

PHYSIOLOGICAL ROLE OF INTESTINAL COPPER TRANSPORTER ATP7A
IN IRON METABOLISM

By

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To my wife and parents

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LIST OF ABBREVIATIONS

AAS	Atomic absorbance spectroscopy
Amu	Atomic mass unit
Atp7a	ATPase Cu ⁺⁺ transporting, alpha polypeptide
Atp7b	ATPase Cu ⁺⁺ transporting, beta polypeptide
Bnip3	BCL2/adenovirus E1B 19 kDa interacting protein 3
BSA	Bovine serum albumin
Cp	Ceruloplasmin
Ctr1	Copper transporter 1
Cyc	Cyclophilin
Cybrd1	Duodenal cytochrome B
DFO	Desferoxamine
DMEM	Dulbecco's modified eagle's medium
Dmt1	Divalent metal transporter 1
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FAC	Ferric ammonium citrate
FAS	Ammonium iron (III) sulfate
FOX	Ferroxidase
Fpn1	Ferroportin 1
Ftn	Ferritin
Hamp	Hepcidin
Hb	Hemoglobin
HCP1	Heme carrier protein 1

Hct	Hematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
HIF2a	Hypoxia inducible factor 2 alpha
HRP	Horseradish peroxidase
HRE	Hypoxia regulator element
IEC-6	Rat intestinal enterocyte cell-6
ICP-MS	Inductively coupled plasma mass spectrometry
IRP	Iron-regulatory protein
IRE	Iron-responsive element
mRNA	Messenger RNA
Mo ^{Br/y}	Atp7a mutant Brindled male mice
Mt1	Metallothionein
NTBI	Non-transferrin-bound iron
RBC	Red blood cell
ROS	Reactive oxygen species
SFM	Serum-free medium
shRNA	Small hairpin RNA
TBST	Tris-buffered saline, Tween-20
TEER	Transepithelial electrical resistance
Tf	Transferrin
Tfr1	Transferrin receptor 1
qPCR	Quantitative polymerase chain reaction

WT

Wild-type littermates

Abstract of Dissertation Presented to the Graduate School
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The Menkes Copper-Transporting ATPase (Atp7a) is an intestinal copper (Cu) transporter that is essential for assimilation of dietary Cu. Studies showed that Atp7a is induced in the rodent intestine during iron (Fe) deficiency, when serum and liver Cu levels increase. Thus, to test the hypothesis that Atp7a is important for Fe absorption and maintain Fe balance in body during Fe deficiency, I initiated an investigation in *Atp7a* mutant (Brindled) male mice. Brindled mice express mutant form of Atp7a with reduced function. Because mutant mice die perinatally of severe Cu deficiency, Cu was administered intraperitoneally at 7 and 9 days-of-age. After 3 month recovery period, rescued mutant mice and wild-type littermates were deprived of dietary Fe for 3 weeks, and Fe homeostasis was studied. Results showed that Brindled mice were able to appropriately regulate Fe absorption in response to Fe deprivation; however, unlike the situation in wild-type littermates, subsequent changes in intestinal and liver Cu levels in the mutants may have been necessary to support Fe homeostasis. An essential role for Atp7a in Fe absorption in these mice was thus not clearly identified, but possible residual Atp7a function and the complex Fe - and Cu -deficient phenotype of the mutant mice complicated data interpretation. Next, I evaluated a possible role for Atp7a in Fe

absorption by developing Atp7a knock down (KD) rat intestinal epithelial (IEC-6) cells using shRNA technology. In Atp7a KD cells, Cu loading increased intracellular Cu levels, consistent with defective Cu export function of Atp7a. Expression of Fe transport-related genes was altered in KD cells. Expression of hephaestin (Heph), ferroxidase important for Fe export from intestine, was strongly repressed in KD cells. Heph downregulation was associated with a reduction in ferroxidase activity in KD cells. Conversely, expression of ferroportin 1 (Fpn1), basolateral Fe exporter, increased in KD cells. Importantly, increased Fpn1 expression correlated with enhanced transepithelial Fe transport observed in the Atp7a KD cells. This increase in transport suggested a minimal role for Heph in Fe absorption. Atp7a silencing thus altered Fe flux, suggesting that Atp7a function or intracellular Cu levels are important to maintain cellular Fe homeostasis.

CHAPTER 1 LITERATURE REVIEW

The first part of this chapter will provide a general overview of the function and utilization of iron in the body and the regulatory mechanisms of iron homeostasis. The second part will focus on the role of copper in the body and regulation of copper metabolism. The third part will describe interactions between iron and copper and copper influences iron homeostasis.

Main Functions of Iron

Iron is the second most abundant transition metal in earth's crust (137). Iron is group 8 metal on periodic table. Its atomic number is 26, and standard atomic weight is 55.845. The origin of the name of iron came from the Anglo-Saxon word "*iren*" and the symbol Fe derives from "*ferrum*" (141). Important areas of iron biology relate to how organisms control iron metabolism, how iron utilization occurs in body, and how iron is involved in disease pathology. Iron is an essential for living organisms and it has the ability to donate or accept electrons (29). This feature makes iron an essential component of oxygen-binding molecules including hemoglobin, myoglobin and cytochromes in the electron transport chain (13). Iron-sulfur containing cytochrome P-450 is involved in steroid hormone biosynthesis (140). Iron is important contributor in signaling controlling of neurotransmitters such as dopamine and serotonin (113). In eukaryotes, mitochondria are main consumers of iron. Iron is involved in mitochondrial electron transport. Iron containing protein complex I- III in mitochondria plays a role energy production in cell (40). The amount of iron in cells is tightly controlled due to harmful effects of free iron, which can mediate the Fenton reaction. In this reaction, H_2O_2 is converted to the highly reactive hydroxyl radical ($OH\bullet$ and $OOH\bullet$) (63). Hydroxyl

radicals have harmful effects on cell membranes, proteins, and nucleic acids (DNA or RNA), resulting in cellular dysfunction (13, 94).

Iron in Food and Iron Deficiency

There are two forms of iron, heme and non-heme (inorganic), found in foods. Non-heme iron is present in fruits, vegetables, dried beans, nuts, grain products, and meat. Heme iron is found only in animal products such as meat, fish and poultry (59). Typical daily dietary iron intake of humans is ~ 10 to 15 mg, but only ~1 to 2 mg is absorbed. The average person's body contains ~ 3-4 g of iron. Tight control of dietary iron absorption is necessary to maintain iron within the normal range to reduce risk of iron deficiency (4). Iron deficiency is the most common nutritional deficiency worldwide, and it has negative effects on cognitive development in infants, children, and adolescents. Maternal iron deficiency anemia may cause low birth weight and preterm delivery (51, 109). In 2002, iron-deficiency anemia (IDA) was considered to be among the most important factors contributing to the global burden of disease (47). Iron deficiency anemia affects ~1.62 billion people, which corresponds to 24.8% of the world population. Iron deficiency is also the most common nutritional deficiency in the United States, affecting 7.8 million adolescent girls and women of childbearing age and 700,000 children aged one to two years (43). Therefore, prevention of iron deficiency is crucial and important for these groups.

Iron Metabolism

Systemic iron homeostasis is controlled by intestinal iron absorption, erythropoietic iron utilization, recycling of iron from senescent erythrocytes, and storage of iron in hepatocytes and macrophages (72). Most body iron is used by bone marrow to synthesize hemoglobin of red blood cells. This iron required for erythropoiesis is

supplied predominantly by stored iron in the reticuloendothelial (RE) system that is responsible for the destruction of senescent erythrocytes (71). Iron is stored in macrophages of spleen and liver, with the liver serving as the central control point of whole-body iron regulation (91).

Intestinal Iron Absorption and Utilization

As no active excretory mechanisms exist, body iron content is determined by regulation of iron transport across enterocytes of the proximal small intestine, as shown Figure-1. Non-heme (inorganic) and heme iron are absorbed by different mechanisms. Inorganic iron exists in ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms in living organisms and in the diet. The mechanisms involved in intestinal non-heme iron absorption, are summarized in Figure 1 (A and B).

Most of the inorganic iron is found as the Fe^{3+} in the diet. In the first step of intestinal iron absorption, Fe^{3+} is reduced to Fe^{2+} at least in part by duodenal cytochrome B (Cybrd1), which is abundantly expressed on the apical membrane of enterocytes (84). Fe^{2+} can then be taken up by the transmembrane protein, divalent metal transporter 1 (Dmt1), which is located at the brush-border surface of enterocytes (32). It has also been proposed that dietary iron might be absorbed via endocytosis into enterocytes (79). Briefly, Fe^{2+} bound to Dmt1 protein is in early endosome and this endosome complex migrates through the cytosol. Fe^{2+} is converted to Fe^{3+} by the multicopper ferroxidase protein, Hephaestin, (Heph). Fe^{3+} is then exported to the portal circulation by the iron export protein, ferroportin1 (Fpn1) (Figure-1; Part A) (79). However, the relative contribution of intestinal iron transport via the endocytosis mechanism has not been established.

With the second mechanism of intestinal iron transport, reduced iron is transported into cytosol directly by Dmt1. Inside the enterocyte, iron is oxidized by ferritin (Ftn) and stored in Ftn protein or it is used for the metabolic needs of the cells. At the basolateral membrane, iron is transported from enterocytes into the portal blood circulation by Fpn1 (27). Fe^{2+} must be oxidized to Fe^{3+} by Heph and iron is loaded on the transferrin (Tfr) protein, which is an iron carrier protein in blood (Figure-1; Part B) (136).

As described part C of Figure 1, heme iron can enter enterocytes via heme carrier protein 1 (Hcp1) within membrane bound vesicles and then iron is released from the heme group by heme oxygenase (143). Subsequently, iron enters the same intracellular pool as newly absorbed inorganic iron and can be stored in Ftn or transported from enterocytes via Fpn1 as described above.

After Fe^{3+} is released from intestinal enterocytes, iron is then bound to Tf in the blood and transported to peripheral tissues including liver, bone marrow, and spleen. Around 25 mg of iron is used daily for heme biosynthesis in the production of new red blood cells (RBCs) in the bone marrow (11). When RBCs reach the end of their life span, they are ingested by splenic macrophages. Iron is exported from macrophages by Fpn1 and is oxidized by the circulating multicopper oxidase ceruoplasmin (Cp) (55). Tf-bound iron in blood can be recognized by the transferrin receptor (Tfr) and this complex is taken up into hepatocytes via receptor mediated endocytosis. Iron is released from by acidification of the endosome and then can be transported into the cytoplasm by Dmt1 in the endosomal membrane of liver (85). Hepatocytes stores around 12% to 25% of

total body iron in healthy adult man (5). Most of iron in liver is stored in ferritin or hemosiderin (54).

Regulation of Iron Homeostasis

Intracellular iron homeostasis is predominantly controlled by posttranscriptional mechanisms (Figure-2). These regulatory mechanisms depend upon interaction between iron regulatory proteins (IRPs) and iron response elements (IREs) of iron regulated mRNAs. IRPs are iron-sensing intracellular proteins that can bind IREs, which are short, conserved stem-loop structures found in the untranslated regions (UTRs) of mRNAs (95). Two forms of IRP protein have been characterized in mammalian cells including IRP1 and IRP2. IRP1 and IRP2 has ~ 60 % amino acid identity compared to IRP1 (114). IRP1 is a long-lived protein and its degradation is not affected by intracellular iron, whereas IRP2 is degraded in cells during iron repletion (50). IRP1 shares sequence homology with mitochondrial aconitase and it is closely related to intracellular iron homeostasis (68). Aconitase is an iron-sulfur (Fe-S) containing protein and these minerals are necessary for its function. When intracellular iron level increases, IRP1 assembles with 4 Fe-S clusters and this results in the loss of its IRE binding capacity (69).

Fpn1 and Ftn have IRE sequences in the 5' UTR of their mRNAs, while Tfr1 and Dmt1 have an IRE sequence in the 3' UTR of their mRNAs. As described in Figure-2, during iron deficiency, IRP binds to the IRE in the 5' UTR of Ftn and to the 3' UTR of Tfr1. This leads to decreased translation of Ftn protein and increased stability of Tfr1 mRNA. During Fe overload, Fe binds to IRP and inhibits binding to IRE. As a result, Tfr1 mRNA levels decrease by degradation and Ftn protein levels increase (86).

The Dmt1 transcript is alternatively spliced into 2 transcripts, but only one has a conserved IRE structure in their 3' UTRs (58). The IRE form of Dmt1 is readily upregulated in duodenum of iron-deficient animals (49). This suggests that Dmt1 mRNA stability is also IRP dependent (49). Furthermore, Dmt1 expression is regulated differently in different cell types. The IRE form of Dmt1 mRNA is slightly increased in the human hepatoma cell line (Hep3B), whereas it is markedly upregulated in the human colorectal cancer cell line (CaCo2) (48) during iron deficiency. Additionally, Dmt1 mRNA levels are not affected by iron depletion in human cervical cancer cells (HeLa) (48) or human intestinal epithelial cells (HT29) (125). Dmt1 regulation and regulatory factors have not been completely elucidated to date.

Transcriptional regulation plays a role in regulating iron-related genes. Transcriptional factors bind to specific promoter sequence of genes and control the level of mRNA transcript production. One of the best known transcription factor families that control iron-related gene expression is hypoxia inducible factors (HIFs). HIF1 α , HIF2 α , and HIF3 α are regulatory subunits of these heterodimeric trans-acting factors. HIF α interacts with HIF β and this complex is transported into the nucleus and binds to hypoxia responsive elements (HREs) sequence of genes (23). During hypoxia or iron depletion, Hif1 α and 2 α degradation is inhibited, whereas normoxia or iron repletion lead to degradation of Hif1 α and 2 α (116). Hif2 α is closely related to intestinal iron homeostasis. Knocking out Hif2 α in mouse intestine leads to reduced mRNA expression of iron-related genes including Dmt1, Fpn1, Tfr1 and as a result, intestinal iron absorption is decreased (117). As an in vitro model, iron chelation in CaCo2 cells only

stabilizes Hif2 α (not Hif1 α) and induces expression of intestinal iron-regulated genes (56).

Post-translational regulation is also important for protein function (131). Post-translational modifications such as glycosylation play roles in regulation of Tfr1. Human Tfr1 protein has three N-linked and one O-linked glycosylation sites. Deletion of the O-linked glycosylation site at thr-104 leads to generation of soluble form of Tfr1. O-linked glycosylation of Tfr1 might prevent proteolytic cleavage (61). Another example for post-translational regulation is degradation of Fpn1 by hepcidin protein. Systemic iron levels are regulated by hepcidin, which is an antimicrobial peptide synthesized by the liver (38). Hepcidin is a negative regulator of iron uptake from the small intestine and of iron release from RE macrophages. Hepcidin binds to Fpn1 and causes its internalization and degradation. Elevated systemic iron levels and inflammation increase hepcidin levels and decrease iron in the circulation by degradation of Fpn1 (91).

Factors That Influence Iron Absorption

Depletion of iron stores and low dietary iron absorption lead to iron-deficiency. Several dietary factors can influence intestinal iron absorption. Ascorbic acid and protein enhance iron absorption, whereas plant components in vegetables, tea and coffee (e.g., polyphenols, phytates), and calcium decrease Fe absorption (155). Genetic factors can also modulate intestinal iron absorption and lead to iron deficiency or overload. Studies of genetic factors related to iron deficiency demonstrate a defect in iron uptake into reticulocytes and enterocytes due to mutation of the *Dmt1* gene (32). Another important factor effecting iron absorption is erythropoiesis. Iron is a constituent of hemoglobin (Hb) within red blood cells and Hb production directly relates to body iron levels. β -Thalassemia is a genetic disorder that leads to decreased and defective

production of hemoglobin. Low hemoglobin levels result in increased iron absorption in patients with β -Thalassemia (31).

Iron Excretion

There is no physiological mechanism for iron excretion and iron loss level is thus very limited. Losses of endogenous iron in male subjects are ~ 1 mg/day. Iron losses occur predominantly via the intestine from sloughed enterocytes, biliary secretion and, blood loss (44).

Main Functions of Copper

Copper is a trace element with atomic no 29 and atomic weight ~ 63 . It is believed that the name of copper come from Roman era. Copper was first called “*cyprium*” (metal of Cyprus) and then it was changed to “*cuprum*” (30). Copper-containing proteins play roles in iron metabolism, signal transduction, aerobic respiration, neuropeptide production, and collagen maturation (53). For instance, copper is important for cross-linking of collagen and elastin that are essential for the formation of strong, flexible connective tissues (12). Copper is also involved in cardiac function. It has been shown that human copper deficiency leads irregular heart function (130). During inflammation, copper level increases in the human body. Copper is an important factor for generation of CD4 positive cells and mitogen-induced production of interleukin-2 (92). Furthermore, the central nervous system cells have tyrosinase, peptidylglycine α -amidating mono-oxygenase, copper/zinc superoxide dismutase, and dopamine- β -hydroxylase enzymes. These are copper-dependent proteins and copper is essential their functions in brain (73).

Copper in Food and Copper Deficiency

Copper is an essential trace element in body and the average adult male contains ~ 100 mg of copper (42). Daily net copper absorption in the gut is approximately 0.6-1.6 mg/day. The average serum copper level in human is ~ 0.5- 1.5 µg/mL (~ 8-24 µM). Total body copper in adults male is ~ 4.5 mg/kg body weight and recommended daily dietary intake is 0.9 mg/day for average person (101). Rich copper sources are shellfish, nuts, seeds, legumes, germ portions of grains, and liver (120). Abnormal intestinal copper absorption leads to copper deficiency in humans, which is seen in Menkes Disease (MD) (128). The importance of copper is clearly illustrated in this fetal, neurodevelopmental disorder. MD is a recessive, X-linked disorder caused by mutation of the *Atp7a* gene (128). Various mutations in *Atp7a* result in different phenotypes including classical MD and occipital horn syndrome (OHS) (34, 64). Mutation of the *Atp7a* gene leads to a mucosal block to Cu absorption and Cu accumulation in enterocytes (45).

Copper Metabolism

Metabolic copper balance is provided by absorption of dietary copper and hepatic excretion. Copper is excreted in proportion to the amount that is ingested from diet and the amount of copper in the body is maintained at a steady state level. Copper in excess has potentially deleterious effects due to its participation in the production of reactive oxygen species (ROS) (16). Thus, body copper levels are controlled tightly. Most copper is used for cuproenzymes production. Collectively, these cuproenzymes are important for human physiology and copper is involved as a redox catalyst for a number of oxidases (78).

Intestinal Copper Absorption

Most dietary Cu is in the cupric (Cu^{+2}) form and it must be reduced to the cuprous (Cu^{+1}) form before being taken up by intestinal epithelial cells. Dcytb is a candidate cupric reductase (147). In the enterocytes, the cuprous form of copper is transported by copper transporter 1 (Ctr1), which is localized on the apical surface of enterocytes (Figure 3, Part A). Another proposed copper import mechanism is Dmt1-dependent copper transport (Figure 3, Part A). It has been shown that knocking down DMT1 in CaCo2 cells result in inhibition of iron uptake by 80% with 47% reduction of copper uptake. This observation suggests that DMT1 might be involved in copper transport (7). Copper is then bound to intracellular chaperone proteins in order to minimize the possible harmful effects of this reactive metal (104). The copper chaperone for superoxide dismutase (Ccs) binds copper with high affinity in the cytoplasm of intestinal epithelial cells and transfers Cu to superoxide dismutase (Sod1) (66). Another copper chaperone, antioxidant protein 1 (Atox1), delivers copper to the $\text{P}_{1\text{B}}$ -type ATPase, Atp7a. Atp7a transports copper into the *trans*-Golgi network for cuproenzyme synthesis in the secretory pathway (101). Elevated copper levels in intestinal cells results in translocation of Atp7a to the basolateral membrane for copper export from enterocytes (67). Copper can be stored in cytosolic metallothionein (Mt) in intestinal epithelial cells or it is transported primarily bound to albumin to the liver. Copper in the portal venous system enters the liver via Ctr1 expressed on the surface of hepatocytes (76). Once in the liver, copper is delivered to the multi-copper ferroxidase, ceruloplasmin (Cp) in the *trans*-Golgi, by the P-type ATPase, Atp7b. Absence or dysfunction of Atp7b results in impaired biliary copper excretion and increased copper levels in the liver, leading to a clinical condition termed Wilson's disease (25).

