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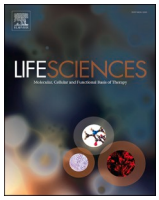

A spotlight on the role of copper in the epithelial to mesenchymal transition

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ABSTRACT

The complex process known as epithelial to mesenchymal transition (EMT) plays a fundamental role in several biological settings, encompassing embryonic development, wound healing, and pathological conditions such as cancer and fibrosis. In recent years, a bulk of research has brought to light the key role of copper, a trace element with essential functions in cellular metabolism, cancer initiation and progression. Indeed, copper, besides functioning as cofactor of enzymes required for essential cellular processes, such as energy production and oxidation reactions, has emerged as an allosteric regulator of kinases whose activity is required to fulfill cancer dissemination through the EMT. In this comprehensive review, we try to describe the intricate relationship between the transition metal copper and EMT, spanning from the earliest foundational studies to the latest advancements. Our aim is to shed light on the multifaceted roles undertaken by copper in EMT in cancer and to unveil the diverse mechanisms by which copper homeostasis exerts its influence over EMT regulators, signaling pathways, cell metabolic reprogramming and transcription factors ultimately contributing to the spread of cancer. Therefore, this review not only may contribute to a deeper comprehension of copper-mediated mechanisms in EMT but also supports the hypothesis that targeting copper may contribute to counteract the progression of EMT-associated pathologies.

1. EMT: an overview

EMT is a dynamic and finely regulated biological process in which, following the rewiring of cell gene expression, polarized epithelial cells assume a mesenchymal-like phenotype. It can be subdivided into three types, depending on its role in different biological contexts.

Type I EMT occurs during the embryo implantation into the uterus, during gastrulation [1]. In this context, the fulfillment of EMT leads to the formation of mesoderm and endoderm from ectoderm [2] (Fig. 1A) promoting the embryo transformation from a single layer to three germ layers. Thus, mesoderm and ectoderm participate in the generation of many tissues of the adult organism and in turn undergo several rounds of EMT and mesenchymal to epithelial transition (MET) [1]. Furthermore, Type I EMT contributes to vertebrate nervous system development allowing the detachment of neural crest cells from the neuroepithelium prompting their subsequent migration to form sensory and autonomic neurons, neuroendocrine cells, glia, and melanocytes [3,4]. Type I EMT is also required for heart development, where it participates in the creation of cardiac valves and various cardiac cell types [5] (Fig. 1B).

Type II EMT is a physiological process, required for tissue repair and regeneration in wound healing, but it can led to pathological conditions, such as fibrosis [6,7]. Wound healing is a dynamic process organized into four stages: i) hemostasis (activated immediately upon tissue damage); ii) inflammation (including the release of inflammatory cytokynes such as IL6 and TNFα); iii) cell proliferation (epithelial cells proliferate and migrate over the matrix within the wound, implying the activation of Type II EMT cascade) and iv) tissue remodelling [8,9] (Fig. 1C). Fibrosis is a pathological evolution of wound healing, characterized by a disproportionate replacement of parenchymal tissue with connective tissue leading to a significant remodelling of the surrounding environment resulting in excessive scarring. In this context, epithelial, mesothelial and endothelial cells, as well as macrophages, pericytes, adipocytes, bone marrow precursor tissue and resident fibroblasts undergo a Type II EMT generating myofibroblasts [7]. In fibrotic tissues, myofibroblasts accumulate and excessively secrete collagen, culminating in the formation of fibrous tissue. This process compromises organ function and can eventually lead to organ failure [10] (Fig. 1C).

Type III EMT occurs during tumor spreading, prompting the acquisition of mesenchymal traits by epithelial cancer cells. This enables them to dissociate from the primary tumor site, invade adjacent tissues, enter

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the bloodstream, and ultimately establish secondary tumors in distant organs where the mesenchymal to epithelial transition (MET) takes place [6]. Thus, this fine-tuned process has profound clinical implications, as it underlies the dissemination of cancer cells contributing not only to metastasis formation, but also favoring the acquisition of resistance to treatment and resulting in poor prognosis (Fig. 1D).

1.1. Conditions prompting EMT activation in cancer

In the field of cancer research the role of EMT is subject of extensive interest. The activation of EMT often occurs at the invasive tumor front, as observed in colorectal (CRC) [11] and breast cancer [12]. Its fulfillment results in the loss of desmosomes, adherens, tight and gap junctions, due to the downregulation of epithelial proteins such as E-cadherin, occludin and EpCam, and the acquisition of mesenchymal markers (*e.g.*, vimentin, N-cadherin and fibronectin) and the subsequent

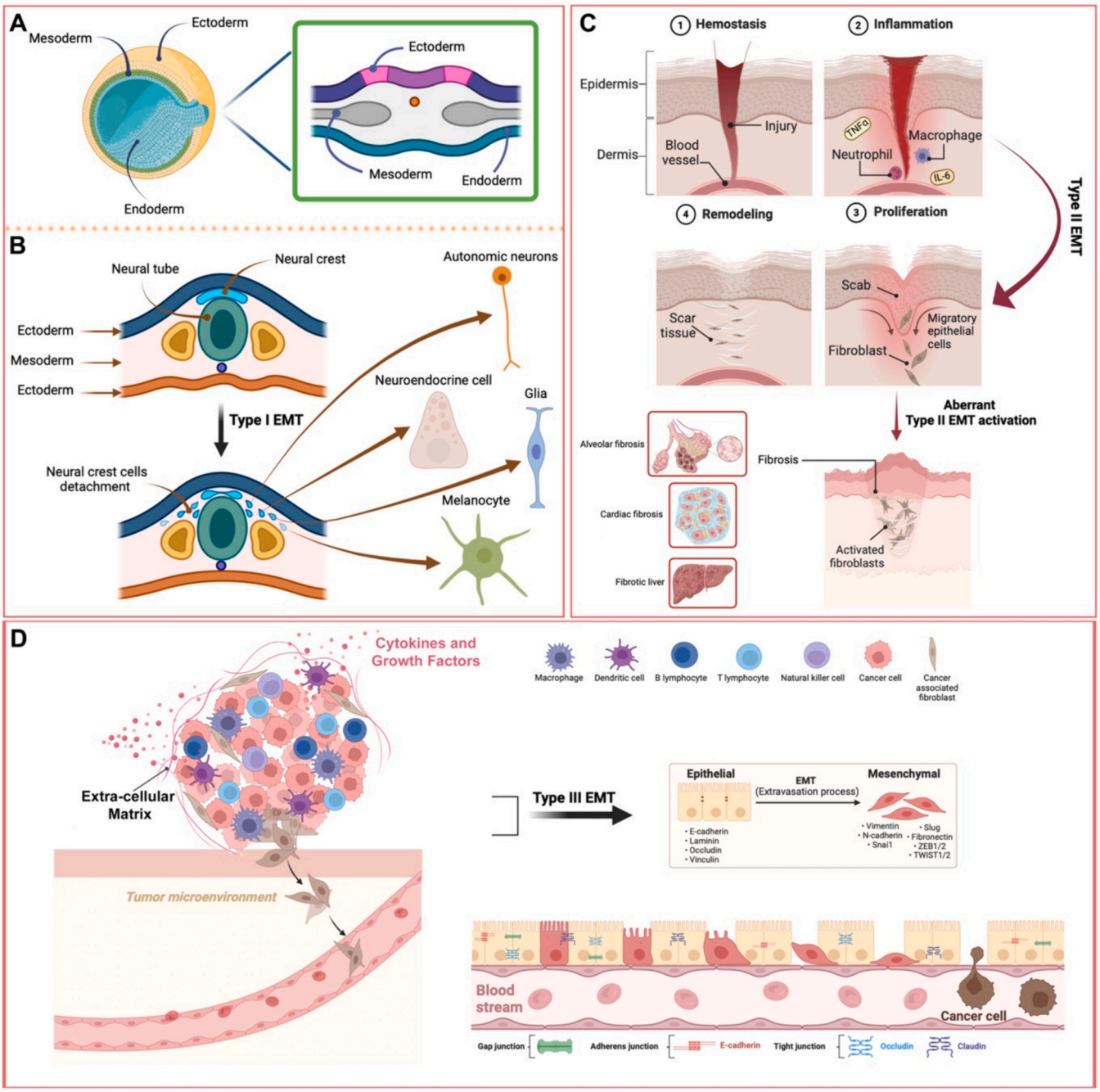

Fig. 1. Different subtypes of EMT. Type I EMT is a physiological process required during (A) gastrulation, promoting the formation of mesoderm and endoderm from ectoderm, and for (B) the nervous system development inducing the detachment of the neural crest cells from the neuroepithelium and their subsequent migration to form neurons, neuroendocrine and glia cells, and melanocyte. Similarly, Type II EMT (C) is a physiological process involved in wound healing, but its aberrant activation underlies pathological processes that lead to the formation of fibrotic tissue. On the contrary, type III EMT (D) is a mechanism occurring exclusively under pathological conditions. Its activation is favored by the tumor microenvironment through the secretion of proinflammatory cytokines and growth factors. It results in the acquisition of mesenchymal features from the epithelial tumor cells allowing them the detach from the primary tumor mass, degrade the extracellular matrix prompting their entry into the bloodstream, to eventually colonize distant organs. Created in BioRender.com.

increased metastatic potential of cancer cells [13–15] (Fig. 1D). In particular, it proceeds upon engagement of several receptors [*i.e.*, tyrosine kinase receptors (TKRs), TGF-β receptor I and II (TGFBRI/II), Notch, Frizzled receptors] by their soluble ligands, which includes growth factors (FGF, EGF, VEGF and PDGF), inflammatory cytokines (TGFβ and TNF-α) and interleukins (IL-1, IL-6 and IL-8), Wnt, Bone Morphogenetic Protein (BMP) and integrins [16].

