

# Connecting copper and cancer: from transition metal signalling to metalloplasia

Eva J. Ge, Ashley I. Bush<sup>1</sup>, Angela Casini<sup>1</sup>, Paul A. Cobine<sup>1</sup>, Justin R. Cross, Gina M. DeNicola, Q. Ping Dou, Katherine J. Franz, Vishal M. Gohil<sup>1</sup>, Sanjeev Gupta, Stephen G. Kaler, Svetlana Lutsenko, Vivek Mittal<sup>1</sup>, Michael J. Petris, Roman Polishchuk<sup>1</sup>, Martina Ralle<sup>1</sup>, Michael L. Schilsky, Nicholas K. Tonks, Linda T. Vahdat, Linda Van Aelst, Dan Xi, Peng Yuan<sup>1</sup>, Donita C. Brady<sup>1</sup> and Christopher J. Chang<sup>1</sup>

**Abstract** | Copper is an essential nutrient whose redox properties make it both beneficial and toxic to the cell. Recent progress in studying transition metal signalling has forged new links between researchers of different disciplines that can help translate basic research in the chemistry and biology of copper into clinical therapies and diagnostics to exploit copper-dependent disease vulnerabilities. This concept is particularly relevant in cancer, as tumour growth and metastasis have a heightened requirement for this metal nutrient. Indeed, the traditional view of copper as solely an active site metabolic cofactor has been challenged by emerging evidence that copper is also a dynamic signalling metal and metalloallosteric regulator, such as for copper-dependent phosphodiesterase 3B (PDE3B) in lipolysis, mitogen-activated protein kinase kinase 1 (MEK1) and MEK2 in cell growth and proliferation and the kinases ULK1 and ULK2 in autophagy. In this Perspective, we summarize our current understanding of the connection between copper and cancer and explore how challenges in the field could be addressed by using the framework of cuproplasia, which is defined as regulated copper-dependent cell proliferation and is a representative example of a broad range of metalloplasias. Cuproplasia is linked to a diverse array of cellular processes, including mitochondrial respiration, antioxidant defence, redox signalling, kinase signalling, autophagy and protein quality control. Identifying and characterizing new modes of copper-dependent signalling offers translational opportunities that leverage disease vulnerabilities to this metal nutrient.

Cell proliferation is a fundamental process that is essential for the development and homeostasis of multicellular organisms and leads to the exponential growth of tissue. In this context, aberrant elevations and/or deficiencies in cell proliferation are major contributors to injury, ageing and disease. Indeed, a prime example of uncontrolled cell proliferation is cancer, in which survival, proliferation and implantation in distant tissues are highly dependent on the ability to acquire adequate oxygen and nutrients within a range of hostile environments<sup>1</sup>.

Copper is a mineral nutrient that is increasingly implicated in cell proliferation and death pathways. The inherent oxidation–reduction (redox) property of copper makes it both beneficial and potentially toxic to the cell. Cu(II) and Cu(I) are the two physiologically relevant oxidation states of copper, with Cu(I) understood to be the predominant form in the reducing environment of the cell cytosol. Copper is a required cofactor for enzymes that mediate a host of essential cellular functions, including mitochondrial

respiration, antioxidant defence and the biosynthesis of hormones, neurotransmitters and pigments, but at the same time dysregulation of copper stores can induce oxidative stress and cytotoxicity<sup>2–4</sup>. This traditional view of copper as solely an active site cofactor has been challenged by emerging evidence that copper is a dynamic signalling metal and metalloallosteric regulator that governs and coordinates biological activities in response to external stimuli<sup>5,6</sup>. Rapid progress in the field has forged new links between researchers in different disciplines that can help translate basic research in the chemistry and biology of copper into potential clinical therapies that exploit copper-dependent disease vulnerabilities, particularly in cancer.

To discuss our current understanding of the connection between copper and cancer and to explore how challenges in the field could be addressed, a group of leading researchers with chemical, biological and clinical perspectives on this topic were brought together for an interdisciplinary conference, the Copper Cancer Consortium, organized by Linda Vahdat, Mick Petris and Nick Tonks at the Banbury Center at Cold Spring Harbour Laboratory on 1–4 March 2020. Several common threads emerged from the meeting that are of general interest to the cancer and broader biomedical community. In particular, one unifying theme that crystallized from these discussions is the concept of cuproplasia, a newly recognized form of regulated copper-dependent cell proliferation.

## Definition of cuproplasia

‘Cuproplasia’ is defined as copper-dependent cell growth and proliferation. This term encompasses both neoplasia and hyperplasia, describes both primary and secondary effects of copper via signalling pathways and includes both enzymatic and non-enzymatic copper-modulated activities. Cuproplasia can be pharmacologically targeted: copper signalling can be repressed by copper-selective chelators or can be activated with metal ionophores that elevate copper levels or spatially and temporally redistribute copper stores across cellular and subcellular pools. Cuproplasia can also be modulated by genetic and/or pharmacological manipulation of proteins

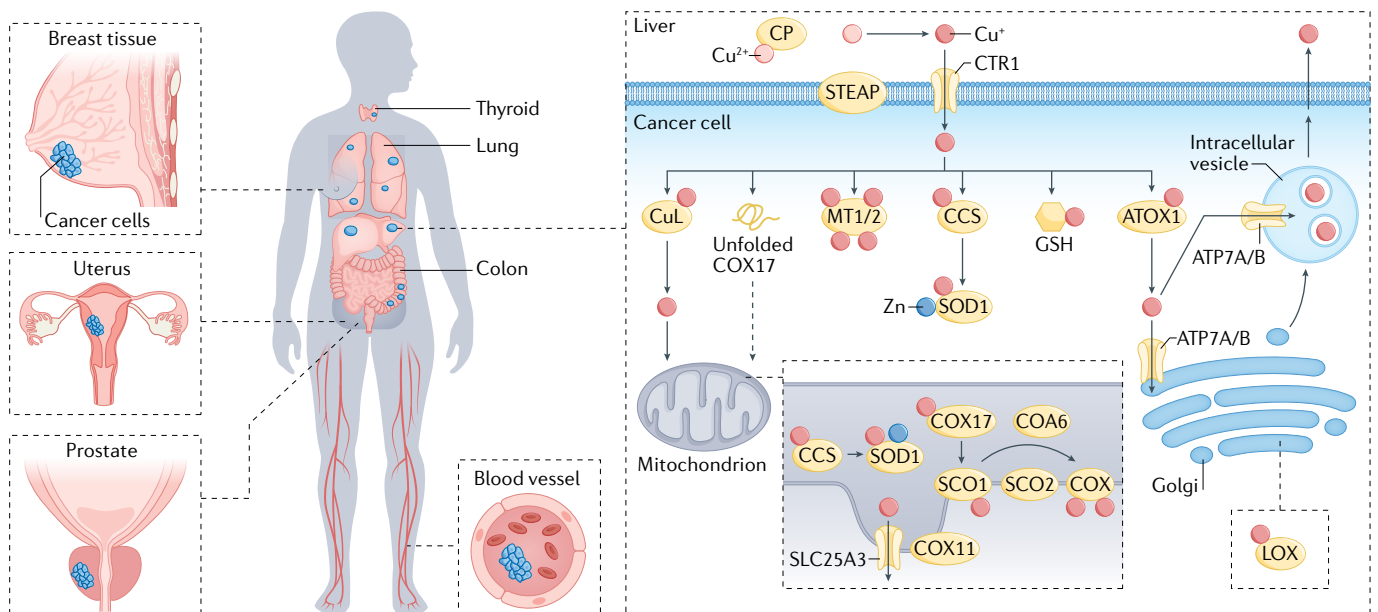
involved in copper homeostasis. We propose that cuproplasia is one example of different forms of metalloplasia, as other metals, such as iron, can also regulate signalling pathways to promote iron-dependent cell proliferation (ferroplasia), akin to iron-dependent cell death (ferroptosis)<sup>7,8</sup>. Along the same lines, copper can also mediate cell death through cytotoxicity induced by increased mitochondrial-dependent energy metabolism and accumulation of reactive oxygen species (ROS) in a process termed ‘cuproptosis’.

