoporosis [14].

The disorder of copper homeostasis can also go in the opposite direction, that is, copper overload, which will also endanger human health [15]. Specifically, Wilson disease is a rare genetic disorder in which copper accumulates in the liver, brain, and other vital organs [16]. High levels of copper have been found in senile plaques of patients with Alzheimer disease, and copper dyshomeostasis may play a role in the pathogenesis of neurodegenerative disease [17]. Chronic copper toxicosis is characterized by the gradual accumulation of copper in the liver, which can lead to liver degeneration, damage, cirrhosis, and potentially even death [18]. In addition, high serum copper levels have been observed in various cancers in humans and may increase the risk of breast, lung, liver, colorectal, prostate, oral, or thyroid cancers [19]. Preclinical studies have shown that mildly elevated copper levels promote tumor initiation and progression *in vitro* and *in vivo* [19–29] (Figure 1). Mechanistically, elevated copper not only induces reactive oxygen species (ROS) production and exacerbates genomic instability, but also affects various tumor-associated signal transduction events. As expected, copper chelators can help prevent tumor formation [30]. In contrast, in established tumors, copper-based compounds have shown encouraging anticancer activity by inducing

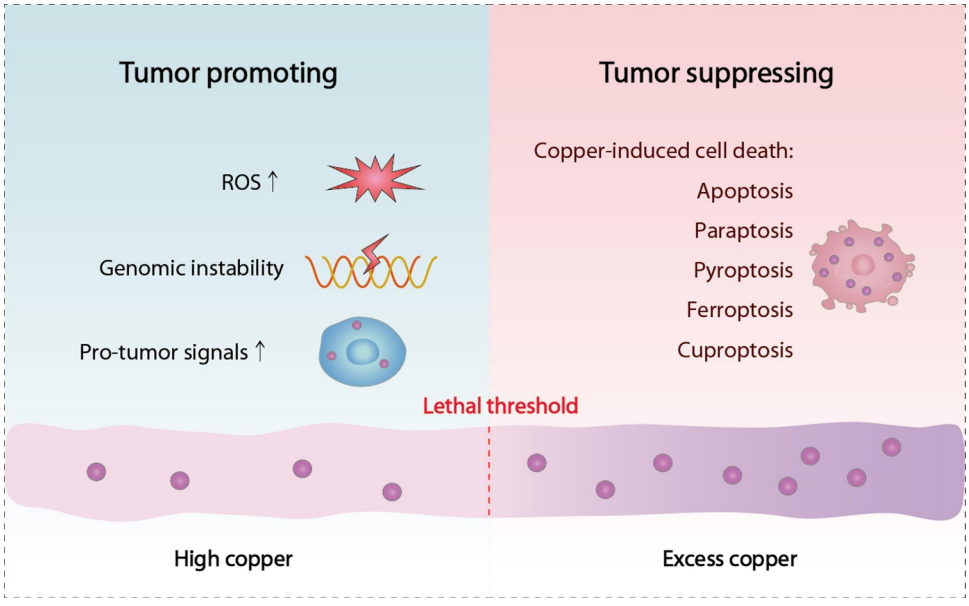

Figure 1. Dual role of copper in cancer development. On the one hand, elevated copper levels can promote tumor growth by inducing ROS production, exacerbating genomic instability, and affecting various tumor-associated signal transduction events. On the other hand, excessive copper concentrations can induce tumor cell death when they exceed a certain threshold limit.

various types of cell death when the concentration of copper exceeds a certain threshold limit [31] (Figure 1). Further understanding the signaling, process, and regulation of copper-induced cell death is important for the development of novel anticancer strategies.

In this review, we not only summarize the key steps of copper metabolism, but also discuss the recent progress in our understanding of how copper triggers various types of regulated cell death, including apoptotic and non-apoptotic cell death [32]. In addition, we highlight how macroautophagy (hereafter referred to as autophagy), a fundamental cellular homeostasis program [33], selectively degrades cell death regulatory proteins to promote or inhibit copper-associated cytotoxicity. These emerging concepts will facilitate the development of copper-based drugs for tumor therapy.

Copper metabolism

Copper homeostasis is regulated at multiple dimensions, including intestinal or tissue cell absorption, blood circulation, and tissue cell utilization, excretion or export. Below, we outline the processes and key regulators of cellular copper metabolism in humans (Figure 2).

Copper uptake

Copper is a metallic substance that occurs naturally on earth, both in elemental form and in native metal deposits. While copper is an essential nutrient, it is also widely used in industry and can become an environmental pollutant at high concentrations. In the digestive tract of mammals, dietary copper is absorbed by the small intestine. Extracellular copper ions are present in the small intestine in the form of divalent copper ions. Cu(II) cannot be taken up directly by enterocytes until it is reduced to Cu(I) by binding to the STEAP family of metalloreductases. Cu(I) enters the intestinal tract or other somatic cells mainly through SLC31 copper osmosis family transporter SLC31A1/CTR1 (solute carrier family 31 member 1) and SLC31A2/CTR2 (solute carrier family 31 member 2) [1]. There are currently two possible mechanisms that affect the expression of SLC31A1, which regulates the absorption of copper. First, the gene expression of *SLC31A1* is regulated by SP1 (Sp1 transcription factor) [34]. Second, elevated copper induces the endocytosis and degradation of SLC31A1 protein [35]. In addition, copper uptake by SLC11A2/DMT1 (solute carrier family 11 member 2) may be a compensatory mechanism for SLC31A1 deficiency [36]. It will be important to further determine whether these copper transporters at the plasma membrane play tumor type-specific roles in mediating copper uptake.

Copper utilization

Once the copper ions enter the cell, copper chaperones carry copper to different cell compartments, including the cytoplasm, mitochondria, Golgi apparatus, and nucleus to mediate different cellular processes.

In the cytoplasm, CCS (copper chaperone for superoxide dismutase) is a copper chaperone that functions in the delivery of copper to specific proteins, such as SOD1 (superoxide

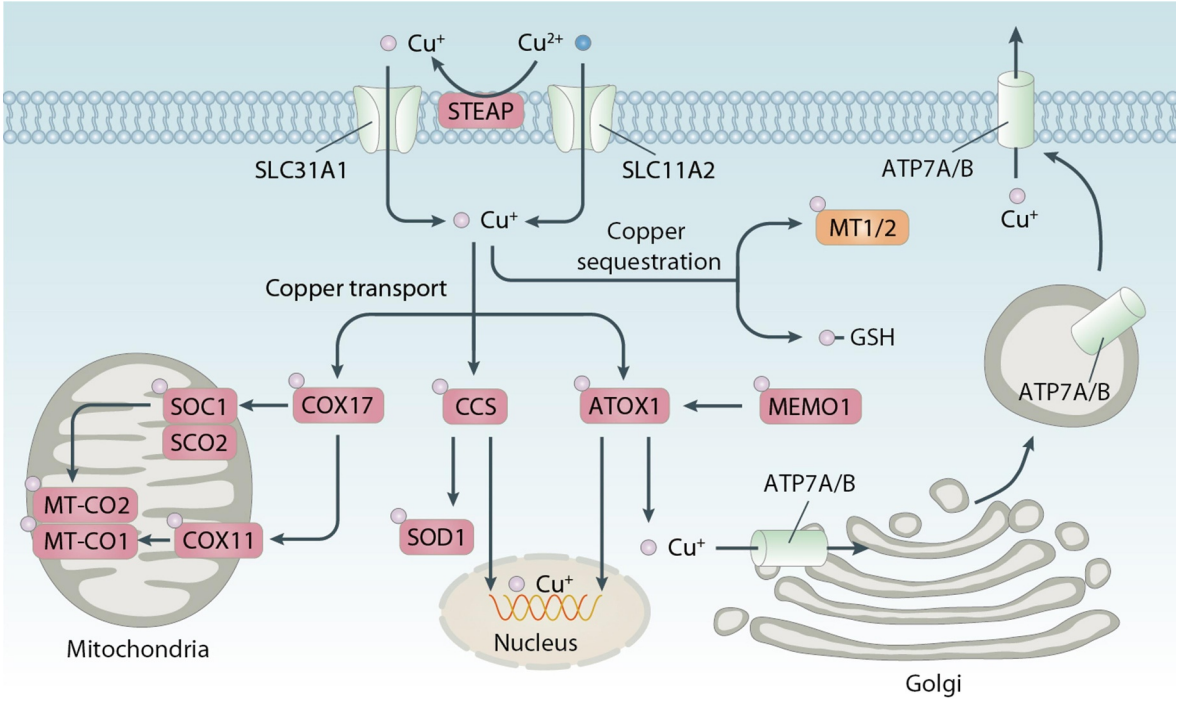

Figure 2. Molecular mechanisms of copper metabolism. Copper metabolism is a complex dynamic process regulated at the cellular and organ level by multiple molecules. The uptake of copper ions is mediated by SLC31A1 and SLC31A2, while the export of copper is driven by ATP7A and ATP7B. In cells, copper is transported to different subcellular organelles for bioavailability by several copper-binding proteins, including COX17, CCS, and ATOX1. Furthermore, the binding of MT1, MT2 and GSH to copper can limit the cytotoxicity of copper excess.

dismutase) [37]. SOD1 is an antioxidant protein located mainly in the cytoplasm, but a small portion of SOD1 is present in the mitochondrial intermembrane space. Abnormal expression of SOD1 is closely related to the growth and development of cancer [38–40]. CCS also regulates the distribution of SOD1 between the intermembrane space and the cytoplasm in an oxygen-dependent manner [41]. This regulatory mechanism maintains the stability of ROS in the body and alleviates ROS generated by the electron transport chain (ETC), thereby avoiding the oxidant damage caused by copper overload [41].

