

# Effect of dietary iron deficiency and overload on the expression of ZIP metal-ion transporters in rat liver

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**Abstract** The mammalian ZIP (Zrt-, Irt-like Protein) family of transmembrane transport proteins consists of 14 members that share considerable homology. ZIP proteins have been shown to mediate the cellular uptake of the essential trace elements zinc, iron, and manganese. The aim of the present study was to determine the effect of dietary iron deficiency and overload on the expression of all 14 ZIP transporters in the liver, the main site of iron storage. Weanling male rats ( $n = 6/\text{group}$ ) were fed iron-deficient (FeD), iron-adequate (FeA), or iron-overloaded (FeO) diets in two independent feeding studies. In study 1, diets were based on the TestDiet 5755 formulation and contained iron at 9 ppm (FeD), 215 ppm (FeA), and 27,974 ppm (3% FeO). In study 2, diets were based on the AIN-93G formulation and contained iron at 9 ppm Fe (FeD), 50 ppm Fe (FeA), or 18916 ppm (2% FeO). After 3 weeks, the FeD diets depleted liver non-heme iron stores and induced anemia, whereas FeO diets resulted in hepatic iron

overload. Quantitative RT-PCR revealed that ZIP5 mRNA levels were 3- and 8-fold higher in 2% FeO and 3% FeO livers, respectively, compared with FeA controls. In both studies, a consistent downregulation of ZIP6, ZIP7, and ZIP10 was also observed in FeO liver relative to FeA controls. Studies in H4IIE hepatoma cells further documented that iron loading affects the expression of these ZIP transporters. Overall, our data suggest that ZIP5, ZIP6, ZIP7, and ZIP10 are regulated by iron, indicating that they may play a role in hepatic iron/metal homeostasis during iron deficiency and overload.

**Keywords** SLC39 · Iron deficient · Iron overload · Zinc

## Introduction

ZIP proteins are integral membrane proteins that transport metal ions from the extracellular environment or the lumen of intracellular organelles into the cytosol. The name “ZIP” derives from the first two family members: Zrt1, zinc-regulated transporter 1, and IRT1, iron-regulated transporter 1. Zrt1 is plasma membrane protein that accounts for the high-affinity uptake of zinc by yeast (Zhao and Eide 1996). The amino acid sequence of yeast Zrt1 is 30% identical to IRT1, a protein in the root of *Arabidopsis thaliana* that mediates the uptake of iron from the soil (Kautz et al. 2008; Korshunova

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et al. 1999). The expression of IRT1 is highly responsive to iron; mRNA levels of IRT1 increase during iron deficiency and decrease after iron loading (Connolly et al. 2002). A similar regulation of Zrt1 occurs in response to zinc deficiency or excess (Zhao and Eide 1996). Since the identification of these first ZIP proteins, over 90 family members have been identified in species ranging from bacteria, yeast, *Arabidopsis thaliana*, *Drosophila*, *Caenorhabditis elegans*, mouse, rat, and human (Eide 2004; Taylor and Nicholson 2003).

The mammalian genome encodes for 14 ZIP transporters (Lichten and Cousins 2009). Although most mammalian ZIP proteins have been characterized nearly exclusively by their ability to transport zinc, we have shown that one ZIP transporter—ZIP14—is able to mediate the cellular uptake of iron in addition to zinc (Liuzzi et al. 2006; Pinilla-Tenas et al. 2011). The transport of iron by ZIP14 is noteworthy given that, to date, only one other transmembrane iron import protein has been identified (i.e., divalent metal transporter 1, DMT1 (Gunshin et al. 1997)).

ZIP14 is most abundantly expressed in liver, the principal iron storage organ in the body. How the liver takes up iron is incompletely understood. Under normal circumstances and during iron deficiency, the liver acquires iron via receptor-mediated endocytosis of transferrin, the circulating iron transport protein (Morgan et al. 1986). During conditions of iron overload, the iron-carrying capacity of transferrin can become exceeded, giving rise to non-transferrin-bound iron (NTBI), which is rapidly taken up by the liver (Craven et al. 1987). Studies in hepatic cell lines demonstrate that NTBI uptake is mediated, at least in part, by ZIP14 at the cell surface (Gao et al. 2008; Liuzzi et al. 2006). More recently, we have found that ZIP14 may also participate in the assimilation of iron from transferrin (Zhao et al. 2010). Given the significant homology of the ZIP family of proteins (Taylor and Nicholson 2003), it is possible that ZIP proteins other than ZIP14 may play a role in iron metabolism as well. The aim of the present study was to screen the expression of all 14 ZIPs in the liver in an effort to identify those that are regulated by dietary iron deficiency or iron overload. This approach is based on the hypothesis that iron-regulated ZIP transporters represent the best candidates for playing a role in iron metabolism.