**Copper homeostasis**

A central tenet regarding elemental nutrients is that they can be neither created nor destroyed, and like all metals, the total pool of intracellular copper is divided into two subsets: a tightly bound protein pool (micromolar) and a bioavailable labile pool (subfemtomolar)<sup>9,10</sup>. Advances in the biometals field have revealed key molecular pathways that regulate copper acquisition, trafficking, storage and export<sup>11–17</sup> (FIG. 1). Key targets in mammalian

copper homeostasis include ceruloplasmin as the predominant protein carrier for exchangeable copper in blood plasma, CTR1 (also known as SLC31A1) and related ion transporters for cellular copper uptake, cytoplasmic metallochaperones (ATOX1) and cytoplasmic–mitochondrial metallochaperones (CCS, SCO1, SCO2, COX11 and COX17) for targeted insertion of copper into metalloenzymes and the copper-dependent ATPases ATP7A and ATP7B, which possess both copper export and metallochaperone functions. Metallothionein 1 (MT1) and MT2, two of three isoforms of thiol-rich proteins with high affinity for binding multiple copper ions, are the presumptive analogues of the iron-storing protein ferritin. Together, these proteins maintain appropriate intracellular copper bioavailability and ensure the metallation of copper-dependent enzymes, including cytochrome *c* oxidase, superoxide dismutase and various oxygenase/oxidase enzymes, including tyrosinase, lysyl oxidase (LOX), dopamine β-hydroxylase and copper amine oxidases (TABLE 1).

**Systemic metabolism.** As an essential nutrient, copper is absorbed in the gastrointestinal tract from dietary sources. The recommended daily intake for adults is 0.9 mg, which is typically met or exceeded by the average Western diet<sup>18</sup>. Foods rich in copper include shellfish, seeds, nuts, organ meats and chocolate<sup>19</sup>. Dietary copper absorption occurs mainly in the small intestine, where copper uptake across the apical membrane of enterocytes is dependent on CTR1 and copper export across the basolateral membrane requires the protein ATP7A<sup>20</sup> (FIG. 1). Within the portal circulation, serum proteins such as albumin deliver copper from the intestine to the liver, where excess copper is stored in hepatocytes by MT1 and MT2. The liver is also the major site of copper removal from the body via hepatobiliary excretion across the bile canalicular membrane of hepatocytes, which occurs via the copper exporter ATP7B<sup>21,22</sup>. The bulk of copper exported from hepatocytes into the systemic circulation is initially bound to ceruloplasmin, a secreted multicopper oxidase that receives copper



**Fig. 1 | Overview of systemic and cellular copper homeostasis.** Human copper homeostasis involves a number of key molecular targets. Ceruloplasmin (CP) is the major protein carrier for exchangeable copper in blood plasma for circulation and delivery to organ and tissue systems. At the cellular level, the STEAP family of metalloreductases and copper ion channel copper transporter 1 (CTR1) enable high-affinity copper uptake, with a diverse array of cytoplasmic and mitochondrial metallochaperones (antioxidant protein 1 (ATOX1), copper chaperone for superoxide dismutase (CCS), synthesis of cytochrome oxidase 1 (SCO1), SCO2, copper chaperone for cytochrome *c* oxidase 11 (COX11), COX17, ATPase 7A (ATP7A) and ATP7B) working in concert to ensure targeted insertion of copper into metalloprotein. The ATP-driven transmembrane copper ion pumps ATP7A and ATP7B perform both copper export and metallochaperone functions. The thiol-rich proteins metallothionein 1 (MT1) and MT2 bind multiple

copper ions and can serve as a copper storage reservoir. In addition, the abundant peptide and antioxidant glutathione (GSH) can also participate directly or indirectly in regulating cellular copper pools. Within the mitochondria, cytochrome *c* oxidase assembly factor 6 (COA6) and SCO2 help maintain the redox balance of SCO1 and in turn its copper binding and delivery to cytochrome *c* oxidase (COX). Together, these proteins maintain appropriate intracellular copper bioavailability and ensure metallation of copper-dependent enzymes, including COX, superoxide dismutase 1 (SOD1) and oxygenase/oxidase enzymes, including tyrosinase, lysyl oxidase (LOX), dopamine β-hydroxylase (DBH) and copper amine oxidases. Aberrant elevations in copper levels have been reported in tumours or serum of animal models and patients with various cancers, including breast, lung, gastrointestinal, oral, thyroid, gall bladder, gynaecologic and prostate cancers.

Table 1 | Genes involved in copper homeostasis and cuproplasia

Gene	Name	Function
<i>CP</i>	Ceruloplasmin	Major exchangeable plasma Cu carrier
<i>CTR1</i> (also known as <i>SLC31A1</i> )	Copper transporter 1	High-affinity Cu importer
<i>CTR2</i> (also known as <i>SLC31A2</i> )	Copper transporter 2	<i>CTR1</i> regulator
<i>ATOX1</i>	Antioxidant protein 1	Cytosolic Cu metallochaperone
<i>CCS</i>	Copper chaperone for superoxide dismutase	Cytosolic Cu metallochaperone
<i>COX11</i>	Copper chaperone for cytochrome c oxidase 11	Mitochondrial Cu metallochaperone
<i>COX17</i>	Copper chaperone for cytochrome c oxidase 17	Mitochondrial Cu metallochaperone
<i>SCO1</i>	Synthesis of cytochrome oxidase 1	Mitochondrial Cu metallochaperone
<i>SCO2</i>	Synthesis of cytochrome oxidase 1	Mitochondrial Cu metallochaperone
<i>COA6</i>	Cytochrome c oxidase assembly factor 6	Mitochondrial Cu metallochaperone
<i>SLC25A3</i>	Phosphate carrier protein	Mitochondrial Cu importer
<i>ATP7A</i>	ATPase 7A	Cu exporter/Golgi apparatus Cu chaperone
<i>ATP7B</i>	ATPase 7B	Cu exporter/Golgi apparatus Cu chaperone
<i>MT1</i> and <i>MT2</i>	Metallothionein	Cu/Zn storage protein
<i>COX1</i> and <i>MT-CO2</i>	Subunits 1 and 2 of cytochrome c oxidase	Respiratory O <sub>2</sub> reduction
<i>SOD1</i>	Superoxide dismutase 1	Superoxide scavenger
<i>TYR</i>	Tyrosinase	Tyrosine oxidation
<i>LOXL2</i>	Lysyl oxidase like-protein 2	Lysine oxidation
<i>DBH</i>	Dopamine β-hydroxylase	Dopamine oxidation
<i>AOC3</i> (also known as <i>VAP1</i> )	Amine oxidase 3 (vascular adhesion protein 1)	Amine oxidation
<i>MEK1</i> and <i>MEK2</i> (also known as <i>MAP2K1</i> and <i>MAPK2</i> )	Mitogen-activated protein kinase kinase 1/2	Protein kinase
<i>ULK1/ULK2</i>	Unc51-like kinase 1/2	Protein kinase
<i>PKD1</i>	3-Phosphoinositide dependent protein kinase 1	Protein kinase
<i>PDE3B</i>	Phosphodiesterase 3B	Cyclic AMP degradation
<i>UBE2D1</i> , <i>UBE2D2</i> , <i>UBE2D3</i> and <i>UBE2D4</i>	E2D ubiquitin conjugating enzyme E2 D1, D2, D3 and D4	Ubiquitin conjugation to protein target
<i>H3C1</i> and <i>HC14</i>	Histone H3.1 and Histone H4 in the H3/H4 tetramer	Copper reductase
<i>VEGFA</i>	Vascular endothelial growth factor A	Growth factor
<i>PD1L1</i> (also known as <i>CD274</i> )	Programmed cell death 1 ligand 1	Immune response control

