CHARACTERIZATION OF GLYCOSYLPHOSHATIDYLINOSITOL-ANCHORED CERULOPLASMIN IN MULTIPLE RODENT ORGANS FOLLOWING DIETARY COPPER DEFICIENCY

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

ELISE JUNE MOSTAD

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

DR. JOSEPH R. PROHASKA

OCTOBER 2010

Acknowledgements

I express my sincere gratitude to those who have contributed to the completion of this project:

I would like to thank Margaret Broderius for the continuous support, assistance, and guidance, and for helping me become a better scientist. I would also like to thank Katie Lassi for her ongoing support and assistance with various projects.

I convey my deepest appreciation to Dr. Prohaska for sharing his vast knowledge and for his insight and continued guidance throughout this project. I hope that I may be as passionate about my future work as he is.

Thank you to Dr. Berry for allowing me to start my research career as an undergraduate in his lab and for being a member of my thesis committee.

Dr. Drewes also deserves my gratitude for being a past professor and a member of my thesis committee.

I would also like to thank my parents, Ben and Laurel, for their support and for trusting me and my decisions throughout my academic career.

Funding for this research was provided, in part, by National Institutes of Health grants [R01-HD039708 and R03-HD055423] and the Integrated Biosciences Graduate Program.

Abstract

Copper is a necessary metal cofactor in many enzymes that catalyze key reactions in both prokaryotes and eukaryotes. Ceruloplasmin (Cp) is a copper-dependent enzyme that acts as a ferroxidase, oxidizing FeII to FeIII for systemic iron mobilization. Cp is expressed as both a secreted plasma (sCp) enzyme and a membrane-bound glycosyl-phosphatidylinositol-anchored (GPI-Cp) splice variant enzyme. sCp is the most abundant copper-binding protein in mammalian plasma. The ferroxidase activity of Cp is essential for iron mobilization, as *Cp* null humans and mice exhibit selective tissue-specific iron overload. Dietary copper deficient (CuD) rodents have near total loss of Cp activity, severe loss of Cp protein, and anemia. The impacts of dietary copper deficiency on GPI-Cp has not been previously evaluated. Studies were conducted in Holtzman and Sprague-Dawley rats, albino mice, and *Cp* -/- mice, to investigate the copper-iron interaction and further characterize GPI-Cp. Purified membrane extracts of these rodent tissues detected immunoreactive Cp protein, especially enriched in spleen and kidney, but not in membranes from *Cp* -/- mice. Immunoreactive Cp protein was released with phosphotidylinositol-specific phospholipase C treatment and released protein exhibited ferroxidase activity. These data suggest that the membrane-bound Cp immunoreactivity detected is GPI-anchored. Following perinatal and postnatal copper restriction, GPI-Cp was markedly lower in spleen and modestly lower in liver of CuD rats and mice, compared to copper-adequate (CuA) rodents. Livers of CuD mice contained elevated liver non-heme iron (NHI), while spleen NHI was lower in CuD than CuA rats, and not different in CuD mice, implying that lower GPI-Cp was not correlated with augmented NHI levels in CuD rodent spleens. Spleen and liver membranes of CuD rats expressed augmented levels of ferroportin, the iron efflux transporter, which may compensate for the loss of GPI-Cp in iron efflux. Copper deficient rats and mice both develop severe anemia but only in rats is plasma iron lower than normal, consistent with impaired Cp function. As multicopper oxidases like Cp are thought to be the major metabolic link between copper and iron, additional research is needed to determine the impact, if any, of lower GPI-Cp on iron flux and the development of anemia when copper is limiting.

Table of Contents

ACKNOWLEDGEMENTS…… i

ABSTRACT…… ii

LIST OF TABLES…….. iv

LIST OF FIGURES…… v

ORGANIZATION OF THESIS……..…. 1

CHAPTER 1……..... 2

CHAPTER 2……... 10

CHAPTER 3……..... 37

CHAPTER 4……..... 43

LITERATURE CITED……... 46

List of Tables

Table Page

1-1 Proteins important in the copper-iron interaction…….……..... 9

2-1 Characteristics of rats and mice following copper deficiency…….…… 28

2-1S Comparison of rat organ mRNA levels..…… 29

3-1 Spleen non-heme iron and liver total iron values.....…… 42

List of Figures

Figure and Title Page

2-1 Characterization of rodent GPI-Ceruloplasmin……. 30

2-2 Verification of copper status in multiple rodent tissues by CCS ……. 31

2-3 Impact of copper status on spleen GPI-Cp in male rats…….… 32

2-4 Impact of copper status on liver GPI-Cp in male rats ……..... 33

2-5 Impact of copper status on GPI-Cp in female rats...……...… 34

2-6 Impact of copper status on GPI-Cp in male mice…….....…. 35

2-7 Impact of postnatal copper deficiency in male rats on GPI-Cp abundance…..…. 36

3-1 Impact of copper deficiency and repletion on GPI-Cp in older male rats...…..…. 40

3-2 Characterization of rodent transferrin receptor…….…... 41

Thesis Organization

Chapter 1 is an introduction to the overall importance of copper in biological systems, copper-dependent enzymes, and the copper-iron interaction. Chapter 2 is a manuscript that has been submitted to the journal *Experimental Biology and Medicine* for review. Chapter 3 contains additional supporting results determined to be pertinent to the copper deficiency studies. Chapter 4 is an expanded discussion focusing on the implications of our findings and future directions.

CHAPTER 1

Copper in proteins

Essential trace metals including copper and iron are present in most tissues and cells within plants and mammals and in prokaryotes. Copper, in particular, is critical in all biological systems, acting as a catalytic cofactor in many metalloproteins. Copper exists in two oxidation states within a cell, CuII and CuI; this contributes to its ability to play a central role in many biological processes requiring single electron transfer reactions (Halliwell 1984). In biological systems, under physiological conditions, all copper tends to be bound to protein, chelators, or metallochaperones, as it is toxic in its free form (Linder & Hazegh-Azam 1996). Copper-dependent proteins have been discovered in mammals, yeast, plants, and bacteria; these proteins have homologues with similar functions in each of the kingdoms (Harrison *et al.* 2000).

In prokaryotes, copper is bound to proteins, most often blue copper proteins, which function in the electron transport chain as single-electron carriers (Metzler & Metzler 2003). Bacteria, such as *Psuedomonas aeruginosa,* contain many copper-dependent proteins that act as oxidases. Ten copper-containing proteins have been characterized in prokaryotes, including cytochrome c oxidase (CCO), Cu, Zn-superoxide dismutase (SOD), nitrosocyanin, and plastocyanin (Ridge *et al.* 2008). Studies have found that bacterial multicopper oxidases play a critical role in FeII acquisition under aerobic conditions, oxidizing FeII to FeIII (Huston *et al.* 2002).

In plants, copper is ubiquitous and acts as a structural element in regulatory proteins that participate in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism, and hormone signaling (Pilon *et al.* 2006). Some of the copper-dependent proteins within plants include SOD, CCO, amine oxidase, laccase, and plastocyanin (Yruela 2005). At the cellular level, copper also plays an essential role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation, and iron mobilization (Clarkson 1995). Copper-dependent laccase-like ferroxidases that allow systemic iron mobilization are predominantly expressed in plant vascular systems and are thought to be required for iron uptake systems (Hoopes & Dean 2004).

In mammals, copper is present in all body tissues and is one of the highly essential metal ions necessary for normal development and function. It is a cofactor for about a dozen enzymes (cuproenzymes) in the mammalian body including ceruloplasmin (Cp), hephaestin, SOD, the copper chaperone for SOD (CCS) (Prohaska 2006) and the recently discovered zyklopen (Chen *et al.* 2010). Within these cuproenzymes, copper assists the catalysis of redox reactions and scavenging free radicals. Mammalian cuproenzymes can have multiple roles, but mostly act as oxidases reducing molecular oxygen, or participating in electron transfer (Linder & Hazegh-Azam 1996). Some of the specific physiologic functions of cuproenzymes include energy production, connective tissue formation (O'Dell 1981), iron metabolism (ferroxidase), neurotransmitter synthesis and metabolism (Feller *et al.* 1982), and antioxidant defense (Linder & Hazegh-Azam 1996), among others. The important roles copper plays in the many cuproenzymes in mammals demonstrate the need for proper dietary copper intake.

Baker's yeast, *Saccharomyces cerevisiae,* has been used as a model to study copper transport, distribution, and detoxification in mammals (Labbe *et al.* 1997). Many of the proteins in yeast that are involved in these processes are the same or homologous to those in mammals. Ctr1 is a protein in both yeast and mammals responsible for high-affinity copper transport across the membrane (Dancis *et al.* 1994). Copper transporters homologous to those in humans then transport copper to different cellular locations, with a final insertion into a copper-dependent ferroxidase, Fet3p (Valentine & Gralla 1997). Studies on Fet3p have provided much insight into its human homologue, Cp (De Silva *et al.* 1995).

The many multicopper oxidases can contain any number of the six or more different types of copper centers (types I, II, and III, Cu_Z, Cu_A, and Cu_B) (Abolmaali *et al.* 1998). These specialized copper centers provide long-range electron transfer reactivity and oxygen binding. Cp requires copper for safe handling of oxygen in the metabolic pathways of mammals. It is classified in the blue multicopper oxidase family and contains six total coppers; three occupy mononuclear type I sites (single copper surrounded by neutral molecules), and the remaining three form a trinuclear center (three coordinated coppers in the center of the protein) (Bento *et al.* 2007). Of the three coppers

in the trinuclear center, two are identified as coupled type III copper ions and the third is a type II copper ion. The type I blue copper proteins are known to function in electron transfer, type II copper centers occur in proteins that assist in oxidization, and type III copper centers are present in oxidases and oxygen-transporting proteins (Dwulet & Putnam 1981, Owen 1982); each of these coppers is necessary for the effectiveness of the ferroxidase activity of Cp.

Copper-iron interaction

Iron is another essential trace metal present in every organism which acts with heme proteins as the main carrier for oxygen to all of the body's cells in mammals, plants, and fungi. Iron is a functional component in both nonheme-containing proteins and hemoproteins. In particular, iron-dependent proteins catalyze reactions in energy metabolism, respiration, and oxygen delivery to tissues, as well as assist DNA synthesis. Iron is also associated with transferrin (Tf), a protein that complexes iron and transports it in the circulation (Comporti 2002).

The metabolic links between copper and iron metabolism are complex and have been an area of great interest, with certain aspects still unknown (Fox 2003). The molecular details of the copper-iron interaction were first characterized in baker's yeast, *Saccharomyces cerevisiae*, in 1993 (Askwith *et al.* 1994, Dancis et al. 1994). Yeast contains homologous proteins to humans that participate in copper and iron metabolism and transport, allowing a better understanding of the copper-iron interaction in humans. Transport of copper into the cell requires the high-affinity copper-binding protein, Ctr1, which is localized to the plasma membrane. Yeast strains with a *Ctr1* gene deletion (*Ctr1*-/-) display characteristics of copper deficiency and are defective in ferrous iron uptake, which causes the failure of the multicopper oxidase, Fet3p, to receive sufficient copper (Askwith et al. 1994, Dancis et al. 1994). Fet3p is homologous to human serum Cp and plays a similar role in its copper-dependent ferroxidase activity. The oxidation of ferrous to ferric iron by Fet3p then allows for ferric iron transport by Ftr1p (Askwith & Kaplan 1998). The similarities of yeast and mammalian pathways of iron transport on homologous multicopper ferroxidases suggest the universal nature of many aspects of the copper-iron interactions in different eukaryotic systems. However, yeast Fet3p mediates

iron influx, while mammalian Cp mediates cellular iron efflux and transport (Askwith & Kaplan 1998).

In mammals, both iron and copper are absorbed in the upper small bowel, where iron is bound to Tf for delivery to the liver and copper is bound to albumin or alpha-2 macroglobulin (Liu *et al.* 2007). Most copper leaves the liver Cp-bound and iron may be stored in hepatocytes or secreted Tf-bound. From the liver, iron and copper are delivered to the necessary tissues of the body. Most iron is delivered to the bone marrow for hemoglobin production. Iron leaves cells in the form of FeII with the help of an iron exporter, like ferroportin (Fpn), but it must be oxidized to FeIII at the cell surface by a ferroxidase such as Cp before it can bind to Tf for distribution (Osaki *et al.* 1971); thus, this ferroxidase activity of Cp is crucial for the proper mobilization of iron from storage cells (Gray *et al.* 2009). Early studies on Cp ferroxidase activity by Osaki *et al*. showed that Cp markedly increased the rate of iron loading into Tf and that Cp was the plasma factor that oxidized both iron and paraphenylenediamine (PPD) (Osaki *et al.* 1966).

Two forms of Cp exist, a membrane-bound glycophosphatidylinositol form (GPI-Cp) and the circulating serum form (sCp), both possessing very important ferroxidase activity. Ferroxidase activity of Cp is required for iron release from macrophages as *Cp-/-* humans and mice eventually accumulate iron in macrophages of the spleen, liver, and brain (Harris *et al.* 1999, Xu *et al.* 2004). Decreased Cp ferroxidase activity leads to defects in mobilization of liver iron and recycling of iron from erythrocytes by Kupffer cells, ultimately leading to hepatic iron accumulation (Chen *et al.* 2006). Ferroxidase activity is also present in the membrane-bound Cp homologues hephaestin and zyklopen. Hephaestin is present predominately on the enterocytes and is required for efficient dietary iron absorption. Mutations in hephaestin lead to iron retention in enterocytes due to decreased intestinal iron efflux and an overall systemic iron deficiency (Chen *et al*. 2006). Zyklopen is a recently discovered membrane-bound ferroxidase located in the placenta and mammary gland (Chen *et al.* 2010). The fact that more than one copper-dependent ferroxidase exists within an organism suggests the importance of this iron-oxidizing activity.

In 1966, Osaki *et al*. proposed a model in which the ferroxidase activity of sCp played a central role in driving iron from the gut, through the plasma, to the erythroid marrow for hemoglobin synthesis and RBC formation. By facilitating the binding of iron to Tf, an iron gradient is created which increases cellular iron efflux (Osaki et al. 1966). This model was the first molecular link between copper and iron metabolism in mammals.

Copper and iron homeostasis are linked through multicopper ferroxidases in bacteria (Huston et al. 2002), yeast (De Freitas *et al.* 2003), plants (Hoopes & Dean 2004), and mammals (Kosman 2002, Nittis & Gitlin 2002). Decreased or absent ferroxidase activity due to copper deficiency or a genetic defect (as in aceruloplasminemia) leads to many physiological defects as well as iron metabolism issues in different organisms (Chen et al. 2006).