## Materials and methods

### Animals, diets, and experimental designs for studies 1 and 2

Weanling male Sprague–Dawley rats were obtained from Charles River Laboratories. After 3 days acclimation, rats were randomly assigned to three dietary groups ( $n = 6/\text{group}$ ): iron deficient (FeD), iron adequate (FeA), and iron overload (FeO). In study 1, rats were fed modified TestDiet<sup>®</sup> 5755 purified rodent diets (TestDiet) formulated to contain no added iron (FeD), 200 ppm iron as ferric citrate (FeA), or 30,000 ppm iron as carbonyl iron (FeO) (Sigma-Aldrich). Iron concentrations of the diets, as determined by inductively coupled plasma mass spectroscopy (ICP-MS), were found to be 9 ppm (FeD), 215 ppm (FeA), and 27,974 ppm (3% FeO). In study 2, rats were fed modified AIN-93G purified rodent diets (Reeves et al. 1993) formulated to contain no added iron (FeD), 35 ppm iron as ferric citrate (FeA), or 20,000 ppm iron as carbonyl iron (FeO). Iron concentrations of the diets were determined to be 10 ppm (FeD), 50 ppm (FeA), and 18,916 ppm (2% FeO). The AIN-93G diet was also modified by adding 20% sucrose instead of 10% in an effort to make the iron-loaded diet more palatable. To adjust for the increased sucrose in the diets, the amount of cornstarch was reduced accordingly. In addition, Avicel<sup>®</sup> was used instead of cellulose to lower the amount of any contaminant iron. Concentrations of iron, zinc, copper, manganese, and cobalt in the diets used in studies 1 and 2 are shown in Table S1. In both studies, animals were given free access to the diets and distilled water for 3 weeks. Body weights were determined every third day. At the end of the studies, rats were anesthetized after an overnight fast, and blood and livers were collected. Animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida.

### Determination of iron status and liver mineral concentrations

Hemoglobin was measured in heparinized blood by using a HemoCue 201 + hemoglobin analyzer (HemoCue). Hematocrit was determined by using a Clay Adams Readacrit centrifuge. Liver non-heme

iron concentrations were determined colorimetrically after acid digestion of tissues (Torrance and Bothwell 1968). ICP-MS was used to measure hepatic concentrations of total iron (non-heme and heme), zinc, copper, manganese, and cobalt. Parallel analysis of a sample of Bovine Liver Standard Reference Material 1577b (National Institute of Standards and Technology, NIST) confirmed the accuracy of liver mineral measurements by ICP-MS (data not shown).

#### Cell culture and iron treatment

H4IIE rat hepatoma cells were obtained from the American Type Culture Collection (Manassas, Va). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. Cells were maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>. To load cells with iron, the cell culture medium was supplemented with 50 µM Fe-nitritoltriacetate (molar ratio of 1:4) for 8 h (Knutson et al. 2003).

#### Measurement of relative mRNA levels

Total RNA was isolated by using RNABee (TelTest) according to the manufacturer's protocol. Any residual DNA was removed by using the Turbo DNA-free kit (Ambion). RNA integrity was confirmed by denaturing gel electrophoresis. cDNA was synthesized by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative RT-PCR was performed by using Power SYBR Green PCR Master Mix and an Applied Biosystems 7300 realtime PCR system. Quantitation of mRNA was determined by comparison to standard curves generated by four 10-fold serial dilutions of standard cDNA. Transcript levels were normalized to the average of cyclophilin B, a commonly used internal control gene, and RPL13a, which has recently been identified as one of the most stably expressed genes (de Jonge et al. 2007). Normalization to the average of more than one housekeeping gene has been shown to improve the reliability of expression measurements, especially when subtle biological differences are present (Vandesompele et al. 2002). Gene-specific primer sequences are shown in Table 1. Dissociation curve analysis of PCR products revealed

single peaks, indicating specific amplification products. All PCR amplification efficiencies were  $100 \pm 10\%$ .

#### Statistical analysis

Results are presented as means  $\pm$  standard error. Data were analyzed by one-way ANOVA and Tukey's post-hoc test using PRISM (version 4.02 for Windows, GraphPad) software. Data sets with unequal variances were ln transformed to normalize variance prior to statistical analysis.

