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Metabolic crossroads of iron and copper

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Abstract

Interactions between the essential dietary metals, iron and copper, have been known for many years. This review highlights recent advances in iron-copper interactions with a focus on tissues and cell types important for regulating whole-body iron and copper homeostasis. Cells that mediate dietary assimilation (enterocytes) and storage and distribution (hepatocytes) of iron and copper are considered, along with the principal users (erythroid cells) and recyclers of red cell iron (reticuloendothelial macrophages). Interactions between iron and copper in the brain are also discussed. Many unanswered questions regarding the role of these metals and their interactions in health and disease emerge from this synopsis, highlighting extensive future research opportunities.

Keywords

iron; copper; ceruloplasmin; hephaestin; anemia

Introduction

Optimal nutritional support is critical for mammalian development and health. One global nutrient deficiency, the trace element iron, illustrates the potential consequences of inadequate nutrition on human development. In infancy, for example, lack of sufficient iron intake can result in cognitive limitations that persist into adulthood.¹ It is therefore important to identify and correct iron deficiency as early as possible. Moreover, recent dietary intake data available online from *What We Eat In America, NHANES*² reports daily iron consumption several mg below the current RDA of 18 mg. Does this pose a threat to pregnant and lactating women who require more iron and may not consume adequate dietary iron or supplemental iron? Copper intake, in contrast, appears to be near or exceed the RDA of 0.9 mg. The extra copper needed for pregnancy and lactation however is often not met by diet alone and many prenatal supplements no longer contain copper. It thus seems likely that both iron and copper deficiency can occur in selected populations consuming normal diets. In fact, anemia due to copper deficiency has been frequently reported in adults.³

The tools available to assess iron status of neonates rely on hemoglobin measurements and serum iron, which are typically low in iron-deficient infants. There may be other factors that result in the same clinical phenotype. Notably, copper deficiency in infants when first detected was associated with low hemoglobin levels and low serum iron.³ Is this because of dual deficiencies of both copper and iron, or rather an iron deficiency induced by copper limitation? Recently, it has been shown in copper-deficient rat pups that iron injection (without copper) restored low serum iron and low hemoglobin levels to normal, demonstrating that in early life copper deficiency results in iron deficiency.⁴ Conversely, it is well known that iron fails to reverse anemia in older copper-deficient rodents.^{5, 6} Thus, the understanding of the copper-iron interaction is critically important in order to treat the *apropos* deficiency with the correct nutrient. For example, treating the anemia of copper deficiency with iron is like treating the anemia of B₁₂ deficiency with folate. The anemia disappears but the underlying problems of specific nutrient deficiencies do not.

An overview of whole-body iron and copper homeostasis highlights points of interaction between the two metals (Figure 1). Specific molecular interactions that occur between the two metals within various cell types, along with normal cellular homeostasis of each metal, are detailed in Figure 2. Cell types include the absorptive epithelium of the intestinal tract (enterocytes) and those managing metals in the liver (hepatocytes). Also shown are cells involved in iron recycling and storage (reticuloendothelial, RE, macrophages) and those that utilize the vast majority of iron for hemoglobin production (erythroid cells). Not shown in Figure 2, but discussed in the text, is copper and iron homeostasis in brain cells, which is relevant to many pathological states whereby these metals are deposited in various cell types within the brain causing oxidative stress and associated damage. Also discussed in the text are important observations that have been recently noted regarding the differences and similarities in iron and copper homeostasis among commonly used laboratory rodent models.

As seen in Figure 1, dietary iron and copper are absorbed in the upper small bowel. Absorption of both minerals is regulated. Iron absorption, which is controlled by the liver-derived, peptide hormone hepcidin, is modulated to suit the body's demand for iron, particularly for erythropoiesis. Hepcidin regulates iron absorption by inhibiting iron efflux from enterocytes. Hepcidin also inhibits iron release from important storage depots in the body, including autocrine action on hepatocytes and endocrine action on RE macrophages. Conversely, the precise control of copper transport in the gut is not currently understood, but absorption is modulated in relation to intake levels, with percent absorption being higher with low intakes.⁷ Under certain circumstances, a mucosal block to metal absorption may occur via sequestration of iron and copper by ferritin and metallothionein, respectively, with the net effect being the loss of the metals as enterocytes are sloughed off into the intestinal lumen. Important iron-copper interactions in the gut include the regulation of hephaestin (HP; a multi-copper ferroxidase necessary for iron efflux from enterocytes) by dietary copper levels and the regulation of the Menkes copper Atpase (ATP7A; a protein necessary for copper efflux) expression by iron levels. There are no regulated excretory mechanisms for iron, whereas copper is excreted in the bile (as mediated by the copper exporter, ATP7B) and then passes out of the body with the feces. Iron binds to transferrin (Fe-TF) in the portal blood for delivery to the liver, and copper binds to albumin or α_2 -macroglobulin.⁸

After absorption and transport to the liver, iron may be stored in hepatocytes or secreted on transferrin (Fe-TF) for distribution to body tissues. Most copper leaves the liver within the ceruloplasmin (CP) molecule, but other copper-binding proteins must exist in the serum as copper is efficiently distributed in the absence of CP (e.g. during aceruloplasminemia).⁹ The absence of CP, however, leads to iron accumulation in the pancreas, retina, and brain, indicating that the ferroxidase activity of circulating CP is critical for normal iron

homeostasis.¹⁰ Liver copper levels vary inversely according to iron status for unexplained reasons, exemplifying another important interaction between these two metals.

Iron and copper are delivered from the liver to all organs/tissues/cells of the body, where they are required for cellular metabolism. The ferroxidase activity of a GPI-anchored form of CP (GPI-CP) is necessary for iron release from brain and RE macrophages. This process may thus be affected by body copper levels as CP (and GPI-CP) activity requires copper as a prosthetic group. Most iron is delivered to the bone marrow for hemoglobin production. Interestingly, an unknown aspect of bone marrow iron utilization is copper dependent, for during copper deficiency, hemoglobin production is inefficient despite normal serum iron levels.¹¹

The purpose of this review is to critically review recent data on copper-iron interactions with a focus on overall body homeostasis of these metals. It is our hope to stimulate research to reveal the mechanism for this interaction known since the 19th century.¹² Many believe the interaction is due to CP, a circulating, copper-dependent enzyme capable of oxidizing ferrous to ferric iron. However, aceruloplasminemia in humans does not result in frank anemia.¹³ Others, suggest the copper-iron interaction involves HP, a CP homolog expressed in the intestine with similar catalytic properties.¹⁴ Intestinal block in iron uptake could lead to anemia. However, despite a modest elevation in enterocyte iron following copper deficiency whole body iron is normal, thus challenging the concept of limitation in iron uptake causing anemia.¹¹ Clearly, the mechanism(s) for anemia in copper deficiency is not known.

