Biometals (2012) 25:633–642
DOI 10.1007/s10534-012-9521-2

Copper deficiency has minimal impact on ferroportin expression or function

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Received: 6 December 2011 / Accepted: 7 January 2012 / Published online: 1 February 2012
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Abstract Interactions between copper and iron homeostasis have been known since the nineteenth century when anemia in humans was first described due to copper limitation. However, the mechanism remains unknown. Intestinal and liver iron concentrations are usually higher following copper deficiency (CuD). This may be due to impaired function of the multicopper oxidases hephaestin or ceruloplasmin (Cp), respectively. However, iron retention could be due to altered ferroportin (Fpn), the essential iron efflux transporter in enterocytes and macrophages. *Fpn* mRNA is controlled partially by intracellular iron and IRE dependence. CuD should augment Fpn based on iron level. Some argue that Fpn stability is controlled partially by membrane ferroxidase (GPI-Cp). CuD should result in lower Fpn since GPI-Cp expression and function is reduced. Fpn turnover is controlled by hepcidin. CuD results in variable *Hamp* (hepcidin) expression. *Fpn* mRNA and protein level were evaluated following dietary CuD in rats and mice. To correlate with Fpn expression, measurements of tissue iron were conducted in several rodent models. Following CuD there was little change in *Fpn* mRNA. Previous work indicated that under certain circumstances Fpn protein was augmented in liver and spleen following CuD. Fpn levels in CuD did not correlate with either total iron or non-heme iron (NHI), as iron levels in CuD liver were higher and in spleen lower than copper adequate controls. Fpn steady state levels appear to be regulated by a complex set of factors. Changes in Fpn do not explain the anemia of CuD.

Keywords Copper deficiency · Rodents · Iron · Ferroportin · Anemia

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Introduction

Copper is recognized as an essential dietary trace metal because limitations in copper uptake lead to many pathophysiological consequences (Prohaska 2006). One such consequence is anemia, reflected primarily in reduced erythrocyte numbers and lower hemoglobin per cell (Fox 2003). This phenotype is similar to dietary iron deficiency. In fact many argue that anemia of copper deficiency is caused by an impairment in iron uptake across the intestinal absorptive cell membrane (Chen et al. 2006; Reeves et al. 2005). This argument was based on iron tracer kinetics and the known requirement for iron efflux by the multicopper oxidase (MCO) hephaestin whose mutation in mice results in sex-linked anemia (*Sla*) (Reeves and DeMars 2004; Vulpe et al. 1999). However, *Sla* mice as adults are not anemic (Chen et al. 2009). Further, iron injection to anemic post weanling copper deficient rats does not reverse

anemia, challenging the hephaestin iron trap theory (Reeves and DeMars 2006).

In addition to the intestinal MCO hephaestin, iron egress across the basolateral membrane has an absolute requirement for ferroportin (*FPN*), SLC 40A1 (Donovan et al. 2005). Fpn expression is highly regulated (Knutson 2010). Rodent Fpn is regulated transcriptionally by iron (Aydemir et al. 2009). Fpn is regulated post-transcriptionally via an IRE-dependent mechanism (Lymboussaki et al. 2003). Fpn is regulated post-translationally by the hepatic hormone hepcidin that binds to Fpn and results in internalization and degradation (Nemeth et al. 2004). Dietary copper deficiency can alter hepcidin expression and this may impact Fpn and thus iron transport (Chen et al. 2006; Jenkitkasemwong et al. 2010). In addition, some suggest that Fpn stability, via a hepcidin independent pathway, depends on membrane MCO activity. Experiments have shown that the glycosylphosphatidylinositol (GPI)-linked splice variant of ceruloplasmin (Cp) is required for Fpn to remain in the cell surface (De Domenico et al. 2007). Thus, copper deficiency, known to impact Cp activity may result in less Fpn and contribute to iron retention.

The purpose of the following experiments and review was to test the hypothesis that copper deficiency can impact Fpn expression. Studies were conducted in male Holtzman and Sprague–Dawley rats as well as C57BL6 and albino outbred male mice using semi-purified diets low in copper. Emphasis was placed on evaluating tissues critical to iron uptake (small intestine) and iron mobilization from stores (liver and spleen).