via ATP7B-driven copper transport into the secretory pathway<sup>23</sup>. As ceruloplasmin is rapidly degraded when it is metal-free, its abundance in plasma is a biomarker of systemic copper deficiency<sup>24</sup> that has been widely used in clinical trials of therapeutic copper depletion in patients with cancer. However, as ceruloplasmin is also an acute phase reactant, its use as a biomarker of systemic copper may be confounded during inflammatory conditions<sup>25</sup>. Despite constituting the vast bulk of copper in plasma, copper-bound ceruloplasmin is not essential for copper acquisition by peripheral tissues<sup>26</sup>. Rather, low molecular weight copper ligands such as amino acids, including cysteine, histidine, methionine, aspartic acid and glutamic acid, are thought to serve as plasma donors of copper to the uptake machinery in tissues<sup>25,27</sup>. Copper uptake in most tissues is thought to occur

via the copper permease CTR1. As CTR1 is highly specific for reduced Cu(I)<sup>28,29</sup>, CTR1-dependent copper uptake is likely facilitated by the STEAP family of metalloreductases, which maintain copper in the reduced state<sup>30</sup>. Cellular copper balance is crucial for cellular maintenance and metabolism. This requirement is illustrated by Menkes disease, where mutations in *ATP7A* prevent the export of dietary copper from enterocytes into the bloodstream, causing a systemic copper deficiency characterized by developmental defects, including stunted growth, hypopigmentation, neurodegeneration and connective tissue defects<sup>31</sup>. By contrast, mutations in *ATP7B* cause Wilson disease, which is characterized by progressive hepatic copper overload that may induce liver failure or neuropsychiatric disease secondary to excess copper in the brain<sup>32</sup>. Although Menkes disease and

Wilson diseases are rare, these disturbances in copper metabolism have firmly established the contribution of this metal to critical aspects of cellular pathophysiology.

### Copper status in cancer

In the context of cancer, although numerous risk factors for hepatocellular carcinoma have been described, the increased incidence of hepatocellular carcinoma in patients with Wilson disease and animal models of Wilson disease evokes the possibility that aberrant copper accumulation may promote malignant transformation via an unknown mechanism<sup>33</sup>. Indeed, connections between copper and cancer have been noted for more than a century, with numerous observations pointing to a requirement for higher levels of copper for tumours compared with healthy tissue<sup>34</sup>.

Once growth and development are complete in mammals, the rates of

gastrointestinal absorption and biliary excretion are balanced to maintain systemic copper homeostasis<sup>35</sup>. However, anabolic states such as pregnancy require significantly higher intakes of dietary copper to meet the metabolic demands for growth and development<sup>36</sup>. Maternal copper deficiency is known to cause early embryonic death or congenital malformations in mammals, depending on the timing and severity of the deficiency<sup>37</sup>. This situation is due, in part, to the requirement for copper as a cofactor of mitochondrial cytochrome *c* oxidase, which is necessary to meet the energy demands of rapidly dividing cells. Cancer cells thus have a higher demand for copper compared with non-dividing cells<sup>38</sup>. Elevated copper concentrations have been reported in the tumours or serum of animal models and patients with many types of cancers, including breast<sup>39–44</sup>, lung<sup>45–47</sup>, gastrointestinal<sup>48–53</sup>, oral<sup>54</sup>, thyroid<sup>55</sup>, gall bladder<sup>56</sup>, gynaecologic<sup>52,53</sup> and prostate<sup>57</sup> cancers. Copper imbalance can not only impact mitochondrial respiration but can also lead to changes in glycolysis, insulin resistance and lipid metabolism<sup>58–60</sup>. Beyond mitochondrial function, copper pathways, for example, the ATOX–ATP7A–LOX pathways, promote metastatic expansion<sup>61</sup>. In addition, copper regulation of autophagy via ULK1 and ULK2 (REF.<sup>62</sup>) and/or protein quality control via UBE2D2 (REF.<sup>63</sup>), provide new copper-dependent targets that can influence tumour growth and progression. Copper is also able to promote blood vessel formation that contributes to tumour

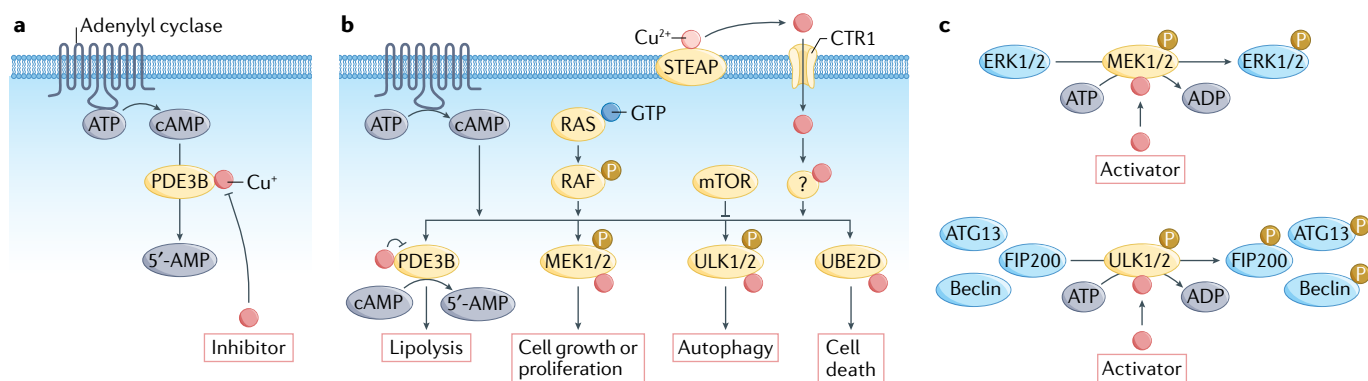
initiation, growth and metastasis. This metal nutrient directly activates several proangiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), tumour necrosis factor (TNF) and interleukin-1 (IL-1).

### Emerging mechanisms in cuproplasia

Although decades of research highlight the interplay between copper homeostasis, biology and medicine, the repertoire of known copper-dependent enzymes does not yield a complete molecular explanation for how biological systems convert copper abundance into distinct cellular functions. The ability of cells to initiate and/or maintain cellular processes is governed by signalling networks that facilitate intracellular and intercellular communication and in turn guide cellular responses. Whereas the signalling networks that integrate fluctuations in the abundance of growth factors, nutrients and metabolites are well established, the discovery of signalling molecules capable of mediating similar functions depending on copper availability is rudimentary. An emerging facet in nutrient sensing and protein regulation, termed ‘metalloallostery’<sup>66</sup>, whereby protein activity is regulated by dynamic copper binding at non-catalytic sites, has expanded our knowledge of the contributions of copper to signalling events and cellular processes. Indeed, identification of targets of copper metalloallostery and other mechanisms of dynamic metal nutrient regulation has given rise to

a new field of transition metal signalling<sup>6</sup>. These advances have, in part, built upon foundational work in the development of copper-responsive fluorescent probes that have enabled identification of rapid changes in labile copper pools in response to biological stimuli across many different cell types<sup>64–73</sup>.