## Results

#### Body weights

In study 1, weanling rats were fed modified TestDiet® 5755 diets containing different amounts of iron for 3 weeks. Final body weights of FeD rats were 15% lower than FeA controls (Fig. 1a). Body weights of 3% FeO rats after 3 weeks were markedly lower than both the FeA and FeD animals (107.8 g vs. 224.3 g and 192.4 g, respectively). Because this marked growth suppression introduces a potential confounding variable, a second study was performed with 2% carbonyl iron in the FeO group. Previous studies have shown that 2% carbonyl iron diets have less of an impact on growth than do 3% carbonyl iron diets (Mackinnon et al. 1995). In study 2, the basal diets were also changed to the more standard American Institute of Nutrition formulation, AIN-93G (Reeves et al. 1993). After 3 weeks of feeding modified AIN-93G diets containing different amounts of iron, body weights of FeD and 2% FeO rats were 20% lower than FeA rats (Fig. 1b). In contrast to study 1, however, body weights did not differ between FeD and 2% FeO rats.

#### Indicators of iron status

FeD rats in both studies became anemic due to iron deficiency, as indicated by low concentrations of hemoglobin, hematocrit, and liver non-heme iron (Table 2). In the 3% FeO rats, concentrations of liver non-heme iron were 60 times higher than those in FeA group. In the 2% FeO rats, concentration of liver non-heme iron were also 60 times higher than those

**Table 1** Primers used for qRT-PCR

mRNA	Forward primer	Reverse primer
ZIP1	5'-TCTGGACTTGCTGCCTGACTAC-3'	5'-CGTCACATGAAGAGCCTCCAA-3'
ZIP2	5'-GCCTGAGGCTGGTGAAGATC-3'	5'-CACAGGAGACATGAGAGCTAAGGA-3'
ZIP3	5'-GGCACACCGCTCCAAGAA-3'	5'-AAGACGCCACCCCAAA-3'
ZIP4	5'-ATGCCGGGCTGACTGTGA-3'	5'-TGAGCGCTGAGGCCAGAT-3'
ZIP5	5'-CCTCAGCACTACCCTAGCAGTCTT-3'	5'-TGCAAAGTCACCCAGTTCATG-3'
ZIP6	5'-CACGTTGGCCTGGATGGT-3'	5'-GGCCGTCGCTGAAATTGT-3'
ZIP7	5'-TTGACTGCAATTGGAGCATTG-3'	5'-CTGCCCTCCCTCAGTGA-3'
ZIP8	5'-CCAGATAACCAGCTCGAACTTCA-3'	5'-TGTTGGATCCTCACAGGGATGA-3'
ZIP9	5'-CAGCTGCATGCCTACATTGG-3'	5'-CCACGAGCAACATGAAAACG-3'
ZIP10	5'-GGCCCTTCAACAGAGACCAA-3'	5'-CTCCTGACCTTCCCTGACTTCA-3'
ZIP11	5'-GGGTCTCGCTGTCGGTGTAG-3'	5'-TCGAAGGTGGCAGATGCA-3'
ZIP12	5'-AATGTGCCAGCCTCCAACA-3'	5'-GGACCATAACAGCCAACAAGCT-3'
ZIP13	5'-GGTCCGGAACCTCAAAGTCA-3'	5'-TGGGTGAAGTTGTCAATGGTATTG-3'
ZIP14	5'-CCTCACGAGCTGGGAGACTTC-3'	5'-AGAGGGCCTGCTGGATACTCA-3'
BMP6	5'-CGCCGCAATCCTCTCTT-3'	5'-CTTTTGCATCTCCCGCTTCT-3'
TFR1	5'-GAATACGTTCCCGTTGTTGA-3'	5'-ATCCCCAGTTCCTAGATGAGCAT-3'
Cyclophilin B	5'-CGCACAGCCGGGACAA-3'	5'-TTCGATCTTGCCACAGTCTAC-3'
RPL13A	5'-GCATTTTTTGGCGCACTG-3'	5'-GCCTGGCCTCTTTTGGTCTT-3'