Materials and methods

Animal care and diets

Pregnant Holtzman rats, weanling Sprague–Dawley rats and Swiss Webster pregnant mice were purchased commercially (Harland Sprague–Dawley, Indianapolis, IN, USA). C57BL6 mice were derived from a colony provided originally by David Grahn, Argonne National Laboratory. Rodents were offered a copper-deficient diet (Teklad Laboratories, Madison, WI, USA) similar to the AIN-76A diet but modified by omitting cupric carbonate from the AIN-76A mineral mix (Prohaska and Heller 1982). This diet contained 0.4 mg Cu/kg and 47 mg Fe/kg by chemical analysis. Copper adequate (CuA) rodents were given deionized water with copper sulfate (20 mg Cu/L) to drink. Copper deficient (CuD) rodents drank copper-free deionized water.

Perinatal and postweanling nutritional paradigms were studied. Rats were placed on the diet treatment at postnatal day 21 (P21) and killed 5 weeks later P56. Mouse dams began treatments at embryonic day 17 (E17) or on the day of parturition; and rat dams began treatment on E7 similar to established recent protocols (Pyatskowit and Prohaska 2008a). On postnatal day 2 (P2), litter sizes were culled to ten pups. At P21, rodent pups were either killed or transferred to stainless steel cages. C57BL mice were killed after 7 weeks of treatment, three lactational via dams and four postweaning at P49. All protocols were approved by the University of Minnesota Institutional Animal Use and Care Committee.

Upper small intestine (15 cm), livers, kidney, heart, brain, spleen, thymus and blood were harvested from pups. Trunk blood was collected in a heparinized tube. Intestinal lumens were flushed with saline to remove contents and blotted with tissue paper prior to metal analyses. In other experiments, tissues were weighed and either processed for biochemical analysis or frozen in liquid nitrogen and stored at −75°C until used. Enterocytes, in selected experiments, were harvested as described elsewhere (Chen et al. 2003).

Biochemical analyses

Hemoglobin was determined spectrophotometrically as metcyanhemoglobin. Plasma activity of the cuproprotein ceruloplasmin (EC 1.16.3.1) was measured by following oxidation of *o*-dianisidine at 37°C (Prohaska 1983). Tissues and diet were wet-digested with HNO_3 (Trace Metal Grade, Fisher Scientific) and residue was dissolved in 0.1 mol/L HNO_3. Samples were analyzed for total copper and iron by flame atomic absorption spectroscopy (AAS) (model 1100 B, Perkin–Elmer).

PCR methods

Enterocytes were isolated from the upper small intestine of four P21 male CuD and CuA rats and mice using an EDTA treatment method (Chen et al. 2003). Total RNA was isolated from these cells and

other tissues and quality established spectrophotometrically and by RNA gels (Prohaska and Brokate 1999). The relative mRNA content of enterocyte and tissue ferroportin (*Fpn*), copper transporter *Ctr1,* and *cyclin D1* (a transcript sensitive to the copper chaperone Atox1 (Itoh et al. 2008)) were determined by q-PCR using the reference gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as described previously (Broderius et al. 2010). *Fpn* primer pairs were 5′ GGT GGT GGC AGG CTC TGT 3′ (forward) and 5′ TTT GAA CCA CCA GGG ACG TC 3′ (reverse); for *Ctr1 5′ GGA GAA ATG GCT GGA GCT TTT 3′ (forward) and 5′ CGG GCT ATC TTG AGT CCT TCA 3′* (reverse); for *cyclinD1 5′ CGT GGC CTC TAA GAT GAA GG 3′ (forward) and 5′ CTG GCA TTT TGG AGA GGA AG 3′ (reverse);* for *Gapdh* 5′ TTC CTA CCC CCA ATG TAT CCG 3′ (forward) and 5′ ACC ACC CTG TTG CTG TAG CCA 3′ (reverse).

Statistics

Dietary treatment effects were evaluated by Student's *t*-test after variance equality was tested by *F*-test, $\alpha = 0.05$. Data were analyzed using Microsoft Excel™.