Recent studies illustrate the capacity of copper to serve as a positive or negative allosteric regulator of enzymatic activity (FIG. 2). One of the first examples of metalloallostery was uncovered in the search for mechanistic features that drive aberrant lipid metabolism in the context of diet-induced copper deficiency or liver copper hyperaccumulation in Wilson disease. Specifically, the level of cyclic AMP (cAMP) generated downstream of G protein-coupled receptor activation was potentiated in response to copper in a 3T3-L1 white adipocyte model<sup>67</sup>. Mechanistically, copper inhibited cAMP degradation by directly binding to a conserved cysteine residue in the phosphodiesterase 3B (PDE3B), which is responsible for cAMP-dependent breakdown of triglycerides into fatty acids and glycerol, leading to higher cAMP levels and lipolytic activity through copper-dependent PDE3B inhibition. In contrast to allosteric copper-mediated inhibition of enzyme function, ablation of *CTR1* (also known as *SLC31A1*) reduced canonical RAF–mitogen-activated protein kinase kinase (MEK)–ERK signalling output at the level of ERK1/2 phosphorylation in response



**Fig. 2 | Copper metalloallostery and signalling promotes cell growth/proliferation and autophagy pathways.** Besides the traditional role of copper as a static cofactor for protein function, emerging evidence shows that copper is able to serve as both a negative allosteric regulator and a positive allosteric regulator of enzyme activity to influence foundational cellular pathways. As an example of negative metalloallostery, copper binds and inhibits phosphodiesterase 3B (PDE3B) to inhibit cyclic AMP (cAMP) degradation (part a) and promote cAMP-dependent lipolysis (part c), the breakdown of triglycerides into fatty acids and glycerol that is essential for fat metabolism. In the context of positive metalloallostery, copper acts on mitogen-activated protein kinase kinase 1 (MEK1) and MEK2 and enhances

their ability to phosphorylate extracellular signal-regulated kinase 1 (ERK1) and ERK2) (part b), stimulating RAF–MEK–ERK signalling (part c). Unc51-like kinase 1 (ULK1) and ULK2 provide a second example of copper-dependent kinase regulation, with copper able to relieve ULK1 and ULK2 inhibition and increase kinase activity in response to amino acid starvation (parts b,c). Finally, recent work has identified a role for copper signalling in promotion of protein degradation by positive allosteric activation of the E2 conjugating enzyme clade UBE2D1–UBE2D4 (part c). Therefore, copper-dependent kinase signalling can regulate cell growth/proliferation through MEK1 and MEK2 and autophagy through ULK1 and ULK2 (part c). mTOR, mechanistic target of rapamycin.

to proliferative signals in melanoma cell lines. More recently, copper treatment of cell cultures resulted in increased phosphorylation of the receptor tyrosine kinases TRKB, EGFR and MET upstream of MAPK signalling<sup>74–76</sup>. Epistatic and biochemical analysis revealed that copper acts on MEK1 and MEK2 and allosterically enhances the ability of MEK1 and MEK2 to phosphorylate ERK1 and ERK2 in a dose-dependent manner<sup>77</sup>. In addition, ablation in flies and mouse cells of *Ctr1* (also known as *Ctr1A*) in flies) to decrease the protein levels or introduction of surface accessible mutations in *Mek1* (also known as *Map2k1*) that disrupt copper binding decreased *Braf*<sup>V600E</sup>-driven signalling and tumour growth<sup>5,77</sup>. Furthermore, targeting the copper-dependent kinase activity of MEK1 and MEK2 with tetrathiomolybdate (TTM), a copper chelator used for the treatment of patients with Wilson disease, dampened the tumorigenesis of treatment-naive and resistant forms of *Braf*<sup>V600E</sup>-driven melanoma in mice<sup>5</sup>. Indeed, copper bioavailability was shown to be a KRAS-dependent vulnerability in colorectal cancer mouse models<sup>78</sup>. Beyond this first example of copper as a positive regulator of mammalian kinase activity, ULK1 and ULK2, downstream targets of the major nutrient-sensing kinase mechanistic target of rapamycin (mTOR), are also copper-dependent kinases<sup>62</sup>. Relief of ULK1 and/or ULK2 inhibition by the drug MRT68921 or by TTM or amino acid starvation-induced elevated kinase activity induced autophagy to salvage building blocks for macromolecular biosynthesis and bioenergetics that are scarce due to the nutrient-poor and oxygen-poor tumour microenvironment. Indeed, hepatic copper accumulation in Wilson disease is paralleled by activation of autophagy<sup>79</sup>. In addition, PDK1 activity is copper dependent, as shown in the colon cancer cell line DLD1 (REF.<sup>80</sup>). Finally, copper metalloallostery promoted protein degradation by allosteric activation of the E2 conjugating enzyme clade, UBE2D1–UBE2D4 (REF.<sup>63</sup>). As a result, numerous proteins became ubiquitin-tagged and degraded, notably including p53. Thus, the diminution of p53 by the oversupply of copper, as occurs in malignancy, could play a role in the inability of malignant cells to commit to programmed cell death.

Copper homeostasis has been shown to influence epigenetic regulators at the level of both chromatin modifications and transcription factors. Exposure of Hep3B human hepatoma cells to Cu<sup>2+</sup> resulted in hypoacetylation on histones H3 and H4 via

Table 2 | Pharmacological agents for modulating copper

Therapy	Type	Status	Refs
D-Penicillamine	Chelator	Wilson disease (approved)	175
Trientine	Chelator	Wilson disease (approved) Melanoma (preclinical)	84,86
Tetrathiomolybdate	Chelator	Wilson disease (phase II) Breast cancer (phase II)	87,88, 95,176
ATN-224	Chelator	Wilson disease (phase III) Breast cancer (phase II)	89,177
Disulfuram	Ionophore	Alcohol abuse (approved) Glioblastoma (phase I/II)	96,97, 100,178
Elesclomol	Ionophore	Melanoma (phase II)	98,99, 101
Diacetylbis(N <sup>4</sup> -methylthiosemicarbazonato) copper(II)	Ionophore	Amyotrophic lateral sclerosis (phase II)	179
Glyoxalbis(N <sup>4</sup> -methyl-3-thiosemicarbazonato)copper(II)	Ionophore	PET (preclinical)	180
Clioquinol	Ionophore	Fungal infection (approved)	181
<sup>64</sup> Cu	Radiotherapy	Melanoma, glioblastoma, prostate cancer (preclinical)	146,150, 151,182
DC_AC50	ATOX1/CCS inhibitor	Melanoma (preclinical)	168

PET, positron emission tomography.

inhibition of histone acetyltransferases<sup>81</sup>. Excess copper can also damage mitochondria, a defect often observed in Wilson disease, with mitochondrial abnormalities disrupting the production of metabolites used for epigenetic regulation<sup>82,83</sup>.

Taken together, the discoveries described are beginning to mine the diverse mechanisms of copper sensing in which signalling components are, in part, dependent on copper. This dependency ensures that the activity of associated cellular processes is restricted to optimal environmental conditions<sup>17</sup>. These findings presage broader roles for metalloallostery and transition metal signalling in the proteome, particularly in the context of cancer.

### Potential therapeutic strategies

Current clinical management of inherited disorders of copper dysregulation such as Menke disease, Wilson disease or CTR1 deficiency provides a foundation based on which safe, effective and rational approaches for the pharmacological modulation of cuproplasia can be considered. Coupled with recent advances in characterizing new copper-dependent signalling targets and pathways, there is growing interest in translating therapeutic strategies that can leverage copper-dependent disease vulnerabilities. In the context

of cancer, we summarize the two major current approaches: chelators for copper depletion to inhibit cuproplasia and ionophores for copper supplementation to promote cuproptosis. In addition, we also forecast inhibitors and activators of copper metalloallostery as a new class of therapeutics that will emerge from basic science studies. Exploiting disease-dependent vulnerabilities of an endogenous nutrient such as copper for pharmacology has potential advantages for mitigating side effects associated with current chemotherapies. Indeed, chelation therapy is well tolerated for long-term use in chronic genetic diseases of copper misregulation<sup>84</sup>, whereas copper supplementation strategies can rely on native metal homeostasis pathways to tune therapeutic windows relative to exogenous metalotherapies such as platinum-based therapies<sup>85</sup>.