The tumor microenvironment is crucial for EMT induction, orchestrating the interactions between immune cells, fibroblasts, and the extracellular matrix and the secretion of signaling molecules [6,17]. In breast cancer, the tumor microenvironment, particularly cancer-associated fibroblasts (CAFs) and immune cells, secretes factors like TGFβ, TNF-α, and IL-6 [18] that can induce EMT in neighbouring epithelial cancer cells [19]. In pancreatic cancer, the dense stroma, characteristic of this tumor, is involved in EMT activation. Pancreatic stellate cells and immune cells secrete factors like TGF-β and connective tissue growth factor (CTGF), driving EMT.

The induction of EMT may be promoted also by the acquisition of genetic alterations such as mutations in key regulatory genes like TP53 which destabilize cellular equilibrium and enhance cellular adaptability [20]. In particular, in the context of EMT, it has been found that mutated TP53 loses the ability to form a complex with the ubiquitin ligase Mdm2 leading to the stabilization of the EMT transcription factors (EMT-TFs) [21].

Oncogenic mutations affecting RAS and Myc can influence EMT through the modulation of downstream signaling pathways [22,23]. Indeed, in the pancreatic cancer cell line Panc-1 the silencing of RAS resulted in the impairment of EMT induction upon TGFβ treatment. In contrast, in the cervical carcinoma cells HeLa, the transfection of constitutively active RAS leads to the TGFβ mediated induction of EMT [24]. These findings suggested that mutations activating RAS collaborate with the TGFβ signaling pathway to promote EMT, contributing to tumor progression and metastasis.

Another level of EMT modulation is achieved thanks to the activity of various microRNA (miRNAs). The contribute of miRNAs in the modulation of EMT has been demonstrated in gliomas [25,26], in CRC [27,28] and in metastatic breast cancer [27].

Hypoxia is another process triggering alterations that support EMT in cancerous tissues [29]. Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor regulating the transcription of genes required for fundamental biological processes, including glucose metabolism, cell proliferation, migration and angiogenesis. In the tumor tissues, characterized by aberrantly proliferating cells, oxygen balance is highly impaired, and the core of solid tumors become hypoxic. Besides promoting cancer cells adaptation to low oxygen environment by inducing the shift of cancer cell metabolism towards the glycolytic pathway (the Warburg effect) and neo-angiogenes [30], HIF-1α propels EMT by binding to the promoter regions of the EMT-TFs ZEB1 [31] and SNAI1 [32], amplifying their expression and transcriptional capabilities.

1.2. Signaling cascades orchestrating EMT (canonical and non-canonical pathways)

The TGFβ signaling pathway is the main EMT inducer in cancer. This pro-inflammatory cytokine is produced by the tumor cells or by cells resident in the tumor microenvironment, such as stromal cells, macrophages and platelets [33]. TGFβ signaling proceeds through the formation of a heterotetrameric complex with its type I and type II serine/threonine kinase receptors (TGFβRI/TGFβRII). Upon cytokine binding, the TGFβRII phosphorylates and activates TGFβRI [34] initiating disjointed signaling cascades, so called "canonical" and "not-canonical", culminating in transcriptional changes promoting EMT [35].

The "canonical" pathway involves the Smad family members: Smad2 and Smad3 undergo phosphorylation and subsequently form a a ternary complex with Smad4. The Smad complex translocates into the nucleus, where it activates the EMT-TFs SNAI1, SLUG, TWIST1/2 and ZEB1/2 [14,36] (Fig. 2A).

TGFβ activates also "non-canonical" EMT pathways, involving the kinases RAS/RAF/MEK/ERK [37]. This activation may occur upon engagement of TKRs (Tyrosine Kinase Receptors) by growth factors triggering the phosphorylation of the adaptor Shc, which in turn interacts with Grb2 (Growth Factor Receptor-bound protein 2). The Shc/Grb2 complex recruits Sos (Son of sevenless) leading to RAS activation and the downstream ERK signaling [38]. ERK1/2 are serine-threonine kinases activated upon phosphorylation by MEK1/2. Phosphorylated ERK translocates into the nucleus, where it modulates gene expression and regulates various transcription factors [38]. Dysregulation of the RAF/MEK/ERK pathway is closely associated with oncogenesis, contributing to tumor proliferation, survival, invasion, metastasis, extracellular matrix degradation, and angiogenesis [39]. Mutations in components of this pathway are frequently observed in various cancer types. The mutation of RAF, particularly BRAF, assumes significant clinical importance. The $BRAF^{V600E}$ mutation is commonly found in melanoma, papillary thyroid carcinoma, CRC, lung and ovarian cancer [40]. These mutations lead to the constitutive activation of the signaling cascade, promoting oncogenesis and leading to chemoresistance (Fig. 2A) [41].

The main "non-canonical" pathway involved in the induction of EMT is the PI3K/Akt/mTOR cascade that is activated both by TGFβ, upon engagement of a TKR, or G-protein-coupled receptors (GPCR), which triggers the recruitment and activation by phosphorylation of class I Phosphatidylinositol 3-kinase (PI3K) [14,36,37]. Upon PI3K activation, two crucial residues on AKT are phosphorylated: Thr308, within the activation loop of the kinase core, and Ser473, located in a C-terminal hydrophobic motif. The Thr308 residue is phosphorylated by the phosphoinositide-dependent protein kinase 1 (PDK1) [42,43], while the phosphorylation of the Ser473 residue, which stabilizes Thr308 phosphorylation finalizing the activation of AKT, is performed by mTORC2 [44]. Once activated, AKT phosphorylates downstream substrates including glycogen synthase kinase-3β (GSK3β) and forkhead box O (FOXO) transcription factor [45]. Additionally, once PI3K is activated, it phosphorylates cell membrane lipids, generating phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3, in turn, operates through a positive feedback mechanism, further activating Akt (Fig. 2A). The PI3K/Akt/mTOR axis is frequently mutated and hyperactivated in cancer. Mutations in this pathway are reported in several types of cancers [46].

Another "non-canonical" EMT signaling pathway activated by TGFβ is the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway [14,37]. This cascade is induced also by other cytokines, such as IL-6, IFN-α and IFN-γ, and involves JAK family kinases (JAK1, JAK2, JAK3, and TYK2) and STAT transcription factors (STAT1, STAT3, STAT5, and others) [47]. The role of IL-6/JAK/STAT3 pathway in EMT has been investigated in various cancer types, including prostate, breast and pancreatic cancer [48] (Fig. 2B).

The Wnt/β-catenin pathway also falls in the "non canonical" EMT signaling cascade, which proceeds alongside TGFb. It has been shown that Wnt levels increase during cancer progression conferring to this network tumor-promoting properties [49]. The activation of this pathway, results in the disruption of the β-catenin destruction tertiary complex leading to the cytoplasmic accumulation and stabilization of β-catenin. Afterwards, β-catenin translocates into the nucleus, where it modulates the expression of EMT-related genes [49]. The ability of Wnt/β-catenin pathway to drive transcriptional changes promoting EMT has been demonstrated in breast cancer [50] and in CRC cells [51] (Fig. 2B).

Both the PI3K/Akt and the Wnt/β-catenin pathways converged to GSK3β, a serine-threonine kinase, whose activity is modulated by phosphorylation and dephosphorylation events [52,53]. In particular, the Wnt/b-catenin and PI3K/Akt cascades phosphorylate and then suppress GSK3β activity. Thus, dysregulation of these pathways, due to inactivating mutations or aberrant activation, can lead to the inactivation of GSK3β, which in turn results in the activation of the EMT-TF SNAI1 [54].

