

The Multiple Faces of the Metal Transporter ZIP14 (SLC39A14)

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Abstract

The SLC39A family of metal transporters was identified through homologies with the Zrt- and Irt-like (ZIP) proteins from yeast and plants. Of all the ZIP transporters, ZIP14 is arguably the most robustly characterized in terms of function at the integrative level. Mice with a global knockout of *Zip14* are viable, thus providing the opportunity to conduct physiologic experiments. In mice, *Zip14* expression is highly tissue specific, with the greatest abundance in the jejunum > liver > heart > kidney > white adipose tissue > skeletal muscle > spleen > pancreas. A unique feature of *Zip14* is its upregulation by proinflammatory conditions, particularly increased interleukin 6 (IL-6) and nitric oxide. The transcription factors AP-1, ATF4, and ATF6 α are involved in *Zip14* regulation. ZIP14 does not appear to be zinc-regulated. The *Zip14* knockout phenotype shows multiple sites of ZIP14 function, including the liver, adipose tissue, brain, pancreas, and bone. A prominent feature of the *Zip14* ablation is a reduction in intestinal barrier function and onset of metabolic endotoxemia. Many aspects of the phenotype are accentuated with age and accompany increased circulating IL-6. Studies with ^{65}Zn , ^{59}Fe [nontransferrin-bound iron (NTBI)] and ^{54}Mn show that ZIP14 transports these metals. At a steady state, the plasma concentrations of zinc, NTBI, and manganese are such that zinc ions are the major substrate available for ZIP14 at the cell surface. Upregulation of ZIP14 accounts for the hypozincemia and hepatic zinc accumulation associated with acute inflammation and sepsis and is required for liver regeneration and resistance to endoplasmic reticulum (ER) stress. *Zip14* ablation in mice produces a defect in manganese excretion that leads to excess manganese accumulation in the brain that produces characteristics of Parkinsonism. *J Nutr* 2018;148:174–184.

Keywords: metal transporter, zinc, iron, manganese, nontransferrin-bound iron, inflammation

Introduction

Coordination of the functions of the nutritionally required trace metals by mammals was significantly stimulated by the identification of specific transporters. Relevant to this review was the initial description of the SLC39A family of transporters based on homologies with the Zrt- and Irt-like (ZIP) proteins from yeast and plants (1). These are commonly referred to as the ZIP transporters. In the following 2 decades, numerous reviews have provided updates on progress in our understanding of the roles the ZnT and ZIP proteins play in trace metal transport. That progress was achieved through the utilization of an array of approaches and has helped define roles for these transporters

in dietary acquisition, systemic metabolism, and more recently, targeted functions at specific subcellular sites. This review will leave that pattern and instead will focus on one metal transporter (i.e., ZIP14; SLC39A14). Of all of the ZIP transporters, ZIP14 is arguably the most robustly characterized in terms of physiologic function at the integrative level. This has arisen in part because the *Zip14* null mutation in mice is not lethal, thus providing the opportunity to conduct physiologic experiments with the use of these models.

Identification and Initial Characterization

SLC39A14 (ZIP14) was first identified in an effort to accumulate information on the coding sequences of uncharacterized human genes (2). In that screening study, cDNA clones that carry unreported sequences at the 5'-ends were isolated from human cDNA libraries and the sizes of their inserts were then compared with those corresponding transcripts by Northern hybridization. After sequencing those inserts, ZIP14 was named KIAA0062, among 40 newly identified coding sequences. Of the tissue RNAs sampled, KIAA0062 was most highly expressed in

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Abbreviations used: CRC, colorectal cancer; DKO, double knockout; ER, endoplasmic reticulum; Fe-TF, transferrin-bound iron; GH, growth hormone; HCC, hepatocellular carcinoma; HFE, hereditary hemochromatosis protein; IR, insulin receptor; LR, liver regeneration; Mt, metallothionein; NTBI, nontransferrin-bound iron; Pca, prostate cancer; PTP1B, tyrosine-protein phosphatase nonreceptor type 1; TJP, tight junction protein; siRNA, small, interfering RNA; SLC39A, solute carrier 39A; WAT, white adipose tissue; WT, wild-type; ZIP, Zrt- and Irt-like.

the liver. With the use of differential display, it was found to be induced by IL-1 α in human epithelial cells (3).

Sequence alignment studies showed that ZIP14 has 9 homologs, all with sequence homology to the protein produced by the estrogen-related gene *LIV-1* (4). It was originally proposed that these proteins should be called the LZT subfamily of the ZIP (ZRT/IRT-related protein) transporter family proteins because of this sequence homology (4). These homologs are now referred to as group IV of the 14-member ZIP family (5). Analyses of ZIP14 secondary structure prediction showed that it has 8 transmembrane domains, a long extracellular N-terminus, a short extracellular C-terminus, and numerous histidine-rich repeats (6). Multiple alignment analyses also showed an important conserved motif (HEXPHE). This motif localized in TM domain V is similar to the HXXXE motif in TM domain V of other ZIP proteins of this subfamily. The motif is thought to be part of the intramembrane metal-binding site, which forms the pore region required for zinc transport function (6). The one exception in the subfamily is ZIP14, which has a glutamic acid replacement of the first histidine residue of the conserved HEXPHE motif. This replacement may allow for the transport of ions in addition to zinc (7). ZIP14 was shown to run as a trimer in an SDS-PAGE with the use of nonreducing conditions (4). Similarly, numerous other studies have shown that immune-detectable ZIP14 can migrate as multiples of the molecular mass of the primary structure of 52 KDa. The ability of the ZIP transporter family to function primarily as dimers has been documented (8). This ability to form complexes is consistent with the formation of ion channels. In the future, these multiple forms may be shown to reflect specific functions for ZIP14 monomers and dimers or specific interactions with other ZIP proteins or other protein partners. Such interactions are under investigation.

Tissue Specificity of Zip14 Expression

The earliest reports on ZIP14 documented marked tissue specificity of expression. At the mRNA level, an array of human tissues of undefined origin showed the greatest mRNA abundance in liver followed by pancreas > thyroid > heart (9). In mice, we showed that the greatest *Zip14* mRNA abundance was in the duodenum followed by jejunum > liver > heart > kidney > white adipose tissue (WAT) > skeletal muscle > spleen > pancreas (10, 11). These mice were at steady state—that is, under normal dietary and environmental conditions and no treatments. Separate experiments showed that mouse *Zip14* mRNA levels in liver and brain are refractory to changes in dietary zinc intake (12). The marked sensitivity of *Zip14* expression in mice to proinflammatory conditions will be discussed in the sections that follow.

