

THE FUNCTION OF INTESTINAL ZIP8 IN MANGANESE METABOLISM

By

Yuze Wu

Copyright © Yuze Wu 2023

A Thesis submitted to the Faculty of the

SCHOOL OF NUTRITIONAL SCIENCES AND WELLNESS

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

Graduate College

THE UNIVERSITY OF ARIZONA

2023

THE UNIVERSITY OF ARIZONA

GRADUATE COLLEGE

As members of the Master's Committee, we certify that we have read the thesis prepared by Yuze Wu, titled the function of intestinal zip8 in manganese metabolism and recommend that it be accepted as fulfilling the dissertation requirement for the Master's Degree.


Ningning Zhao

Date: 5/5/2023


Kirsten Limesand

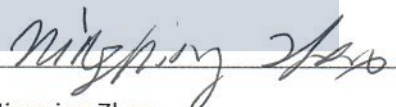
Date: 5/6/23


Ashley Snider

Date: 05/07/2023

Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the Master's requirement.


Ningning Zhao

Date: 5/5/2023



Master's Thesis Committee Chair
School of Nutritional Sciences and Wellness

ARIZONA

Table of Contents

Title: The Function of intestinal ZIP8 in manganese metabolism.....	1
Abstract	5
Chapter 1: Introduction	6-8
Chapter 2: literature review	9-17
2.1 Manganese	9
2.1.1 Manganese as nutrients in humans.....	9
2.1.2 Manganese homeostasis (Uptake, distribution, and excretion)	9
2.1.3 Manganese in tissues	9-12
2.1.3.1 Manganese in liver	10
2.1.3.2 Manganese in brain	10-11
2.1.3.3 Manganese in bone	11
2.1.3.4 Manganese in intestine	11-12
2.1.4 Manganese and disease	12-15
2.1.4.1 Manganese deficiency	12-13
2.1.4.2 Manganese overload	13-15
2.1.4.3 Manganese and cancer	15
2.2 ZIP8	15-17
2.2.1 ZIP8 and disease	15-16
2.2.2 ZIP8's function in manganese metabolism	16-17
Chapter 3: Methodology	18-21
3.1 Animals	18
3.2 Genotyping	19
3.3 Metal analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)	19
3.4 Culture of Caco2 cell line.....	19-20
3.5 Western blotting analysis	20
3.6 Statistical Analysis	20-21
Chapter 4: Results	21-28
4.1 ZIP8 is localized to the apical membrane in Caco-2 cells	21

4.2 ZIP8 is a manganese importer in Caco-2 cells	21-22
4.3 ZIP8 is highly expressed in the large intestine	22-23
4.4 <i>Zip8</i> intestine-specific knock out mice (<i>Zip8</i> -ISKO) have decreased manganese levels in the liver and bone	24-26
4.5 <i>Zip8</i> -ISKO& <i>Zip14</i> ^{-/-} double knock out mice (DKO) have decreased manganese levels in the brain and blood compared with <i>Zip14</i> ^{-/-} mice	27-28
Chapter 5: Discussion	29-31
Chapter 6: Supplemental Figures	32
6.1 Supplemental Figure 1. ZIP8 is highly expressed in the apical side of the cell membrane	32
6.2 Supplemental Figure 2. ZIP8 expression in Caco-2 cells.....	33
6.3 Supplemental Figure 3. ZIP8 expression levels in the small intestine (SI2) and large intestine (LI3) of wild-type male mice.....	34
6.4 Supplemental Figure 4. ZIP8 expression levels in the small intestine (SI2) and large intestine (LI3) of wild-type female mice.....	35
6.5 Supplemental Figure 5: Confirmation of intestinal ZIP8 deletion in <i>Zip8</i> -ISKO mice. 36	
6.6 Supplemental Figure 6: Effect of intestinal ZIP8 inactivation on liver ZIP8 expression in <i>Zip8</i> -ISKO mice.....	37
6.7 Supplemental Figure 7: Confirmation of intestinal ZIP8 and ZIP14 inactivation in DKO mice.....	38
Chapter 7: Reference	39-44

Abstract

Manganese is an essential trace element required for various biological processes. An imbalance of body manganese content can have detrimental effects on human health. This study investigated the role of intestinal ZIP8 in regulating systemic manganese homeostasis and the potential for targeting ZIP8 to manage manganese-overload disorders.

