Biometals
DOI 10.1007/s10534-012-9526-x

Physiologic implications of metal-ion transport by ZIP14 and ZIP8

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Received: 31 December 2011 / Accepted: 19 January 2012

Abstract Zinc, iron, and manganese are essential trace elements that serve as catalytic or structural components of larger molecules that are indispensable for life. The three metal ions possess similar chemical properties and have been shown to compete for uptake in a variety of tissues, suggesting that they share common transport proteins. Two likely candidates are the recently identified transmembrane proteins ZIP14 and ZIP8, which have been shown to mediate the cellular uptake of a number of divalent metal ions including zinc, iron, manganese, and cadmium. Although knockout and transgenic mouse models are beginning to define the physiologic roles of ZIP14 and ZIP8 in the handling of zinc and cadmium, their roles in the metabolism of iron and manganese remain to be defined. Here we review similarities and differences in ZIP14 and ZIP8 in terms of structure, metal transport, tissue distribution, subcellular localization, and regulation. We also discuss potential roles of these proteins in the metabolism of zinc, iron, manganese, and cadmium as well as recent associations with human diseases.

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Published online: 09 February 2012

Keywords SLC39 · Iron · Zinc · Manganese · Cadmium · Hemochromatosis

Introduction

ZIP14 (SLC39A14) and ZIP8 (SLC39A8) are transmembrane proteins that belong to the ZIP (SLC39) family of metal-ion import proteins. Initially described as zinc transporters, ZIP14 and ZIP8 have recently been shown to mediate the uptake of a variety of nutritionally important divalent metals (e.g., zinc, iron, and manganese) as well as the toxic heavy metal cadmium. Although closely related, ZIP14 and ZIP8 differ with respect to tissue distribution, cellular expression, and subcellular localization. These differences, along with peculiarities in regulation, have important implications for the roles of these proteins in cellular and whole-body metal homeostasis. The aim of the present review is to summarize the similarities and differences between ZIP14 and ZIP8 in an effort to evaluate their potential physiologic roles in metal metabolism.

Structural aspects of ZIP transporters and unique properties of ZIP14 and ZIP8

ZIP proteins are generally predicted to have eight transmembrane (TM) domains with a long extracellular N-terminal region and a short extracellular C-terminus

(Eide 2004). Most ZIPs also have histidine-rich repeats $(HX)_n$ in the long intracellular loop between predicted TM domains III and IV. The 14 mammalian ZIP family members have been classified into four subfamilies (Fig. 1a). The largest group, consisting of nine proteins including ZIP8 and ZIP14, is known as the LIV-1 subfamily (Taylor and Nicholson 2003). The name "LIV-1" refers to ZIP6, which was first identified as LIV-1, an estrogen-regulated gene (Manning et al. 1994). LIV-1 family members are distinguished by a signature sequence (HEXPHEXGD) in TM domain V that is not found in other ZIP transporters. The first part of this sequence fits the consensus sequence HEXXH of a zinc-binding motif found in zinc metalloproteases (Hooper 1994). Interestingly, in ZIP14 and ZIP8 (Fig. 1b), the first histidine in this motif is replaced by a glutamic acid (E), which has been suggested to confer an ability to bind/transport metal ions other than zinc (Taylor et al. 2007). Among the ZIP family members, ZIP14 and ZIP8 are most closely related: they are similar in length (489 vs. 462 a.a.'s); they have identical amino acids in about 50% of positions; and they each