Copper deficiency

The necessity of copper in multicopper oxidases like Cp warrants investigating the effects of altering body copper levels. Because of the importance of the copper-dependent ferroxidase activity in iron efflux, copper deficiency is likely to affect ferroxidase activity and thus iron homeostasis. Induced dietary copper deficiency studies in rodents cause symptoms of iron deficiency anemia, including low red blood cell count and low hemoglobin, hematocrit, and serum iron (Chen et al. 2006, Reeves *et al.* 2004). The decreased copper availability causes an inherent reduction in the abundance of copper-dependent proteins, such as Cp, which leads to larger overall copper and iron mobilization issues.

Studies on copper deficiency in mice and rats have generally shown some unique differences. Copper deficient mice have lower tissue copper concentrations, higher tissue-specific iron concentrations, lower serum Cp activity, and lower hephaestin activity in enterocytes compared to control mice (Chen et al. 2006). Pyatskowit and Prohaska reported that anemic copper deficient mice do not have low plasma iron concentrations. In contrast, copper deficient rats on the same dietary treatment exhibit anemia and low plasma iron levels (Pyatskowit & Prohaska 2008a). Anemic copper deficient rats also display higher ferroportin (Fpn), the iron exporter, levels in liver and

spleen. Interestingly, copper deficient mice do not display higher Fpn levels (Jenkitkasemwong *et al.* 2010).

Cp-/- humans and animals have no Cp activity, which leads to an accumulation of iron in the spleen, liver, and brain but with an overall systemic iron deficiency (Harris et al. 1999, Xu et al. 2004). Copper deficiency causes decreased Cp activity and protein and, under certain circumstances, leads to selective iron accumulation. This iron accumulation can be reversed by intravenous administration of active Cp, releasing iron into circulation from the liver (Tran *et al.* 2002). Likewise, multiple groups in the 1930s reported that the increase in tissue iron in rats during copper deficiency can be reversed by copper administration, which stimulated release of iron from storage tissues to plasma hemoglobin (Cook & Spilles 1931, Josephs 1932). The iron deficiency experienced by copper deficient rat pups can be lessened by injection with iron, restoring low serum iron and low hemoglobin levels to normal (Pyatskowit & Prohaska 2008b). Iron injection fails to reverse anemia in older copper deficient rodents, however (Reeves & DeMars 2006).

Copper deficiency in humans is rare, although slightly more common in children (Levy *et al.* 1985). Studies on nutritionally anemic infants and children with low copper levels were performed in the 1930s. Treatment with copper and iron was determined to be effective, much more so than iron treatment alone, particularly in infants with low hemoglobin (Josephs 1932). Copper deficiency in humans may be induced by excess zinc intake or through long-term parenteral nutrition. Because intake of copper and iron through the gut is important for proper absorption, bypassing the gut completely can cause copper deficiency. In these cases, oral copper therapy returns hematological parameters to near-normal levels (Karpel & Peden 1972). Zinc is also known to cause copper deficiency by inhibiting copper absorption and increasing copper secretion (Prasad *et al.* 1978). Zinc toxicity may be due to taking zinc supplements or, more recently discovered, using denture cream daily, which contains high levels of polymethyvinylether maleic acid calcium-zinc salt. Discontinuation of zinc intake returns hematologic values to normal (Willis *et al.* 2005).

Perturbations in systemic iron metabolism may arise because the metabolic pathways involving copper and irons are linked at least in part through multicopper ferroxidases like Cp, hephaestin, and zyklopen. The following studies were performed to analyze the presence of the membrane-anchored form of Cp (GPI-Cp) in multiple organs in response to dietary copper deficiency, as it was previously believed to be present in a limited number of cell-types. Ferroxidase activity of GPI-Cp in the spleen was verified and the response of the iron status marker, TfR, was analyzed to try to determine further the relationship between copper, ferroxidases, and iron in the mammalian body.

Table 1-1. Proteins important in the copper-iron interaction

Protein	Putative function in copper-iron interaction	Reference(s)
GPI-Cp	membrane-bound ferroxidase	(Patel *et al.* 2000)
sCp	circulating ferroxidase	(Osaki et al. 1966)
hephaestin	transmembrane ferroxidase predominantly in intestinal enterocytes	(Vulpe *et al.* 1999)
zyklopen	membrane-bound ferroxidase in placenta and mammary gland	(Chen et al. 2010)
CCO	Copper- and iron-dependent inner-mitochondrial membrane terminal respiratory chain oxidase	(Hamza & Gitlin 2002)
Ctr1	High affinity membrane Cu transporter	(Dancis et al. 1994)
Fet3p	transmembrane ferroxidase in yeast	(Askwith et al. 1994)

Glycolsylphosphatidylinositol-anchored ceruloplasmin, GPI-Cp; secreted ceruloplasmin, sCp; cytochrome *c* oxidase, CCO; copper transporter 1, Ctr1; ferro-O_2-oxidoreductase, Fet3p

CHAPTER 2

Glycosylphosphatidylinositol-linked ceruloplasmin is expressed in multiple rodent organs and is lower following dietary copper deficiency

Elise J. Mostad and Joseph R. Prohaska

Department of Biochemistry and Molecular Biology, University of Minnesota Medical School Duluth, USA

Address correspondence to:

Joseph R. Prohaska

Department of Biochemistry and Molecular Biology

University of Minnesota Medical School Duluth

1035 University Drive

Duluth, MN 55812

Phone: 218 726-7502

Fax: 218 726-8014

E-mail: jprohask@d.umn.edu

Short title: GPI-ceruloplasmin and copper status

Abbreviations used: Cp, ceruloplasmin; GPI-Cp, glycosylphosphatidylinositol-ceruloplasmin; CuA, copper adequate; CuD, copper deficient; CCS, copper chaperone for superoxide dismutase; NHI, non-heme iron; P24, postnatal day 24.

ABSTRACT

Ceruloplasmin (Cp), a multicopper ferroxidase, is expressed as both a secreted (sCp) plasma enzyme from liver and a membrane-bound glycosylphosphatidylinositol-anchored (GPI-Cp) splice variant protein. Cp is thought to be essential for iron mobilization as selective iron overload occurs in aceruloplasminemia in humans and in Cp null mice. Dietary copper-deficient (CuD) rodents have near total loss of Cp activity, severe loss of Cp protein, and develop anemia. Hepatic iron augmentation is often observed. Impact of CuD treatment on GPI-Cp has not previously been evaluated. In these studies, GPI-Cp was detected in purified membranes of multiple organs of rats and mice but not *Cp* -/- mice. Immunoreactive Cp protein was released with phosphatidylinositol phospholipase C treatment and expressed ferroxidase activity. Following perinatal and postnatal copper restriction, GPI-Cp was markedly lower in spleen and modestly lower in liver of CuD rats and mice, compared to copper-adequate (CuA) rodents. However, spleen non-heme iron was lower in CuD than CuA rats, and not different in CuD mice. Hepatic iron was higher only in CuD mice. Spleen and liver membranes of CuD rats expressed augmented levels of ferroportin, the iron efflux transporter, which may explain lower non-heme iron content in spleen of CuD rats despite a greater than 50% lower level of the multicopper ferroxidase GPI-Cp.

Key words: copper deficient, rat, mice, GPI-ceruloplasmin, iron

INTRODUCTION

Copper is an essential cofactor for numerous cuproenzymes known to catalyze key biochemical reactions. In general, these cuproenzymes participate in redox chemistry and impact many physiological processes such as pigmentation, catecholamine synthesis, superoxide metabolism, mitochondrial energy production, connective tissue integrity, and hematopoiesis (Prohaska 2006). Seminal work in rats described a copper requirement for growth and hemoglobin formation (Hart *et al.* 1928). Later it was shown that iron metabolism requires multicopper oxidases with ferroxidase activity to convert ferrous to ferric iron to facilitate binding to transferrin (Tf), the plasma iron transporter (Osaki 1966). It is believed that the anemia (low hemoglobin) observed in copper deficient rats is due to hypoferremia (Lee *et al.* 1968). An interaction between iron and copper was described in humans in the 19th century, but specific details still remain unknown (Collins *et al.* 2010, Fox 2003). For example, dietary copper deficiency in mice also leads to profound anemia but plasma iron levels are normal (Pyatskowit & Prohaska 2008a). Key in the molecular copper-iron hypothesis is the cuproenzyme ceruloplasmin (Cp).

Cp is an α-2-globulin that binds six atoms of copper which represents about 95% of the copper found in plasma. This glycoprotein was first characterized in 1947 by Holmberg and Laurell and determined to be synthesized and secreted into plasma by hepatocytes (Holmberg & Laurell 1947). Cp can oxidize several diamines but is thought to be essential for the ferroxidase pathway of iron mobilization (Osaki 1966). Consistent with this hypothesis was the observation that humans lacking *Cp* (aceruloplasminemia) accumulated excess iron in several organs including liver and brain (Harris *et al.* 1998). Experiments with *Cp*-/- mice confirmed these observations and challenged the idea that Cp was involved in copper transport and delivery because liver, spleen, and brain accumulated iron but had normal copper levels (Harris *et al.* 1999). Collectively these studies strongly supported the Cp ferroxidase hypothesis. Further, when copper is limiting in the diet, Cp activity is markedly lower and liver iron is elevated (Lee et al. 1968). However, *Cp* -/- mice, despite tissue-specific iron overload, do not display severe anemia (Cherukuri *et al.* 2004, Harris et al. 1999, Patel *et al.* 2002, Yamamoto *et al.* 2002). Thus, the mechanism for anemia in copper deficiency remains unknown.

Interestingly, accumulation of iron in the brain in aceruloplasminemia suggested the existence of another form of Cp, since plasma Cp presumably does not cross the blood-brain barrier. Previously Cp was characterized strictly as a secreted protein found in the plasma. It is now known that two isoforms of Cp exist, a secreted form (sCp) and a membrane-bound glycosylphosphatidylinositol-anchored form (GPI-Cp), first described in brain astrocytes (Patel & David 1997). GPI-Cp is generated by alternative splicing of exons 19 and 20, encoding a 1084 amino acid protein, whereas sCp has 1059 amino acids (Patel *et al.* 2000). GPI-Cp has also been reported on the plasma membrane of Sertoli cells and on the surface of leptomeningeal cells in the central nervous system (Fortna *et al.* 1999, Mittal *et al.* 2003). GPI-Cp in brain astrocytes and Sertoli cells of testes suggests a role for GPI-Cp in metabolism of iron at the blood-brain barrier and blood-testis barrier (Hellman & Gitlin 2002). However, little is known about GPI-Cp in other tissues or its precise role in iron biology and its role, if any, in the mechanism of anemia associated with copper deficiency.

Recent studies confirmed that dietary copper deficiency in rats and mice resulted in a 60-90% decrease in sCp protein abundance (Broderius *et al.* 2010). Perhaps this is due to enhanced degradation of unstable apoceruloplasmin (Holtzman & Gaumnitz 1970). A key objective of the current experiments was to assess the affect of dietary copper deficiency on membrane-bound GPI-Cp. A more thorough investigation of the impact of GPI-Cp on iron retention seemed prudent, as iron imbalance can have very serious physiological consequences besides anemia including diabetes, dementia, oxidative stress, and impaired cognitive development (Madsen & Gitlin 2007). The primary cells involved in iron recycling are splenic macrophages and liver Kupffer cells. *Cp*-/- humans and mice accumulate iron in these macrophages. It is not clear whether reduction of sCp observed in dietary copper deficiency or a putative alteration in GPI-Cp might also impact iron export from spleen and liver.

Current experiments were designed to evaluate GPI-Cp in multiple organs using rodents as models with an emphasis on organs where iron is stored and excreted, spleen and liver. Impact of dietary copper limitation and related variables (age, gender, species, and rat strain) were analyzed. Data demonstrated GPI-Cp is expressed in multiple organs

of rodents, including liver, spleen, kidney, heart and brain. Furthermore, following dietary copper deficiency GPI-Cp expression in spleen, and to a lesser extent liver, was lower. However, it was also determined that lower GPI-Cp was not correlated with augmented non-heme iron (NHI) levels.

MATERIALS AND METHODS

Animal care and dietary treatments

Sperm-positive Holtzman rats were purchased from Harlan Laboratories Inc. and weanling male and sperm-positive Sprague Dawley rats and sperm-positive Swiss Webster mice were purchased from Charles River. Rodents were offered either a copper-deficient (CuD) or copper-adequate (CuA) dietary treatment consisting of a CuD modified AIN-76A diet, or CuD and CuA modified AIN-93G diet (Teklad Laboratories). The modified AIN-76A diet contained 0.32 mg Cu/kg and 47 mg Fe/kg by analysis. All Holtzman dams and offspring were fed this CuD diet. CuA groups drank water supplemented with cupric sulfate, 20 mg Cu/L, and CuD groups drank deionized water. This perinatal Holtzman rat experiment is referred to as Experiment 1 (Exp. 1). Sprague Dawley rats and Swiss Webster mice were fed the modified AIN-93G diet containing 0.46 mg Cu/kg and 78.3 mg Fe/kg (CuD diet) or 8.73 mg Cu/kg and 80.3 mg Fe/kg (CuA diet). Rodents on the modified AIN-93G diets were given deionized water to drink. The Sprague Dawley rat experiment is referred to as Experiment 2 (Exp. 2). A third experiment (Exp. 3) began with weanling male Sprague Dawley rats offered CuA, CuD, or an additional modified AIN-93G iron deficient (FeD) diet, containing 6.2 mg Fe/kg and 7.0 mg Cu/kg. Further details of the diets used and animal husbandry are published elsewhere (Bastian *et al.* 2010, Pyatskowit & Prohaska 2008a). All animals were maintained at 24 ˚C with 55% relative humidity on a 12-h light cycle (0700-1900 h). All protocols were approved formally by the University of Minnesota Animal Care Committee.

Holtzman rat dams (Exp. 1) were placed on dietary treatments on embryonic day 7, Sprague Dawley rat dams (Exp. 2) on embryonic day 2, and Swiss Webster mouse dams on embryonic day 17. Offspring in all cases were weaned at postnatal day 20 (P20) and

continued on dietary treatment of their respective dams. Exp. 3 male weanling rats began their respective postnatal CuD, CuA, or FeD diets at P21.

One representative Exp. 1 male rat pup from each litter (n=4, CuA and CuD) was anesthetized by ketamine/xylazine injection and killed by cardiac puncture on P25, and on P26 a sister was killed from each litter in the same manner. For Exp. 2, CuA and CuD P25 (n=4 each) male rat pups were killed by decapitation. For Swiss Webster mice, CuA and CuD males (n=4 each) were killed on P27 by decapitation. For Exp. 3, CuD and CuA male rats (n=3 each) were killed at one, two, and four weeks after onset of dietary treatments. At P35, three FeD rats were killed. These rats were anesthetized with ketamine/xylazine injection and killed by cardiac puncture. Appropriate tissues from all rodents were harvested, quick frozen in liquid nitrogen, and stored at -70 ˚C for analyses. Dr. Z. L. Harris, Vanderbilt University, kindly provided tissues and plasma from C57BL mice of two genotypes ceruloplasmin null (*Cp*-/-) and wild-type controls (*Cp*+/+) (Harris et al. 1999).

