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Age-related changes in iron homeostasis in mouse ferroxidase mutants

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Abstract Disorders of iron metabolism are a significant problem primarily in young and old populations. In this study, We compared 1-year-old C57BL6/J mice on iron deficient, iron overload, or iron sufficient diets with two similarly aged genetic models of disturbed iron homeostasis, the *sla* (sex-linked anemia), and the ceruloplasmin knockout mice ($Cp^{-/-}$) on iron sufficient diet. We found tissue specific changes in *sla* and nutritional iron deficiency including decreased liver *Hamp1* expression and increased protein expression of the enterocyte basolateral iron transport components, hephaestin and ferroportin. In contrast, the $Cp^{-/-}$ mice did not show significantly increased *Hamp1* expression despite increased liver iron suggesting that regulation is independent of liver iron levels. Together, these results suggest that older mice have a distinct response to alterations in iron metabolism and that age must be considered in future studies of iron metabolism.

Keywords Hepcidin · Ceruloplasmin · Ferroportin · Hephaestin · Ferritin

Abbreviations

Hamp1	Hepcidin gene
Cp	Ceruloplasmin
FPN1	Ferroportin
Hp	Hephaestin

Introduction

Iron is essential for life. Multiple proteins in mammals take advantage of the ability of iron to interconvert between Fe^{3+} and Fe^{2+} in order to carry out their biological function (Dunn et al. 2007; Mackenzie et al. 2008). Iron deficiency therefore results in multiple adverse consequences related to the role of these proteins. However, this same capability of iron can lead to the production of toxic oxygen radicals. As a result, organisms have developed elaborate regulatory systems for controlling iron bioavailability to meet metabolic needs and prevent iron deficiency while preventing excess iron accumulation that could represent a risk.

The regulation of iron metabolism throughout life is particularly challenging as mammals lack a known

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regulated mechanism of organismal iron excretion (Anderson 2007). As a consequence, mammals tightly control iron absorption from the diet. Hepcidin, a liver derived peptide, plays a central role in this regulation. A lack of hepcidin expression results in systemic iron loading in mice (Nicolas et al. 2001) and people (Majore et al. 2002). Similarly, mutations in *HFE*, *TfR2* and *hemojuvelin* (*HJV*) are characterized by reduced hepcidin expression (Ahmad et al. 2002; Bridle et al.2003; Kawabata et al. 2005; Nemeth et al. 2005; Papanikolaou et al. 2005), increased intestinal iron absorption and result in increased body iron load. Conversely, overexpression leads to severe iron deficiency and refractory anemia (Weinstein et al. 2002; Roy et al. 2007). Hepcidin has been shown to inhibit iron release from a number of cell types, including macrophages, enterocytes and hepatocytes (Ganz 2005; Rivera et al. 2005) and the peptide has been demonstrated to bind to ferroportin and facilitate its internalization and degradation (Nemeth et al. 2004; De Domenico et al. 2007a, b) although recent cell culture and in vivo experiments suggest that this mechanism may be specific to macrophages and not play a role in regulation of intestinal iron transport (Chaston et al. 2008). Other studies have found decreased *FPN1* mRNA expression in inflammatory conditions associated with increased Hepcidin levels (Yeh et al. 2004). Hepcidin expression responds to changes in body iron levels and increases with iron loading and decrease with iron deficiency (Pigeon et al. 2001; Frazer et al. 2002; Nicolas et al. 2002). The mechanism of hepcidin regulation in response to body iron remains uncertain although HFE, TFR2 and HJV likely play a role (De Domenico et al. 2007a, b). HJV can influence hepcidin expression by acting through the BMP/SMAD signaling pathway (Wang et al. 2005; Anderson and Frazer 2006; Babitt et al. 2006, 2007; Milward et al. 2007) while the role of HFE and TFR2 remains to be elucidated.

Perhaps as a consequence of this physiological requirement for regulation of body iron at the level of uptake, iron levels generally increase with normal aging. Total body iron increases with age in C57BL/6 J mice (Massie et al. 1983; Sohal et al. 1999) and rats (Masson and Roome 1997; Cook and Yu 1998). In men and post-menopausal women, particular attention has been given to tissue specific changes especially in the liver and brain (Levenson and Tassabehji 2004; Rouault and Cooperman 2006; de Lima et al. 2008; Xu et al. 2008). Iron has been proposed to play a role in the pathogenesis of age-related disorders including Parkinson disease, Alzheimers, stroke and cardiovascular disease (Rouault and Cooperman 2006). Despite the known association of age and iron status, most studies of dysregulated iron homeostasis in model organisms focus on young animals.