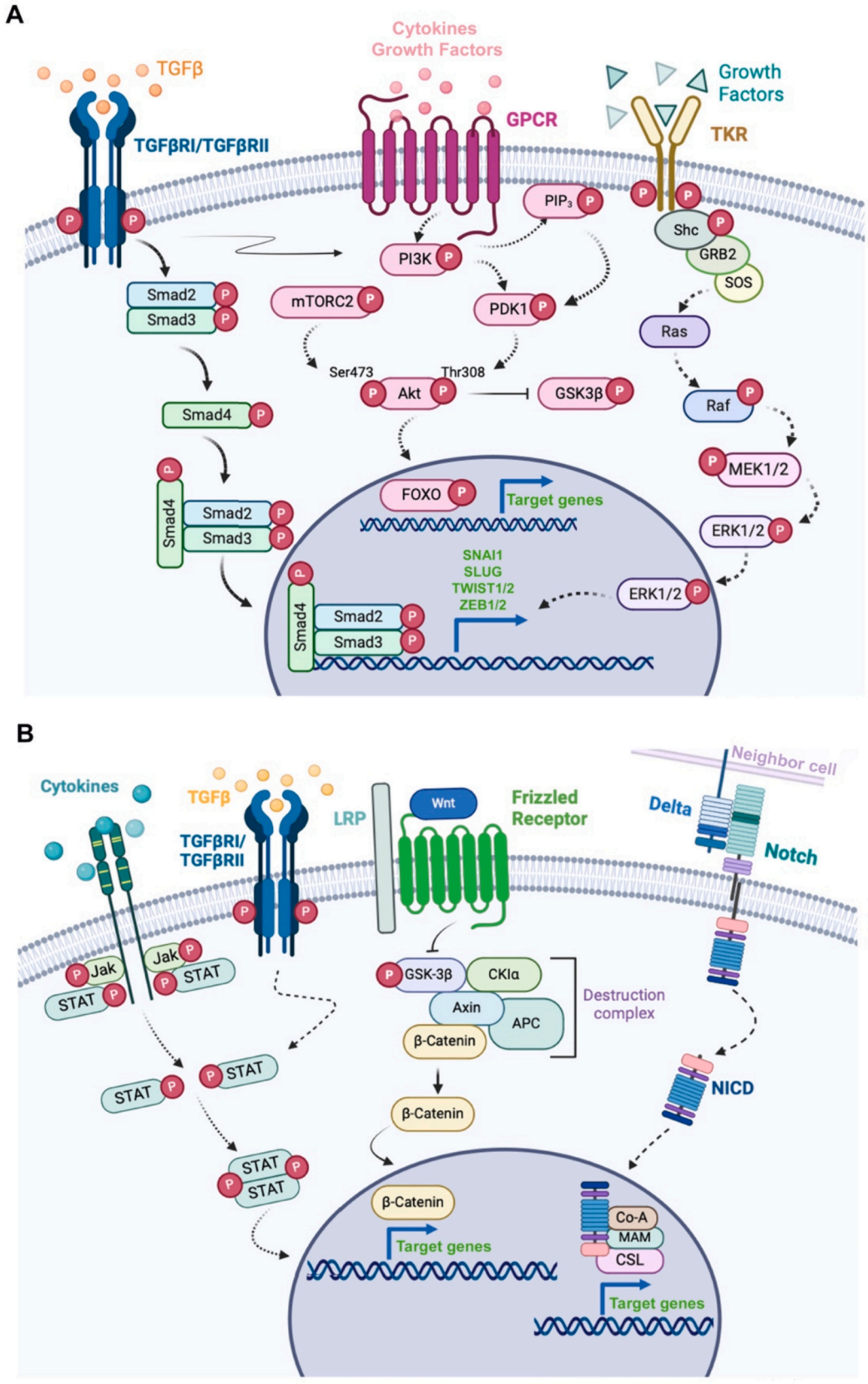

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Fig. 2. Signaling cascades activating EMT, canonical and non-canonical pathways. (A) The canonical TGFb pathway proceeds through engagement of the TGFbRI/II and subsequent activation, through phosphorylation, of the receptor dimer, which in turn recruits and activates SMAD2/3. SMAD2 and 3 form a ternary complex with SMAD4 that translocates into the cell nucleus promoting the activation of the EMT transcription factors (EMT-TFs) SNAI1, SLUG, TWIST1/2 and ZEB1/2. TGFb could also activate the G protein-coupled recetor (GPCR) leading to the phosphorylation of Akt, the inhibition through phosphorylation of GSK3b activity and the translocation in the cell nucleus of the phosphorylated transcription factor FOXO. The activation of tyrosine kinase receptors (TKRs) by growth factors, including TGFb, results in the initiation of the mitogen activated protein kinases (MAPK) cascade of RAS/RAF/MEK/ERK that concludes with the translocation of phosphorylated ERK into the cell nucleus where it modulates the transcription of EMT-related genes. (B) Another "non canonical" network activated by TGFb, together with proinflammatory cytokines, is the JAK/STAT signaling that results in the translocation of phosphorylated STAT dimers acting as transcription factor activating also EMT-related genes. The Wnt/b-catenin cascade is classified as a "non-canonical" EMT pathway not activated by TGFb. The engagement of the extra-cellular N-terminal cysteine-rich domains of the Frizzled G-protein-coupled receptor, following the binding of the cysteine-rich glycoproteins of the Wnt family, lead to the binding of the receptor to its co-receptor: the low-density-lipoprotein-receptor-related-protein (LRP). Subsequently, GSK3b is inhibited through phosphorylation leading to the disruption of the β-catenin destruction tertiary complex, composed of axin, APC (Adenomatous Polyposis Coli), CK1α, and GSK3β. As a result, β-catenin is stabilized and accumulates in the cytoplasm. β-catenin then translocates into the nucleus, where it also modulates the expression of EMT-related genes. Also the Notch signaling does not require TGFb for its activation and it is included in the "non canonical" EMT networks. Notch receptors interact with Delta and Serrate/Jagged family ligands, resulting in the cleavage and nuclear translocation of the Notch Intracellular Domain (NICD). NICD moves in the cell nucleus activating the transcription of target genes by displacing the co-repressor complex of the SLC transcription factor and mediating the recruitment of co-activators like (Co-A), mastermind proteins (MAML 1–3) and CREB-binding protein (CBP)/p300. Created in BioRender.com.

Aberrant Notch signaling is often associated with EMT and cancer metastasis and belongs to the "non canonical" signaling cascade inducing EMT (Fig. 2B) [55]. Notably, some of the target genes of this cascade, including c-myc, cyclinD1, and p21/Waf1, have been implicated in cancer pathogenesis [56].

Overall, the complex regulation of EMT becomes even more evident when we consider the interplay between different signaling pathways, underscoring the multifaceted control of EMT. This includes interactions between Notch-mediated signaling, Wnt, ERK, TGF-β, PI3K, and hypoxia signaling that collectively contribute to the regulation of EMT, playing crucial roles in cancer progression [57,58].

1.3. EMT transcription factors: roles and redundancies

The ultimate goal of the activation of the above discussed transduction pathways is the modulation of the activity of the EMT-TFs SNAI1, SLUG, TWIST1/2 and ZEB1/2, among others (Fig. 2A) [59].

The SNAIl family of EMT-TFs comprises SNAI1, SLUG, and SNAI3, which act as transcriptional repressors. SNAI1 and SLUG, drive EMT by inhibiting the expression of the gene coding for *E*-cadherin (*CDH1*), through the direct binding to *CDH1* promoter [60,61], or by reducing the expression of the epithelial markers EpCAM and KRT18 [62]. In addition to the *CDH1* gene, SNAI1 has been shown to bind other EMT-related genes, such as *OCLN*, the gene coding for occludin [63].

Another group of EMT-TFs is that of the basic helix-loop-helix (bHLH) transcription factors which includes TWIST1/2 (Fig. 2B). These proteins bind to E-box DNA responsive elements, inducing EMT and promoting cancer metastasis [64]. TWIST1/2 activation leads to the downregulation of epithelial markers, such as E-cadherin [65], while promoting the expression of mesenchymal markers like N-cadherin, vimentin, and fibronectin [66]. Moreover, in circulating tumor cells (CTCs) it has been found that TWIST1-mediated changes may confer resistance to anoikis, a form of cell death triggered by loss of anchorage, and enable immune evasion strategies [67].

The ZEB family of EMT-TFs includes ZEB1 and ZEB2, zinc finger TFs that actively repress epithelial cell markers (*E*-cadherin and EpCAM) while activating mesenchymal biomarkers (N-cadherin and vimentin), thereby driving EMT [68]. Their expression is associated with poor clinical outcomes and survival in various cancer types [69,70]. Overexpression of SNAI1, and TWIST1/2 in human mammary epithelial cells induces EMT and in turn increases ZEB1 levels, suggesting that ZEB1 acts downstream of these transcription factors [71]. Additionally, in melanoma cells SLUG directly controls ZEB1 transcription by binding to E-box fragments in the ZEB1 promoter [72].

Several studies have suggested the presence of redundant functions within the family of homologous EMT-TF members. Chen et al. conducted a comprehensive investigation into the dynamics of SNAI1 and SLUG expression during the differentiation process of the ATDC5 cell line, a well-established model for chondrogenic differentiation in tissue culture [73]. They found that during ATDC5 cells differentiation, both the mRNA and protein expression levels of SNAI1 and SLUG exhibited a notable increase. This simultaneous upregulation of both genes during chondrogenic differentiation strongly suggests a functional overlap or compensatory role between them in this specific context. On the other hand, other studies have highlighted non-redundant roles for specific EMT-TFs. Specifically ZEB1 and ZEB2 exerted different roles in melanocytes homeostasis and during melanoma progression [74]. ZEB2 favors the expression of MITF (Microphthalmia-associated transcription factor), which is essential for melanocytes differentiation, growth, and migration [75]. In contrast, ZEB1 becomes relevant when MITF levels are reduced, potentially promoting the survival and progression of melanoma cells in such circumstances [74].