Using the human intestinal cell line Caco-2 cells as a model for enterocytes, we identified the localization of ZIP8 on the apical side of the enterocyte membrane. Manganese uptake experiments carried out in wild-type and *ZIP8* knockout Caco-2 cells indicated that the absence of ZIP8 reduced cellular manganese accumulation. These results suggest that ZIP8 is present in enterocytes responsible for the apical manganese absorption in the intestine.

We then investigated ZIP8 expression across different regions of the mouse intestine, finding it highly expressed in sections of the large intestine. To examine the physiological role of intestinal ZIP8, we generated intestine-specific *Zip8* knockout mice (*Zip8*-ISKO) to further explore ZIP8's function in vivo. These mice showed significantly decreased manganese levels in their livers and bones that are two major organs for manganese storage, pointing to the critical role of intestinal ZIP8 in regulating systemic manganese homeostasis. We further generated double knockout (DKO) mice by crossing *Zip8*-ISKO mice with *Zip14* knockout mice (*Zip14*^{-/-}), a known mouse model for manganese overload, to examine the effects of intestinal *Zip8* knockout in a high manganese environment. The DKO mice exhibited reduced manganese levels in the brain and blood when compared with *Zip14*^{-/-} mice, suggesting that decreasing ZIP8 in the intestine can reduce the body's manganese burden under manganese-overload conditions.

In conclusion, the present study has identified a novel role for intestinal ZIP8 in maintaining systemic manganese homeostasis. Our results have advanced the understanding of ZIP8's function and its role in manganese metabolism. Moreover, our findings may inform future strategies for managing manganese-related disorders, such as reducing manganese intake to decrease manganese accumulation in patients with manganese overload.

Chapter 1: Introduction

Manganese is an essential nutrient required in trace amounts for normal growth, development, and overall health. A balanced diet must provide sufficient levels of manganese to avoid its deficiency or overload. Key dietary sources of manganese encompass whole grains, legumes, nuts, seeds, leafy green vegetables, and certain fruits such as pineapple and raspberries (1-3). The recommended amounts of daily intake for manganese are 2.3 mg/day for adult men and 1.8 mg/day for adult women (4).

Manganese absorption mainly takes place in the small intestine, with a portion occurring in the stomach (5). The exact process of manganese absorption remains unclear. Once absorbed, manganese travels through the bloodstream, binding to proteins including albumin and beta-1 globulin (6). From there, it is distributed to different tissues and organs, with the highest concentrations found in the liver, pancreas, kidneys, bone and brain (5). Manganese is essential for normal brain function, as it can cross the blood-brain barrier (7, 8). To enter cells, manganese relies on various transporters, including the divalent metal transporter 1 (DMT1), which is also involved in iron uptake (9). A tightly controlled cellular manganese level is critical for maintaining cellular functions and avoiding toxicity. Manganese elimination primarily occurs through bile secretion into the small intestine, followed by excretion in feces (10, 11). A smaller amount of manganese is also excreted through the urine (10). The body can regulate manganese levels by adjusting the rate of biliary excretion (11, 12).

Manganese plays several important roles in the human body, including protein metabolism (13), antioxidant defense (14), wound healing (15), nervous system function (16), reproduction (17), and blood sugar control (18). These functions are carried out through manganese-containing enzymes. For example, arginase, a manganese-dependent metalloenzyme, exists in two isoforms. Arginase I is mainly present in the liver, while arginase II is predominantly located in the kidneys and various other tissues throughout the body. Arginase plays a crucial role in the urea cycle by converting arginine into urea and ornithine, a process essential for the elimination of ammonia from the body (19). Manganese superoxide dismutase (MnSOD) is an important antioxidant enzyme that requires manganese for its activity. MnSOD is primarily located within the mitochondria, the energy-producing organelles in cells. Its main function is to neutralize reactive oxygen species (ROS), specifically the superoxide radical (O_2^-), which is a harmful by-product generated during normal cellular respiration and other metabolic processes (20).