ZIP14 and Metal Transport

The transport properties of human ZIP14 were shown in 2005 with the use of human *ZIP14* transfected cells (9). This activity agrees with the plasma membrane localization of ZIP14 (9, 13). Concurrently, the zinc transport function of the mouse ortholog of ZIP14 was shown with the use of mouse *Zip14* transfected HEK293 human kidney cells and with multiple approaches to measure zinc uptake in the presence of 10 μ M extracellular zinc (13). In the same study, the plasma membrane localization of ZIP14 was shown with the use of liver tissue sections and

isolated murine liver parenchymal cells. We further reasoned, as discussed below, that physiologic regulation of ZIP14 expression could influence the metabolism of zinc and perhaps other metals. Focusing on iron, we showed that ZIP14 could transport nontransferrin-bound iron (NTBI) (10). Later, it was shown with the use of canine kidney epithelial cells that ZIP14 could transport cadmium and manganese via metal/bicarbonate symporter activity (14). Kinetic studies that used the *Xenopus* oocyte system provided further evidence that ZIP14 could transport multiple metals, albeit with different affinities (15).

ZIP14 and Zinc Transport

ZIP14-mediated zinc transport in the liver. Hypozincemia is one of the consequences of an acute inflammatory response and occurs concurrently with hypoferrremia (16). Hypozincemia is thought to be part of a host defense mechanism; the exact physiologic role of hypozincemia is not known. Two possibilities are as follows: 1) deprivation in the availability of zinc for utilization by pathogenic micro-organisms (17) or 2) the provision of more zinc for cellular needs. During acute inflammation, a large number of acute-phase response (APR) proteins are synthesized by the liver. A reduction in circulating zinc was observed in IL-1 α -injected rats (18) and was concurrent with an increase in liver zinc uptake. This differential was likely the result of enhanced zinc transporter expression.

To delineate which transporter or transporters were responsible for hepatic zinc uptake during acute inflammation, differential expression of Zip and ZnT transporter mRNAs were measured in the liver of mice after the administration of LPS (13). Hepatic metallothionein (Mt) mRNA levels were significantly higher in LPS-injected mice than in control mice, suggesting that hepatic zinc uptake was increased and activated metal responsive transcription factor 1 (MTF-1). The most responsive zinc transporter mRNA was found to be *Zip14*, with a 3.1-fold increase in mRNA levels in response to the LPS injection. Among the cytokines that are produced in response to an LPS injection, IL-6 is viewed as the main proinflammatory cytokine responsible for downstream effects of LPS (19, 20). Studies with IL-6 knockout mice showed that, after LPS, elevated hepatic *Zip14* and *Mt* expression and hypozincemia were not detected, suggesting that *Zip14* expression was IL-6 dependent. (13). Similarly, when primary hepatocytes that were isolated from wild-type (WT) and inducible NO synthase (*iNOS*) knockout mice were treated with IL-1 β (21), ZIP14 expression and zinc uptake were increased in WT hepatocytes, whereas there was no change in *iNOS* knockout hepatocytes. These experiments suggested that NO might be a cytokine-stimulated downstream mediator of *Zip14* expression. Treatment of isolated mouse hepatocytes with the NO donor, *S*-nitroso-*N*-acetylpenicillamine, resulted in increased ZIP14 abundance at the plasma membrane. Chromatin immunoprecipitation showed that NO increased transcription of *Zip14* via activation of transcription factor AP-1 (21). These results showed 3 important concepts: 1) *Zip14* was a cytokine-regulated gene, 2) ZIP14 must be the main transporter to facilitate hepatic zinc uptake during inflammation, and 3) *Zip14* transcription is activated by AP-1.

Functional significance of ZIP14-mediated zinc transport is also implicated in other studies. For example, repeated psychological stress in male rats caused sequential increases in serum cortisol, IL-6, and liver *Zip14* mRNA expression that were coincident with an elevated liver zinc concentration and hypozincemia (22). In another study, *Zip14* expression and zinc