Results and discussion

Impact of dietary copper deficiency on tissue iron levels

After 5 weeks on CuD treatment using a modified AIN-76A mineral formula male CuD rats were smaller, anemic, exhibited cardiac hypertrophy and had near total loss of plasma Cp activity (Table 1). These features are consistent with earlier studies conducted two decades ago (Prohaska and Heller 1982). Seven organs from these CuD and CuA rats were analyzed for metal content by flame AAS (Fig. 1). Copper concentration of all seven organs was markedly lower in CuD organs compared to CuA. Even brain of CuD rats was about 30% lower than CuA (Fig. 1a). In contrast, iron concentration was altered in an organ specific manner (Fig. 1c). Liver iron was higher in CuD than CuA rats. Intestinal iron was not altered by CuD and iron content of the remaining five organs was significantly lower in CuD than CuA rats. These results seem confusing if dealing with a common copper-iron interaction as liver, intestine, and spleen were all different in their response to CuD. Also while others report similar iron data in CuD liver, kidney, and spleen; intestinal iron was reported to be higher in CuD rats (Reeves et al. 2005). Dietary copper deficiency was also studied in postnatal rats using a new modified AIN-93G, described previously; both CuA and CuD diets contained nearly twice the iron content of AIN-93G or AIN-76A diets (Bastian et al. 2010). After 4 weeks of treatment the small intestine copper level was greatly diminished in CuD rats 0.60 ± 0.13 μg/g compared to 1.79 ± 0.04 for CuA rats, $P < 0.01$. However, iron content of the intestine was not impacted by diet, CuD 40.4 ± 9.39 compared to CuA 53.6 ± 14.5. Thus, despite higher exposure of dietary iron the CuD rats did not experience intestinal iron retention in this model.

Similar metal data was collected previously from CuD and CuA mice (Prohaska and Lukasewycz 1990). These studies were done on 7 week old male C57BL6 mice and a subset of data from those studies are summarized (Table 1). CuD mice exhibited many features of CuD rats except a lack of growth impairment. Copper levels were lower in liver,

Table 1 Characteristics of 8 week old male rats and 7 week old male mice following dietary copper deficiency

Characteristic	Rats		Mice	
	CuA	CuD	CuA	CuD
Body weight (g)	297 ± 8.9	263 ± 5.7*	22.2 ± 0.41	21.0 ± 0.42
Hemoglobin (g/L)	138 ± 0.26	96.3 ± 1.2*	159 ± 2.2	128 ± 8.4*
Ceruloplasmin (units/L)	130 ± 11.2	0.49 ± 0.30*	50.8 ± 1.19	0.91 ± 0.37*
Heart/body (mg/g)	3.19 ± 0.05	4.49 ± 0.16*	4.95 ± 0.18	7.33 ± 0.2*

Values are means ± SEM ($n = 6$ minimum). Treatment was from age 3–8 weeks for rats and from birth to 7 weeks for mice. An asterisk indicates Cu-deficient (CuD) means differ from Cu-adequate (CuA) within species, $P < 0.05$ (Student's *t*-test)