It's important to note that while manganese is essential for health, too much of it can be harmful. Manganese overload in the human body can occur due to various reasons, including environmental exposure (21), ingestion of contaminated food or water, genetic disorders, medical conditions, and medications. People working in industries of welding, mining, and battery production may be exposed to high levels of manganese in the air, leading to excessive accumulation in the body (22). Certain genetic disorders, such as loss function mutations of *SLC39A14* gene, also known as *ZIP14*, can cause the body to absorb too much manganese from food, leading to accumulation in the body (23, 24). Some medical

conditions such as liver disease, chronic renal failure, and neurological disorders can impair the body's ability to excrete manganese, leading to manganese accumulation in the body (25). Moreover, manganese may be administered as part of parenteral nutrition (PN) to patients who are unable to receive adequate nutrition through the gastrointestinal tract. However, excessive manganese administration in PN can lead to manganese toxicity, which can cause neurological symptoms such as tremors and impaired cognitive function (26, 27).

Manganese deficiency is generally caused by inadequate dietary intake or malabsorption disorders that prevent the body from absorbing sufficient manganese from the diet. However, rare genetic disorders such as *SLC39A8* deficiency, caused by mutations in the *SLC39A8* gene (also known as *ZIP8*), can cause manganese deficiency. The protein encoded by the *ZIP8* gene is a multi-transmembrane protein with the ability to transport various divalent cations such as cadmium, zinc, iron, and manganese (28-30). Mutations in the *ZIP8* gene have been associated with various health conditions in humans, including a rare genetic disorder called congenital disorder of glycosylation type IIc (CDG-IIc) or leukocyte adhesion deficiency type II (LAD-II). This disorder is characterized by developmental delay, intellectual disability, impaired immune function, hypotonia, and dysmorphic facial features. The neurological symptoms observed in affected individuals may be partly attributed to altered manganese homeostasis resulting from the *ZIP8* mutations (31).

Previous studies have employed various *Zip8* knockout mouse models to examine the role of ZIP8 in manganese metabolism and overall health. Mice with global *Zip8* knockout exhibited embryonic lethality, severe anemia, and growth retardation, emphasizing the significance of ZIP8 in embryonic development. Researchers generated *Zip8*-inducible global-knockout (*Zip8*-iKO) and liver-specific-knockout (*Zip8*-LSKO) mice and observed significantly decreased manganese levels in multiple organs and whole blood in both mouse models. To further explore this relationship, they re-expressed ZIP8 through liver-specific overexpression of human ZIP8 (adeno-associated virus-ZIP8 [AAV-ZIP8]), leading to increased tissue and whole blood manganese levels. The expression of ZIP8 was found to be localized on the hepatocyte canalicular membrane, the apical side of the cells, highlighting a critical role for hepatic ZIP8 in manganese homeostasis and demonstrating that ZIP8 is responsible for reclaiming manganese from bile (12).

ZIP8 is known to be expressed in the small intestines of humans (32). However, the function of ZIP8 in the intestine is unknown. Investigating the role of ZIP8 in intestinal manganese absorption could help elucidate how the body maintains necessary manganese levels and how disruptions of ZIP8 function may lead to health problems. In this study, we aimed at investigating the function of intestinal ZIP8. We determined the localization of ZIP8 in Caco2 cells and found that ZIP8 is enriched on the apical membrane of these cells. Based on this finding, we hypothesized that ZIP8 may play a role in manganese absorption at the apical side of enterocytes. To test this hypothesis, we generated a Caco2 cell line with ZIP8 inactivation (Caco2-*ZIP8*KO cells) and conducted manganese uptake experiments. These experiments revealed significantly decreased manganese accumulation in Caco2-*ZIP8*KO cells compared with Caco2-WT cells. To assess the physiological relevance of this finding, we generated intestine-

specific *Zip8* knockout (*Zip8*-ISKO) mice, which exhibited markedly decreased manganese levels in their livers and bones. Additionally, we crossed the *Zip8*-ISKO mice with *Zip14*^{-/-} mice, finding that the double knockout mice showed decreased manganese accumulation in the brains and blood of *Zip14*^{-/-} mice. In summary, our findings suggest that intestinal ZIP8 plays a crucial role in controlling systemic manganese homeostasis.