Enterocytes

The mechanisms by which dietary iron and copper are absorbed in the upper small intestine have been elucidated in recent years. Several transport proteins and chaperone molecules have been identified by investigations principally in mutant rodent models and knockout mice. Dietary non-heme iron (the major source of iron in most human populations) is predominantly in the ferric oxidation state and must be reduced for absorption (Figure 2A). This may occur by cytochrome B reductase 1 (CYBRD1).¹⁵ *Cybrd1* is a strongly induced gene during iron deficiency in laboratory rodents,^{16, 17} along with divalent metal transporter 1 (*Dmt1*), transferrin receptor 1 (*TfR1*) and other genes with proven roles in iron absorption, suggesting it may have an important physiological role in intestinal iron homeostasis. *Cybrd1*-null animals however were found to not have an iron-deficient phenotype.¹⁸ Although these observations suggest that this reductase is dispensable for intestinal iron absorption, the animals were fed ferrous iron and direct measurements of ferric iron uptake by the intestine were not made; nor was iron deficiency induced in the null animals. Thus, the exact role of *Cybrd1* in iron absorption remains unresolved. Other candidate intestinal ferrireductases include cytochrome b (558) ferric/cupric reductase¹⁹ and Steap2.²⁰ After reduction, ferrous iron (Fe²⁺) is transported into enterocytes by DMT1,²¹ coupled to the electrochemical H⁺ gradient from outside to inside cells. The important role of this transporter in intestinal iron uptake is best exemplified by the intestinal-specific *Dmt1* knockout mouse, which has a severe iron-deficient phenotype due to impaired vectorial iron transport in the upper small intestine.²²

Dietary iron can also be in the heme form, obtained principally from meat sources. A recent report described the identification of an apically expressed intestinal heme transporter (heme carrier protein 1; HCP1),²³ but current evidence suggests it functions mainly as an intestinal folate transporter (renamed proton-coupled folate transporter; PCFT),²⁴ as mutation of the gene in humans causes hereditary folate malabsorption. There is however recurring debate regarding the possible physiological role of PCFT/HCP1 in intestinal heme transport.²⁵ The

scientific community thus awaits the positive identification of the intestinal heme transporter.

Once inside enterocytes, it is not exactly clear how iron is handled. One candidate iron chaperone that delivers iron to ferritin has been recently identified,²⁶ but its role in the intestinal epithelium is currently unknown. One theory that has been proposed is the “transcytosis” of iron, whereby absorbed iron atoms remain within intracellular vesicular structures which may fuse with vesicles derived from the basolateral surface for export out of cells or delivery of iron to other intracellular compartments.²⁷ Iron may also be bound by cytosolic ferritin, amounting essentially to a mucosal block to iron absorption, as ferritin-bound iron is ultimately lost when enterocytes are sloughed off into the intestinal lumen.

Iron is transported across the basolateral membrane via ferroportin (FPN).^{28,29} Once ferrous iron has been effectively pumped out of cells, it is oxidized by HP,³⁰ a multi-copper ferroxidase that localizes to the basolateral membrane of enterocytes in close proximity to FPN.³¹ HP is required for intestinal iron absorption, as evidenced by the anemic phenotype of the sex-linked anemic (*sla*) mouse.³⁰ Once iron is oxidized, it may bind with apo-transferrin (Apo-TF) in the lamina propria for transport in the portal blood to the liver. Another circulating, multi-copper ferroxidase of hepatic origin, CP, may also participate in intestinal iron transport, particularly during states of hematopoietic stress.³²

The mechanism by which dietary copper is absorbed is distinct from that of iron, although complementary mechanisms seem plausible given the atomic properties of the two highly reactive and structurally similar transition metals. Dietary copper, like iron, must be reduced (Cu^{2+} to Cu^{+}) for transport across the apical membrane into enterocytes. Candidate proteins that can accomplish this include cytochrome b (558) ferric/cupric reductase,¹⁹ Steap 2,²⁰ and CYBRD1,³³ which can all reduce copper in addition to iron. Once reduced, the metal is likely transported into the enterocyte by copper transporter 1 (CTR1).³⁴ The precise mechanism of this transport process is not currently clear, as intestine-specific knockout of *Ctr1* leads to increased enterocyte copper levels, with the copper being apparently trapped in an intracellular compartment where it is not biologically available.³⁴ These mice develop severe systemic copper deficiency, indicating an essential role for Ctr1 in copper absorption. Interestingly, CTR1 has been detected on the basolateral surface of enterocytes as well,^{35, 36} creating some controversy regarding its precise role in intestinal copper transport. It is also possible that Dmt1 is involved in dietary copper absorption as *in vitro* evidence has shown that it can transport copper;³⁷ the precise role Dmt1 in the absorption of metals besides Fe (and Mn) however remains controversial.³⁸ A potential role for Dmt1 in copper transport certainly seems plausible during dietary iron deprivation, when Dmt1 mRNA and protein levels are very strongly induced in the setting of no competing iron atoms.^{17, 39, 40} Another possible mechanism for copper transport into enterocytes is an ATP-dependent mechanism recently described in Belgrade rats, a *Dmt1*-deficient rodent model.⁴¹

Once in cells, copper is bound by one of several chaperone proteins that deliver copper to the mitochondria (possibly COX17; a chaperone for cytochrome c oxidase),⁴² to the *trans*-golgi network (ATOX1; a chaperone for the Menkes copper ATPase [ATP7A]),⁴³ or to the cytosol for Cu, Zn-superoxide dismutase (SOD1) expression (copper chaperone for SOD1 [CCS]).⁴⁴ A possible nuclear copper chaperone protein representing an alternatively spliced form of the *ATP7A* transcript has also been hypothesized.⁴⁵ ATOX1 has additionally been described to be a copper-activated transcription factor involved in cell cycle control.^{46, 47} Whether ATOX1 is involved in enterocyte proliferation and differentiation however is not known, but this is certainly an intriguing possibility. Excess copper may also be bound in cells by metallothionein (MT),⁴⁸ which would presumably lead to copper loss when enterocytes are sloughed off into the lumen. Finally, copper may be transported out of

enterocytes by ATP7A, where it is then bound to albumin or α_2 -macroglobulin for delivery to the liver via the portal circulation.