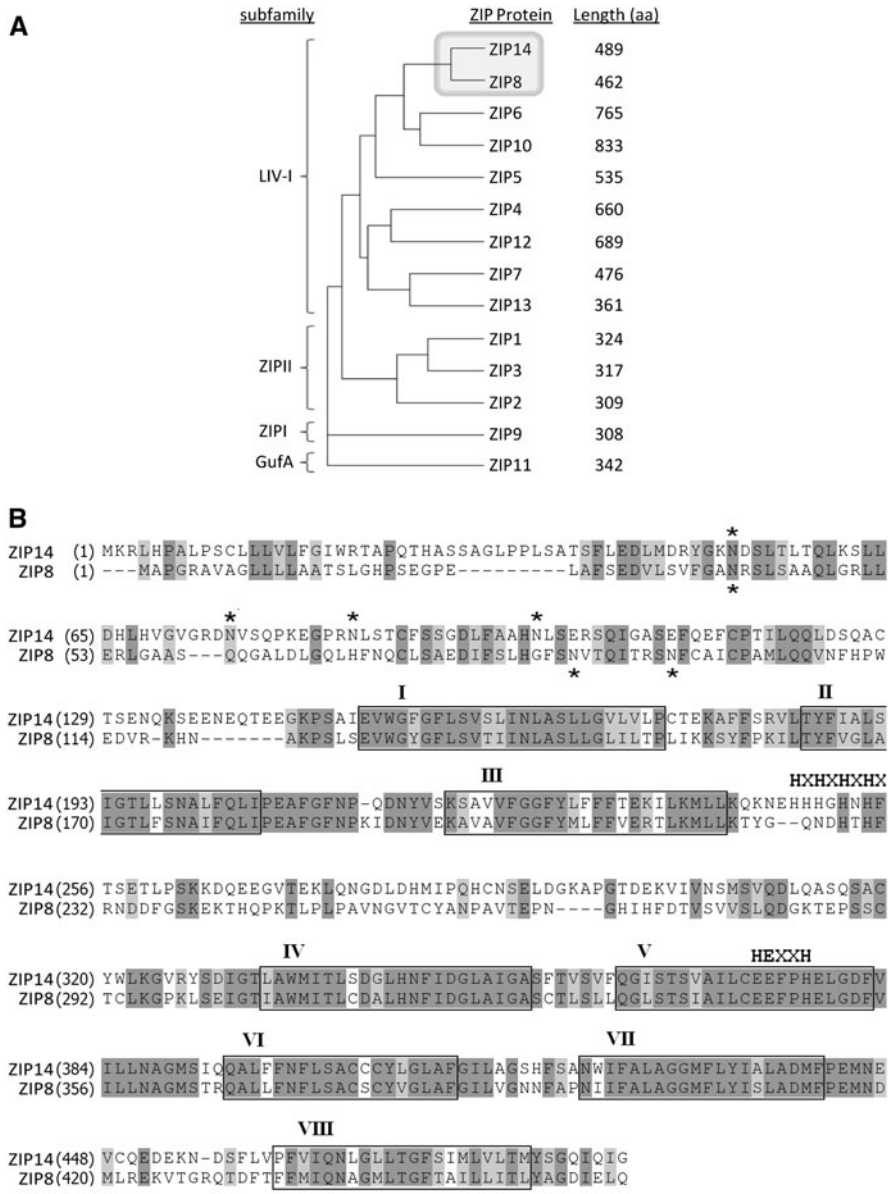

Fig. 1 Similarity of ZIP14 and ZIP8 proteins. **a** Simplified dendrogram showing relationships of the ZIP family proteins. Dendrogram was generated from murine ZIP protein sequences by using ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2). ZIP subfamily groupings and amino acid lengths are also indicated. **b** Amino acid sequences for murine ZIP14 (NP_001128623.1) and ZIP8 (NP_001128621.1) were obtained from GenBank and aligned by using Vector NTI. *Dark gray shading* indicates identical amino acids; *light gray shading* indicates conservative substitutions. Putative transmembrane (TM) domains, indicated by *Roman numerals*, were predicted by using MEMSAT-SVM and predictions by Taylor et al. (2005). Also indicated are the histidine-rich repeat region (HXHXHXHX) between TM domains III and IV, the metalloprotease motif in TM domain V (HEXXH), and the experimentally verified *N*-linked glycosylation sites (*) for ZIP14 and ZIP8 (unpublished data, N. Zhao, S. Jenkitkasemwong, and M.D. Knutson)

have at least three *N*-linked glycosylation sites in the N-terminal region (Fig. 1b). The degree of similarity between the two proteins is notably evident in putative TM domains. The very highly conserved TM domains IV and V, with their metal-binding histidine and glutamic residues, have been proposed to comprise part of an ion channel (Eng et al. 1998). Although the function of the histidine-rich region in the cytoplasmic loop between TM domains III and IV in ZIP14 $(HX)_4$ and ZIP8 $(HX)_2$ is unknown, it may play a role in ubiquitination and degradation, such as in ZIP4 (Mao et al. 2007), or in metal-binding/transport (Kerkeb et al. 2008). Recently, the cytosolic HXHXHX motif in LIT1, a ZIP transporter in *Leishmania amazonensis*, was shown to be essential for iron transport (Jacques et al. 2010).

Metal transport by ZIP14 and ZIP8

ZIP8 was first identified by screening genes induced during immune activation of monocytes (Begum et al. 2002). A role for this protein in zinc binding/transport was investigated because its amino acid sequence contained a metalloprotease-like motif and shared significant homology with ZIP1 and ZIP2. CHO cells transfected with ZIP8 accumulated more zinc than mock-transfected controls, suggesting a role for ZIP8 in zinc uptake/retention. Subsequent studies showed that ZIP8 overexpression also stimulated the accumulation of cadmium and manganese (Dalton et al. 2005; He et al. 2006). Metal transport by ZIP14 was first examined in 2005 (Taylor et al. 2005). Similarly to ZIP8, transfection of CHO cells with ZIP14 cDNA resulted in cellular zinc accumulation, consistent with a role in metal uptake. Later it was shown that transfection of HEK 293T cells with ZIP14 cDNA enhanced the cellular accumulation of not only zinc, but also iron, suggesting that ZIP14 could function as an iron import protein as well (Liuzzi et al. 2006). This finding was of particular significance because at that time only one other mammalian transmembrane iron uptake protein was known, namely divalent metal-ion transporter 1, DMT1 (Gunshin et al. 1997). A role for ZIP14 in the cellular uptake of cadmium and manganese was additionally suggested by studies of MFF cells expressing ZIP14 (Girijashanker et al. 2008).