1.4. The importance of the hybrid epithelial/mesenchymal phenotype of cancer cells

One of the most noteworthy recent developments that has further complicated the study of the role of EMT in cancer, is the discovery of the significant role played by the hybrid epithelial/mesenchymal phenotype (E/M phenotype). This phenomenon, also known as a partial, intermediate, or incomplete EMT phenotype, consists of the presence of tumor cells that maintain the expression of epithelial markers while simultaneously acquiring mesenchymal traits. This situation contrasts with the complete fulfillment of EMT, which involves the total loss of epithelial markers in favor of the exclusive gaining of mesenchymal hallmarks by tumor cells. Kisoda and colleagues [76] examined a range of head and neck squamous cell carcinoma (HNSCC) cell lines, each representing unique facets of EMT. Notably, they demonstrated that partial EMT phenotypes exhibited a remarkable capacity for invasion and migration, suggesting that the hybrid E/M phenotype could be identified as critical driver of cancer aggressiveness. The same evidence has been obtained in breast cancer. Indeed, it has been shown that tumorigenicity depends on individual cells residing in this E/M hybrid state and cannot be replicated by mixing two cell populations that stably remain at the extremes of the spectrum, *i.e.*, in the purely E or M states [77]. Luond and coworkers settled a protocol to trace EMT during tumor progression, either partial or full, in a transgenic mouse model of metastatic breast cancer. They found that in primary tumors cancer cells rarely underwent full EMT but rather showed partial EMT [78,79]. Additionally, they demonstrated that the partial EMT was the main phenotype contributing to the development of lung metastases and chemoresistance. In contrast, the fully mesenchymal cancer cells lose their ability to colonize distant organs. This evidence has been further corroborated by a recent paper from Saini et al. In this work, through a sophisticated single-cell RNA sequencing analysis, the authors demonstrated that the attainment of a fully mesenchymal state by tumor cells

results in the reduction of their tumorigenic capacity [80]. Even in melanoma it has been found tha the heterogeneity of the E/M phenotype of cancer cells drives metastasis. In particular, it has been described a biphasic progression of melanoma: at first, melanoma cells maintain their epithelial features and simultaneously up-regulate their mesenchymal traits, but subsequently the levels of their mesenchymal markers tend to decline following a further de-differentiation of the cells. This type of behaviour reflects also the kinetic of melanocyte development [81].

2. Copper: cell uptake and intracellular distribution

Copper is an essential transition metal as it is a cofactor for enzymatic processes critical for cell survival, such as mitochondrial respiration and oxidation reactions. However, due to its high redox potential, its intracellular homeostasis is finely regulated [82]. Copper (CuII), upon reduction catalyzed by a family of metalloreductase (STEAP1-6) [83], is internalized into cells through the high affinity Copper Transporter 1 (CTR1) [84]. As demonstrated by the embryonic lethality resulting from its deletion [84], CTR1-mediated copper uptake is essential for life, and investigations have disclosed elevated intracellular copper levels in various tumors characterized by heightened CTR-1 expression [85]. Remarkably, exposure to elevated copper levels triggers the internalization and subsequent degradation of CTR1, establishing a feedback loop that negatively modulates the CTR1–copper axis [86]. This mechanism prevents the re-localization of internalized CTR1 to the plasma membrane, effectively curbing excessive copper uptake and potential toxicity [87,88]. Retromer-mediated CTR1 endocytosis enables the elimination of copper cytotoxicity in response to heightened extracellular copper levels. Conversely, when extracellular copper levels decrease, CTR1 undergoes recycling back to the cell membrane with the assistance of the retromer protein [89] (Fig. 3).

Together with CTR1, it has been demonstrated that the CRISPR-Cas9 knockout of the divalent metal transporter 1 (DMT1) influences copper distribution in the NSCLC cell line H1299 [90] (Fig. 3). The study unveiled significant changes in cytosolic and mitochondrial copper levels, providing insights into the complex interplay between these transporters in the modulation of the intracellular copper homeostasis. Cytosolic copper levels significantly decreased, with CTR1KO cells exhibiting higher copper levels than DMT1KO cells, indicating DMT1's compensatory role in the absence of CTR1 function [91]. Notably, mitochondrial copper concentration remained relatively stable, suggesting DMT1's potential involvement at the outer membrane in facilitating copper transport from the cytosol to the mitochondrial intermembrane space [92].

As previously mentioned, unbound copper is endowed with a potent redox reactivity and could participate in the Fenton and Haber–Weiss reactions alongside hydrogen peroxide (H_2O_2) and superoxide anions

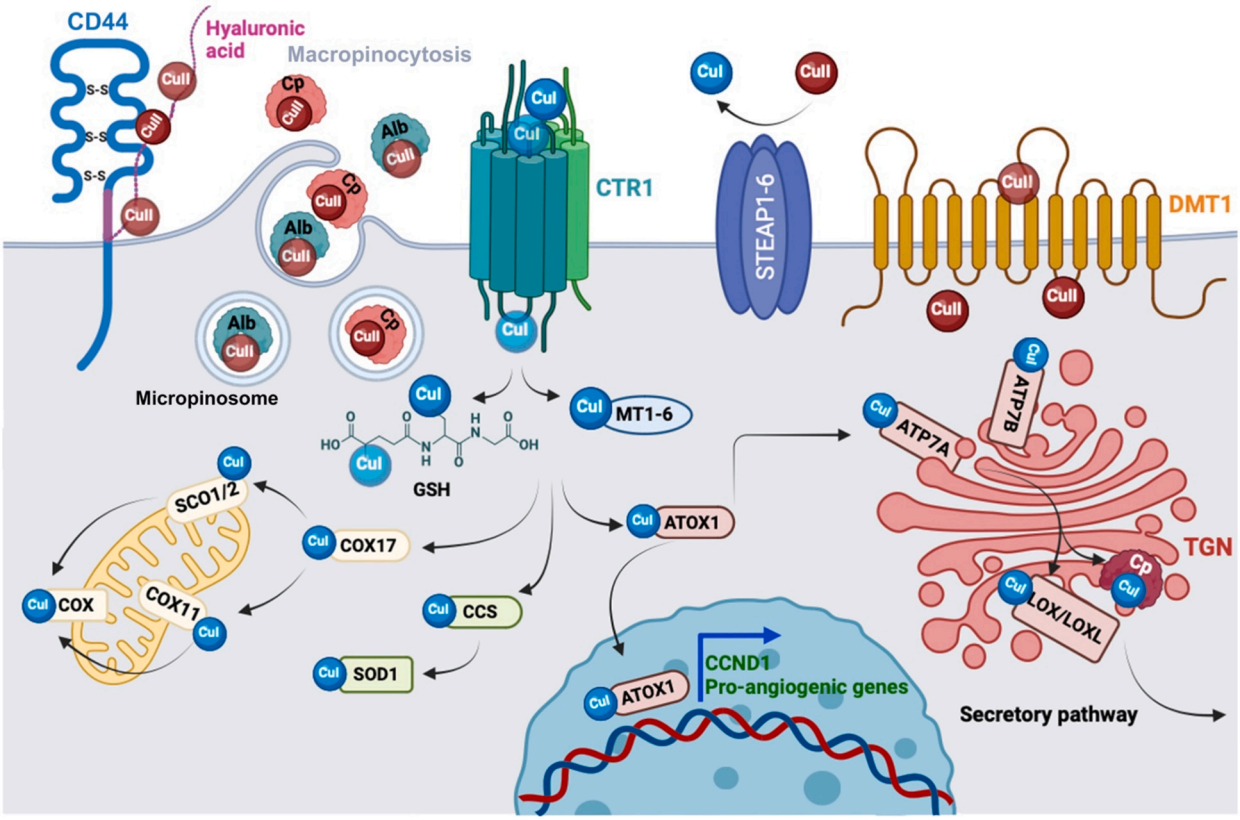

Fig. 3. The roads transporting copper within the cells. Cu(II), upon reduction from the metalloreductase family members STEAPs to Cu(I), is transported into the intracellular compartment by the high affinity copper transporter CTR1. Once inside the cell, the transition metal is immediately transferred to metallothioneins (MT1-6) and the tripeptide glutathione (GSH), forming the so called labile copper pool. Afterwards, copper is carried by specific copper chaperones: the copper chaperone for superoxide dismutase 1 (CCS) delivering copper to SOD1; the cytochrome *c* oxidase copper chaperone (COX17), which in turn transfer copper to the mitochondrial proteins SCO1, SCO2 and COX11 to refurnish of copper the mitochondrial subunit II of the complex IV, the cytochrome *c* oxidase (COX); the Antioxidant-protein 1 (ATOX1) that, besides acting as a copper-dependent transcription factor of pro-angiogenic genes and of the *CCND1* gene, coding for cyclin D1, delivers copper to the P-type ATPases ATP7A and ATP7B located in the trans-Golgi network (TGN). In particular, ATP7A participates in the secretory pathway of copper by transferring the transition metal to the lysyl oxidase family members (LOX/LOXL2) as well as to ceruloplasmin (Cp). In condition of CTR1 impairment, it has been shown the ability of the divalent metal transporter (DMT1) to transport Cu(II) inside the cells. Besides these "canonical" routes for the intracellular import of copper, it has been shown the involvement of macropinocytosis in the intracellular uptake of copper probably bound to Cp or albumin (Alb). Additionally, the receptor of hyaluronic acid, CD44, it has been recongized as a new copper transporter. Created in BioRender.com.