The interplay between iron and copper homeostasis in the intestinal epithelium has recently been described at the molecular level. One such example is the multi-copper ferroxidases, HP and CP, which both play important roles in intestinal iron transport. HP activity is dependent upon normal copper levels, as exemplified by the fact that decreased HP activity has been documented in the intestine of copper-deficient mice, which correlates with systemic iron deficiency.⁴⁹ Additional studies have shown that copper deficiency reduces iron absorption in rats,⁵⁰ via a mechanism thought to involve decreased activity of duodenal hephaestin.⁵¹ Furthermore, repletion of copper-deficient rats with dietary copper restores intestinal iron absorption and Hp protein levels.⁵² These observations support earlier studies which also documented decreased iron transport in copper-deficient swine.⁵³ Moreover, the same phenomenon was shown in differentiated Caco-2 cells (a widely accepted model for the human intestinal epithelium); copper deficiency led to decreased HP protein and activity levels, presumably resulting in the documented decrease in iron efflux from cells.⁵⁴ Another recent investigation confirmed that HP is necessary for iron efflux in colorectal cancer cell lines (HT29 and WiDr), as manipulation of the levels of CDX2, a *trans*-acting factor implicated in *HP* gene expression, altered not only HP expression but also intracellular iron levels.⁵⁵ This study thus revealed a regulatory pathway connecting cellular iron levels to iron export mediated by *CDX2*-induction of *HP* expression.

On the other hand, CP, a circulating multi-copper ferroxidase of hepatic origin, has not historically been strongly associated with basolateral iron flux. A recent study however demonstrated that Cp plays a redundant role in iron absorption to Hp during times of stress.³² These studies carried out on *Cp*^{-/-} mice, revealed a marked impairment of iron absorption in acutely bled *Cp*-null mice, suggesting that Cp plays an important physiological role in iron transport for which Hp apparently cannot compensate. Moreover, Cp was found to relocalize from the duodenal epithelium into the lamina propria when wild-type mice were bled. These observations suggest that the role of Cp in basolateral iron efflux needs to be reconsidered and investigated further.

Interestingly, *in vivo* studies have demonstrated that there might be changes in the absorption of one of these trace minerals by perturbations in the homeostasis of the other. For example, copper absorption may be increased during states of iron deficiency, as suggested by increased metallothionein (*Mt*) mRNA expression (>20-fold)^{17, 56} and ATP7A mRNA and protein expression in the duodenal epithelium,^{17, 39} and increased copper levels in the intestinal mucosa and liver.^{39, 56} These observations may be indicative of enhanced copper transport across the apical membrane of enterocytes, although the precise mechanism by which this could occur has not been delineated. Another potential iron-copper interaction in the gut relates to the proposed role of ATOX1 in control of cell proliferation and differentiation. ATOX1 translocation to the nucleus and its *trans*-activating capabilities are stimulated by copper in endothelial and cancer cells.⁴⁷ As mentioned, copper accumulates in enterocytes during iron deficiency³⁹. Moreover, there are documented morphological changes in the intestinal epithelium during iron deficiency, including increased villus height and width, crypt depth and overall mucosal thickness, and increased numbers of mitotic cells in the crypts.^{57, 58} It was also shown that enterocytes in the lower half of the villus absorbed iron in iron-deficient rats as compared to higher up the villus in controls.⁵⁸ These observations are overall consistent with alterations in duodenal cell proliferation and differentiation during iron deficiency. It is thus conceivable that ATOX1 is involved in the adaptive response of the intestinal epithelium to iron deficiency, perhaps further linking iron and copper metabolism.

Of additional interest is the recent description of the regulation of the iron transport-related genes *Dmt1* and *Cybrd1* by the hypoxia inducible factor, Hif-2 α .^{40, 59} This observation is of particular import because the HIF factors are stabilized by copper, resulting in enhanced expression of HIF-responsive genes (e.g. Cp in the liver by Hif-1 α).^{60, 61} This then raises the possibility that increased copper levels in enterocytes,³⁹ as well as in the liver and in the body fluids,^{57, 62} may play a role in enhancing HIF activity, which in turn regulates genes related to intestinal (and body) iron homeostasis.

Finally, studies in the Caco-2 cell model have shown that depletion of iron or copper led to increased uptake of both metals into cells,⁶³ and that depletion of either metal led to enhanced iron flux from the apical to the basolateral cell culture chamber. It was also noted that copper supplementation enhanced expression of iron transport-related genes in Caco-2 cells. The authors of this study suggested that copper repletion induced intracellular iron depletion ultimately resulting in increased transepithelial iron flux.⁶⁴ Although the precise molecular mechanisms underlying these observations are not known, they further exemplify the intimate connections between iron and copper in the intestinal epithelium and demonstrate the need for additional investigations in this area.

Hepatocytes

After absorption by the duodenum, iron (Fe) in portal blood is bound to transferrin (Fe-TF) and transported to the liver (Figure 1). Fe-TF is taken up by hepatocytes (Figure 2B) in proportion to the amount of transferrin receptor 1 (TFR1).⁶⁵ The liver may also take up Fe-TF via TFR2, a TFR1 homologue that has been shown to enhance Fe-TF uptake by cells.⁶⁶ After endocytosis of the Fe-TF/TFR complex, vesicular acidification causes Fe-TF to release its iron, which is reduced and transported into the cytosol. It is presumed that DMT1 mediates the transfer of iron from the endosome to cytosol,⁶⁷ but *Dmt1*^{-/-} mice are able to assimilate iron into the liver (including hepatocytes), indicating that this protein is dispensable for hepatic iron uptake. In iron overload conditions, the carrying capacity of plasma TF becomes exceeded, giving rise to non-TF-bound iron (NTBI), which is rapidly cleared, mainly by the liver. Uptake of NTBI into hepatocytes is mediated, at least in part, by ZIP14,^{68, 69} and perhaps DMT1.⁷⁰ Iron taken up by the hepatocyte is either used for synthesis of iron-requiring proteins, stored in ferritin, or exported via FPN.