The transport of copper ions into mitochondria mainly depends on COX17 (cytochrome c oxidase copper chaperone COX17). COX17 transports Cu(I) from the cytoplasm to mitochondrial membrane proteins SCO1 (synthesis of cytochrome C oxidase 1) and SCO2 (synthesis of cytochrome C oxidase 2), which inserts copper into MT-CO2/COX2 (mitochondrially encoded cytochrome c oxidase II) [42]. Another pathway for COX17 to transport copper from the cytoplasm to MT-CO1/COX1 (mitochondrially encoded cytochrome c oxidase I) is through delivering copper to COX11 (cytochrome c oxidase copper chaperone COX11) [42]. MT-CO1 and MT-CO2 are two copper binding subunits of complex IV, which transfers electrons from CYCS (cytochrome c, somatic) and drives the electrochemical production of adenosine triphosphate (ATP) [43]. Thus, cellular copper pools are closely linked to mitochondrial oxidative phosphorylation. COX17 is essential for the activity of MT-CO1 and MT-CO2 in the mitochondrial respiratory chain, which plays a critical role in tumor growth, invasion, and metastasis [44]. Accordingly, COX17 is a potential molecular target for the treatment of solid or blood cancers [45,46].

Copper can be carried to the trans-Golgi by the copper chaperone ATOX1 (antioxidant 1 copper chaperone). In the cytoplasm, ATOX1 binds Cu(I) and delivers it to one of the copper-dependent ATPases, ATP7A (ATPase copper transporting alpha) and ATP7B (ATPase copper transporting beta), in the Golgi network [47]. MEMO1 (mediator of cell motility 1) enhances Cu(I) binding to ATOX1, preventing excess copper-induced ROS induction [48]. In response to elevated copper, ATP7A and ATP7B traffic from the Golgi to post-Golgi sites or lysosomes to facilitate efflux of excess copper [49,50]. The upregulation of ATOX1 mediates resistance of cancer cells to genotoxic drugs [51]. Coordination of two copper regulators, ATOX1 and ATP7A, plays a role in mediating the migration of breast cancer cells [52,53]. As a result, the inhibition of copper transport to the trans-Golgi is a potential strategy to suppress tumor migration and invasion.

Copper is transported by the CCS to the nucleus, where it can activate the transcription factor HIF1 (hypoxia inducible factor 1) [54]. Alternatively, ATOX1 can transport copper into the nucleus and itself acts as a copper-dependent transcription factor [55,56]. Pharmacological inhibition of CCS or ATOX1 induces copper accumulation and attenuates tumor growth *in vitro* and *in vivo* [57–60]. Likewise, histone H3-H4 tetramer acts as an oxidoreductase to catalyze the reduction of Cu(II) to Cu(I), thereby regulating nuclear events [61]. These advances build in part on fundamental work in the development of copper-related strategies to interfere with gene expression in cancer by modulating transcription factor activity or altering chromosomal function.

Unbound free copper ions generate ROS or cytotoxicity in cells, and this process is blocked by proteins that sequester intracellular Cu(I). For instance, excess intracellular copper ions are sequestered by MTs (metallothioneins) and glutathione (GSH). MTs are a family of low-molecular-weight proteins that contain metal – thiolate clusters bound to heavy metals. MT1 and MT2 chelate a large amount of copper ions transported by SLC31A1, and the addition of metal ions such as copper can induce MT expression [62]. MTs are heterogeneously expressed in different tumor cells and regulate tumor growth, differentiation, and metastasis [63]. GSH is the most abundant non-protein thiol and may be the first receptor to bind excess free copper ions prior to MT-mediated copper binding [64]. HIF1-induced GSH represses copper-dependent MAP2K1/MEK1 activity, which contributes to the breast cancer stem cell phenotype [65]. Together, MTs and GSH constitute an endogenous line of defense against copper-induced cytotoxicity in a variety of cells, including cancer cells. However, identifying the copper-independent functions of MT and GSH remains a key hurdle in the development of anti-oxidative stress strategies.

Copper export

The copper-transporting ATPases ATP7A and ATP7B play a central role in the extracellular export of copper ions [66]. The deficiency of ATP7A and ATP7B caused by genetic mutation results in copper transport disorders Menkes and Wilson diseases, respectively [67]. Under basal conditions with low copper levels, ATP7A and ATP7B are localized in the trans-Golgi network. In response to increased copper exposure, ATP7A and ATP7B translocate to the plasma membrane or intracellular vesicular compartments. At the same time, ATP7A and ATP7B transport copper from the trans-Golgi network to post-Golgi vesicles. These copper-loaded vesicles can fuse with the plasma membrane and release copper into the extracellular environment. In most cells, including cancer cells, copper ions transport copper out of the cell in this way [68]. High expression of ATP7A protects KRAS (KRAS proto-oncogene, GTPase) mutant colorectal cancer cells from excess copper-induced toxicity [69]. Based on these findings, targeting ATP7A represents a synthetic lethal approach to killing KRAS-driven colorectal cancer [69]. Notably, ATP7A and ATP7B also regulate platinum drug efflux. Thus, enhanced expression of ATP7A and ATP7B contributes to platinum resistance in ovarian cancer [70]. Collectively, these findings suggest a broader role for ATP7A and ATP7B in tumor biology.

Role of copper in cell death

Copper is an essential component of various enzymes, including MT-CO1 and SOD1, which are involved in the electron transport chain and antioxidant system in mammalian cells. Both copper deficiency and excess can lead to abnormal

cellular function and eventually cell death. Cell death, including accidental cell death and regulated cell death, is an important process involved in the regulation of cell number under physiological or pathological conditions [32]. There are two forms of regulated cell death, apoptotic and non-apoptotic cell death (Figure 3). The discovery of novel forms of non-apoptotic cell death has greatly increased our understanding of injury diversification and immune responses in tumor development and therapy [71,72]. In recent years, disturbances in copper metabolism have played a new role in affecting the susceptibility of cancer cells to cell death (especially apoptosis, paraptosis, pyroptosis, ferroptosis, and cuproptosis), as described below.

Apoptosis

Apoptosis is a form of programmed cell death usually manifested by the externalization of phosphatidylserine and the activation of the endogenous caspases [73]. There are two kinds of apoptotic pathways, namely intrinsic and extrinsic pathways, lead to the initiating activation of caspases. The extrinsic pathway is mediated by the interaction of FASL (Fas ligand) and FAS (Fas cell surface death receptor), which engages the adaptor molecule FADD (Fas associated via death domain) to recruit and activate CASP8 (caspase 8) [74]. The intrinsic pathway is the mitochondrial pathway initiated by mitochondrial injury signaling, such as excessive ROS and overloaded calcium ion. A critical event for mitochondrial apoptosis is the release of CYCS or AIFM1/AIF (apoptosis inducing factor mitochondria associated 1) from the mitochondria, which mediates apoptosis through activating caspases (e.g., CASP9 [caspase 9]) or endogenous endonucleases, respectively [75,76]. The activation of upstream initiating CASP8 and CASP9 trigger cascade activation of effector caspases (e.g., CASP3 [caspase 3]), which are mainly responsible for the definite cleavage of cellular components to induce

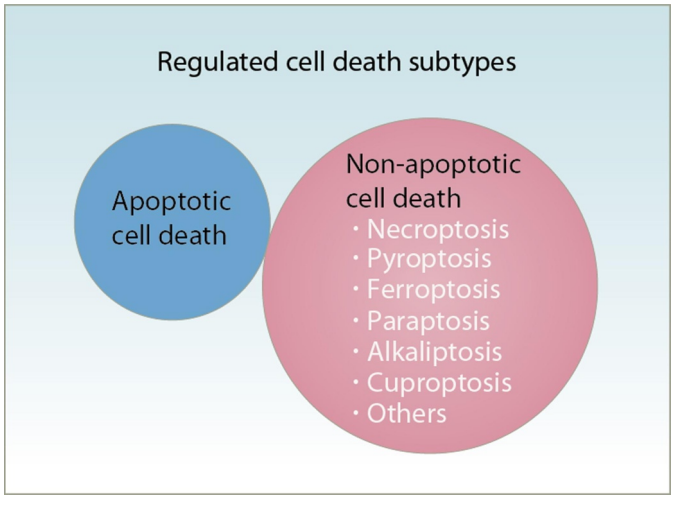

Figure 3. Types of regulated cell death. Regulated cell death is a biologically controlled process involved in various physiological or pathological events. It can be divided into apoptotic and non-apoptotic cell death. Compared with apoptosis, which generally requires the activation of caspase proteases, non-apoptotic cells are mostly caspase-independent and have the morphological characteristics of necrosis.

apoptotic cell death [77]. The release of mitochondrial proteins is determined by the mitochondrial membrane potential, which is tightly regulated by the BCL2 protein family [78]. For example, BAX (BCL2 associated X apoptosis regulator) and BAK1 (BCL2 antagonist/killer 1) can promote apoptosis though oligomerization at the mitochondrial surface to decrease the mitochondrial membrane potential [79,80]. BID (BH3 interacting domain death agonist), a pro-apoptotic BCL2 family protein, is cleaved by CASP8 upon stimulation by extrinsic apoptosis-inducing signals, thereby further promoting mitochondrial apoptosis [81].

Excessive copper ions induce apoptosis through a variety of mechanisms (Figure 4A). First, as a redox active metal, copper ions participate in the Fenton reaction to produce ROS [82]. Copper-induced ROS causes the release of CYCS and AIFM1 from the mitochondria into the cytoplasm, leading to the activation of caspases and DNA fragmentation in PC12 pheochromocytoma cells [83]. Copper-induced ROS also increase lipid peroxidation and deplete GSH, rendering cells more vulnerable to oxidative damage [84]. Second, copper accumulated in the nucleus can bind DNA, resulting in DNA damage in several biological systems [85]. A potential binding site for copper is proposed to involve guanine and cytosine of the opposite strands [86]. Copper also induces base substitution mutants by generating oxidative damage upon interaction with DNA [87]. Third, copper binds to thiol groups and inactivates thiol-containing enzymes, such as proteasome subunits in various types of cancer [88]. Cu(II) inhibits the chymotrypsin-like activity of the purified 20S proteasome or 19S proteasome deubiquitinases [89,90]. These studies provide a copper-based approach to target the ubiquitin-proteasome system for the treatment of human cancers.