We therefore undertook an examination of iron homeostasis and tissue iron levels in mature mice models with either nutritional or genetic causes of iron deficiency and iron overload. Defects in two multi-copper ferroxidases, ceruloplasmin and hephaestin, lead respectively to the contrasting phenotypes of iron overload and deficiency in mice. Previous studies of the sex-linked anemia (*sla*), a genetic mouse model of iron deficiency with a defect in hephaestin, found that hemoglobin levels gradually increase with age (Bannerman and Cooper 1966; Bannerman and Pinkerton 1967) although they continue to be iron deficient (Pinkerton et al. 1970). In contrast, 10–12 week old ceruloplasmin-deficient mice had increased spleen and liver iron (Harris et al. 1999). We determined tissue iron levels in multiple tissues in these two mouse models in comparison to nutritional models of iron deficiency and iron overload. We similarly examined the hepcidin response and regulation of the intestinal proteins involved in iron export in these mice in response to hepcidin.

Materials and methods

Animal experiments

The *sla* mice are from a colony maintained by Greg Anderson at QIMR and are maintained on a C57BL/6 J background. C57BL/6 J *Cp* knockout (Cp^{tm1Hrs}/Cp^{tm1Hrs}) (Harris et al. 1999) were originally a kind gift of Dr. Leah Harris and are maintained on C57BL/6 J background. Three separate diets were used and diets were based on the AIN- 93 M formulation (Reeves et al. 1993) and contained 50 mg Fe/kg for control, 12 mg Fe/kg for iron deficiency and 2% carbonyl iron for the iron overload diet. Six-week-old C57BL/6 J male mice were separated into three groups of seven mice each. Mice were fed either a

control diet (Ctrl), iron deficient diet (Fe^{-}) for 12 months, or an iron sufficient diet for 6 months followed by an iron overload diet (Fe^{+}) for an additional 6 month period. C57BL/6 J *sla* and C57BL/6 J $Cp^{-/-}$ mice were fed the control diet for 12 months. The mice were allowed unlimited access to their respective diets and distilled water and were housed in cages designed to minimize coprophagy and environmental iron contamination (stainless steel grid bases used instead of bedding material and silicon stoppers in water bottles). All mouse protocols were in accordance with the National Institute of Health guidelines and approved by the Office of Lab Animal Care at the University of California, Berkeley.

Mouse tissue collection and iron status measurements

At the end of the dietary treatment, mice were anesthetized with Aerrane (Isoflurane) following overnight fasting. Blood was collected by cardiac puncture and brain, heart, liver, spleen, kidney, upper small intestines and right tibia were dissected. A small aliquot of blood was removed for hemoglobin and hematocrit levels which were measured by the Mouse Pathology Laboratory at the University of California, San Francisco Cancer Center, and remaining blood was collected by Serum-gel tubes (SARSTEDT). Iron content of tissues were measured using a Vista AX Simultaneous ICP-AES (Varian) after nitric acid digestion method (Chen et al. 2003). Enterocyte isolation was collected as described (Chen et al. 2003). Enterocyte and a part of liver were snap-frozen in liquid nitrogen and stored at −80°C for subsequent RNA and protein analysis.

Northern blot analysis

Northern blot analysis of mice liver mRNA was done as described (Chen et al. 2003) using a mouse *Cp* probe corresponding to positions 268–788 (Genbank NM_007752), a *Fpn1* (ferroportin1 cDNA) probe corresponding to nucleotides 929–1605 (Genbank AF231120), a *Hamp1* probe corresponding to positions 51–361 (Genbank BC021587), and a mouse *β-actin* cDNA probe purchased from Clontech. The *β-actin* signal was used as a loading control.

Hp, FPN1 and ferritin antibodies

Polyclonal rabbit anti-mouse Hp IgG was raised to the domain two amino acids (SDPASVDKED-GAFQDSNRM) of Hp as described in (Chen et al. 2003). Rabbit anti-FPN1 (CGKQLTSPKDTEPK-PLEGTH) was made using the same protocol, rabbit anti-mouse ferritin (recognizes both L and H subunits) was purchased from Roche, and rabbit anti-actin polyclonal antibody (H300) was purchased from Santa Cruz Biotechnology. Peroxidase-labeled anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology.