Absorbed copper is bound to α_2 -macroglobulin or albumin and transported to the liver (Figure 1). Studies in the human hepatoma cell line, HepG2, provide evidence that α_2 -macro-globulin delivers copper primarily to CTR1, whereas albumin donates the metal to a different, yet undefined, uptake mechanism.⁷¹ Assimilation of Cu²⁺ from either protein requires a reduction step because CTR1 transports only Cu⁺.⁷¹ Targeted deletion of hepatocyte *Ctr1* in mice demonstrated the importance of this protein in hepatic copper uptake *in vivo*.⁷³ Once inside the cell, copper is bound to chaperones and distributed to copper-requiring proteins (Figure 2B). ATP7B pumps copper into the TGN where it is incorporated into CP⁷⁴ and other cuproproteins. Excess copper stimulates the translocation of ATP7B from the TGN to the canalicular membrane of the hepatocyte, facilitating the secretion of copper into the bile.⁷⁵ Biliary secretion of copper appears to require the interaction between ATP7B and COMMD1 (Copper metabolism MURR1 domain), although the mechanism for this effect remains poorly understood.⁷⁶ COMMD1 levels are regulated by the ubiquitin ligase XIAP (X-linked inhibitor of apoptosis).⁷⁷

Hepatocyte iron and copper metabolism are linked through CP, a copper-containing enzyme that catalyzes the oxidation of Fe²⁺ to Fe³⁺, the form of iron that binds to Apo-TF. CP exists as a soluble secreted form and a glycosylphosphatidylinositol (GPI)-anchored form (GPI-CP).⁷⁸ Efficient iron release from hepatocytes requires CP, as formally demonstrated by

hepatocyte iron accumulation of *Cp*^{-/-} mice.⁷⁹ Copper-deficient rodents also accumulate iron in hepatocytes,⁸⁰ most likely because of reduced Cp activity.

The Steap (six-transmembrane epithelial antigen of the prostate) family of proteins have recently emerged as another potential link between iron and copper homeostasis.⁸¹ Steap proteins share distant though significant sequence homology with yeast FRE (Fe³⁺ reductase) proteins. Yeast use FRE proteins not only for the uptake of iron, but also for the reduction and uptake of copper. Similarly, 3 of the 4 known mammalian Steap proteins (Steap2, Steap3, and Steap4) have been shown to reduce both iron and copper, enhancing their cellular uptake.²⁰ Epitope-tagged Steap proteins have been localized to endosomes and the plasma membrane.^{20, 82} Analysis of *Steap3*^{-/-} mice demonstrated that this reductase was required for efficient Fe-TF uptake by erythroid cells (discussed below).⁸² Steap3 is most abundantly expressed in liver (and fetal liver), suggesting that it may serve as a reductase in this organ as well. If so, one would predict lower hepatic copper/iron concentrations in mice lacking Steap3. Interestingly, mice harboring a mutation in Steap3 that decreases its ferrireductase activity were found to have not lower but higher concentrations of hepatic copper and iron.⁸³ Although this study does not clarify the function of Steap3 in the liver, it does suggest a role for this metallo-reductase in hepatic copper/iron metabolism. Future studies need to evaluate tissue iron and copper status in Steap3-null animals.

In addition to its cupric/ferric reductase activity, Steap3 appears to play an essential role in the secretion of exosomes.⁸⁴ First discovered as membrane vesicles that extruded TFR1 from maturing reticulocytes, exosomes are now recognized as important cellular and physiologic mediators.⁸⁵ Indeed, defective exosome formation leading to deregulation of TFR1 shedding in Steap3-null mice has been proposed as an alternative explanation for the anemia of these animals.⁸⁴ A number of iron/copper-related proteins have been shown to undergo exosomal secretion, including TFR2,⁸⁶ GPI-CP,⁸⁷ ATP7A, and ATP7B.⁸⁸ The contribution of these secreted proteins to the regulation of cellular/systemic iron and copper homeostasis remains to be determined.

Since the 1960's, numerous studies have documented that hepatic copper concentrations vary inversely with iron status. Iron-deficient rodents have elevated hepatic copper levels,^{39, 57, 89-93} whereas iron-loaded animals have decreased levels.⁹⁴ The molecular basis for this relationship remains unknown. In iron deficiency, it is possible that elevated hepatic iron concentrations result from increased absorption of dietary copper. This possibility is supported by the observation that iron deficiency causes dramatic increases in intestinal expression of the basolateral copper exporter *Atp7a* and *Cybrd1*,^{17, 39} which can function as a cupric reductase.³³ Studies in rats, however, found no increase in copper absorption after the induction of iron deficiency by diet or bleeding.⁹⁵ Alternatively, it is possible that iron deficiency decreases biliary secretion of copper, incorporation of copper into secreted copper-containing proteins (e.g. CP), or the mobilization of copper from the liver.

Erythroid Cells

Erythroid cells are the most avid consumers of iron in the body. Each day, erythroid precursors of the bone marrow take up 20-25 mg of iron as they mature into erythrocytes. Unlike hepatocytes or enterocytes, which can rapidly assimilate NTBI, erythroid cells can acquire iron only from Fe-TF (Figure 2C). At the cell surface, TFR1 binds to circulating Fe-TF, leading to internalization of the Fe-TF/TFR1 complex into endosomes. The acquired ferric iron is then reduced to Fe²⁺ by Steap3 and transported into the cytosol by DMT1. That Steap3 serves as the endosomal ferrireductase in erythroid cells was revealed by studies of Steap3 mutant and Steap3-null animals.¹⁹ The essential role of DMT1 in iron assimilation by erythroid cells was demonstrated by mice engineered to lack *Dmt1* in hematopoietic

cells,²² as well as by studies of reticulocytes from anemic Belgrade rats, which express a poorly functional mutant form of the protein.⁹⁶

Nearly all the iron taken up by developing erythroid cells is rapidly directed to mitochondria, where it is used for heme biosynthesis and Fe-S cluster protein assembly.⁹⁷ Mitochondrial iron assimilation requires mitoferrin, an iron transporter located on the inner mitochondrial membrane.⁹⁸ The iron chaperone, frataxin, is thought to deliver iron to the site of Fe-S formation and heme synthesis.⁹⁹ In the developing erythroid cell, the overwhelming majority of newly synthesized heme is destined for hemoglobin in mature erythrocytes, ultimately accounting for 70% of total body iron.