More recently, metal uptake by ZIP8 and ZIP14 has been investigated by using RNA-injected *Xenopus* oocytes, a powerful expression system in which to characterize membrane transport proteins. Among several advantages of using *Xenopus* oocytes, heterologous expression of exogenous proteins is very robust whereas endogenous transport activities are, in general, extremely low (prophase-arrested oocytes survive virtually independently of exogenous nutrients). When expressed in *Xenopus* oocytes, Zip8 was shown to transport zinc and cadmium, but other metals were not tested (Liu et al. 2008). We used the *Xenopus* oocyte system to assess the metal-ion substrate profile of ZIP14 and found that it was able to transport any of several divalent metals (Zn^{2+}, Fe^{2+}, Mn^{2+}, and Cd^{2+}), but not Cu^{2+} or Cu^{1+} (Pinilla-Tenas et al. 2011). We have additionally found that ZIP8 can transport the same metals as ZIP14 (Wang et al. 2012) although the affinities may differ. Zip8 and Zip14 share in common some mechanistic features. Zip8 and Zip14 transport metal ions at optimal pH $\approx$ 7.5 and both transporters are electroneutral, indicating that the transport activity requires the symport or antiport of additional ionic species (Girijashanker et al. 2008; He et al. 2006; Liu et al. 2008; Pinilla-Tenas et al. 2011; Wang et al. 2012). The activity of both transporters is stimulated by extracellular bicarbonate, but the conclusion that they operate by a metal-bicarbonate symport mechanism (Girijashanker et al. 2008; Liu et al. 2008) is not supported by any experimental evidence. Collectively, these observations indicate that ZIP14 and ZIP8 are both broad-scope metal-ion transporters that can mediate the cellular uptake of nutritionally important metals as well as the toxic heavy metal cadmium. That Zip8 and Zip14 share basic mechanistic features suggests that their differential regulation will be key to understanding their cellular roles.

Tissue, cellular, and subcellular distribution of ZIP14 and ZIP8

Table 1 summarizes the studies that have assessed the tissue distributions of ZIP14 and ZIP8. Among human tissues, ZIP14 is most abundantly expressed in liver followed by pancreas and heart. Among mouse tissues also, ZIP14 expression is most abundant in liver; however, one study reported highest expression in duodenum (Liuzzi et al. 2006). Immunohistochemical analysis of human liver revealed that ZIP14 localized

Table 1 Summary of studies of the tissue distribution of ZIP14 and ZIP8

Protein	Tissue distribution	Number of tissues examined	Technique	References
hZIP14	Widespread: Liver > Heart = Pancreas > Placenta = Lung = Brain = Kidney = Skeletal muscle = Small intestine > Spleen = Peripheral blood lymphocytes	16	Northern blotting	(Nomura et al. 1994)
hZIP14	Widespread: Liver > Pancreas > Heart > Thyroid gland > Small intestine > Other tissues	>30	Multi-tissue expression array	(Taylor et al. 2005)
mZIP14	Liver ≫ White adipose tissue/Heart/Brain/Kidney/Skeletal muscle. Almost undetectable in lung, testis, and brown adipose tissue	9	qRT-PCR	(Tominaga et al. 2005)
mZIP14	Small intestine > Liver > Heart > Kidney/Spleen/Pancreas	7	qRT-PCR	(Liuzzi et al. 2006)
mZIP14	Liver > Duodenum > Kidney > Lung/Testis/Brain	6	qRT-PCR (copy number)	(Girijashanker et al. 2008)
mZIP14	Liver > Duodenum > Jejunum = Kidney > Testis > Other tissues	14	RT-PCR	(Fujishiro et al. 2009)
hZIP8	Pancreas > Placenta > Lung > Liver > Thymus > Other tissues. Very low in prostate, brain and kidney	16	Northern blotting	(Begum et al. 2002)
mZIP8	Lung > Kidney > Liver = Testis	N/A	Northern blotting	(Dalton et al. 2005)
mZIP8	Lung > Testis = Kidney ≫ Liver > Brain = Small intestine	6	Northern blotting	(Wang et al. 2007)
mZIP8	Lung = Testis > Kidney > Liver > Brain/Duodenum	6	qRT-PCR (copy number)	(Girijashanker et al. 2008)
mZIP8	Widespread: Testis > Liver = Kidney > Lung > Stomach > Spleen > Other tissues	14	RT-PCR	(Fujishiro et al. 2009)

hZIP14 human ZIP14, *mZIP14* mouse ZIP14, *hZIP8* human ZIP8, *mZIP8* mouse ZIP8

to the plasma membrane of hepatocytes (Franklin et al. 2011), similar to its expression in primary mouse hepatocytes (Liuzzi et al. 2006).

ZIP8 shows a much different expression pattern than ZIP14. The majority of studies have reported that ZIP8 is most abundantly expressed in lung, testis, and kidney (Table 1). One study, however, noted highest ZIP8 expression in pancreas and placenta followed by lung and liver (Begum et al. 2002). In a side-by-side comparison of mRNA copy numbers in six tissues (duodenum, lung, liver, kidney, testis, and brain), ZIP14 transcript levels were roughly 10-fold higher than ZIP8 in the liver and duodenum, whereas ZIP8 was 10-fold more abundant than ZIP14 in the lung and testis (Girijashanker et al. 2008).

A number of studies have investigated the subcellular localization of ZIP14 and ZIP8 (Table 2) in isolated cells or cell lines. Most of these studies, which have relied on overexpression of epitope- or fluorescently tagged proteins, have detected ZIP14 and ZIP8 at the plasma membrane, consistent with their proposed role in transporting metals from the extracellular space into the cytosol. However caution is warranted when interpreting protein localization from robust overexpression systems because detection at the plasma membrane may indicate a failure to recycle to the cytosol or saturation of a trafficking pathway. In an effort to circumvent the potential problems associated with overexpression systems, we generated a human hepatoma cell line (HepG2) that expresses FLAG-tagged ZIP14 from its endogenous locus, and localized endogenously produced ZIP14 by using extensively validated FLAG monoclonal antibodies (Zhao et al. 2010). We found that ZIP14 was detectable at the cell surface, but it also partially colocalized with EEA1 and LAMP1, suggesting that it