In addition to the BCL2 protein family, TP53 is another important regulator of apoptosis through the transcription of multiple apoptotic target genes [91,92]. The activation of the TP53 pathway is involved in copper-induced apoptosis (Figure 4B). In human breast cancer MCF7 cells, copper ions increase the expression of TP53 [93]. Copper-induced trans-activation of TP53 increases the expression of BAX, leading to the opening of the mitochondrial permeability transition pore and the generation of ROS [93]. Other TP53 target genes, including *CDKN1A/p21* (cyclin dependent kinase inhibitor 1A), *PMAIP1/NOXA* (phorbol-12-myristate -13-acetate-induced protein 1), and *BBC3/PUMA* (BCL2 binding component 3), coordinately regulate copper-induced apoptosis. Collectively, these findings provide an example of how copper enhances TP53 transcriptional activity to mediate apoptosis, although a TP53-independent pathway may occur [94].

Intracellular copper also plays a role in preventing cancer cell death in the tumor microenvironment. Inflammatory cytokine IL17 (interleukin 17) increases intracellular copper by inducing STEAP4 (STEAP4 metalloreductase) in colon cancer [95] (Figure 4C). The elevated intracellular copper level leads to the activation of XIAP (X-linked inhibitor of apoptosis), which acts as an apoptotic brake through suppressing CASP3 activity [95]. Consequently, IL17-induced copper uptake contributes to

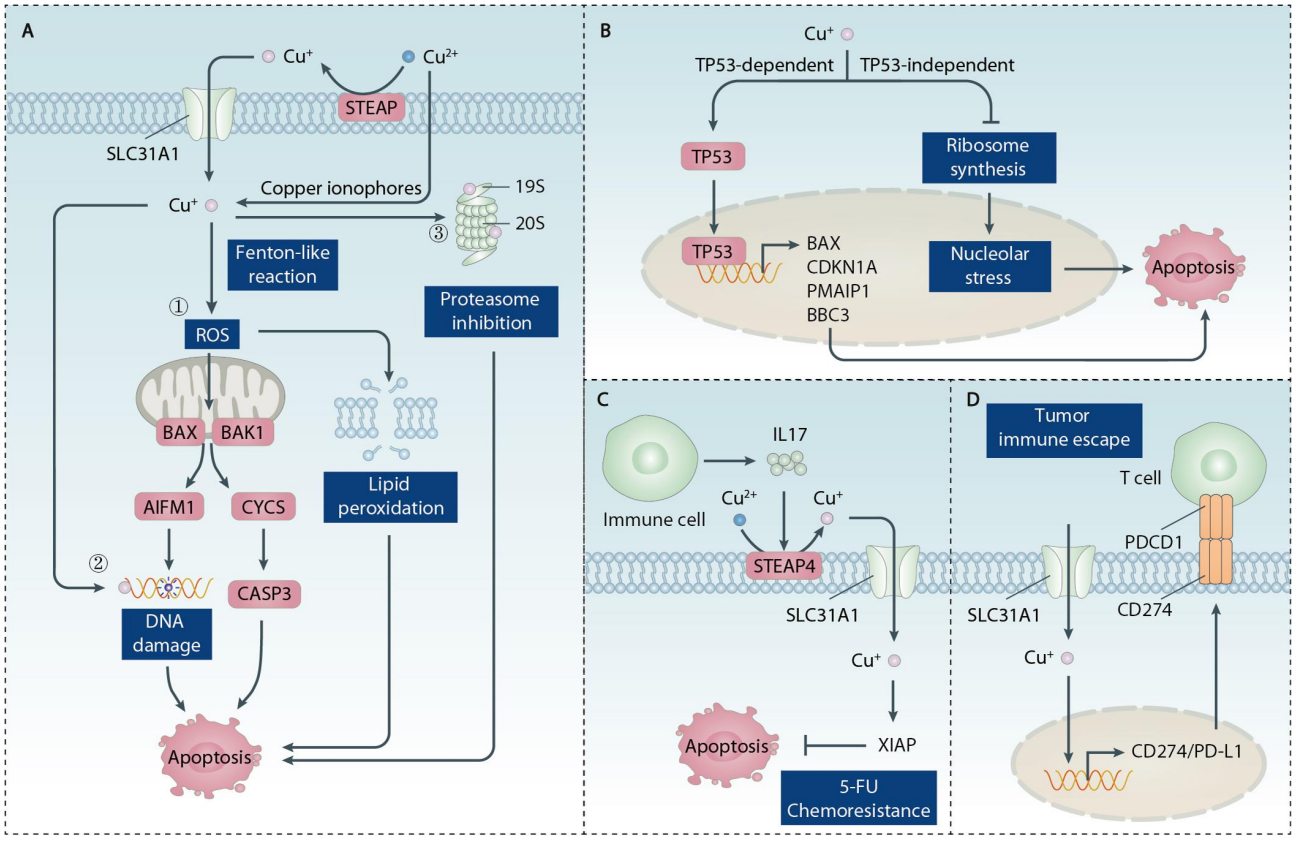

Figure 4. Role of copper in apoptosis. (A) Mechanism of copper-induced apoptosis. Copper induces apoptosis primarily through the induction of ROS, DNA damage, and proteasome inhibition. The apoptotic process is initiated by mitochondria-intrinsic apoptotic signals. CYCS-activated CASP9 propagates the apoptotic cascade by activating downstream CASP3, a key apoptosis execution protein that participates in the cleavage of multiple substrates. Furthermore, AIFM1 is released from mitochondria and induces caspase-independent apoptosis by attacking DNA. (B) the role of TP53 in copper-induced apoptosis. Copper induces TP53-dependent apoptosis by activating transcription of TP53 target genes, including BAX, CDKN1A/p21, PMAIP1/NOXA, and BBC3/PUMA. Copper also induces TP53-independent apoptosis by inhibiting ribosome synthesis and inducing nucleolar stress. (C, D) the anti-apoptosis role of copper. IL17 released by immune cells can increase STEAP4-mediated intracellular copper levels, leading to 5-fluorouracil (5-FU) resistance by activating the anti-apoptotic XIAP protein. Copper triggers the upregulation of CD274/PD-L1, which induces tumor immune escape by binding with PDCD1/PD-1 on activated T cells.

5-fluorouracil chemoresistance in human colon cancer cells, which is effectively reversed by copper chelators or XIAP inhibitors [95]. In addition, copper ions increase tumor immune escape by upregulating the expression of CD274/PD-L1 (CD274 molecule), which acts as an immune checkpoint in cancer cells [96]. Copper influx transporters SLC31A1 and CD274 expression are positively correlated in neuroblastoma [97] (Figure 4D). Indeed, copper supplementation increases the expression of CD274 at the mRNA and protein levels in SH-SY5Y and U87 cancer cells [97]. In contrast, copper chelators inhibit the upregulation of CD274 expression and increase the number of tumor-infiltrating immune cells in neuroblastoma xenograft mouse models [97]. These findings establish an integrated role for copper in the tumor microenvironment to affect apoptosis sensitivity and immune responses.

Paraptosis

Paraptosis is a form of non-apoptotic programmed cell death which is driven by CASP9 but independent of APAF1 (apoptotic peptidase activating factor 1) [98]. The main characteristic of paraptosis is extensive vacuolation derived from the endoplasmic reticulum (ER) and mitochondria and the absence of features of apoptosis (such as apoptotic body formation and nuclear fragmentation) [99]. An ER stress-induced intracellular Ca^{2+} imbalance is involved in the vacuolation [100,101]. Accordingly, ER stress marker proteins, including ATF4 (activating transcription factor 4), EIF2AK3/PERK (eukaryotic translation initiation factor 2 alpha kinase 3), EIF2A (eukaryotic translation initiation factor 2A), and DDIT3/CHOP (DNA damage inducible transcript 3), are required for the occurrence of paraptosis [102–104]. However, autophagic vacuoles are usually not observed in paraptosis. Furthermore, paraptosis is caused by proteasomal inhibition [105], ROS production (e.g., TXNRD1 [thioredoxin reductase 1] inhibition) [106,107], or MAPK (mitogen-activated protein kinases) activation [108], indicating that the induction signals of paraptosis are multiplexed and nonspecific. It is also speculated that phosphorylation of CASP9 by MAPK may switch CASP9 from a proapoptotic function to a pro-paraptotic function [108].

Copper-induced paraptosis (Figure 5) offers a potential approach for the treatment of apoptosis-resistant tumors [109,110]. Paraptosis contributes to copper toxicity induced by pyrazole-pyridine copper complexes in HT-1080 cells, resulting from copper-mediated ER stress and CASP3

inhibition [111]. The inhibition of the ubiquitin-proteasome system is another possible cause of copper-induced ER stress and subsequent paraptosis. For example, increased copper by a hinokitiol copper complex triggers paraptosis through targeting the 19S proteasomal deubiquitinase in A549 and K562 cells [112]. ATF4-mediated ER stress, but not caspase activation, is required for hinokitiol copper-induced paraptosis [112]. Similarly, paraptosis induced by copper complexes is associated with the inhibition of 20S proteasome activity and ER stress in cancer cells [101,113]. Another study also found that intracellular copper overload triggers caspase-independent paraptosis through the induction of ROS and the unfolded protein response in HeLa cells [114]. Although the current research on copper-induced paraptosis mostly relies on the use of copper-binding agents, the direct impact of copper on the molecular mechanisms of paraptosis remains to be further studied.

Pyroptosis

Pyroptosis is a form of lytic programmed cell death initiated by inflammasomes, which is driven by the activation of caspase family proteins, including classical CASP1 (caspase 1) and non-classical CASP4/CASP11 (caspase 4) or CASP5 (caspase 5) [115,116]. Various microbial infections and noninfectious stimuli induce the activation of CASP1, leading to classical pyroptosis. Activated CASP1 cleaves GSDMD (gasdermin D) into *N*- and C-terminal components and promotes the maturation and release of inflammatory cytokines, such as IL1B (interleukin 1 beta) [117–119]. The N-terminal domain of GSDMD then binds to the cell membrane, forming oligopores, which further leads to water inflow, dissolution, and cell death [120,121]. In the non-classical pyroptosis pathway, CASP4/5/11 can be activated by intracellular LPS, leading to the cleavage of GSDMD into the N-terminal domain, promoting pyroptosis [122–124].