Human erythrocytes contain approximately 2.5 $\mu\text{g Cu/g}$.¹⁰⁰ As with other cell types, it is probable that erythroid cells obtain their copper via CTR1. Reduction of Cu^{2+} at the plasma membrane may be facilitated by the metalloreductases Steap2, Steap3 or Steap4, which are all expressed in erythroid cells.^{20, 82} The contribution of Steap3 to copper uptake can (and should) be readily assessed by measuring copper concentrations in erythrocytes of Steap3-null mice. An estimated 60% of the copper in mature erythrocytes is associated with SOD1.¹⁰¹ Copper is delivered to SOD1 in the cytosol by the copper chaperone CCS. Levels of CCS in erythrocytes have proved to be a reliable indicator of copper deficiency in various species.^{102, 103} Copper also traffics to the mitochondrial matrix where it becomes part of a bioactive pool of copper that is used for the metallation of proteins such as cytochrome c oxidase (CCO). Also known as complex IV of the electron transport chain, CCO contains two copper atoms as well as two heme molecules. The delivery of copper to CCO requires COX17.¹⁰⁴

In terms of iron-copper interactions, no cell type has been studied more than the erythroid cell. Indeed, the demonstration by Hart et al. in 1928¹⁰⁵ that copper has an important function in hemoglobin synthesis is acknowledged as the beginning of the iron/copper field.¹² In their classic studies, Hart, Elvehjem and colleagues showed that rats fed an all-milk diet developed an anemia that could not be corrected by supplementation with iron salts.¹⁰⁵ The anemia was, however, readily corrected by supplementation with copper, revealing an essential role for copper in hemoglobin formation. Why does copper deficiency lead to anemia? It is frequently cited that copper-deficiency anemia results from diminished activities of CP and HP, the two copper-containing proteins that are required for efficient iron release from stores and for iron absorption.^{49, 106} According to this model, anemia ensues because sufficient iron cannot be mobilized from stores or absorbed from the diet. However, several studies have documented that iron administration—including intravenous iron injection, which bypasses any defect in iron absorption or release from stores—fails to correct the anemia in copper-deficient animals.^{106, 107} Moreover, copper-deficient mice can become anemic despite having normal plasma iron concentrations,¹⁰⁸ indicating that the anemia does not result from insufficient iron availability to developing erythroid cells of the bone marrow. These studies rather indicate that copper deficiency affects iron utilization by erythroid cells. Defective erythroid iron utilization in copper deficiency may reflect a defect in any one or a combination of these processes: cellular uptake, intracellular mobilization, uptake into mitochondria, or heme/hemoglobin synthesis. Studies of copper-deficient rat reticulocytes have documented that iron accumulates in ferritin in intracellular vesicular bodies, but its uptake into mitochondria is impaired, even under conditions of iron loading.¹⁰⁹ Other studies of copper-deficient swine reticulocytes found that the uptake of iron from Fe-TF was 50% lower than normal, with a 67% reduction in heme synthesis.¹¹⁰

RE Macrophages

Because of their key role in iron recycling (Figure 1), iron handling by RE macrophages has been thoroughly studied.¹¹¹ Although RE macrophages can take up iron from Fe-TF, these cells *in vivo* receive most of their iron by phagocytosis of senescent erythrocytes (Figure 2D). The ingested erythrocyte and its hemoglobin are degraded in the phagolysosome, releasing heme. Heme oxygenase cleaves the protoporphyrin ring to liberate iron, which is either utilized by the cell, stored in ferritin, or released from the cell via FPN. Efficient recycling of erythrocyte iron *in vivo* has recently been shown to involve Nramp1 (Natural resistance-associated macrophage protein 1), a divalent metal ion transporter that localizes to the late phagolysosome.¹¹² RE macrophages additionally serve as a large reservoir of storage iron, normally holding approximately half of total body iron reserve.¹¹¹

In contrast to our understanding of RE macrophage iron metabolism, comparatively little is known about how these cells handle copper. A small, and perhaps significant, amount of copper is obtained via phagocytosis of red cells, which contain the metal as part of SOD1. RE macrophages may also take up copper via CTR1. A recent study in RAW264.7 cells, a macrophage-like cell line, found that hypoxia increased cellular uptake of copper and the levels of Ctr1.¹¹³ The influx of copper resulted in an increase in Atp7a and its translocation from the TGN to cytoplasmic vesicles near the plasma membrane; a decrease in the levels of CCS, SOD1, and CCO; and an increase in the abundance and activity of Cp secreted into the culture medium. These observations suggest that, during hypoxia, macrophages divert the extra copper preferentially into Cp. *In vivo*, hypoxia has been shown to markedly increase Cp mRNA levels in mouse liver.⁶¹ Cell culture studies revealed that the induction of CP expression by hypoxia (or iron deficiency) requires HIF-1 α and HIF-1 β .¹¹⁴ Because the erythropoietic demand for iron is increased in both hypoxia and iron deficiency, the increase in CP resulting from either condition likely serves to enhance the release of iron from stores for mobilization to the bone marrow. Moreover, Atp7a has recently been shown to be involved in bacterial killing in a macrophage cell line,¹¹⁵ implicating copper in macrophage function.

As in the hepatocyte, copper and iron metabolism in the RE macrophage are linked via CP. Cp deficiency, resulting from dietary copper deprivation or genetic ablation, causes iron to accumulate in the RE cells of the spleen and liver.^{79, 80} The iron accrues secondary to impaired cellular iron efflux via FPN. De Domenico et al.¹¹⁶ demonstrated that the GPI-anchored form of Cp was required for cell-surface FPN expression and iron release from cells. They also showed that murine bone marrow macrophages express GPI-Cp, and that copper depletion prevented cell-surface localization of GPI-Cp and Fpn. These data suggest that GPI-linked, cell-bound Cp is more critical than is circulating Cp for iron release from RE cells. Selective deletion of the GPI-Cp variant will be required to better define its role in RE cells, as well as in other cell types, *in vivo*.