Table 2 Summary of studies of the subcellular localization of ZIP14 and ZIP8

Protein	Localization	Tissue/cell line	Mode of expression	Technique	References
mZIP14	PM/cytoplasm	Liver, HEK 293T	Endogenous/Transient transfection	IF	(Liuzzi et al. 2005)
hZIP14-V5	PM	CHO	Transient transfection	IF	(Taylor et al. 2005)
mZIP14-EGFP	PM	K562	Transient transfection	IF	(Tominaga et al. 2005)
mZIP14	PM	Sf9	Transient transfection	IF	(Liuzzi et al. 2006)
hZIP14-V5	PM	MCF-7	Transient transfection	IF	(Taylor et al. 2007)
mZIP14-HA	PM	MDCK	Transient transfection	IF	(Girijashanker et al. 2008)
mZIP14	PM	Primary hepatocytes	Endogenous	IF	(Lichten et al. 2009)
hZIP14-Flag	PM/Endosome/Lysosome	HepG2	Endogenous	IF	(Zhao et al. 2010)
hZIP14	PM/Cytoplasm	Liver, HepG2	Endogenous	IHC	(Franklin et al. 2011)
mZIP14-V5	PM	Primary chondrocytes	Lentiviral transfection	IF	(Hojyo et al. 2011)
mZIP14-EGFP	PM	*Xenopus* oocytes	Transient transfection	IF	(Pinilla-Tenas et al. 2011)
hZIP8-EGFP	Lysosome	HEK 293 Cos-7 RK-13 MCF-7	Stable expression	IF/cell fractionation	(Begum et al. 2002)
mZIP8-HA	PM	MFF	Retroviral transfection	IF	(Dalton et al. 2005)
mZIP8-HA	PM	MDCK	Transient transfection	IF	(He et al. 2006)
mZIP8	PM/cytoplasm	Kidney tissue	Transgenic	IHC/IF	(Wang et al. 2007)
hZIP8-DsRed	PM/mitochondria	BEAS-2B	Transient transfection	IF/cell fractionation	(Besecker et al. 2008)
mZIP8-HA	PM	MDCK	Retroviral transfection	Cell-surface labeling	(Liu et al. 2008)
mZIP8	PM	RBCs	Endogenous	IF	(Ryu et al. 2008).
hZIP8	PM/lysosome	T-cells	Endogenous	IF	(Aydemir et al. 2009)

hZIP14 human ZIP14, *mZIP14* mouse ZIP14, *hZIP8* human ZIP8, *mZIP8* mouse ZIP8, *PM* plasma membrane, *IF* immunofluorescence, *IHC* immunohistochemistry

is present in early endosomes and lysosomes, respectively. The detection of ZIP14 in cytosolic structures agrees with other studies that have examined endogenous ZIP14 (Liuzzi et al. 2005; Franklin et al. 2011). Similar to ZIP14, endogenous ZIP8 has been detected in the cytosol (Besecker et al. 2008) and lysosomes (Aydemir et al. 2009), although other subcellular compartments were not examined. Lysosomal localization of ZIP8 was also reported for four different cell lines stably expressing ZIP8 (Begum et al. 2002). One study additionally found that ZIP8 was present in mitochondria, as assessed by immunofluorescence and cell fractionation (Besecker et al. 2008).

Regulation of ZIP14 and ZIP8

As Zn/Fe/Mn transport proteins, one would predict that ZIP14 and ZIP8 would be regulated by tissue concentrations and/or by dietary deficiency or excess of these metals. A few studies have provided limited evidence for this, but the data are inconsistent. For example, ZIP14 mRNA levels have been reported to be upregulated in the duodenum of iron-deficient Belgrade rats (Yeh et al. 2011), iron-deficient pigs (Hansen et al. 2009), and *Hfe* knockout mice (Coppin et al. 2007). A microarray analysis of iron-deficient rat duodenum, however, did not identify ZIP14 as being differentially expressed (Collins et al. 2005). Interestingly in that study, ZIP8 was found to be downregulated by iron deficiency. In rat liver, ZIP14 and ZIP8 mRNA levels were unaffected by iron deficiency or overload (Nam and Knutson 2011). A similar lack of hepatic ZIP14 regulation by iron was reported in pigs (Hansen et al. 2009) and calves (Hansen et al. 2010). Zinc status also does not appear to alter the expression of ZIP14 and ZIP8, at least in the mouse intestine (Liuzzi et al. 2004).

Inflammation is well known to affect the metabolism of zinc and iron. In a screen of hepatic ZIP transcripts induced by inflammatory stimuli in mouse liver, ZIP14 mRNA levels were most affected, being

2–3-fold higher after administration of turpentine or lipopolysaccharide (LPS) (Liuzzi et al. 2005). By contrast, ZIP8 transcript levels were unaffected by turpentine and 50% lower after LPS. The induction of ZIP14 expression by turpentine or LPS was prevented in mice lacking IL-6, suggesting that IL-6 is involved in regulating ZIP14 abundance. Additional studies in primary mouse hepatocytes demonstrated direct stimulatory effects of IL-6 (Liuzzi et al. 2005), as well as IL-1β and nitric oxide on ZIP14 expression (Lichten et al. 2009). The induction of ZIP14 by turpentine was not observed in the small intestine or spleen, indicating tissue-specific regulation. ZIP14 expression has also been demonstrated to be upregulated by inflammation in mouse lung after an ovalbumin challenge, a model of asthma (Lang et al. 2007). As in liver, inflammation of the lung resulted in a downregulation of ZIP8. In cultured sheep pulmonary artery endothelial cells, ZIP14 but not ZIP8 mRNA levels increased after treatment with LPS (Thambiayya et al. 2011).