Biochemical analyses

A 5 μL aliquot of blood from each rodent was mixed with Drabkin's reagent to measure hemoglobin spectrophotometrically (Prohaska 1991). A piece of liver was wet-digested with HNO_3 (Trace Metal grade; Fisher Scientific) and analyzed for total copper and iron content by flame atomic absorption spectroscopy (Model 1100B, Perkin-Elmer) (Prohaska 1991). Plasma was treated with hot trichloroacetic acid and iron was measured by flame atomic absorption spectroscopy (Pyatskowit & Prohaska 2008c). Non-heme iron (NHI) levels in spleen and liver were determined colorimetrically after acid extraction of tissues (Torrance & Bothwell 1968). This is necessary, particularly for spleen, where much of the iron is bound to heme from hemoglobin degradation. Protein concentration of membrane and cytosolic extracts was determined using a modified Lowry method (Markwell *et al.* 1978). Plasma Cp activity was assessed by measuring plasma diamine oxidase activity using *o*-dianisidine as substrate (Prohaska 1991). Ferroxidase activity was determined on spleen membrane proteins digested with phospholipase C as described below after separation on non-denaturing gels and transfer to PVDF membranes as described previously for plasma (Broderius et al. 2010).

Preparation of membrane fractions

Membrane samples for Western blot analysis were prepared by homogenizing frozen rat or mouse spleen, liver, kidney, heart, and cerebellum in 9 volumes of homogenization buffer containing 10 mM HEPES, 0.1 M KCl, 0.04 M mannitol and 1 mM EDTA (pH 7) with the addition of protease inhibitors (Protease Inhibitor Cocktail; Sigma Aldrich) using a T25 high speed digital homogenizer (IKA Works, Inc., Wilmington, NC, USA). Homogenates were centrifuged at 2000 ***g*** for 10 min at 4 ˚C and supernatant fractions were centrifuged again at 100000 ***g*** for 30 min at 4 ˚C. Supernatant from this spin was saved as the cytosolic extract and the pellet was washed with homogenization buffer and recentrifuged at 100000 ***g*** for 30 min at 4 ˚C, discarding supernatant. Pellets were resuspended in 10 mM potassium phosphate buffer (pH 6.8) containing 4% SDS and 0.2% Triton X-100 and are referred to as membrane extracts.

PI-PLC treatment

Selected membrane pellets were suspended in 100 µL of 50 mM Tris-HCl (pH 7.4) containing 10 mM EDTA. These solutions were treated with either 0.7 units of specific phopholipase C (PI-PLC) (P5542, Sigma Aldrich) or 0.1 µg of bovine serum albumin (BSA) as negative control (Sigma Aldrich) (Shen *et al.* 1994). Pellet suspensions were incubated at 37 ˚C for 1 hr with mixing every 15 min. Extracts were spun at 100000 ***g*** for 15 minutes and the supernatant was removed and saved as the released protein fraction. The treated membrane pellet was washed, the supernatant discarded, and the pellet was resuspended in membrane extraction buffer. Aliquots of the resuspended treated membrane fractions and released extracts were used for protein determination and Western immunoblots.

Analysis of mRNA expression in rat tissues

Total RNA was isolated chemically from fast frozen rat tissues using TRI reagent (Ambion, Austin, TX, USA) following manufacturer recommendations including suggested optional steps. Concentration of purified RNA was measured with a Nanodrop spectrophotometer and integrity was evaluated by denaturing agarose gel electrophoresis. DNAse treatment used DNA-free kit (Ambion) and cDNA was synthesized with Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). Tissue cDNA was

amplified and quantified by qRT-PCR using Rotor-Gene SYBR Green PCR Kit (Qiagen) and Corbett RotorGene RG-3000 to determine C_T values. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Agarose gel analysis showed each primer pair to yield a single band of the expected size. Each PCR reaction contained cDNA synthesized from 15 ng total RNA in a 12.5 µl total reaction volume. sCp and GPI-Cp primers were those used previously for rat kidney experiments (Wiggins *et al.* 2006). C_T values were confirmed with another set of specific primers for sCp and GPI-Cp used for mouse retina (data not shown) (Stasi *et al.* 2007). Hephaestin primers designed for qRT-PCR based on NM_133304, were reverse: 5'-TCC CAG CTT CTG TCA GGG CAA TAA-3' and forward: 5'-TGT TCC AGT CAT CAA GAC AGC CCA-3'. Amplicon size was 107 bp. Forward and reverse primers used for GAPDH analysis produced an 87 bp product (Vandesompele *et al.* 2002).

SDS-PAGE and immunoblotting

Membrane proteins were mixed with SDS Laemmli sample buffer without boiling and electrophoretically fractionated by SDS-PAGE on 10% gels. Cytosolic samples were treated similarly, but first boiled for 5 min and fractionated on 15% gels. Separated proteins were transferred to a Protran BA 83 nitrocellulose membrane (Whatman) and treated as described previously to detect the appropriate antigens (Broderius et al. 2010). Immunoreactivity was visualized using SuperSignal chemiluminescence substrate (Thermo Scientific). Chemiluminescence detection and densitometry were carried out using the FluorChem™ system (Alpha Innotech, San Leandro, CA, USA).

Antibodies

The following antibodies were purchased commercially and used at appropriate dilutions: anti-mouse actin (MAB1501, Millipore); mouse anti-rabbit sodium potassium ATPase (Na/K ATPase) (ab7671, Abcam); mouse anti-human transferrin receptor (TfR) (13-6800, Zymed Laboratories); goat anti-human ceruloplasmin (C 0911, Sigma Aldrich); and goat anti-rabbit lactate dehydrogenase (LDH) (AB1222, Millipore). Affinity purified rabbit anti-human copper chaperone for superoxide dismutase (CCS) and rabbit anti-rat/mouse ferroportin (Fpn) were characterized previously (Jenkitkasemwong *et al.* 2010, West & Prohaska 2004).

Statistical analysis

Means ± SEM were calculated. Student's unpaired two-tailed *t*-test was used for comparison of data between two dietary treatments (α=0.05) and *F*-test was used to determine variance equality. Data were processed using Microsoft Excel™. Experiments comparing CuD, FeD, and CuA data were evaluated by one-way ANOVA and Tukey's test after variance equality was evaluated by Bartlett's test (KaleidaGraph, Synergy Software).

RESULTS

Characterization of membrane-anchored Cp

Membranes were isolated from multiple organs of two CuA mouse dams and detergent-extracted proteins were separated by SDS-PAGE and Cp was detected by immunoblotting. When probed with polyclonal anti-human ceruloplasmin (Cp) antibody, a robust band in both mice was detected with an apparent molecular weight of 130 kDa for spleen, liver, kidney, and heart. Cp protein was more abundant in spleen and kidney than in liver and heart (Figure 2-1A). Mobility of the Cp in membrane extracts was faster than plasma Cp, used as a control, indicating unique features. The immunoblot was reprobed for lactate dehydrogenase (LDH) and no bands were detected, suggesting the organ preparations were not contaminated with plasma (data not shown). No immunoreactive Cp was detected in isolated rat erythrocyte ghosts (data not shown) suggesting that splenic Cp was not due to red cell membrane contamination. Further studies on a pair of four month old male mice, *Cp*+/+ or *Cp*-/-, were done to confirm that immunoreactivity was indeed Cp. Membranes from multiple organs of these mice were extracted and immunoreactivity was not detected in *Cp*-/- mouse membranes or plasma (Figure 2-1B). Cerebellum and heart tissues have less dense membrane Cp bands and spleen the most dense. Actin (44 kDa) was used as a loading control for this blot and all other Cp blots. However, robust actin bands were not detected for male rat liver membrane preparations. Actin density was tissue-specific, but not impacted by Cp genotype (Figure 2-1B).

Next, additional experiments were done with rat membranes to extend mouse data and to determine if the detected Cp protein was GPI-anchored. Spleen, liver, kidney, and heart membrane pellets from a P25 CuA male rat, Exp. 2, were treated with either PI-PLC or a negative control protein (BSA) (Figure 2-1C). Membrane fractions were processed to separate the treated membrane fraction pellet from released supernatant extracts. Resuspended PI-PLC-treated membrane fractions displayed less remaining immunoreactive Cp than BSA-treated fractions (Figure 2-1C). GPI-Cp released from the membrane fraction was recovered in the extract of these four rat organs and suggests that immunoreactive Cp was GPI-anchored in these membranes. Released extracts from spleen and kidney possessed ferroxidase activity in a gel/membrane assay (data not shown) (Broderius et al. 2010). Actin abundance was not impacted by PI-PLC treatment. Note weak actin signal in the liver.

Relative mRNA expression levels for three multicopper oxidases including both the secreted form (sCP) and the GPI-anchored form of Cp were determined in multiple organs of a representative P27 male CuA rat by qRT-PCR. Primers, designed by others, specific to each of the forms of Cp were used (Supplemental Table 2-1). Secreted Cp, as expected, was expressed highest in the liver. Importantly, GPI-Cp was expressed in all of the organs tested and had its highest expression in the kidney, consistent with immunoblot data. Hephaestin, another multicopper oxidase, had highest expression in enterocytes, consistent with previous data (Vulpe *et al.* 1999).

Confirmation of copper deficiency

One mouse model and three rat models of dietary copper deficiency were examined to determine if alterations observed in sCp levels might occur with GPI-Cp (Broderius et al. 2010). The perinatal models of Cu deficiency (mouse experiment and rat Exps. 1 and 2) produced CuD pups with features consistent with severe deficiency. CuD pups were smaller in size than CuA rodents (Table 2-1). Hemoglobin levels for these CuD rodents were significantly lower than their CuA counterparts, ranging between 57-71% of CuA values. Liver copper levels were markedly lower in all CuD rodents. Liver iron was 104% higher in CuD than CuA mice. For CuD rats, the liver iron data were less clear. In fact, the reason that CuA rats in Exp. 1 had lower liver iron than CuD rats may be due to

lower dietary iron rather than copper deficiency, since male P25 CuA pups in Exp. 2 had iron levels greater than CuA pups in Exp. 1. Serum iron concentration was significantly lower in CuD male rats but not altered in CuD mice. Plasma diamine oxidase activity of all CuD rodents was non-detectable (data not shown). Collectively, CuD rats and mice displayed signs characteristic of CuD rodents noted previously (Pyatskowit & Prohaska 2008a).

Copper chaperone for superoxide dismutase (CCS) immunoblots were prepared using the cytosolic fraction of the membrane preparations to evaluate the copper status of spleen and cerebellar tissue as limited sample size prevented metal determination (Figure 2-2). Previous work indicated that tissue CCS levels were higher following copper deficiency (West & Prohaska 2004). CCS bands (37 kDa) from CuD samples were markedly denser than corresponding bands from CuA samples, verifying copper deficiency in the CuD rodents. Actin density confirmed equal loading for each immunoblot and was not impacted by copper status.

Impact of copper deficiency on GPI-Cp in rats

Liver and spleen were organs of particular interest to evaluate regarding copper status and GPI-Cp expression. Spleen membranes from CuD and CuA male rat pups from Exp. 1 (Figure 2-3A) and Exp. 2 (Figure 2-3B, C, D) were examined. Immunoblots from both experiments showed a significant decrease in GPI-Cp abundance in CuD rats compared to CuA controls, 63% and 49% lower respectively (Figure 2-3A and 2-3B). These very similar results were obtained using two different CuD diets and two different rat strains. Interestingly, non-heme iron (NHI) was significantly lower in spleen of CuD rats to a similar degree in both experiments, suggesting a disconnect between the NHI and GPI-Cp function. Spleen membranes from another set of littermates in Exp. 2 were used to study release of GPI-Cp and were also probed for GPI-Cp, actin, and ferroportin (Fpn). Spleens of these CuD rats have lower GPI-Cp, 64%, but higher Fpn, 37%, than CuA rats (Figure 2-3C). The PI-PLC released material was detected with anti-Cp antibody following separation on a non-denaturing gel. Ferroxidase activity could be detected in the CuA but not CuD samples confirming both lower protein abundance and impaired activity of GPI-Cp following copper deficiency (Figure 2-3D).

The response of GPI-Cp to dietary copper deficiency was also examined in male rat liver for both Exp. 1 and 2 (Figure 2-4). Results were not as definitive as those for the spleen. Abundance of GPI-Cp tended to be lower in CuD rats in both experiments, but was not statistically different. Sodium-potassium ATPase was used as a loading control for the liver membrane samples but it was apparent that proper loading could not be determined measuring this protein. Tubulin also appeared to be impacted by diet (data not shown). Recall that actin blotting also was not acceptable for male rat liver membranes. Ponceau S staining suggested equal loading of both immunoblots (data not shown). Membrane extracts were also probed for Fpn from livers of rats in Exp. 2 (Figure 2-4B). Robust enhancement of Fpn expression, 74%, was detected in CuD compared to CuA rats, $P < 0.05$.

Membrane extracts from P26 Exp. 1 female rats were evaluated to compare with male rat data (Figure 2-5). GPI-Cp density was 45% lower in the CuD female spleen. Liver GPI-Cp membrane extracts followed the same trend as the young male rats, appearing lower but not statistically significant. Cerebellum samples from P26 females showed no difference in GPI-Cp expression between CuD and CuA rats. CCS results verified the extent of copper deficiency in the cerebella of CuD female rats (Figure 2-2). Actin was used as a loading control for all female membrane preparations and was not impacted by copper status.

Impacts of perinatal copper deficiency on GPI-Cp in mice

Spleens and livers of P27 CuD and CuA male Swiss Webster mice were evaluated for GPI-Cp following perinatal copper deficiency to compare with rat data. Spleen GPI-Cp mouse expression confirmed data in rats; levels in CuD mice were 44% lower than CuA (Figure 2-6A). Spleen NHI levels were not significantly affected by copper deficiency in mice. Like CuD rat liver, CuD mouse liver tended to have a modest reduction in GPI-Cp, though not significant (Figure 2-6B). However, an overall statistical evaluation of liver immunoblot densities for GPI-Cp of all CuD and CuA rodent comparisons resulted in a robust difference ($P< 0.01$) between the treatment groups, consistent with spleen data. Actin was an effective loading control in mouse tissues and demonstrated equal loading.