Brain

Brain metal homeostasis is carefully regulated by an elegant series of influx and efflux transporters because, while essential, excess copper and iron lead to neuropathology.^{117, 118} These transporters are especially critical in brain where metal flux requires passage through multiple membranes of the neurovascular unit, often called blood-brain-barrier (BBB). There is another cellular barrier separating cerebral spinal fluid (CSF) from blood lined by polarized cells of the choroid plexus, often called blood-CSF-barrier (BCB). Both copper and iron transporters are enriched in cells lining these barriers compared to brain parenchyma.^{119, 120}

Current working models suggest copper is taken up from blood by CTR1 and transported across the BBB by ATP7A.¹²¹ Likewise, copper is taken up from CSF by CTR1 which is enriched on the apical surface of the choroid plexus.¹²² Choroid plexus Ctr1 location and abundance appear to be enhanced following copper restriction.^{122, 123} Transport of copper across BCB is greater than across BBB.¹²⁰ Brain also contains ATP7B but it is not known if it is involved in copper transport or metallation of GPI-CP in astrocytes in parallel to its role in hepatocytes.

Transport of iron from blood to brain is somewhat controversial regarding the role of Fe-TF vs. NTBI particularly with respect to the presence of DMT1 in capillary endothelial cells.^{119, 124-126} There is some consensus that neurons acquire their iron via TFR1 and glia via DMT1. Ferritin seems more abundant in glia than neurons. Recent data suggest plasma ferritin may be a source of brain iron.¹²⁷

Iron flux from CSF to blood across the BCB appears to involve DMT1 whereas movement from blood to CSF requires TFR1 and FPN.¹²⁸ Similar to copper restriction, iron deprivation upregulates the iron transport machinery of brain.¹²⁹

It is not clear if copper modulates iron flux or iron modulates copper flux. It is clear that proper regulation of both copper and iron transport are critical to avoid neurological disturbances associated with metal imbalance including Alzheimer's disease, Parkinson's disease, Huntington's disease and diseases specifically associated with copper (Menkes and Wilson's disease) or iron (Friedreich's Ataxia and aceruloplasminemia).¹¹⁸ As noted earlier, copper deficiency results in elevated iron levels in the liver, due in part to diminished CP activity. In contrast, in brain (at least in rats), iron concentration is lower following dietary copper deficiency.¹³⁰ This seems to be due to the accompanying lower serum iron as injection of copper-deficient rats with iron reverses the serum and brain iron deficit.⁴ Brains of copper-deficient rats behave as though iron deficient with robust enhancement of TfR1 expression.¹³⁰

Brains of young rats subject to iron deficiency have elevated copper levels.¹³¹ It is tempting to speculate that this may be due to higher Dmt1 expression known to accompany iron deficiency, since some believe that Dmt1 can transport copper.²⁹ However, in a parallel study, brain iron deficiency was induced by high dietary manganese supplementation resulting in lower brain iron and higher Dmt1.¹³² Interestingly, brain copper was not augmented, suggesting that iron deficiency induced by diet rather than by Dmt1 overexpression is responsible for copper augmentation.

Brain copper-iron interactions may involve CP. In fact the brain, like liver, accumulates excess iron when CP is missing, for both humans with aceruloplasminemia and *Cp*^{-/-} mice exhibit high brain iron as adults.^{13, 133} Thus, adequate dietary copper is necessary to ensure proper transport of iron from the diet to ferric iron TF loading and for metallation of brain CP to perform its ferroxidase function in astrocytes.

The impact of copper and iron loading in rats has also been evaluated. Following a lactational challenge with high dietary copper administered to dams, weanling rats have markedly higher liver copper but no significant changes in plasma or brain copper.¹³⁴ The same study demonstrated a reduction in liver iron, higher plasma iron, but no alteration in brain iron following copper loading. Similar pups from iron-loaded dams had no change in liver copper, but lower plasma and brain copper. Iron loading resulted in higher liver and plasma iron levels but no change in brain iron concentration. Thus, the only impact on brain was when iron loading lowered both plasma and brain copper. Mechanisms for this observation are unknown.

Variables

Copper-iron interactions are influenced by age and species. Space does not permit an exhaustive review but selected examples will be given to stimulate thinking, and further research to confirm, refute, or extend current dogma.

As needs of an animal for copper and iron depend on stage of development, it is not surprising that homeostatic mechanisms differ between infants and adults. For example, iron uptake in the intestine of nursing mammals (mice and humans) may depend on lactoferrin rather than DMT1.¹³⁵ Thus, even if DMT1 transports copper, it may not be operative during lactation. Uptake and retention of brain copper and iron are both greater in sucking rodents than adults.^{130, 136} This is likely regulated by enhanced uptake transporters in developing brain. Enhanced brain copper was not observed when iron was deprived post-weaning in iron-deficient pups following lactational deprivation.¹³⁷ However, the elevated hepatic copper phenotype was observed in both paradigms. Thus, timing of iron deficiency can impact copper in different organs. Moreover, copper-deficient rodents are born iron deficient, likely due to impaired iron transport across placenta.^{138, 139}

The response to copper limitation in mice appears different than rats in some aspects of iron homeostasis. For example, copper-deficient rats and mice both develop severe anemia but only in rats is there a reduction in plasma iron.¹¹ This fact likely explains why these young copper-deficient mice do not have lower brain iron whereas copper-deficient rat pups do. The disparate response of plasma iron between copper-deficient rats and mice with a common severe hypohemoglobinemia suggests strongly that lack of iron in plasma cannot explain the anemia of copper deficiency.

Unanswered Questions

The numerous iron-copper interactions described in this review, many of which have been discovered within the last few years, exemplify the strong continued interest of metal biologists in this area of research. What becomes clear is that there are many unanswered questions regarding the interactions of these two metals that provide ample opportunity for future investigations.

- Is the decrease in intestinal iron absorption in copper deficiency solely due to a decrease in HP activity? Does CP, which also decreases in copper deficiency, play a role? CP was shown to be important during iron depletion, but does it have an important, unappreciated role in intestinal iron homeostasis under normal circumstances?
- What is the effect of enhanced copper uptake into enterocytes during iron deficiency? Copper can alter the activity of various factors involved in regulating iron homeostasis, including the hypoxia inducible factors which are increasingly being recognized as important in mediating intestinal metal ion homeostasis. Moreover, the copper-dependent chaperone ATOX1 has recently been identified as a transcription factor involved in cell proliferation. Could ATOX1 play a role in the apparent cellular proliferation in the intestine of iron-deficient animals?
- What are the roles of Steap proteins in iron/copper metabolism? Steap2, Steap3, and Steap4 have been shown to reduce both iron and copper. Steap3 clearly plays a role in iron uptake by erythroid cells, but what is its role in the liver, where it is most abundantly expressed? Why do Steap3-null mice accumulate liver iron and copper? Does intestinal Steap2 serve as an apical cupric/ferric reductase?