Although LPS has been reported to downregulate or not affect ZIP8 mRNA levels in liver (Liuzzi et al. 2005) and pulmonary artery endothelial cells (Thambiayya et al. 2011), it does markedly upregulate ZIP8 expression in human monocytes (Begum et al. 2002) and peripheral blood mononuclear cells (Aydemir et al. 2009). The apparent discrepancies between these studies may relate to differences in sampling time points as ZIP8 induction by LPS is time dependent (Begum et al. 2002). Such a possibility seems likely given that ZIP8 expression is induced by the inflammatory cytokine TNFα (Begum et al. 2002; Besecker et al. 2008), which is induced by LPS (Dumitru et al. 2000). In contrast to its effect on ZIP8, TNFα does not increase the expression of ZIP14 (Besecker et al. 2008; Liuzzi et al. 2005). An induction of ZIP8 expression has also been observed in monocytes infected with live or heat-killed *Mycobacterium* or a bacterial cell wall derivative (Begum et al. 2002). Related to the immune response, the activation of human T cells is accompanied by an upregulation of ZIP8 and ZIP14 (Aydemir et al. 2009).

Potential roles of ZIP14 and ZIP8 in zinc metabolism

Whole-body zinc homeostasis is maintained by balancing zinc absorption and excretion in the gastrointestinal tract. At the cellular level, zinc homeostasis is thought to be regulated by the 14 members of the ZIP family of import/influx proteins and the 10 members of the ZNT family of export/efflux proteins (Fukada and Kambe 2011). Among the ZIP proteins, ZIP4 has the most clearly defined role; it is located at the apical surface of enterocytes where it mediates the intestinal uptake of dietary zinc (Andrews 2008). Mutations in *ZIP4* result in zinc deficiency and the clinical condition known as acrodermatitis enteropathica. Interestingly, zinc deficiency in patients with this disorder can be overcome with oral zinc supplements, suggesting that other, though much less efficient, mechanisms of intestinal zinc uptake exist (Wang et al. 2002). It is possible that ZIP14 and ZIP8 could play such a backup role as they are expressed in the duodenum and both have been shown to localize to the apical membrane of polarized cells (Girijashanker et al. 2008; He et al. 2006). Future studies will need to determine the localization and function of ZIP14 and ZIP8 in the duodenum.

As noted above, the inflammatory mediators turpentine and LPS markedly increase the expression of ZIP14 in the liver. The upregulation of hepatic ZIP14 was associated with lower serum zinc levels, suggesting that ZIP14 plays a role in the hypozincemia of inflammation (Liuzzi et al. 2005). A role for ZIP14 in this process was further supported by the observation that turpentine injection did not cause hypozincemia in mice lacking IL-6, an inducer of ZIP14.

Our understanding of the physiologic roles of ZIP14 has been markedly enhanced by the recent generation of *Zip14* knockout mice (Hojyo et al. 2011). Mice lacking ZIP14 exhibit growth retardation, shortened long bones, and other skeletal abnormalities. A role for ZIP14 in bone was suggested by its abundant expression in chondrocytes, cartilage cells in the growth plate that proliferate during the process of bone elongation. Chondrocytes from ZIP14 knockout mice were found to have significantly reduced levels of zinc and lower levels of cAMP resulting from enhanced phosphodiesterase activity. Expression of ZIP14 in chondrocytes from *Zip14* knockout mice rescued the cellular zinc levels and increased cAMP levels, thus identifying an indispensable function of ZIP14. The lower cAMP levels in *Zip14*-knockout chondrocytes resulted in impaired parathyroid hormone 1 receptor (PTH1R) signaling, which regulates chondrocyte differentiation. Similarly, low zinc and cAMP levels were found in the pituitary and liver of

Zip14 knockout mice, resulting in reduced signaling via growth hormone releasing hormone receptor (GHRHR) and glucagon receptor (GCGR) respectively. As PTH1R, GHRHR, and GCGR are all G-protein coupled receptors (GPCRs), it was concluded that ZIP14 plays a unique role in GPCR-mediated signaling by importing zinc into cells and maintaining basal cAMP levels. With respect to zinc metabolism, hepatic zinc concentrations were decreased by only about 25% in *Zip14* knockout animals (Hojyo et al. 2011), indicating that the liver has other mechanisms of zinc uptake. Based on relative abundances of ZIP mRNAs expressed in the liver (unpublished data, H. Nam and M.D. Knutson), we propose that the most likely candidates are ZIP1, ZIP8, and ZIP4.