Immunoblot analysis

Mouse livers were homogenized and mice enterocytes were lysed as described (Chen et al. 2003). For all studies, except when FPN1 analysis was carried out, samples containing 50–100 mg protein were denatured by boiling for 5 min in 2X SDS sample buffer; however, samples for FPN1 Western blot analysis were not boiled (Chen et al. 2003) before electrophoresis. The proteins were separated by SDS–PAGE (7.5% acrylamide running gel) and transferred to nitrocellulose membranes. Blots were first incubated for 1 h with blocking buffer (containing PBS, 0.1% Tween-20, and 10% nonfat dry milk), and then incubated with primary antibodies for 1 h at room temperature. Primary antibodies were used at the following concentrations: 1:2000 for rabbit anti-Hp D2, 1:1000 for rabbit anti-FPN1, 1:500 for rabbit anti-ferritin, and 1:1000 for rabbit anti-actin. Blots were then washed three times in 0.1% PBS-T, incubated for 1 h at room temperature with 1: 20,000 diluted peroxidase-labeled anti-rabbit secondary antibodies, and signals were visualized by enhanced chemiluminescence.

Statistical analysis

All of the results are presented as means ± standard deviation. Statistical analysis of hematologic paramaters was calculated by one factor ANOVA followed by multiple comparisons with a control (MCC) procedure by Dunnett implemented in S-plus (built-in). Statistical inference was based on 95% simultaneous confidence intervals. Other data were analyzed by one-way ANOVA and differences among groups

by the Bonferroni post-hoc test. In any row, numbers not sharing a common superscript are significantly different ($P < 0.05$).

Results and discussion

We carried out analysis of iron levels and molecular changes in older mice with nutritional and genetic causes of iron deficiency and iron overload. We focused on two genetic mouse models which have iron deficiency and iron overload due to defects in the related ferroxidases, hephaestin and ceruloplasmin which are expressed in different tissues. These studies represent the first detailed examination of tissue-specific effects of these mutants in older mice. We compared the effects of these mutants on tissues with the effects of nutritional iron deficiency and iron overload to provide insight into the respective roles of these proteins in older mice.

Hematology and tissue iron levels in 1 year old mice

We measured hematologic indices (Table 1) and tissue iron (Table 2) of 1 year old *sla*, $Cp^{-/-}$ as well as C57Bl6/J mice on iron deficient (Fe^{-}), iron overload (Fe^{+}) or control diets (Ctrl). Mean corpuscular hemoglobin (MCH) were decreased consistent with microcytic hypochromia without anemia in *sla* mice and $Cp^{-/-}$. MCV, MCHC and RDW were also significantly decreased in $Cp^{-/-}$. The anemia of the *sla* mouse which contains a defect in hephaestin has been previously reported to ameliorate with age (Bannerman and Cooper 1966; Bannerman and Pinkerton 1967). We similarly found that hematologic

Table 1 Hematologic parameters for mice models

	sla	Ctrl	Fe^{-}	Fe^{+}	$Cp^{-/-}$
RBC (M/μl)	9.5 ± 1.6	8.5 ± 0.4	8.9 ± 0.4	8.6 ± 2.0	9.9 ± 0.8
Hb (g/l)	12.3 ± 1.6	12.5 ± 0.4	12.0 ± 1.9	13.9 ± 0.8	12.2 ± 0.8
HCT (%)	41.3 ± 4.3	40.0 ± 1.9	41.6 ± 2.8	44.0 ± 5.0	40.5 ± 3.0
MCV (fl)	43.8 ± 4.2	47.0 ± 1.1	46.5 ± 1.6	51.5 ± 6.0	41.6 ± 1.9*
MCH (pg)	13.4 ± 2.0*	14.8 ± 0.6	14.2 ± 0.2	17.2 ± 0.3*	12.6 ± 0.8*
MCHC (g/dl)	30.2 ± 2.4	31.5 ± 0.8	30.3 ± 1.0	30.4 ± 0.6	29.6 ± 1.0*
RDW (%)	19.6 ± 3.0	17.9 ± 0.6	17.8 ± 0.4	18.8 ± 0.2	21.0 ± 1.5*