- Why do hepatic copper levels increase in iron deficiency and decrease during iron overload? Do these changes in copper levels have important implications for hepatic and/or overall body iron and/or copper homeostasis?
- Why do copper-deficient mice become anemic? The anemia does not result from insufficient supply of iron to the bone marrow, but rather from a defect in erythroid iron utilization. Hopefully new research on erythropoiesis following copper deficiency will clarify this most important copper-iron interaction.
- What is the role of GPI-CP in iron metabolism? GPI-CP is required for iron export from cells in the central nervous system, but does it also play a role in iron efflux from RE macrophages, hepatocytes, or other cell types? Is GPI-CP required for cell-surface expression of FPN *in vivo*?
- Why is brain iron concentration decreased by dietary copper deficiency, in contrast to what occurs in the liver where iron levels increase under these circumstances? Moreover, why does copper deficiency have the opposite effect in young rats, where brain iron levels are elevated during copper deficiency? These documented copper-iron interactions in brain are at least partially mediated by CP; in the absence of Cp, brain iron levels are increased.

It becomes increasingly apparent that there are numerous copper-iron interactions in all organs (and cell types within those organs) that are primarily responsible for managing overall body metal levels. In numerous cases, these interactions are of proven (or hypothesized) physiological relevance. Many of these interactions have been reported within the last 4-5 years, reflecting the continued interest by the scientific community in this area of research. It is our hope that this review will encourage new areas of experimental pursuit and will further stimulate interest in this field. There is no question that the next 5-10 years hold great promise as scientists and clinicians with interests in metal biology seek to further understand the complex interactions between dietary metals and how these are important in human health and disease.

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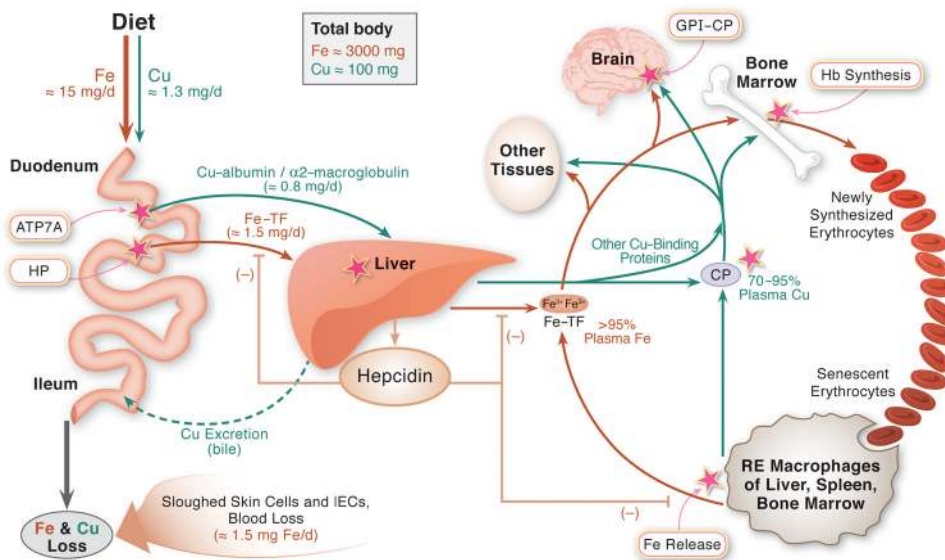


Figure 1. Overview of whole body iron and copper homeostasis, highlighting points of interaction

The principal organs controlling body iron and copper homeostasis are shown with points of interaction between the two metals indicated by a star (★). Dietary iron and copper are absorbed in the duodenum, where two points of interaction occur; namely, the copper-dependence of HP (★), and the strong induction of ATP7A (★) during iron deficiency. Absorbed copper is transported by albumin or α₂-macroglobulin in the portal blood to the liver, where it is incorporated into another ferroxidase, CP (★) or excreted into the bile. Absorbed iron binds to TF (Fe-TF) for transport to the liver. Iron may be stored in hepatocytes bound to ferritin or released into the circulation, where it is distributed by Fe-TF to the cells of the body. Copper is released from the liver principally on CP, which functions to deliver copper to other organs/tissues and which is important for iron release from certain tissues. Copper is efficiently delivered to these other tissues in the absence of CP, so other copper-binding proteins/ligands must exist in the serum. Systemic Fe-TF delivers iron predominantly to the bone marrow for red cell hemoglobin production; iron utilization by these cells is copper dependent (★). Iron is also taken up by other tissues, including the brain, where iron release is dependent upon GPI-CP (★). Iron contained within erythrocytes eventually is recycled by RE macrophages, where it can be stored until times of need. The release of iron from RE macrophages is a copper-dependent process, again involving CP and GPI-CP (★). Iron homeostasis is controlled by the liver-derived peptide hormone hepcidin, which functions to inhibit iron absorption from the diet, and iron release from storage sites in the liver and RE macrophages. There are no active excretory mechanisms for iron, but some iron is lost by sloughing of skin and enterocytes and by blood loss. ATP7A, ATPase, Cu⁺⁺ transporting, alpha polypeptide; CP, ceruloplasmin; Fe-TF, iron-transferrin; GPI-CP, glycosylphosphatidylinositol-linked CP; HP, hephaestin; RE, reticuloendothelial; TF, transferrin.