The pancreas contains a high amount of zinc, particularly in β-cells where it is a cofactor for insulin biosynthesis (Dodson and Steiner 1998). In pancreatic α-cells, zinc serves as a signaling ion for glucagon secretion (Robertson et al. 2011), and in acinar cells, zinc is secreted in zymogen granules as a component of digestive enzymes. Despite the dynamic zinc metabolism in the pancreas, the transporters responsible for zinc uptake by various pancreatic cell types remain poorly defined. Both ZIP14 and ZIP8 are likely to play a role as they are abundantly expressed in pancreas (Begum et al. 2002; Taylor et al. 2005). In a screen of ZIP transporter transcripts expressed in α-cells, ZIP14 was the second most abundant transcript, whereas ZIP8 was nearly undetectable (Gyulkhandanyan et al. 2008). Immunohistochemical analysis of human pancreas has also detected ZIP14 in α-cells as well as in β-cells (Mohanasundaram et al. 2011). In β-cells, ZIP8 mRNA levels were found to increase in response to glucose, an effect that was dramatically magnified by adding diazoxide, a pharmacologic agent that blocks insulin secretion (Bellomo et al. 2011). These observations suggest that ZIP8 plays an important role in zinc homeostasis of the β-cell.

Potential roles of ZIP14 and ZIP8 in iron metabolism

In contrast to zinc, whole-body iron balance is maintained chiefly by regulating the absorption of iron (Knutson 2010). Intestinal iron absorption is mediated by DMT1, the apical iron transport protein, and ferroportin, the basolateral iron exporter. DMT1 is required for intestinal iron absorption because intestine-specific *Dmt1* knockout mice fail to absorb sufficient dietary iron (Shawki et al. 2012) and become progressively iron deficient and anemic (Gunshin et al. 2005). Anemia and impaired iron absorption are also characteristic of the Belgrade rat, which harbors a point mutation in *Dmt1* that markedly diminishes the iron transport function of the protein (Fleming et al. 1998). Although DMT1 is required for intestinal iron uptake by the adult rat, radiotracer studies in the Belgrade rat have shown that it is not essential for iron absorption from milk during early development (Thompson et al. 2007). The high expression of ZIP14 in duodenum raises the possibility that ZIP14 could contribute to iron assimilation during the neonatal period. An alternate mechanism for iron absorption is also suggested by the observation that the anemia of intestine-specific *Dmt1* knockout mice is partially ameliorated by crossing the mice with *Hfe* knockout mice, which hyperabsorb iron (Gunshin et al. 2005). It should be pointed out, however, that ZIP14 transports iron poorly at pH < 6.5 (Pinilla-Tenas et al. 2011; Zhao et al. 2010) and therefore would likely not function well in the acidic microclimate of the brush border in the adult, but it could function at the brush border in suckling animals prior to maturation of the acidic microclimate (Collins et al. 1997).

In the studies that first demonstrated that ZIP14 could transport iron (Liuzzi et al. 2006), the iron was provided to cells as ferric citrate, a physiological form of non-transferrin-bound iron (NTBI) present in the plasma of patients with iron overload such as hemochromatosis or β-thalassemia. Given that ZIP14 is most abundantly expressed in the liver, pancreas, and heart—the three tissues that preferentially accumulate iron in iron overload disorders—it was hypothesized that ZIP14 mediated the uptake of NTBI into these tissues. Consistent with this proposed role are studies in AML12 mouse hepatocytes and human hepatoma HepG2 cells which showed that siRNA-mediated suppression of ZIP14 expression reduced the uptake of NTBI (Liuzzi et al. 2006; Gao et al. 2008). Moreover, the iron transport characteristics of ZIP14, as determined by our functional studies in *Xenopus* oocytes, are in close agreement with NTBI uptake studies reported in isolated hepatocytes and perfused liver (Pinilla-Tenas et al. 2011).

Another link with iron overload was suggested by the observation that overexpression of the hemochromatosis protein, HFE, downregulated ZIP14 in HepG2 cells and reduced NTBI uptake (Gao et al. 2008). Interestingly, overexpression of HFE was also associated with reduced uptake of iron from transferrin, suggesting that transferrin-bound iron (TBI) and NTBI uptake share a common pathway in HepG2 cells. The possibility that ZIP14 plays a role in TBI uptake was revealed by studies showing that transfection of cells with ZIP14 increased the uptake of TBI and suppression of ZIP14 reduced its uptake (Zhao et al. 2010). Moreover, ZIP14 was found to colocalize with transferrin in early endosomes and was able to transport iron at pH 6.5, the pH at which iron dissociates from the transferrin–transferrin receptor complex in endosomes. Together, these data provide strong evidence that ZIP14 plays a role not only in the uptake of NTBI but also TBI.

Most TBI is taken up by developing erythroid cells of the bone marrow. It is unlikely that ZIP14 plays a role in TBI uptake by erythroid cells because it is only weakly expressed in bone marrow (Taylor et al. 2005) and because it cannot compensate for loss of DMT1 (Gunshin et al. 2005). On the other hand, ZIP14 may play a role in TBI uptake by the liver, the second largest consumer of TBI, as suggested by three lines of evidence. First, as noted above, ZIP14 is required for TBI uptake by HepG2 hepatoma cells. Second, ZIP14 is abundantly expressed in liver whereas DMT1 is not (Gunshin et al. 1997). Third, DMT1 knockout mice are born with elevated hepatic non-heme iron levels (three times control)—indicating that an alternate iron-uptake pathway must exist in the liver (Gunshin et al. 2005). It was recently reported that hepatic iron concentrations did not differ between *Zip14* knockout mice and controls (Hojyo et al. 2011); however, total iron (heme plus non-heme iron) was measured, and mice consumed non-purified rodent chow diet, which contains roughly 10 times the amount of iron as in standard AIN-93 purified diets for laboratory rodents (Reeves et al. 1993). Future studies will need to measure hepatic concentrations of non-heme iron, an indicator of iron stores, in mice fed normal amounts of iron.