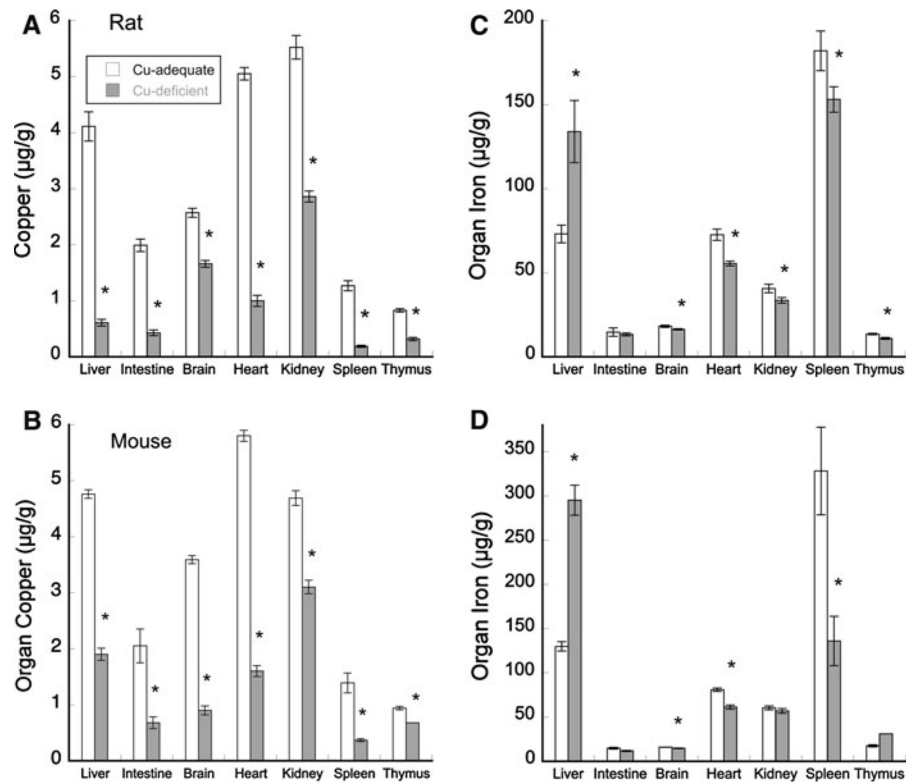

Fig. 1 Copper (**a**) and iron (**b**) concentrations in 8 week old male Sprague–Dawley rats. Copper (**c**) and iron (**d**) concentrations in 7 week old male C57BL6 mice. Values are means ± SEM. Cu-deficient means were significantly different than Cu-adequate, $*P < 0.05$ (Student's *t*-test)

intestine, brain (75%), heart, kidney, spleen and thymus of CuD mice (Fig. 1b) whereas iron was higher in liver, not impacted in intestine, and lower in spleen similar to CuD rats (Fig. 1d). Splenomegaly was evident in the CuD mice but calculation of total spleen iron suggests lower iron content in CuD mice, 13.6 µg, compared to CuA mice, 21.1 µg. However, in more current studies with other strains of rodents there are many differences between CuD rats and CuD mice regarding GPI-Cp, Fpn, hepcidin, and non-heme iron (NHI).

Importantly, another key difference between mice and rats is the response of plasma iron level (holo-transferrin). CuD mice, in many circumstances, do not develop hypoferremia but rats do, yet both CuD mice and rats are anemic (Table 1) (Pyatskowit and Prohaska 2008a). This fact suggests transferrin iron is not the limiting factor in the anemia associated with copper deficiency (Prohaska 2011). The mechanism for normal plasma iron in CuD mice is more complicated. Plasma ferroxidase activity was reported to be non-detectable in CuD rats, mice, and *Cp−/−* mice using a gel based method (Broderius et al. 2010). However, another group has reported residual ferroxidase in *Cp−/−* mice using spectrophotometric methods (Gray et al. 2009). Further research will be necessary to characterize the ferroxidase activity of mouse plasma as early work failed to detect ferroxidase activity associated with Cp following gel permeation chromatography (Prohaska 1981).

Small intestine response to copper deficiency

Ferroxidase theory suggests that Fpn function requires a MCO to facilitate iron efflux (Prohaska 2011). Thus, for intestine of CuD rats and mice there should be iron retention if hephaestin function is compromised. Previous work summarizing six experiments from our lab suggested that small intestinal Fe was rarely augmented with the exception of the upper duodenum (Pyatskowit and Prohaska 2008c). Others have reported higher iron levels in intestine of CuD rats (Reeves et al. 2005). At birth CuD pups are iron-deficient (FeD) based on fetal Fe content of liver or whole rat pup iron content (Andersen et al. 2007; Pyatskowit and Prohaska 2008c). After weaning when dietary iron content rises sharply as pups switch from milk to solid food (50 mg Fe/kg), intestinal iron has

been reported to become augmented in CuD rat pups compared to CuA controls (Pyatskowit and Prohaska 2008c). Limited studies on CuD mice suggest they retain iron in intestine after lactational Cu deficiency (Chen et al. 2006). Although, our data using the same strain of mice and a similar diet did not confirm this (Fig. 1d). Thus, overall there is some evidence for iron retention in intestine following dietary copper deficiency.

The mechanism for this retention is consistent with reduction in the function of MCO hephaestin. Both in CuD mice and CuD rats there is evidence of lower hephaestin protein (Chen et al. 2006; Reeves et al. 2005). Further, supplementation of CuD rats with dietary copper can restore hephaestin level and reverses impaired intestinal iron transport (Reeves and Demars 2005).