Chapter 2: literature review

2.1 Manganese

Manganese is the 25th element in the periodic table with a standard atomic weight of 54.94u (33). Manganese has 10 oxidation states which are 7, 6, 5, 4, 3, 2, 1, -1, -2, -3. Manganese 2+ (Mn II) and 3+ (Mn III) are most common oxidation states in the human body (34).

2.1.1 Manganese as a nutrient in humans

Manganese is an essential trace element that plays a crucial role in various biological processes in humans. It serves as a cofactor for many enzymes involved in metabolism, antioxidant defense, and bone development. The recommended daily allowance (RDA) of manganese for adult men and women is 2.3 mg/day and 1.8 mg/day, respectively, with slightly higher requirements for pregnant and lactating women (4). Manganese is primarily obtained through the diet, with whole grains, nuts, legumes, and leafy green vegetables being rich sources of this nutrient (1-3).

2.1.2 Manganese homeostasis (absorption, distribution, and excretion)

Manganese homeostasis is maintained through the coordinated processes of absorption, distribution, and excretion. A balanced diet and proper regulation of manganese transporters are crucial for ensuring adequate manganese levels in the body and preventing its deficiency or toxicity (35). The absorption of manganese occurs in the intestine. The exact process of manganese absorption remains unclear. Once absorbed, manganese is distributed to different organs and tissues in the body, such as the liver, pancreas, kidneys, and brain. Manganese is transported in the bloodstream primarily bound to proteins, such as albumin and transferrin (36). Manganese is utilized in various cellular processes, including serving as a cofactor for enzymes involved in metabolism, antioxidant defense, and bone development (36). The primary route of manganese excretion is through the bile, which is secreted into the gastrointestinal tract and eventually eliminated in feces (36). A smaller fraction of manganese can be excreted through the urine (36). The excretion of manganese is essential for maintaining proper manganese homeostasis and preventing its hyperaccumulation in the body.

2.1.3 Manganese in tissues

Once ingested, manganese is absorbed in the small intestine through various transporters, then distributed to different organs and tissues, such as the liver, pancreas, kidneys, and brain, where it participates in several critical cellular processes (5). However, the absorption process in the intestine is not clear.

2.1.3.1 Manganese in the liver

The liver is a vital organ for manganese metabolism, as it is involved in the uptake, distribution, and excretion of manganese, as well as in the regulation of manganese homeostasis (36). Following absorption in the small intestine, manganese is transported to the liver through the portal circulation, primarily bound to proteins such as albumin and transferrin (6, 36). In the liver, hepatocytes take up manganese via ZIP14 (24, 37-40). Once inside hepatocytes, manganese can be stored in the form of metalloproteins, such as metallothioneins, or sequestered within intracellular organelles like mitochondria and lysosomes (41). The liver also plays a crucial role in the distribution of manganese to other tissues and organs, either through the release of manganese-bound proteins into the systemic circulation or via the synthesis of manganese-containing enzymes, such as manganese superoxide dismutase (MnSOD) (42). Manganese serves as a cofactor for several enzymes essential for liver function. One of the most critical manganese-dependent enzymes in the liver is MnSOD, which is responsible for neutralizing reactive oxygen species and protecting cells from oxidative damage (42). The liver is the primary organ responsible for manganese excretion, which occurs mainly through biliary secretion (36). Hepatocytes transport manganese into bile canaliculi through ZNT10 (43). Manganese is then secreted into the gastrointestinal tract along with bile and eventually eliminated in feces. Moreover, hepatocytes re-uptake manganese from bile via ZIP8 (12) .