What role(s), if any, could ZIP8 play in iron metabolism? Although ZIP8, like ZIP14, can transport iron, its distinct tissue distribution suggests that it does not function redundantly. Most notably, ZIP8 is abundantly expressed in the placenta (Begum et al. 2002), where it may function in the materno-fetal transfer of metals, such as iron, across the placenta. The two other known mammalian transmembrane iron transport proteins, DMT1 and ZIP14, are dispensable in placenta because *Dmt1* and *Zip14* knockout mice are born with adequate amounts of iron (Gunshin et al. 2005; Hojyo et al. 2011). Therefore, an alternative iron transport pathway in the placenta must exist. In an effort to generate a *Zip8* knockout mouse, it was found that the incorporation of a neomycin resistance cassette into the *Zip8* gene markedly downregulated the expression of ZIP8 in the placenta, yolk sac, and fetus of homozygote mice, resulting in animals not surviving past the neonatal period (Wang et al. 2011). Thus, ZIP8 appears to play an essential role during early development. Accordingly, it is predicted that ablation of the ZIP8 gene would be lethal to the developing embryo (Wang et al. 2011). The high expression of ZIP8 in the lung, kidney, testis, and pancreas (Table 1) implies that ZIP8 may contribute to iron uptake in these tissues, but at present, little is known about iron metabolism in these organs.

Potential roles of ZIP14 and ZIP8 in manganese metabolism

Compared with zinc and iron, the molecular mediators of manganese metabolism are not well understood, mainly because unique mammalian manganese transport proteins have not been identified. It has long been known, however, that manganese can compete for iron absorption (Rossander-Hulten et al. 1991), implying that the two metals share a common intestinal transporter (i.e., DMT1). Indeed, DMT1-mutant Belgrade rats were found to have impaired manganese absorption, indicating that DMT1 mediates the bulk of intestinal uptake of manganese in addition to iron (Chua and Morgan 1997). These studies further suggest that ZIP14 and ZIP8, which are expressed in the duodenum, cannot compensate for the loss of functional DMT1.

Manganese is found in blood plasma bound to alpha 2-macroglobulin, albumin, and transferrin or possibly as free Mn^{2+} (Roth 2006). Studies of the uptake of injected radiolabeled manganese by tissues of the Belgrade rat have been particularly useful in identifying DMT1-dependent and DMT1-independent

uptake pathways for manganese (Chua and Morgan 1997). When given as Mn-transferrin, the uptake of manganese was impaired in kidney, brain, and bone of Belgrade rats. However, when given as $MnCl_2$ mixed with serum, the uptake of manganese into Belgrade rat kidney and brain was not impaired. Collectively, these observations suggest that the normal uptake of manganese from transferrin requires DMT1, whereas the uptake from other serum complexes does not. Most interestingly, the uptake of both forms of injected manganese was enhanced in the liver of Belgrade rats, suggesting that the mechanism of manganese uptake is different in the liver than in other organs. Alternatively, it is possible that a manganese transporter, such as ZIP14 or ZIP8, is upregulated in the liver of Belgrade rats. ZIP14 appears to be the most likely candidate because it is more abundant in the liver and because it promotes the assimilation of iron—and possibly manganese—from transferrin (Zhao et al. 2010).

Whole-body manganese homeostasis, like zinc, is regulated primarily by the hepatic excretion of manganese into the bile (Roth 2006). However, this homeostatic mechanism can be overwhelmed if manganese chronically enters the body via the lungs, such as in workers occupationally exposed to manganese (e.g., miners and welders). Transpulmonary transport of manganese is unimpaired in Belgrade rats (Heilig et al. 2006), indicating that the lung possesses DMT1-independent mechanisms of manganese transport. It is possible that ZIP8 contributes to manganese transport in the lung because it has been reported to have a moderately high affinity for Mn^{2+} uptake (He et al. 2006) and is highly expressed in this tissue (Table 1). Moreover, manganese is a potent inducer of inflammation in the lung (Rice et al. 2001), which would likely increase ZIP8 (and ZIP14) expression through inflammatory mediators.

Manganese that enters the body via the lungs can ultimately accumulate in the brain, leading to manganism, a disorder that resembles Parkinson's disease. Many studies have aimed to determine the molecular mechanisms of manganese uptake into the brain (reviewed by Yokel 2009). The uptake of intravenously injected manganese from Mn-transferrin was found to be $\sim$30% lower in Belgrade rats, suggesting that DMT1 plays a role (Chua and Morgan 1997). However, manganese uptake from serum was unaffected in Belgrade rats and total brain manganese levels did not differ from controls. Furthermore, in situ brain perfusion studies found no impairment of manganese uptake into nine different brain regions of the Belgrade rat (Crossgrove and Yokel 2004). It therefore appears that DMT1 does not play a major role in manganese uptake into the brain. ZIP8 and ZIP14 are both detected in the brain and have recently been proposed as possible mediators of brain manganese uptake and homeostasis (Rivera-Mancia et al. 2011). Inhaled manganese can also be absorbed into the body via the nasal respiratory epithelium and olfactory receptor neuron dendrites, where ZIP14 and ZIP8 are readily detected (Genter et al. 2009), thus bypassing the blood–brain barrier and entering the brain directly.