Fpn has not been as thoroughly studied following CuD treatments as has hephaestin. One study reported augmented intestinal *Fpn* mRNA and Fpn protein in C57BL mice following 6 weeks of CuD treatment from birth (Chen et al. 2006). In contrast CuD Hsd:ICR (CD-1) outbred mice born to and nursed by CuD dams from E17 had normal *Fpn* mRNA at P27 (Fig. 2). Intestinal iron was not assessed in this study but prior work suggested a modest augmentation in intestinal Fe following perinatal CuD (Chung et al. 2004). In that study, intestinal *Fpn* mRNA was not augmented in CuD mice. It is not apparent why one study reported higher *Fpn* while the other did not, assuming iron level is critical to the augmented transcription abundance of *Fpn*. Current data also failed to detect augmented *Fpn* mRNA in CuD mice and CuD rats (Fig. 2). Interestingly, both CuD rodents did display modest reduction in the *cyclin D1* mRNA, a transcript dependent on the copper chaperone Atox1 (Itoh et al. 2008). This suggests that the tissue was indeed copper deficient. Intestinal Ctr1 expression evaluated using q-PCR protocols failed to detect any change in CuD mouse intestine and a modest reduction in CuD rat intestine (Fig. 2). Similar results using Northern blot techniques were published previously (Lee et al. 2000). Fpn protein abundance, of course, is the true potential indicator of Fpn function. Only one published study has examined intestinal Fpn protein, that in CuD mice. It was reported that robust augmentation parallel to mRNA data existed following lactational/postweaning copper restriction (Chen et al. 2006).

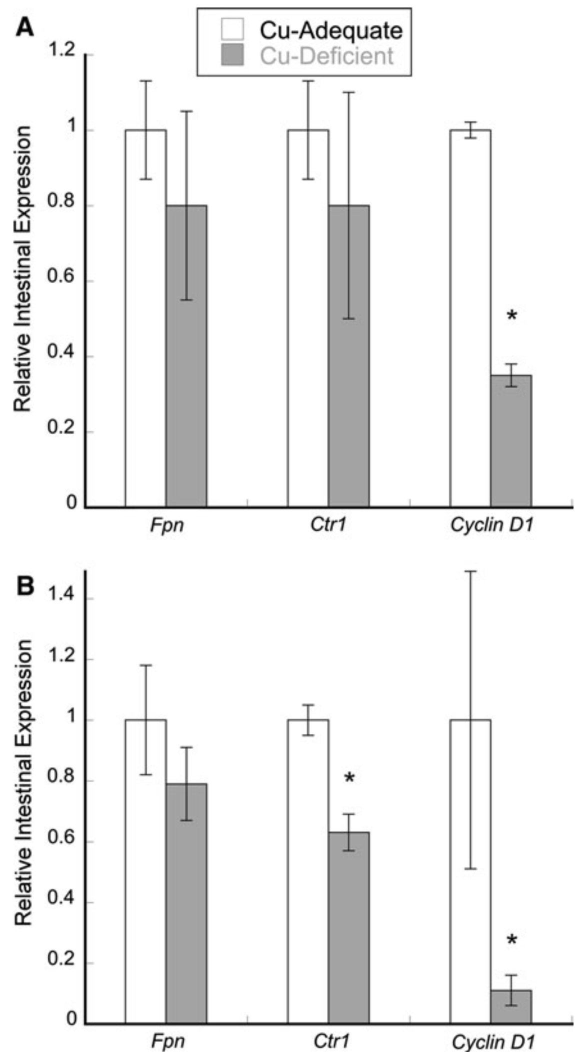

Fig. 2 Mouse (**a**) and rat (**b**) relative gene expression in small intestine. Values are means ± SEM ($n = 4$). Mice were age P27 and rats P21. Cu-deficient means were significantly different than Cu-adequate, $*P < 0.05$ (Student's *t*-test)

Genetic models in mice have revealed further information about intestinal iron homeostasis and iron efflux transporters. When hepcidin null mice were evaluated there was significant augmentation in Fpn protein, consistent with the theory that when hepcidin is present it causes a decrease in Fpn abundance via accelerated degradation (Viatte et al. 2005). *Sla* mice and *Cp−/−* mice both exhibit higher intestinal iron than controls, and higher Fpn protein (Chen et al. 2009). However, the mechanism must differ as *Sla* mice display lower and *Cp−/−* mice normal levels of *Hamp*, the hepcidin gene. Overall one can conclude

that intestinal iron retention is not likely due to loss of intestinal Fpn; perhaps it is due to lower hephaestin function. Intestinal hephaestin was not evaluated in *Hamp* null mice. *Cp*−/− mice express higher intestinal hephaestin than control mice (Chen et al. 2009). Recall *Cp*−/− mice have augmented intestinal iron. This suggests that Cp in addition to Fpn and hephaestin may play a role in intestinal iron egress. Perhaps too ferritin H levels can explain this disparate observation, since this protein is also required for proper intestinal iron flux (Vanoaica et al. 2010). Unfortunately, neither Fpn or hephaestin were evaluated in Ferritin H null mice. Suffice it to say the mechanism for intestinal iron retention in CuD rodents and *Cp*−/− mice requires further evaluation.