Copper Excretion

Hepatic excretion into bile is the major excretory mechanism and ~ 97% copper is lost via feces. Copper excretion is mediated by Atp7b protein expressed on the canalicular surface of hepatocytes (115). Healthy humans excrete only 10- 30 µg/day in urine, but renal tubular defects increase urinary copper losses (129). Other unregulated routes of excretion are from hair and nail losses, sloughed epithelial cells, but collectively they contribute little to total copper loss (138).

Iron and Copper Interactions

Copper metabolism can be affected by iron, as first described in 1927 (139). Copper is necessary to form hemoglobin and copper deficiency leads to cholorosis, which is a form of iron deficiency-like anemia. It was shown that cholorosis could be corrected in young women after copper injection (not iron injection) (46). This was the first established relationship between copper and iron in human disease. Moreover, low copper levels in rats cause decreased ferritin (Ftn) protein expression and this decreases inorganic iron content in cells (126). Iron deficiency induces copper uptake, while giving high copper markedly decreases iron uptake in CaCo-2 cells (77). These data suggest that the absorption of these two metals may be closely related. Furthermore, it was reported that a patient with MD presented with low serum copper levels and serum Cp activity with iron deficiency. Copper was injected to rescue the copper deficiency in this patient, however, iron deficiency anemia was still observed (26).

In the intestine, Heph is the best characterized link between iron and copper, where it functions in the mobilization of iron to other tissues (35, 118). Another copper-dependent ferroxidase is Cp, which plays a role in iron release from body storage sites

including Kupffer cells of the liver and splenic macrophages (136). The Heph and Cp ferroxidases are thus important for overall body iron utilization. A mutant form of Heph leads to sex-linked anemia (*s/a*) in mice and causes decreased iron export from intestinal epithelial cells (19). The lack of Cp leads to aceruloplasminemia in humans and results in iron accumulation in the liver and brain (55). Furthermore, it has been shown that iron deficiency induces Cp expression and activity in rodents, possibly by copper loading in hepatocytes (107). It was also observed that iron deficiency increases copper levels in the intestinal mucosa (110). These observations suggest that increased liver, blood, and intestinal copper levels are important for the compensatory response to iron deficiency.

Efficient iron transport from the intestine during iron deficiency relates to elevated intracellular copper levels and delivery of copper for the synthesis of copper-dependent proteins, which play important roles in intestinal iron absorption. It has been recently shown that Atp7a mRNA and protein levels are elevated during iron deficiency (110). This suggests that Atp7a might be another link between iron and copper homeostasis.

The Menkes Atp7a Gene and Protein

The Atp7a gene is approximately 150 kb with 23 exons and is located on chromosome Xq13.2-13.3. (22). The open reading frame is ~ 4.5 kb long and it encodes a full protein ~ 180 kDa (28). The P- type ATPase, Atp7a protein, is type 2 membrane protein and its N- and C- terminal ends are localized in the cytosol (132). The N-terminal end has six repeats of GMXCXXC repeats and they are encoded by exons 2, 3, 4, and 7. Cysteine residues (CPC motif) of this conserved repeat are involved in copper binding (132). The Atp7a protein has eight transmembrane α -helical segments, and two cytoplasmic loops that are formed by extension of the transmembrane α -

helices into the cytosol. These loops play a role in phosphorylation of the Atp7a protein that is important for its physiological function (6). Atp7a transport activity depends upon hydrolysis of the gamma phosphate group of the ATP molecule. This phosphate is transferred to an aspartate residue of the conserved DKTG motif in the Atp7a protein. Dephosphorylation of aspartate leads to the transfer of copper from one side to the other side of the membrane. After dephosphorylation, the Cu- ATP7ase returns to its initial state (6). Under normal physiological copper concentration, Atp7a is primarily localized on the trans-Golgi network (TGN). When intracellular copper level is elevated, Atp7a traffics to the cytoplasm and the basolateral membrane of enterocyte (67, 134).

Regulation of Atp7a

Atp7a expression is detected in various organs, including intestine, placenta, brain, heart, lung, muscle, kidney, and pancreas, but not in liver. Liver does however express Atp7a during the perinatal period (90). It has been shown that Atp7a expression is regulated differently in various by different stimuli. For instance, Atp7a mRNA level is increased in the human monocytic cell line (THP-1) after treatment with phorbol-12-myristate-13-acetate (PMA), which induces neovascularization. Thus, Atp7a is involved in blood vessel growth and development (2). The proinflammatory molecules interferon gamma and lipopolysaccharide (LPS) induced Atp7a protein expression by increasing intracellular copper levels (1). Hypoxia is also closely related to Atp7a expression. It has been shown that oxygen limitation promotes Atp7a expression in the mouse macrophage cell line (RAW262.7) via increasing stability of Hif1 α transcription factor, and low oxygen tension triggers migration of Atp7a from the TGN to the plasma membrane (144). Furthermore, it has been recently noted that Atp7a mRNA level is significantly increased during iron deficiency in rat intestine (110). Hypoxia is also

involved in intestinal iron absorption by upregulation of Hif2 α in mouse intestine. It has been shown that the Atp7a promoter region has Hif2 α binding sites (150). This suggests that Atp7a might be involved in hypoxia regulation of intestinal iron absorption.

In conclusion, preliminary observations regarding regulation of Atp7a during iron deficiency and hypoxia led to the hypothesis that the intestinal copper exporter Atp7a might be involved in intestinal iron transport. It is thus a goal of this proposal to gain a better understanding of the physiologic role of Atp7a in iron metabolism and iron transport across the intestinal epithelium. To achieve this, two specific aims were pursued:

AIM I- To examine the role of the Atp7a in iron homeostasis in Atp7a mutant

Mottled Brindled (Mo^{Brly}) mice. *Hypothesis: Atp7a protein is involved in intestinal iron absorption and necessary for induction of Cp activity during iron deficiency.*

1. Determine how the Atp7a mutation affects the expression of iron- and copper-related genes.
2. Investigate the effect of Atp7a on hepatic Cp ferroxidase activity .
3. Determine if Atp7a contributes to iron absorption, especially under conditions of iron deficiency

AIM II- To test whether Atp7a is involved in iron transport in an in vitro model of

intestinal cells (rat IEC-6 cells) *Hypothesis: Atp7a protein affects cellular iron transport in rat enterocytes.*

1. Determine the effect of Atp7a knockdown on the expression of iron transport-related genes.
2. Investigate the relation between Atp7a knockdown and Heph enzyme activity.
3. Assess the effect of Atp7a knockdown on iron transport during iron deficiency.

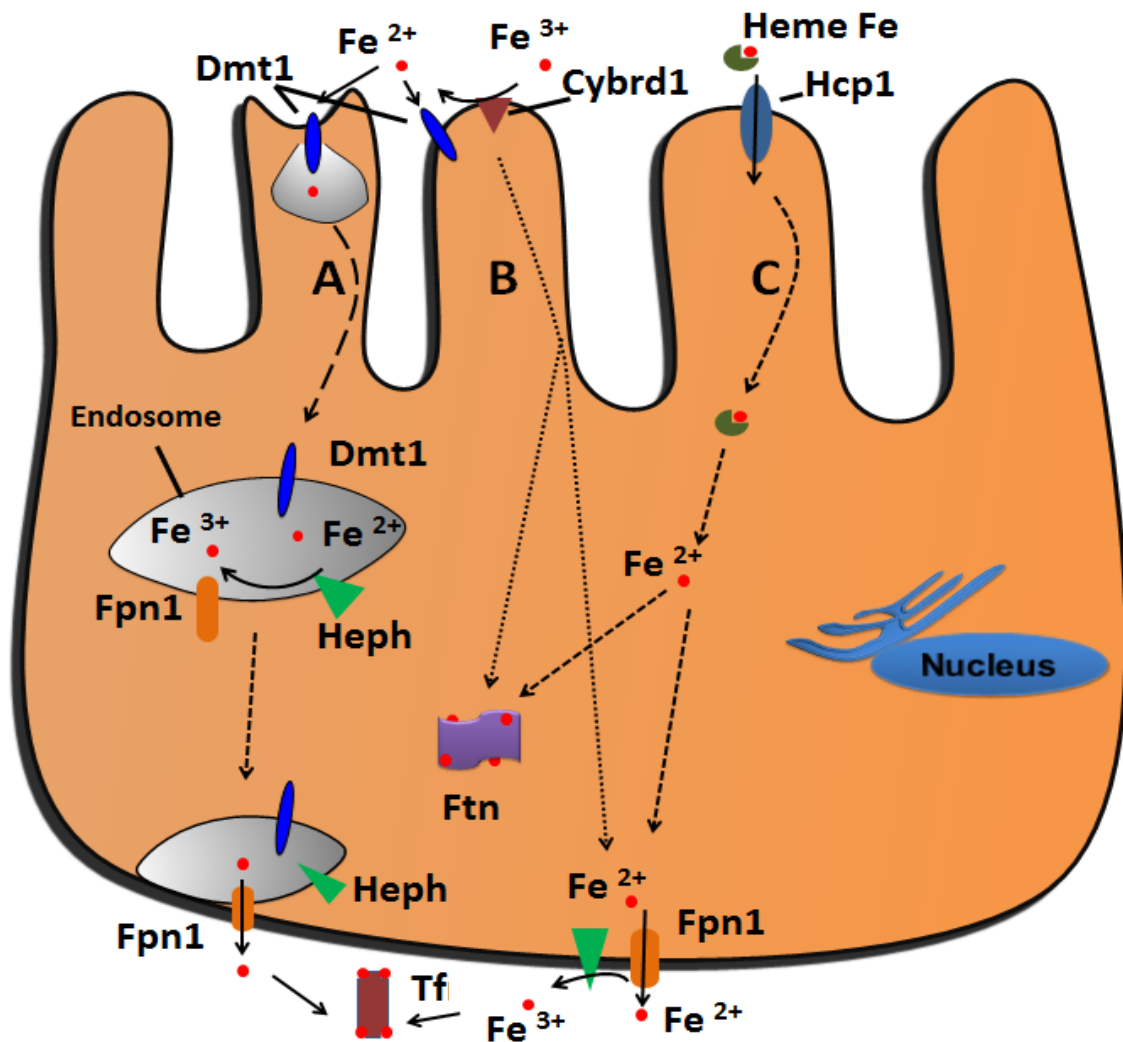


Figure 1-1. Intestinal non-heme and heme iron absorption. Three different pathways (A,B,C) are shown. (A) Reduced iron can be taken by Dmt1 into endosome complex and then oxidized by Heph. Fpn1 delivers iron to Tf at basolateral side of enterocyte. (B,C) Dietary non-heme iron and heme iron are taken up by Dmt1 and Hcp1 respectively. After iron releases from heme group, it enters intracellular iron pool. Intracellular Fe^{2+} then can be stored in Ftn or it is exported by Fpn1 from enterocyte. Fe^{2+} is oxidized by Heph to Fe^{3+} form. Fe^{3+} is taken up by Tf in blood circulation. Abbreviations: Dmt1, divalent metal transporter1; Fpn1, ferroportin1; Heph, hephaestin; Cybrd, cytochrome b reductase1; Ftn, ferritin; Hcp1, heme carrier protein1; Tf, transferrin.

Control of mRNA Stability

Control of Translation

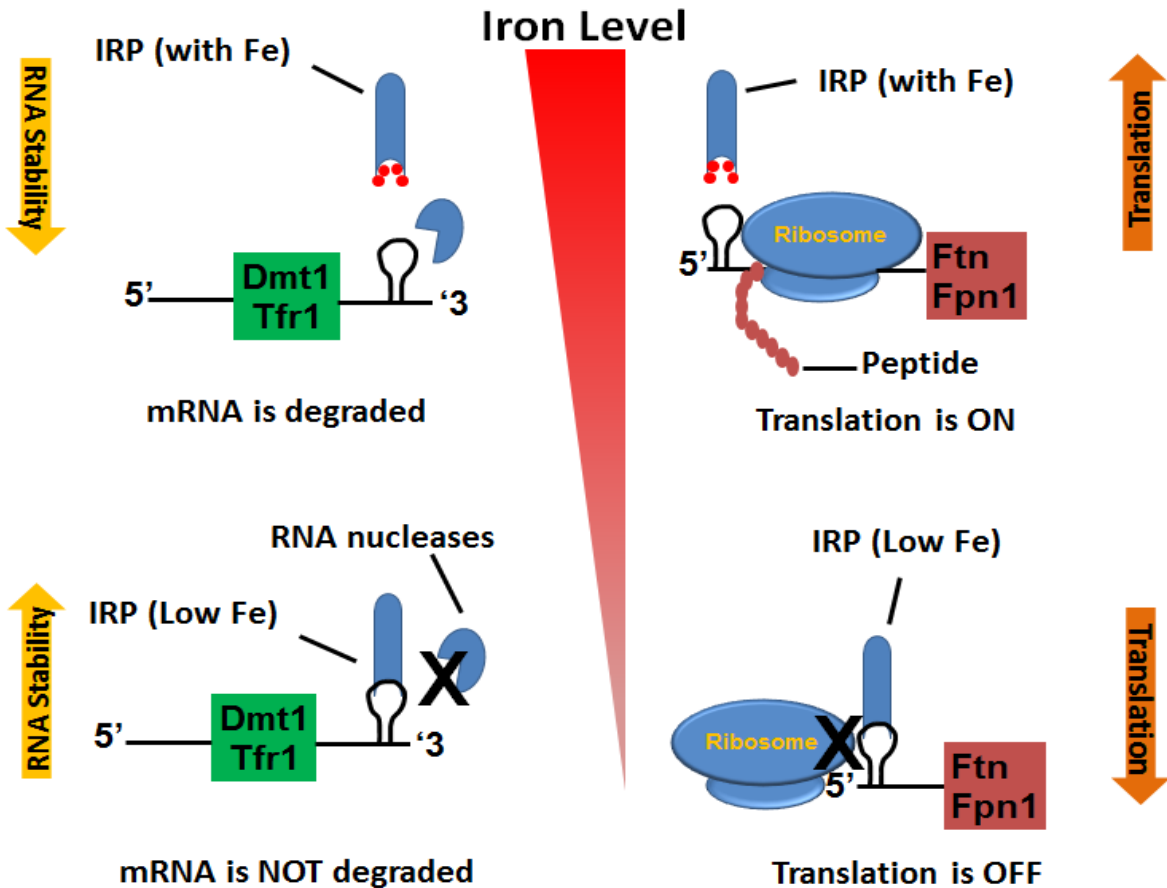


Figure 1-2. Intracellular regulation of iron metabolism. Expression of iron regulated-genes is controlled by posttranscriptional mechanisms. DMT1 and Tfr1 mRNAs have specific motif (IRE) on 3'-prime site, whereas Ftn and Fpn1 mRNAs have 5'-prime IRE site. Intracellular iron level controls binding of iron response protein (IRP) to IRE sequence of mRNA. This interaction changes translation machinery or stability of mRNA against to RNA nucleases. Abbreviations: Dmt1, divalent metal transporter1; Fpn1, ferroportin1; Ftn, ferritin; IRP, iron-responsive element binding protein; Tfr1, transferrin receptor1.

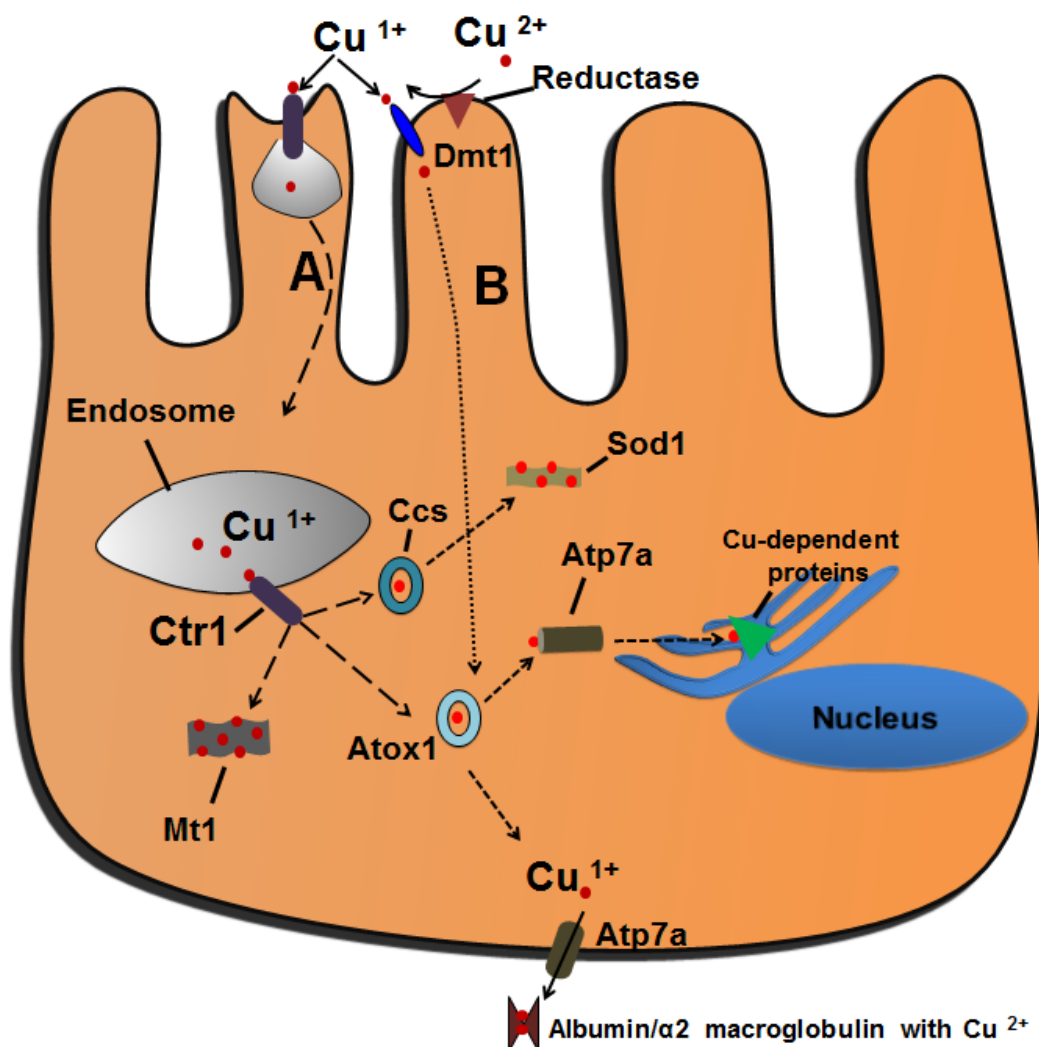


Figure 1-3. Intestinal copper absorption. Two pathways are shown for intestinal copper transport. (A) Dietary copper is reduced to Cu^{1+} and is taken up by Ctr1. (B) In the second possible pathway, Cu^{1+} might enter into cell via DMT1. Intracellular Cu can be stored in Mt1 or taken up by Cu chaperones, Ccs and Atox1. Cu is delivered to Sod1 by Ccs and to Atp7a by Atox1. Atp7a provides Cu for cuproproteins in the *trans*-Golgi or exports Cu to the bloodstream. Cu is taken up by albumin or alpha-macroglobulin in blood. Abbreviations: ATP7A, ATPase, Cu^{++} transporting, alpha polypeptide; Atox1, anti-oxidant protein 1; CCS, copper chaperone for Sod1; Ctr1, copper transporter; Dmt1, divalent metal transporter1; Mt1, metallothionein1; Sod1, superoxide dismutase1

CHAPTER 2 MATERIALS AND METHODS

Rescue of the Cu-Deficient Phenotype of Brindled Mice

Mottled Brindled ($Mo^{Br/y}$) mice on a *CBA/ C3H* background were provided by Dr. Julian Mercer (Deakin University, Australia) and a breeding colony was established at the University of Florida. The X-linked gene, *Atp7a*, has a six bp mutation in $Mo^{Br/y}$ mice resulting in a two amino acid deletion in the 4th transmembrane domain, abolishing activity of the phosphatase domain (45). Brindled mice have been used widely as a model of Menkes disease in humans (14). Brindled ($Mo^{Br/+}$) females were bred to wild type (Wt) males ($Mo^{+/y}$). Screening of the Brindled mutation was done by examination of the coat color; hemizygous males displayed a markedly hypopigmented coat and curly whiskers whereas heterozygous brindled females only had a mottled coat. Newborn Brindled mice were rescued by 2 injections of $CuCl_2$ (50 μg $CuCl_2$ in 20 μL 0.9% of NaCl) into the scruff of the neck at 7- and 9-days-of-age. After Cu injection, mice were observed during the rescue period and experiments were performed when they were adult (~3 months old). Using Brindled mice for these studies have a number of attractive features: 1) Brindled mice are the closest clinical model to human Menke's Disease; 2) This is the first investigation to use Brindled mice to study iron metabolism; 3) The initial study was the first descriptive report regarding whether the *Atp7a* protein plays an important role during iron deficiency in mice.