(O_2^-) leading to the generation of the hydroxyl radical (OH•), which can inflict damage upon lipids, proteins, and nucleic acids, ultimately leading to cell death. To counteract this peril, once entered in the cells copper is readily bound to protective proteins with metal-binding domains, such as those found within the metallothionein family (MT1-4), and to the tripeptide glutathione (GSH). Copper bound to MTs or GSH constitutes the so called "labile copper pool". From this compartment copper is transferred to higher affinity proteins, the metallochaperones, that facilitate the conveyance of copper to cuproenzymes [82]. The main cytosolic chaperones for copper are the Copper Chaperone for Superoxide dismutase, CCS, which delivers copper to the cuproenzyme Superoxide dismutase 1 (SOD1) and ATOX1 (Antioxidant-protein 1, also known as HAH1) delivering copper to the P-type ATPases ATP7A and ATP7B located in the trans-Golgi network (TGN). In particular, ATP7A is required to transfer copper to newly synthesized cuproenzymes that will be secreted outside the cells, such as ceruloplasmin (Cp) and the members of the family of lysyl oxidase and lysyl oxidase like (LOX/LOXL) cuproenzymes, forming the "secretory pathway" of copper [82] (Fig. 3).

The mitochondrial copper pool is controlled through the activity of the cytochrome *c* oxidase copper chaperone (COX17), together with other mitochondrial proteins (*e.g.*, SCO1, SCO2 and COX11), which provide copper to the primary cuproenzyme found in these organelles, the cytochrome *c* oxidase (COX), a multi-subunit protein forming the complex IV within the respiratory chain, crucial in both cell respiration and ATP production [82,93] (Fig. 3).

2.1. Copper-EMT crosstalk in cancer

In the last decade a bulk of evidence has extended the role of copper beyond that of an essential micronutrient, acting as cofactor of enzyme required in metabolic processes. Indeed, it has become clear its involvement in tumor onset and progression. In particular, copper homeostasis and cuproproteins are required to sustain tumor growth and progression by favoring a plethora of mechanisms including cancer cells adaptation to hypoxic environments [94], extracellular matrix (ECM) remodelling, neovascularization, EMT, and thus ultimately metastasis occurrence [82,93]. Ongoing investigation on the role of copper-dependent enzymes and pathways in the context of cancer constantly discovers genes and proteins which may represent potential innovative targets for cancer therapy.

Copper homeostasis influences multiple mechanisms involved in the growth and progression of cancer. The intracellular bioavailability of copper is able to influence the early stages of tumor growth represented by the secretion of pro-inflammatory cytokines and growth factors. It has been shown that in tumor-associated macrophages (TAMs) the intracellular depletion of copper, obtained through a novel synthetic copper chelator, namely CuNG, resulted in TAMs reprogramming [95]. The reduction of TAMs intracellular copper content led to an increased secretion of IL-12, while decreasing the secretion of TGFβ and IL-10, modifying TAMs immunosuppressive qualities and reactivating T cell immune responses against tumor cells [95].

The importance of copper in the regulation of TGFβ production has been described by the work of Guo et al. in 2021 [96]. They demonstrated (in murine models of pulmonary fibrosis) that $CuSO_4$ treatment resulted in the activation of the EMT through the upregulation of both TGFβ1 mRNA and protein levels. Accordingly, a study conducted by Kim and collaborators found that in glioblastoma multiforme (GBM) cells the specific copper chelator D-Penicillamine suppressed TGFβ expression, confirming its dependence on copper [97]. As further confirmation, an interesting paper has recently shown the ability of copper to modulate the epigenetic state and transcription mechanism of activated monocyte-derived macrophages [98]. The reduction of the mitochondrial copper pool induced the rewiring of the inflammatory signature of macrophages significantly decreasing the inflammatory genes at both the RNA and protein levels [98].

Besides the regulation of cytokines, copper coordinates the production, and subsequent secretion, of different growth factors required to sustain tumor progression. In a 3D culture system it $CuSO_4$ stimulates the secretion of both the fibroblast growth factor (FGF) and of the vascular endothelial growth factor (VEGF) [99]. Of note, copper is also indirectly involved in the modulation of VEGF and tumor necrosis factor-alpha (TNFα) levels through the ATOX1-ATP7A-LOX axis [100] (Fig. 3). Additionally, copper regulates the expression of VEGF and Fibroblast Growth Factor-1 (FGF-1) by also modulating the activity of the hypoxia-inducible factor-1 alpha (HIF-1α). Feng and coworkers showed that in a HepG2 human hepatoma cell line, under hypoxic conditions, copper inhibits the activity of Factor Inhibiting HIF-1 (FIH-1), thereby retaining the capacity of HIF-1α to accumulate and translocate into the nucleus, where it interacts with its cofactors to assemble the HIF-1 transcriptional complex [101]. Furthermore, the authors demonstrated that the expression or stability of HIF-1α were related to the nuclear localization of the copper chaperone CCS. Silencing the *CCS* gene suppressed HIF-1 activity [101]. In human umbilical vein endothelial cells (HUVEC) it has been shown that the conditioned medium from copper-treated breast cancer cells activated HIF-1α and the G-protein estrogen receptor (GPER) signaling promoting cell migration and tube formation [102]. However, the specific role of copper in the induction of angiogenesis through VEGF is debated [103]. The interplay between copper-activated HIF-1α and the activation of hypoxia-responsive elements (HREs) in targetting genes involved in EMT (*e.g.*, *CDH1*, *FN1*, *etc.*) has also been demonstrated. Notably, in the human breast cancer cell line MCF-7, copper depletion strongly inhibits hypoxia-induced EMT by down-regulating the expression of mesenchymal genes under the regulatory influence of the HIF-1α-SNAI1/TWIST signaling axis such as those coding for vimentin and fibronectin [104].

3. EMT signaling cascades modulated by copper

An increasing number of studies have highlighted the role of this mineral micronutrient as a "dynamic signaling metal" and as a "metalloallosteric regulator" [105] of signaling networks essential for the cancer cells proliferation as well as for tumor progression, for the acquisition of drug resistance and for mechanisms sustaining cancer cells evasion from the immune system defenses. To date, the role of copper has expanded so greatly that the term "cuproplasia" has been coined to describe its involvement in both neoplasms and hyperplasias. This term allows us to encompass both the direct and indirect effects of copper on signaling, including both the modulation of copper-dependent enzymatic and non-enzymatic activities [105].

3.1. Copper and the RAS/RAF/MEK/ERK pathway

One of the first copper-dependent signaling pathways identified and characterized, in the context of cancer, is the RAS/RAF/MEK/ERK signaling cascade [106]. In particular, within this cascade, the direct binding of copper to MEK1 allows the interaction of MEK1 to ERK and eventually ERK phosphorylation. It has been demonstrated that MEK1 contains a high-affinity copper-binding motif, rich in histidine and methionine residues (H188, M187, M230, and H239) that specifically binds two atoms of Cu(II) [107] (Fig. 4). However, the process of copper loading onto MEK1 remains unclear. The copper-dependence of ERK phosphorylation has been further confirmed using the specific copper chelators tetrathiomolybdate (TTM) and bathocuproine disulfonate (BCS), that significantly impaired ERK activation [108]. Furthermore, a recent work by Grasso and colleagues [109] shed light on the mechanism providing copper to MEK1 demonstrating the pivotal role of CCS in delivering copper to MEK1. The transfection of the HEK cell line with CCS mutants that affect Cu(I) uptake and exchange, as well as the cells treatment with a CCS small-molecule inhibitor, resulted in the decrease of the copper-activated MEK1 kinase activity (Fig. 4).