2.1.3.2 Manganese in the brain

The brain is a crucial target for manganese, as it is involved in numerous neurological functions, such as neurotransmitter synthesis, neural development, and protection against oxidative stress (44, 45). Manganese transport into the brain occurs through the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) (46). Several transporters are implicated in manganese uptake across these barriers, including ZNT10, ZIP8 and ZIP14 (8, 47-50). Once in the brain, manganese is distributed to various regions, including the cortex, hippocampus, striatum, and cerebellum (45). Manganese is taken up by neurons and glial cells, where it can be stored in the form of metalloproteins or sequestered within intracellular organelles (51). Manganese can also be transported between brain cells via gap junctions, allowing for the coordination of manganese-dependent processes within the neural network (44). Manganese is also required for the proper function of enzymes such as glutamine synthetase, which plays a role in the regulation of glutamate and glutamine (Gln)/glutamate (Glu) - γ -aminobutyric acid (GABA) neurotransmitter systems, and pyruvate carboxylase, which participates in the tricarboxylic acid cycle and energy metabolism (52-54). Maintaining proper manganese homeostasis in the brain is critical for overall neurological health. Manganese deficiency can lead to impaired synaptic transmission,

reduced cognitive function, and abnormal motor coordination (55). Conversely, excessive manganese levels can cause neurotoxicity, characterized by cognitive deficits, movement disorders, and psychiatric symptoms, collectively referred to as manganism (56-58). Manganism shares some clinical features with Parkinson's disease, such as bradykinesia, rigidity, and tremor, suggesting a potential link between manganese toxicity and neurodegenerative processes (59). Several factors can influence manganese homeostasis in the brain, including dietary intake, environmental exposure, and genetic predisposition. Occupational exposure to manganese through welding or mining activities can lead to excessive manganese accumulation in the brain, increasing the risk of neurotoxicity (60). Genetic mutations affecting manganese transporters, such as DMT1, ZIP8, and ZIP14, can result in disrupted manganese homeostasis and contribute to the development of manganese-related disorders (9, 31, 61).

2.1.3.3 Manganese in the bone

Manganese is a crucial element for maintaining bone health. Similar to zinc, manganese partially stimulates growth through somatomedin activation (62). Bone alterations believed to result from manganese deficiency have been observed in patients receiving total PN (63). Manganese plays a vital role in maintaining adult bone health as well. In oophorectomized rats, those given manganese supplements experienced slower bone loss compared to rats without supplementation (64). The manganese levels in rat bones decrease after oophorectomy but normalize during estradiol treatment (65). Estrogen, in addition to directly inhibiting bone resorption, likely influences manganese deposition. Utilizing an absorption spectrophotometric method, researchers discovered a positive relationship between serum manganese levels and bone mineral density, as well as a negative correlation between serum manganese and the number of fractures in a group of 40 postmenopausal women (66). These findings suggest that manganese may contribute to adult bone health.

2.1.3.4 Manganese in the intestine

The intestine plays a critical role in maintaining manganese homeostasis, as it is the primary site for both absorption and excretion of this essential trace element. Despite its importance, our understanding of manganese metabolism within the intestine remains limited. One crucial finding from earlier research is the identification of the metal transporter ZIP14 as a manganese transporter located on the basolateral side of enterocytes, which are the absorptive cells lining the intestinal epithelium (24). This transporter is responsible for the reuptake of manganese from the bloodstream back into the enterocyte, thereby playing a critical role in regulating manganese levels within the body. In contrast, other metal transporters such as DMT1 have been shown not to be the primary transporters of manganese, despite their roles in the transport of other divalent metal ions (9, 67).