Induction of Iron Deficiency

Mice were fed semi-purified AIN-93G rodent diets (Dyets Inc., Bethlehem, PA) for 21 days prior to sacrifice. The control diet contained 198 ppm iron and the low-iron diet contained 3 ppm iron, with the diets being otherwise identical. These are diet

formulations that we have used extensively in the past (62); 198 ppm iron is similar to the iron content of standard rodent chow and 3 ppm is well below the absolute dietary requirement of ~50 ppm iron for laboratory rodents. Facial vein bleeding was performed to induce more severe iron deficiency, in conjunction with iron-deficient diet. Briefly, a sterile 18 gauge needle was aligned at the far side of the mouse's face. Needle was inserted into the facial vein of the mouse and then blood was collected into a container. Blood samples were taken on 3 consecutive weeks and hemoglobin levels were measured to monitor the degree of the iron deficiency. All experimental studies in animal subjects were performed with prior approval of the University of Florida IACUC.

Blood and Tissue Collections

Mice were euthanized by CO₂ narcosis followed by cervical dislocation. Blood was collected for hemoglobin and hematocrit determination. Hemoglobin was measured with a HemoCue 201+ hemoglobin analyzer (HemoCue). Hematocrit was determined by centrifugation of blood collected in heparinized microcapillary tubes (Fisher). Tissues including intestine, liver, spleen, and kidney were collected. For total RNA isolation, mucosal layers of duodenum and upper jejunum were scraped on a chilled glass plate to eliminate RNA degradation. Duodenum with upper jejunum, liver and spleen samples were frozen in liquid nitrogen and stored at -80°C for RNA, protein, and further studies.

Non-heme Iron and Copper Determination

Iron and copper in enterocytes were determined by the Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University. Enterocyte samples were dry-ashes and digested with nitric acid. The digested tissue samples were then diluted and analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Mineral measurement of liver tissue was performed in house by using atomic

absorption spectroscopy (AAS). Briefly, wet-ashes of tissue samples were incubated with nitric acid at 90⁰ C for 3 hrs. After samples were digested, they were diluted with deionized water and mineral levels were measured. Absorbance values were normalized to total protein concentration measured by RC/DC assay (BioRad). For serum mineral level determination, blood was collected by cardiac puncture under CO₂. Blood samples were incubated at 4⁰ C for least 2 hr and serum was separated by a two-stage centrifugation at 2000 x g for 10 minutes, and samples were diluted by deionized water in 1mL total volume and mineral levels were measured by AAS.

RNA Isolation and Quantitative RT-PCR

All experimental steps were done at 4⁰ C. Small pieces of tissue from samples were cut on a chilled glass plate and post-confluent IEC-6 cell were used to isolate total RNA. 1 ml trizol reagent was added to tissue or cell samples. Tissue samples were homogenized by using a tissue grinder and cells were broken apart by repeated pipetting followed by adding 400 µL chloroform. Homogenates were centrifuged at 16,000 X g for 10 min and around 400 µL the supernatant was collected in ice-cold tubes. 400 µL iso-propanol was then used to precipitate by removing water from RNA samples followed by adding 400 µL cold 75% ethanol by centrifugation at 12,000 X g for 7 min. After removing alcohol, RNase-free sterile water used to dissolve sample pellets and total RNA was quantified by optical density at 260 nm (Nanodrop instrument). 1 µg of RNA was converted to cDNA using the Bio-Rad iScript cDNA synthesis kit (Biorad) containing poly A and random hexamer primers, in a 20 µl reaction. One µL of the cDNA reaction was used for PCR with 5 µl of SYBR Green master mix (Bio-Rad), 0.75 µl (0.25 pmol) of each forward and reverse gene-specific primers, in a 20-µl reaction. Primers

were designed to span large introns to minimize the chances of amplification from genomic DNA (listed in Supplementary Tables 2-1 and 2-2). Mean fold changes of mRNA were calculated by using the $2^{-\Delta\Delta Ct}$ analysis method as previously described (24).

Protein isolation

Membrane and cytosolic protein isolation: Frozen tissue samples/ IEC-6 cells were homogenized in buffer 1 (0.05 M Tris-HCl, pH 7.4 at 4° C, 0.05 M NaCl) with 1X protease inhibitor (PI) cocktail (Pierce, Rockford, IL) and centrifuged at 16,000 x g for 15 min. After removal of cell debris, the supernatant was recentrifuged at 110,000 x g for 1 h. After ultra-centrifugation, supernatant containing cytosolic proteins was collected in ice-cold tubes. The resulting pellet contained membrane proteins, was resuspended in buffer 2 [buffer 1 plus 0.25% (v/v) Tween-20] containing 1X PI. Then, the suspension was sonicated for 10 sec in an ice water slurry twice, with 5 sec chilling in between, at an output power of 2 watts (RMS), followed by centrifugation at 16,000 x g for 30 min.

Total protein isolation: Some experiments were done by using total protein isolation. For total lysates, samples were homogenized in ice-cold RIPA buffer (50 mM Tris- HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate) containing 1X PI. Samples were centrifuged at 16,000 X g for 15 min to remove cell debris and supernatant was collected in ice-cold tubes. Protein concentration was determined by colorimetric BCA protein assay (Pierce) according to manufacturer's' instruction.

Immunoblotting

Anti-rat Atp7a antibody was generated in rabbits against a carboxy terminal peptide NH₂-KHSLLVGDFREDDDTTL-COOH, and against the amino-terminal region NH₂-KKDRSANHLDHKRE-COOH. Rabbits were immunized three times and the 10 week bleed was used for the experiments described here (110). After protein isolation, fifty micrograms of cytosolic extract, membrane or total protein was mixed with 6X sample buffer (350 mM Tris, pH 6.8, 600 mM dithiothreitol (DTT), 10% sodium dodecyl sulfate (SDS), 0.1 mg bromophenol blue, and 30% Glycerol), and incubated at 70° C for 15 min. Denaturated proteins were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA) for 1 hr under ice-cold conditions. Protein transfer was confirmed by Ponceau Red staining (0.25% Ponceau stain red, 40% Methanol and 15% Glacial acetic acid). The proteins on membranes were blocked with 5% non-fat dry milk in TBS-T for 3 hrs at room temperature or overnight at 4° C. After blocking, membranes were incubated with a 1:1000 dilution of anti-Atp7a antibody was used for 3 hours, subsequently 1:5000 dilution of secondary beta-tubulin antibody (Abcam) in 5% non-fat dry milk in TBST buffer conjugated to horseradish peroxidase was applied for one hour at room temperature. After three washes with TBS-T for 15 minutes each, immunoreactivity was visualized by enhanced chemiluminescence (Solution A: 100 mM Tris, pH 8.5 containing 90 mM cumaric acid and 250 mM luminol in DMSO. Solution B: 100 mM Tris, pH 8.5 containing 30% hydrogen peroxide. Working solution was made by mixing solution A and B (1:1). The optical density of immunoreactive bands on film was quantified using the digitizing software UN-SCAN-IT (Silk Scientific) and the average

pixel numbers were used for normalization and comparison. The intensity of immunoreactive bands on film was normalized to the intensity of beta tubulin or to total proteins on stained blots.

Gavage Study

Mice were fasted overnight but allowed water *ad libitum* prior to gavage feeding of $^{59}\text{Fe-HCl}$ (500 μCi , 1.85 MBq, Perkin Elmer). Anesthesia was used to decrease possible stress in mice during gavage. Pharmacological grade isoflurane was used for mice as an anesthetic agent. It was delivered at known percentages (1-3% for maintenance; up to 5% for induction) in oxygen from a precision vaporizer. During the process, animals were observed closely and anesthesia was discontinued when any unexpected condition was observed from animals. Approximately 2.5 μCi (9.2510, 4 Bq) of $^{59}\text{Fe-HCl}$ diluted into a 0.2 mL of a solution of 0.5 M ascorbic acid, 0.15 M NaCl plus 5 μg of unlabeled Fe (FeSO_4) was used for gavage (22, 65). The iron test solution was administered to each animal with an olive-tipped gavage needle. After 7 hours, control diet was given to all experimental animals. The 24 hour time point was chosen as intestinal transit time in mice is ~ 11 hr (10). 24 hours after gavage, mice were euthanized by CO_2 inhalation and cervical dislocation. Tissue samples were rinsed and intestine was flushed with 1X PBS. Radiolabelled iron content in blood and tissues were analyzed by a γ -counter (Perkin Elmer). Percent iron absorption was calculated as $100 \times (\text{iron in blood, organs, plus carcass} \div ^{59}\text{Fe administered by gavage})$. Radio labeled iron percent utilization in blood, and liver, spleen was measured and values were normalized to mass of tissue and to mL volume sample for blood.

In Vivo and in Vitro Oxidase Activity Assay for Cp and Heph

Ferrozine assay was performed to determine oxidase activity (for Cp) in serum from experimental animals. Ferrozine interacts with Fe^{2+} and gives a pink-colored complex which absorbs light at ~ 570 nm. 1.0 mL reaction mix containing 0.125 M CH_3COONa buffer (pH 7.0), 50 μM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, plus equal quantities of 200 μg serum samples were mixed and incubated at 37 °C. Samples were allowed to read for different time periods, and the reaction was terminated with 3 mM ferrozine and absorbance was read at 570 nm in μQuant spectrophotometer (Biotek). Absorbance of experimental samples was compared to a reference solution (without serum samples) and results were given as changes in comparison to the reference solution (ΔA_{570}). Transferrin-coupled ferroxidase assay was performed in cytosolic and membrane protein samples from rat intestinal cell line (IEC-6) cells. 200 μg cytosolic and 100 μg membrane protein samples in 0.125 M CH_3COONa buffer, pH 5.0, were mixed with 50 μM bovine apo-transferrin (Sigma-Aldrich, St. Louis, MO). Reaction was initiated by adding $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ to a 50 μM final concentration, in a 200 μl total volume. Initial velocities from 30 to 120 s were obtained by following dA/dt at 460 nm in a spectrophotometer (Implen GmbH), at room temperature. Blanks were run for each experiment. Inhibition experiment was done by adding 0.01 % SDS to reaction solution and incubating them for 30 min at 37 °C. After incubation, absorbance was measured as described above.

Creating Atp7a knockdown IEC-6 Cell line

IEC-6 was purchased from ATCC (Manassas, VA). IEC-6 cells are grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum, 10 U/ml insulin and antibiotics (100 U/ml penicillin and streptomycin). The cells

were maintained on sterile 100 mm cell culture dishes and in a humidified atmosphere at 37⁰ C with 5% CO₂. The passage number was kept between 15-30.

Small hairpin RNA (shRNA) technology is a well-established system to knock down target mRNAs in cultured cells (96, 148). shRNAs are transcribed from a plasmid vector containing a Pol III promoter. The shRNA contains the sense and antisense sequences from a target gene connected by a loop. The transcribed shRNAs are transported from the nucleus into the cytoplasm where the Dicer enzyme processes them into small interfering RNAs (siRNAs). At this point, the shRNAs proceed to reduce protein expression of the target genes. shRNAs targeting rat Atp7a mRNAs and negative control shRNA were purchased from Invitrogen (Table 2-3). All shRNAs in the block-iT vector system were transfected into cell individually or in combination. Atp7a knockdown stable IEC-6 cell line was created under 250 µg/ mL zeocine (Invitrogen) selection according to the manufacturer's protocol.

Differentiation of the Rat Intestinal Cell (IEC-6)

IEC-6 cells were used for in vitro studies. IEC-6 cells are described as a homogenous population of epithelial-like cells. Structural features of the IEC-6 cells include Golgi with membrane-limited granules, microvilli in a perinuclear region, numerous mitochondria, and ribosomes both free and attached to the endoplasmic reticulum (146). These cells have been used for studying mechanisms of differentiation (93), wound healing (83), and stimulation of the cells proliferation by growth factors and hormones (154). It has been shown that differentiated IEC-6 cells grown on extracellular matrix form an enterocyte-like phenotype (17). This morphological change is correlated with development of cell-surface alkaline phosphatase enzymatic activity (146). 1X10⁵ IEC-6 cells were plated on collagen-coated 0.4 micron and 1.25 cm² transwell inserts

(Corning, NY) and monitored at days 8-10. Cell monolayer integrity was tested by measuring transepithelial electric resistance (TEER) by using an evom meter instrument (World Precision Instruments). Characteristic transepithelial resistance of the IEC-6 monolayer cell is around 30-40 $\Omega \cdot \text{cm}^2$ after 6- 8 days postconfluence (9, 103).

Induction of Iron Deficiency and CuCl_2 Treatment

1×10^5 IEC-6 cells were plated on 0.4 micron collagen-coated transwell inserts (Corning, MA). When they reached full confluence, they were incubated for 6-8 additional days at 37 °C with 5% CO_2 to allow differentiation. DFO is an iron chelator and can bind to iron ions and thereby it induces iron deficiency in cultured cells (126). DFO functions by reducing bioavailability of iron (20). 200 μM DFO was used to mimic the iron-deficient condition and 50 $\mu\text{g}/\text{mL}$ ferric ammonium citrate (FAC) was used as an iron-loading condition for 24 hours. IEC-6 cells were loaded with 100 μM CuCl_2 for 3 hours or 16 hours to confirm Atp7a knockdown at the functional level.

Iron and Copper Measurements in IEC-6 Cells

Cells were washed with a solution containing, 150 mM NaCl , 10 mM HEPES, 1mM EDTA (3) and then were digested with 0.2% SDS in 0.2 M NaOH (15) at 60° C for 30 min. Intracellular iron and copper content was determined by using AAS. Total protein concentrations were measured by RC/DC assay kit (BioRad), and then levels of copper and iron were normalized by total protein concentration.

Uptake and Transport of ^{59}Fe

IEC-6 cells were trypsinized (0.025% trypsin-EDTA) and 1×10^5 cells were seeded on collagen-coated, 0.4 μm pore size 1.12 cm^2 transwell inserts (Corning). Transport studies were conducted on IEC-6 monolayers after 6-8 days in culture. Formation of a tight monolayer was monitored by measuring transepithelial electrical resistance with a

World Precision Instrument as described under differentiation of the rat intestinal cell section. Cellular ^{59}Fe uptake and transfer of the metal from the apical chamber to the basolateral chamber (transport) were determined as described previously (77, 89). Briefly, cells were incubated with uptake buffer (130 mmol/L NaCl, 10 mmol/L KCl, 1 mmol/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mmol/L glucose and 50 mmol/L HEPES, pH 7.0) at 37°C in a humidified atmosphere of 95% air-5% CO_2 for 2 hrs. After pre-incubation with uptake buffer, TEER was measured to determine that cells had tight gap junctions. 500 μL uptake buffer containing 0.5 μM ^{59}Fe -ferric citrate with 1 M ascorbic acid was added to the apical side and 500 μL uptake buffer was added in basolateral side for 90 min. Cells were washed three times with ice-cold wash solution (150 mM NaCl_2 , 10 mM HEPES, 1 mM EDTA) to remove any surface bound iron. Radioactivity was determined by γ -counting cells which were lysed by 0.2 N NaOH containing 0.2% SDS and the basolateral solution. Uptake and transport were expressed as cpm/mg of protein.

Statistical Analysis

All results were expressed as mean $\pm$ SD. All analyses were performed and figures were made in GraphPad Prism (version 5.0 for Windows, GraphPad). Blood parameters, individual gene expression, mineral levels in mice samples, and radiolabeled iron levels in mice and in cells were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Data sets with unequal variances were in transformed to normalize variance prior to statistical analysis. Atp7a mRNA, protein expression were analyzed by student-t test. Relative FOX activity in serum and in IEC-6 cells, and TEER measurements were compared by two-way ANOVA followed by Bonferroni's multiple comparisons test between experimental groups. Pearson

correlation and linear regression analysis were performed to compare serum Fox enzyme activity to liver and serum copper levels.

Table 2-1. List of Mouse qPCR primers

Gene Symbol	Forward/Reverse	Primer Sequence
Atp7a	F R	5'- GCAGTACAAAGTCCTCATTGG-3' 5'- GCCTCAGGTTTCACAGTATCAGC-3'
Ctr1	F R	5'- GGGGCTTGGTAGAAGTCCGTAC-3' 5'- TGGTAATGTTGTCGTCCGTGTGG-3'
Cybrd1	F R	5'- CGTGTTTGATTATCACAATGTCCG-3' 5'- CACCGTGGCAATCACTGTTCC-3'
Cyclophilin	F R	5'- TGGAGATGAATCTGTAGGACGAGTC-3' 5'- CTCCACCCTGGATCATGAAGTC-3'
Dmt1	F R	5'- GTGATCCTGACCCGGTCTATCG-3' 5'-TGAGGATGGGTAGAGCAAAGG-3'
Fpn1	F R	5'- AAGGGACTGGATTGTTGTTGTGG-3' 5'- GCTGGTCAATCCTTCTAATGGTAGC-3'
Ftn	F R	5'- CCAGAACTACCACCAGGACGC-3' 5'- TCAGAGCCACATCATCTCGGTC-3'
Hamp	F R	5'- CCTGAGCAGCACCAACACCTAT-3' 5'- AATGTCTGCCCTGCTTTCTTCCC-3'
Heph	F R	5'- ACACCTTTGTGACGGCCATC-3' 5'- TTATAAATTGCCTGCATCCCTTC-3'
Mt1a	F R	5'- TCTCCTCACTTACTCCGTAGCTCC-3' 5'- CAGTTCTTGCAGGCGCAGGA-3'
Tfr1	F R	5'-AACTTACCCATGACGTTGATTGAACC-3' 5'- ACAGCCACTGTAGACTTAGACCCATATC-3'

Table 2-2. List of Rat qPCR Primers

Gene Symbol	Forward/Reverse	Primer Sequence
Atp7a	F R	5'- TGAACAGTCATCACCTTCATCGTC -3' 5'- GCGATCAAGCCACACAGTTCA -3'
Atp7b	F R	5'- TTAGCATCCTGGGCATGACTTG -3' 5'- TTGGTGTGTGAGGAGTCCTCTAGTGT -3'
Cybrd1	F R	5'- CGTGTTTGATTATCACAATGTCCG-3' 5'- CACCGTGGCAATCACTGTTCC -3'
Cyclophilin	F R	5'- CTTGCTGCAATGGTCAACC-3' 5'- TGCTGTCTTTGGAACCTTGTCTGC-3'
Dmt1+IRE	F R	5'- GCATCTTGGTCCTTCTCGTCTGC -3' 5'- AACACACTGGCTCTGATGGCTCC-3'
Fpn1	F R	5'- TCGTAGCAGGAGAAAACAGGAGC-3' 5'- GGAACCGAATGTCATAATCTHGC-3'
Ftn	F R	5'- ACTCGGAGGCTGCCATCAAC -3' 5'- GAAGATTCGTCCACCTCGCTG-3'
Heph	F R	5'- ACACTCTACAGCTTCAGGGCATGA -3' 5'- CTGTCAGGGCAATAATCCCATTCT -3'
Mt1a	F R	5'- CTTCTTGTCGCTTACACCGTTG-3' 5'- CAGCAGCACTGTTTCGTCACCTTC-3'
Tfr1	F R	5'- ATTGCGGACTGAGGAGGTGC -3' 5'- CCATCATTCTCAGTTGTACAAGGGAG -3'

Table 2-3. Atp7a mRNA target shRNA and negative control (NC) shRNA sequences

shRNA name	shRNA Sequences
shRNA1 –NM052803	
Top oligo	5'-CACCGCAACGAACAAAGCACATATTCGAAAATATGTGCTTTGTTTCGTTGC-3'
Bottom oligo	3'-AAAAGCAACGAACAAAGCACATATTTTCGAATATGTGCTTTGTTTCGTTGC-5'
shRNA2 –NM052803	
Top oligo	5'-CACCGGACGAGTCTATGATTGAACACGAATGTTCAATCATAGACTCGTC-3'
Bottom oligo	3'-AAAAGGACGAGTCTATGATTGAACATTTCGTGTTCAATCATAGACTCGTCC-5'
shRNA3 –NM052803	
Top oligo	5'-CACCGCCTCTGACCCAAGAAGTTGTCGAAACAACTTCTTGGGTCAGAGG-3'
Bottom oligo	3'-AAAAGCCTCTGACCCAAGAAGTTGTTTCGACAACTTCTTGGGTCAGAGG-5'
NC shRNA	
Top oligo	5'-CACCGTCTCCACGCGCAGTACATTTTCGAAAAATGTACTGCGCGTGGAGA-3'
Bottom oligo	3'-AAAAGTCTCCACGCGCAGTACATTTTCGAAATGTACTGCGCGTGGAGA-5'

CHAPTER 3
INVESTIGATION OF IRON METABOLISM IN A MOUSE MODEL OF MENKES
DISEASE (BRINDLED; Mo^{Brly})

Introduction

Iron is an essential trace mineral that serves as a cofactor for enzymes, which mediate diverse biochemical reactions including oxygen delivery, energy metabolism and immunity. Overall body iron homeostasis is primarily controlled by absorption of dietary iron in the upper small bowel, as no regulated excretory pathways exist in mammals. Properly managing body, tissue and cellular iron levels is critical as free iron can generate oxygen free-radicals, causing damage to biological molecules (e.g. DNA) and membranes. A detailed understanding of molecular mechanisms mediating transepithelial iron transport is thus critical to develop therapeutic approaches to modulate iron absorption in humans with pathologies that perturb iron homeostasis (e.g. iron overload- and iron deficiency-related disorders).