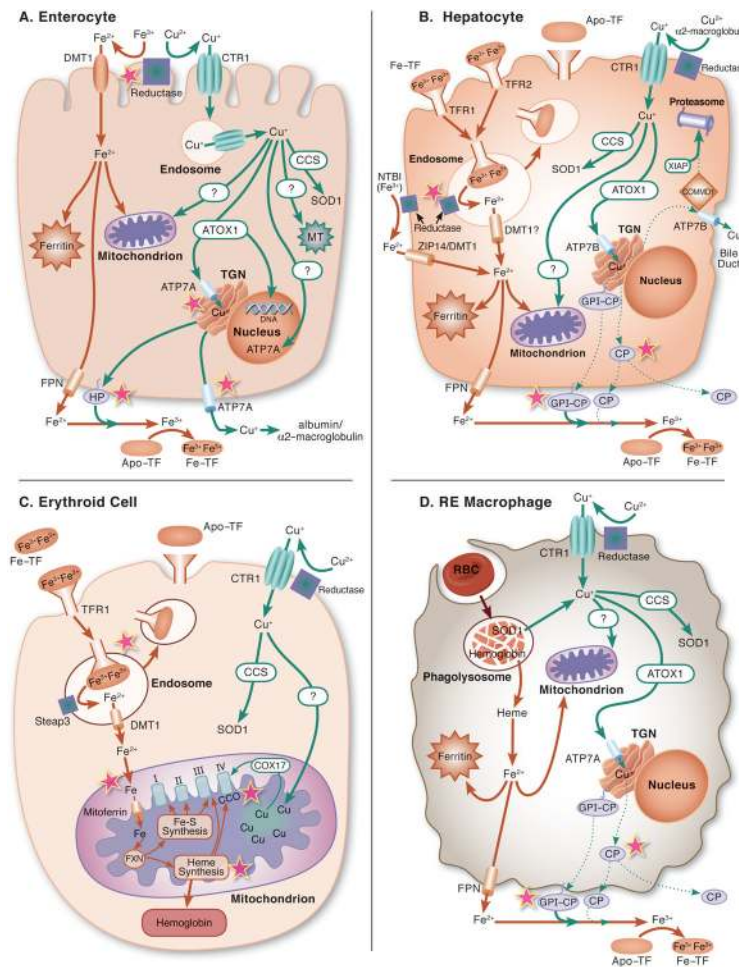


Figure 2. Main pathways of cellular iron and copper transport, highlighting points of interaction

The principal cells controlling body iron and copper homeostasis are shown with points of interaction between the two metals indicated by a star (★). (A) Duodenal enterocytes absorb dietary iron and copper via transporters located at the apical membrane. Absorption of oxidized forms of the metals requires the action of a cell-surface reductase. In the cytosol, chaperone proteins deliver copper to various proteins and organelles; excess copper is sequestered by MT. How cytosolic iron is transported is less well characterized, but may also involve chaperone proteins, at least for delivery of iron to ferritin. Iron and copper are exported across the basolateral membrane by FPN and ATP7A, respectively. Copper deficiency reduces levels of the copper-containing ferroxidase, HP (★), required for efficient enterocyte iron efflux and loading onto TF (Fe-TF). Iron deficiency markedly increases levels of basolateral ATP7A (★), perhaps increasing copper efflux. (B) Hepatocytes control iron metabolism by producing the iron-regulatory hormone hepcidin (not shown). These cells also control copper homeostasis, primarily by regulating the secretion of copper into the bile. Hepatocytes can take up Fe-TF via TFR1 or TFR2 cycling through the endocytic pathway. In the endosome, iron carried on Fe-TF is reduced and transported into the cytosol by mechanisms that are not well understood. During iron overload conditions, hepatocytes can also take up NTBI via ZIP14 or perhaps DMT1. Copper taken up via hepatocyte CTR1 is delivered to proteins including ATP7B, which pumps copper into the TGN for incorporation into GPI-CP and CP. These proteins serve an analogous function as HP in the enterocyte. Accordingly, copper deficiency or genetic ablation of CP, including GPI-CP (★), causes hepatocyte iron accumulation. High copper levels induce the translocation of ATP7B

from the TGN to the bile duct, facilitating biliary copper excretion, a process that requires interaction between ATP7B and COMMD1. Proteasomal degradation of ATP7B is regulated by XIAP. (C) Developing erythroid cells, the most avid consumers of iron in the body, acquire iron exclusively through the Fe-TF/TFR1 cycle. Endosomal Steap3 probably catalyzes the reduction of iron prior to DMT1-mediated transport into the cytosol. Virtually all the iron in these cells is directed to the mitochondria, where it is transported into the matrix via mitoferrin. The chaperone, FXN, is thought to deliver iron to the sites of Fe-S cluster assembly and heme synthesis. Fe-S and heme are incorporated into proteins comprising complexes I-IV of the electron transport chain. Most copper taken up by erythroid cells is delivered by CCS to SOD1. Copper is also directed to the mitochondrion for incorporation into complex IV (CCO) (★), in which two copper atoms work in concert with two heme groups to transport electrons. Mitochondrial copper is delivered to CCO by the chaperone COX17. Copper deficiency has been shown to decrease the assimilation of iron from Fe-TF (★), impair the uptake of iron by mitochondria (★), and reduce heme synthesis (★). Molecular details for these iron-copper interactions in erythroid cells remain to be elucidated. (D) RE macrophages play a central role in iron metabolism by phagocytosing senescent red blood cells and recycling their iron. Red cell hemoglobin is degraded within the phagolysosome, releasing heme, which is further catabolized to liberate iron. The freed iron is either exported, stored, or utilized by the cell. Although RE macrophages can take up copper via CTR1, they also accrue copper by phagocytosis of red cells, which contain the metal as part of SOD1. Efficient release of iron from RE macrophages, as well as cell-surface expression of FPN, requires GPI-CP/CP (★). Apo-TF, apo-transferrin; ATP7A, ATPase, Cu⁺⁺ transporting, alpha polypeptide; ATP7B, ATPase, Cu⁺⁺ transporting, beta polypeptide; ATOX1, antioxidant protein 1; COMMD1, copper metabolism (Murr1) domain containing 1; CCS, copper chaperone for SOD1; COX17, cytochrome c oxidase copper chaperone 17; CP, ceruloplasmin; CTR1, copper transporter 1; DMT1, divalent metal transporter 1; Fe-TF, iron-transferrin; FPN, ferroportin; FXN, frataxin; GPI-CP, glycosylphosphatidylinositol-linked CP; HP, hephaestin; MT, metallothionein; NTBI, non-transferrin-bound iron; RE, reticuloendothelial; SOD1, superoxide dismutase 1; Steap3, six transmembrane epithelial antigen of the prostate 3; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2; TGN, trans-Golgi network; ZIP14, Zrt- and Irt-like protein 14; XIAP, X-linked inhibitor of apoptosis protein.