**Copper chelation.** Copper chelators, which are typically used to lower elevated copper levels in the plasma of patients with Wilson disease, have been evaluated as antitumorigenic drugs in preclinical cancer models and phase I/II clinical trials<sup>86–89</sup> (TABLE 2). These drug-repurposing pursuits were attempted following observations that (1) preventing blood vessel development with antiangiogenic agents limits cancer growth as demonstrated in

patients<sup>90,91</sup>, (2) copper supplementation is sufficient to promote angiogenesis in rabbit models<sup>92,93</sup> and (3) limiting copper availability diminishes the ability of known angiogenic factors to stimulate blood vessel development<sup>87</sup>. Indeed, expression of VEGF is sensitive to copper, presumably through its ability to generate ROS<sup>94</sup>. On the basis of these findings, copper chelation as an antiangiogenic treatment has been translated in nearly every solid tumour and a few blood cancers<sup>86–89,95–101</sup> (TABLE 2).

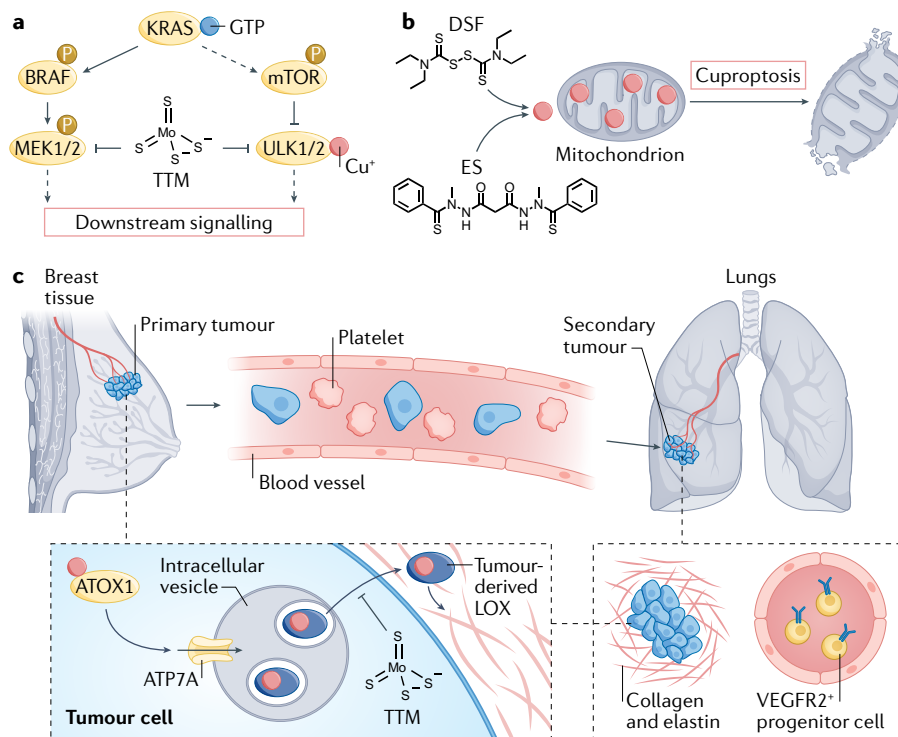
The kinases MEK1 and MEK2 are attractive targets to exploit copper-dependent cancer vulnerability, as the RAF–MEK–ERK signalling cascade is one of the most well defined axes to promote cell proliferation and is mutated in most human cancers. Indeed, 40–50% of melanomas are initiated by

mutations in the *BRAF* gene typically at V600, which constitutively activate the downstream kinases MEK1 and MEK2 to stimulate the MAPK pathway and promote cancer<sup>102</sup>. Given that the oncogenic effects of *BRAF* mutations are dependent on copper-stimulated MEK1 and MEK2 kinase activity, combination approaches that target both copper and kinase nodes are attracting interest<sup>103</sup> (FIG. 3).

Another copper-dependent cancer vulnerability is autophagy, which in a cancer context salvages building blocks for macromolecular biosynthesis and bioenergetics that are scarce due to the nutrient-poor and oxygen-poor tumour microenvironment<sup>104</sup>. In both lung adenocarcinoma and pancreatic cancer, oncogenic mutations in *KRAS* are associated with metabolic reprogramming, including

upregulated autophagy that sustains unrestricted tumour growth. Although autophagy inhibitors have had some clinical success for patients harbouring *KRAS* mutations, success is limited by the lack of potent inhibitors and the onset of resistance<sup>105,106</sup>. Of significance, traditional MAPK pathway inhibition at the level of *KRAS*, MEK or ERK results in the upregulation of protective autophagy and signalling in an ULK-dependent fashion in *KRAS*-mutant and *BRAF*-mutant cancers. By exploiting the copper dependence of both RAF–MEK–ERK signalling and ULK1-driven and ULK2-driven autophagy, preclinical studies have shown that limiting availability of this metal is an effective strategy for blocking *KRAS*-driven tumour growth and survival<sup>107,108</sup>. In addition to kinase pathways, chelators such as DPM-1001 link copper and signalling via inhibition of PTP1B<sup>109</sup>. These findings chart new ground by defining copper bioavailability as a metabolic Achilles heel that can be exploited to target multiple nodes of vulnerability in oncogenic signalling pathways.

In parallel to preclinical models centred on blocking primary tumour growth, translational efforts in modulating cuproplasia have focused on strategies to achieve copper depletion in metastasis, where modifying the tumour microenvironment by lowering copper levels can create an unwelcoming niche for tumour metastases (FIG. 3). TTM suppressed spontaneous mammary tumour development in mouse models, but tumours returned within weeks of TTM therapy being stopped, suggesting that long-term copper depletion maintains neoplastic tumour cells in a state of dormancy rather than suppressing neoplastic transformation<sup>110,111</sup>. Speculated mechanisms of action include inhibition of copper enzyme activities, suppression of angiogenesis and inhibition of kinases that function in autophagy and mitogenic signalling<sup>5,62,112–115</sup>. Clinical work has focused on patients with triple-negative breast cancer (TNBC; lacking all three receptors), who, despite accounting for only 15–20% of all patients with breast cancer, represent 50% of patients with metastatic disease<sup>116,117</sup>. TNBC systemically generates metastasis-promoting local microenvironments in distant organs referred to as the ‘premetastatic niche’, which provide the optimal infrastructure for disseminated tumour cells to colonize and grow into lethal macrometastases. A long-term phase II clinical trial was



**Fig. 3 | Therapeutic strategies to target cuproplasia in cancer.** Copper status can be leveraged as a cancer vulnerability, where the two major current treatment approaches targeting this nutrient include Cu(I) chelators to deplete copper pools that drive tumour proliferation and metastasis pathways (part **a**) or copper ionophores to supplement copper and drive cuproptosis, an oxidative stress-inducing form of cell death triggered by excess copper (part **b**). **a** | Copper chelators such as tetrathiomolybdate (TTM) can be used in combination therapy to augment the efficacy of kinase inhibitor drugs for oncogenic signalling pathways, particularly BRAF-driven MAPK signalling. **b** | Copper ionophores such as disulfiram (DSF) or elesclomol (ES) can be used to induce cuproptosis by inducing oxidative stress by overwhelming native antioxidant systems such as in mitochondria. **c** | TTM can also be used to deplete copper in the primary tumour and/or the metastatic niche to impede copper-dependent tumour metastasis without impairing the function of healthy tissue, as shown by long-term clinical trials in patients with triple-negative breast cancer. This chelator targets a key antioxidant protein 1 (ATOX1)–ATPase 7A (ATP7A)–lysyl oxidase (LOX) copper nexus that drives invasion and metastasis by impacting collagen deposition and structure and decreases the number of VEGFR2<sup>+</sup> endothelial progenitor cells in circulation that prime the metastatic niche. MEK, mitogen-activated protein kinase; mTOR, mechanistic target of rapamycin.

undertaken in a population of patients with breast cancer (49% with TNBC) after they had undergone standard chemotherapy including patients with no evidence of disease but high risk of relapse<sup>95</sup>. By treatment with TTM, patients were copper depleted to a level that impeded copper-dependent tumour-associated processes without impairing the function of healthy tissues. Specifically, TTM-treated patients exhibited a reduction in the levels of circulating VEGFR2<sup>+</sup> endothelial progenitor cells and LOX activity, which are both essential for metastatic niche priming. With more than 12,000 weeks of treatment given over a decade, TTM has been safe and well tolerated, with limited severe side effects. A larger randomized phase II clinical trial of copper depletion as a therapeutic strategy in high-risk TNBC is currently in development.