Previous investigations have noted that copper influences iron homeostasis (18, 35, 70). During iron deficiency, in many mammalian species, body copper levels increase (121, 145), including in the intestinal mucosa (110), the liver (119) and in serum (153). Consistent with this, we noted induction of a copper exporter, the Menkes copper-transporting ATPase (Atp7a), in duodenal tissue extracted from iron-deficient rats (24). Atp7a pumps copper into the trans-Golgi network of intestinal epithelial cells (IECs) cells for cuproenzyme synthesis and upon copper excess, traffics to the basolateral membrane to mediate copper efflux (67). Upregulation of Atp7a is thus consistent with documented alterations in intestinal and body copper levels during iron deficiency.

Atp7a is necessary for copper absorption, as exemplified in patients with Menkes disease (135) and in mice harboring *Atp7a* mutations (82), which present with copper loading in enterocytes (and other tissues) and severe systemic copper deficiency. Given its necessity for assimilation of dietary copper, Atp7a may mediate increases in body copper levels during iron deficiency. Moreover, Atp7a (and copper) may be a key player in the compensatory response of the intestinal epithelium to increase body iron acquisition during states of deficiency. The role of copper in iron homeostasis is best exemplified by the multi-copper ferroxidases (FOX), hephaestin (Heph) and ceruloplasmin (Cp). Heph is a membrane-bound FOX expressed in enterocytes, and is important for iron efflux (74), while Cp is a liver-derived, circulating FOX that mediates iron release from stores in liver and spleen (41). During iron deficiency, Cp expression and activity increases, possibly due to increased metallation of the *apo*-enzyme in copper-loaded hepatocytes (107). Heph is also induced during iron deficiency (19), but whether copper accumulation in enterocytes directly influences Heph expression or activity is not known.

The current investigation was undertaken to test the hypothesis that Atp7a function is important to maintain body iron homeostasis during states of deficiency. Atp7a could directly influence enterocyte or liver copper levels, potentially increasing expression or activity of the multi-copper FOXs. Our approach was to utilize Brindled (*Mo^{Brly}*) mice, which have a 6 base-pair deletion in the *Atp7a* gene (45), resulting in a protein with significantly reduced functional activity (28). To assess the role of Atp7a specifically in iron homeostasis, neonatal mutant mice were rescued by copper injection, allowing them to live beyond 14-days-of-age when they would normally die

from severe copper deficiency. Once mutant mice recovered, they along with wild-type (WT) littermates were deprived of dietary iron, and then body iron and copper homeostasis was studied. Results showed that rescued Brindled mice suffered from copper-deficiency anemia, in which hemoglobin, hematocrit and body copper is low, but iron levels are normal. When mutant mice were deprived of iron, they had a similar ability as WT mice to upregulate intestinal iron absorption. However, unlike the situation in WT mice, where no alterations in copper levels were noted, increases in enterocyte and liver copper content and serum ferroxidase activity occurred concomitantly with enhanced iron absorption in mutant mice.

Results

Copper Rescue of Mutant Mice

Mo^{Brly} mice were born at ratios significantly below predicted Mendelian ratios (based upon the breeding scheme) of 1 Mo^{+ly}:1 Mo^{Brly}. The actual ratio was closer to 3 Mo^{+ly}:1 Mo^{Brly}. This investigation utilized ~35 total Mo^{Brly} mice, which resulted from >250 live births from ~ 12 breeding pairs. Except for using some heterozygous females for breeding, WT females and excess WT males were routinely sacrificed in the perinatal period. Copper treatment greatly improved viability of the mutant mice, none of which survived past ~14 days without treatment. Within 24 hours of injection, strikingly, grey colored hair growth occurred (mutant mice were initially pinkish) (Fig. 1A/B). The changes were even more dramatic after the 2nd copper injection (Fig. 1C). A lesion was noted on the upper back marking the site of injection, which gradually became less distinct and was hardly noticeable 10 weeks later (panel E). The coat color never matched that of the WT mice, but copper-treated Mo^{Brly} mice were healthy, showed

normal behavior and were similar in size to WT littermates at 3-months-of-age (panels D and E).

Blood Parameters and Iron Levels in Experimental Mice

Mo^{Brly} mice were anemic as compared to WT littermates (Hb and Hct decreased by ~18%; Table 1). Dietary iron deprivation lowered Hb and Hct levels in both genotypes of mice, with the reduction being more dramatic in the Mo^{Brly} mice (~ 24% from WT levels). Although Mo^{Brly} mice were anemic, they had normal enterocyte, serum and liver iron levels, and hepcidin mRNA expression (Fig. 2). Iron-deprived mice of both genotypes however had significant decreases in tissue iron levels and Hamp mRNA expression was dramatically reduced (~ 95%; Fig. 2).

Quantification of Gene Expression in Mouse Duodenal Mucosa and Liver

Expression of Cybrd1, Dmt1, Fpn1, Tfr1 and Atp7a increased in both genotypes upon dietary iron deprivation (Fig. 3). Fold increases however did not achieve statistically significant differences between genotypes. Interestingly, expression of these genes was unchanged in Mo^{Brly} mice (as compared to WT littermates), despite the fact that they were anemic. Mt1 expression was increased in Mo^{Brly} mice as compared to WT mice (2-fold), and furthermore, induction was noted in both genotypes upon iron deprivation, as compared to control-diet fed mice (Fig. 3). Mt1 induction was greater in the mutant mice (~1.8 X higher). Expression of ferritin (Ftn), Heph and copper transporter1 (Ctr1) mRNA was not different between genotypes or dietary groups (data not shown).

In the liver, Tfr1 mRNA expression was increased by iron deprivation in both genotypes (~ 5 fold in WT mice and 3.5-fold in Mo^{Brly} mice) (Fig. 4). Moreover, expression of two known hypoxia-responsive gene, Bnip3, was upregulated in all mice

that had low circulating hemoglobin levels (i.e. Mo^{Brly} mice and both genotypes consuming the low-iron diet). Furthermore, hepatic expression of ceruloplasmin (Cp), Dmt1 and Fpn1 was not significantly different amongst all groups of mice (data not shown).

Copper Levels in Serum and Tissues of Mice, and Serum Cp Activity

Enterocyte copper levels were similar in WT mice in both dietary treatment groups and in Mo^{Brly} mice consuming the control diet (Fig. 5). In FeD Mo^{Brly} mice however, the enterocyte copper content was increased ~3-fold. Serum copper levels were reduced ~ 55% in Mo^{Brly} mice consuming both diets, as compared to wild-type mice in both dietary treatment groups. Furthermore, liver copper content, while not differing between WT mice consuming either diet, was reduced ~46% in Mo^{Brly} mice consuming control diet and ~ 25% in Mo^{Brly} mice consuming the low-iron diet (both as compared to WT mice) (Fig. 5).

Serum FOX activity was not different when comparing the WT mice on either dietary treatment, but was reduced in both groups of Mo^{Brly} mice (Fig. 5). FOX activity was however higher in the Mo^{Brly} mice consuming the low-iron diet, as compared to those consuming the control diet. For example, at the 60 min time point, serum FOX activity was reduced from control values ~54% in Mo^{Brly} mice but the reduction was only ~29% in FeD Mo^{Brly} mice. At the 90 min time point, in Mo^{Brly} mice on control or low-iron diets, the reductions were ~66% and ~50%, respectively.

⁵⁹Fe Absorption and utilization after dietary iron deprivation with or without concurrent phlebotomy

In mice used for iron uptake studies, Hb and Hct levels showed similar reductions to data presented in Table 1 (Fig. 6). Mice that were concurrently bled however

displayed more significant Hb and Hct reductions. Iron absorption (% of ^{59}Fe dose), and ^{59}Fe in blood, liver and spleen was increased to a similar extent in FeD mice of both genotypes, and furthermore, did not vary between WT mice and Mo^{Brly} mice consuming the control diet (Fig. 6).

Discussion

Although copper homeostasis has been thoroughly examined in Brindled mice (as cited above), to our knowledge, iron homeostasis has not been investigated. Suckling mice were previously injected with iron (and compared to copper injection) and killed a few days later for analysis (98, 102); however, mechanisms of absorption are different in neonatal mice, likely involving non-specific nutrient absorption via pinocytosis and paracellular flux (80). Direct comparison to the current studies using adult Mo^{Brly} mice is thus not meaningful.

Brindled mice die perinatally of severe copper deficiency (81, 112). Early studies on Mo^{Brly} mice demonstrated that they could be rescued by IP injection of copper around the 8th day of life (122). Copper-treated, mutant mice developed and grew normally but still had persistent perturbations in body copper levels; 53 days post-injection, Cu levels in kidney were high and levels were low in brain and serum (82). We treated mice twice with copper chloride in the perinatal period, allowed mice to recover for 7-8 weeks and then deprived them of dietary iron. Wild-type littermates did not undergo copper injections, as we predicted it would be without effect since mice were studied ~11 weeks after treatment of the mutants. Earlier studies treated WT mice with copper, and although liver copper levels were very high 11 days post-injection (7X higher than untreated controls), they had normalized 2 weeks later (82), and no other persistent perturbations in copper levels were noted. Furthermore, although the copper

treatment likely produced an acute inflammatory response, when mice were studied at 12-weeks-of-age, there was no sign of inflammation, as exemplified by normal hepatic hepcidin (Hamp) mRNA expression. Hamp is known to be strongly induced at the transcriptional level by pro-inflammatory cytokines (e.g. IL-6) (39), and as such, Hamp gene expression serves as a sensitive marker of the acute-phase response and was used as an indirect indicator for testing inflammation in this study.

Adult, Mo^{Brly} mice were similar in size to and just as active as WT littermates, had pigmentation defects and were anemic, but had no abnormalities in enterocyte, serum, and liver iron levels and no changes in Hamp expression. The mutant mice did however have perturbations in body copper levels including decreased hepatic and serum copper, indicative of severe systemic copper deficiency (see Supplemental Table 2 for a comprehensive overview of all data from this study). Low hepatic copper levels have been noted in Brindled mice (14), although this is thought to be a secondary phenomenon related to copper being avidly taken up by and trapped within other tissues (81). Serum FOX (i.e. Cp) activity was also reduced in the rescued mutants, consistent with previous observations. In the current investigation, a significant relationship between serum and liver copper and serum FOX activity was documented, when considering all groups of experimental mice together ($r = 0.8852$ for serum copper vs. serum FOX activity, and $r = 0.9552$ for liver copper vs. serum FOX activity) (Figure 3-7). This is consistent with our previous observations in rats consuming various iron- and copper-deficient diets (107). Given the documented role of Cp in iron release from liver and other tissues (55), it is not readily apparent why liver iron levels were not increased in the mutant mice. In sum, these observations demonstrated that copper-

treated, Brindled mice suffered from copper-deficiency anemia, displaying many known symptoms of copper deprivation in mice (97). Interestingly, this is in contrast to phenotypical changes associated with copper deficiency in other mammalian species, including swine, rats and humans (75, 88, 100), in which perturbations in iron homeostasis are noted. These differences could be species-related or due to the underlying genetic defect in Mo^{Brly} mice.

The main goal of this investigation was to define the role of Atp7a and copper in the compensatory response of the intestinal epithelium to iron deprivation. Accordingly, iron absorption studies were performed in all experimental mice, including WT, iron-deficient (FeD) WT, Brindled and FeD Brindled mice. FeD WT mice had the classical iron-deficient phenotype. Although body copper levels were not altered in the FeD WT mice, as is commonly noted in other mammalian species (e.g. human, rat etc.), metallothionein and Atp7a gene expression increased in duodenal enterocytes perhaps indicating subtle alterations in copper homeostasis. As expected, iron absorption in WT FeD mice was significantly enhanced by iron deprivation and was further increased by concurrent phlebotomy.

Data interpretation from the Mo^{Brly} mice was more complex given the underlying copper deficiency associated with both dietary treatment groups. The mutants consuming a standard chow diet were copper deficient, but had no observable perturbations in iron homeostasis, in contrast to previous studies on dietary copper deficiency in mice (8, 20) and rats (111), in which hypoferremia was noted. It was hypothesized that Heph expression/activity was decreased by copper deprivation and that this reduced iron release from enterocytes causing hypoferremia, consistent with

the phenotype of *Heph* mutant (sex-linked anemia [*sla*]) mice (3). In the case of the Mo^{Brly} mice, although they had systemic copper deficiency, enterocytes were not depleted of copper (probably due at least in part to the decreased copper export function of Atp7a), perhaps explaining why iron absorption was not affected. This could also explain why the Mo^{Brly} mice did not have low serum iron and were not iron deficient, although such a conclusion would require experimental validation. Past studies have provided conflicting results in regards to iron homeostasis in the setting of dietary copper deprivation in mice (60, 99, 152).

Despite the anemia and probable intestinal hypoxia in Mo^{Brly} mice, documented Hif2 α targets in duodenal enterocytes (e.g. Dmt1, Fpn1, Atp7a) were not induced. Known hypoxia-responsive genes were indeed induced in liver of the mutant mice (Bnip3 (108)), consistent with decreased blood hemoglobin levels (and tissue hypoxia). However, when Mo^{Brly} mice were deprived of dietary iron, Hif2 α target genes were induced (as in WT FeD mice), consistent with previous observations (117, 124). These observations demonstrate that the signal for Hif2 α -mediated induction of iron and copper homeostasis-related genes in mice is low intracellular iron (i.e. hypoxia in the setting of normal intracellular iron levels did not trigger the response).

Of further note is the fact that although circulating FOX (i.e. ceruloplasmin) levels increased during FeD of Mo^{Brly} mice, no changes in hepatic *Cp* mRNA expression were observed. This is in contrast to the reported Hif1 α -mediated transcriptional induction of the *Cp* promoter in HepG2 cells (87), but is consistent with our previous study in rats in which iron deprivation increased hepatic *Cp* protein expression and serum *Cp* activity without effect on *Cp* mRNA expression (107). Thus, in vivo upregulation of *Cp* in rats

and mice during iron-deficiency anemia (and hypoxia) likely does not involve HIF signaling in the liver.

In summary, the compensatory response of the WT mice used in this study (CBA/C3H) to iron deprivation does not involve concurrent alterations in enterocyte or liver copper levels or changes in serum FOX activity. This exemplifies a perhaps unique aspect of iron homeostasis in this strain of mice, as altered copper homeostasis typifies iron deficiency in humans and other mammalian species. Conversely, in the same strain of mice harboring a 6 bp deletion in the *Atp7a* gene, enhanced iron absorption upon iron deprivation was associated with increased enterocyte and hepatic copper levels and serum FOX activity. The well-established interrelationship between iron and copper is thus preserved in the Mo^{Brly} mice. These findings raise two important questions: 1) are alterations in copper homeostasis necessary for the Mo^{Brly} mice to appropriately upregulate intestinal iron absorption, and 2) how can hepatic copper levels and serum FOX activity increase in the absence of an essential intestinal copper exporter? In regards to question 1, we speculate that a threshold of enterocyte and hepatic copper levels is required to maintain iron homeostasis and given that Mo^{Brly} mice were copper deficient, increases in copper levels during iron deprivation were necessary to achieve that threshold (and support iron homeostasis). Question 2 has two possible explanations, first that residual *Atp7a* function could explain increased hepatic copper levels, or secondly that hepatic copper loading is independent of copper absorption and relates to altered copper excretion, as mediated by the *Atp7b* copper-transporting ATPase expressed on the canicular surface of hepatocytes. Although definitive answers to these puzzling questions await further experimentation, these novel studies provide

additional support of the concept that copper plays an important role in the maintenance of mammalian iron homeostasis.

Table 3-1. Analysis of blood collected from WT and Brindled ($Mo^{Br/y}$) mice consuming control and iron-deficient diets. Hemoglobin (Hb) and hematocrit (Hct) levels were measured from blood samples taken from experimental mice. (a,b,c) indicates value are statistically different from one another ($p < 0.05$). n= 8 animals per group.

	Hemoglobin (g/dL)		Hematocrit (%)	
	WT	$Mo^{Br/y}$	WT	$Mo^{Br/y}$
Ctrl	15.7 $\pm$ 0.71 ^a	13.4 $\pm$ 0.57 ^b	50.9 $\pm$ 1.83 ^a	46.2 $\pm$ 1.91 ^b
FeD	14.4 $\pm$ 0.86 ^b	11.9 $\pm$ 0.59 ^c	47.6 $\pm$ 3.15 ^b	41.4 $\pm$ 2.88 ^c

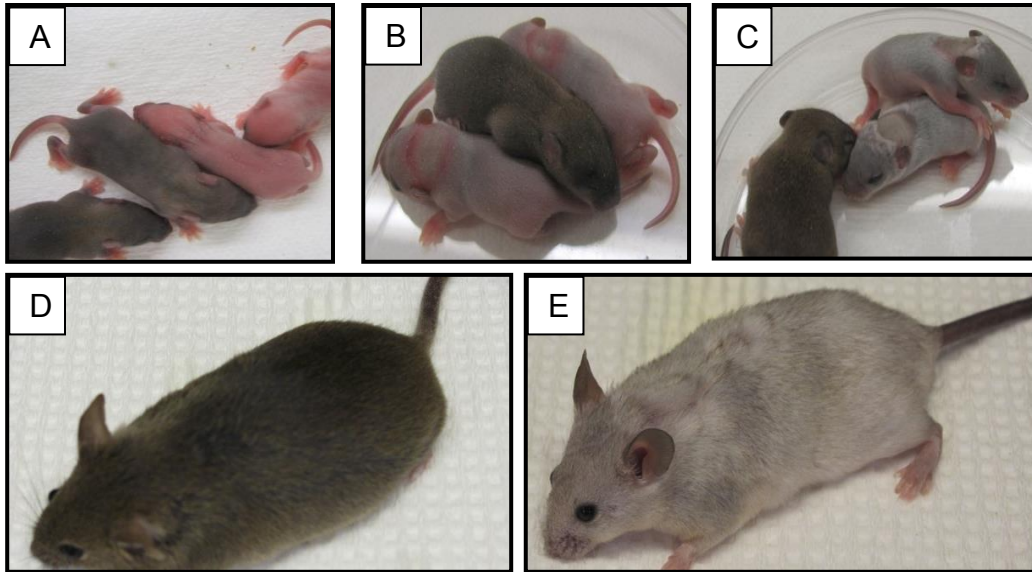


Figure 3-1. Phenotypic response of $Mo^{Br/y}$ mice to copper injection. Mice were photographed before and after copper treatment to exemplify the dramatic phenotypical changes that occur in the mutants. (A) 7-day-old male mice prior to treatment. Mutant males are pink. (B) 8-day-old mice after first Cu injection. (C) 9-day-old mice after second Cu injection. (D) 3-month-old WT male. (E) 3-month-old copper-treated $Mo^{Br/y}$ male. In panels A, B & C, untreated WT littermates are shown for comparison.