ROS production may also contribute to copper-induced pyroptosis (Figure 6). Copper exposure increases the expression CASP1 and pyroptosis-related genes, such as IL1B and NLRP3 (NLR family pyrin domain containing 3), in hepatocytes [125]. N-acetylcysteine (NAC; an ROS scavenger) and Z-YVAD-FMK (a caspase inhibitor) attenuate excessive copper induced hepatocyte pyroptosis [125]. Therefore, copper-induced ROS may activate NLRP3 inflammasome, leading to the induction of pyroptosis. Similarly, Cu(II) treatment increases pyroptosis-related genes, such as NLRP3, CASP1, GSDMD, and IL1B, in jejunal epithelial cells [126]. This type of treatment induces pyroptosis mainly through the activation of the ER stress pathway [126] (Figure 6). Inhibitors of ER stress, such as 4-PBA and MKC-3946, decrease copper-induced pyroptosis in jejunal epithelial cells [126]. However, it is still unclear whether copper is capable to cause pyroptosis in tumors.

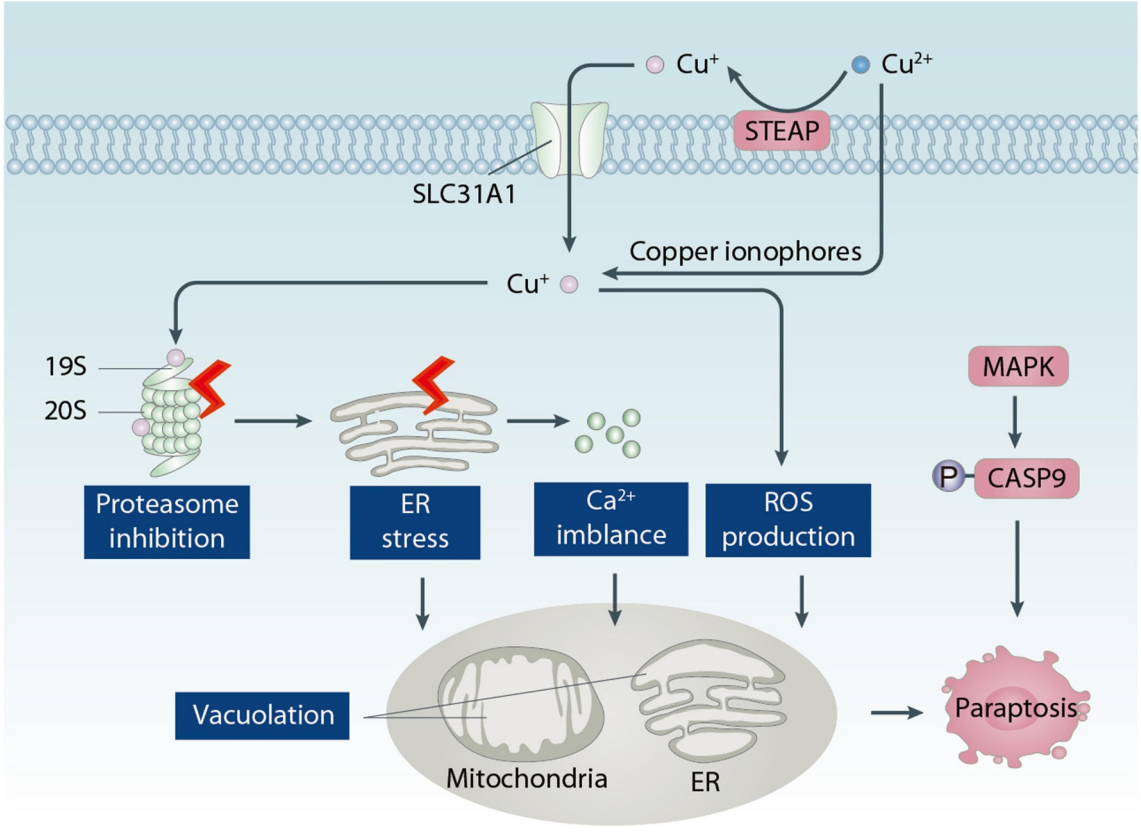

Figure 5. Role of copper in paraptosis. Paraptosis is a form of regulated necrosis characterized by vacuolation of mitochondria or ER. Copper promotes paraptosis by inducing proteasome inhibition, ER stress, Ca^{2+} imblance, and ROS production.

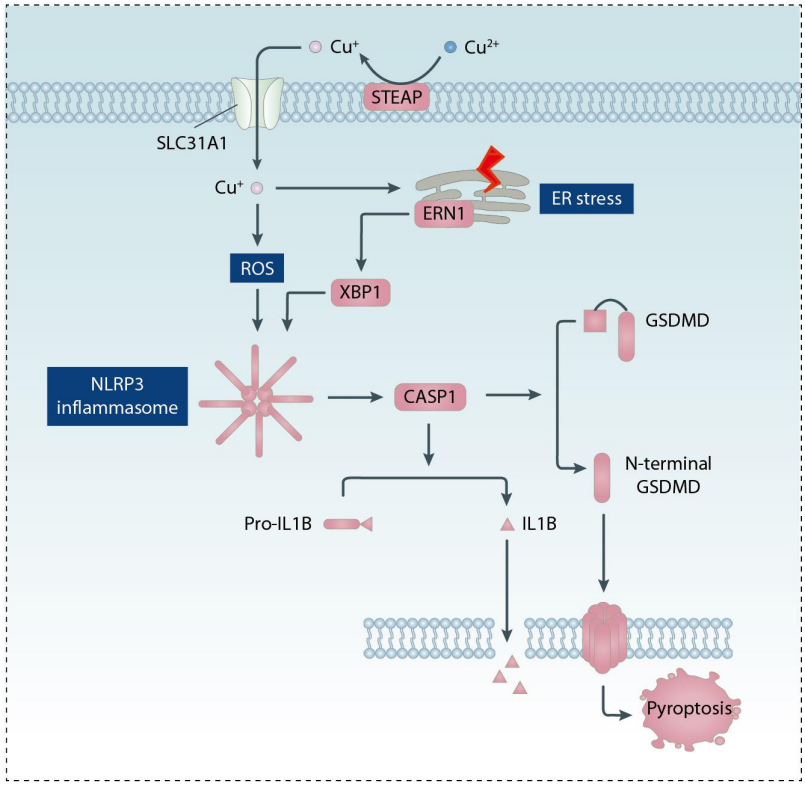

Figure 6. Role of copper in pyroptosis. Copper promotes pyroptosis by inducing ROS production and ER stress, which leads to the formation of the NLRP3 inflammasome and the creation of membrane pores through the action of GSDMD.

Ferroptosis

Ferroptosis is an iron-dependent regulated cell death mechanism driven by unrestricted lipid peroxidation [127,128]. Ferroptosis can be caused by both intrinsic and extrinsic pathways [129,130]. The extrinsic ferroptotic pathway is mainly mediated by inhibiting the amino acid reverse transporter system xc^-, whereas the intrinsic ferroptotic pathway is induced by inhibiting the GPX4 (glutathione peroxidase 4) protein. System xc^- is a cystine/glutamate antiporter on the plasma membrane, which mediates cystine absorption and promotes subsequent GSH synthesis [131]. GPX4 is the main antiferroptotic enzyme, which directly prevents the accumulation of lipid hydroperoxide in a GSH-dependent manner [132]. During ferroptosis, polyunsaturated fatty acids (PUFAs) are the primary substrates for the initiate of lipid peroxidation, which causes damage to the lipid bilayer and influences membrane function [133]. ACSL4 (Acyl-CoA synthetase long chain family member 4), LPCAT3 (lysophosphatidylcholine acyltransferase 3), and SLC47A1 (solute carrier family 47 member 1) are required for lipid peroxidation due to their role in the biosynthesis and remodeling of PUFA phospholipids (PUFA-PL) or PUFA esterified cholesterol esters (PUFA-CE) in cell membranes [134–137]. ALOX (arachidonate lipoxygenase) or POR (cytochrome P450 oxidoreductase) drive enzyme-mediated oxidation of PUFA [133,138–140]. NFE2L2/NRF2 (NFE2 like BZIP transcription factor 2) is a critical mitigator of lipid peroxidation by promoting the expression of multiple anti-ferroptosis genes [141]. In addition to GPX4, AIFM2 (apoptosis inducing factor mitochondria associated 2) plays a role in mediating the conversion of coenzyme Q 10 (CoQ_{10}) to the reduced form ($CoQ_{10}H_2$), which directly prevents lipid peroxidation or indirectly activates the endosomal sorting complex required for transport-III (ESCRT-III) membrane repair system [142–144]. Thus, high ROS levels and low antioxidant capacity predispose cancer cells to ferroptosis, although the uniqueness of the ferroptosis mechanism remains controversial [145,146].