Primers were designed by using Primer Express, version 3.0, Applied Biosystems

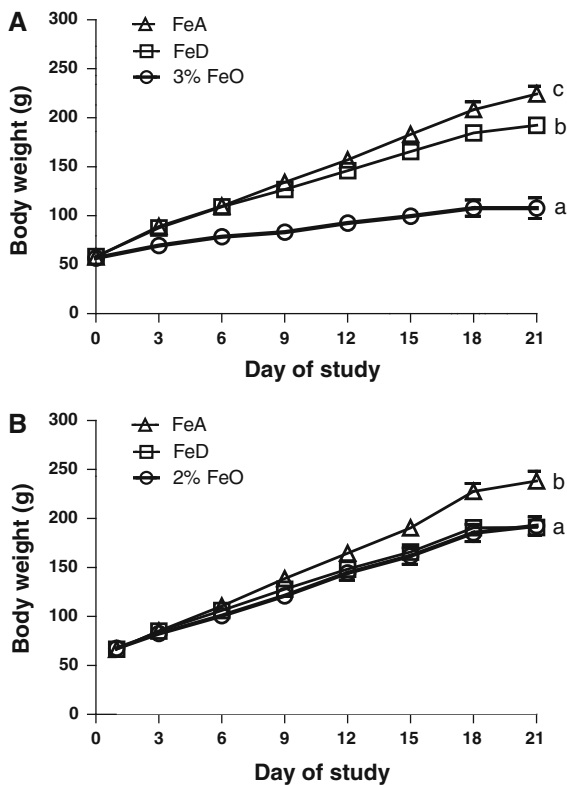
in the FeA group, but the degree of iron loading (1813 µg/g) was less severe than that obtained from the 3% FeO diet (5224 µg/g).

#### Liver mineral concentrations

ICP-MS was used to measure the concentrations of total iron, zinc, copper, manganese, and cobalt in the livers of FeD, FeA, and FeO rats (Table 3). In both studies, total iron concentrations were lower in FeD rats and higher in FeO rats, consistent with differences observed with non-heme iron concentrations (Table 2). The 3 and 2% FeO animals had slightly elevated hepatic zinc concentrations (21 and 14%, respectively) compared to FeA animals. In addition, FeD rats had higher hepatic copper concentrations when compared to FeA controls in both studies. In study 1, hepatic concentrations of manganese were elevated by approximately 20% in both the FeD and 3% FeO rats compared to FeA controls. The 3% FeO animals also had 41% lower hepatic concentrations of cobalt. The higher hepatic cobalt concentrations in study 1 than in study 2 is likely due to differences in the concentrations of cobalt in the different diet formulations (Table S1).

#### Effect of FeD and FeO on hepatic ZIP transporter mRNA levels

We used qRT-PCR to assess relative mRNA levels of all fourteen ZIP transporters in the livers of FeD, FeA, and FeO rats. All ZIPs were measured except for ZIP2 and ZIP12, which were not detectable, even when multiple primer sets were tested. In study 1, expression levels of four ZIP transporters were affected by iron status (Fig. 2a). Relative to FeA controls, ZIP5 mRNA levels were 8-fold higher in 3% FeO liver, whereas mRNA levels of ZIP6, ZIP7, and ZIP10 were 24–45% lower. As a control for iron-responsive gene expression, we measured hepatic BMP6 (bone morphogenetic protein 6) mRNA levels, which are positively regulated by iron supplementation and negatively regulated by iron deficiency (Jenkitkasemwong et al. 2010; Kautz et al. 2008). In study 2, we observed differential expression of the same ZIP transporters as in study 1. Relative to FeA controls, ZIP5 mRNA levels were 3-fold higher in 3% FeO liver, whereas mRNA levels of ZIP6, ZIP7, and ZIP10 were 28–40% lower (Fig. 2b). In study 2, however, we additionally found that mRNA levels of ZIP14 were 50% higher in FeD liver compared to FeA liver. Transcript levels of



**Fig. 1** Body weights of rats fed iron-adequate (FeA), iron-deficient (FeD) and iron-overloaded (FeO) diets. **a** In study 1, weanling male Sprague–Dawley rats were fed modified TestDiet® 5755 basal diets that contained 9 ppm Fe (FeD), 215 ppm Fe (FeA) or 27,974 ppm Fe (3% FeO) for 3 weeks. **b** In study 2, weanling male Sprague–Dawley rats were fed modified AIN-93G diets that contained 9 ppm Fe (FeD), 50 ppm Fe (FeA) or 18916 ppm Fe (2% FeO) for 3 weeks. Body weights were measured every 3 day. Final body weights were compared by one-way ANOVA followed by Tukey’s multiple comparison test. Values represent mean ± SE, n = 6. Means without a common letter differ, *P* < 0.05

the internal control genes, cyclophilin B and RPL13a, correlated strongly (*r* = 0.91) and did not vary among the dietary treatment groups (data not shown).