Another attractive target of copper chelation therapy is the immune checkpoint protein programmed cell death 1 ligand 1 (PDL1), which cancer cells overexpress to protect themselves from antitumour immune response. Bioinformatic analyses of pan-cancer gene expression profiles from [The Cancer Genome Atlas \(TCGA\) database](#) compared with normal tissue samples from the Genotype-Tissue Expression (GTEx) database showed strong correlation between expression of *CTRI* and *PDL1* (also known as *CD274*) in many cancers, but not in normal tissues. Copper supplementation was shown to enhance PDL1 expression, while copper chelation promoted ubiquitin-mediated degradation of PDL1 in colon cancer DLD1 cell lines. In an immunocompetent transgenic neuroblastoma mouse model, Th-MYCN, treatment with copper-chelating drugs slowed tumour growth and increased survival. These findings suggest that copper chelation therapy could potentially synergize with and enhance antitumour immune response<sup>118</sup>.

The use of small-molecule copper chelators to treat tumours could be further optimized by use of existing strategies for tissue-specific drug delivery, for example glucose-modified therapeutics for GLUT1 targeting in the brain and pancreas, and prostate-specific membrane antigen (PSMA) for the prostate using glutamate-urea-modified modalities<sup>119</sup>. However, identifying the most effective clinical settings remains a key barrier in this drug-repurposing strategy and offers future opportunities.

**Copper supplementation.** Although copper is essential for cell survival and proliferation and constitutes an exploitable

dependency in cancer, excess copper can cause toxicity via production of deleterious ROS<sup>120</sup>. As certain cancers exhibit constitutively elevated oxidative stress and show dependence on antiapoptotic ROS signalling, they are uniquely susceptible to further increases in ROS levels<sup>121–124</sup>. Therefore, copper ionophores, such as disulfiram (DSF) and elesclomol, are being pursued as cancer therapeutics to induce cuproptosis, a copper-dependent form of cell death<sup>96,125,126</sup> (TABLE 2).

DSF is a member of the dithiocarbamate family and was approved by the FDA in 1951 as the first drug to treat alcohol dependence through inhibition of aldehyde dehydrogenase (ALDH)<sup>96,127,128</sup>. Many studies over the past 30 years have shown that DSF in combination with cupric ion (Cu(II)) has potential to treat various human cancers<sup>96</sup>. In acidic environments such as the stomach, DSF is reduced to diethyldithiocarbamate (DDTC), a potent chelator of divalent transition metal ions, including Cu(II)<sup>129</sup>. DDTC–Cu(II) complexes have been reported to possess strong anticancer activities<sup>130–133</sup> with multiple identified potential mechanisms of action, including inhibition of the ubiquitin protein pathway, suppression of nuclear factor- $\kappa$ B, ROS generation, activation of the MAPK pathway, inhibition of superoxide dismutase activity, alterations in non-protein thiols and chemosensitization, in addition to irreversible inhibition of ALDH<sup>134–137</sup>. In this context, ALDH<sup>+</sup> cancer stem cell populations harbour strong self-renewal and tumour-initiating capabilities that develop resistance to chemotherapy and radiotherapy<sup>138–140</sup>. Preclinical studies revealed that the combination of DSF and Cu(II) selectively targets and kills these cancer stem cells, contributing to the inhibition of tumour recurrence<sup>138–140</sup>. As an FDA-approved drug, DSF has well-studied pharmacokinetics and an excellent safety profile. Clinical trials confirm the anticancer and/or chemosensitizing effects of DSF or Cu–DSF<sup>127,137</sup>, particularly in glioblastomas owing to the penetrance of DSF across the blood–brain barrier. A phase I clinical trial (NCT01907165) showed that DSF in combination with temozolomide had an acceptable safety profile and produced increased progression-free survival in patients with glioblastoma<sup>97</sup>, and a recent phase II study (NCT03034135) showed that addition of Cu–DSF to temozolomide for treatment of patients with otherwise-resistant glioblastoma appears well tolerated but requires further efforts to select responsive populations<sup>97</sup>.

Elesclomol is another copper-binding compound originally identified through a cell-based phenotypic screen for small molecules that enhance the antitumour activity of paclitaxel<sup>141</sup>. Elesclomol is a bis(thiohydrazide) amide compound that forms a 1:1 complex with Cu(II)<sup>142</sup>. Initial in vitro experiments showed that elesclomol strongly induced ROS in cancer cells, leading to unmitigated oxidative stress and cell death<sup>125</sup>. However, recent studies aimed at uncovering small-molecule vulnerabilities in the context of proteasome inhibitor-resistant multiple myelomas that exhibit elevated mitochondrial metabolism provided new mechanistic insights into elesclomol and DSF efficacy. Specifically, a high-throughput screen of small molecules in cancer cells with reduced 19S subunit expression or cultured in galactose to establish mitochondrial metabolism dependence revealed selective sensitivity to either elesclomol treatment or DSF treatment. Elesclomol generates ROS by selectively transporting Cu(II) from the extracellular environment into mitochondria, where it is reduced to Cu(I)<sup>143</sup>. Subsequent mechanistic studies that used a CRISPR–Cas9 deletion screen revealed that the mitochondrial protein ferredoxin 1 is responsible for reduction of elesclomol-bound Cu(II) to Cu(I) and cancer cell sensitivity to elesclomol<sup>126</sup>. Reaction of Cu(I) released in mitochondria with molecular oxygen is expected to yield superoxide that would dismutate to produce H<sub>2</sub>O<sub>2</sub>, which can further react with Cu(I) to generate the even more damaging and highly reactive hydroxyl radical<sup>120</sup>. Therefore, tumours relying on mitochondrial metabolism are likely to be particularly susceptible to elesclomol. Elesclomol in combination with paclitaxel has been evaluated in a number of clinical trials mostly targeting advanced-stage melanomas<sup>98,99,144</sup>. A phase I clinical trial showed that the combination of elesclomol and paclitaxel was well tolerated with a toxicity profile similar to that of paclitaxel alone<sup>144</sup>. A phase II study showed that addition of elesclomol to paclitaxel yielded a doubling of median progression-free survival, a 41.7% risk reduction for disease progression/death and increased overall survival rates<sup>98</sup>. Although in a large, randomized, double blind, phase III study of patients with chemotherapy-naïve advanced melanoma, elesclomol and paclitaxel combination therapy did not achieve its progression-free end point, a prospectively defined subgroup analysis revealed a statistically significant improvement in patients with normal

baseline levels of lactate dehydrogenase<sup>99</sup>. As increased lactate dehydrogenase activity is associated with tumour hypoxia, this finding is consistent with the requirement of active mitochondrial respiration for the action of elesclomol<sup>126</sup>. Taken together, the findings suggest that ionophore-mediated copper delivery to intracellular compartments could be a promising therapeutic strategy for a subset of tumours.