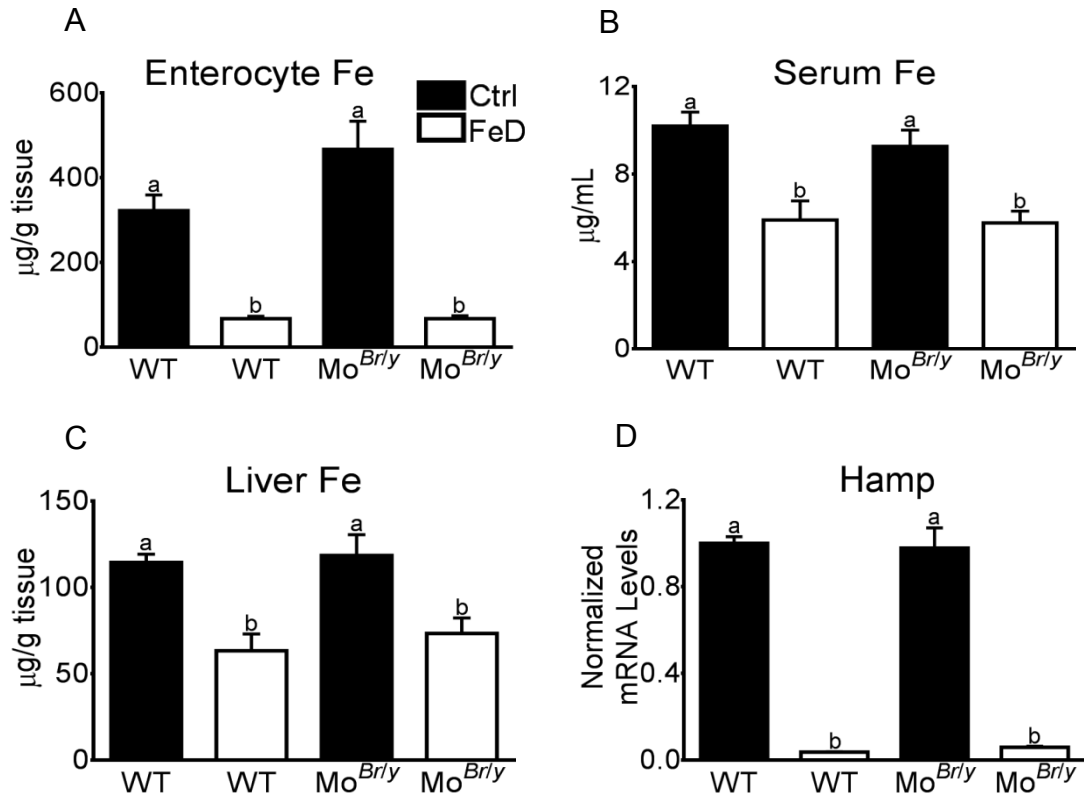


Figure 3-2. Tissue Fe levels and hepatic hepcidin (Hamp) gene expression. Iron levels were determined as described in Methods. Results are depicted graphically, with filled bars representing data from mice fed the control diet and open bars depicting data from FeD mice. Genotype is indicated beneath each bar. Iron levels were normalized by mass of tissue or volume of serum. (A) Enterocyte Fe content, $n=3$ per group; (B) Serum Fe content, $n=4$ for WT mice and 3 for Mo^{Brly} mice; (C) Liver Fe content, $n=10$ mice per group. Also depicted is hepatic Hamp mRNA expression (D), normalized to cyclophilin mRNA levels (which did not vary significantly). $n=8$ per group. ^{a,b}Statistically different from one another within each panel ($p < 0.05$). WT, wild-type. Bars (A-D) depict mean $\pm$ SD.

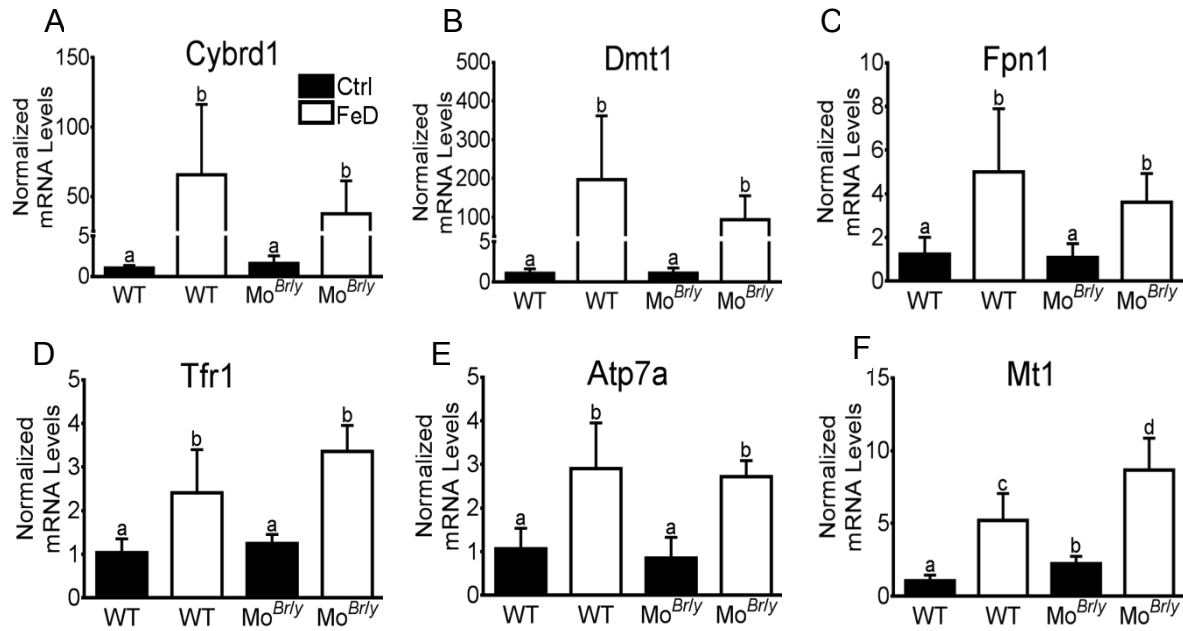


Figure 3-3. Intestinal qRT-PCR analysis of iron and copper homeostasis-related genes. Expression of key genes (indicated in each panel) was determined by standard methods, with each experimental gene being normalized to expression of mouse cyclophilin mRNA (which did not vary significantly). Filled bars represent data from mice fed the control diet and open bars represent data from mice consuming the low-iron diet. Genotype is indicated below each bar. ^{a,b,c,d}Statistically different from one another within each panel (p < 0.05). n = 4 for each genotype consuming the control diet and n = 6 for each genotype fed the low-iron diet. WT, wild-type. Bars (A-F) depict mean ± SD.

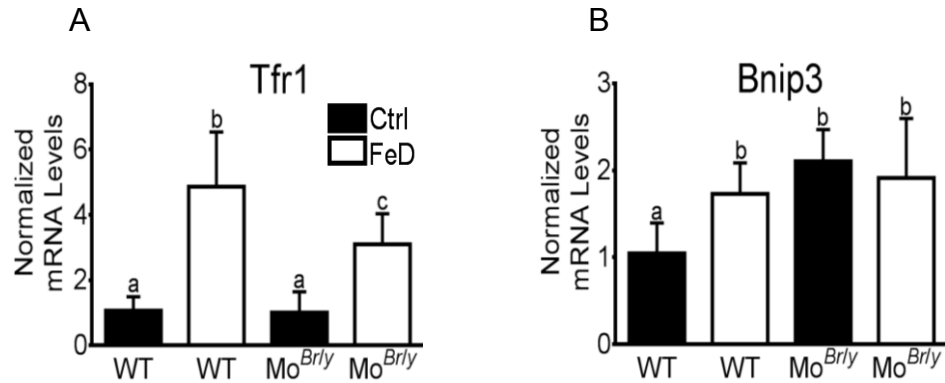


Figure 3-4. Liver qRT-PCR analysis of iron- and hypoxia-related genes. Expression of key genes (indicated in each panel) was determined by standard methods, with each experimental gene being normalized to expression of mouse cyclophilin mRNA (which did not vary significantly). Filled bars represent data from mice fed the control diet and open bars represent data from mice consuming the low-iron diet. Genotype is indicated below each bar. ^{a,b,c,d} Statistically different from one another within each panel ($p < 0.05$). $n=6$ for all groups. WT, wild type; Bnip3, BCL2/adenovirus E1B 19 kDa interacting protein 3. Bars (A-B) depict mean $\pm$ SD.

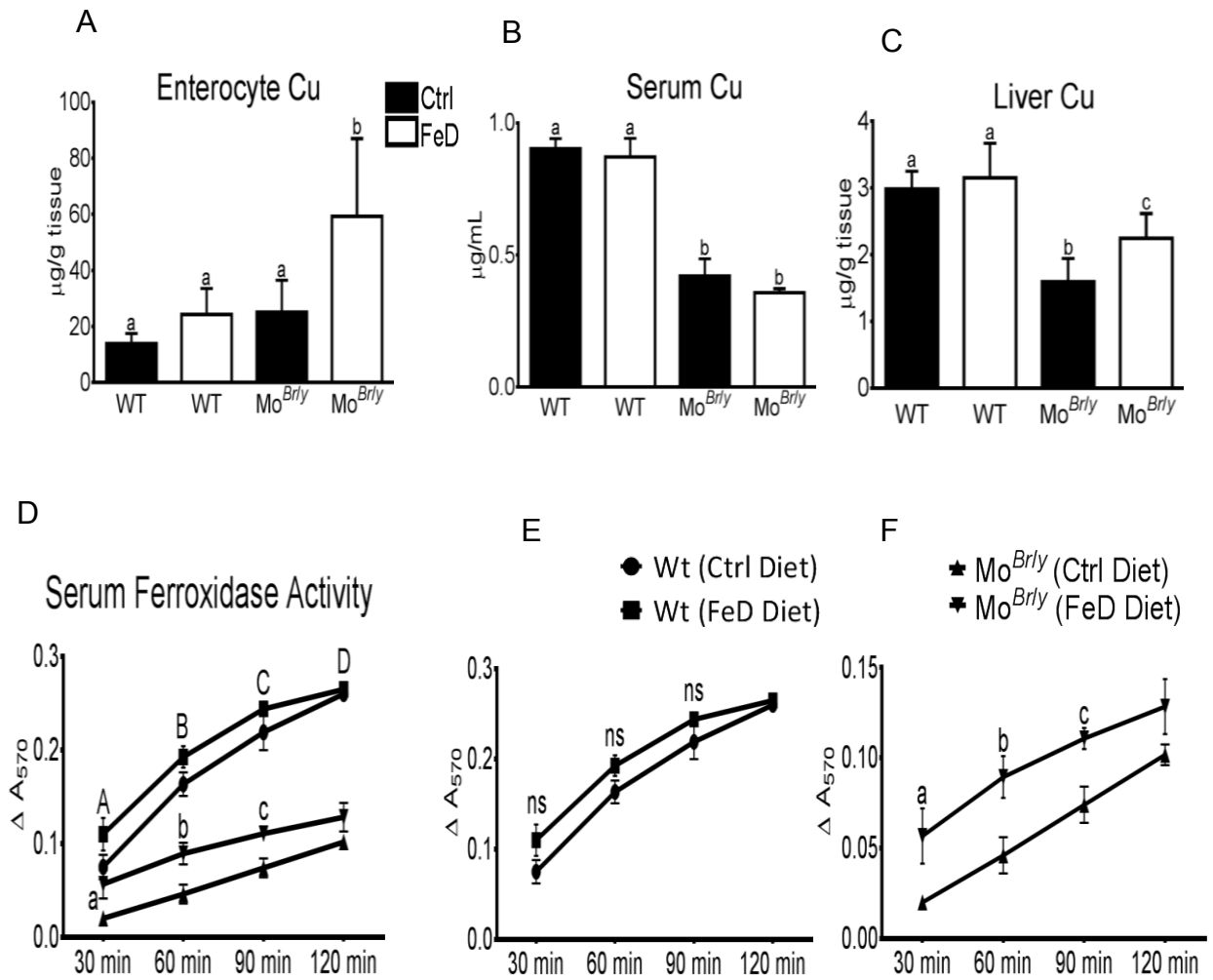


Figure 3-5. Tissue and serum Cu levels and serum FOX activity in experimental mice. Copper levels were determined as described in Methods (A-C). Results are depicted graphically, with filled bars representing data from mice fed the control diet and open bars depicting data from FeD mice. Genotype is indicated beneath each bar. Copper levels were normalized by mass of tissue or volume of serum. (A) Serum copper levels, $n=4$ for both groups of WT mice and $n=3$ for both groups of Mo^{Br/y} mice; (B) Enterocyte copper levels, $n=3$ for all groups; (C) Liver Fe levels, $n=10$ for all groups. ^{a,b}Statistically different from one another within each panel ($p < 0.05$). Also depicted is quantification of serum FOX activity (D-F), as determined by ferrozine assay. Composite data from all 4 groups is shown in panel D, while (E) shows data from WT mice consuming control or low-iron diets and (F) depicts data from Mo^{Br/y} mice on both diets. (D) Uppercase letters indicate that data from both control groups statistically differ from data from both groups of Mo^{Br/y} mice, while lowercase letters indicate significance between the Mo^{Br/y} mice on the different diets ($p < 0.05$ for both); (E) ns, not-significant; (F) Lowercase letters indicate significance between groups at individual time points ($p < 0.05$). (D-F) Statistics by 2-way ANOVA followed Bonferroni's multiple comparisons test. $n=4$ for both control groups and $n=3$ for both groups of Mo^{Br/y} mice. Bars (A-F) depict mean $\pm$ SD.

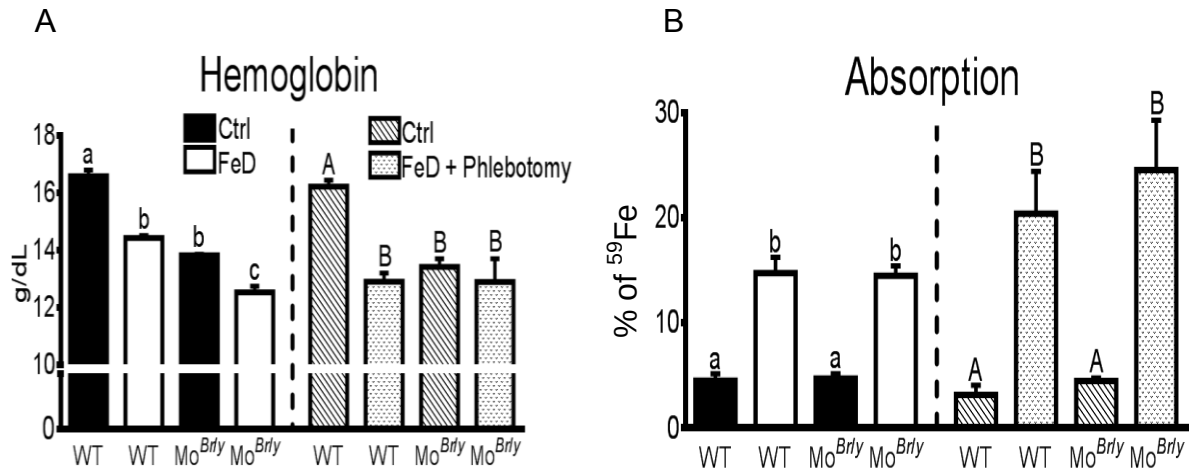


Figure 3-6. Intestinal iron absorption and blood, liver, spleen iron levels in experimental mice deprived of dietary iron with or without concurrent phlebotomy. Mice were fed an iron-deficient diet for three weeks with or without concurrent once weekly bloodletting. Subsequently, ⁵⁹Fe was administered by oral gavage and the distribution of radioactivity was assessed 24 hours later. Blood hemoglobin levels are depicted (A) as well as % of ⁵⁹Fe dose absorbed (B), as an indicator of intestinal iron absorption, and radioactive counts in blood, liver and spleen (C-E). In all panels, the left side depicts data from dietary iron deprivation only, while the right panel shows data from dietary iron deprivation plus bleeding. Each bar is defined in panel A, and is consistent throughout all panels. a, b and A, B indicate statistical significance between groups within each one half of an individual panel ($p < 0.05$). Genotypes are indicated below each bar. For dietary treatment groups, $n=3$ for all; for dietary treatment plus phlebotomy groups, $n=5$ for WT mice fed the control diet, $n=6$ for WT mice consuming the low-iron diet, and $n=4$ for Mo^{Br/y} mice consuming both diets. Bars (A-E) depict mean $\pm$ SD.

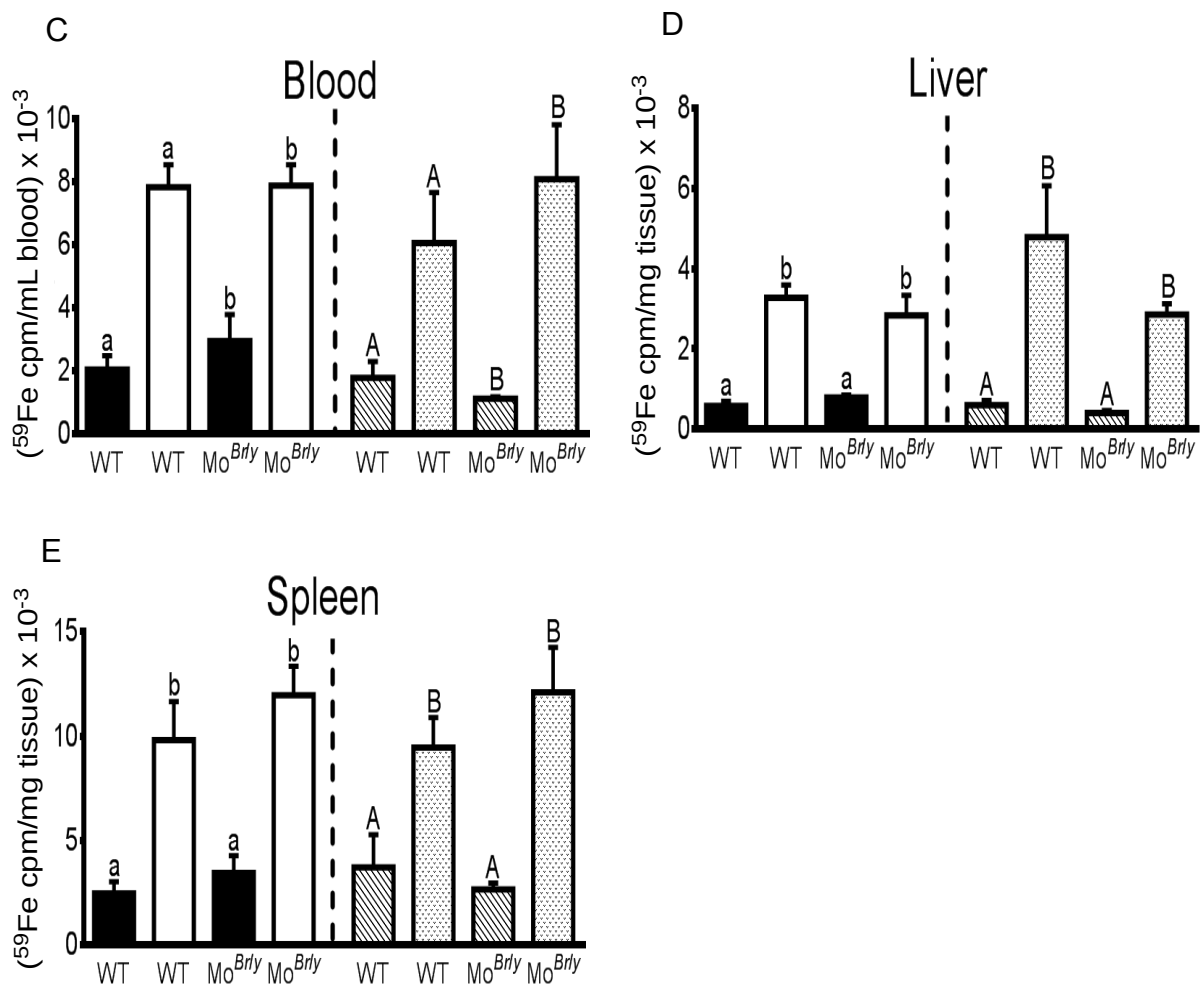


Figure 3-6. Continued.