Effect of iron loading on ZIP transporter mRNA levels in H4IIE rat hepatoma cells

The two feeding studies suggested that the hepatic expression of ZIP5, ZIP6, ZIP7, and ZIP10 are regulated by FeO. To determine if iron loading per se affects the expression of these ZIP transporters, we incubated H4IIE cells, a rat hepatoma cell line, with or without 50 μM Fe-NTA for 8 h and measured mRNA levels of ZIP5, ZIP6, ZIP7, and ZIP10. As a positive control for iron loading, we measured mRNA levels of TFR1 (transferrin receptor 1), which decrease under high iron conditions (Lu et al. 1989). For comparison, we also measured ZIP3 mRNA levels, which were unaffected by FeO in rat liver. Consistent with FeO liver (Fig. 2), iron-loaded H4IIE cells had 30–45% lower mRNA levels of ZIP6, ZIP7, and ZIP10 compared to controls, whereas ZIP3 mRNA levels were unaffected (Fig. 3). In contrast, levels of ZIP5 mRNA, which were 3- to 5-fold higher in FeO liver, were 50% lower in iron-loaded H4IIE cells compared to controls. The 75% lower levels of TFR1 mRNA in Fe-NTA-treated cells relative to controls indicate that the cells became loaded with iron.

Discussion

This is the first report of hepatic ZIP transporters whose expressions are affected by dietary FeD or FeO. In two independent feeding studies, FeO rat liver consistently demonstrated differential expression of ZIP5, ZIP6, ZIP7, and ZIP10. Among these four ZIPs, the expression of ZIP5 was most affected by FeO; its mRNA levels were 3- and 8-fold higher in 2% FeO and 3% FeO livers, respectively, compared with FeA controls. The higher relative ZIP5 levels in

**Table 2** Hemoglobin, hematocrit, and liver non-heme iron concentrations in FeD, FeA, and FeO rats

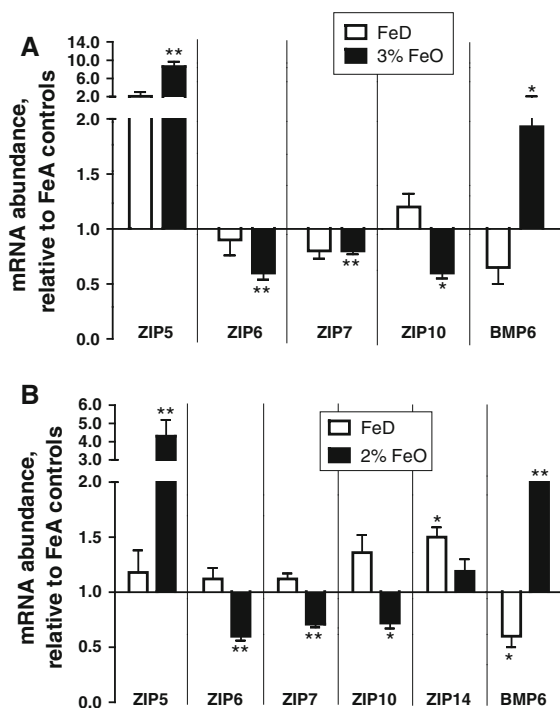
	Study 1			Study 2		
	FeD	FeA	3% FeO	FeD	FeA	2% FeO
Hb (g/dl)	7.2 ± 0.4 <sup>a</sup>	13.3 ± 0.3 <sup>b</sup>	14.5 ± 0.4 <sup>b</sup>	4.6 ± 0.2 <sup>a</sup>	13.2 ± 0.2 <sup>b</sup>	14.6 ± 0.5 <sup>c</sup>
HCT (%)	29.4 ± 1.6 <sup>a</sup>	44.8 ± 0.6 <sup>b</sup>	49.5 ± 1.3 <sup>c</sup>	18.7 ± 1.0 <sup>a</sup>	42.4 ± 0.9 <sup>b</sup>	44.8 ± 1.3 <sup>b</sup>
NHI (μg/g)	15.0 ± 4.6 <sup>a</sup>	87.4 ± 16 <sup>b</sup>	5224 ± 345 <sup>c</sup>	12.9 ± 0.5 <sup>a</sup>	30.1 ± 5.0 <sup>b</sup>	1812 ± 380 <sup>c</sup>