**Copper imaging and replacement.** Finally, in addition to chelation and ionophore therapies, the general propensity of tumours to accumulate elevated copper levels relative to adjacent tissue has been exploited to develop copper-based targeted anticancer agents. Of note, following this strategy, the radioisotope <sup>64</sup>Cu has been applied for *in vivo* tumour imaging and therapy (TABLE 2). With a half-life of 12.7 hours and decay characteristics that enable imaging by positron emission tomography (PET), <sup>64</sup>Cu shows significant promise for the detection and treatment of primary cancer and its metastases<sup>145</sup>. Unlike most conventional radiopharmaceuticals, which require complexation between a radioisotope and a targeting ligand, <sup>64</sup>Cu in the form of simple <sup>64</sup>CuCl<sub>2</sub> has theranostic potential as both a radiotracer for PET and a targeted radiotherapeutic agent<sup>145</sup>. PET of mouse cancer models after intravenous injection of <sup>64</sup>CuCl<sub>2</sub> has been successfully used to detect different types of tumours, including melanoma, breast cancer, prostate cancer, glioblastoma, colorectal cancer, ovarian cancer, lung cancer, and head and neck cancer<sup>146–149</sup>. At elevated doses, <sup>64</sup>CuCl<sub>2</sub> was shown to exhibit radiotherapeutic activity in various mouse models of cancer, including melanoma<sup>146</sup>, glioblastoma<sup>150</sup> and prostate cancer<sup>151</sup>. In humans, PET of <sup>64</sup>CuCl<sub>2</sub> has been used in the staging of prostate cancer<sup>152</sup>, and was recently found to outperform <sup>18</sup>F-labelled choline in detecting relapse of prostate cancer in patients with low levels of prostate-specific antigen<sup>153</sup>. In a study of patients with glioblastoma, PET of intravenously administered <sup>64</sup>CuCl<sub>2</sub> readily identified brain lesions with excellent agreement with standard diagnostic magnetic resonance images<sup>154</sup>. Importantly, <sup>64</sup>CuCl<sub>2</sub> may be able to detect metastatic brain lesions associated with other primary cancers, such as lung and breast cancer<sup>154</sup>.

An important number of copper-based compounds have been studied as experimental anticancer agents *in vitro*, and in some cases *in vivo*<sup>155–157</sup>. Thus, various families of Cu(I) and Cu(II) complexes have been designed and have shown promising

pharmacological effects against different cancer types<sup>155,158–161</sup>. While the mechanisms of cytotoxic action of these metallodrugs have not been fully elucidated and may also involve effects triggered by the Cu(I)/Cu(II) coordinating ligands, the results obtained point towards a multimodal spectrum of biological activity, involving intracellular redox reactions triggered by the metal centre.

Overall, the aforementioned examples of either copper chelators or copper-based compounds further demonstrate that the selective accumulation of copper by cancer cells has immense potential in nuclear medicine for the development of diagnostic and therapeutic interventions.

### Assessing clinical copper status

As therapeutics targeting copper and copper-dependent signalling pathways are developed and tested, a parallel need is to assess functional ‘copper status’ in patients to minimize side effects and any impact on essential biological processes and to determine whether therapeutic goals are reached. The potential for unintended adverse events and consequences of perturbing copper balance includes alterations in physiologic processes and metabolism that are dependent on patient age and/or the stage of development.

As clinical signs and symptoms of altered copper homeostasis often appear later than biochemical evidence for copper deficiency or excess, there is a need to establish best methods and practices to evaluate a patient’s ‘copper status’ to prevent clinically significant complications. Standard testing measures total copper concentration in the circulation (serum or plasma) and in the urine<sup>32</sup>. Less frequently performed is measurement of tissue copper content due to the more invasive nature of obtaining samples for measurement from organs. Indirect estimation of copper status can be accomplished by measurement of the activity of cuproenzymes as a reduction in enzymatic activity can occur in copper-depleted states. Indirect detection of copper depletion is possible on routine blood counts, because copper depletion is reflected in a decreased count of white blood cells (mainly neutrophils). Platelet counts may also be lower and a sideroblastic anaemia may occur. In copper-deficient states, ceruloplasmin may not acquire its full complement of copper. Due to the short plasma half-life of ceruloplasmin without copper (apoceruloplasmin), ceruloplasmin detection by immunoassay or by oxidase activity is lower in copper-deficient

states. In untreated patients with Wilson disease, there may be elevation of ‘non-ceruloplasmin-bound copper’ (NCC) deposition in other tissues, in particular in the liver and the central nervous system, where it causes tissue injury. Treatment goals for Wilson disease include reduction of NCC levels using copper chelation or with zinc, which indirectly inhibits copper absorption across the gut. Methods are in development that directly measure the copper in ceruloplasmin, permitting a more accurate determination of NCC that should help with treatment monitoring. Alternatively, administration of radiolabelled copper and measurement of its accumulation in tumours and/or secondary appearance in the circulation as radiolabelled ceruloplasmin offers another companion diagnostic approach. As new agents that target copper are developed for treatment of cancer and other disorders, determination of which biomarker of ‘copper status’ provides the most accurate and reliable therapeutic target will be needed in addition to its measurement for safety monitoring.

### Conclusions and future prospects

Mounting observations connect copper signalling to cell proliferation as well as tumour growth and metastasis in cancer. However, more foundational mechanistic information is needed to establish causal rather than correlative relationships and further link copper-dependent targets and pathways to copper-dependent disease vulnerabilities. In particular, technical approaches to measuring and manipulating the actions of copper within biological systems produce data that are often difficult to interpret and require continued development of new chemical probes to monitor labile copper, particularly in terms of subcellular resolution and oxidation state specificity, as well as the ability for comparable profiling of the total and labile copper status of different cell populations. Indeed, *in vivo* imaging of copper and other metals will help advance the field<sup>162–164</sup>. The diverse chemical properties of metals offer several features to consider in the design of metal-specific sensors, ionophores and chelators, including their abundance, binding affinities and complexes formed with other competing ligands in the cell. For detection, the emergence of activity-based sensing<sup>70,165</sup>, which relies on reactivity rather than recognition, can provide a complementary approach to binding-based approaches to achieve selectivity and sensitivity. Challenges remain in distinguishing between relevant oxidation



states of copper<sup>69,71</sup>, as well as achieving specificity for copper over more abundant alkali and alkaline earth metals and other transition metals in biological environments. In this regard, thioethers<sup>72,166</sup> and phosphine sulfides<sup>167</sup> offer privileged ligand donors for copper-selective binders.

Likewise, the discovery and characterization of the first examples of metalloallostery, as shown by copper as a negative allosteric regulator of PDE3B and a positive allosteric regulator of MEK1, MEK2, the kinases ULK1 and ULK2 and the E2 ligase UBE2D2, provide motivation for studies to provide a more comprehensive picture of the metalloproteome and copper-sensitive signalling targets, as well as basic biochemical mechanisms of how allosteric metal binding regulates protein activity and function. Indeed, nutrient sensing of copper and the intersection between copper and cancer metabolism warrant further studies in various aspects of cancer biology, including tumour initiation, growth and metastasis, particularly in stem cell niches and inflammatory response. Bringing to bear advanced sequencing, proteomics, metabolomics and other analyses will uncover new metallobiology relevant to cancer and other diseases.

In terms of pharmacology, increasing the specificity of therapeutics to target cuproplasia is an important next challenge to address. The possibility to directly target the copper homeostasis machinery, as illustrated by lead candidates that block the copper metallochaperone machinery<sup>168,169</sup>, or leverage gene therapy as has been shown in treatment of genetic disorders of copper dysregulation such as Menkes disease<sup>16,170</sup> show promises in this regard. Delivery of therapeutic agents to specific cell populations and even subcellular compartments (for example, by targeted ionophore metal supplementation for liver-specific copper delivery<sup>171</sup> and lipid nanoformulations for the delivery of Cu(II) complexes to the tumour site<sup>172,173</sup>), organelle-specific copper depletion (for example, with nanoparticles for lowering mitochondrial copper levels<sup>164</sup>) and biochemically and environmentally sensitive reagents to uncage copper-sensitive reagents<sup>174</sup> offer but a few strategies to increase specificity and reduce off-target effects. Indeed, the data show that both depleting copper and supplementing copper to toxic levels are viable strategies to block cuproplasia pathways.