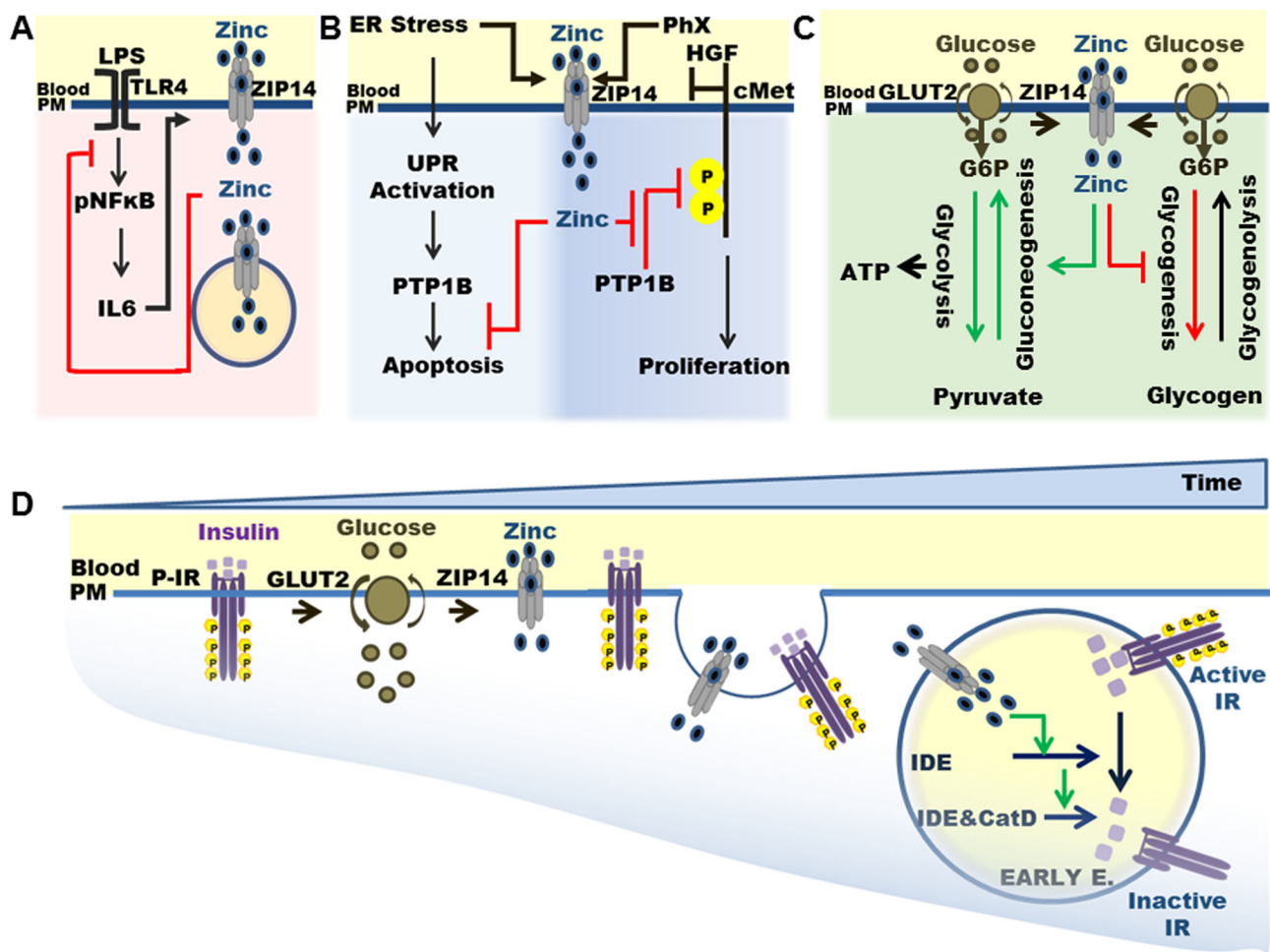


FIGURE 1 Function of ZIP14-mediated zinc transport in different physiologic events. (A) An inflammation-induced increase in intracellular zinc concentration via ZIP14-mediated transport provides a negative feedback by inhibiting TLR4 signaling for NF- κ B activation. (B) ZIP14-mediated zinc transport is needed for modulation of PTP1B phosphatase activity during regulatory pathways for hepatic ER stress and regeneration. (C) Hepatic ZIP14 expression and zinc uptake are upregulated during glucose uptake. Increased intracellular zinc involves differential regulation of hepatic glucose metabolism. (D) Hepatic ZIP14 along with transporter-bound zinc is endocytosed with active IR. ZIP14-bound (then released) zinc in early endosomes is necessary for the inactivation of IR via activation of zinc-requiring enzymes, IDE, and CatD required for disassociation and degradation of insulin. Green and red arrows indicate positive and negative regulation of zinc, respectively. CatD, cathepsin D; cMet, tyrosine-protein kinase Met; Early E., early endosome; ER, endoplasmic reticulum; GLUT2, glucose transporter 2; G6P, glucose-6-phosphate; HGF, hepatocyte growth factor; IDE, insulin-degrading enzyme; IR, insulin receptor; PHx, partial hepatectomy; P-IR, phosphorylated insulin receptor; PM, plasma membrane; pNFkB, phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells; PTP1B, tyrosine-protein phosphatase nonreceptor type 1; TLR4, Toll-like receptor 4; UPR, unfolded protein response; ZIP14, Zrt- and Irt-like 14.

uptake were upregulated in the liver of magnesium-deficient male rats (23). Those authors speculated that the *Zip14* upregulation was due to the increase in serum IL-6 and IL-1 β caused by magnesium deficiency (24). In contrast, decreased hepatic ZIP14 expression, along with hepatic zinc deficiency and increased serum and urinary zinc, was shown in male mice in response to chronic alcohol exposure (25).

We also explored a role in liver regeneration (LR), because this process is initiated by proinflammatory cytokines (26, 27). IL-6-induced expression of hepatocyte growth factor (HGF) is a major regulator of LR. During LR, we found that there was an influx of hepatic zinc early after partial hepatectomy that was concurrent with increased ZIP14 expression (28). After partial hepatectomy, *Zip14* knockout mice did not accumulate zinc and experienced delayed and perhaps defective LR as shown by depressed proliferation markers. The increased hepatic zinc was shown to depress tyrosine-protein phosphatase non-receptor type 1 (PTP1B) activity. PTP1B regulates c-Met phosphorylation, which is required for regeneration after acti-

vation by HGF. PTP1B regulates c-Met phosphorylation, thus regulating ERK1/2, a downstream regulator of proliferation (29). It has long been known from in vitro studies that zinc is a potent inhibitor of PTP1B activity [reviewed in (30)]. We proposed that ZIP14-transported zinc functions to inhibit PTP1B activity and thus regulates the hepatocyte proliferation signaling pathway during LR. To our knowledge, these data represent the first demonstration of a link between function and ZIP14-mediated zinc transport (Figure 1B).

When the *Zip14* knockout mouse model became available (31), we revisited the hypothesis that ZIP14 was the main transporter to facilitate hepatic zinc uptake during LPS-induced acute inflammation. We found that, after LPS administration, hypozincemia was fully prevented and hepatic zinc uptake was impaired in female *Zip14* knockout mice (32). Similar to the response of ZIP14 expression to LPS, polymicrobial sepsis in mice of mixed sexes leads to expression of multiple ZIP transporters, with ZIP14 having the most sustained expression on a temporal basis (33). Features of polymicrobial sepsis, such as serum

IL-6, TNF- α , and IL-10 concentrations, are markedly elevated in *Zip14* knockout mice. The knockout mice also exhibited reduced white blood cell counts. Such experiments suggest that defects in ZIP14 function extend beyond the liver. These findings collectively prove our long-standing hypothesis that ZIP14-mediated tissue zinc uptake is the primary driving force needed for the hypozincemia that occurs with acute endotoxemia (13) (Figure 1A).

Our initial characterization of *Zip14* knockout mice showed a phenotype with multiple metabolic abnormalities, suggesting that ZIP14-mediated zinc transport influences metabolic pathways (32). ZIP14 is highly expressed in the liver, the major metabolic organ responsible for the utilization and production of glucose and for energy balance. Hepatic glucose metabolism is regulated by insulin. Upon insulin binding to the insulin receptor (IR; active), insulin-bound IR is internalized by endocytosis. Insulin is then released from IR (inactivation) and degraded by zinc-dependent endosomal enzymes, insulin-degrading enzyme (IDE) and cathepsin D (CatD). In experiments that used female mice, we observed that during hepatic glucose uptake, zinc was distributed to multiple sites in hepatocytes through sequential translocation of ZIP14 from the plasma membrane to early and late endosomes, suggesting ZIP14 internalization along with IR (34) (Figure 1C, D). Hepatic endosomes from *Zip14* knockout mice were zinc-depleted based on analysis with the fluorophore FluoZin3. Activities of IDE and CatD were impaired; hence, IR inactivation was prevented (Figure 1D).