FeD iron deficient, FeA iron adequate, FeO iron overloaded, Hb hemoglobin, HCT hematocrit, NHI liver non-heme iron. Values are means ± SE of 6 rats per group. Data were analyzed by one-way ANOVA. In any row, values not sharing a common superscript are different (*P* < 0.05)

**Table 3** Liver mineral concentrations in FeD, FeA, and FeO rats

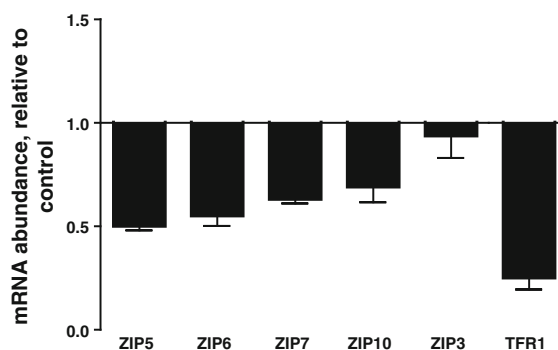
	Study 1			Study 2		
	FeD	FeA	3% FeO	FeD	FeA	2% FeO
Fe	143 ± 14.0 <sup>a</sup>	471 ± 77.9 <sup>b</sup>	12836 ± 1282 <sup>c</sup>	93.2 ± 6.7 <sup>a</sup>	267.8 ± 63.8 <sup>b</sup>	4966 ± 275.5 <sup>c</sup>
Zn	99.7 ± 3.1 <sup>a</sup>	98.2 ± 4.3 <sup>a</sup>	119 ± 6.2 <sup>b</sup>	105.8 ± 4.8 <sup>a</sup>	128.7 ± 3.0 <sup>b</sup>	146.8 ± 1.5 <sup>c</sup>
Cu	31.3 ± 3.3 <sup>b</sup>	17.8 ± 0.7 <sup>a</sup>	17.0 ± 0.9 <sup>a</sup>	56.2 ± 6.1 <sup>c</sup>	22.3 ± 0.8 <sup>b</sup>	6.2 ± 0.7 <sup>a</sup>
Mn	10.4 ± 0.2 <sup>b</sup>	7.9 ± 0.1 <sup>a</sup>	9.8 ± 0.8 <sup>b</sup>	11.2 ± 0.4 <sup>b</sup>	10.4 ± 0.6 <sup>b</sup>	8.4 ± 0.4 <sup>a</sup>
Co	2.0 ± 0.1 <sup>b</sup>	1.7 ± 0.1 <sup>b</sup>	1.0 ± 0.1 <sup>a</sup>	0.03 ± 0.0 <sup>a</sup>	0.03 ± 0.0 <sup>a</sup>	0.03 ± 0.0 <sup>a</sup>

Liver mineral concentrations (ppm) were determined by ICP-MS. Liver Fe values reflect both heme and non-heme iron. Values are means ± SE of 6 rats per group. Data were analyzed by one-way ANOVA. In any row, values not sharing a common superscript are different ( $P < 0.05$ )



**Fig. 2** Effect of FeD and FeO on the expression of hepatic ZIP transporters in **a** study 1 and **b** study 2. Total RNA was isolated from liver and transcript abundances of all fourteen ZIP transporters were determined by using qRT-PCR. Shown above are the ZIP transporters whose expression differed compared to FeA controls. Relative transcript abundances of each gene were normalized to the average of two housekeeping genes (cyclophilin B and RPL13A). Transcript levels of BMP6, an iron-regulated gene, were measured to demonstrate iron-related changes in gene expression. Values are means ± SE, n = 6. Asterisks indicate difference relative to FeA controls (\* $P < 0.05$ , \*\* $P < 0.01$ )

the 3% versus the 2% carbonyl iron FeO rat livers suggest a dose-responsive effect of iron. In contrast to ZIP5, mRNA levels of ZIP6, ZIP7, and ZIP10 were