Spleen response to copper deficiency

Splenic macrophages are key for recycling iron from hemoglobin as red cells are removed from circulation. Several copper-iron interactions can impact iron efflux but especially the MCO Cp working with Fpn (Collins et al. 2010). Recently, we reported that the GPI-Cp splice variant of Cp was widely expressed in rat and mouse tissues (Mostad and Prohaska 2011). Previously, emphasis on this membrane associated MCO was brain astrocytes; the organ and cell type where it was first identified (Patel and David 1997). Spleen GPI-Cp is much greater in abundance than cerebellum GPI-Cp. Furthermore abundance of spleen GPI-Cp was greatly reduced by dietary Cu deficiency in both CuD rats and mice (Mostad and Prohaska 2011).

Some believe that GPI-Cp, or some membrane MCO, is necessary for Fpn stability (De Domenico et al. 2007; Kono et al. 2010). Thus, it would follow that spleen of CuD rats and mice with very low GPI-Cp protein and ferroxidase activity should have lower Fpn; but, in fact CuD spleen Fpn protein levels are either not impacted or augmented (Jenkitkasemwong et al. 2010; Mostad and Prohaska 2011). Spleen *Fpn* mRNA content was not altered in postweanling CuD rats (Fig. 3). Splenic Fpn protein levels of CuD mice were not impacted by diet (Jenkitkasemwong et al. 2010). As mentioned previously, spleen Fe levels of CuD Sprague–Dawley rats and C57BL6 CuD mice are lower than their CuA controls (Fig. 1b, d). Analysis of spleen NHI in several models confirmed lower levels in CuD rats (Mostad and Prohaska 2011). However, in the same study CuD mice consuming the newer diet with extra iron exhibited spleen NHI concentration equivalent to CuA controls. However, splenomegaly was not apparent in these P27 male mice and relative spleen weight (data not shown) and total spenic NHI level was actually lower in CuD mice, 2.98 μg compared to 5.23 μg for CuA mice, $P < 0.01$. Spleen NHI of CuD rodents is not correlated with Fpn as NHI levels are lower than control values and spleen FPN level is either augmented or not impacted by copper limitation. It seems clear from in vivo data, however, that spleen Fpn level in CuD rodents is not compromised by low GPI-Cp ferroxidase.

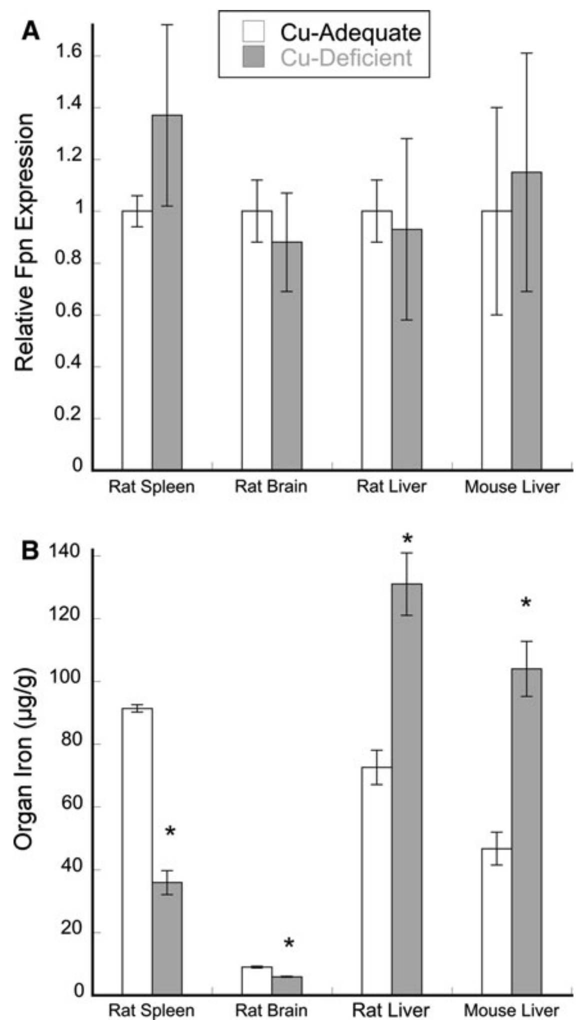

Fig. 3 Relative ferroportin (Fpn) expression (**a**) and iron concentration (**b**) in selected organs of rats and mice. Values are means ± SEM ($n = 4$). Rats for spleen data were age P49, for brain P26, for liver P53, for mouse liver P20. Cu-deficient means were significantly different than Cu-adequate, $*P < 0.01$ (Student's *t*-test)