Due to the strong oxidation-reduction potentials, both iron and copper could induce the generation of hydroxy radical (·OH) via Fenton or Fenton-like reactions. Although previous studies have shown that iron is the only metal ion that triggers ferroptosis [147], there is growing evidence that copper can promote ferroptosis in some conditions (Figure 7A). Copper-binding agents or their copper complexes, such as disulfiram-copper and elesclomol-copper disrupt mitochondrial homeostasis and lead to oxidative stress, thus resulting in ferroptosis in liver cancer and colorectal cancer, respectively [148–150]. This is consistent with the observation that excessive copper accumulation produces a large number of ROS in cancer cells [151]. In addition, copper promotes ferroptotic cell death by inducing autophagic degradation of GPX4 in pancreatic cancer [152]. Copper chelators specifically reduce cancer cell sensitivity to ferroptosis, but not other types of cell death, such as apoptosis, necroptosis, and alkaliptosis (a form

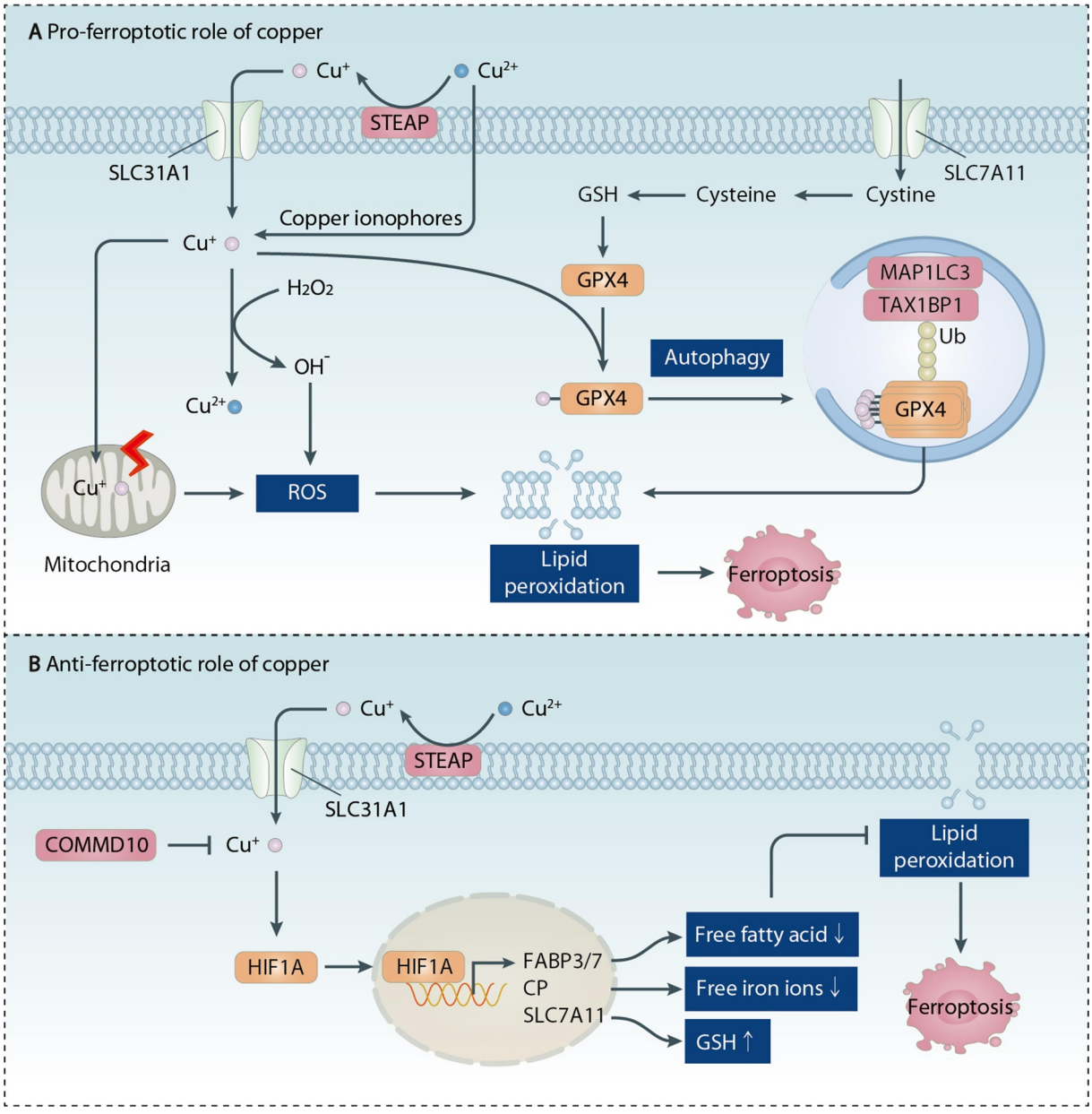

Figure 7. Role of copper in ferroptosis. (A) Pro-ferroptotic role of copper. Copper increases intracellular ROS through Fenton-like reactions or mitochondrial damage. Subsequently, increased ROS lead to lipid peroxidation of the plasma membrane or membrane structures. Furthermore, copper directly binds to GPX4 and induces GPX4 oligomerization, ultimately promoting the autophagic degradation of GPX4 mediated by the autophagy receptor TAXIBP1. (B) Anti-ferroptotic role of copper. COMMD10 is a key copper metabolism protein that reduces intracellular copper levels. Copper promotes HIF1A stabilization, thereby increasing transcription of HIF1A target genes, including FABP3, FABP7, CP, and SLC7A11. Copper-mediated upregulation of these HIF1A target genes suppresses lipid peroxidation and ferroptosis.

of cell death induced by intracellular alkalinization) [152]. Conversely, exogenous copper increases the formation of GPX4 aggregates and subsequent GPX4 ubiquitination by directly binding to GPX4 protein cysteines (C107 and C148) [152]. TAX1BP1 (Tax1 binding protein 1) is required for copper-mediated ferroptosis by acting as an autophagic receptor for GPX4 degradation [152]. Consequently, copper enhances the antitumor activity of ferroptosis inducers in a mouse model of pancreatic cancer tumors [152]. Copper-containing nanoparticles provide a viable copper delivery method to induce ferroptosis in colorectal cancer cells [153]. Thus, ferroptosis is a metal-dependent mode of cell death, involving iron and copper.

Of note, copper ions may play an anti-ferroptosis role in certain contexts (Figure 7B). Intracellular copper could stabilize the HIF1A (hypoxia inducible factor 1 subunit alpha) protein via inhibiting PHD (prolyl-4-hydroxylase domain) enzymes in liver cancer cells [154]. HIF1A antagonizes ferroptosis by upregulating lipid metabolism-related genes (e.g., FABP3 [fatty acid binding protein 3] and FABP7 [fatty acid binding protein 7]) in cancer cells [155]. Therefore, we can speculate that intracellular copper may inhibit ferroptosis by enhancing the expression of HIF1A. This hypothesis is supported by a study showing that copper promotes radioresistance in liver cancer cells by inhibiting ferroptosis [156,157]. COMMD10 (COMM domain containing 10), a key protein in

copper metabolism, inhibits HIF1A stability by decreasing intracellular copper to enhance ferroptosis-mediated radiosensitization [156]. Moreover, copper-induced upregulation of HIF1A enhances the transcription of additional targets, such as CP (ceruloplasmin) and SLC7A11 (solute carrier family 7 member 11), which suppress ferroptosis in hepatocellular carcinoma cells [156]. Likewise, the copper chelator bathocuproinedisulfonic acid (BCS) enhances ferroptosis in the dermal papilla cells [158]. Thus, copper ions occupy a crucial functional role as signaling molecules that determine ferroptosis sensitivity.

Cuproptosis

More recently, cuproptosis was termed copper-dependent cell death driven by mitochondrial stress and damage [159] (Figure 8). The copper ionophore elesclomol produces cytotoxicity by forming an elesclomol-copper complex with Cu(II) to carry Cu(II) into the cell [159]. This type of cell death depends on impaired mitochondrial respiration and subsequent mitochondrial protein stress, rather than mitochondrial oxidative stress [160]. Accordingly, cuproptosis is limited by inhibitors of respiratory chain complexes I and III (rotenone and antimycin A, respectively), and an inhibitor of the mitochondrial pyruvate transporter (UK5099) [159,161]. Mitochondrial energy depletion activates AMP-activated protein kinase (AMPK) to promote cuproptosis in liver and pancreatic cancer cells, further supporting cuproptosis as a form of metabolic cell death [162].

In addition to impairment of energy metabolism, cuproptotic death displays other mitochondrial changes, such as increased mitochondrial protein (e.g., DLAT [dihydrolipoamide S-acetyltransferase]) lipoylation, elevated disulfide bond-dependent aggregation of lipoylated DLAT, and decreased stabilization of Fe-S cluster proteins [159]. At the molecular level, FDX1 (ferredoxin 1) is a direct target of elesclomol and is required for elesclomol-induced cuproptosis [159,163]. Cell-free assays have shown that FDX1 acts as a reductase to reduce Cu(II) to Cu(I), although whether this function is conserved is controversial. Whether the mitochondrial quality control system (e.g., mitophagy) limits cuproptosis remains to be investigated.

Copper metabolism regulatory proteins are expected to affect the sensitivity of cancer cells to cuproptosis. Copper itself has the same cytotoxicity effect as elesclomol-Cu in SLC31A1-overexpressing cells [159]. As GSH can bind with Cu(I) by the thiol group, the inhibitor of GSH synthesis buthionine sulphoximine (BSO) increases cuproptosis [159]. The copper exporter ATP7A decreases cuproptosis sensitivity in KRAS-mutant colorectal cancer cells [69]. Similarly, lacking ATP7B causes excess copper accumulation and cuproptotic damage [159]. However, the expression of ATP7A and ATP7B and their impact on multidrug resistance have not been thoroughly investigated.

Although the well-known alarm molecule HMGB1 (high mobility group box 1) is a mediator of cuproptotic injury-induced sterile inflammation [162], the immune consequences of cuproptosis in the tumor microenvironment remain unknown. It also remains speculative whether mitochondrial danger/damage-associated molecular patterns/DAMPs, such as mitochondrial DNA, participate in pathogenesis of cuproptosis-induced inflammation.