Postnatal copper deficiency

The preceding studies were performed on rodents with severe copper deficiency following perinatal treatments. CuD rodents had frank anemia and reduced body weights (Table 2-1). Exp. 3 was conducted with older postweanling rats to determine how quickly a change in GPI-Cp might occur following a CuD or iron deficient (FeD) diet. Following four weeks of treatment, CuD rats had modest signs of copper deficiency, mild anemia, and no differences in body weight compared to the CuA rats (data not shown). Rats were sampled one, two, and four weeks after diet treatment. Spleen and liver membrane extracts were evaluated at P35 (Figure 2-7A). Spleen GPI-Cp abundance was markedly lower (65%) in CuD samples following just two weeks of treatment, confirming and extending data in the perinatal rat and mouse models. In liver, but not in spleen, GPI-Cp abundance was actually higher in FeD rats, $P < 0.05$. Iron regulation of transferrin receptor (TfR) appears to be proper only in liver as a robust enhancement is evident in the FeD samples, consistent with the very low NHI levels (Figure 2-7A). Interestingly, in spleen, despite low levels of NHI in both CuD and FeD rats, TfR expression was not augmented. Reduction of spleen GPI-Cp abundance in CuD rats to less than 50% of levels in CuA rats occurred less than one week after diet-induced copper deficiency (Figure 2-7B). After 4 weeks on the CuD diet, spleen GPI-Cp of the CuD rats was less than 20% of the CuA values. Liver copper content displayed a similar response as spleen GPI-Cp results. After the first week on the CuD diet, liver copper levels dropped to less than 60% of the CuA values and after 4 weeks, liver copper levels were less than 40% of CuA values. Although liver GPI-Cp for P35 CuD rats was similar to CuA levels (Figure 2-7A), levels for CuD rats at P49 were 53% lower than CuA values $P < 0.05$ (data not shown). The copper deficient state of both spleen and liver from CuD rats was verified when cytoplasmic CCS levels were evaluated (Figure 2-7C). Cp diamine oxidase activity was not detectable in the CuD rat plasma. Cp activity in the P35 FeD rats, 78.2 ± 7.3 units/L, was not significantly different than CuA rats, 90.2 ± 7.5, $P > 0.05$.

DISCUSSION

Numerous investigations have probed factors that impact expression and function of secreted plasma ceruloplasmin (sCp). Results in the current studies with rat and mouse

tissues clearly demonstrate both mRNA and protein for the membrane bound form of this multicopper oxidase (GPI-Cp). Previous studies detected Cp mRNA in numerous tissues, including lung, spleen, testes, placenta, yolk sack, in addition to robust expression in liver (Hellman & Gitlin 2002). Our data confirm and extend those observations, and those made by others, who have reported the presence of the GPI-anchored form of Cp in selected cells. In particular, GPI-Cp has been reported in brain astrocytes and leptomeningeal cells, murine retina, rat Sertoli cells, rat kidney parietal epithelial cells, and human peripheral lymphocytes, especially Natural Killer cells (Banha *et al.* 2008, Fortna et al. 1999, Patel & David 1997, Stasi et al. 2007, Wiggins et al. 2006). It was speculated that the presence of GPI-Cp in brain, kidney, and retina corresponds to blood-tissue barrier trafficking of iron. Data from the current investigation suggests that GPI-Cp is present in tissues known to have a role in iron trafficking, liver and spleen macrophages. It is well known that the iron exporter ferroportin (Fpn) is critical for iron efflux. It was clearly demonstrated that the *Fpn* knockout mouse accumulated iron in the absorptive enterocyte, splenic macrophage, and liver Kupffer cell (Donovan *et al.* 2005). It is proposed that GPI-Cp helps facilitate iron efflux in macrophage-like cells in conjunction with Fpn in a parallel manner to the multicopper oxidase hephaestin, a Cp homologue, found predominantly in enterocytes (Collins et al. 2010). Mutations in hephaestin, documented in the *sla* mouse, lead to iron retention in enterocytes presumably due to a block in intestinal iron efflux (Vulpe et al. 1999).

Liver is a major homeostatic organ for copper biology. One important function is to synthesize and secrete sCp. It is well known that the liver of copper deficient rats and mice accumulate iron predominately either as ferritin or hemosiderin (Welch *et al.* 2007). As mentioned previously, aceruloplasminemia leads to a lack of iron recycling through Kupffer cells because of a decrease in ferroxidase function. Recent data from our lab on both copper deficient rats and mice confirmed earlier work that the predominant form of Cp in the plasma following copper deficiency is the apo form of the protein lacking ferroxidase activity (Broderius et al. 2010). Earlier work in copper deficient rats and in mice with a deletion of the copper transporter Ctr1 in intestine had convincingly shown that apo-Cp was the major plasma form following copper limitation (Holtzman &

Gaumnitz 1970, Nose *et al.* 2006). Limited sCp function may restrict iron efflux. Data in the current experiments further extend the possibility that iron retention in the liver is due to a decrease in GPI-Cp. Lower GPI-Cp abundance in hepatic tissue of both CuD rats and mice is reported, albeit only modestly impacted. Perhaps though, the GPI-Cp that is detected does not possess full ferroxidase activity as suggested by the current data on spleen membrane GPI-Cp. This apparent decrease in hepatic GPI-Cp abundance is likely not related to the iron content of the hepatocytes. Two perinatal rat experiments were conducted and there was a difference in liver iron concentration in only one; yet, both showed a similar reduction of liver GPI-Cp abundance.

In the current experiments, liver iron was only augmented in CuD mice. This was verified by measurement of total iron and reflected in the decreased expression of TfR in the CuD mouse liver (not shown). Diets used in Exp. 2 and 3 contained more iron and may explain why there was no apparent difference in liver iron in those rats. It is possible that the iron in CuD rat liver is trapped in hemosiderin and not available for rapid export (Welch et al. 2007). Though this liver iron data is somewhat confusing, it confirms recent interesting observations of differing responses to copper deficiency between rats and mice in regard to iron homeostasis. For example, copper deficient rats experience hypoferremia whereas copper deficient mice do not (Pyatskowit & Prohaska 2008a).

Splenic macrophages are key in iron recycling that occurs when phagocytized erythrocytes are processed by the spleen (Collins et al. 2010). Current data suggest that spleen contains robust expression of GPI-Cp and that abundance is dependent on adequate copper. Significant (~50%) reductions in the expression of GPI-Cp in spleen were observed in several different CuD rat and mouse models ranging from severe to modest copper deficiency. Though the cell specific expression was not confirmed in these studies, we presume that GPI-Cp is macrophage associated. Previous studies suggested that mouse macrophage bone marrow cells express GPI-anchored Cp (De Domenico *et al.* 2007). Recent work in CuD rats and mice detected residual levels of sCp in plasma but was unable to detect ferroxidase activity (Broderius et al. 2010). It is possible that the residual GPI-Cp in spleen of CuD rodents has ferroxidase activity but

our gel assay detected none. Interestingly, in the current studies, with diminished levels of GPI-Cp in spleen, there was no concomitant retention of iron. In fact, lower NHI was detected in all CuD splenic samples from rats. There was no difference in NHI in spleens of CuD mice. Once again, it is not known which cells contain the majority of non-heme iron in spleen, but it is likely to be splenic macrophages, the same cells which express GPI-Cp. Perhaps this disconnect in CuD rats between the abundance of GPI-Cp, a relative reduction, and non-heme iron, a relative reduction, can be explained by the recent observation that Fpn abundance is higher in spleen of copper deficient rats (Jenkitkasemwong et al. 2010). That same study showed that mouse Fpn abundance was not changed following copper deficiency. Our current data also detected higher Fpn expression in both CuD liver and spleen using a new diet that eliminated hepatic iron differences.

Higher expression of splenic and hepatic Fpn was observed concurrently with decreased expression (and presumably activity) of the multicopper oxidases sCp and GPI-Cp (current data) (Broderius et al. 2010, Jenkitkasemwong et al. 2010). Others based on *in vitro* experiments suggest that Fpn abundance depends on active multicopper oxidases, so as to prevent internalization and degradation via the proteosome (De Domenico et al. 2007). That theory would predict lower Fpn abundance in CuD tissues. An alternate hypothesis was suggested that higher Fpn in the spleen and liver of copper deficient rats was due to near total loss of hepcidin expression (Jenkitkasemwong et al. 2010). Hepcidin binding to Fpn is also a mechanism to control the steady state levels of Fpn protein and iron flux (Nemeth *et al.* 2004). In support of the disconnect between lower multicopper oxidase and higher Fpn expression is work with copper deficient mice from others that showed a higher expression of enterocyte Fpn concomitant with a decrease in hephaestin, another multicopper oxidase, and also lower hepcidin mRNA (Chen *et al.* 2006).

GPI-Cp was first characterized in mammalian brain astrocytes (Patel & David 1997). Current experiments detected a relatively low abundance of GPI-Cp in rat cerebellum. Further, unlike spleen and liver, abundance of GPI-Cp in rat cerebellum was not different between CuD and CuA samples. Tissue culture studies with brain glial cells treated with

the copper chelator, bathocuproine disulfonate (BCS), demonstrated loss of membrane bound Cp (De Domenico et al. 2007). Thus, potentially a severe copper deficiency in brain could result in decreased expression of GPI-Cp as apparently it does in liver and spleen. Ferroxidase function of brain GPI-Cp may still be compromised by dietary copper deficiency even though the steady state levels of protein are not impacted. This decreased function may compromise iron efflux from astrocytes to neurons and create a severe functional iron deficiency in brain. Further work on this speculative hypothesis is necessary. Additional work is also necessary to explore the putative function of GPI-Cp in organs such as heart and kidney that express GPI-Cp and currently are not known to have major functions in iron recycling. Research is needed to determine the mechanism for lower steady-state levels of GPI-Cp protein following copper deficiency. Seminal work on sCp and copper deficiency would suggest that transcriptional control is unlikely (McArdle *et al.* 1990, Gitlin *et al.* 1992). Studies on turnover rates of GPI-Cp have not been published.

As recently reviewed, it is well known that following iron deficiency there is copper accumulation in the liver (Collins et al. 2010). Could this accumulation of hepatic copper have an impact on ceruloplasmin synthesis, secretion, and expression? Others found changing iron status in a rat model had little or no effect on levels and activity of sCp nor expression of mRNA in the liver (Tran *et al.* 2002). Current studies evaluated an acute model of iron deficiency in the growing rat. These animals were anemic and had very low levels of iron in liver and spleen. Interestingly sCp activity was not statistically impacted; however, the abundance of liver GPI-anchored Cp was actually higher in these rats. The state of iron deficiency was readily evident by the upregulation of TfR in liver, but not in spleen despite very low non-heme iron content. This suggests the possibility that the TfR in spleen is reflecting a lymphocyte population rather than a macrophage population.

Aceruloplasminemia in humans and deletion of *Cp* in mice both lead to eventual tissue iron overload (Harris 2003). However, 10-12 week old *Cp* -/- mice do not display elevated iron in spleen, whereas at one year of age there is nearly 5-fold higher spleen iron (Meyer *et al.* 2001). Thus, it is not too surprising that spleen of four week old CuD

rodents in the current experiments also did not display augmented iron levels, despite marked reduction in spleen GPI-Cp expression. Plasma sCp levels are lower by 60-90% in these CuD rodents (Broderius et al. 2010). Also, somewhat confusing for the ferroxidase hypothesis of iron efflux is the observation that CuD and *Cp* -/- mice have normal plasma iron levels (Harris et al. 1999, Pyatskowit & Prohaska 2008a). Clearly, further research is needed to determine the impact, if any, of lower GPI-Cp on iron flux and the development of anemia when copper is limiting.

Author contributions: EM and JP participated in the design of the studies. EM conducted most the technical aspects with help from JP. Both EM and JP evaluated data and wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Dr. Z. Leah Harris, Vanderbilt University, for the generous gift of *Cp* +/+ and *Cp* -/- tissues, Dr. Mitchell Knutson for ferroportin antibody, and Margaret Broderius for technical assistance. This work was supported, in part, by National Institutes of Health grants [R01-HD039708 and R03-HD055423] and the Integrated Biosciences Graduate Program.

Table 2-1. Characteristics of Rats and Mice Following Copper Deficiency

	CHARACTERISTIC				
Diet	BW (g)	Hb (g/L)	L-Cu (μg/g)	L-Fe (μg/g)	S-Fe (μg/mL)
			Exp. 1 Male rats		
CuA	78.6 ± 3.1	108 ± 3.6	6.86 ± 0.81	32.3 ± 2.35	4.66 ± 0.68
CuD	59.1 ± 8.4*	69.4 ± 3.0*	0.40 ± 0.11*	60.1 ± 6.2*	0.78 ± 0.12*
			Exp. 1 Female rats		
CuA	71.5 ± 4.1	124 ± 8.3	6.86 ± 0.64	76.2 ± 8.9	N/A
CuD	53.2 ± 3.2*	70.5 ± 6.1*	0.42 ± 0.10*	68.3 ± 5.3	N/A
			Exp. 2 Male rats		
CuA	73.2 ± 3.8	128 ± 6.2	8.80 ± 0.69	60.3 ± 18.0	5.34± 0.57
CuD	62.8 ± 5.9	90.6 ± 3.8*	0.63 ± 0.02*	55.1 ± 4.2	1.02± 0.32*
			Male mice		
CuA	22.7 ± 1.4	138 ± 3.4	4.25 ± 0.16	113 ± 17	3.53± 0.10
CuD	15.2 ± 0.7*	82.3 ± 8.3*	1.85 ± 0.27*	230 ± 43*	4.24± 0.66