Potential roles of ZIP14 and ZIP8 in cadmium metabolism

With respect to the substrates transported by ZIP14 and ZIP8, the non-essential metal cadmium has been the most extensively studied, most likely because of its well-known toxicity. Humans are exposed to cadmium through the environment, diet, occupational exposure (e.g. smelting, battery manufacturing, welding), and cigarette smoke (Jarup 2002). Cadmium can enter the body through the intestine and lung and accumulates in the liver and kidney, causing organ damage. Cadmium is also associated with damage to the lung, pancreas, testis, and bone. A link between ZIP8 and cadmium toxicity was first established in studies of mice resistant to cadmium-induced testicular damage (Dalton et al. 2005). Cadmium resistance in these mice was mapped to a genetic locus containing the *Zip8* gene. The gene product was then cloned and expressed in cells where it was shown to enhance the cellular uptake of cadmium. Proof that ZIP8 contributed to cadmium-induced testicular damage was provided by the generation of ZIP8 transgenic mice in which enhanced testicular expression of ZIP8 resulted in enhanced sensitivity to cadmium (Wang et al. 2007). The abundant expression of ZIP8 in the lung suggests that it may play a role in the pulmonary absorption of cadmium. The bioavailability of cadmium in the lung is estimated to be 10–50%, compared to only a few percent in the intestine (Jarup 2002).

In addition to demonstrating that ZIP14 could transport cadmium, it was found that overexpression

of ZIP14 decreased the survival of cells exposed to the metal (Girijashanker et al. 2008), suggesting that ZIP14 contributed to cadmium toxicity. Consistent with this possibility are studies showing that mRNA levels of ZIP14, like ZIP8, are dramatically downregulated in cadmium-resistant cells (Fujishiro et al. 2009). *Zip14* knockout mice will be useful in establishing the relative contribution of ZIP14 to cadmium toxicity.

Associations of ZIP14 and ZIP8 with diseases

Three recent studies have associated ZIP14 with cancer. One study found that ZIP14, which is normally abundant in liver, was downregulated in hepatocellular cancer (HCC) cells (Franklin et al. 2011). HCC cells also consistently showed low levels of zinc, supporting the hypothesis that ZIP14 downregulation contributes to zinc depletion and cancer progression. The other two studies reported alternative splicing of the *ZIP14* gene (*SLC39A14*) in colorectal cancer (Sveen et al. 2011; Thorsen et al. 2011). In this type of cancer, the transcript variant referred to as *SLC39A14-exon4B* was found to be selectively enriched, raising the possibility that it could be used as a colorectal cancer-specific biomarker.

In line with its apparent role in immunity, ZIP8 has been associated with diseases related to infection and/or inflammation. For example, ZIP8 mRNA levels were found to be highly upregulated in circulating monocytes from HIV-infected individuals (Raymond et al. 2010). The elevated ZIP8 expression in monocytes was associated with higher intracellular zinc concentrations that conferred resistance to apoptosis. Since monocytes are targets of HIV infection and serve as HIV reservoirs, this study suggests that ZIP8 contributes to HIV-infected monocyte survival thereby affecting disease activity and chronicity. ZIP8 mRNA levels have also been found to be elevated in peripheral monocytes of patients with sepsis (Besecker et al. 2011). ZIP14 mRNA levels, on the other hand, were not elevated despite high levels of IL-6, a known inducer of ZIP14 expression (Liuzzi et al. 2005). This is likely because basal levels of ZIP14 in monocytes are relatively low (Besecker et al. 2011).

Genome-wide association studies of large datasets from human populations have revealed unexpected associations between a single nucleotide polymorphism (SNP) in the *ZIP8* gene (*SLC39A8*) and body mass index/obesity (Speliotes et al. 2010), coronary artery disease risk (Waterworth et al. 2010), and schizophrenia (Carrera et al. 2012). The SNP, rs13107325, is located in exon 8 of the *SLC39A8* gene and causes an alanine-to-threonine substitution at amino acid position 391. This alanine is conserved in mZIP8 and is located between putative TM domains VI and VII (Fig. 1b). With respect to coronary artery disease risk, the T allele (8% frequency) is associated with lower circulating levels of HDL cholesterol (Waterworth et al. 2010). The authors of that study speculate that ZIP8 might be related with HDL cholesterol through inflammation.

Questions and future directions

- Although the *Zip14* knockout mouse shows that ZIP14 is required for normal zinc uptake into the liver, pituitary and chondrocytes, our understanding of its physiologic roles is far from complete. Does ZIP14 play a role in zinc, iron, or manganese uptake in any other tissues? With respect to iron, does it participate in the uptake of NTBI by the liver, heart, and pancreas or in TBI uptake?
- What are the normal physiologic roles of ZIP8? Does it participate in the transfer of metals across the placenta? Global and tissue-specific knockouts will be needed to answer these questions.
- The pancreas, a site of dynamic zinc metabolism, takes up NTBI during iron overload, resulting in β-cell destruction. As ZIP14 and ZIP8 are both abundantly expressed in the pancreas, it will be important to determine the contribution of these transporters to the uptake of zinc and iron into the various pancreatic cell types.
- What effect if any does the disease-associated A391T polymorphism in ZIP8 have on the function or expression of the protein?
- Most ZIPs have been characterized nearly exclusively by their ability to transport zinc. Future studies will need to more fully examine the range of metals (including cobalt) that are transported by individual ZIP proteins.

Acknowledgment Supported by National Institutes of Health Grant R01 DK080706 and R01 DK080047.

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