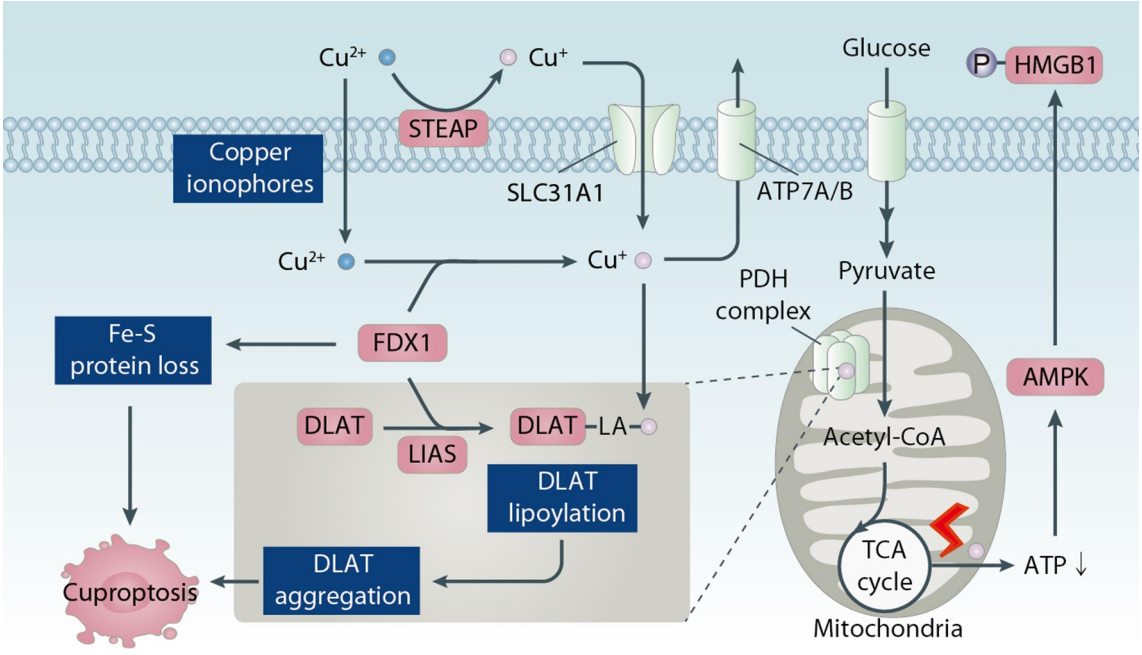

Figure 8. Role of copper in cuproptosis. Cuproptosis is induced by enrichment of copper through SLC31A1 uptake or copper ionophores. ATP7A and ATP7B have copper efflux functions, thereby inhibting cuproptotic cell death. The cell death process is related to mitochondrial proteotoxic stress caused by lipoylation of subunites of the PDH complex, such as DLAT. FDX1 plays a key role in reducing Cu(II) to the toxic form, Cu(I), increasing DLAT protein lipidation, and promoting Fe-S cluster protein loss. Furthermore, copper-mediated damage to the mitochondrial respiratory chain causes hyperactivation of the energy sensor AMPK, which accelerates cuproptosis and the release of the pro-inflammatory mediator HMGB1.

Role of copper in autophagy

Autophagy is a conserved degradation and recycling system in organisms driven by autophagy-related (ATG) proteins and their partners [164,165]. It is a dynamic process associated with the formation of membrane structures, such as phagophores, autophagosomes, and autolysosomes. Initiating kinase signaling includes the activation of AMPK or the inhibition of MTOR (mechanistic target of rapamycin kinase). AMPK acts as an energy sensor and promotes autophagy by phosphorylating MTOR complex 1 (MTORC1), ULK1 (unc-51 like autophagy activating kinase 1), and the BECN1 (beclin 1) component of PIK3C3/VPS34 (phosphatidylinositol 3-kinase catalytic subunit type 3) complexes [166–169]. Active MTORC1 inhibits autophagy by binding and phosphorylating the ULK complex composed of ULK1, ULK2 (unc-51 like autophagy activating kinase 2), and ATG13 (autophagy related 13) proteins [170]. The activated ULK1 complex recruits the BECN1-PIK3C3 complex to the site of autophagosome formation [171]. ATG proteins including MAP1LC3 (microtubule associated protein 1 light chain 3) are responsible for mediating the expansion and completion of phagophores to form autophagosomes [172,173]. Autophagy receptors, such as SQSTM1/p62 (sequestosome 1), that bind with both targets and MAP1LC3 on the phagophore, determine the selectivity of autophagic degradation of cargo [174]. Autophagy plays a dual role in cancer development [175,176]. On the one hand, autophagy can suppress cancer by reducing the levels of ROS, genomic instability, or intracellular damaged organelles and toxic substances. On the other hand, autophagy can also promote tumor growth and metastasis by providing metabolic support, or inhibiting apoptosis of tumor cells.

The cytotoxicity of heavy metals, including copper, is mainly mediated by ROS generated by the Fenton or Fenton-like reaction. In addition to triggering cell death, ROS-induced activation of the autophagy pathway can enhance cellular defense against damage. Once overactivated, the autophagic machinery may also positively feed back to accelerate cell death through the selective degradation of anti-damage or antioxidant proteins [177]. For example, recent studies have highlighted that ferroptosis is a type of autophagy-dependent cell death due to autophagy-mediated degradation of anti-ferroptotic factors [178,179], such as ferritin [180,181], lipid droplets [182], GPX4 [152,183–185], or CDH2 (cadherin 2) [186]. Targeting autophagy-dependent ferroptosis offers a new approach to treating cancers with high levels of autophagy and multidrug resistance [187–189].

Copper can perturb the autophagy process through multiple mechanisms (Figure 9). Mechanistically, copper can induce autophagy through increasing ATG (e.g., ATG5 [autophagy related 5], SQSTM1, and MAP1LC3) expression, regulating the AMPK-MTOR pathway, or inducing oxidative stress in various types of cells [190–192]. The loss of the copper exporter ATP7B or copper overload leads to autophagy through activation of TFEB (transcription factor EB), which acts as a regulator of lysosomal biogenesis [193,194]. Specifically, copper induces autophagy flux through directly binding to and activating the autophagic kinases ULK1 and ULK2 in $KRAS^{G12D}$-driven lung cancer, whereas copper chelator inhibits the kinase activity of ULK1 and ULK2 [9]. Copper-mediated autophagy contributes to $KRAS^{G12D}$-driven lung adenocarcinoma and the deletion of the copper transporter SLC31A1 suppresses the growth of lung tumors [9]. Interestingly, the inhibition of copper absorption by copper chelator or the knockdown of SLC31A1 increases

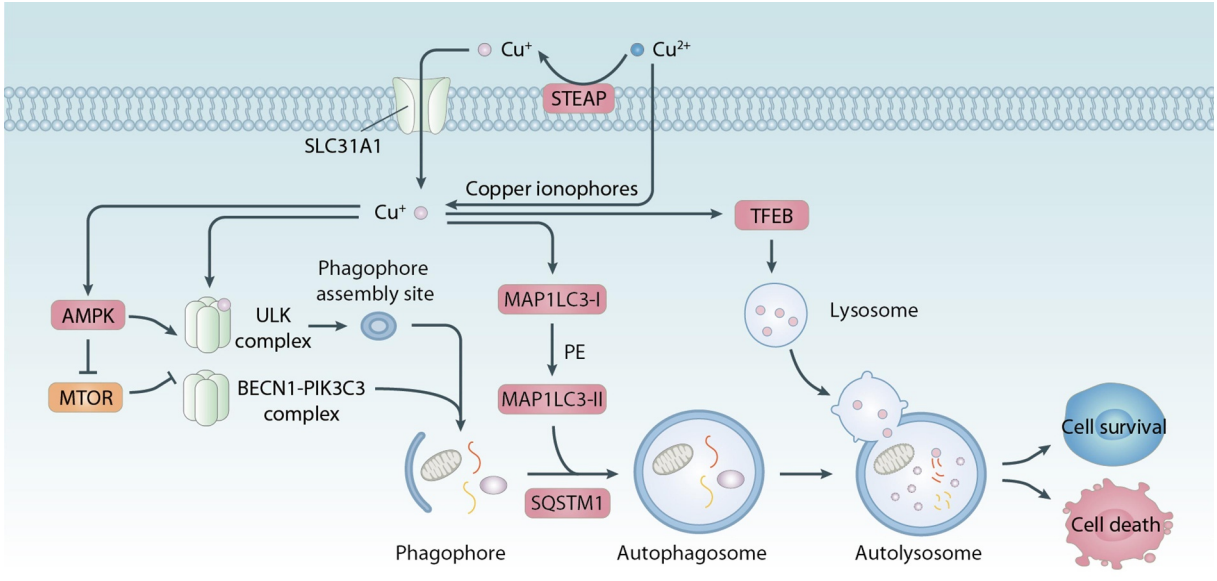

Figure 9. Role of copper in autophagy. Autophagy is a lysosome-mediated degradation process characterized by the formation of multiple member structures such as phagohpores, autophagosomes, and autolysosomes. Copper can induce autophagy initiation by activating AMPK or inhibiting the MTOR kinase pathway or directly binding to ULK1 or ULK2 kinase. Copper-mediated increase in MAP1LC3 and activation of TFEB transcription factor contribute to the formation of autophagosomes and autolysosomes, respectively. Functionally, copper-induced autophagy can lead to a protective response and autophagic cell death, respectively, depending on the strength of the stimulus and the type of substrate being degraded.

autophagy in pancreatic cancer cells [195], suggesting that copper deficiency may also activate autophagy.

Autophagy can act as both a suppressor and promoter of cell death in response to copper-induced toxicity. Copper induces autophagy in normal and cancer cells, which can serve as a cellular defense against copper-mediated toxicity [191,196–198]. Copper-mediated activation of autophagy can protect cells from apoptosis, such as in hepatocytes of a Wilson disease mouse model [193]. However, while most evidence suggests that copper-activated autophagy protects against cell death, some copper compounds such as copper oxide nanoparticles and Cu(II) complexes have been shown to induce autophagy-dependent cell death in cancer cells, which can be ameliorated by autophagy inhibitors [199,200]. Furthermore, copper can promote ferroptotic cell death in pancreatic cancer cells by triggering TAX1BP1-mediated autophagic degradation of GPX4 protein [152]. Key autophagy receptors that distinguish autophagy-mediated survival from death remain to be identified under various stresses, including copper overload or copper deficiency. Given the selectivity of autophagy substrates, clinical studies evaluating their therapeutic utility against cancer are urgently needed.

Copper-based agents for cancer treatment

Currently, copper-based agents used for tumor therapy mainly include copper chelators, copper ionophores, and copper-containing compounds. Since copper-containing compounds have been reviewed in detail elsewhere [201,202], this review focuses on the mechanisms of action of copper chelators and ionophores in cancer therapy (Figure 10).