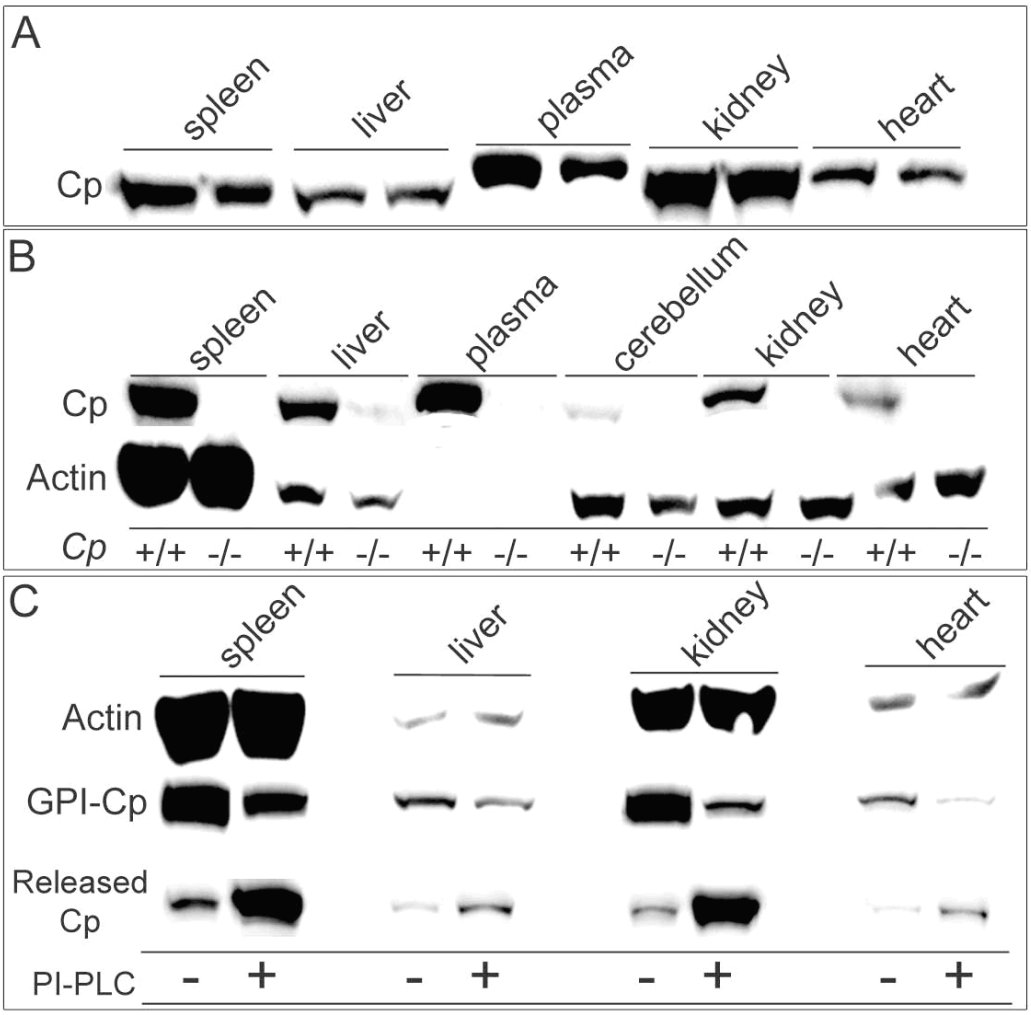
Values are means ± SEM (n=3 or 4) for P25-P27 rats and mice. Body weight (BW), hemoglobin (Hb), liver copper (L-Cu), liver iron (L-Fe), and serum iron (S-Fe) were determined in copper-adequate (CuA) or copper-deficient (CuD rodents as described in Methods. *Different from Cu-adequate (CuA) within group, $P < 0.05$ (Student's *t*-test).

Supplemental Table 2-1. Comparison of rat organ mRNA levels of secreted-ceruloplasmin, GPI-ceruloplasmin, hephaestin, and glyceraldehyde 3-phosphate dehydrogenase

Organ	*sCp*	*GPI-Cp*	*Heph*	*GAPDH*
Spleen	21.7	19.6	26.8	19.2
Liver	14.7	19.0	28.0	15.6
Cerebellum	22.4	19.1	26.3	15.6
Kidney	21.5	17.7	25.6	15.7
Heart	24.0	20.6	25.6	14.5
Testes	22.1	20.1	26.3	17.2
Enterocyte	19.9	23.1	22.0	16.6
Muscle	24.7	21.1	27.6	13.8

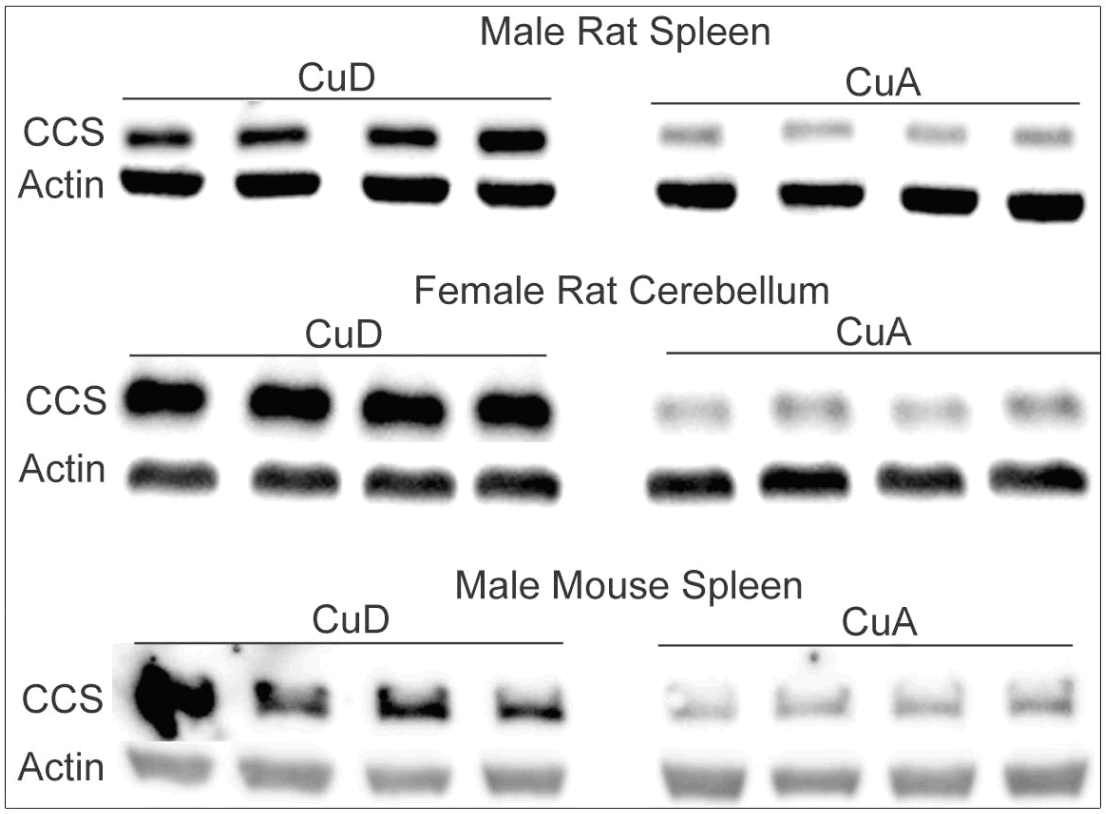
RNA was extracted from organs of a representative month old male copper adequate Sprague Dawley rat that weighed 98g. Total RNA was isolated from 8 organs and mRNA abundance was quantified using qRT-PCR. C_T values were determined for the secreted form of ceruloplasmin (sCp), for GP-anchored ceruloplasmin (GPI-Cp), for hephaestin (Heph) and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using primers specific for each mRNA.

Figure 2-1 Characterization of rodent GPI-Ceruloplasmin



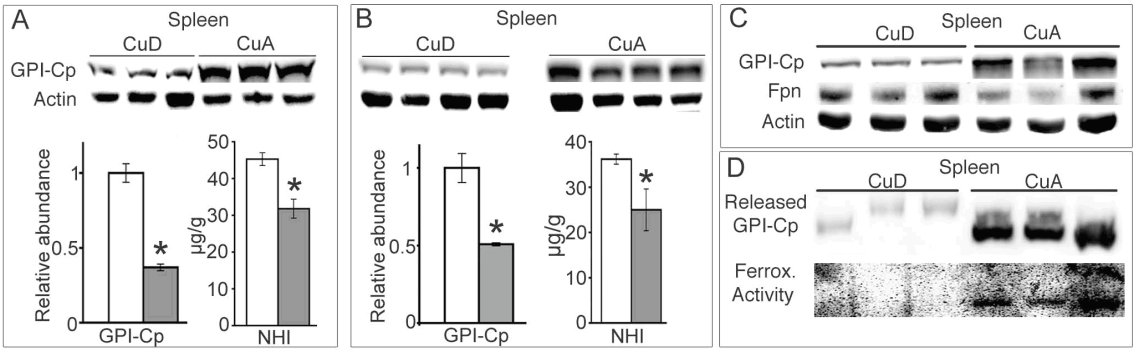
(**A**) Membranes from multiple organ samples from two CuA mouse dams were isolated and membrane extracts (50 μg/lane) were separated by 10% SDS-PAGE. Cp (130 kDa) abundance was analyzed by immunoblot. (**B**) Multiple organs from two male mice, *Cp* +/+ and *Cp* -/-, were evaluated as described above. Actin (44 kDa) was used as a loading control. Mouse plasma 1 μL was also processed for each blot. (**C**) Exp. 2 P25 male rat multiple membrane samples were incubated with either 0.7 units of PI-PLC (+) or negative control (-) for 1 hr. The treated membrane fractions (GPI-Cp) and the released protein fractions (Released Cp) were subjected to immunoblot analysis of Cp.

Figure 2-2 Verification of copper status in multiple rodent tissues by CCS abundance



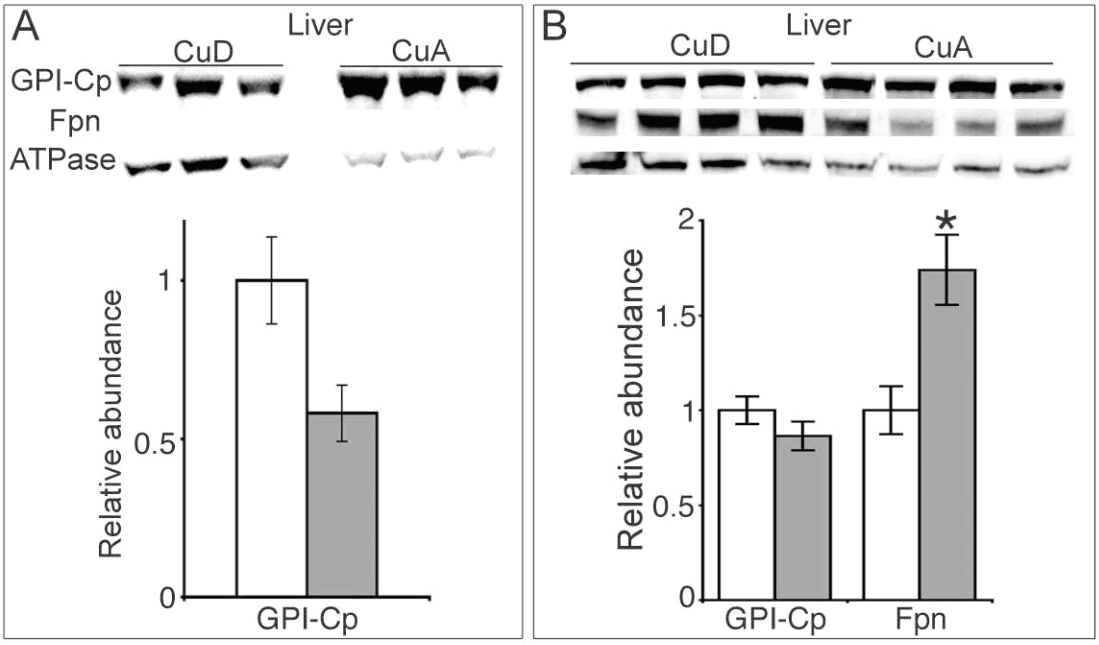
Cytosolic fractions (20 µg/lane) were separated on 15% SDS-PAGE and subjected to Western immunoblot protocols. Exp. 2 P25 male spleens, Ex. 1 P26 female cerebellum, and P27 mouse samples were compared for abundance of copper chaperone for superoxide dismutase (CCS) (37 kDa) to verify copper status. Blots were reprobed for actin.

Figure 2-3 Impact of copper status on spleen GPI-Cp in male rats



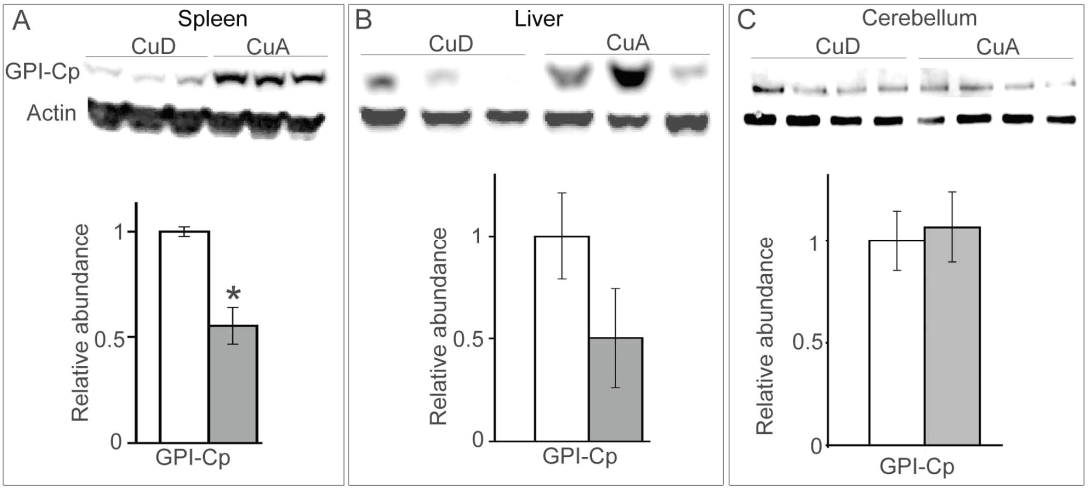
Spleen membrane extracts (50 µg/lane) from (**A**) CuD and CuA (Exp. 1 P25) and (**B**) CuD and CuA (Exp. 2 P25) were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were reprobed for actin. Non-heme iron (NHI) was determined spectrophotometrically. White bars represent CuA rats, gray bars represent CuD rats in all bar graphs. An asterisk indicates significant mean differences, $P < 0.05$. (C) Spleen membrane extracts (50 µg/lane) from additional male rats in Exp. 2 were subjected to Western immunoblot protocols for GPI-Cp, Fpn, and actin. Abundance of GPI-Cp was significantly lower and Fpn higher in CuD than CuA samples, $P < 0.05$. (D) Spleen membranes from the same 6 rats were treated with PI-PLC and the released protein was fractionated on non-denaturing gels, transferred to PVDF membranes and assayed for ferroxidase activity. Membrane was then probed for Cp protein abundance.