RBC Red blood cell, *Hb* hemoglobin, *HCT* hematocrit, *MCV* mean cell volume, *MCH* mean cell hemoglobin, *MCHC* mean cell hemoglobin concentration, *RDW* red cell distribution width, *sla* sex-linked anemia, *Ctrl* control, Fe^{-} iron deficient, Fe^{+} iron overload, $Cp^{-/-}$ ceruloplasmin knockout. Values are means ± SD for seven mice per group

* Significant differences (adjusted *P*-value < 0.05)

Table 2 Iron concentration in mouse tissues

	sla	Ctrl	Fe^{-}	Fe^{+}	$Cp^{-/-}$
Serum (μg/ml)	1.62 ± 0.42^{ab}	1.96 ± 0.69^{b}	1.45 ± 0.17^{ab}	4.41 ± 0.45^{c}	0.99 ± 0.19^{a}
Brain (μg/g)	86.7 ± 5.6^{a}	86.2 ± 5.6^{a}	81.7 ± 3.1^{a}	85.2 ± 7.8^{a}	80.1 ± 3.0^{a}
Heart (μg/g)	304 ± 28^{a}	374 ± 22^{bc}	327 ± 47^{ab}	420 ± 66^{c}	412 ± 18^{c}
Spleen (μg/g)	901 ± 170^{a}	1687 ± 218^{b}	1207 ± 76^{ab}	3524 ± 281^{c}	4867 ± 825^{d}
Liver (μg/g)	234 ± 50^{a}	415 ± 48^{a}	202 ± 19^{a}	4684 ± 528^{b}	2766 ± 445^{c}
Kidney (μg/g)	221 ± 40^{a}	284 ± 13^{b}	280 ± 7.9^{b}	501 ± 54^{c}	266 ± 27^{ab}
Intestine (μg/g)	177 ± 23^{a}	133 ± 29^{b}	33.5 ± 5.7^{c}	871 ± 73^{d}	188 ± 12^{a}
Bone (μg/g)	88.7 ± 27^{a}	198 ± 55^{b}	67.5 ± 8.8^{a}	402 ± 66^{c}	48.9 ± 18^{a}

Values are means ± SD of seven mice per group. Data were analyzed by one-way ANOVA. In any row, numbers not sharing a common superscript (a, b, c, or d) are significantly different ($P < 0.05$)

parameters were similar to control mice at 1 year of age. We saw a distinct set of tissues affected in each mouse model with some tissues showing decreased iron while others showed increased iron levels. Not surprisingly, tissue iron concentration was increased in liver, spleen, kidney, intestine and bone in mice fed an iron overload diet. The *sla* mice remain profoundly iron deficient with dramatically decreased heart, spleen, liver, kidney and bone marrow iron levels. The very low iron in most tissues especially liver, spleen, and bone marrow in *sla* mice indicate very low body iron stores and suggest rapid recycling of iron may account for the maintenance of hematologic parameters.

$Cp^{-/-}$ mice showed increased liver and spleen iron but significantly decreased iron in plasma and bone marrow. The Cp deficient mouse similarly had persistent defects in iron homeostasis. There has been controversy over whether brain iron is increased in this mutant (Harris et al. 1999; Patel et al. 2002) and whether anemia is present (Harris et al. 1999; Patel et al. 2002; Yamamoto et al. 2002; Cherukuri et al. 2004). We found no change in whole brain iron levels; however, region or cell specific changes have been found by others (Jeong and David 2006). We also noted very low serum and bone marrow iron levels which were lower than nutritional iron deficiency and *sla* mice. Despite these very low levels, RBC, Hb, and Hct are maintained which as with the *sla* mice suggest robust mechanisms to maintain erythropoeisis in $Cp^{-/-}$. We found dramatically increased cardiac iron comparable to the nutritional iron overloaded mice and suggests that Cp plays an important role in mobilizing iron from the heart. A recent study found expression of both Hp and Cp in the heart but only Cp was responsive to iron levels (Qian et al. 2007). As has been previously noted, the $Cp^{-/-}$ mouse has elevated spleen and liver iron.

Liver gene and protein expression of selected iron metabolism genes

Northern blot analysis of liver from *sla* mice fed control diet (*sla*) and C57BL/6 J mice fed control (Ctrl), iron deficient (Fe^{-}) or iron overload (Fe^{+}) diets and $Cp^{-/-}$ mice fed control diet ($Cp^{-/-}$) is shown in Fig. 1a. The same blots were sequentially hybridized to probes for *Cp*, *Fpn1*, *Hamp1* and *β-actin.* The *actin* band was used as a loading control.