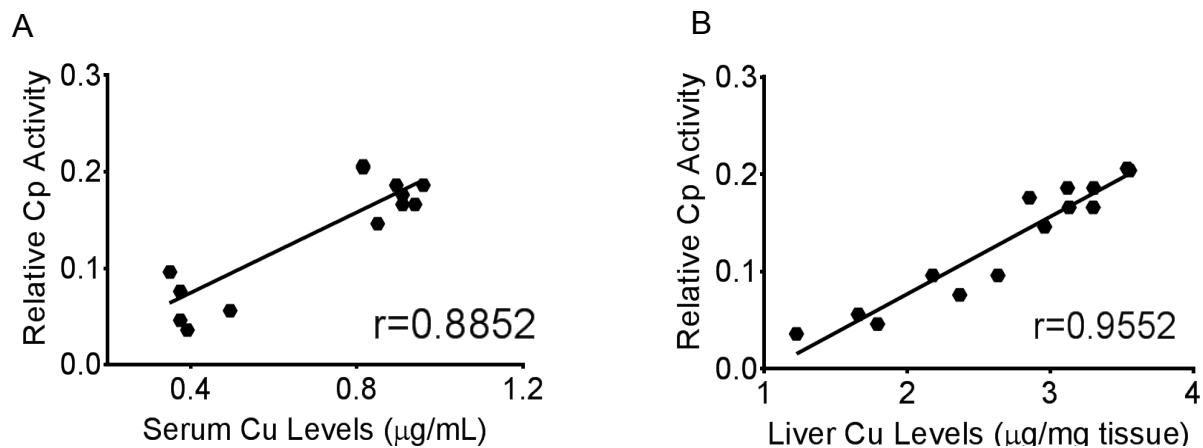


Figure 3-7. Relative Cp activity as a function of serum and liver copper levels. Plots show the relationship between serum (A) and liver (B) copper and Cp activity. Lines fitting the data were derived by linear regression for Cp activity versus serum and liver copper. In panel A and B, $P < 0.0001$. r , Pearson correlation coefficient.

Table 3-2. Summary of Iron/Copper Related Parameters in Experimental Mice

Parameters for Investigation	WT ^{&}	WT (FeD)	Mo ^{Brly}	Mo ^{Brly} (FeD)
Hemoglobin*	100%	92%	85%	76%
Hematocrit	100%	94%	91%	81%
Serum Fe	100%	57%	100%	57%
Enterocyte Fe	100%	21%	100%	21%
Liver Fe	100%	60%	100%	60%
Hamp mRNA Expression	100%	5%	100%	5%
Serum Cu	100%	100%	41%	41%
Enterocyte Cu	100%	100%	100%	4X
Liver Cu	100%	100%	53%	75%
Serum FOX Activity (60 min)	100%	100%	46%	71%
Serum FOX Activity (90 min)	100%	100%	50%	66%
Enterocyte Gene Expression				
Cybrd1	1X	45X	1X	45X
Dmt1	1X	140X	1X	140X
Fpn1	1X	4X	1X	4X
TfR1	1X	3X	1X	3X
Atp7a	1X	3X	1X	3X
Metallothionein	1X	5X	2X	4X
Hepatic Gene Expression				
TfR1	1X	5X	1X	3X
Bnip3	1X	2X	2X	2X
Iron Absorption Studies**				
Hemoglobin	100%	83%	87%	86%
Hemoglobin (+bleeding)	100%	81%	81%	81%
Fe Dose Absorbed	1X	3X	1X	3X
Fe Dose Absorbed (+bleeding)	1X	6X	1X	6X
Iron in Blood (both groups)	1X	4X	1X	4X
Iron in Liver (both groups)	1X	6X	1X	6X
Iron in Spleen (both groups)	1X	4X	1X	4X

*All values rounded to the nearest whole number

**Changes in FeD mice compared to appropriate control group

[&] Identical values between groups indicates no statistical differences (data were averaged between the groups that were not different)

CHAPTER 4
SMALL HAIRPIN RNA (shRNA) KNOCK-DOWN OF THE MENKES COPPER-
TRANSPORTING ATPASE (ATP7A) ALTERS IRON HOMEOSTASIS IN RAT
INTESTINAL EPITHELIAL (IEC-6) CELLS

Introduction

Iron is a transitional mineral involved in a variety physiological pathways and disease processes, and it is toxic when in excess (33, 133). Low dietary iron intake reduces production of hemoglobin and utilization of oxygen in body, resulting in iron-deficiency anemia. Iron homeostasis is principally controlled by regulation of intestinal absorption as no active excretory mechanisms exist. Therefore, efficient intestinal iron absorption is essential to protect from development of anemia. Dietary iron absorption occurs in epithelial cells of the duodenum. There are two main steps for iron transport in enterocyte. First, dietary ferric iron is reduced by duodenal cytochrome reductase B1 (Cyrb1) followed uptake of iron into cells via divalent metal transporter 1 (Dmt1) (37). Next, ferrous iron is exported by ferroportin1 (Fpn1) from enterocytes and then it is oxidized via Hephaestin (Heph) (20). Finally, iron binds to transferrin (Tf), which is the main iron carrier in the blood (142). Heph is a copper-dependent ferroxidase protein that is anchored to the cell membrane by a single C-terminal domain and it functions in tandem with Fpn1 to support iron needs for body (136).

The importance of Heph function was first illustrated in mice with sex-linked anemia (*sla*) (21). These mutant mice had a frame shift mutation in the *Heph* gene resulting in a truncated form of the Heph protein, which showed reduced ferroxidase activity relative to the wild-type (WT) protein (21, 123). Heph is expressed in a subcellular compartment where copper could be delivered to Heph by the Menkes Copper-transporting ATPase (Atp7a) (74). This situation would be analogous to the

situation in liver whereby the Wilson's Copper-Transporting ATPase (Atp7b) delivers copper for ceruloplasmin synthesis in the trans-Golgi network (68). During iron deficiency, Heph translocates to the basolateral membrane of enterocytes where it oxidizes ferrous iron for binding to transferrin (74). Efficient iron transport from the intestine during iron deficiency occurs when intracellular copper levels are elevated (121). Intracellular copper increases might lead to greater metallation of Heph, resulting in higher ferroxidase activity, given that copper is necessary for the electron transfer reaction.

Iron- copper interaction was also shown to be related to human disease. Copper is an important mineral to allow proper formation of hemoglobin and copper deficiency results in iron deficiency-like anemia, historically called cholorosis, which could be corrected by only copper injection (46) . Still today, the precise role of copper in hemoglobin formation is not understood, but the copper-dependent step likely occurs in bone marrow where red blood cells are produced. In addition to anemia, copper deficiency decreases Heph activity and intestinal iron absorption in a rat model (111).

Given the importance of iron for many physiologic functions, it is essential to understand the cellular and molecular mechanisms that are involved in intestinal iron absorption. It was recently noted that Atp7a mRNA and protein levels are elevated during iron deficiency in rat (110) and mouse enterocytes (Gulec, and Collins, unpublished observation). In addition to intestine, Atp7a is expression in various organs of neonatal rats, including intestine, placenta, brain, heart, lung, muscle, kidney, and pancreas (90). Atp7a protein is primarily localized in the trans-Golgi network (TGN) during normal physiological circumstances; however, if copper levels are increased in

cytoplasm, Atp7a traffics to the plasma membrane and functions in copper export (67). Not only is Atp7a regulated during iron deficiency, but it is also induced during hypoxia, which is an important signaling mechanism for intestinal iron absorption. These facts implicate Atp7a as another possible candidate for regulation of the enterocyte iron homeostasis. Intestinal hypoxia inducible factor 2 alpha (Hif2 α) knocked out mouse model showed reduced mRNA levels of iron transport-related genes including Dmt1, Fpn1 and Tfr1, and as a result, intestinal iron absorption was decreased (117). Atp7a was also shown to be a Hif2 α target gene in rats, which may mediate induction during iron deprivation (150). Regulation of Atp7a during hypoxia or iron-deficiency anemia suggests that Atp7a might be involved in intestinal iron metabolism. However, to date, there have been no functional studies which show possible physiological effects of Atp7a on intestinal iron metabolism.

To understand the possible physiological role of Atp7a in iron homeostasis, we created an Atp7a knock down rat intestinal cell line (Atp7a KD IEC-6). Reduction of Atp7a protein increased intracellular copper levels presumably due to a decrease in the copper export function of Atp7a. Unexpectedly, Atp7a KD decreased Heph mRNA levels, whereas Fpn1 mRNA levels were induced and further enhanced in KD cells by DFO treatment. Furthermore, Atp7a KD reduced ferroxidase (FOX) activity in membrane and cytosol fractions of IEC-6 cells and thus, FOX activity correlated with Heph mRNA expression patterns. Furthermore, Iron transport studies were done in the Atp7a KD model. IEC-6 cells grown on collagen-coated membranes can form an enterocyte-like phenotype (17). Interestingly, Atp7a KD IEC-6 cells grown on collagen-coated membranes showed higher iron uptake and transport compared to control cells.

These results demonstrate that lack of Atp7a function decreases intestinal Heph-dependent FOX activity. However, this does not affect intestinal iron absorption in this *in vitro* model.

Results

Atp7a KD in IEC-6 Cells

Atp7a mRNA levels were significantly reduced (~75%, $p < 0.001$) in cells transfected with Atp7a-target shRNAs relative to cells transfected with a negative control shRNA (Fig. 4-1A). This reduction of Atp7a mRNA was correlated with Atp7a protein expression in the KD cells (~90%, $p < 0.001$; Fig. 4-1B/C). Copper-loading experiments showed that intracellular copper levels were significantly elevated in Atp7a KD cells (1.5-fold for 3 hr, 2.5-fold for 16 hr) (Fig. 4-1D/E).

Quantification of Gene Expression in IEC-6 Cells

Iron- and copper- regulated gene expression were affected by Atp7a KD and DFO treatment. Atp7a mRNA expression was induced by DFO treatment and decreased >65% in untreated and DFO-treated KD cells (Fig. 4-2A). Tfr1 mRNA was used a control for iron deficient condition in experiment and mRNA of Tfr1 was significantly induced in both Ctrl and Apt7a KD cells during DFO treatment (Fig. 4-2B). Surprisingly, expression of Fpn1 mRNA (3.5-fold, $p < 0.005$) (Fig. 4-2C) and Mt1 (9-fold, $p < 0.001$) was induced in Atp7a KD IEC-6 cells; induction of both genes was however partially attenuated by iron chelation (Fig. 4-2C/D). Conversely Atp7a KD reduced Heph mRNA expression (~70%, $p < 0.001$) under both conditions (Fig. 4-2E). Atp7a KD resulted in a slight increase in Dmt1 mRNA expression (data not shown). Atp7a KD and DFO treatment did not affect expression of Ctr1 or ferritin and furthermore, Atp7b mRNA was not detected in IEC-6 cells under any conditions (data not shown).

Membrane and Cytosolic Fractionation of IEC-6 Cells

Membrane and cytosolic proteins were isolated from Ctrl and Atp7a KD IEC-6 cells and were analyzed for relative purity of membrane and cytosolic fractions by western blotting (Fig. 4-3). The membrane protein Atp7a was only detected in membrane fractions and Atp7a protein level was reduced in Atp7a target shRNA-transfected IEC-6 cells. Data also indicated that no significant protein contamination occurred between membrane and cytosol fractions, consistent with previous observations by us (106).

Membrane and Cytosolic FOX Activity

FOX activity was detected in membrane and cytosolic fractions isolated from the IEC-6 cells (Figure 4-4 and 4-5). This is consistent with previous studies in which we detected robust FOX activity in duodenal enterocytes isolated from rats and mice (105). In membrane fractions, FOX activity was decreased in Atp7a KD cells (30-50%). DFO treatment slightly decreased membrane-derived FOX activity in Ctrl cells (10-15%), but had no effect in Atp7a KD cells. Similar results were noted in cytosolic fractions with Atp7a KD partially attenuating activity, and DFO causing modest reductions in activity in both Ctrl and KD cells. Further experiments showed that FOX activity in both fractions was abolished by pretreatment of samples with SDS (Fig. 4-6), consistent with previously published observations (105).

⁵⁹Fe Transport in IEC-6 Cells

IEC-6 cells differentiate when grown on cell culture inserts for several days post-confluence, as evidenced by alkaline phosphatase expression (146), which typifies fully differentiated enterocytes. In our studies, phenol red addition to apical chambers indicated that IEC-6 monolayers had fully formed tight junctions, indicated by a lack of

phenol red flux across cell culture inserts containing confluent monolayers (8 days post-confluence) (Fig. 4-7A). Further, TEER measurements were consistent with previous studies where IEC-6 cells were utilized for transport studies (Fig. 4-7B) (17, 103). Cells cultured under identical conditions were subsequently utilized for iron transport studies. ^{59}Fe uptake was significantly lower with DFO treatment in Ctrl and KD cells (~60-65% decrease; Fig. 4-7C). The KD cells however accumulated more ^{59}Fe under both conditions (1.5-fold higher with no treatment and 1.8-fold with DFO treatment). Furthermore, transepithelial iron flux was assessed by measuring accumulation of ^{59}Fe in the basolateral chambers of culture cells. DFO treatment increased iron flux in Ctrl and KD cells (~1.4-fold in Ctrl cells and ~1.9-fold in KD cells; Fig. 4-7D). Iron flux was significantly higher in KD cells under both conditions (~1.2-fold with no treatment and ~1.7-fold with DFO treatment).

Discussion

This investigation was undertaken to test the hypothesis that Atp7a influences iron flux across duodenal enterocytes. The rationale for these studies was based upon our observation that Atp7a is induced in the rodent intestinal epithelium during iron deficiency in parallel with genes involved in iron transport (e.g. Dcytb, Dmt1, Fpn1) (24). Moreover, we recently demonstrated that the mechanism of Atp7a induction during iron deprivation relates to a hypoxia-inducible factor (HIF2 α) (150), which also mediates induction of Dcytb, Dmt1 and Fpn1 (117). Proven co-regulation of Atp7a with these other genes thus provided the impetus to consider a potential role for Atp7a in intestinal iron transport, despite the fact that it has been principally associated with intestinal copper homeostasis to date (62, 149).

A logical approach to assess the influence of Atp7a on iron transport is to utilize an in vivo or in vitro model in which Atp7a expression is reduced or absent. *Atp7a* mutations underlie Menke's disease in humans (128), and mouse models are available in which the Atp7a gene is mutated, producing a protein with reduced activity (28). However, these mouse models are complicated by the fact that Atp7a mutation disturbs copper homeostasis, with copper accumulation in some tissues in the setting of severe systemic copper deficiency (80, 81). These perturbations in copper metabolism confound analysis of iron homeostasis given the well-recognized interactions between these two essential trace minerals ((8, 20); Gulec and Collins, submitted). Our approach was thus to model Atp7a regulation in vitro. As the original description of Atp7a induction during iron deficiency was made in rats, we chose the IEC-6 cell model for these studies. IEC-6 cells are derived from rat jejunum and are known to differentiate in post-confluent culture (146). Moreover, IEC-6 cells have been used as a model of intestinal transport (127), and have been shown to express an inducible iron transport system (89). We also previously documented that Atp7a mRNA expression in IEC-6 cells is induced by iron deprivation (151), consistent with observations in iron-deficient rodents.

Atp7a knock down in IEC-6 cells was accomplished by transfecting cells with plasmids expressing Atp7a-specific shRNAs. Stable transfectants were selected for by antibiotic treatment. Cells were also stably transfected with an shRNA plasmid expressing a negative control (i.e. unrelated) shRNA. Atp7a KD cells had significant reductions in Atp7a mRNA and protein expression. Moreover, KD cells accumulated copper when extra copper was added to the medium, indicating a defect in the Atp7a

copper export function. KD cells also showed increased expression of an intracellular copper-binding protein (Mt1) under normal cell culture conditions, likely reflecting increases in intracellular copper. The in vitro model was thus validated and further experiments were undertaken to assess a possible influence of Atp7a KD on iron homeostasis.

Unexpectedly, significant reductions in Heph mRNA expression were noted in Atp7a KD cells. To determine the functional significance of this, FOX assays were performed in membrane and cytosolic samples derived from control and KD cells. We previously noted that rodent enterocytes have notable FOX activity in membrane and cytosolic fractions (106), and immunoreactive Heph protein was detected in both fractions (106). Relative purity of fractions was confirmed in the current studies by western blotting with an Atp7a-specific antiserum, using an identical purification protocol. Consistent with the Heph mRNA expression data, FOX activity was significantly reduced in both fractions, suggesting that Atp7a KD reduced Heph FOX activity. A logical prediction would have been that Atp7a influences Heph activity via its copper delivery action in the trans-Golgi (where biosynthesis of Heph could occur). How Atp7a KD could directly influence Heph mRNA expression however and whether intracellular copper is involved in this effect is unclear.

Another unexpected consequence of Atp7a KD was an increase in Fpn1 mRNA expression. Previous studies have provided conflicting results regarding Fpn1 regulation during alterations in copper homeostasis (60, 99). Importantly, increases in Fpn1 mRNA expression correlated nicely with iron transport data, particularly during DFO treatment when iron egress from KD cells increased >2-fold over control cells. It thus

seems likely that changes in Fpn1 expression in Atp7a KD cells have biologically relevant functional consequences.

These studies have revealed unanticipated alterations in cellular iron metabolism related to disruption of Atp7a function. Whether these alterations relate to the copper transport function of Atp7a is not immediately clear. The most important findings can be summarized as follows: 1) Atp7a KD reduces Heph mRNA expression and membrane and cytosolic FOX activity, but does not appear to influence trans-epithelial iron flux; and 2) Reduction in Atp7a function increases Fpn1 mRNA expression, resulting in increased iron efflux across the basolateral surface of cells. Regarding point 1, Heph function, while important for iron homeostasis during the rapid post-natal growth phase in rodents (36), is not essential for iron transport. Secondly, significant cytosolic FOX activity remains in Heph knockout mice (105), which may be sufficient to support iron absorption under most circumstances. The current data also support the contention that Heph activity is not essential, as iron absorption was not impaired by reduction in Heph expression and activity. Perhaps the most significant result that derives from this investigation relates to point 2 above, namely the induction of Fpn1 expression and activity in Atp7a KD cells. As Fpn1-mediated iron export represents the rate-limiting step in dietary iron acquisition, influence of this activity by Atp7a or copper is of potential significance. Also of potential relevance is the increase in iron uptake by Atp7a KD cells. The mechanism of this induction however remains unclear, as Dmt1 mRNA expression was only slightly increased between control and KD cells.

In summary, consistent with previous predictions, Atp7a function influences enterocyte iron homeostasis. We speculate that this could relate to a direct influence of

intracellular copper on expression and activity of iron transport-related genes.

Interestingly, studies in Atp7a mutant mice (Brindled) mice support this contention, as the mutant mice are able to properly upregulate intestinal iron absorption during iron deprivation, but concurrent increases in enterocyte copper levels may support this function (Gulec and Collins, submitted). In the current studies, in the setting of Atp7a knock down and increases in intracellular copper, iron uptake and Fpn1-mediated iron export activity increases. Intracellular copper may thus directly influence iron absorption, perhaps providing a plausible explanation for the observation that copper levels increase in many mammalian species during iron deficiency.