Figure 2-4 Impact of copper status on liver GPI-Cp in male rats



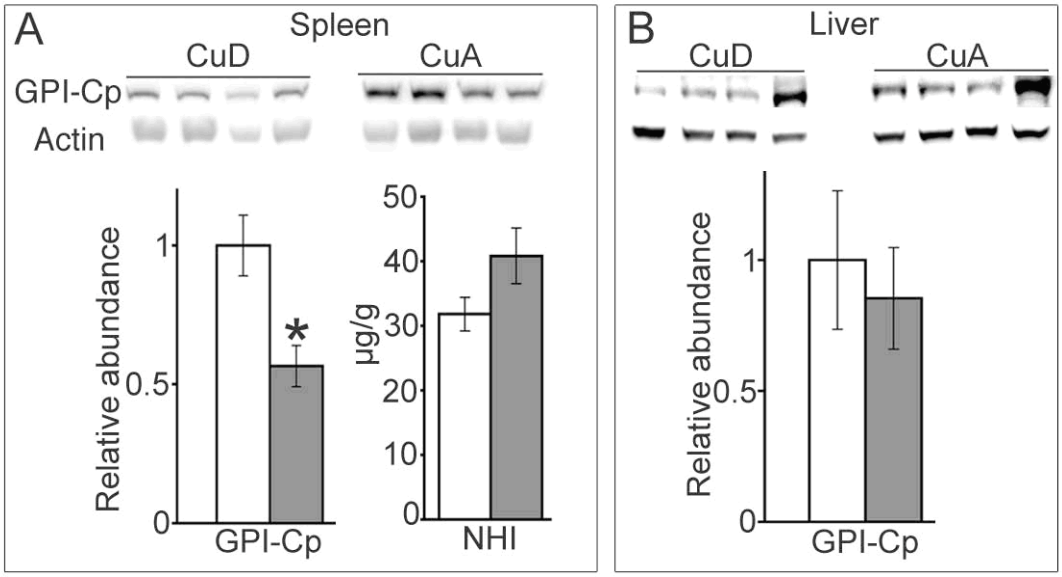
Liver membrane extracts (50 µg/lane) from (**A**) CuD and CuA (Exp. 1 P25) and (**B**) CuD and CuA (Exp. 2 P25) were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were reprobed for Na/K ATPase. Membranes from Exp. 2 were also probed for Fpn. White bars represent CuA rats, gray bars represent CuD rats in all bar graphs. An asterisk indicates significant mean differences, $P < 0.05$.

Figure 2-5 Impact of copper status on GPI-Cp in female rats



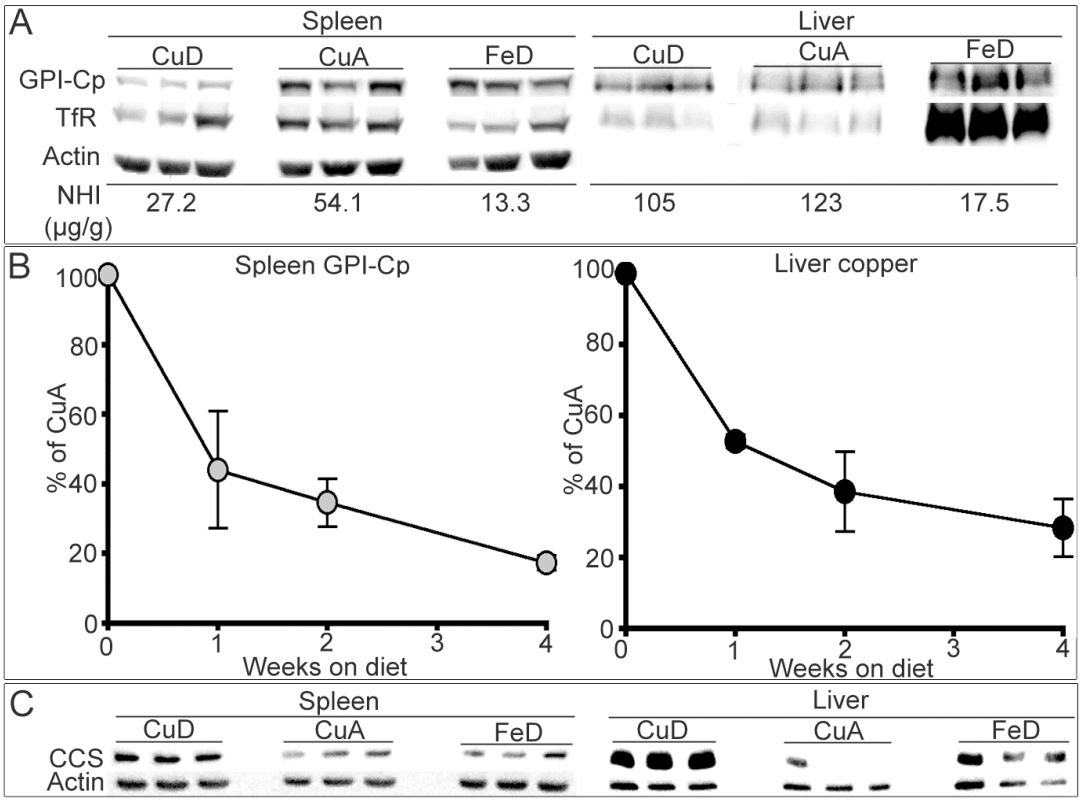
(**A**) Spleen, (**B**) Liver, and (**C**) Cerebellum membrane extracts (50µg/lane) from CuD and CuA female (Exp. 1 P26) rats were subjected Western immunoblot protocols for GPI-Cp abundance. All blots were reprobed for for actin. White bars represent CuA rats; gray bars represent CuD rats in all bar graphs. An asterisk indicates significant mean differences, $P < 0.05$.

Figure 2-6 Impact of copper status on GPI-Cp in male mice



(**A**) Spleen and (**B**) Liver membrane extracts (50µg/lane) from CuD and CuA P27 male mice were subjected Western immunoblot protocols for GPI-Cp abundance. Both blots were reprobed for actin. Non-heme iron (NHI) was determined spectrophotometrically. White bars represent CuA rats; gray bars represent CuD rats in all bar graphs. An asterisk indicates significant mean differences, $P < 0.05$.

Figure 2-7 Impact of postnatal copper or iron deficiency in male rats on GPI-Cp



(**A**) Spleen and liver membrane extracts (50µg/lane) from CuD, CuA, or FeD (Exp. 3 P35) male rats were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were stripped and reprobed for TfR (95 kDa) and actin. Non-heme iron (NHI) was determined spectrophotometrically. Mean values for spleen and liver are listed. (**B**) Relative changes in spleen GPI-Cp and liver copper concentration in CuD rats, compared to CuA, following dietary copper deficiency. Standard error (SE) bars are shown at each time point. CuD means were significantly lower than CuA for all time points in both comparisons, $P < 0.01$. (**C**) Spleen and liver cytosolic fractions (20 µg/lane) were separated on 15% SDS-PAGE and subjected to Western immunoblot protocols. CuD, CuA, and FeD samples were compared for abundance of CCS to verify copper status. Both blots were reprobed for actin. CCS was augmented in CuD extracts in both organs and not in FeD extracts, $P < 0.05$.

CHAPTER 3

Additional experiments were performed to further explore the effects of dietary copper manipulation in older male rats as well as the impact of copper deficiency on transferrin receptor (TfR) abundance in male and female rats and male mice.

Copper deficiency studies on P49 male rats were completed, and after dietary copper repletion for two weeks (P63), data from the two studies were compared.

Western immunoblots probed for GPI-Cp abundance were subsequently reprobed for TfR to determine the effect of copper deficiency, and iron deficiency in P35 Exp. 3 rats, on TfR abundance, a well-characterized marker of iron status.

Copper deficiency and repletion

Six male Sprague-Dawley rats from Exp. 3 (n=3, CuA and CuD) were sampled after five weeks (P49) on diet. At P49, all remaining rats were offered lab chow (5001) containing copper (11.8 mg/kg), so that CuD rats began copper repletion and CuA rats continued on as controls. After two weeks (P63), six male rats (n=3, CuA and CuR) were sampled. These rats were anesthetized with ketamine/xylazine injection and killed by cardiac puncture.

Transferrin receptor abundance

Exp. 1 male and female rats, Exp. 2 P25 male rats, Exp. 3 P35, P49, and P63 male rats, and P27 male mice spleen and liver membrane extracts were separated on 10% SDS-PAGE (50μg/lane) and subjected to Western immunoblot protocols for transferrin receptor (TfR) abundance, following established protocols previously mentioned (Chapter 2).

Results and Discussion

In the comparison blots of P49 vs. P63 spleen and liver GPI-Cp, P49 CuD rats have decreased GPI-Cp compared to CuA rats for both spleen and liver (Figure 3-1A). The effect of copper deficiency has disappeared in the P63 copper repleted (CuR) male rats, as these previously CuD rats have Western blot Cp band densities very similar to their CuA counterparts after two weeks of repletion. Actin probing on both blots shows no discrepancies in loading, as actin appears equivalent between treatment groups. Probing spleen cytosol samples for CCS from these same rats illustrates the great degree of

copper deficiency in the P49 CuD rats (Figure 3-1B). The liver cytosol CCS blot shows a slightly lesser degree of copper deficiency, but both the spleen and liver cytosol blots illustrate reversal of copper deficiency in the P63 CuR rats.

The measurement of spleen NHI in both P35 and P63 rats was performed (Figure 3-1C). In P35 CuD male rats, spleen NHI was significantly lower than the CuA rats, in agreement with spleen data on other CuD rats (Chapter 2). P63 CuR rats appear to have a recovery in spleen NHI, with values similar to the CuA counterparts. Let it be assumed that a subsequent measurement of P49 spleen NHI would produce equivalent or greater differences in NHI values. Both the significant difference in the P35 mean NHI values and the recovery of NHI in the spleens of the P63 CuR rats were expected and verified the effects of copper deficiency and subsequent repletion.

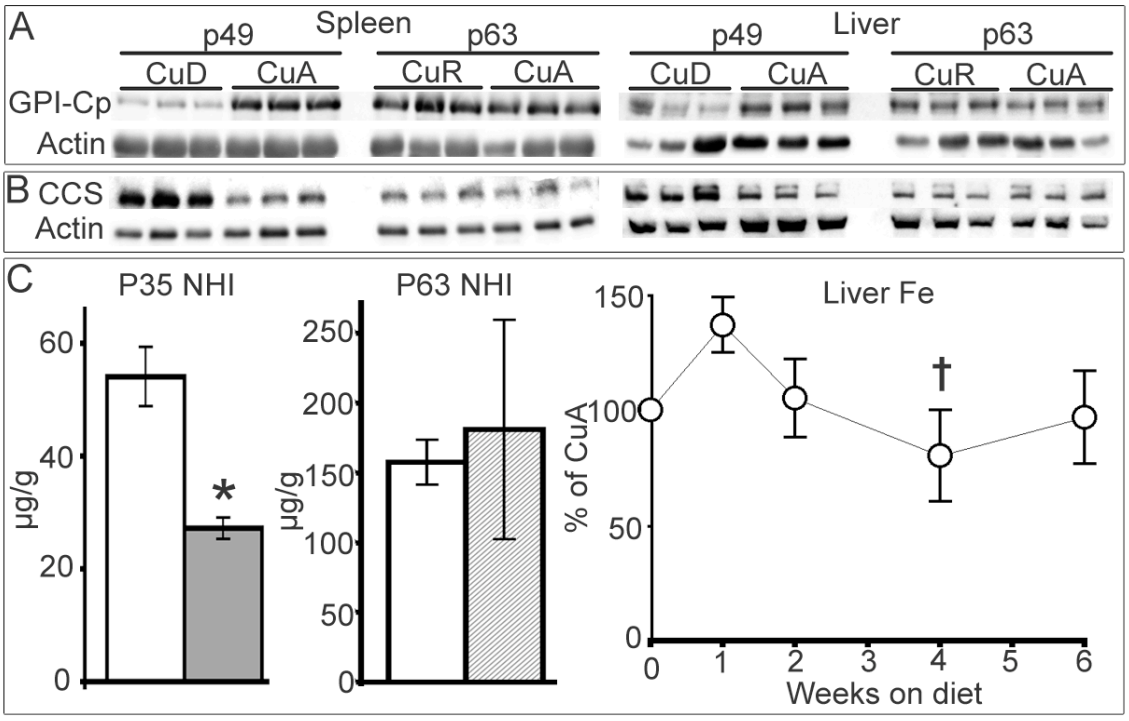
Liver iron was measured by atomic absorption after wet ashing and includes both NHI and heme iron. In liver, the primary pool is ferritin-bound NHI. Liver iron concentration of the Exp. 3 male rats is shown as percent of CuA (Figure 3-1C). After one week on the copper deficient diet, CuD rats appear to have much more liver iron (~150%) than CuA, however, this was non-significant due to one low value in the CuA group. Liver iron did double from weanling (P21) to P28 (data not shown). Even after 4 weeks on CuD diet, iron content is decreased, not increased, and statistically not different than CuA levels, illustrating no obvious trend, unlike that of the liver copper content (Figure 2-7B). While convention suggests hepatic iron accumulation develops in dietary CuD rodents, this was not the case in these rats, nor was it the case in studies by Bastian et al., where hepatic iron accumulation was not present in adult rat dams on the modified AIN-93G diet (Bastian et al. 2010). The added iron in this modified AIN-93G diet appears to provide the necessary amount to support proper hemoglobin production and block hepatic iron overload.

Transferrin receptor (TfR) is a 95 kDa membrane-bound protein that binds the iron-bound transferrin (Tf) to internalize iron in the cell. Generally, in response to increased iron concentration, TfR has a reduced abundance. Based on a comparison of Western blot TfR band densities and corresponding spleen and liver iron concentration values (Table 3-1), TfR does not appear to be regulated in the spleen of rats, but overall, behaves

as expected in rodent liver samples as well as mouse spleen samples. Data of P35 FeD rats illustrates this best (Table 3-1). Despite very low spleen NHI, there was no augmentation in TfR, whereas, in FeD liver samples TfR abundance illustrates a dramatic rise in response to low iron levels. Of the rats with spleen iron measurements, all indicate slightly lower iron concentrations in CuD samples; this is not reflected in the TfR Western immunoblots, however. Exp. 2 P25 males do reflect this, but the difference between CuD and CuA iron concentrations was not significant, as the blot suggests. Mouse P27 livers had a slightly higher iron concentration in CuD samples, which is dramatically reflected in the Western immunoblot (Figure 3-2D).