**Fig. 3** Effect of iron loading on the expression of ZIP5, ZIP6, ZIP7, ZIP10, and ZIP3 in H4IIE cells. Cells were incubated in medium supplemented with or without 50  $\mu$ M Fe-NTA for 8 h. Total RNA was isolated and levels of ZIP5, ZIP6, ZIP7, ZIP10, and ZIP3 mRNA were determined by qRT-PCR and expressed as relative to control. Levels of TFR1 were measured to confirm iron loading of cells. Values are means ± SE of 3 independent experiments

all lower (by 24–45%) in FeO livers relative to FeA controls. FeD resulted in differential expression of only one ZIP transporter, ZIP14, but the effect was modest (50% higher relative to FeA) and not consistently observed between the two studies. It should be noted that ZIP5, ZIP6, ZIP7, ZIP10, and ZIP14 are all members of the ZIP subfamily referred to as LIV-1 (Taylor and Nicholson 2003). LIV-1 family members are highly conserved, particularly in putative transmembrane domains IV and V, which are thought to form a metal-ion translocation pore. All LIV-1 family members also contain a cytosolic histidine-rich region that has been proposed to play a role in metal binding/transport. Recently, the histidine-rich region of the ZIP transporter LIT1 in *Leishmania amazonensis* has been shown to be essential for iron transport (Jacques et al. 2010).



Although the present studies suggest that mRNA levels of ZIP5, ZIP6, ZIP7, and ZIP10 are regulated by iron, it is alternatively possible that they are regulated by secondary alterations in hepatic concentrations of other metals. For example, in both studies, hepatic concentrations of zinc were higher (by 14–21%) in FeO livers compared to FeA controls. The finding of elevated zinc levels in FeO rat liver is in agreement with previous studies of iron-loaded rats and mice (Vayenas et al. 1998; Zhang et al. 2009) and patients with hereditary hemochromatosis (Adams et al. 1991). The higher zinc concentrations in FeO livers may result from either increased dietary zinc absorption by the intestine or increased hepatic uptake/retention of zinc. FeO and/or FeD also resulted in different hepatic concentrations of manganese and cobalt relative to FeA controls (by 20–42%), but the differences were not consistent between the two studies. Although FeD in both studies resulted in elevated hepatic copper concentrations, similar to many previous reports (Collins et al. 2010), the higher hepatic copper concentrations were not associated with consistent differences in the expression of any ZIP transporters. Overall, it therefore seems that hepatic concentrations of iron and/or zinc are affecting the expression of ZIP5, ZIP6, ZIP7, and ZIP10, whereas Cu, Mn, and Co have little effect.

The only ZIP whose expression was higher in FeO liver was ZIP5, a plasma membrane protein that localizes to the basolateral surfaces of various cell types (Wang et al. 2004b; Dufner-Beattie et al. 2004). The basolateral localization of ZIP5 in acinar cells of the pancreas has led to the hypothesis that ZIP5 functions to transport zinc from the blood into acinar cells (Dufner-Beattie et al. 2004). Zinc transport ability of ZIP5 was demonstrated by showing enhanced uptake of zinc in HEK293 cells overexpressing ZIP5 (Wang et al. 2004b). In our studies, the upregulated expression of ZIP5 in FeO livers was associated with higher concentrations of hepatic zinc. Thus, it is possible that upregulated expression of ZIP5 may contribute to hepatic zinc accumulation in FeO livers. Future studies will need to identify which cell types of the liver express ZIP5, as well as the subcellular localization of ZIP5 in hepatic cells.

ZIP6, which was downregulated by 40 and 45% in FeO liver, was first identified in 1988 as an estrogen-

induced gene in a human breast cancer cell line (Manning et al. 1988). ZIP6 localizes to the plasma membrane and has been shown to transport zinc into the cytosol (Taylor et al. 2003). The protein was originally named LIV-1, the founding member of the LIV-1 subfamily of ZIP transporters. A rapidly growing number of studies have documented associations between ZIP6 expression and breast cancer (reviewed by Taylor (2008)). ZIP6 has additionally been linked to prostate cancer (Zhou et al. 2008), pancreatic cancer (Unno et al. 2009), and cervical cancer (Zhao et al. 2007). The apparent involvement of ZIP6 in cancer progression is likely related to the fact that ZIP6 regulates the nuclear translocation of the transcriptional repressor Snail, a master regulator of the epithelial to mesenchymal transition (EMT) (Yamashita et al. 2004). During EMT, cells undergo extensive remodeling of the cytoskeleton and display an increased propensity for migration, which is considered to be a key event in malignant tumor progression and metastasis. Suppression of ZIP6 expression in cancer cell lines has been shown to downregulate Snail and limit cell growth and prevent cell migration (Zhao et al. 2007). Future studies will need to assess downstream effects of lower ZIP6 expression in FeO liver.