Emerging evidence indicates that manganese has a significant role in promoting gut health and regulating gut microbiota (68). As an essential nutrient for numerous microorganisms, manganese can

affect their immune system and functionality (69). Some studies have revealed that imbalanced gut manganese levels can cause dysbiosis, a condition marked by an unhealthy alteration in the composition and function of the gut microbiota (70). Gut permeability is crucial for regulating systemic levels of microbial metabolites, and manganese overload's impact on gut integrity could increase the entry of bioactive bacterial metabolites into the systemic circulation and eventually the brain (70). Manganese exposure elevates A β and Tau production in the brain, potentially triggering neurotoxicity by increasing inflammation in both peripheral blood and the central nervous system (CNS) (71). Fecal microbiome transplantation from healthy rats may alleviate manganese-induced neurotoxicity by suppressing A β and Tau expression and inhibiting cerebral NLRP3 inflammasome activation (71). Another study group found manganese overload reduced intestinal flora diversity, and transplantation of gut microbiota from normal rats can attenuate neuroinflammation and reduce the number of degenerated neuronal cells by shaping the gut microbiota (72). These imbalances can heighten susceptibility to gastrointestinal infection and inflammation, negatively affecting overall health (71, 73).

Further investigation into the precise mechanisms and regulations of manganese transport across the intestinal epithelium will not only enhance our understanding of manganese homeostasis but also provide valuable insights into potential therapeutic targets for conditions related to manganese deficiency or toxicity. Moreover, an improved understanding of manganese metabolism in the intestine may facilitate the development of more effective dietary interventions and supplementation strategies for individuals at risk of manganese-related health issues. In summary, the intestine is a key tissue involved in maintaining manganese homeostasis through absorption and excretion processes. While previous research has identified ZIP14 as a manganese transporter in enterocytes and excluded DMT1 as a major manganese transporter, further studies are necessary to fully elucidate the complex mechanisms and regulation of manganese metabolism within the intestine.

2.1.4 Manganese and disease

2.1.4.1 Manganese deficiency

Manganese deficiency is relatively rare, as the mineral is widely distributed in various food sources. However, when manganese deficiency does occur, it can lead to a range of health issues, underscoring the importance of adequate manganese intake. Several factors can contribute to manganese deficiency, including poor dietary intake, malabsorption due to gastrointestinal disorders, interactions with other nutrients, and genetic factors that affect the absorption, transport, or utilization of manganese (31, 35). Manganese deficiency can manifest in various ways, depending on the severity and duration of the deficiency. Symptoms and health implications may include impaired bone health, impaired glucose metabolism, neurological issues, dermatological issues, and reproductive issues.

In 2015, the first inherited disorder associated with manganese deficiency was identified, resulting from bi-allelic mutations in *ZIP8* (31, 74). Systemic manganese deficiency manifests in various symptoms, such as developmental delay, intellectual disability, failure to thrive, short stature, dwarfism, cranial asymmetry, seizures, hypotonia, dystonia, strabismus, and deafness. Affected individuals have characteristically low blood manganese levels and display an abnormal glycosylation pattern, consistent with a type II congenital disorder of glycosylation. This can be attributed to the impaired function of manganese-dependent enzymes like β -1,4-galactosyltransferase, which is essential for the galactosylation of glycoproteins (31, 74, 75). Furthermore, *ZIP8* mutations in affected individuals lead to decreased mitochondrial manganese levels, reduced activity of mitochondrial MnSOD, and increased oxidative stress. As an essential trace metal, manganese serves as a cofactor for various enzymes and is a component of metalloenzymes (31, 76). Thus, the pathology of *ZIP8* deficiency can be explained by a multitude of enzyme deficiencies.

Zip8 knockout mice studies have confirmed the transporter's primary role in regulating manganese homeostasis. Loss-of-function in these mice resulted in significantly reduced tissue manganese levels, while levels of zinc (Zn) and iron (Fe) remained unchanged (12). Liver-specific knockout also caused systemic manganese deficiency, indicating the liver as the primary target of *ZIP8* function (12). This hypothesis is supported by the finding that *ZIP8* localizes to the apical surface of hepatocytes, where it absorbs manganese from bile, consequently reducing biliary manganese excretion (12).