Intriguingly, it has been highlighted the addiction to copper of

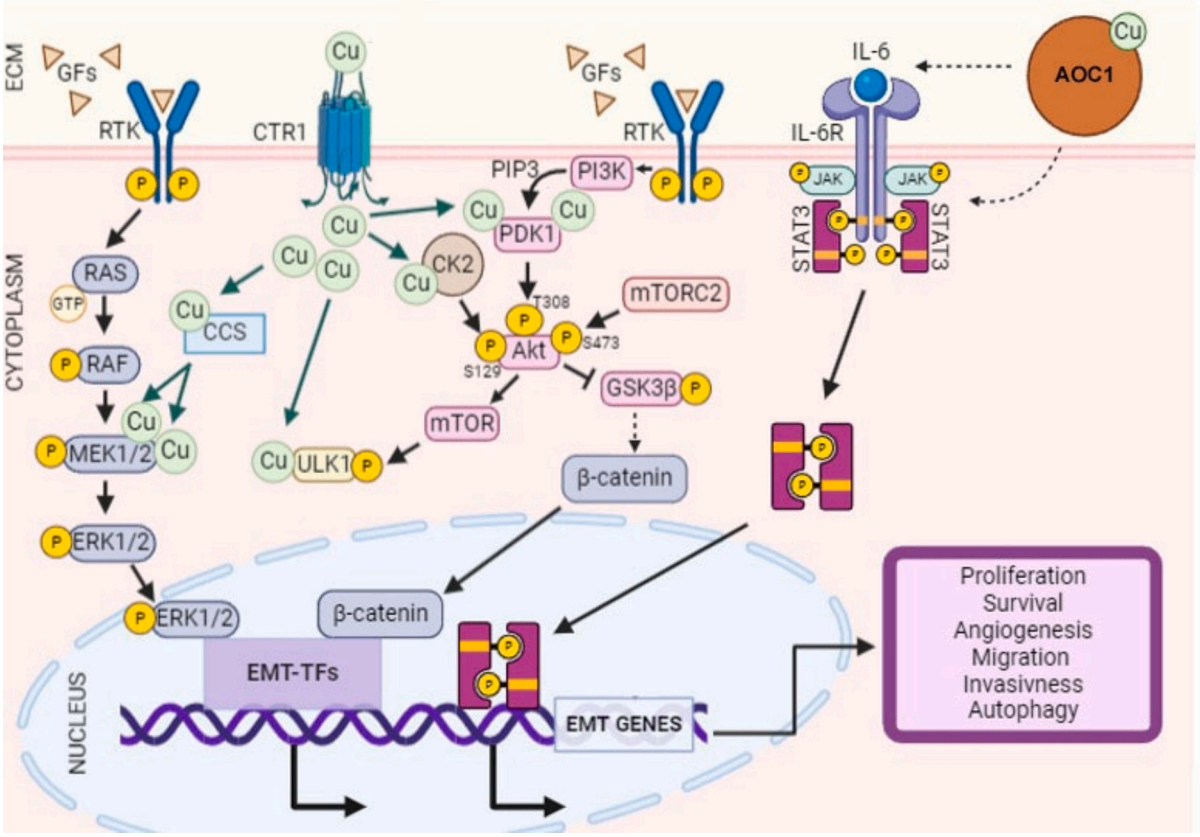

Fig. 4. Copper-dependent EMT signaling cascades. The copper chaperone for superoxide dismutase (CCS), besides delivering copper to SOD1, also transfers two atoms of the transition metals to the Mitogen Activated Kinase Kinase (MEK1/2) allowing its interaction and subsequent phosphorylation of ERK1/2, eventually leading to the activation of the EMT transcription factors (EMT-TFs). Additionally, the casein kinase 2 (CK2) and the phosphoinositide-dependent protein kinase 1 (PDK1) require copper to phosphorylate two different residues on Akt: Ser129 (S129) and Tyr308 (T308), respectively. These phosphorylation events contribute to the final activation of Akt and to the translocation of b-catenin into the cell nucleus where it prompts the activation of EMT-TFs. Also, the JAK/STAT pathway is affected by copper bioavailability. The copper-dependent amino oxidase (AOC1) modulates the activation of the JAK/STAT pathway upon engagement of the IL6 receptor. Lastly, the Unc-51-like kinase 1 (ULK1) requires the binding of copper to efficiently induce the autophagic process, as well as tumorigenesis. Created in BioRender.com.

$BRAF^{V600E}$-mutated cancers. This mutation is a missense activating mutation resulting in the constitutive activation of the signaling cascade leading to uncontrolled cancer cell proliferation [40]. The $BRAF^{V600E}$ mutation has been identified in several cancers including melanoma, CRC and non-small cell lung cancer (NSCLC) [110] and the copper-dependence of this mutated signaling network has been proved in all of these different neoplasms The pioneering work of Brady and colleagues [107] have shown that in a mouse model of $BRAF^{V600E}$-positive lung cancer, as well as in melanoma cell lines, the intracellular reduction of copper content, achieved through the silencing of the copper transporter CTR1 or following treatment with TTM, reduces $BRAF^{V600E}$-driven tumorigenesis, both *in vivo* and *in vitro* (Fig. 4). In $BRAF^{V600E}$-driven lung adenocarcinoma the role of copper in sustaining cancer progression has been further corroborated by the finding of a copper-dependent autophagic process. In particular, the BRAF-mutated lung adenocarcinoma cells require the binding of copper to Unc-51-like kinase 1 (ULK1) to achieve efficient autophagy, clonogenic survival and tumorigenesis both *in vitro* and *in vivo* models [111]. In CRC, the requirement for copper in the $BRAF^{V600E}$-positive HT29 cell line has been clearly demonstrated, as opposed to its BRAF wild-type counterpart represented by the HCT116 CRC cell line [112]. The reduction of the intracellular bioavailability of copper particularly hindered the proliferation, survival, and migration of $BRAF^{V600E}$-mutated cells. However, partially in contrast with these findings, it has been found that CRC cells carrying the KRAS mutations, including the previously mentioned HCT116 together with the DLD-1, SW480 and SW620 cells, were strictly dependent on copper. The impairment of the intracellular copper level obtained through TTM particularly affected the tumorigenesis and growth of KRAS-mutated CRC models, both *in vitro* and *in vivo* [113].

3.2. Copper and the PI3K/Akt/mTOR signaling cascade

The first evidence regarding the implication of copper in the modulation of the PI3K pathway dates back to 2002, when Ostrakhovitch and colleagues demonstrated that the treatment of human skin fibroblasts or of the ovarian cancer cells HeLa with Cu(II) led to a dose- and time-dependent activation of the antiapoptotic kinase Akt resulting in the phosphorylation of its downstream target GSK3β [114]. The stimulation of this signaling was independent of the copper-mediated production of ROS from which, usually, depends on the induced-activation of tyrosine kinase receptor (TKR). Twenty years later, Guo and coworkers partially unveiled the copper-dependent mechanism modulating the Akt network [115]. They demonstrated, in both CRC and breast cancer cell lines, the direct binding of copper to the Hys117 and Hys203 residues of phosphoinositide-dependent protein kinase 1 (PDK1), enhancing PDK1 binding and the consecutive activation of its downstream substrate Akt through phosphorylation at the Thr308 (Fig. 4). The use of both TTM, or the ablation of CTR1, strongly affected Akt activation [115].

Copper acts on this transduction pathway through an additional level of modulation. To fulfill Akt activation, together with the Thr308

phosphorylation, it is required the phosphorylation of the Ser473 residue of the kinase [116]. Besides this phosphorylation event, which is accomplished by the mTOR complex 2 (mTORC2) [44] and DNA-dependent protein kinase (DNA-PK) [117], Chojnowski et al. demonstrated the requirement of the copper-dependent casein kinase 2 (CK2) for phosphorylating Akt at Ser129 residue [118]. This latter phosphorylation results in the hyperphosphorylation of Akt and eventually in the up-regulation of β-catenin transcriptional activity [119] (Fig. 4).

The impact of this copper-mediated tuning of the PI3K/Akt network during EMT has been investigated for the first time in our laboratory [120]. We have analysed the effect of the specific copper chelator TRIEN in TGFβ-induced EMT in different subtypes of breast cancer cell lines. Of note, we found a different modulation of the EMT, depending either on the time of cells exposure to the copper chelator and on the subtype of breast cancer cells studied. We observe a copper-mediated effect on EMT only in the breast cancer cells lines lacking HER2 expression. In particular, the short-term treatment with TRIEN (24 h) of the triple negative breast cancer (TNBC) cells MDA-MB-231 and SUM159, as well as of the luminal A breast cancer cell line T47D, pushed the cells towards a more mesenchymal and aggressive phenotype. On the contrary, prolonged exposure to TRIEN, up to 6 days, led to a drastic reduction of the cells metastatic potential, once again only in the cell lines lacking HER2 expression (TNBC and luminal A). Our initial observation that HER2-negative breast cancer cells become more aggressive following the decrease of intracellular copper availability, added a further level of complexity to the copper-mediated modulation of cancer progression. Our data were in accordance with a previous study demonstrating that, for the efficient establishment of metastases, TNBC cells require sustained oxidative phosphorylation, which is supported by copper [121]. Moreover, we demonstrated that copper depleted TNBC cells became insensitive to the induction of EMT prompted by TGFβ. Copper deprivation affected not only the levels of EMT hallmarks but also counteracted the lipidome changes induced by TGFβ [120]. Surprisingly, when we analysed the mechanisms underlying these events, we observed that in the HER2-negative breast cancer cells the "non-canonical" EMT signaling PI3K/Akt pathway was hyperactivated following both the short- and long-term TRIEN treatments. Specifically, we observed the hyperphosphorylation of Akt at the Ser473 residue, but not at the Thr308 residue, confirming the dependence on copper of this phosphorylation event. However, the copper-independent activation of the Ser473 residue was sufficient to accomplish the kinase activity. In prolonged treatments, this phenomenon was also associated to a reduction of STAT phosphorylation levels, belonging to the "non-canonical" TGFb orchestrated EMT cascade. While the latter finding can be easily explained by the discovery of the copper-dependent amine oxidase AOC1 (Amine oxidase copper containing 1) required for the activation of the IL6/JAK/STAT3 axis [122] (Fig. 4), the data concerning Akt was particularly striking. The hyperactivation of Akt, upon 24 h of copper depletion, resulted, as expected, in an increase of the metastatic behaviour of TNBC cells, while, following 6 days of copper reduction, even in the presence of increased SNAI1 levels, we found a strong reduction of cancer cells aggressiveness and an irresponsiveness to TGFβ signaling.

Our findings on the copper-mediated modulation of Akt signaling cascade during EMT, have been further confirmed by our team using a natural-based copper chelating agent. Specifically, Perta et al. [123] investigated the copper-complexing properties of hydroxytyrosol (HDT), the major polyphenolic metabolite of oleuropein, and the effects of the resulting HDT-copper complex on the levels of EMT hallmarks and cancer cell aggressiveness in TNBC cells. Of note, it is already known that some polyphenols, including catechin [124] and curcumin [125], and of the secoiridoid oleuropein [126], form complexes with copper thus acting as chelators. Notably, following the characterization of the HDT-copper complex, the TNBC cell lines MDA-MB-231 and MDA-MB-468 were treated with HDT and we observed an increase of CCS levels and a simultaneous decrease of the level of the subunit II of cytochrome *c* oxidase, indicative of the reduction of the intracellular copper bioavailability. These effects were accompanied by a significant reduction in the TNBC cells aggressiveness due to the reduction of the levels of mesenchymal markers. Most importantly, in this case, in accordance with previous data in literature [127], we observe a decrease of both PDK1 and Akt (Ser473) phosphorylation levels, that in turn resulted in the downregulation of the mRNAs coding for the EMT-TFs SLUG, TWIST and ZEB1 [123].