In *Zip14* knockout mice, cytosolic zinc content was less than in WT mice, whereas the liver glycogen content was significantly greater (34). When fed a zinc-supplemented diet, the liver glycogen concentration in *Zip14* knockout mice returned to the same level as in WT mice. This suggests that ZIP14-mediated hepatic zinc transport during glucose uptake might provide a negative feedback for glycogen synthesis. By measuring the activity of the enzymes glycogen synthase kinase 3 and glycogen synthase (regulate glycogen metabolism), we determined that, in HepG2 cells, the transient increases in cytosolic zinc concentration during glucose uptake, and additional zinc treatment, both caused suppression of glycogen synthesis. *Zip14* knockout mice exhibited impaired gluconeogenesis and glycolysis, most likely related to low cytosolic zinc concentrations (34). Expression of the enzymes of the gluconeogenic pathway, phosphoenolpyruvate (Pepck) and glucose-6-phosphatase (G6Pase), was lower in *Zip14* knockout mice. When mice were fed a zinc-supplemented diet, the expression of *Pepck* and *G6Pase* was enhanced in both WT and *Zip14* knockout mice. Moreover, the pyruvate tolerance test showed that impaired gluconeogenesis in *Zip14* knockout mice was rescued by dietary zinc supplementation. Glucose metabolism is largely dependent on mitochondria to generate energy. The mitochondrial zinc content was reduced in HepG2 cells during glucose uptake (34). The constant mitochondrial zinc content appeared to be regulated by the reciprocal action of ZIP14 and ZIP8, because there was no change between WT and *Zip14* knockout genotypes in mitochondrial ATP concentration. In contrast, cytosolic ATP was lower in *Zip14* knockout mice due to impaired glycolysis. Impaired glycolysis in the knockout mice was recovered by dietary zinc supplementation. The conclusion was that ZIP14-mediated zinc transport contributed to the regulation of endosomal insulin receptor activity and glucose homeostasis in hepatocytes. These experiments answer questions about the influence of zinc on glucose homeostasis that go back decades (35–37). We believe that this study shows the potential to modulate zinc transport for therapeutic strategies.

Zinc is essential for both maintaining normal endoplasmic reticulum (ER) function and resolving ER stress (38). Induction of zinc deficiency through injection of the metal chelator TPEN [N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine] or injection of the ER stress inducer tunicamycin resulted in increased expression of *Zip* mRNAs, including *Zip14*, along with markers of ER stress in livers of mice (39). It was proposed that ZIP14-mediated zinc transport influences superoxide dismutase 1 conformation, leading to progression to ER stress. Our experiments failed to show that a nutritional zinc deficiency in mice causes hepatic ER stress (40). During both a pharmacologically (tunicamycin) or high-fat-diet-induced hepatic ER stress, an increase in ZIP14 expression was followed by an elevation of hepatic zinc uptake in WT mice, whereas *Zip14* knockout mice exhibited impaired hepatic zinc uptake (41). The impaired hepatic zinc uptake in *Zip14* knockout mice during ER stress coincided with a greater expression of proapoptotic pathway ER stress proteins (p $\text{eIF}2\alpha$, ATF4, and CHOP) and a lower expression of the ER chaperone protein GPR94. Furthermore, in *Zip14* knockout mice, liver sections showed a greater number of Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling assay (TUNEL)-positive cells, indicative of apoptosis, and also increased serum alanine amino transferase, indicative of liver damage, respectively. When ER stress was induced, the *Zip14* knockout mice showed greater levels of hepatic steatosis. PTP1B is a zinc-regulated phosphatase (28, 30) with expression and activity that are increased during ER stress to help modulate the hepatic ER stress response (42–44). We proposed that ZIP14 transport activity provides zinc ions to inhibit PTP1B activity (41). In support of that notion, PTP1B activity was greater in *Zip14* knockout mice due to a deficiency of zinc in a cellular pool that interacts with the enzyme (Figure 1B). The effect of zinc on the ER stress response was confirmed with the use of HepG2 cells. Similar to data derived from mouse liver, HepG2 cells treated with tunicamycin had impaired hepatic zinc uptake; a greater amount of phospho (p)- $\text{eIF}2\alpha$, ATF4, and CHOP; and higher PTP1B activity and apoptosis when *Zip14* was knocked down by small, interfering RNA (siRNA). Zinc treatment rescued the knocked-down phenotype in tunicamycin-treated HepG2 cells. Chromatin immunoprecipitation studies showed that ER stress caused an increase in *Zip14* gene expression via activation of the transcription factors ATF4 and ATF6 α . These results collectively show the importance of zinc trafficking and functional ZIP14 transporter activity for adaptation to hepatic ER stress associated with chronic metabolic disorders.

ZIP14-mediated zinc transport and function in the intestine. The highest *Zip14* mRNA expression of those tissues examined in mice was in the duodenum and jejunum (10). Zinc is required for intestinal health and cell renewal as well as for barrier function. Therefore, we asked whether ZIP14-mediated zinc transport has a role in intestinal zinc metabolism, barrier function, or both? A most important finding in that regard was that intestinal ZIP14 was localized at the basolateral membrane of enterocytes along most of the villus (45). When ^{65}Zn was administered via subcutaneous injection to label the endogenous zinc pool, a greater accumulation of ^{65}Zn was found in the small intestines of *Zip14* knockout mice than in WT mice. This unexpected result led to the hypothesis that ZIP14 might be associated with endosomes; hence, the absence of ZIP14 might prevent endosomal zinc mobilization, thus trapping zinc in the endosomes. To test this concept, crude endosomes were isolated from the small intestine of WT and *Zip14* knockout mice. The

presence of ZIP14 was confirmed, and higher zinc concentrations were found in endosomes isolated from *Zip14* knockout mice. These results suggested that zinc was trapped in endosomes of enterocytes of the *Zip14* knockout mice, causing an intracellular zinc-deficient environment. The mechanism for the accumulation of endosomal zinc in the absence of ZIP14 is currently under investigation. Zinc deficiency can lead to intestinal inflammation (46). Interestingly, expressions of IL-6 and TNF- α were greater in the jejunum of *Zip14* knockout mice. Inflammation has been reported to cause an increase in intestinal permeability (47). Intestinal permeability was assessed in *Zip14* knockout mice with the use of both oral fluorescein isothiocyanate–dextran and serum endotoxin content. Amounts of both fluorescein isothiocyanate–dextran in plasma and endotoxin in serum were greater in *Zip14* knockout mice, indicating impaired intestinal barrier function with leakage into the systemic circulation. Intestinal barrier function is regulated by tight junction proteins (TJPs) (48–50). The reduced threonine phosphorylation of the TJP occludin and the respective decrease and increase in abundance of TJPs claudin 1 and 2 in the intestine of the knockout mice are highly supportive of a role for ZIP14-mediated zinc transport in the maintenance of intestinal barrier function (45). In addition, in light of the current finding that zinc is an important regulator of the gut microbiota (46), basolaterally localized ZIP14 might be required to supply zinc to the intestine for renewal of the gut epithelium or microbial host defense and intestinal barrier function.