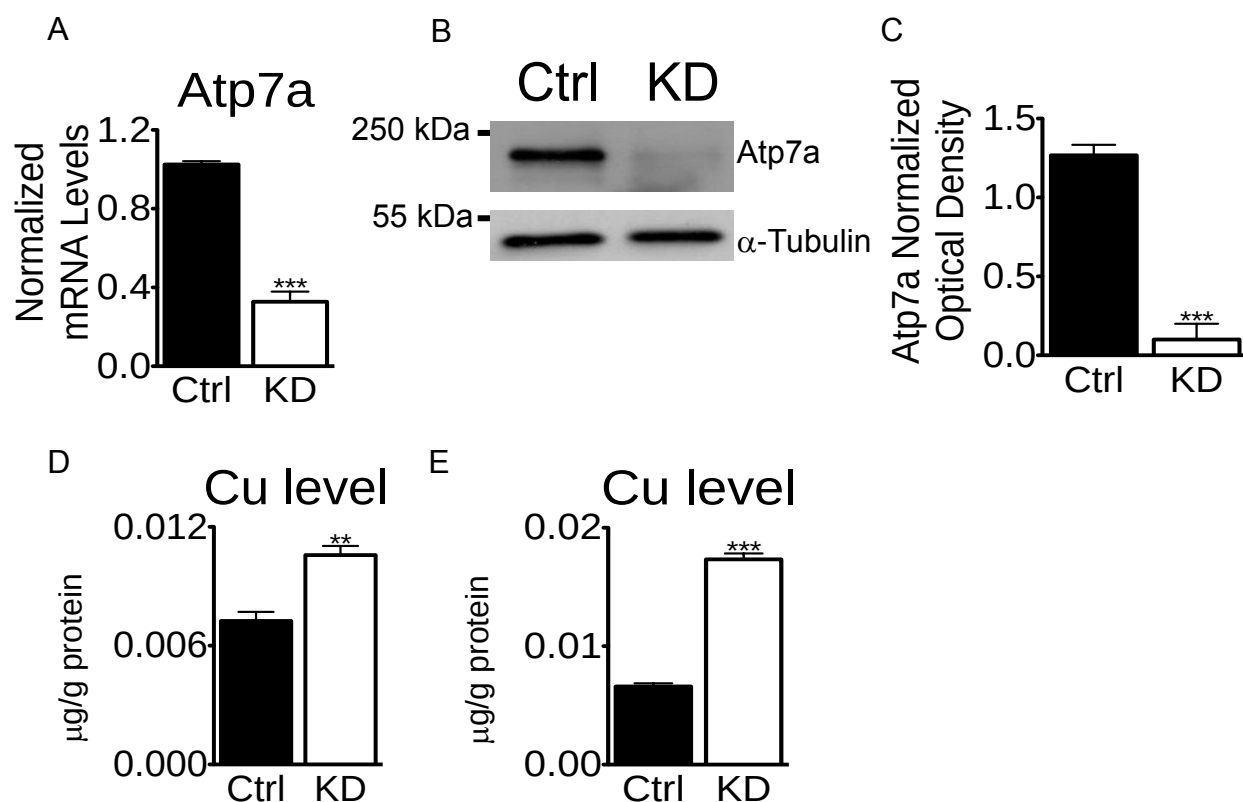


Figure 4-1. Confirmation of Atp7a KD in IEC-6 Cells. Atp7a KD IEC-6 cells were selected with zeocin treatment to create cell lines stably transfected with Atp7a or negative control shRNA-expressing plasmids. Atp7a KD was confirmed at the mRNA and protein levels. Cells were loaded with 100 μ M CuCl₂ and intracellular copper levels were measured. (A) Atp7a mRNA levels by qRT-PCR (n=4 per group); mRNA expression was normalized to cyclophilin mRNA levels; (B) a representative western blot using the anti-Atp7a (54-10) antiserum; (C) optical densitometry results of western blots (n=4); (D) copper level in cells after 3 hr treatment (n=4); (E) copper levels in cells after 16 hr treatment (n=4). **Statistically different from one another within each panel ($p < 0.001$). All results in Atp7a KD IEC-6 cells were compared to negative control shRNA-transfected IEC-6 cells and given as mean $\pm$ SD.

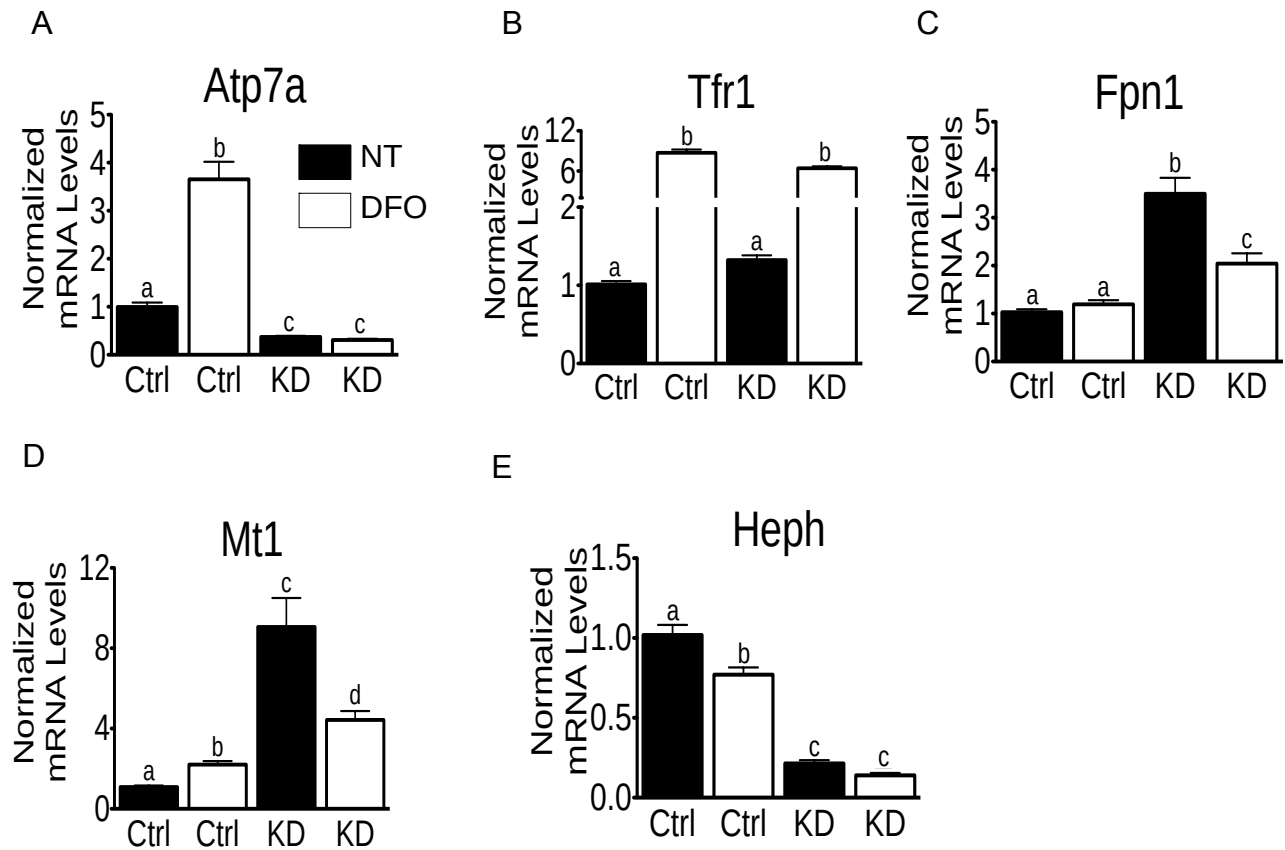


Figure 4-2. Enterocyte qRT-PCR Analysis of Iron and Copper Homeostasis-Related Genes. Expression of genes was determined by standard SYBR Green methods and relative mRNA expression for each experimental gene was normalized to cyclophilin mRNA levels, which were not affected by treatment or Atp7a KD. Filled bars indicate data from control (Ctrl) and Atp7a knock down (KD) IEC-6 cells, while open bars represent Ctrl and Atp7a KD IEC-6 cells treated with 200 μ M DFO for 24 hrs. ^{a,b,c,d} Statistically different from one another within each panel ($p < 0.05$). n=8 for all groups and treatments. Ctrl; negative shRNA-transfected IEC-6 cells, KD; Atp7a target shRNAs transfected IEC-6 cells, Atp7a; Menkes copper-transporting ATPase, Tfr1; Transferrin receptor1, Fpn1; Ferroportin1, Mt1; Metallothionein1, Heph; Hepheastin. (A-E) depict mean $\pm$ SD.

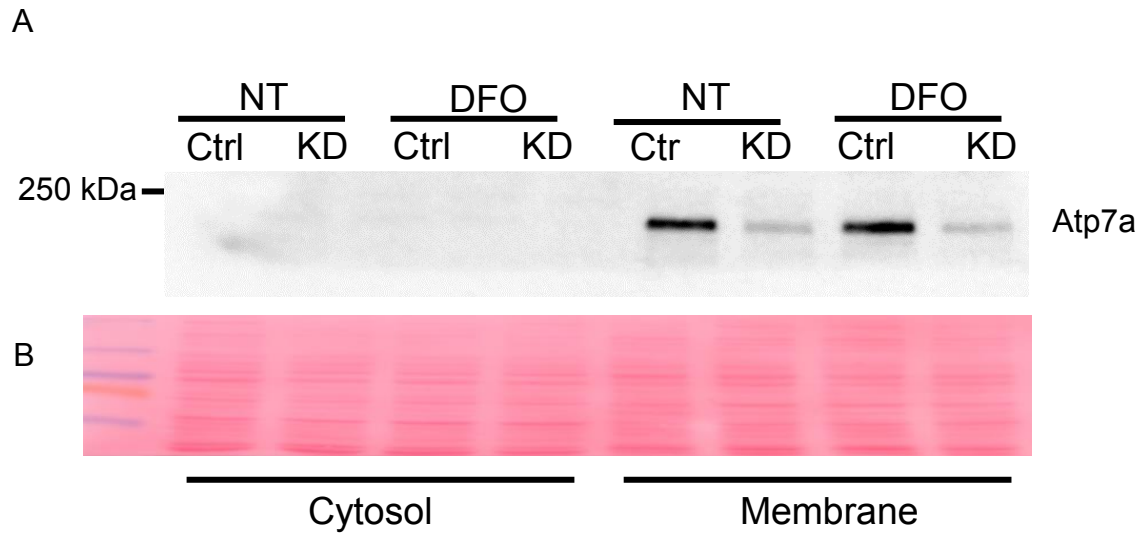


Figure 4-3. Western blot analysis of cytosol and membrane protein samples. Cytosolic and membrane proteins were isolated from Ctrl and Atp7a KD IEC-6 cells as described in *Methods*. After proteins were separated by SDS-PAGE, they were transferred onto PVDF membranes. (A) Proteins were probed with rat anti-Atp7a antibody and visualized on X-ray film. (B) Ponceau S-staining of proteins on membranes was utilized to show equal protein gel loading and efficient transfer of proteins. Data are representative of three independent experiments.

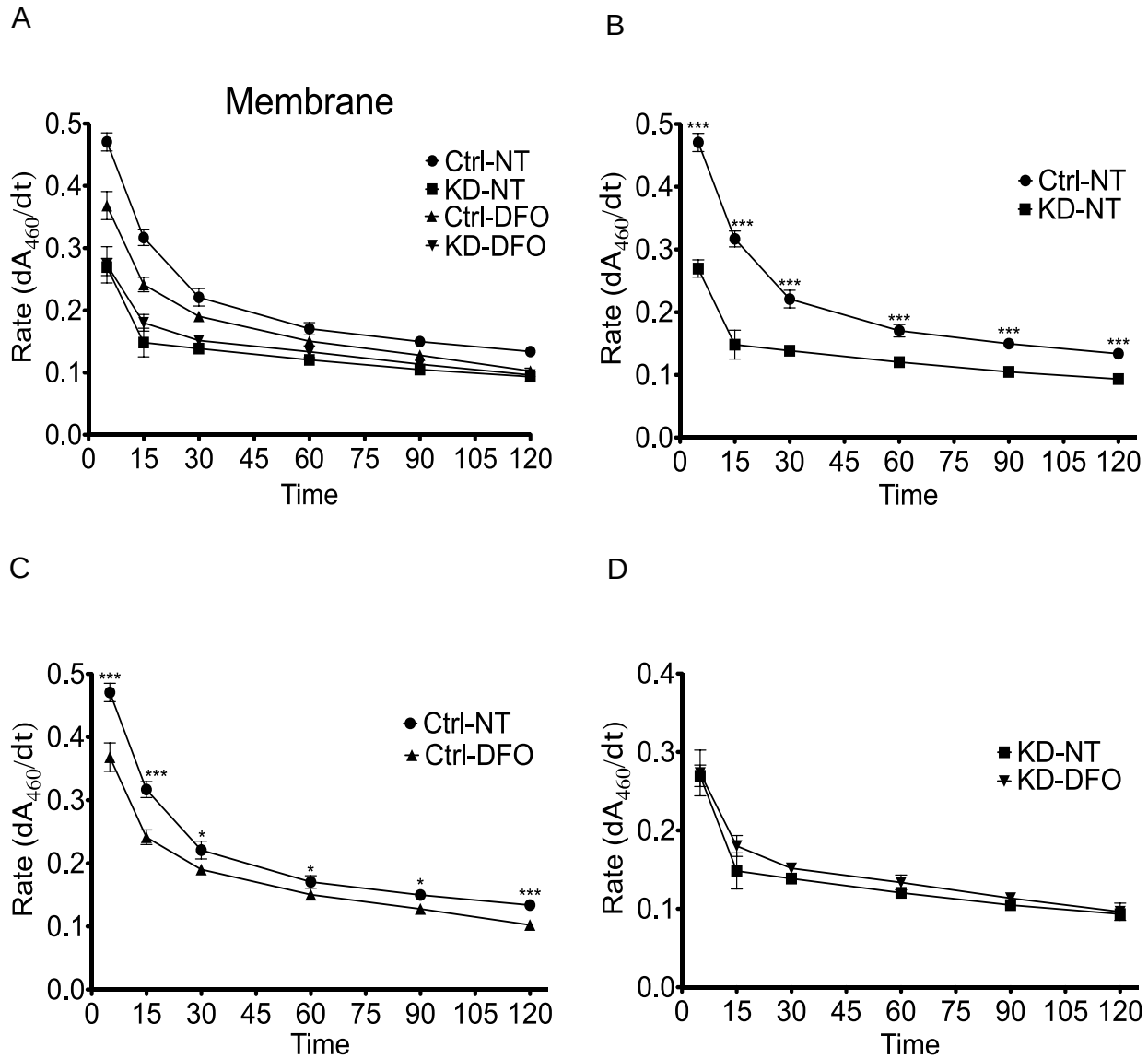


Figure 4-4. FOX Activity in Enterocyte Membrane Fractions. Relative FOX activity was measured in membrane fractions by a transferrin-coupled assay as described in *Methods*. (A) FOX activity was measured in membrane samples from Ctrl and Atp7a KD IEC-6 cells and in the same treated with 200 μ M DFO for 24 hours. $n=6$ per group and treatment. Individual graphs are also presented for groups that showed statistical differences in FOX activity (panels B-C). ***Statistically different from one another within each panel ($p < 0.05$). Data points (A-D) depict mean $\pm$ SD. NT, no treatment.

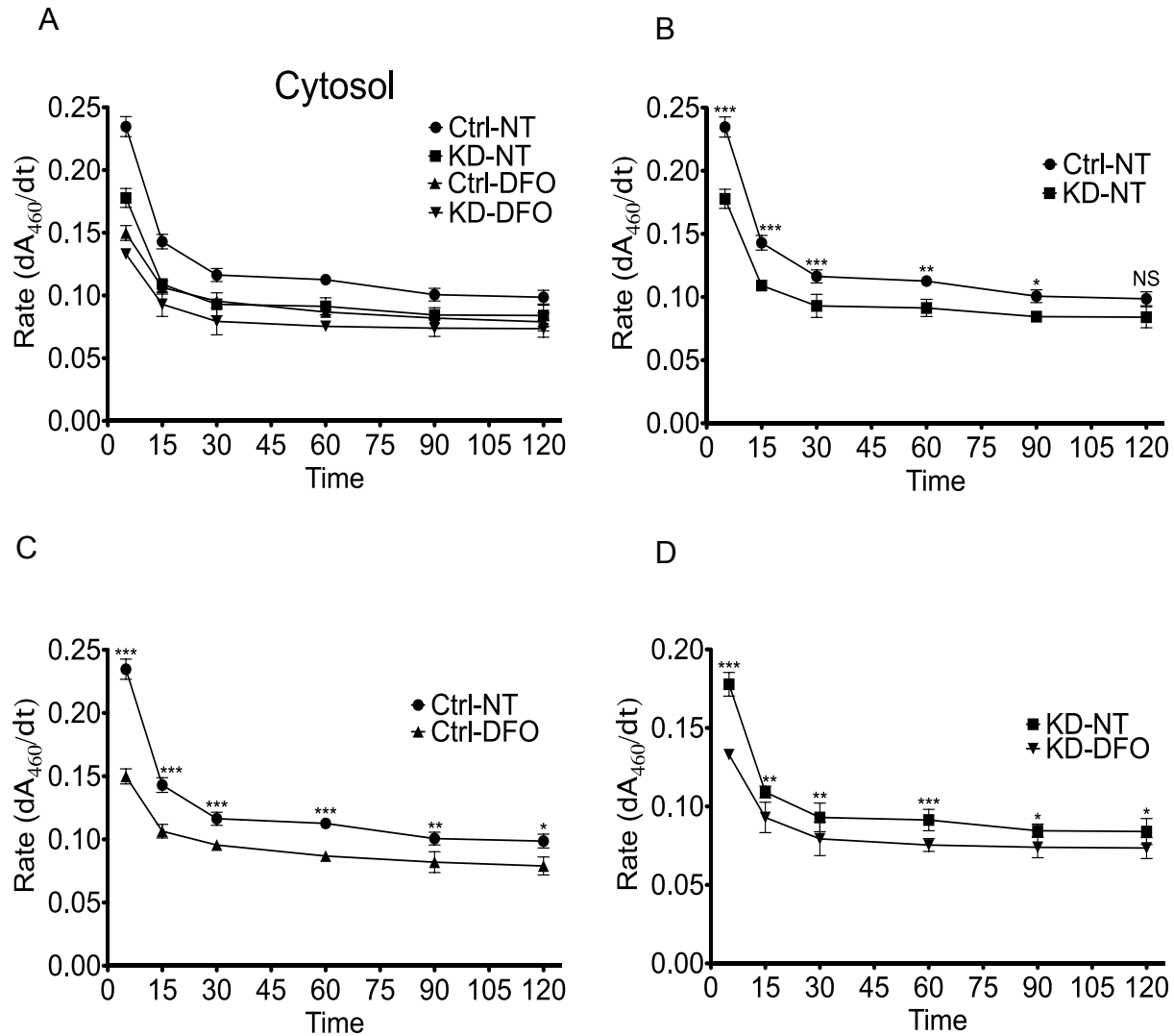


Figure 4-5. FOX Activity in Cytosolic Fractions. Relative FOX activity was measured in membrane fractions by a transferring-coupled assay as described in *Methods*. (A) FOX activity was measured in cytosolic samples from Ctrl and Atp7a KD IEC-6 cells or the same treated with 200 μ M DFO for 24 hours, n=6 per groups and treatment. Individual graphs are also presented for groups that showed statistical differences in FOX activity (panels B-D). ***Statistically different from one another within each panel ($p < 0.05$). Data points (A-D) depict mean $\pm$ SD. NT, no treatment.