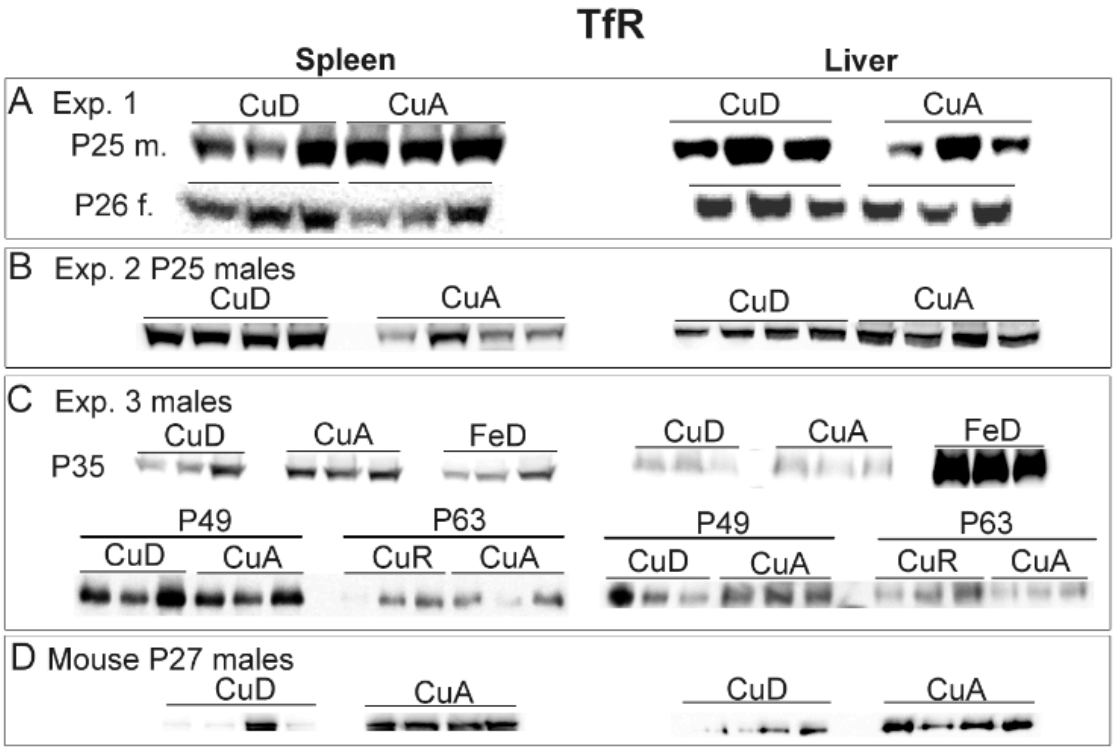
Generally, in the liver blots, the expected TfR response was obtained for male and female rats and male mice. Mouse liver samples have a proper TfR response, as iron concentrations for CuD were twofold higher than CuA and the Western immunoblot has very low CuD TfR band density compared to CuA (Figure 3-2D). All rat samples, excluding Exp. 1 P25 males and Exp. 3 P35 FeD males, had little differences in liver iron concentrations; this is reflected nicely in each of the TfR immunoblots. The significant elevation of TfR in the livers of Exp. 3 P35 FeD rats was expected, but there was no notable difference in the spleens of these same animals (Figure 3-2C), verifying the disconnect between iron concentration and TfR response in the spleens of male rats. If spleen TfR is localized specifically on the lymphocytes, but GPI-Cp is localized on the spleen macrophages, TfR would not correctly represent the GPI-Cp status of the liver. Most of the rodents used in this copper deficiency study were very young (P24-P35); this allows for very little NHI production overall. A study on older rodents may yield more accurate TfR results when compared to measured iron values.

Figure 3-1 Impact of copper deficiency and repletion on GPI-Cp in older male rats



(A) Spleen and liver membrane extracts (50µg/lane) from Exp. 3 P49 CuD and CuA and P63 CuR and CuA male rats were subjected to Western immunoblot protocols for GPI-Cp abundance. Both blots were stripped and reprobed for actin. (B) Cytosolic fractions (20 µg/lane) were separated on 15% SDS-PAGE and subjected to Western immunoblot protocols for CCS abundance to verify copper status. Blots were reprobed for actin. (C) Spleen NHI was determined spectrophotometrically for both P35 and P63 Exp. 3 male rats. An asterisk indicates significant mean differences, $P < 0.05$. Relative changes in liver iron concentration in CuD rats compared to CuA, following dietary copper deficiency and repletion (onset at cross). There was no significant difference in mean liver iron concentration over the course of 6 weeks of treatment in these CuD and CuA Exp. 3 male rats.

Figure 3-2 Characterization of rodent transferrin receptor



Spleen and liver membrane extracts (50 µg/lane) were prepared and separated on 10% SDS-PAGE and subjected to Western immunoblot protocols for transferrin receptor (TfR) abundance (95 kDa) in: (A) Exp. 1 CuD and CuA P25 male and P26 female rats (B) Exp. 2 P25 CuD and CuA male rats (C) Exp. 3 P35 CuD, CuA, and FeD male rats; P49 CuD and CuA, and P63 CuR and CuA male rats and (D) P27 male mice.

Table 3-1 Spleen non-heme iron and liver total iron values in male and female rats and male mice

Group	Spleen non-heme iron (µg/g)	Liver total iron (µg/g)
Exp. 1 P25 male rats		
CuD	32*	60.1*
CuA	45	32.3
Exp. 1 P26 female rats		
CuD	ND	68.3
CuA		76.2
Exp. 2 P 25 male rats		
CuD	25*	55.1
CuA	36	60.3
Exp.3 P35 male rats		
CuD	27.2*	117
CuA	54	112
FeD	13*	17*
Exp. 3 P49 male rats		
CuD	ND	81
CuA		102
Exp. 3 P63 male rats		
CuR	180	79
CuA	157	82
P27 male mice		
CuD	40.8	230*
CuA	31.8	113

Spleen non-heme iron (µg/g) was measured spectrophotometrically following established protocols (Chapter 2). Liver total iron (µg/g) was measured by atomic absorption after wet ashing; this measurement is NHI and heme iron inclusive, although primarily NHI. Values are presented as the mean of three or more rodents in each dietary group. An asterisk indicates significant mean differences from CuA, $P < 0.05$ (Student's *t*-test). ND indicates no data was collected.

CHAPTER 4

The necessity of copper and iron in all biological systems justifies further examination of the relationship between these two important metals. Copper and iron are vital components of redox reactions and act as cofactors in metalloproteins that participate in many essential biological processes within yeast, plants, mammals, and bacteria. In both prokaryotes and eukaryotes, copper is necessary for transport, energy production, and detoxification processes (Koch *et al.* 1997), while iron is required for energy metabolism, respiration, oxygen delivery to tissues, and DNA synthesis (Comporti 2002). The complex molecular relationship between these metals was first characterized in yeast, with studies on the copper transporter Ctr1 and *Crt1*-/- yeast strains. A defective *Ctr1* gene causes a failure of the multicopper oxidase, Fet3p, to receive sufficient copper to perform its ferroxidase function (Askwith et al. 1994). Fet3p in yeast is analogous in function to serum ceruloplasmin (Cp) in mammals (Askwith & Kaplan 1998). Loss of ferroxidase activity due to insufficient cellular copper content is thought to cause systemic issues with iron homeostasis.

Multiple multicopper ferroxidases homologous to Cp have also been found in mammals, including hephaestin and zyklopen. Hephaestin is present predominately on the enterocytes (Chen *et al.* 2004), while zyklopen is recently discovered to be localized in the placenta and mammary gland (Chen et al. 2010); both are membrane-bound, like GPI-Cp The fact that more than one copper-dependent ferroxidase exists within an organism suggests the importance of this iron-oxidizing activity.

The ferroxidase of focus in the current studies was Cp, present in mammals in two different forms, a circulating serum form (sCp) and a GPI-anchored form (GPI-Cp). sCp is synthesized in the liver and has been well-characterized as a circulating multicopper oxidase, responsible for the oxidation of FeII to FeIII for proper iron homeostasis. The membrane-bound form of Cp, GPI-Cp, has a much more widespread abundance than previously thought; it had only been characterized, in vitro, in a few specific cell-types in brain, testes, retina, and kidney, as well as in macrophages (Banha et al. 2008, Fortna et al. 1999, Patel & David 1997, Stasi et al. 2007, Wiggins et al. 2006). Current studies reported GPI-Cp in additional tissues including spleen and liver. The membrane-bound

nature of GPI-Cp was verified by its release from the membrane when PI-PLC was used to specifically cleave the GPI anchor, as well as the complete absence of the protein in membrane preparations of multiple tissues from *Cp-/-* mice. Data show that GPI-Cp is abundant in the spleen and that there is a marked protein decrease during dietary copper deficiency in male and female rats and male mice, similar to reduction of sCp.

The role of GPI-Cp in ferrous iron efflux has been thoroughly examined in the astrocytes (Jeong & David 2003). Its exact role in other organs remains unclear, though it is likely GPI-Cp plays a similar role in other organs. The ferroxidase activity of GPI-Cp could be important for iron efflux from tissues, as *Cp-/-* humans and mice eventually accumulate iron in macrophages of the spleen, liver, and brain (Harris et al. 1999). Recent data are confusing, however, as *Cp-/-* mice have normal plasma iron and no spleen iron overload at 10 weeks of age (Meyer et al. 2001). Also interesting is that the spleen does not accumulate iron in copper deficient rats with decreased GPI-Cp abundance. Perhaps the augmentation in the iron exporter ferroportin, Fpn, in copper deficient rats compensates for lower levels of the multicopper oxidase GPI-Cp, helping to regulate iron homeostasis in spleen. Studies on copper deficient mice have shown decreased GPI-CP abundance, but no augmented Fpn levels nor lower NHI levels in the spleen (Jenkitkasemwong et al. 2010). Another study on copper deficient mice demonstrated that Fpn protein levels were determined to be higher in enterocytes, while hephaestin, a multicopper oxidase that colocalizes with Fpn, was reduced (Chen et al. 2006). It has been theorized that multicopper oxidase activity was essential for the stability and expression of Fpn. Tissue culture studies with brain glial cells treated with the copper chelator, bathocuproine disulfonate (BCS), demonstrated loss of membrane-bound Cp, with a subsequent decrease in Fpn abundance (De Domenico et al. 2007). More recent in vivo studies, along with the current studies, show that lower GPI-Cp associated with copper deficiency does not correspond with a reduction in Fpn in spleen and liver (Jenkitkasemwong et al. 2010). Does multicopper oxidase activity really determine Fpn concentrations? It is more likely that Fpn is regulated by hepcidin, a negative regulator that binds Fpn, causing the degradation of the hepcidin-Fpn complex

in the lysosome (Jenkitkasemwong et al. 2010). Further in vivo studies should be performed to verify Fpn stability in response to decreased membrane-bound oxidases.

A detrimental iron accumulation is seen in the brains of *Cp-/-* mice; however, neonatal CuD rats have decreased iron concentrations in the brain. In these very young rats, iron may be trapped in astrocytes creating low iron conditions in the other brain cells like neurons and overall iron deficiency in the brain. This could mean that GPI-Cp does not function well in iron efflux from the astrocytes. GPI-Cp protein is present in membrane preparations of the cerebellum but shows no change in abundance during copper deficiency. Further work is needed to evaluate GPI-Cp results found in the brain.

Limitations of the current study include the age of the rodents used for experiments and the inability to distinguish specific cell types within the organs. The ability to detect iron overload in tissue macrophages would require older rodents. For example *Cp* -/- mice did not show iron augmentation at 2 months, but did at 2 years of age (Meyer et al. 2001). Macrophages of interest could not be differentiated from other cells in each organ, such as whole spleen lymphocytes in the spleen. Future studies could then include cell-type differentiation after membrane extracts are prepared. Older male and female rodents (older than P40) should also be used in the copper deficiency studies, using the modified AIN-93G diet. This could allow the full effects of the deficiency in specific tissues to be observed, such as tissue-specific iron accumulation and more notable copper and iron concentration differences.

Preliminary data on GPI-Cp mRNA abundance (Chapter 2) suggests a more ubiquitous distribution than sCp abundance, which was enriched in liver. The function of GPI-Cp in cells not noted for iron efflux remains to be established.

LITERATURE CITED

Abolmaali, B., Taylor, H. V. and Weser, U. (1998) Evolutionary aspects of copper binding centers in copper proteins. *Structure and Bonding,* **91,** 91-190.

Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M. and Kaplan, J. (1994) The FET3 gene of S. cerevisiae encodes a multicopper oxidase required for ferrous iron uptake. *Cell,* **76,** 403-410.

Askwith, C. and Kaplan, J. (1998) Iron and copper transport in yeast and its relevance to human disease. *Trends Biochem Sci,* **23,** 135-138.

Banha, J., Marques, L., Oliveira, R., Martins Mde, F., Paixao, E., Pereira, D., Malho, R., Penque, D. and Costa, L. (2008) Ceruloplasmin expression by human peripheral blood lymphocytes: a new link between immunity and iron metabolism. *Free Radic. Biol. Med.,* **44,** 483-492.

Bastian, T. W., Prohaska, J. R., Georgieff, M. K. and Anderson, G. W. (2010) Perinatal iron and copper deficiencies alter neonatal rat circulating and brain thyroid hormone concentrations. *Endocrinology,* **151,** 4055-4065.

Bento, I., Peixoto, C., Zaitsev, V. N. and Lindley, P. F. (2007) Ceruloplasmin revisited: structural and functional roles of various metal cation-binding sites. *Acta Crystallogr D Biol Crystallogr,* **63,** 240-248.

Broderius, M., Mostad, E., Wendroth, K. and Prohaska, J. R. (2010) Levels of plasma ceruloplasmin protein are markedly lower following dietary copper deficiency in rodents. *Comp Biochem Physiol C* **151,** 473-479.

Chen, H., Attieh, Z. K., Su, T. et al. (2004) Hephaestin is a ferroxidase that maintains partial activity in sex-linked anemia mice. *Blood,* **103,** 3933-3939.

Chen, H., Attieh, Z. K., Syed, B. A. et al. (2010) Identification of Zyklopen, a New Member of the Vertebrate Multicopper Ferroxidase Family, and Characterization in Rodents and Human Cells. *J Nutr*.

Chen, H., Huang, G., Su, T., Gao, H., Attieh, Z. K., McKie, A. T., Anderson, G. J. and Vulpe, C. D. (2006) Decreased hephaestin activity in the intestine of copper-deficient mice causes systemic iron deficiency. *J. Nutr.,* **136,** 1236-1241.

Cherukuri, S., Tripoulas, N. A., Nurko, S. and Fox, P. L. (2004) Anemia and impaired stress-induced erythropoiesis in aceruloplasminemic mice. *Blood Cells Mol. Dis.,* **33,** 346-355.

Clarkson, T. (1995) Health effects of metals: a role for evolution? *Environ Health Perspect,* **103 Suppl 1,** 9-12.

Collins, J. F., Prohaska, J. R. and Knutson, M. D. (2010) Metabolic crossroads of iron and copper. *Nutr. Rev.,* **68,** 133-147.

Comporti, M. (2002) Introduction-serial review: iron and cellular redox status. *Free Radic Biol Med,* **32,** 565-567.

Cook, S. F. and Spilles, N. M. (1931) Some factors regulating the utilization of splenic iron. *Am J Physiol,* **98,** 626-635.