2.1.4.2 Manganese overload

Manganese overload can result from various factors, such as occupational exposure, environmental exposure, long-term parenteral nutrition (PN) and genetic predisposition.

Workers in industries like mining, welding, and battery manufacturing may be exposed to high levels of manganese through inhalation of manganese-containing dust or fumes. Additionally, individuals living near industrial areas or consuming water contaminated with high levels of manganese might be at risk for manganese overload (21, 22).

Long-term (PN) is a medical intervention used to provide essential nutrients directly into a patient's bloodstream when oral or enteral feeding is not possible or insufficient. However, when PN is administered for an extended period, excessive manganese levels can accumulate in the body, leading to manganese overload. Manganese overload in the context of long-term PN is due to the fact that the primary route of manganese excretion is through the bile, and the liver is responsible for regulating manganese levels. In patients receiving long-term PN, especially those with compromised liver function, the excretion of manganese can be significantly impaired, leading to the accumulation of the metal in the body (77).

Genetic factors, such as mutations in the *SLC30A10* (*ZNT10*) gene and *SLC39A14* (*ZIP14*) gene, can impair the body's ability to regulate manganese levels, leading to accumulation and overload.

Hypermnangesemia with dystonia 1 (HMNDYT1) is caused by bi-allelic mutations in the *ZNT10* gene and is the first identified inherited manganese transporter defect (78, 79). Over 30 patients have been reported to date, with the disorder characterized by a unique syndrome involving hypermnangesemia, polycythemia, dystonia, chronic liver disease (ranging from asymptomatic steatosis to cirrhosis with liver insufficiency), and depletion of iron stores. Blood manganese levels in these patients are significantly elevated, averaging ten times higher than normal. *ZNT10* is primarily expressed in the liver, gastrointestinal tract, and brain (50, 80). Studies on zebrafish and mice with loss-of-function mutations in *Znt10* exhibit similarities to the human phenotype, including the accumulation of manganese in the blood, liver, and brain (50, 81). Tissue-specific knockout mice studies have shown that, under normal physiological conditions, *ZNT10* efflux activity is required in both the liver and gastrointestinal tract—but not the brain—to maintain normal brain manganese levels. This suggests that in addition to biliary excretion, manganese homeostasis is maintained by luminal excretion of manganese by enterocytes. However, when manganese exposure increases, *ZNT10* activity in the brain is necessary to protect against manganese-induced neurotoxicity (50). Loss-of-function mutations in the *Znt10* gene in zebrafish lead to locomotor abnormalities associated with impaired GABAergic and dopaminergic signaling. Mutant zebrafish exhibit increased expression of *atp2c1*, a Golgi-expressed manganese transporter. It appears that *atp2c1* overexpression protects mutant embryos from manganese toxicity during early development, suggesting a potential treatment target for individuals with manganese overload (81).

In 2016, bi-allelic mutations in *SLC39A14* (*ZIP14*) were identified in individuals who presented with typical features of manganese neurotoxicity. *ZIP14* deficiency is generally characterized by evidence of delay or loss of motor developmental milestones, such as delayed walking or gait disturbance, occurring between six months and three years of age. Early in the disease course, children exhibit axial hypotonia, followed by dystonia, spasticity, dysarthria, bulbar dysfunction, and signs of parkinsonism, including bradykinesia, hypomimia, and tremor (82, 83). Individuals with *ZIP14* mutations display hypermnangesemia but do not exhibit systemic features of manganese overload. Blood levels of iron (Fe), zinc (Zn), and cadmium (Cd), divalent metals that can be transported by *ZIP14* in *in vitro* assays, are normal. Liver MRI results are also normal, indicating the absence of hepatic manganese accumulation (82). The lack of hepatic manganese accumulation in affected individuals suggests that *ZIP14* is primarily required for manganese uptake into the liver for subsequent biliary excretion. The buildup of manganese in the brain appears to be secondary to impaired hepatic uptake of the metal. Loss of *ZIP14* function in mice and zebrafish leads to marked manganese accumulation in the brain, accompanied by impaired locomotor behavior (23, 61, 82, 84). *Zip14*^{-/-} mice exhibit reduced hepatic manganese uptake and excretion into the intestine (23). However, hepatocyte-specific *Zip14* knockout mice do not develop a motor phenotype and have normal brain and serum manganese levels, suggesting that manganese overload does not arise solely from impaired hepatic manganese uptake (40). The intestine-specific *Zip14* knockout mice developed markedly increased manganese levels in their livers and brains. *ZIP14* play a role in reuptaking manganese at the basolateral membrane of enterocytes (24). While *ZIP14* has been shown to transport a range of metals *in vitro* (Mn, Zn, Fe, and Cd), loss of *ZIP14* function in