ZIP14-mediated zinc transport and function in adipose tissue. *Zip14* upregulation during adipocyte differentiation *in vitro* was one of the first findings about this gene (51). To our knowledge, however, there had not been any research on adipose tissue and ZIP14 until we reported that ZIP14 alters zinc trafficking in adipose tissue with functional outcomes *in vivo* (11). At steady state, mRNA expression of *Zip14* is low in WAT; however, upregulation of *Zip14* mRNA in response to LPS-induced inflammation is the highest in any tissue examined (11, 32). The *Zip14* knockout phenotype includes elevated serum endotoxin, most likely due to impaired intestinal barrier function, and increased fat or lean body composition (32, 45). The marked induction of ZIP14 in WAT during inflammation, along with the knockout phenotype of increased adiposity and metabolic endotoxemia at steady state, led to the hypothesis that ZIP14 could be critical to the inflammatory response of adipose tissue and ultimately to the metabolic activity of WAT (Figure 1A). Characterization of the phenotypic profile of *Zip14* knockout adipose tissue showed enhanced activation of MyD88, NF- κ B, and STAT3, along with higher IL-6, TNF- α , and IL-1 β and lower adiponectin mRNA expression (11). Furthermore, expression of preadipocytic markers *Pre-1* and *Pref-1*, along with adipogenic enzymes *Acaca* and *Acyl Hmgcr*, were greater, whereas the differentiation marker *Pparg* along with lipolytic markers hormone-sensitive lipase and zinc α 2-glycoprotein were lower in *Zip14* knockout adipose tissue. Higher leptin concentrations were found in the adipose tissue and serum of knockout mice. Overall, the *Zip14* null mutation appeared to enhance WAT inflammation and adiposity and depress adipocyte differentiation. Accordingly, decreased IR phosphorylation at steady state was found in *Zip14* knockout adipose tissue, indicating the development of insulin resistance. Of note, antibiotic treatment reversed the adipose tissue inflammation and insulin-resistance phenotype of *Zip14* knockout, supporting the idea that metabolic

endotoxemia was the underlying cause of this aspect of the phenotype (11).

Zinc metabolism in WAT was altered in *Zip14* knockout mice. After oral ^{65}Zn administration, greater zinc accumulation was observed in WAT of the knockout genotype; however, *Mt* mRNA expression was lower, indicating less available cytosolic zinc (11). Similar results were obtained in 3T3-L1 cells when *Zip14* was knocked down by siRNA. These findings in adipocytes support the “zinc trap” hypothesis associated with *Zip14* deletion, which we showed previously in mouse intestine (45), and described in the section above.

Zip14 mRNA expression was found to be higher in the adipose tissue of obese individuals than in healthy controls (52). When obese individuals were provided with a calorie-restricted diet, *Zip14* expression diminished. The expression pattern of *Zip14* correlated with adipose zinc concentration. Those data support the notion that adipose inflammation induces ZIP14 expression in WAT.

ZIP14-mediated zinc transport and function in other tissues. Zinc is required for normal systemic growth (53). Mice with global *Zip14* deletions are smaller than their WT counterparts (54, 55). *Zip14* knockout mice also exhibited wry neck, slightly radiolucent bones, decreased bone volume and trabecular number, and increased trabecular separation. ZIP14 was highly expressed in the proliferative zone of the growth plate, specifically in primary pituitary cells and chondrocytes of WT mice. Concurrently, intracellular zinc concentrations were significantly decreased in the proliferative zone of the *Zip14* knockout mice growth plate (54). Chondrocytes and pituitary cells are important for bone elongation (56) and growth hormone (GH) production (57), respectively. Experiments with primary chondrocytes showed that zinc transported by ZIP14 was required to maintain the steady state level of parathyroid hormone 1 receptor (PTH1R)–mediated signaling by inhibiting and enhancing PDE and cAMP activity, respectively. Pituitary cells from *Zip14* knockout mice exhibited both low cAMP and zinc concentrations. Accordingly, GH and insulin-like growth factor I (IGF-I) expression and secretion were lower in these cells than in WT cells. GH and IGF-I are regulated by the activity of GH-releasing hormone receptor (GHRHR). Both PTH1R in chondrocytes and GHRHR in pituitary cells are G protein-coupled receptors (GPCRs); thus, these data collectively indicated that ZIP14-mediated zinc transport was selectively involved in the GPCR signaling pathways (54).

In addition to impaired growth, *Zip14* knockout mice also showed metabolic endotoxemia and altered glucose regulation (11, 32, 34, 45). Such phenotypes are associated with aging. Subsequently, we compared the phenotype of young and old male WT and *Zip14* knockout mice (55). *Zip14* deletion reduced growth and increased serum endotoxin in both young and old mice compared with WT mice. Accordingly, higher serum IL-6 was detected in both young and old knockout mice. Of note, aging greatly amplified the differential in serum IL-6 in the *Zip14* knockout mice. IL-6 mRNA levels in the spleen of the old knockout mice were the highest when compared with all groups; this suggests that splenic macrophages could be one site of origin of the elevated IL-6. In support of these data, the highest amount of activated (phosphorylated) NF- κ B and STAT3, master regulators of IL-6 production and secretion, were found in the spleen of old knockout mice.

Both age and *Zip14* deletion influenced bone morphology as measured by micro-computed tomography scanning. Cortical

bone volume decreased, whereas bone surface area increased in old knockout mice, indicating a critical reduction in bone mass by *Zip14* ablation (55). Furthermore, greater loss of femoral trabecular bone was observed in the *Zip14* knockout mice. The old knockout mice showed significant reductions in trabecular bone based on numerical indexes of bone mineral density.

In keeping with these results, when ear mesenchymal stem cells (EMSCs) from *Zip14* knockout mice were cultured in osteogenic medium, there was less mineralization when compared with their WT counterparts, based on Alizarin Red staining (55). In addition, by using these EMSCs we showed that pSmad1/5 (a key transducer of the bone morphogenetic protein pathway) was downregulated in *Zip14* knockout mice (55). ZIP14 upregulation under inflammatory conditions was reported in multiple organisms and tissues, such as mouse lung (58), porcine spleen (59), sheep pulmonary artery endothelial cells (60), primary human macrophages (61), and human peripheral blood mononuclear cells (62), but functional studies were not conducted.