Dancis, A., Haile, D., Yuan, D. S. and Klausner, R. D. (1994) The Saccharomyces cerevisiae copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. *J Biol Chem,* **269,** 25660-25667.

De Domenico, I., Ward, D. M., di Patti, M. C., Jeong, S. Y., David, S., Musci, G. and Kaplan, J. (2007) Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. *EMBO J.,* **26,** 2823-2831.

De Freitas, J., Wintz, H., Kim, J. H., Poynton, H., Fox, T. and Vulpe, C. (2003) Yeast, a model organism for iron and copper metabolism studies. *Biometals,* **16,** 185-197.

De Silva, D. M., Askwith, C. C., Eide, D. and Kaplan, J. (1995) The FET3 gene product required for high affinity iron transport in yeast is a cell surface ferroxidase. *J Biol Chem,* **270,** 1098-1101.

Donovan, A., Lima, C. A., Pinkus, J. L., Pinkus, G. S., Zon, L. I., Robine, S. and Andrews, N. C. (2005) The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metab.,* **1,** 191-200.

Dwulet, F. E. and Putnam, F. W. (1981) Internal duplication and evolution of human ceruloplasmin. *Proc Natl Acad Sci U S A,* **78,** 2805-2809.

Feller, D. J., O'Dell, B. L. and Bylund, D. B. (1982) Alterations in neurotransmitter receptor binding in discrete areas of the copper-deficient rat brain. *J Neurochem,* **38,** 519-524.

Fortna, R. R., Watson, H. A. and Nyquist, S. E. (1999) Glycosyl phosphatidylinositol-anchored ceruloplasmin is expressed by rat Sertoli cells and is concentrated in detergent-insoluble membrane fractions. *Biol. Reprod.,* **61,** 1042-1049.

Fox, P. L. (2003) The copper-iron chronicles: the story of an intimate relationship. *Biometals,* **16,** 9-40.

Gitlin, J. D., Schroeder, J. J., Lee-Ambrose, L. M. and Cousins, R. J. (1992) Mechanisms of caeruloplasmin biosynthesis in normal and copper- deficient rats. *Biochem. J.,* **282,** 835-839.

Gray, L. W., Kidane, T. Z., Nguyen, A. et al. (2009) Copper proteins and ferroxidases in human plasma and that of wild-type and ceruloplasmin knockout mice. *Biochem J,* **419,** 237-245.

Halliwell, B. (1984) Oxygen radicals: a commonsense look at their nature and medical importance. *Med Biol,* **62,** 71-77.

Hamza, I. and Gitlin, J. D. (2002) Copper chaperones for cytochrome c oxidase and human disease. *J Bioenerg Biomembr,* **34,** 381-388.

Harris, Z. L. (2003) Aceruloplasminemia. *J Neurol Sci,* **207,** 108-109.

Harris, Z. L., Durley, A. P., Man, T. K. and Gitlin, J. D. (1999) Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. *Proc. Natl. Acad. Sci. U S A,* **96,** 10812-10817.

Harris, Z. L., Klomp, L. W. and Gitlin, J. D. (1998) Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis. *Am. J. Clin. Nutr.,* **67,** 972S-977S.

Harrison, M. D., Jones, C. E., Solioz, M. and Dameron, C. T. (2000) Intracellular copper routing: the role of copper chaperones. *Trends Biochem Sci,* **25,** 29-32.

Hart, E. B., Steenbock, H., Waddell, J. and Elvehjem, C. A. (1928) Iron in nutrition. VII. Copper as a supplemment to iron for hemoglobin building in the rat. *J. Biol. Chem.,* **77,** 797-812.

Hellman, N. E. and Gitlin, J. D. (2002) Ceruloplasmin metabolism and function. *Annu. Rev. Nutr.,* **22,** 439-458.
Holmberg, C. G. and Laurell, C. B. (1947) Investigations in serum copper; nature of serum copper and its relation to the iron-binding protein in human serum. *Acta Chem. Scand.,* **1,** 944-950.
Holtzman, N. A. and Gaumnitz, B. M. (1970) Studies on the rate of release and turnover of ceruloplasmin and apoceruloplasmin in rat plasma. *J. Biol. Chem.,* **245,** 2354-2358.
Hoopes, J. T. and Dean, J. F. (2004) Ferroxidase activity in a laccase-like multicopper oxidase from Liriodendron tulipifera. *Plant Physiol Biochem,* **42,** 27-33.
Huston, W. M., Jennings, M. P. and McEwan, A. G. (2002) The multicopper oxidase of Pseudomonas aeruginosa is a ferroxidase with a central role in iron acquisition. *Mol Microbiol,* **45,** 1741-1750.
Jenkitkasemwong, S., Broderius, M., Nam, H., Prohaska, J. R. and Knutson, M. D. (2010) Anemic copper-deficient rats, but not mice, display low hepcidin expression and high ferroportin levels. *J. Nutr.,* **140,** 723-730.
Jeong, S. Y. and David, S. (2003) Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. *J Biol Chem,* **278,** 27144-27148.
Josephs, H. W. (1932) Studies on iron metabolism and the influence of copper. *J Biol Chem,* **96,** 559-571.
Karpel, J. T. and Peden, V. H. (1972) Copper deficiency in long-term parenteral nutrition. *J Pediatr,* **80,** 32-36.
Koch, K. A., Pena, M. M. and Thiele, D. J. (1997) Copper-binding motifs in catalysis, transport, detoxification and signaling. *Chem Biol,* **4,** 549-560.
Kosman, D. J. (2002) FET3P, ceruloplasmin, and the role of copper in iron metabolism. *Adv Protein Chem,* **60,** 221-269.
Labbe, S., Zhu, Z. and Thiele, D. J. (1997) Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. *J Biol Chem,* **272,** 15951-15958.
Lee, G. R., Nacht, S., Lukens, J. N. and Cartwright, G. E. (1968) Iron metabolism in copper-deficient swine. *J. Clin. Invest.,* **47,** 2058-2069.
Levy, Y., Zeharia, A., Grunebaum, M., Nitzan, M. and Steinherz, R. (1985) Copper deficiency in infants fed cow milk. *J Pediatr,* **106,** 786-788.
Linder, M. C. and Hazegh-Azam, M. (1996) Copper biochemistry and molecular biology. *Am J Clin Nutr,* **63,** 797S-811S.
Liu, N., Lo, L. S., Askary, S. H. et al. (2007) Transcuprein is a macroglobulin regulated by copper and iron availability. *J Nutr Biochem,* **18,** 597-608.
Madsen, E. and Gitlin, J. D. (2007) Copper and iron disorders of the brain. *Annu. Rev. Neurosci.,* **30,** 317-337.
Markwell, M. A., Haas, S. M., Bieber, L. L. and Tolbert, N. E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.,* **87,** 206-210.

McArdle, H. J., Mercer, J. F., Sargeson, A. M. and Danks, D. M. (1990) Effects of cellular copper content on copper uptake and metallothionein and ceruloplasmin mRNA levels in mouse hepatocytes. *J. Nutr.,* **120,** 1370-1375.

Metzler, D. E. and Metzler, C. E. (2003) *Biochemistry: the chemical reactions of living cells*. Academic Press, San Diego, CA.

Meyer, L. A., Durley, A. P., Prohaska, J. R. and Harris, Z. L. (2001) Copper transport and metabolism are normal in aceruloplasminemic mice. *J. Biol. Chem.,* **276,** 36857-36861.

Mittal, B., Doroudchi, M. M., Jeong, S. Y., Patel, B. N. and David, S. (2003) Expression of a membrane-bound form of the ferroxidase ceruloplasmin by leptomeningeal cells. *Glia,* **41,** 337-346.

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., Ganz, T. and Kaplan, J. (2004) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science,* **306,** 2090-2093.

Nittis, T. and Gitlin, J. D. (2002) The copper-iron connection: hereditary aceruloplasminemia. *Semin Hematol,* **39,** 282-289.

Nose, Y., Kim, B. E. and Thiele, D. J. (2006) Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. *Cell Metab.,* **4,** 235-244.

O'Dell, B. L. (1981) Roles for iron and copper in connective tissue biosynthesis. *Philos Trans R Soc Lond B Biol Sci,* **294,** 91-104.

Osaki, S. (1966) Kinetic studies of ferrous ion oxidation with crystalline human ferroxidase (ceruloplasmin). *J Biol Chem,* **241,** 5053-5059.

Osaki, S., Johnson, D. A. and Frieden, E. (1966) The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. *J Biol Chem,* **241,** 2746-2751.

Osaki, S., Johnson, D. A. and Frieden, E. (1971) The mobilization of iron from the perfused mammalian liver by a serum copper enzyme, ferroxidase I. *J Biol Chem,* **246,** 3018-3023.

Owen, C. A., Jr. (1982) *Biochemical aspects of copper: Copper proteins, ceruloplasmin, and copper protein binding.* Noyes Publications, Park Ridge, NJ.

Patel, B. N. and David, S. (1997) A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. *J. Biol. Chem.,* **272,** 20185-20190.

Patel, B. N., Dunn, R. J. and David, S. (2000) Alternative RNA splicing generates a glycosylphosphatidylinositol-anchored form of ceruloplasmin in mammalian brain. *J. Biol. Chem.,* **275,** 4305-14310.

Patel, B. N., Dunn, R. J., Jeong, S. Y., Zhu, Q., Julien, J. P. and David, S. (2002) Ceruloplasmin regulates iron levels in the CNS and prevents free radical injury. *J. Neurosci.,* **22,** 6578-6586.

Pilon, M., Abdel-Ghany, S. E., Cohu, C. M., Gogolin, K. A. and Ye, H. (2006) Copper cofactor delivery in plant cells. *Curr Opin Plant Biol,* **9,** 256-263.

Prasad, A. S., Rabbani, P., Abbasii, A., Bowersox, E. and Fox, M. R. (1978) Experimental zinc deficiency in humans. *Ann Intern Med,* **89,** 483-490.

Prohaska, J. R. (1991) Changes in Cu,Zn-superoxide dismutase, cytochrome c oxidase, glutathione peroxidase and glutathione transferase activities in copper-deficient mice and rats. *J Nutr,* **121,** 355-363.

Prohaska, J. R. (2006) Copper. In: *Present Knowledge In Nutrition*, (B. A. Bowman and R. M. Russell eds.), Vol. 1, pp. 458-470. International Life Sciences Institute, Washington, DC.

Pyatskowit, J. W. and Prohaska, J. R. (2008a) Copper deficient rats and mice both develop anemia but only rats have lower plasma and brain iron levels. *Comp. Biochem. Physiol. C* **147,** 316-323.

Pyatskowit, J. W. and Prohaska, J. R. (2008b) Iron injection restores brain iron and hemoglobin deficits in perinatal copper-deficient rats. *J Nutr,* **138,** 1880-1886.

Pyatskowit, J. W. and Prohaska, J. R. (2008c) Multiple mechanisms account for lower plasma iron in young copper deficient rats. *Biometals,* **21,** 343-352.

Reeves, P. G. and DeMars, L. C. (2006) Signs of iron deficiency in copper-deficient rats are not affected by iron supplements administered by diet or by injection. *J Nutr Biochem,* **17,** 635-642.

Reeves, P. G., Ralston, N. V., Idso, J. P. and Lukaski, H. C. (2004) Contrasting and cooperative effects of copper and iron deficiencies in male rats fed different concentrations of manganese and different sources of sulfur amino acids in an AIN-93G-based diet. *J Nutr,* **134,** 416-425.

Ridge, P. G., Zhang, Y. and Gladyshev, V. N. (2008) Comparative genomic analyses of copper transporters and cuproproteomes reveal evolutionary dynamics of copper utilization and its link to oxygen. *PLoS One,* **3,** e1378.

Shen, F., Ross, J. F., Wang, X. and Ratnam, M. (1994) Identification of a novel folate receptor, a truncated receptor, and receptor type beta in hematopoietic cells: cDNA cloning, expression, immunoreactivity, and tissue specificity. *Biochemistry,* **33,** 1209-1215.

Stasi, K., Nagel, D., Yang, X., Ren, L., Mittag, T. and Danias, J. (2007) Ceruloplasmin upregulation in retina of murine and human glaucomatous eyes. *Invest. Ophthalmol. Vis. Sci.,* **48,** 727-732.

Torrance, J. D. and Bothwell, T. H. (1968) A simple technique for measuring storage iron concentrations in formalinised liver samples. *S. Afr. J. Med. Sci.,* **33,** 9-11.

Tran, T., Ashraf, M., Jones, L., Westbrook, T., Hazegh-Azam, M. and Linder, M. C. (2002) Dietary iron status has little effect on expression of ceruloplasmin but alters that of ferritin in rats. *J. Nutr.,* **132,** 351-356.

Valentine, J. S. and Gralla, E. B. (1997) Delivering copper inside yeast and human cells. *Science,* **278,** 817-818.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology,* **3,** RESEARCH0034.

Vulpe, C. D., Kuo, Y. M., Murphy, T. L., Cowley, L., Askwith, C., Libina, N., Gitschier, J. and Anderson, G. J. (1999) Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse. *Nat. Genet.,* **21,** 195-199.

Welch, K. D., Hall, J. O., Davis, T. Z. and Aust, S. D. (2007) The effect of copper deficiency on the formation of hemosiderin in sprague-dawley rats. *Biometals,* **20,** 829-839.

West, E. C. and Prohaska, J. R. (2004) Cu,Zn-superoxide dismutase is lower and copper chaperone CCS is higher in erythrocytes of copper-deficient rats and mice. *Exp. Biol. Med.,* **229,** 756-764.

Wiggins, J. E., Goyal, M., Wharram, B. L. and Wiggins, R. C. (2006) Antioxidant ceruloplasmin is expressed by glomerular parietal epithelial cells and secreted into urine in association with glomerular aging and high-calorie diet. *J. Am. Soc. Nephrol.,* **17,** 1382-1387.

Willis, M. S., Monaghan, S. A., Miller, M. L., McKenna, R. W., Perkins, W. D., Levinson, B. S., Bhushan, V. and Kroft, S. H. (2005) Zinc-induced copper deficiency: a report of three cases initially recognized on bone marrow examination. *Am J Clin Pathol,* **123,** 125-131.

Xu, X., Pin, S., Gathinji, M., Fuchs, R. and Harris, Z. L. (2004) Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis. *Ann N Y Acad Sci,* **1012,** 299-305.

Yamamoto, K., Yoshida, K., Miyagoe, Y., Ishikawa, A., Hanaoka, K., Nomoto, S., Kaneko, K., Ikeda, S. and Takeda, S. (2002) Quantitative evaluation of expression of iron-metabolism genes in ceruloplasmin-deficient mice. *Biochim. Biophys. Acta,* **1588,** 195-202.

Yruela, I. (2005) Copper in plants. *Braz J Plant Physiol,* **17,** 145-156.