Copper chelators

Several copper chelators are used to bind copper and reduce copper bioavailability, including tetrathiomolybdate (TM), D-penicillamine, and tetraethylenepentamine (TEPA) (Table 1). Among them, TM is the most studied and widely used copper chelator in various cancers [212–215]. TM is an oral anti-copper agent originally developed for the treatment of Wilson disease [218]. Later, numerous preclinical studies demonstrated that TM acts as an effective anticancer agent through multiple mechanisms. First, TM inhibits the activity of copper-dependent enzymes, such as SOD1, MAP2K1, MAP2K2, and PDK1, that are required for tumor cell proliferation [8,10,22,219]. Second, TM induces tumor cell necrosis or anoikis (a type of cell death that occurs when cells detach from the extracellular matrix) by activating the MAPK14/p38 (mitogen-activated protein kinase 14)-MAPK pathway and downregulating XIAP expression in head and neck cancer cells [220,221]. ATN-224 (a choline salt of TM) also induces cell death in lymphoma by increasing oxidative stress [222]. Third, TM reduces distant metastasis and angiogenesis by inhibiting the expression of pro-angiogenic mediators such as VEGF (vascular endothelial growth factor) and NFKB/NF-κB (nuclear factor kappa B) signaling [223,224]. Fourth, in addition to acting alone, TM can also enhance the activity of chemotherapy drugs such as doxorubicin, nifurtimox, and cisplatin by inducing apoptosis or inhibiting cell proliferation [225–227]. Given the promising preclinical anticancer activity of TM, clinical trials of TM have been

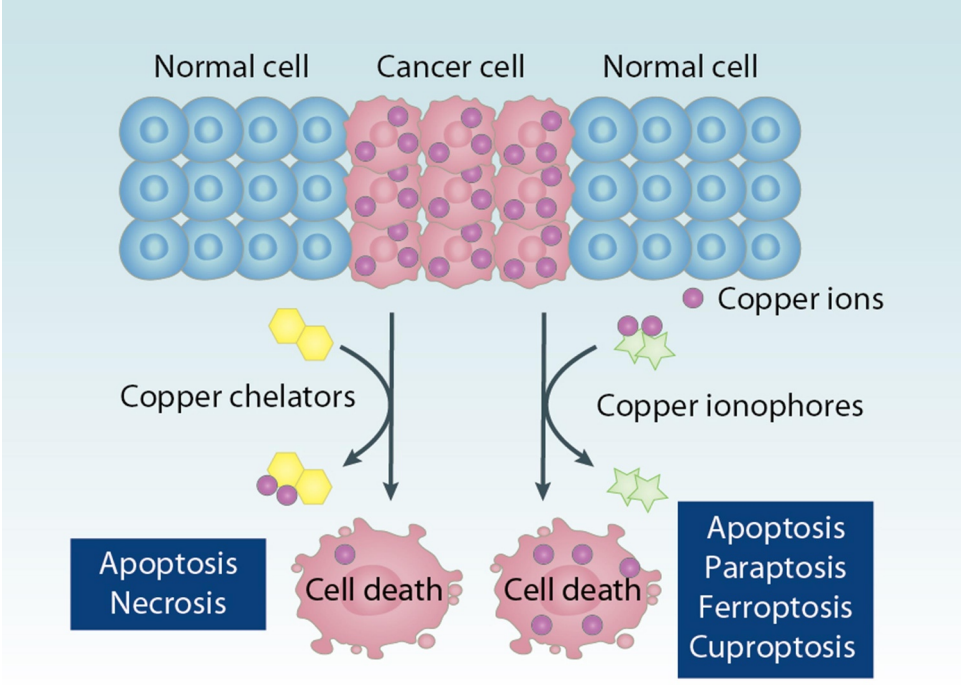

Figure 10. Copper chelators and ionophores in cancer therapy. Because copper supports the rapid growth of cancer cells, copper chelators can inhibit the functions of copper and induce apoptosis or necrosis of cancer cells. In contrast, copper ionophores transport copper into cells and induce copper overload and cytotoxicity. Copper-mediated cell death, including apoptosis, paraptosis, ferroptosis, and cuproptosis, plays a context-dependent role in tumor therapy.

Table 1. Copper chelators for cancer treatment.

Compounds	Tumor types	effects	Refs
2,9-Dimethyl-1,10-phenanthroline (neocuproine)	Lymphoma	Inhibits cell growth	[203]
Di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone (Dp44mT)	Lung cancer, melanoma	Induces ROS production and apoptosis	[204,205]
D-penicillamine	Human leukemia, breast cancer, melanoma, lung cancer, gliosarcoma	Induces ROS production, unfolded protein response, and apoptosis	[206–209]
Tetrakis-[2-pyridylmethyl]-ethylenediamine (TPEN)	Colon cancer	Induces ROS production and apoptosis	[210]
Trientine	Liver cancer	Inhibits angiogenesis and induces apoptosis	[211]
Tetrathiomolybdate (TM)	Lung cancer, breast cancer, thyroid cancer, colon carcinoma, renal cell cancer	Decreases tumor metastases and angiogenesis, inhibits NFKB signaling, induces apoptosis, necrosis, and anoikis	[212–217]
Tetraethylenepentamine (TEPA)	Neuroblastoma, liver cancer	Downregulates CD274/PD-L1 expression	[148,156]

conducted in patients with malignancies [216]. Preliminary studies show that TM is well tolerated and effective in some patients with solid tumors [216].

Another successful drug to treat Wilson disease is D-penicillamine. D-penicillamine chelates copper to generate ROS that are cytotoxic to human leukemia and breast cancer cells [206]. In addition, D-penicillamine rapidly activates the unfolded protein response and mitochondrial pathway of apoptosis in metastatic melanoma cells [207]. D-penicillamine has synergistic anticancer effects with radiotherapy and platinum-based chemotherapy [208,228]. The copper chelator TEPA downregulates the expression of the immune checkpoint protein CD274 and restores antitumor immunity in neuroblastoma in mice [97]. Additional knowledge should enable the development of therapeutics for clinical studies of copper chelators in cancer patients.

Copper ionophores

Copper ionophores transport copper into cells, thereby increasing intracellular copper levels and subsequent cell death. This process can be reversed by copper chelators. As described below, several drugs can act as copper ionophores, including disulfiram, pyrithione, chloroquine, and elesclomol (Table 2).

Disulfiram is one of the clinical drugs approved to treat alcoholism [89]. Disulfiram also has anticancer activity, largely due to its ability to bind copper [237]. Disulfiram is metabolized intracellularly to diethyldithiocarbamate (DTC), which can form a complex with copper to form a DTC-copper complex that induces apoptosis in cancer cells [252]. The combination of disulfiram and copper inhibits tumor growth more than disulfiram or copper alone [89,238]. Disulfiram also suppresses the ubiquitin-proteasome degradation system through inhibiting the chymotrypsin-like activity of the 20S proteasome or targeting NPLOC4/NPL4 (NPL4 homolog, ubiquitin recognition factor), a key player in the degradation of endoplasmic reticulum-associated proteins [89,238]. In addition, disulfiram, as an ALDH (aldehyde dehydrogenase) inhibitor, eliminates stem-like cell populations in a copper-dependent manner in various cancers, including glioblastoma, ovarian, lung, and liver cancers [239–242]. The anticancer activity of disulfiram may also be related to the induction of ROS and the inhibition of NFKB [253]. Disulfiram-copper treatment triggers autophagy in non-small cell lung cancer cells, which impedes apoptosis induced by disulfiram-copper [198]. As a result, blocking autophagy using ATG5 siRNA or 3-Methyladenine can increase the cytotoxicity of disulfiram-copper in non-small cell lung cancer cells [198]. In addition to inducing apoptosis, disulfiram inhibits tumor growth by

Table 2. Copper ionophores for cancer treatment.

Compounds	Tumor types	effects	Refs
2,20-dithiodipyridine (Dpy)	Cervical cancer, lung cancer, liver cancer	Induces ROS production and apoptosis	[229]
3-hydroxyflavone	Lung cancer, liver cancer	Induces GSH depletion, ROS generation, and apoptosis	[230]
Clioquinol	Prostate cancer, myeloma, leukemia	Inhibits HDAC and proteasome activity, induces apoptosis and paraptosis	[231–236]
Disulfiram	Glioblastoma, ovarian cancer, lung cancer, liver cancer	Induces ROS production, apoptosis and cuproptosis, inhibits NFKB signaling	[89,159,237–242]
Elesclomol	Breast cancer, colorectal cancer, lung cancer, uveal melanoma	Induces ROS production, lipoylated protein aggregation, apoptosis, ferroptosis, and cuproptosis	[149,159,243–246]
Hinokitiol	Lung cancer, leukemia, endometrial cancer	Inhibits 19S proteasomal deubiquinases, induces apoptosis and paraptosis	[112,247]
Pyrithione	Breast cancer, liver cancer, myeloma	Inhibits proteasome activity and 19S proteasomal deubiquinases, induces apoptosis	[90]
Pyrrolidine dithiocarbamate (PDTC)	Breast cancer, prostate cancer, leukemia	Inhibits NFKB signaling, proteasome activity, and histone acetylation, induces apoptosis	[248,249]
Salicylaldehyde isonicotinoyl hydrazone (SIH-1)	Liver cancer	Induces ROS generation and apoptosis	[250]
Thiomaltol	Melanoma	Induces lysosomal accumulation of copper and apoptosis	[251]