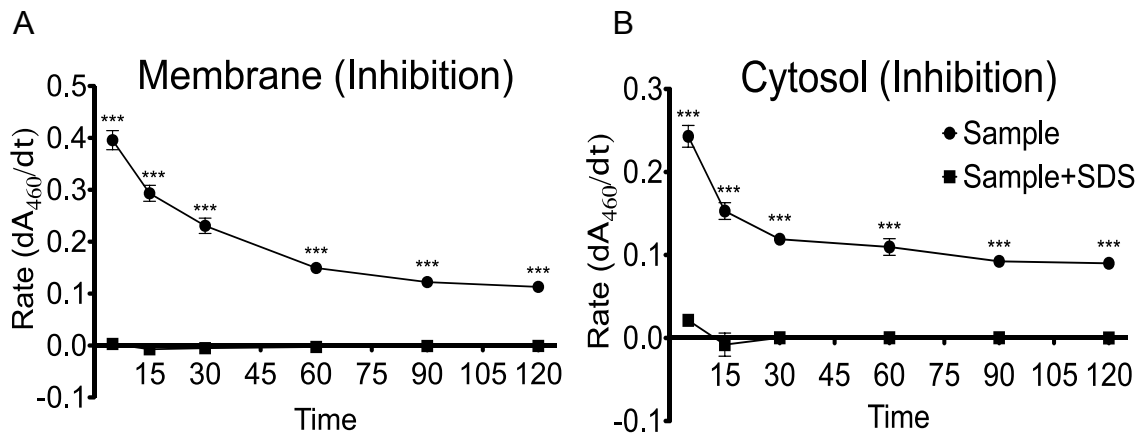


Figure 4-6. Inhibition of FOX Activity in Membrane and Cytosolic Fractions. The effect of 0.01% SDS on FOX activity from cytosolic and membrane samples is shown. Ctrl samples from three independent experiments were selected and incubated with SDS for 30 min followed by measurement of FOX activity in experimental samples. (A) Inhibition assay for cytosol FOX activity, $n=3$; (B) inhibition assay for membrane FOX activity, $n=3$. ***Statistically different from one another within each panel ($p < 0.001$). Data points (A-B) depict mean $\pm$ SD.

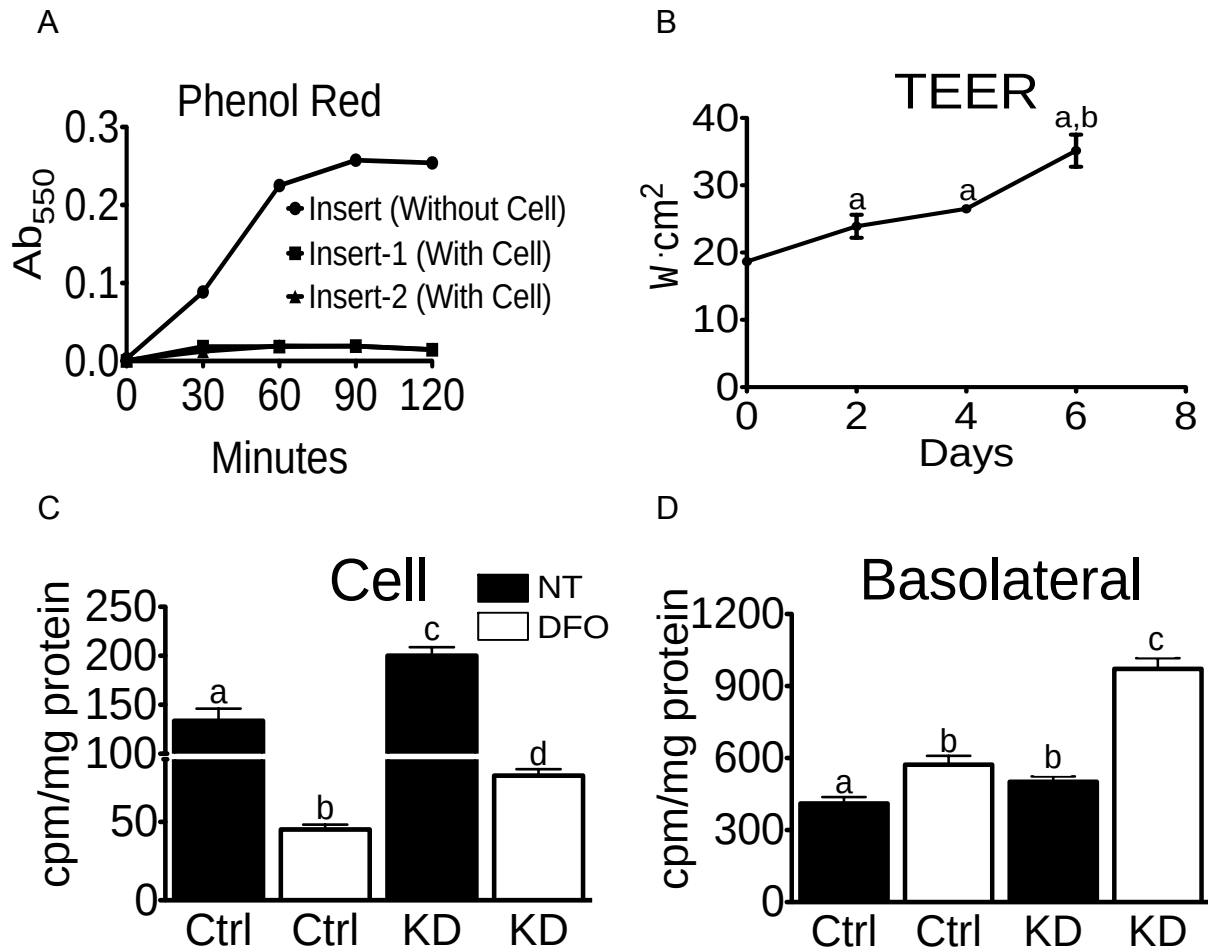


Figure 4-7. ⁵⁹Fe Transport Studies in Atp7a KD IEC-6 Cells Grown on Collagen-Coated Membranes. IEC-6 cells were grown for 8 days post-confluence and TEER was measured to test integrity of monolayers. Phenol-Red was used as a control to test whether IEC-6 cells had intact tight junctions. ⁵⁹Fe was added in transport buffer to the apical side of inserts followed by incubation for 90 minutes at 37° C. ⁵⁹Fe was measured in cells and in the medium in the basolateral compartment. Transport data were normalized to mg protein. (A) Representative data showing phenol red measurements from the basolateral compartment with an insert only (as a negative control) or from inserts with confluent cell monolayers; n=1 for negative control and n=2 for inserts with cells; (B) TEER measurements in IEC-6 cells at different time points. Day “0” represents the first day the cells were confluent; (C) Uptake of ⁵⁹Fe into cells with and without treatment with 200 μM DFO, n=5; (D) ⁵⁹Fe flux from cells to basolateral site of inserts, n=5. ^{a,b,c,d}Statistically different from one another within each panel (*p* < 0.05). Bars (A, B) depict mean ± SD.

CHAPTER 5 CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The general aim of my dissertation was to investigate the physiological role of the intestinal copper exporter Atp7a in intestinal iron homeostasis. To test this investigation *Atp7a* mutant Mottled Brindled ($Mo^{Br/y}$) mice and in parallel in vitro Atp7a KD IEC-6 cells were used. Effects of Atp7a on intestinal iron homeostasis in both experimental models were summarized in the Figure 5-1. As an in vivo, $Mo^{Br/y}$ mice on a *CBA/ C3H* background were used. These mice have a two amino acid deletion in a region of the protein that is responsible for phosphatase activity (45). Mutant animals were evaluated by examination of the coat color. Newborn $Mo^{Br/y}$ mice were rescued by 2 injections of $CuCl_2$ when they were 7 and 9-days-of-age. Hair color and growth were improved by copper treatment within 24 hours of injection. After Cu injection, experiments were initiated when they were adult (~3 months old). Iron deficiency was induced by low-iron diet (3 ppm iron) feeding with and without concurrent bloodletting (for 3 weeks). Several important observations arose from these studies. $Mo^{Br/y}$ mice on control diet were copper deficient and anemic with no changes in serum iron status. Dietary iron deprivation reduced Hb and Hct levels in both genotypes of mice with the reduction being more dramatic in the $Mo^{Br/y}$ mice. $Mo^{Br/y}$ mice were anemic, but they had normal enterocyte, serum and liver iron levels and Hamp mRNA expression. Hamp mRNA expression was dramatically reduced in both genotypes consuming the iron-deficient diet. Dietary iron deficiency increased expression of Cybrd1, Dmt1, Fpn1, Tfr1 and Atp7a in both genotypes, whereas Ftn, Heph and Ctr1 mRNA levels were not different between genotypes or dietary groups. The transcriptional response to iron deficiency in

enterocytes was seen only in WT and Mo^{Brly} mice on low-iron diet, suggesting that the Hif 2 α -mediated induction of iron and copper homeostasis-related genes is triggered by iron levels and not only by hypoxia. Furthermore, Mt1 mRNA expression was increased in Mo^{Brly} mice relative to WT. This induction was more significant during iron deficiency. This observation indicated that a block of copper flux from enterocyte leads to elevate copper level in enterocyte, resulting in increased Mt1 mRNA expression.

In the liver of mice, expression of Tfr1 was induced by iron deprivation in both genotypes and expression of the hypoxia related gene, Bnip3, increased in all experimental group compare to WT mice under control diet. Furthermore, liver Cp, Dmt1 and Fpn1 mRNA levels were not significantly different in all experimental groups. It has been noted that expression of Cp was regulated by hypoxia in the liver (87). However, Cp mRNA was not different in all experimental groups during low-iron diet, whereas Bnip3 was induced. Furthermore, a significant relationship between serum and liver copper and serum FOX activity was found, when considering all groups of experimental mice together ($r = 0.8852$ for serum copper vs. serum FOX activity, and $r = 0.9552$ for liver copper vs. serum FOX activity). In detail, FOX activity was found higher in the Mo^{Brly} mice consuming the low-iron diet, as compared to those consuming the control diet and liver copper levels were positively correlated with FOX activity in experimental groups. Although mice were rescued by copper injections, mutation of Atp7a caused low serum copper and serum Fox activity.

Intestinal iron absorption in FeD WT and Mo^{Brly} mice increased ~2-fold, and ~5-fold with concurrent phlebotomy. However a significant difference was not observed between WT and Mo^{Brly} mice under control diet and iron deficiency or iron deficiency

with phlebotomy. This suggests that Atp7a might not be necessary for intestinal iron absorption in mice. It was believed that Atp7a delivers Cu to make fully functional Heph (74). Heph activity is necessary to release iron from enterocyte and copper deficiency reduces Heph function (20, 136). However, mutant Atp7a did not interfere with Heph function and intestinal iron absorption. Increased level of intracellular copper might be involved in intestinal iron absorption in mutant Brindled mice via another unknown mechanism in this animal model. Another important point is that Atp7a mutation caused systemic copper deficiency and this might activate another copper-related pathway that compensates for lack of Atp7a. This study has evaluated novel aspects of the iron-copper connection in Brindled mice, which were used for the first time as model of genetic copper deficiency in which to investigate iron homeostasis. Studies in Mo^{Brly} mice provide additional support of the concept that copper plays an important role in iron homeostasis.

Published data about regulation of Atp7a during iron deficiency were from a rat animal model (110). Thus, a rat intestinal cell line (IEC-6) was used as an *in vitro* model to investigate Atp7a's role in intestinal iron homeostasis. Atp7a KD IEC-6 cell model was created by using Atp7a mRNA target shRNAs. mRNA and protein levels of Atp7a were significantly reduced in Atp7a KD cells, resulting in increased levels of intracellular copper. Treatment of 200 μ M DFO for 24 hours was used as a condition to induce iron deficiency in IEC-6 cells. Tfr1 mRNA level were used as positive control gene for iron deficiency, and was significantly induced during DFO treatment. DFO induced Atp7a and Mt1 gene expression, whereas expression of Heph was reduced and Fpn1 mRNA

level was not changed in Ctrl IEC-6 cells. Atp7a KD significantly lowered the expression level of the Heph gene, and increased Fpn1 and Mt1 mRNA levels.

Western blot analysis showed that Atp7a was decreased in Atp7a KD cells. Moreover, membrane and cytosolic fractions were separated without cross contamination. FOX activity was measured by a spectrophotometric transferrin-coupled assay. The results indicated that relative FOX activity was significantly (~ 50% in membrane, $p<0.001$; ~ 35% in cytosol, $p<0.001$) decreased in membrane and cytosolic fractions of Atp7a KD cells compared to Ctrl cells. Membrane FOX activity was reduced by DFO treatment and the reduction was more significant (average reduction from 5-15-30 sec, ~55%, $p<0.001$) in Atp7a KD cells. Cytosolic FOX activity was also decreased in Ctrl and Atp7a KD cells during DFO treatment. The inhibition assay showed that FOX activity was completely diminished ($p<0.0001$) by SDS. The finding suggests that Atp7a is an important contributor to enterocyte FOX activity in IEC-6 cells.

DFO treatment increased overall ^{59}Fe transport in Ctrl IEC-6 cells. ^{59}Fe flux was significantly increased in DFO treated Atp7a KD cells, more than other experimental groups (average increase over other experimental samples, 2.5- fold). Important observations from this part of my dissertation research showed that Atp7a KD decreased Heph gene expression and membrane FOX activity. However the reduction in FOX activity did not interfere with ^{59}Fe transport. Moreover, ^{59}Fe transport was increased during Atp7a KD and this might suggest that the rate of intestinal iron absorption might be influenced by Atp7a or intracellular copper during iron deficient condition.

Future Directions

Animal studies in this dissertation research were performed in Atp7a mutant Brindled (Mo^{Brly}) mice to test possible effects of mutant Atp7a protein on intestinal iron metabolism. The mutant form of Atp7a is expressed in spleen, bone marrow, heart, kidney, and pancreases (90). Mutant Atp7a in spleen or bone marrow might affect intestinal iron metabolism indirectly. For instance, macrophages of spleen destroy senescent erythrocytes and iron is released to the systemic iron pool(11). If Atp7a plays a role in macrophage iron flux, this could change amount of circulating iron. Different level of iron in the blood regulates Hmp protein, which is the main systemic regulator for intestinal iron absorption, due to interaction between Hmp and Fpn1. Thus, intestinal iron metabolism should be examined in intestine specific *Atp7a* knock out (*Atp7a* KO) mice. This will eliminate possible effects of other organs on intestinal iron homeostasis. Atp7a and Heph are closely related proteins due to copper requirements for Heph activity (20). Breeding of intestine-specific *Atp7a* KO mice with *Heph* KO mice will help to explain their possible interaction in intestinal iron absorption *in vivo*.

Hypoxia is an important signaling mechanism in intestinal iron absorption and Atp7a is regulated by Hif2 α *in vitro* (150). However, physiological interaction of Atp7a and Hif2 α has not been shown *in vivo*. Breeding of intestine-specific *Atp7a* KO and *Hif2 α* KO animals could demonstrate hypoxia-related Atp7a regulation in intestine and will explain a possible role of Atp7a in intestinal iron absorption during hypoxia.

One of the important observations from this dissertation research was that Mo^{Brly} mice had lower Hb and Hct levels than WT mice under control diet. This result suggests that Atp7a might be involved in production of red blood cells (RBCs). It might be

important to evaluate a possible role of Atp7a in bone marrow in respect to production of RBCs. Furthermore, it was observed that liver of Mo^{Brly} mice was loaded with copper during iron deficiency. This leads to the question, “Did bile copper excretion decrease in liver of Mo^{Brly} mice during iron deficiency?”. To answer this question, bile copper excretion should be tested in liver of Mo^{Brly} mice during iron deficiency.

IEC-6 cells were used to test the physiological role of Atp7a in iron metabolism *in vitro*. Data showed that Heph, Fpn1 and Mt1 gene expression were regulated by Atp7a KD in IEC-6 cells. Most of the genetic KO animal models come from mouse. However, response and regulation of iron homeostasis is different between mouse and rat (60). Atp7a KO IEC-6 cell model can be used as an alternative for rat Atp7a KO model. It might be important to look at regulation of iron-dependent pathways in this model. Microarray is a powerful technique to look at gene regulation in cells (57). A microarray approach for Atp7a KD cells can be utilized to see how iron-related pathways are altered and which signaling pathways are regulated by Atp7a KD.

This dissertation was focused on Atp7a's role in intestinal iron homeostasis in mice and rat intestinal cell models. But, are these finding applicable to humans? Human colorectal adenocarcinoma cells (CaCo-2) or human intestinal epithelial cells (HT29) can be used as an alternative model for human to investigate the role of Atp7a in iron metabolism. Furthermore, reduced levels of FOX activity in Atp7a KD cells did not interfere with iron transport. Atp7a KD increased intracellular copper levels and it has been proposed that copper loading increases iron transport in CaCo-2 cells (52). It has also been shown that iron deprivation loads copper in enterocytes (24) and physiological reason for copper loading has not been completely solved. Role of copper

loading and copper-specific regulation of intestinal iron metabolism during iron deficiency can thus be evaluated in the IEC-6 cell model.

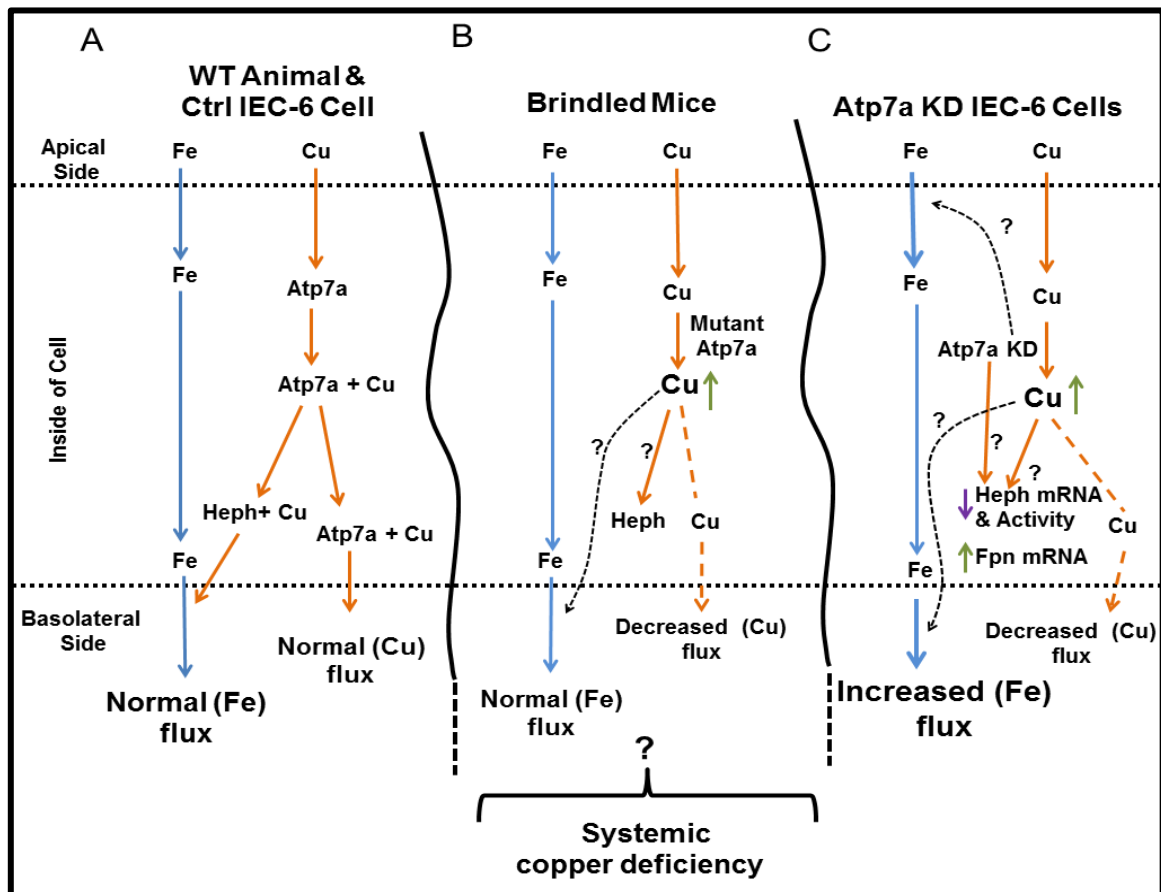


Figure 5-1. Proposed summary of the effect of Atp7 on intestinal iron homeostasis in various experimental models. Copper is an important factor to maintain body iron homeostasis through Heph. (A) Atp7a delivers Cu to Heph that is necessary for iron flux from intestine. (B) Experiments in Brindled mice showed that Heph did not interfere enterocyte iron flux and this might be related increased level intracellular Cu or systemic Cu deficiency in this animal model. (C) Atp7a knockdown caused reduction of Heph mRNA and activity, induced Fpn mRNA, and concomitantly increased enterocyte iron transport. The effects of Atp7a on intestinal iron transport might be related to elevated intracellular Cu level.

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BIOGRAPHICAL SKETCH

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