FeO liver also consistently expressed lower levels of ZIP7 and ZIP10 mRNA. ZIP10 localizes to the cell surface where it is capable of importing zinc (Kaler and Prasad 2007). In rats, ZIP10 mRNA levels in kidney and intestine were found to be positively associated with circulating levels of thyroid hormone in a concentration-dependent manner, suggesting that ZIP10, like ZIP6, is hormonally regulated (Pawan et al. 2007). As many studies have shown that iron overload lowers thyroid hormone levels (Farmaki et al. 2010; Hudec et al. 2008; Delvecchio and Cavallo 2010), it is possible that lower thyroid hormone concentrations may contribute to the downregulation of ZIP10 mRNA levels we observed in FeO liver. Whereas most ZIP transporters localize to the plasma membrane, ZIP7 is unique in that it resides intracellularly, in the endoplasmic reticulum (Taylor et al. 2004) or Golgi apparatus (Huang et al. 2005), where it transports zinc into the cytosol. Similar to ZIP6, both ZIP7 and ZIP10 have been linked to metastatic breast cancer (Taylor et al. 2008, 2007; Hogstrand et al. 2009).

Recently, Kozul et al. (2008) reported that different commonly used laboratory animal diets independently influence gene expression profiles and can thereby confound the effects of experimental treatments. Here we used two different laboratory diets (i.e., TestDiet<sup>®</sup> 5755 versus AIN-93G diet) in two independent studies and found consistent effects of iron status on the expression of ZIP5, ZIP6, ZIP7, and ZIP10. The reproducibility of our findings with different laboratory diets strengthens the conclusion that the expression of these ZIP transporters is altered by iron status. Moreover, the downregulation of ZIP6, ZIP7, and ZIP10 that we observed in FeO liver could be recapitulated by iron loading of H4IIE rat hepatoma cells, suggesting that these ZIP transporters are directly responsive to iron. By contrast, the marked upregulation of ZIP5 in FeO liver was not observed in iron-loaded H4IIE cells. Indeed, iron loading of H4IIE cells resulted in 50% lower mRNA levels of ZIP5. The opposite responses in liver and H4IIE cells may be due to the duration of iron loading (3 weeks in liver vs. 8 h in cells), the magnitude of iron loading, or systemic effects in the whole animal. Alternatively, the upregulation of ZIP5 in FeO liver may reflect changes in hepatic cell types other than hepatocytes (e.g., Kupffer cells, stellate cells, endothelial cells).

Despite the fact that the liver is one of the most active sites of metal metabolism, few studies have investigated the regulation of ZIP transporters in this organ. Liuzzi et al. (2005) studied the expression of hepatic ZIP transporters in response to the inflammatory stimuli turpentine and LPS, which are well known to rapidly induce hypoferrinemia and hypozincemia. They found that mRNA levels of ZIP1, ZIP6, ZIP7, and ZIP14 were higher (60–300%) in response to inflammation, whereas levels of ZIP2 and ZIP8 were 50–60% lower. It has been suggested that hepatic iron overload is an inflammatory condition (Ramm and Ruddell 2010). If so, then one would expect some similarities in the differential expression of ZIP transporters in response to inflammation and hepatic iron overload. This is, however, not what we observed. Liuzzi et al. found that inflammation increased the expression of ZIP6, ZIP7, and ZIP14, whereas we found that iron overload decreased the expression of ZIP6 and ZIP7 and had no effect on ZIP14 expression. We also found no effect of iron overload on the expression of ZIP1, ZIP2, or ZIP8.

These differences clearly indicate that inflammation and iron overload have different effects on the expression of hepatic ZIP transporters.

The altered expression of ZIP5, ZIP6, ZIP7, and ZIP10 by dietary FeO raises the question as to whether these transporters are capable of transporting iron in addition to zinc. Metal competition studies are frequently cited as evidence for specificity of metal transport. For example, in ZIP5-overexpressing HEK293 cells, iron was shown to not compete for zinc uptake (Wang et al. 2004b). From these studies, it was concluded that ZIP5 is a zinc-specific transporter. It is important to note, however, that metal competition studies can only demonstrate inhibition of transport (or lack thereof); they do not demonstrate specificity of transport or transport capacity. To assess metal transport capacity of any ZIP protein, the cellular uptake of radiolabeled metals needs to be measured directly, such as in a heterologous expression system (Pinilla-Tenas et al. 2011). Moreover, some ZIPs are known to be regulated post-transcriptionally (Wang et al. 2004a; Kim et al. 2004; Dufner-Beattie et al. 2004) and therefore screening at the level of protein or subcellular localization may reveal other potentially iron-regulated ZIP proteins.

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