ZIP14 and Iron Transport

Many of the ZIP family member metal transporters have been shown to transport iron as well as other cations (63–66). ZIP14 is unique in the ZIP family in that it has a glutamic acid replacement of the first histidine residue of the conserved HEX-PHE motif. It has been suggested that this replacement may allow for the transport of metal ions in addition to zinc (7). Focusing on iron, we showed that ZIP14 could transport NTBI (10). Overexpression of mouse *Zip14* in HEK293 human kidney and Sf9 insect cells resulted in greater iron accumulation by these cells. Accordingly, lower iron accumulation was found in AML12 hepatocytes when *Zip14* was knocked down by siRNA. We postulated that in pathologic conditions where NTBI concentrations are detectable (e.g., hemochromatosis and repeated transfusions), ZIP14 could lead to excessive hepatic iron accumulation (10).

In vitro support for NTBI transport by ZIP14 was that stable expression of the hereditary hemochromatosis protein (HFE) in HepG2 hepatocytes suppressed NTBI uptake through decreasing ZIP14 stability (67). Overexpression of ZIP14 in HeLa cells caused an increase in NTBI uptake. When HFE expression was induced, with the use of a tetracycline promoter, ZIP14-mediated NTBI uptake by these cells was diminished. Association of ZIP14 with transferrin-bound iron (Fe-TF) assimilation in endosomes was shown in HEK293 cells (67). Partial co-localizations of 3-FLAG ZIP14 and TF and early endosome protein EEA1 were shown in 3-FLAG tagged *Zip14* knock-in HepG2 cells. With *Zip14* knock-down in HepG2 cells, ⁵⁹Fe accumulation from ⁵⁹Fe-TF was lower, suggesting that ZIP14 contributes to the uptake of Fe-TF. However, the same group in their later work showed that hepatic Fe-TF uptake was not different between *Zip14* knockout and WT mice (68).

Dietary iron regulation of ZIP14 expression has been shown (69). ZIP14 protein concentrations were upregulated in dietary iron-loaded rat liver and pancreas. Similarly, ZIP14 protein concentrations were higher and lower when stable *Zip14*-transfected HepG2 cells were iron loaded and depleted, respectively (70). Iron depletion promoted deglycosylation of the ZIP14 protein, and thus proteasome degradation resulted. In contrast, iron overload prevented internalized ZIP14 degradation. Hence, ZIP14 regulation by iron appears to be post-translational.

We conducted the initial evaluation of iron metabolism in *Zip14* knockout mice (adults aged >8 wk). Total serum and liver iron concentrations were not different between genotypes. However, greater absorption of orally administered ⁵⁹Fe without gastrointestinal tract and greater uptake of ⁵⁹Fe by the liver was found in the knockout mice (32). In those studies, ⁵⁹Fe absorption and liver ⁵⁹Fe uptake were calculated as a percentage of whole-body radioactivity corrected for body weight or liver weight, respectively. When total body (without gastrointestinal tract) ⁵⁹Fe absorption and liver ⁵⁹Fe uptake were calculated only as a percentage of whole-body radioactivity of young (4- to 6-wk-old) mice, there was no difference between genotypes (68). Total body and liver ⁵⁹Fe uptake from intravenously injected ⁵⁹Fe-NTBI were both lower in *Zip14* knockout mice, suggesting involvement of ZIP14 in NTBI uptake.

Mutations in both *HFE* and *HFE2* (hereditary hemochromatosis) genes cause an increase in iron absorption and subsequent iron accumulation in body tissues. Jenkitasemwong et al. (68) tested the function of ZIP14 in hereditary hemochromatosis, through crosses of *Zip14* knockouts with *Hfe* knockout as well as *Hfe2* knockout mice to create double knockout (DKO) mice. In both *Hfe* and *Hfe2* knockouts, hepatic nonheme iron and ⁵⁹Fe accumulation were greater when compared with WT mice. However, in the DKO mice, hepatic ⁵⁹Fe accumulation was not seen in mice of the *Hfe* and *Hfe2* knockout genotypes. After dietary iron overload, the elevation in hepatic nonheme iron in WT mice was not observed in *Zip14* DKO mice. These data collectively indicate that ZIP14 contributes to NTBI uptake by murine hepatocytes in diet-induced iron overload and in hereditary hemochromatosis.

Zip14 knockout mice displayed impaired pancreatic ⁵⁹Fe uptake from intravenously injected ⁵⁹Fe-NTBI, whereas there was no difference in transferrin-bound iron (TBI) uptake (68). Mice with double knockout of *Zip14* with *Hfe* and *Hfe2* showed that only the *Hfe* DKO and at a smaller magnitude the *Hfe2* DKO caused an increase in ⁵⁹Fe accumulation in the pancreas. Iron accumulation was in pancreatic acinar cells in *Hfe2* DKO and in inter- and perilobular regions in *Hfe2* DKO mice. Dietary iron-loaded mice showed a similar pattern of iron deposition. These data suggest that both deletion of *Zip14* and dietary iron overload in mice contribute to iron accumulation in the pancreas. ZIP14 was found in β lox5 cells, a human β cell line (71). Increased transport of NTBI by ZIP14-overexpressing β lox5 cells was shown. Moreover, when *Zip14* was knocked down in both β lox5 and primary human islets, NTBI transport was impaired (71). An association of ZIP14 with iron metabolism has been implicated in multiple organisms and tissues, but with little mechanistic explanation (72–78).

ZIP14 and Manganese Transport

The transport of manganese ions by ZIP14 was shown in vitro with the use of transfected HEK293 cells and fetal fibroblasts overexpressing ZIP14 (14). Knockdown of *Zip14* with siRNA reduced manganese uptake by murine kidney tubular cells (79), but not in rat basophilic leukemia cells (80). The murine ear accumulates manganese when injected subcutaneously and also expresses *Zip14* mRNA, but a direct relation to transport was not established (81). An analysis of metal transport with the use of a *Xenopus* oocyte heterologous system expressing mouse ZIP14 compared the transport of manganese with other metals (15). The affinity of ZIP14 for zinc and manganese was such that, at 2 μ M zinc, a manganese concentration of 20 μ M

in 1 mM L-ascorbic acid did not inhibit ^{65}Zn uptake by the oocytes. Conversely, at 2 μM manganese, a concentration of 20 μM zinc completely inhibited ^{54}Mn uptake. Manganese concentrations of this magnitude in plasma of animals would be nonphysiologic; hence, these results may not be reflective of true ZIP14 metal trafficking activity in vivo.

ZIP14 inducibility by IL-6 as a factor in ZIP14-mediated manganese transport was introduced with in vitro studies that used SH-SY5Y neuronal cells (82). IL-6-stimulated neuronal cells had a 3-fold greater manganese accumulation than non-stimulated cells. A direct link between ZIP14 expression and manganese transport was not shown through siRNA inhibition or other methods.