In parallel to developing new therapeutic strategies, dissecting the metal and

metalloproteome landscape may help stratify patients in clinical trials and predict response to targeted therapy. Clinical trials point to the promise of copper depletion on the metastatic microenvironment by rendering the 'soil' of distant tissues less congenial to 'seeds' of primary tumours. Also, new ways to reliably measure total copper inside cells and in plasma and/or bioavailable, labile forms of this metal (for example, non-ceruloplasmin pools in serum), along with identifying and validating new copper-dependent biomarkers, will give rise to companion diagnostics that can identify populations most amenable to copper-responsive therapies. Cuproplasia offers a new lens to view an ancient vulnerability that cancer is, and efforts to target copper-dependent signalling and cell proliferation have the potential to change the natural history of cancer and other diseases. We predict that cuproplasia is the first example of a broader range of potential metalloplasias and provides a starting point to join basic and translational efforts for connecting metal signalling, metabolism and disease.

Eva J. Ge<sup>1</sup>, Ashley I. Bush<sup>2</sup>, Angela Casini<sup>3</sup>, Paul A. Cobine<sup>4</sup>, Justin R. Cross<sup>5</sup>, Gina M. DeNicola<sup>7</sup>, Q. Ping Dou<sup>8,9,10,11</sup>, Katherine J. Franz<sup>12</sup>, Vishal M. Gohil<sup>13</sup>, Sanjeev Gupta<sup>14,15,16,17,18,19</sup>, Stephen G. Kaler<sup>20</sup>, Svetlana Lutsenko<sup>21</sup>, Vivek Mittal<sup>22,23</sup>, Michael J. Petris<sup>24,25,26,27</sup>, Roman Polishchuk<sup>28</sup>, Martina Ralle<sup>29</sup>, Michael L. Schilsky<sup>30</sup>, Nicholas K. Tonks<sup>31</sup>, Linda T. Vahdat<sup>32</sup>, Linda Van Aelst<sup>31</sup>, Dan Xi<sup>33</sup>, Peng Yuan<sup>34,35</sup>, Donita C. Brady<sup>36,37,38</sup> and Christopher J. Chang<sup>1,2</sup>

<sup>1</sup>Department of Chemistry, University of California, Berkeley, Berkeley, CA, USA.

<sup>2</sup>Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, USA.

<sup>3</sup>The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, Parkville, VIC, Australia.

<sup>4</sup>Chair of Medicinal and Bioinorganic Chemistry, Department of Chemistry, Technical University of Munich, Garching, Germany.

<sup>5</sup>Department of Biological Sciences, Auburn University, Auburn, AL, USA.

<sup>6</sup>Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center, New York, NY, USA.

<sup>7</sup>Department of Cancer Physiology, H. Lee Moffitt Cancer Center, Tampa, FL, USA.

<sup>8</sup>Department of Oncology, School of Medicine, Wayne State University, Detroit, MI, USA.

<sup>9</sup>Department of Pharmacology, School of Medicine, Wayne State University, Detroit, MI, USA.

<sup>10</sup>Department of Pathology, School of Medicine, Wayne State University, Detroit, MI, USA.

<sup>11</sup>Barbara Ann Karmanos Cancer Institute, Wayne State University, Detroit, MI, USA.

<sup>12</sup>Department of Chemistry, Duke University, Durham, NC, USA.

<sup>13</sup>Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, USA.

<sup>14</sup>Department of Medicine, Albert Einstein College of Medicine, New York, NY, USA.

<sup>15</sup>Department of Pathology, Albert Einstein College of Medicine, New York, NY, USA.

<sup>16</sup>Marion Bessin Liver Research Center, Albert Einstein College of Medicine, New York, NY, USA.

<sup>17</sup>Diabetes Research Center, Albert Einstein College of Medicine, New York, NY, USA.

<sup>18</sup>Irwin S. and Sylvia Chanin Institute for Cancer Research, Albert Einstein College of Medicine, New York, NY, USA.

<sup>19</sup>Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine, New York, NY, USA.

<sup>20</sup>Center for Gene Therapy, Abigail Waxner Research Institute, Nationwide Children's Hospital, Columbus, OH, USA.

<sup>21</sup>Department of Physiology, Johns Hopkins Medical Institutes, Baltimore, MD, USA.

<sup>22</sup>Department of Cardiothoracic Surgery, Neuberger Berman Foundation Lung Cancer Research Center, Weill Cornell Medicine, New York, NY, USA.

<sup>23</sup>Department of Cell and Developmental Biology, Weill Cornell Medicine, New York, NY, USA.

<sup>24</sup>Department of Biochemistry, University of Missouri, Columbia, MO, USA.

<sup>25</sup>Department of Ophthalmology, University of Missouri, Columbia, MO, USA.

<sup>26</sup>Genetics Area Program, University of Missouri, Columbia, MO, USA.

<sup>27</sup>Christopher S. Bond Life Science Center, University of Missouri, Columbia, MO, USA.

<sup>28</sup>Telethon Institute of Genetics and Medicine, Pozzuoli, Italy.

<sup>29</sup>Department of Molecular and Medical Genetics, Oregon Health and Sciences University, Portland, OR, USA.

<sup>30</sup>Section of Transplantation and Immunology, Division of Digestive Diseases, Department of Medicine and Surgery, Yale University Medical Center, New Haven, CT, USA.

<sup>31</sup>Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

<sup>32</sup>Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA.

<sup>33</sup>National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.

<sup>34</sup>Center for the Investigation of Membrane Excitability Diseases, Washington University School of Medicine, St Louis, MI, USA.

<sup>35</sup>Department of Cell Biology and Physiology, Washington University School of Medicine, St Louis, MI, USA.

<sup>36</sup>Department of Cancer Biology, University of Pennsylvania, Philadelphia, PA, USA.

<sup>37</sup>Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA, USA.

<sup>38</sup>Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

<sup>✉</sup>e-mail: bradyd@penmedicine.upenn.edu; chrischang@berkeley.edu

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#### Author contributions

All authors contributed to discussion of the content. C.J.C. outlined the article. C.J.C., E.J.G., M.J.P., D.C.B., V.M.G., Q.P.D., L.T.V., S.G.K., A.I.B., A.C. and M.L.S. wrote portions of the article. E.J.G., D.C.B. and C.J.C. reviewed and edited the article before submission. C.J.C. and D.C.B. designed the figures.

#### Competing interests

N.K.T. is a member of the Scientific Advisory Board of DepYmed Inc. V.M.G. is listed as an inventor on the patent application PCT/US2019/041571 submitted by Texas A&M University entitled "Compositions for the treatment of copper deficiency and methods of use". D.C.B. holds ownership in Merlon Inc. A.I.B. holds equity in Alterity Biotechnology Ltd, Cogstate Ltd, Mesoblast Ltd and Collaborative Medicinal Development LLC and is a paid consultant for Collaborative Medicinal Development Pty Ltd. L.T.V. is a consultant for Berg Pharma, Osmol Therapeutics and Sema4, serves on the advisory board of Seattle Genetics and Immunomedics/Gilead, and receives research funding from Genentech, Arvinas, and Oncotherapy Sciences. E.J.G., A.C., P.A.C., J.R.C., G.M.D., Q.P.D., K.J.F., S.G., S.G.K., S.L., V.M., M.J.P., R.P., M.R., M.L.S., L.V.A., D.X., P.Y. and C.J.C. declare no competing interests.

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