The findings obtained in our lab have been confirmed and further explored by Poursani et al. [128]. Their investigation showed that copper depletion induced by the copper chelator TEPA led to the downregulation of both the TGFβ-induced EMT "canonical" (TGF-β/SMAD2-3) and "non-canonical" (TGFβ/PI3K/Akt, TGFβ/RAS/RAF/MEK/ERK, and TGFβ/WNT/β-catenin) signaling pathways. This effect was consistent across various cell lines including the MDA-MB-231 TNBC cells, neuroblastoma and diffuse intrinsic pontine glioma. In particular, through RNA-seq analysis, they unveiled the downregulation of the EMT gene set in the MDA-MB-231 cells upon TEPA treatment. Moreover, in a syngeneic TNBC mouse model copper depletion resulted in a notable reduction in lung metastasis [128].

3.3. Copper and the Jak/STAT axis

As reported in the previous paragraph, in 2021 Ding and coworkers [122] identified AOC1 downregulation as the main mechanism responsible for the modulation of the IL6/JAK/STAT3 axis, which in turn reduced hepatocellular carcinoma progression. Our data in TNBC confirmed the copper-dependence of STAT3 cascade, which was abrogated upon cells treatment with the specific copper chelator TRIEN [120] (Fig. 4).

In GBM it has been also demonstrated the interconnection between the JAK/STAT network, integral to the activation of the EMT process, and the modulation of the programmed death ligand 1 (PD-L1) expression [129]. RNA-seq analyses demonstrated that copper controls important signaling pathways governing PD-L1-driven cancer immune evasion [130]. In particular, copper supplementation modulated oxidative and EGFR phosphorylation, prompted STAT3 tyrosine phosphorylation and, following the activation of the NF-kB pathway triggering inflammation, increased PD-L1 expression, at both the mRNA and protein levels [130]. In GBM this inflammatory milieu significantly impacts the immunological properties of the tumor microenvironment, underscoring copper role in influencing key signaling pathways and immune responses, ultimately leading to the upregulation of PD-L1. In contrast, copper chelators induced ubiquitin-mediated degradation of PD-L1, concomitantly reducing STAT3 and EGFR phosphorylation. In addition to slowing tumor development and improving mice survival, copper-chelating medications also markedly raised the number of $CD8^+$ T and natural killer cells that infiltrated the tumor [130].

4. Cuproproteins supporting the EMT process

4.1. Lysyl oxidases and lysyl oxidase like enzymes

Alongside copper's involvement in the modulation of signaling cascades required to accomplish EMT, cuproproteins have been shown to sustain this process by participating to collateral mechanisms. One of the main cuproproteins sustaining EMT is the lysyl oxidases-like 2 protein (LOXL2) [131]. Lysyl oxidases (LOX) and Lysyl Oxidase Like (LOXLs) are a family of enzymes receiving copper from ATP7A and belonging to the secretory pathway of copper (Fig. 3). Their activity is required for the ECM remodelling during EMT: they catalyse the deamination of the amino groups of lysine residues in collagen and elastin monomers favoring the formation of covalent cross-links thus stabilizing collagen and elastin fibers [132]. Overexpression of LOXL2 has been associated *in vitro* with an increased invasion capacity of breast cancer cells [133] and *in vivo*, in a mouse model of breast cancer, to an increased metastatic

potential [134]. A recent work by Chiou et al., demonstrated that exposure of lung cancer cells (A549) to $CuSO_4$ resulted in the upregulation of LOXL2, eventually promoting the occurrence of fibrogenic changes [135].

One of the key mechanisms by which LOX proteins, including LOXL2, promote tumor invasiveness is by enhancing collagen cross-linking, which in turn triggers integrin clustering in focal adhesions, subsequently activating PI3K signaling and promoting tumor cell invasiveness [136,137]. Furthermore, in squamous cells carcinoma, it has been shown that LOXL2 could directly participate to EMT by interacting with SNAI1 stabilizing it [61], and, indirectly, inducing the silencing of *CDH1* through the deamination of trimethylated histone H3 lysine 4, thus promoting EMT [138].

In squamous cell carcinomas (SCC) LOXL2 silencing lead to an increase in cell-cell contacts compared to the control cells. Notably, these cells exhibited a re-expression of the epithelial E-cadherin, typically located at the cell membrane in approximately 50 % of the cell population [139]. Of note, in basal-like breast carcinoma, LOXL2 supports the mesenchymal phenotype promoting the downregulation of genes associated with cell polarity, such as those coding for Lgl2 and Claudin1, required for the metastatic spreading [140].

4.2. The mediator of ERBB2-driven cell motility 1

The Mediator of ERBB2-Driven Cell Motility 1 (MEMO1) has been identified as a copper dependent enzyme [141]. Structurally resembling the bacterial non-heme iron-dependent dioxygenases, MEMO1 contains a metal-binding pocket composed of three His, one Asp, and one Cys. Despite structural similarities, it has demonstrated a unique oxygen-dependent copper-reducing activity, leading to the production of $O2^{-}$ in the presence of Cu(II) [142]. The mutation of the His192 residue with an Ala (MEMO1 H192A mutant) significantly lower the Cu(II)-reducing activity of the cuproprotein. Cu(II) preloading experiments indicated that MEMO1 retained Cu(II), influencing its redox activity [142]. In the TNBC cell line MDA-MB-231, MEMO1 silencing resulted in the development of less invasive cellular structures, accompanied by increased level of markers of cell polarity like the basal laminin V and the apical Golgi marker GM130 [142]. Additionally, the depletion of copper in the same cell line, obtained upon cells incubation with TTM, affected cancer cells invasion. Intriguingly, TTM treatment of MEMO1-knocked down cells did not affect the invasive behaviour of cells, suggesting that MEMO1 might be the major copper-binding target influencing invasion [142].

During heregulin (HRG)-induced ERBB2 activation, cells extend lamellipodia in the direction of migration, with growing microtubules invading this space to propel the cell cortex forward. In response to ERBB2-based signaling, in breast cancer cells MEMO1 promoted the localization at the membrane of the cytoskeletal regulators RhoA and Diaphanous-Related formin 1 (mDia1) during HRG stimulation [143]. The MEMO1/RhoA/mDia1 complex controls lamellipodia formation and represses GSK3b activity, leading to the enrichment of the microtubule-associated proteins APC and CLASP2 at the cell periphery and, simultaneously, to the activation of the EMT. Besides its involvement in promoting breast cancer cell migration, MEMO1 reduces copper-mediated ROS, thus conferring a survival advantage to cancer cells [144]. Consequently, in several breast cancer cell lines MEMO1 altered the redox state of proteins such as RhoA, Shc, and actin and by affecting mRNA levels of redox homeostasis enzymes, including catalase, glutamate-cysteine ligase and glutathione peroxidase.

4.3. ATOX1

Another cuproprotein indirectly supporting EMT is the chaperone Antioxidant Protein-1 (ATOX1). ATOX1 is in charge of delivering copper to the secretory pathway governed by the ATP-dependent pumps ATP7A and ATP7B [145]. Beyond this function as copper chaperone, ATOX1 is also a copper-dependent transcription factor, whose nuclear translocation promotes the transcription of pro-angiogenic genes supporting the development of new blood vessels within the tumor microenvironment [146]. Specifically, in endothelial cells, it has been shown that the angiogenic process, related to the induction of VEGF by ATOX1, proceeds *via* the activation of lysyl oxidases and requires the transfer of copper from ATOX1 to the ATP pump ATP7A. Besides its pro-angiogenic function, it is well described the capacity of ATOX1 to transfer copper to the promotor of the gene coding for cyclin D1 (*CCND1*) resulting in gene transcription [147]. Cyclin D1 functions as a regulator of cell cycle progression, modulating the transition from the G1 to the S phase. Of note, its levels are up-regulated in cancer and is considered a negative prognostic marker [148] (Fig. 4). Moreover, in an ATP7A-independent way, ATOX1 increases ROS-NFκB-VCAM-1/ICAM-1 expression and monocyte adhesion acting as a copper-dependent transcription factor also for NADPH oxidase organizer p47phox. These results revealed a new connection between ATOX1 and NADPH oxidase, which is implicated in inflammatory neovascularization [100].