A major advance in the understanding of the manganese transporting relevance of ZIP14 was the observation that mutations in ZIP14 are concurrent with manganese accumulation in humans with early-onset Parkinsonism dystonia (83). Companion studies in zebrafish showed that these ZIP14 mutations led to excess manganese accumulation and behavioral characteristics corresponding to Parkinsonism. They proposed a model in which ZIP14 facilitates manganese transport from the liver to the biliary excretory route. Furthermore, they showed that mutant ZIP14 has a diminished manganese transporting capability, which leads to tissue manganese accumulation by the zebrafish. We investigated the manganese transporting capabilities in vivo by using a mouse *Zip14* knockout model (84). Our results showed that these knockout mice present with markedly impaired locomotor function, increased intensity of MRI images of the brain indicative of manganese accumulation, impaired manganese (^{54}Mn) elimination, and increased tissue manganese accumulation. Manganese accumulation was markedly greater in male knockout mice, which parallels the greater incidence of Parkinsonism in human males (85). We concluded that normal ZIP14 transport function is necessary for manganese homeostasis in mice fed a commercial feed pellet-type diet. ^{54}Mn radio-tracer experiments showed that the intestine may be a route for some manganese excretion, because ZIP14 is localized to the basolateral membrane of enterocytes from the proximal small intestine (45). These conclusions are in line with the characterization of mammalian manganese homeostasis produced by Cotzias and colleagues (86) in the 1960s. Moreover, our data show that ZIP14 is not required for manganese uptake into neuronal tissues. However, systemic ZIP14 transport activity is essential to prevent manganese-related signatures of neurodegeneration, including, for example, elevated translocator protein (TSPO), activated NF- κ B, and increased TNF- α mRNA in brain. A model based on ZIP14-mediated manganese uptake by hepatocytes with subsequent biliary excretion was proposed to explain the data (84). We further speculated that ZIP14 could be a component of the high-affinity transport system identified for manganese in rat hepatocytes (87). Subsequently, similar studies with another *Zip14* knockout murine model, in which the sex of the mice was not reported, showed similar motor dysfunction and manganese accumulation in the brain and most other tissues (88). A liver-specific *Zip14* knockout did not result in manganese accumulation in the brain or other tissues. Upon feeding a highly nonphysiologic amount of dietary manganese (2400 mg/kg), excess manganese was detected in the brain cortex and pancreas of these liver-specific *Zip14* knockout mice (88).

The function of ZIP14-mediated metal transport in neuronal tissues is currently unknown (89). We know at this juncture that ZIP14 is not essential for manganese uptake into the brain. That does not exclude the potential for ZIP14-mediated delivery of zinc and possibly NTBI and manganese into the

brain. The identification of ZIP14 (SLC39A14), by exome sequencing, as among 32 genes in which variants were associated with intellectual disability, is of significant interest (90). That finding may suggest a beneficial role for ZIP14 in neuronal metal transport.

ZIP14 and Transport of Other Metals

Cadmium is a nonessential, toxic environmental contaminant that is poorly absorbed. Cadmium was found to be transported by ZIP14 in vitro by transfected kidney cells and fetal fibroblasts overexpressing ZIP14 (14). Cadmium exhibited a lower affinity and transport velocity than manganese. Moreover, cadmium uptake could be inhibited by manganese. In rat basophilic leukemia (RBL-2H3) cells, which are very sensitive to cadmium, *Zip14* knockdown with siRNA did not influence cadmium uptake (80). In contrast, the same approach decreased cadmium uptake by mouse kidney proximal cells as did siRNA knockdown of *Zip8* and *Dmt1* (79). It was proposed that ZIP14 is involved in the renal reabsorption and retention of cadmium. ZIP14 and other metal transporter mRNAs are upregulated in the rat placenta by cadmium in a dose-dependent manner (91). Induction of *Zip14* mRNA by LPS was correlated with hepatic cadmium accumulation in mice when cadmium was given by injection (92). Feeding a zinc-deficient diet to those mice accentuated the cadmium accumulation after LPS treatment. Oral cadmium administration to *Zip14* knockout mice decreased hepatic cadmium retention compared with WT mice (93). The reverse situation was found for cadmium accumulation in proximal small intestine, kidney, and lung. No mechanistic explanation was provided by the authors, except that acute shock of the cadmium dose caused upregulation of multiple metal transporter genes based on transcript data.

Cadmium concentrations of erythrocytes from a female Andean population were associated with polymorphisms in *ZIP14* (*SLC39A14*). Specifically, higher concentrations were found in women carrying the *GT* or *TT* genotypes of rs4872479 than those carrying *GG* (94). Such association studies may be of value in studies relating ZIP14 transport functions and phenotype in humans. Experiments with the *Xenopus* oocytes expressing mouse *Zip14*, as described above, did not provide evidence for the transport of copper (either Cu^{1+} or Cu^{2+}) (15).

ZIP14 and Cancer

ZIP14 downregulation in human hepatoma relative to adjacent normal tissue in liver biopsy specimens was detected by microarray (95). This finding supported the long-known (96) observation that zinc concentrations were lower in human hepatomas (97). By using a tissue microarray, the presence and absence of ZIP14 protein in plasma membrane was shown in normal hepatocytes and hepatoma cells, respectively (98). A decrease in ZIP14 was concurrent with the decrease in zinc in hepatomas. A meta-analysis of whole transcriptomes of hepatocellular carcinoma (HCC) was conducted to explore *ZIP14* as a potential biomarker and therapeutic target for HCC (99). Similar to the above microarray studies, *ZIP14* was downregulated in human HCC samples compared with adjacent normal tissue. Furthermore, aberrant splicing in *ZIP14* was found to be associated with HCC.

Transcript variants of *Zip14* were previously reported (14). Transcript variants of *Zip14* were the result of alternative