Liver response to copper deficiency

Conditional deletions of mouse *Fpn* that allowed survival but loss of Fpn function resulted in iron retention in both Kupffer cells and heptatocytes, illustrating the key role of Fpn in hepatic iron homeostasis (Donovan et al. 2005). CuD mice exhibit greater than twofold hepatic iron augmentation even with a new AIN-93G modified diet that eliminates hepatic iron overload in rats (Bastian et al. 2011; Mostad and Prohaska 2011). Mouse liver Fpn level was not reported in those studies. In earlier studies, CuD mice also had hepatic iron overload but no alteration in liver Fpn protein expression (Chung et al. 2004; Jenkitkasemwong et al. 2010). In those studies and current data (Fig. 3a) there is no evidence of augmented liver *Fpn* mRNA in CuD mice.

In CuD rats, liver Fpn protein content is higher compared to CuA rats (Jenkitkasemwong et al. 2010; Mostad and Prohaska 2011). This was observed in two separate rat strains, Holtzman and Sprague–Dawley, with two different CuD diets, modified AIN-76A and modified AIN-93G, that results in hepatic iron increases in young males in one study but not the other (Bastian et al. 2011). This strongly suggests little correlation between liver iron content and liver Fpn content in CuD rats. Further, post weanling CuD male rats with augmented liver iron did not display augmented *Fpn* mRNA (Fig. 3).

Other studies on mice following iron loading had mixed results with respect to the impact on steady state Fpn level. In one study Fpn was reported to be upregulated by iron loading (Muckenthaler et al. 2003). However, another study did not confirm this observation (Kautz et al. 2008). It seems Fpn level is regulated by many factors.

Augmented liver Fpn in CuD rodents correlates best with *Hamp* expression rather than liver iron concentration, liver GPI-Cp, or *Fpn*-mRNA (Jenkitkasemwong et al. 2010; Mostad and Prohaska 2011). *Cp−/−* mice with sixfold higher liver iron levels have augmented liver Fpn protein (Chen et al. 2009). Perhaps lack of GPI-Cp restricts iron efflux in *Cp−/−* and CuD mouse liver. However, data in these two very different mouse models challenge the concept that functional ferroxidase is required for Fpn stability.

Placental and mammary gland response to copper deficiency

Copper is critical for proper development, clearly demonstrated by mouse embryonic lethality when the copper transporter Ctr1 was deleted (Kuo et al. 2001; Lee et al. 2001). Perhaps one requirement for this copper is to utilize iron properly. CuD rodent dams deliver pups that are iron deficient. This was demonstrated by detection of low neonatal liver iron and copper in CuD rat pups (Andersen et al. 2007; Pyatskowit and Prohaska 2008b). Similar studies showed that iron content of newborn CuD mouse pups was lower than CuA pups (Pyatskowit and Prohaska 2008c). Perinatal CuD rat pups also have lower brain iron (Fig. 3) (Prohaska and Wells 1975). Impaired iron transport is a likely mechanism for all these observations.

During lactation CuD rodent dams produce milk with lower than normal iron and pups are FeD (Prohaska 1989; Pyatskowit and Prohaska 2008c). Injection of iron to CuD rat pups was able to elevate hemoglobin to control levels (Pyatskowit and Prohaska 2008b). Recall that iron injection to anemic older CuD rats did not reverse the hemoglobin deficit (Reeves and DeMars 2006). This illustrates a clear difference between neonatal copper deficiency and postnatal copper deficiency in regards to secondary FeD consequences.

Failure to deliver iron across placental, mammary, and other tissue may be due to improper MCO function or perhaps impaired Fpn expression. Interestingly neither CuD dam placental tissue or mammary tissue revealed augmented iron, as might be predicted from reduced MCO function (Andersen et al. 2007; Pyatskowit and Prohaska 2008c).

The MCO that functions in mammary tissue may be GPI-Cp but this has not been established. The MCO in placenta is a newly characterized homolog of Cp called zyklopen (Chen et al. 2010). Like other MCO proteins zyklopen abundance may be lower following CuD and thus impair iron transport. Fpn expression in placenta and mammary tissue of CuD dams has not been published. It seems unlikely that Fpn would be augmented, however, since hepcidin expression is not impacted by CuD in rat dams (unpublished data) in contrast to pups (Jenkitkasemwong et al. 2010). *Fpn* expression in CuD rat brain is not altered compared to CuA levels (Fig. 3).