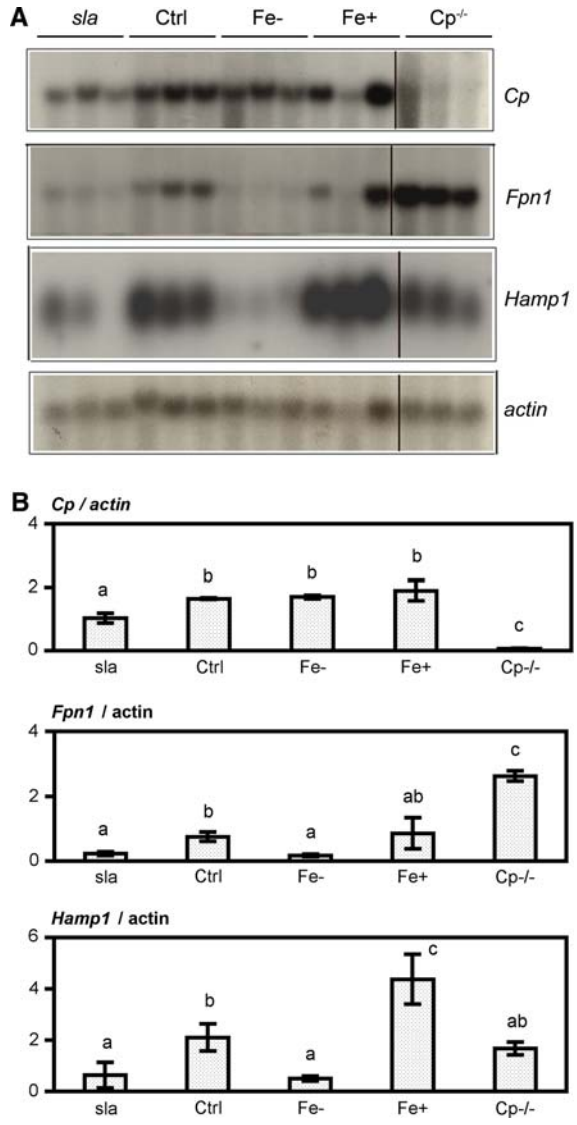

Fig. 1 Liver *Cp*, *Fpn1* and *Hamp1* gene expression. **a** Northern blot analysis of liver from *sla* mice fed control diet (*sla*) and C57BL/6 J mice fed control diet (Ctrl), iron-deficient (Fe^{-}), or iron-overload (Fe^{+}) diets, and $Cp^{-/-}$ mice fed control diet ($Cp^{-/-}$) for 12 months. Blots were sequentially hybridized to probes for *Cp, Fpn1*, *Hamp1* and *β-actin*. The *β-actin* signal was used as a loading control. **b** Relative mRNA levels of *Cp/β-actin* (*upper panel*), *Fpn1/β-actin* (*middle panel*) and *Hamp1/β-actin* (*bottom panel*) were quantified using NIH Image and densitometry values normalized to values obtained for *β-actin*. Data are means ± SD as percent control from three independent experiments. Data were analyzed by one-way ANOVA and differences among groups by the Bonferroni post-hoc test. In each panel, numbers not sharing a common superscript are significantly represented as different ($P < 0.05$). All samples were run on the same gel, we removed three lanes between Fe^{+} and $Cp^{-/-}$ lanes and generated a composite image

Figure 1b show the relative mRNA levels of *Cp* (upper panel), *Fpn1* (middle panel) and *Hamp1* (bottom panel) quantified using NIH Image and normalized to values obtained for *β-actin*. $Cp^{-/-}$ mice show no *Cp* mRNA gene in mice liver, while *Cp* mRNA gene expression was significantly different from other mice only in the *sla* mice. *Fpn1* mRNA levels were significantly decreased in both *sla* and iron deficient mice as compared to control and $Cp^{-/-}$ mice, $Cp^{-/-}$ mice expression was significantly increased compared to the other groups. Liver *Hamp1* mRNA levels were significantly decreased in both of *sla* and iron deficient mice compared to control group and the iron overload group *Hamp1* expression was significantly higher than the other four groups. In contrast and despite the increased liver iron levels, $Cp^{-/-}$ mice did not show significantly increased *Hamp1* expression. Other mouse mutants with iron overload, including the *hfe* knockout (Muckenthaler et al. 2003), and mouse models of beta-thalassemia (De Franceschi et al. 2006) show a similar inappropriately low hepcidin expression despite increased iron levels and our results provide additional support for the hypothesis that liver iron is not the primary determinant controlling hepcidin expression (Bridle et al. 2003). We also noted dramatically increased levels of *Fpn*1 expression in the liver of $Cp^{-/-}$ mice as compared to the *sla* and control mice with similar *Hamp1* expression which suggests a separate mechanism for the upregulation of *Fpn1* transcript levels in response to increased liver iron (McKie et al. 2000). Figure 2a shows mouse liver FPN1 and ferritin protein expression in *sla* mice fed control diet (*sla*) and C57BL/6 J mice fed control (Ctrl), iron deficient (Fe^{-}) or iron overload (Fe^{+}) diets and $Cp^{-/-}$ mice fed control diet ($Cp^{-/-}$). FPN1 and ferritin protein expression were increased in iron overload, $Cp^{-/-}$ mice as compared to *sla*, iron deficient and control group mice and concur with our transcriptional data.