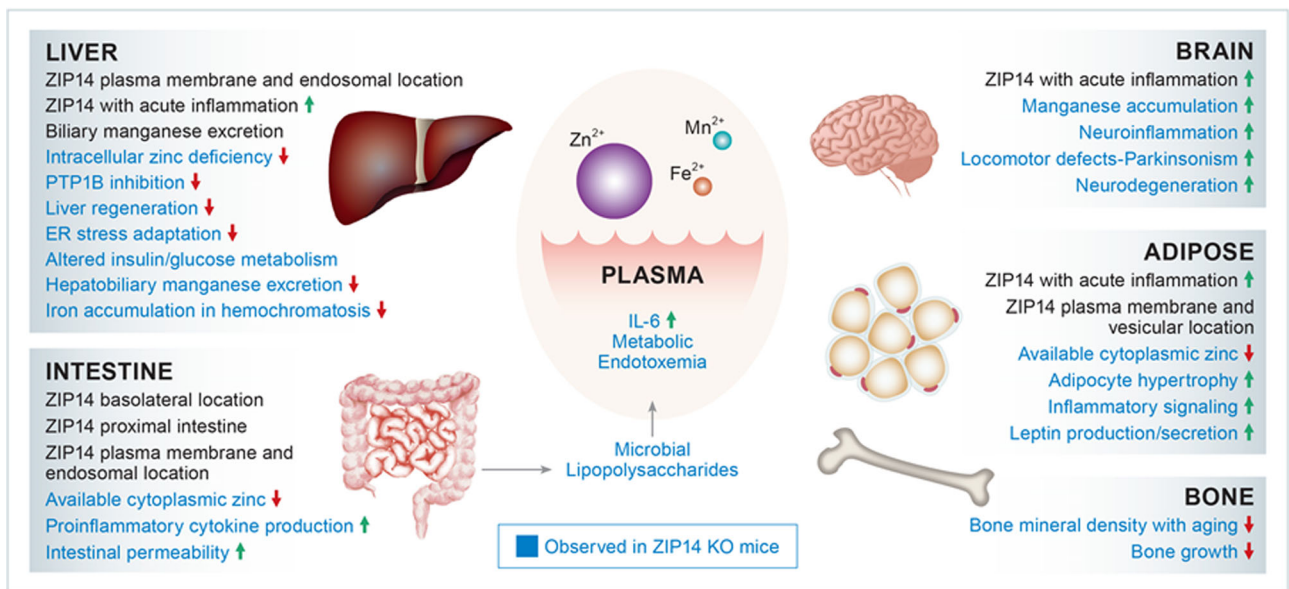


FIGURE 2 Metabolic and intracellular functions of metal transporter ZIP14 (Slc39a14). At steady state, ZIP14 is highly expressed in the jejunum of mice followed by liver > heart > kidney > white adipose tissue > skeletal muscle > spleen > pancreas. Proinflammatory stimuli increase ZIP14 expression in the liver, adipose tissue, and muscle. ZIP14-mediated zinc transport influences cell signaling related to cell proliferation, apoptosis prevention, and suppression of inflammatory pathways. ZIP14 also transports nontransferrin-bound iron and manganese. Circles are approximations of plasma concentrations of Zn²⁺, Fe²⁺, and Mn²⁺ available as substrates for ZIP14 at the cell surface. ZIP14 transport activity can influence the intracellular concentrations of these ions through endocytotic trafficking. Phenotypic changes produced in mice through deletion of *Zip14* are shown in blue. ER, endoplasmic reticulum; KO, knockout; PTP1B, tyrosine-protein phosphatase nonreceptor type 1; ZIP14, Zrt- and Irt-like 14.

splicing of either exon 4A or exon 4B. Mouse *Zip14* exons 4A and 4B are both 170-bp long and share 67% nucleotide identity. Transcripts of ZIP14A and ZIP14B encode 2 different proteins, both with 489 amino acids but different molecular masses. Differing expression of exons 4A and 4B between normal and colorectal cancer (CRC) tumor samples was reported (100). Even though there was no difference in total ZIP14 expression, the 4A-to-4B ratio was significantly lower in adenomas and carcinomas when compared with normal tissues. Due to differential expression of ZIP14 splicing variants in cancer tissue, ZIP14 was suggested as a biomarker for CRC. This idea was further explored by evaluating ZIP14 variants in both CRC biopsy specimens and healthy tissues (101). In that study, high relative expression of ZIP14B compared with ZIP14A in CRC was confirmed. However, ZIP14B expression was also present in healthy tissue, suggesting that the cancer-specificity of ZIP14B was mainly confined to the colon and rectum.

ZIP14 protein and mRNA levels in human prostate cancer (PCa) tissues were lower than those in adjacent noncancerous prostate tissue (102). The decreased expression of ZIP14 protein more frequently occurred in PCa tissues at the advanced clinical stage. Furthermore, the expression of ZIP14 mRNA was identified as one of the predictors of recurrence-free survival in patients with PCa. In vitro, the overexpression of ZIP14 suppressed cell proliferation, invasion, and migration in the PCa cell line LNCaP. Suppression was reversed by the knockdown of ZIP14 (102).

Conclusions and Perspectives

Although the ZIP14 gene was identified >20 y ago, the past decade has seen major developments toward an understanding of this transporter's biological relevance. An appreciation

of this relevance requires an understanding of the strengths and limitations of the in vivo and in vitro methodologies used for research on ZIP14. This is particularly important within the context of the transport potential of multiple metal ions. Factors that must be considered include the steady state compared with induced state of ZIP14 expression, normal plasma concentrations of the metal substrates (zinc, iron, manganese, cadmium, etc.), plasma-binding ligands for those metals and their binding affinities, physiologic conditions that influence those concentrations, comparisons of total tissue metal concentration compared with intracellular metal distributions, as well as phenotypic metal-related abnormalities associated with ZIP14 deletion or mutations. The latter are a likely reflection of biological roles.

To compare the transport options for ZIP14, we considered the metal substrates available to ZIP14 in the systemic circulation. In normal subjects, the plasma zinc concentration is ~15 μM, with zinc ions primarily loosely bound to albumin (103); plasma NTBI is either undetectable or no greater than 0.04 μM (104, 105); and plasma manganese is ~0.07 μM and is tightly bound to transferrin and α2-macroglobulin (106). In some inherited disorders of iron metabolism, as well as in diabetes and occurring transiently after transfusion and iron supplementation (105, 107, 108), NTBI concentrations may increase in plasma and provide iron (Fe²⁺) as a substrate for ZIP14 (10, 68). Portal blood may have somewhat greater concentrations of each metal. Nevertheless, under normal dietary conditions, cells are exposed to plasma, where the concentrations of these metal ions are markedly different [Zn²⁺ >>>> Mn²⁺ > Fe²⁺ (NTBI)]. We propose that Zn²⁺ has an abundance advantage of ~400-fold compared with Fe²⁺ or Mn²⁺. This stoichiometric relation likely explains why, during situations in which ZIP14 expression is increased (e.g., stress, acute infection, or sepsis), liver zinc concentrations increase but those for iron and manganese do not.

Nevertheless, organellar concentrations of these metals may be markedly different through intracellular trafficking and targeted ZIP14 transport activity (34, 67).

We have reviewed the evidence for the multiple faces of ZIP14 (Figure 2). We argue that the primary biological role of ZIP14 is to regulate intracellular zinc homeostasis and functions, such as modulation of cell signaling pathways. Furthermore, we argue that transport roles for NTBI and manganese are secondary, with pathologic or excretory and protective roles, respectively. These roles may be magnified under proinflammatory conditions.

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