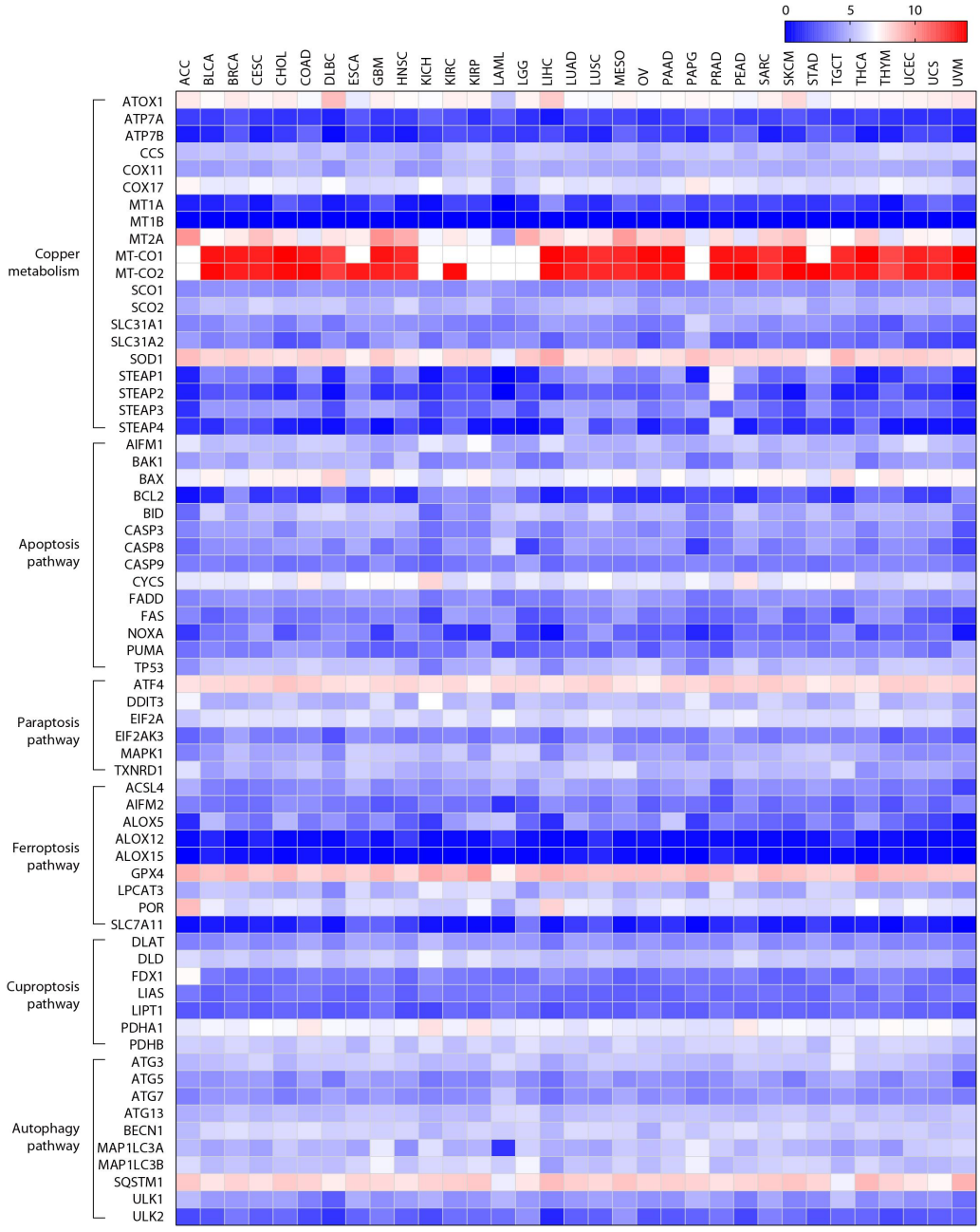

Figure 11. Gene expression changes in cancer patients. The gene expression of key regulators of copper metabolism, cell death, and autophagy pathway in patients with cancer was analyzed using the web server GEPIA (http://gepia.cancer-pku.cn/). Log 2 (TPM+1) was used for log-scale. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

inducing non-apoptotic cell death, including ferroptosis and cuproptosis. Disulfiram or disulfiram-copper induces ferroptosis by upregulating ROS-triggered lysosomal membrane permeabilization and lipid peroxidation in glioblastoma, hepatocellular carcinoma, and nasopharyngeal cancer [148,150,254] and triggers cuproptosis by targeting FDX1-dependent lipoylated tricarboxylic acid/TCA cycle proteins [159]. As an inexpensive and safe drug [255], preliminary data suggest that the use of disulfiram is effective in reducing overall mortality in cancer patients, including colon, prostate and breast cancers [238]. More clinical trials of disulfiram for anticancer applications are ongoing, including breast cancer (NCT03323346), pancreatic cancer (NCT02671890) and multiple myeloma (NCT04521335). These findings suggest that disulfiram may be a promising anticancer drug due to its pharmacokinetic properties and safety profile.

Clioquinol is a metal ionophore that transports copper or zinc into mammalian cells. It has been used for many years as an antimicrobial agent and more recently as a potential treatment for Alzheimer disease. The clioquinol derivative 8-hydroxoquinoline increases intracellular free copper in the cytoplasm, leading to paraptotic cell death in HeLa and PC3 cancer cells [231,232]. Clioquinol binds with copper and subsequently induces apoptosis by blocking the activity of the 20S proteasome [233] or inhibiting HDAC (histone deacetylase) activity in myeloma and leukemia cells [234]. The pro-apoptotic activity of clioquinol is also dependent on the induction of nuclear translocation of cytoplasmic XIAP in a copper-dependent manner in prostate cells [235]. Disruption of the ATOX1 function by clioquinol may further cause the disturbance of cellular copper transport and cancer cell death [236]. Furthermore, clioquinol stimulates autophagy and induces apoptosis in leukemia and myeloma cells by inhibiting MTORC1 [256]. Although these findings suggest that the mechanism by which clioquinol induces cell death is different, the ability to bind divalent metal ions is a key property of clioquinol's anticancer activity.

Pyrithione is considered a copper or zinc ionophore and is an active substance used in anti-dandruff shampoos [257]. Pyrithione-copper complexes target 20S proteasome activity in cancer cells [90]. In addition, the pyrithione-copper complex acts as an inhibitor of 19S proteasomal deubiquitinases, such as USP14 (ubiquitin specific peptidase 14) and UCHL5 (ubiquitin C-terminal hydrolase L5) [90]. The combination of pyrithione and Cu(II) kills tumor cells more effectively than pyrithione or Cu(II) alone, including MCF7, HepG2, and NCI-H929 cells [90]. *In vivo*, the pyrithione-copper complex exhibits potent tumor suppressor activity by inducing apoptosis [90]. Further formulation optimization of the pyrithione-copper complex may focus on improving tissue delivery specificity.

Elesclomol, a copper ionophore, may hold promise in treating Menkes disease caused by copper deficiency [258]. Elesclomol transports copper to mitochondria, damages mitochondrial DNA, and induces ROS production, thereby inducing apoptosis and inhibiting growth of BRCA1 (BRCA1 DNA repair associated)-mutant breast cancer cells and GNAQ/11 (G protein subunit alpha q/11)-mutant uveal melanoma cells [243,259,260]. Elesclomol also potentiates the effect of the conventional chemotherapy drugs (doxorubicin or paclitaxel) by increasing apoptosis-related proteins (e.g., cleaved CASP3, CDKN1A, and CDKN1B/p27 [cyclin-dependent kinase inhibitor 1B]) and reducing NFKB activity in breast cancer cells [244]. The anticancer activity of elesclomol may also be linked to the SLC7A11-related ferroptosis pathways. For example, elesclomol promotes the degradation of the copper transporter ATP7A, leading to copper retention and ROS accumulation in mitochondria, which further promotes the degradation of SLC7A11 and the induction of ferroptosis [149]. Another anticancer function of elesclomol is associated with the induction of cuproptosis in ABC1 lung cancer cells and G402 renal leiomyoblastoma cells [159]. This study found that elesclomol-induced cell death does not involve cleavage or activation of CASP3 activity. In contrast, copper directly binds to mitochondrial lipidated proteins (such as DLAT) in the tricarboxylic acid cycle, leading to lipidated protein aggregation and loss of Fe-S cluster proteins, ultimately promoting proteotoxic stress-induced cuproptosis [159]. Although current evidence has suggested that mitochondria play a key role in cuproptosis, the key executor involved in elesclomol-induced cuproptosis remains unclear. Given that elesclomol is well tolerated, there is an urgent need to evaluate its feasibility alone or in combination with other agents in the treatment of tumors [245].

Conclusions and perspectives

Copper is an essential micronutrient involved in basic life processes, including metabolism, responsiveness, movement, growth, differentiation, and reproduction. However, copper overload leads to oxidative damage and cell death. Disrupted copper homeostasis is associated with various human diseases, including cancer. Abnormalities or reprogramming of copper metabolism lead to increased intratumoral copper and/or altered systemic copper distribution, thereby accelerating the development of tumor hallmarks, a set of functional capabilities acquired by tumor cells [261]. Although preclinical studies have shown encouraging antitumor effects through copper chelation or induction of copper-dependent cell death via copper ionophores, several challenges remain in their clinical translation. Given the diversity of copper-dependent substrates (e.g., enzymes or nuclear transcription factors), it is tempting to speculate that the relative importance of pathogenic mechanisms may vary across different tumor types.

Heterogeneous genetic alterations of copper metabolism-, cell death-, and autophagy-related genes has been observed in various cancers (Figure 11). ATOX1 is expressed at a low level in acute myeloid leukemia and highly expressed in diffuse large B-cell lymphoma, which may provide an indicator of copper metabolism in these two tumor types. Given that the antioxidant enzymes SOD1 and GPX4 are highly expressed in most tumors, copper depletion-induced SOD1 inhibition and copper overload-induced GPX4 degradation may have great potential as a viable anticancer strategy. Clearly, how to minimize the toxic side effects of copper-targeted therapy remains a concern in clinical oncology.

Furthermore, there is no single sensitive and specific test available to diagnose which step of copper metabolism is impaired. The mechanistic specificity of copper-induced cell death is still under debate, although initial studies have shown that cuproptosis is independent of ROS. Technical considerations to produce novel copper-related drugs should incorporate knowledge based on the selective modulation of the biological activity of copper in distinct types of cell death, as distinguished from other metal-induced cell death. A better understanding of the molecular relationship between copper,

cell death, and autophagy in different stages of cancer may pave the way for better prevention and treatment strategies [262].

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Disclosure statement

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