Does Fpn play a role in anemia of copper deficiency

In CuD mice one study reported augmented intestinal Fpn protein and mRNA (Chen et al. 2006). Our current q-PCR data did not detect any change in *Fpn* mRNA in CuD mouse intestine (Fig. 2). Intestinal Fe is higher in CuD mice (Chen et al. 2006; Pyatskowit and Prohaska 2008a). However, severely anemic CuD mice (72% lower hemoglobin) did not have any alteration in whole body iron, suggesting that anemia was not due to an intestinal iron block (Pyatskowit and Prohaska 2008a). Others have reported that whole body iron is not impacted by CuD in rats (Failla and Seidel 1988).

In CuD mice hepatic iron is augmented as discussed previously. However, liver *Fpn* mRNA and protein do not appear to change following CuD (Fig. 3) (Chung et al. 2004; Jenkitkasemwong et al. 2010). Liver Fe accumulation may be due to lower ferroxidase activity of both GPI-CP and Cp (Mostad and Prohaska 2011). *Cp−/−* mice have hepatic iron overload as documented in three separate labs (Prohaska 2011). However, hepatic Fe overload of CuD mice or *Cp−/−* mice cannot explain anemia as CuD mice are very anemic whereas *Cp−/−* mice rarely are (Prohaska 2011). Importantly, CuD mice with anemia have normal plasma iron content whereas *Cp−/−* mice without anemia are hypoferremic.

Spleen Fpn can be augmented by CuD as in post weanling rats or slightly or not augmented as in CuD rat pups (Jenkitkasemwong et al. 2010; Mostad and Prohaska 2011). The common phenotype of CuD rats however is lower splenic NHI (Fig. 1) (Mostad and Prohaska 2011). Is lower splenic iron in CuD rats determined by higher Fpn levels or phagocytosis of hypochromic erythrocytes? Interestingly, in parallel studies spleen of CuD mice also had lower levels of NHI than CuA mice, frank anemia, but control levels of Fpn. This suggests that lower splenic NHI is a result of uptake of hypochromic erythrocytes rather than accelerated Fpn-dependent iron efflux.

Is there any correlation between Fpn and plasma iron? The obvious answer is yes; however, following CuD the answer is not so clear. One study reported augmented intestinal Fpn but hypoferremia in CuD mice (Chen et al. 2006). Our models with two different dietary iron levels rarely detects lower plasma iron in anemic CuD mice (Mostad and Prohaska 2011; Pyatskowit and Prohaska 2008a). Furthermore, we detect no augmentation in Fpn in CuD mouse liver or spleen, likely because hepcidin is not depressed (Jenkitkasemwong et al. 2010). Importantly, the report of augmented intestinal Fpn also reported lower *Hamp* expression (Chen et al. 2006). This is consistent with hepcidin-dependent Fpn turnover (Nemeth et al. 2004).

Further evidence that Fpn changes may impact the anemia of CuD is from the elegant genetic studies on *Fpn* null and conditional knockout mice (Donovan et al. 2005). Data clearly showed that *Fpn* elimination resulted in iron accumulation in intestine, liver, and spleen. Plasma Fe levels, unfortunately, were not reported. However, these *Fpn* knockout mice were barely anemic (non-significant change in hematocrit and a 15% reduction in hemoglobin).

Clearly, anemia of copper deficiency is not the result of an iron limitation to blood caused by impaired Fpn/MCO function (Prohaska 2011). More likely, the anemia is a failure of bone marrow to produce sufficient erythrocytes. Improper function of bone marrow in CuD anemic mice was reported previously (Karimbakas et al. 1998). Incidence of anemia following copper limitation, such as following bariatric surgery to treat obesity, is on the rise in humans (Gletsu-Miller et al. 2012). Thus, understanding the mechanism for this copper deficiency associated anemia is critically relevant for diagnosis and treatment.

Acknowledgments We appreciate the excellent technical assistance of Krista Hemmesch. We thank Dr. Edward Perkins, Mercer University for the primer pairs and sequences used for *cyclinD1*. Research was supported by funding from US Public Health Service NIH HD-39708 and the International Copper Association.

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