Intestinal enterocyte expression

Figure 2b shows enterocyte Hp, FPN1 and Ferritin protein expression in the different groups. Hp expression was increased in both iron deficient and $Cp^{-/-}$ mice as compared to the other groups. FPN levels are comparable in *sla*, iron deficient and $Cp^{-/-}$ and increased relative to control and iron overloaded

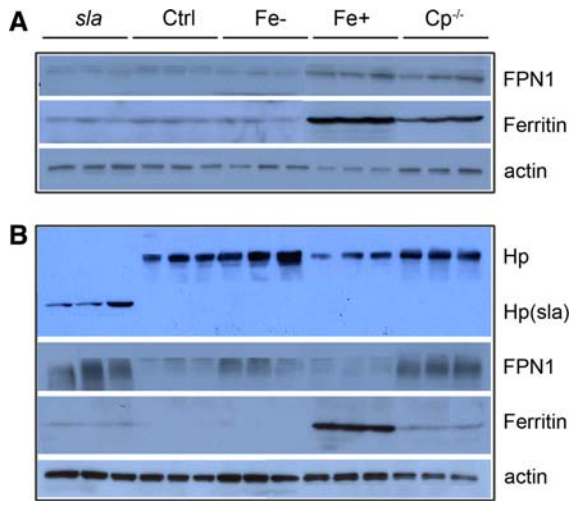

Fig. 2 Ferroportin, Hp, and Ferritin protein expression in liver and enterocytes. **a** Liver FPN1 and Ferritin protein levels expression by western blot analysis from *sla* mice fed control diet (*sla*) and C57BL/6 J mice fed control (Ctrl), iron deficient (Fe^{-}) or iron overload (Fe^{+}) diets and $Cp^{-/-}$ mice fed control diet ($Cp^{-/-}$) for 12 months. **b** Enterocyte Hp, FPN1 and Ferritin protein levels expression by western blot analysis from *sla* mice fed control diet (*sla*) and C57BL/6 J mice fed control (Ctrl), iron deficient (Fe^{-}) or iron overload (Fe^{+}) diets and $Cp^{-/-}$ mice fed control diet ($Cp^{-/-}$) for 12 months. Hp is present in the Ctrl, Fe^{-}, Fe+, and $Cp^{-/-}$ mice as a Mr 155,000 protein while a Mr 130,000 protein is present in the *sla* mice. For comparison of liver and enterocyte levels among different samples, protein concentrations were normalized to yield equivalent actin signal (**a** and **b**)

enterocytes. Iron overloaded mice had very high levels of ferritin. Similarly, *sla* and $Cp^{-/-}$ mouse enterocytes had higher levels than control and iron deficient mice. The very low *Hamp1* expression in *sla* mice in the liver and increased FPN1 protein levels in enterocytes as compared to control mice is consistent with and provides support for persistent iron deficiency in the *sla* mice. The increased expression of Hp and FPN1 in the enterocyte of $Cp^{-/-}$ relative to the control is most similar to the nutritionally iron deficient mouse and further suggests a functional iron deficiency in these mice. Similarly, these results suggest that Cp cannot compensate for Hp in iron uptake as previously suggested (Donovan et al. 2006). Together our study provides additional evidence for the discrete in vivo functions of Cp and Hp in iron metabolism. It also indicates that older mice have a distinct response to alterations in iron metabolism and that age must be considered in future studies of iron metabolism.

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