4.4. X-linked inhibitor of apoptosis protein

X-linked Inhibitor of Apoptosis Protein (XIAP) [149], a cytoplasmic protein with E3 ubiquitin ligase activity, inhibits caspases and consequently prevents apoptotic cell death. XIAP is a copper binding protein whose levels have been found elevated in various cancer [150]. In esophageal squamous cell cancer (ESCC) patients it has been found a tight correlation between the levels of XIAP and the occurrence of lymphatic metastasis. Moreover, patients with higher levels of XIAP were associated with worse overall survival [151]. From a mechanistic point of view the data obtained by Jin and colleagues suggested that XIAP could promote the spreading of ESCC through the activation of the TGFβ-induced EMT. In accordance with this evidence, the data obtained in pancreatic cells by Yi and co-workers, showed that the simultaneous silencing of XIAP and survivin induced a partial mesenchymal-epithelial transition by activating the PTEN/PI3K/Akt network [152]. As further confirmation, the downregulation of XIAP, achieved following the MiR-489 targeting, results in the impairment of ovarian cancer progression through the inhibition of the EMT cascade orchestrated by PI3K/Akt [153]. Interestingly, it has been also demonstrated the up-regulation of XIAP in lung adenocarcinoma with brain metastases, in comparison to lung adenocarcinoma without metastases [154].

5. Copper-dependent metabolic reprogramming during EMT

Copper's integration into various metabolic pathways adds an additional layer of complexity to its role in EMT regulation in cancer [155]. Metabolic reprogramming is a hallmark of EMT, since it supports the increased energy demands of migrating and invasive cancer cells [156]. Metastatic cancer cells, particularly CTCs, exhibit distinct energy requirements at different stages of the metastatic cascade, and a favorable metabolic reprogramming provides a survival advantage by prioritizing energy production [157]. Adaptations in metabolism influence cancer cell motility, detachment from the extracellular matrix and invasion.

Ruiz and colleagues investigated the adaptive responses of mitochondria to mild copper deficiency through intraperitoneal injections of the specific copper chelator bathocuproine disulfonate (BCS) in C57 black mice [158]. This treatment induced a unique and previously undocumented mitochondrial morphology, namely the “butternut squash” mitochondria, together with normal and swollen mitochondria. The study identified an up-regulation of copper-dependent proteins, including ceruloplasmin, ATP7B, and cytochrome *c* oxidase, confirming the copper-deficient condition. This novel mitochondrial morphology was associated with the up-regulation of mitochondrial dynamics proteins such as mitofusins, involved in mitochondrial fusion, and OPA1, which plays a crucial role in inner mitochondrial membrane fusion and maintenance of cristae structure [158]. Additionally, they observed

oxidative phosphorylation (OXPHOS) remodelling marked by supercomplex dissociation, and increased activity of individual complexes I and IV. Despite these alterations, ATP levels remained unaffected. BCS-treated mice exhibited mild anemia without significant health compromise. The study proposed that these adaptive responses, involving changes in mitochondrial dynamics and OXPHOS remodelling, maintain mitochondrial physiology and overall animal well-being in the context of copper deficiency [158].

In this context, fundamental was the study by Ramchandani and coworkers [159] in characterizing the impact of copper dysmetabolism in metastatic tumor cells reliant on OXPHOS. As copper levels decline, using approaches like TTM treatment, a discernible dip in mitochondrial respiration becomes evident through a reduction in the oxygen consumption rate. Complex IV, integral to the mitochondrial electron transport chain, undergoes degradation in conditions of copper depletion, disrupting the assembly and function of the cytochrome *c* oxidase holoenzyme. This influences energy production, with copper depletion causing a significant decrease in mitochondrial ATP production, impacting OXPHOS [121].

In TNBC cells we have observed a metabolic rewiring upon copper depletion, with a prevalence of glycolysis over mitochondrial energetic metabolism and a change in the cells lipidome. In particular, copper deficiency resulted in the accumulation of both neutral and polar lipids. Pre-treatment of TNBC cells with the copper-chelator TRIEN counteracted the metabolic changes induced by TGFβ exposure [120].

6. Cellular import mechanisms providing copper needed to support EMT

Copper homeostasis dysregulation in tumor progression occurs following alteration of both the expression and functions of copper specific transporters, chaperones and pumps.

Besides the key role of CTR1, recent studies have highlighted the significance of macropinocytosis for copper supply in KRAS-mutated cells [160], suggesting a CTR1-independent route for copper uptake (Fig. 3). Intriguingly, in mouse models of KRAS mutated CRC cancer, it has been shown that mice treatment with the selective macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA) lead to a significant reduction of tumor growth. In KRAS-mutated cells decrease in tumor growth was accompanied by an increase of CCS levels compared to control cells, indicating that macropinocytosis inhibition negatively affected copper uptake [113]. An additional effect of macropinocytosis in KRAS mutant CRC cells was the ATP7A stabilization, which accordingly decreased in response to EIPA treatment. These findings underscore the role of macropinocytosis in modulating copper availability and particularly in CRC suggesting the possible uptake of copper from copper-binding proteins, such as ceruloplasmin and albumin, and its subsequent translocation in macropinosomes.

Another route recently discovered and exploited by inflammation to accumulate copper is through the glycoprotein CD44 (Fig. 3). CD44, recognized for its pivotal role in cellular adhesion and migration, serves as a receptor for hyaluronic acid in the extracellular milieu, impacting biological processes such as cell adhesion, migration, and immune cell activation [161]. In addition, CD44 acts as a mediator for copper intracellular uptake, thereby influencing various cellular processes [98]. In particular, the role of CD44 as a copper transporter has been shown in activated monocyte-derived macrophages in which the knockdown of CD44, in contrast to other metal transporters, resulted in the impairment of copper uptake. Moreover, the study demonstrates that the binding of mitochondrial Cu(II) with supformin (LCC-12), a dimer of metformin forming a bimolecular complex with Cu(II), induces a reduction in the reserve of NAD(H). Notably, LCC-12 exhibits interference with cellular plasticity and reduces inflammation in murine models of bacterial and viral infections and interferes also with the EMT process in human non-small cell lung carcinoma cells and mouse pancreatic adenocarcinoma cells [98].

7. Conclusion

The data collected in this review, obtained from *in vitro* cellular models, *ex vivo* samples from oncological patients, clinical trials, and from *in vivo* mouse models of different types of cancers, demonstrate the critical role of intracellular copper homeostasis. Indeed, copper is required not only during tumorigenesis, but especially to support metabolic processes and the activation of pathways fundamental for cancer cells dissemination and the eventual formation of distant metastases. The evidence regarding the central role of copper in supporting oxidative phosphorylation to promote metastasis [121] and its function as allosteric cofactor of kinases whose signaling leads to uncontrolled cancer progression [93], constitutes a solid scientific basis supporting the possible use of copper chelators as adjuvants to conventional chemotherapy. In TNBC it has been demonstrated the ability of intracellular copper bioavailability to control the activation of EMT [120,123,128] modulating signaling cascades orchestrating the partial and/or complete transformation of epithelial tumor cells into mesenchymal cells endowed with a high metastatic potential.

One of the main issues concerning chemotherapy is the development of drug resistance in tumor cells, with the consequent aberrant activation of signaling pathways that further increase cancer proliferation and aggressiveness. The “early” administration of copper chelators could potentially prevent the onset of drug resistance by subtracting to the members of these networks the cofactor needed to support their activation. However, in a phase (II) clinical trial has been demonstrated the effectiveness also of the “late” administration of specific copper chelators to TNBC patients who already underwent surgical interventions and/or chemotherapy, in reducing their risk of patients' relapse [162].

The importance of this transition metal in the metastatic process is also demonstrated by the latest discoveries concerning alternative routes for copper entry into cells. In fact, alongside the canonical import of copper into the intracellular compartment operated by CTR1 and, to a lesser extent, DMT1, new data have shown the prevalence of the macropinocytosis process in experimental CRC models characterized by activating mutations in KRAS [113]. This mechanism is likely to provide a faster and greater copper supply in order to meet the higher demand for copper of these tumor cells. Together with macropinocytosis, the new role of the hyaluronic acid transporter CD44 in promoting copper entry into tumor cells, has also emerged [98]. From this evidence it can be assumed that tumor cells have evolved alternative mechanisms to satisfy the massive need for this metal.

If the repurposing in oncology of copper chelators, such as TRIEN or TM, offers the undisputed advantage of using drugs that have already been tested and approved by the FDA for the treatment of Wilson's disease, a rare genetic disorder characterized by copper overload, on the other hand, could lead to the non-selective systemic chelation of the metal. Considering the fundamental role of copper in physiological metabolic processes, this poses an obstacle to the use of copper chelators as chemotherapeutic agents. It is well known that, even in patients with Wilson's disease, nonspecific systemic copper removal can lead to side effects such as anemia, neuritis, vomiting, and leukopenia [163–165]. This issue could be overcome by the development of nano-based drug delivery systems capable of specifically releasing the copper chelator to the target site. This approach would avoid the occurrence of undesirable effects but also increase the drug's bioavailability at the target site.

In conclusion, research into potential of copper chelators as antitumor agents, particularly for counteracting the metastatic progression of various types of tumors by hindering EMT, is a rapidly evolving field. An increasing amount of experimental data has recently emerged supporting its clinical application and indicating its possible success, as demonstrated in some clinical trials.

Overall, the research reviewed here demonstrates the progress made in understanding copper's role beyond that of a transition element and cofactor for various intracellular enzymes. However, we also highlight the need for significant efforts to successfully implement the use of

copper chelators in oncological clinics.

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Antonio Focaccio: Writing – original draft. **Luisa Rossi:** Writing – review & editing, Supervision, Conceptualization. **Anastasia De Luca:** Writing – review & editing, Validation, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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