humans, mice, and zebrafish has little effect on metals other than manganese. This confirms that the primary function of this transporter is the regulation of manganese homeostasis (23, 61, 84). In a study using *Zip14* knockout mice as a model for ZIP14 deficiency, it was found that these mice were born without manganese loading in the brain but began to hyper-accumulate manganese within three weeks after birth. Decreasing manganese intake in *Zip14*^{-/-} mice was effective in preventing manganese overload that typically occurs in these animals (84). This finding indicates that managing manganese intake during early childhood could be a more effective approach to control manganese accumulation.

2.1.4.3 Manganese and cancer

The cGAS-STING pathway (cyclic GMP-AMP synthase—Stimulator of Interferon Genes) is activated by cytoplasmic double-stranded DNA resulting from viral infections (85, 86). Activation of the cGAS-STING pathway necessitates the release of Mn²⁺ from intracellular organelles, most likely mitochondria (86). Mn²⁺ ions bind to cyclic GMP-AMP synthase (cGAS), increasing its sensitivity to double-stranded DNA and promoting the production of the secondary messenger cyclic GMP-AMP (cGAMP). This process leads to NF-κB activation and an antiviral response (86-90). Recent research has demonstrated the involvement of Mn²⁺ in the anti-tumor immune response triggered by the cGAS-STING pathway (91). Mn²⁺ activates both the innate and adaptive branches of the immune system, suppresses metastasis, and enhances the efficacy of immune checkpoint therapy in mice. A phase 1 clinical trial is currently underway to assess the safety and preliminary efficacy of Mn²⁺-primed anti-PD-1 treatment combined with chemotherapy (91).

2.2 ZIP8

2.2.1 ZIP8 and disease

ZIP8, also known as SLC39A8 (solute carrier 39 family, member 8), is a member of the ZIP family of metal-ion transporter proteins. This family is named after zinc-regulated transporter (ZRT) and iron-regulated transporter (IRT)-like proteins. The *ZIP8* gene encodes a multi-transmembrane protein capable of transporting various divalent cations, including selenium cadmium, zinc, iron, and manganese (28, 92-94). The discovery of human diseases associated with *ZIP8* mutations has significantly advanced our understanding of this transporter's physiological function. In 2015, two clinical studies identified *ZIP8* mutations in humans (31, 74). One study reported recurrent homozygous *ZIP8* mutations resulting in manganese deficiency among a group of children (74). Metal level analysis revealed extremely low to non-detectable blood manganese levels in affected individuals. The clinical phenotypes of these children included intellectual disability, developmental delay, hypotonia, strabismus, cerebellar atrophy, and variable short stature. Another study identified compound heterozygous *ZIP8* mutations in two unrelated patients with congenital disorders of glycosylation (CDG) (31). Manganese was not detectable in blood or urine samples from both patients in this second study. Manganese is essential for

glycosyltransferases, and glycosylation profiles from both patients confirmed a severe defect in the glycosylation of transferrin, a marker for plasma proteins. Similar to the first report, the clinical phenotypes of both patients included skull deformities, severe seizures, short limbs, psychomotor retardation, and hearing loss. The identification of *ZIP8* mutations and their associated human diseases emphasizes the importance of this transporter in maintaining manganese homeostasis and its implications for various physiological processes. The ZIP8 transporter is crucial for proper glycosylation, which plays a fundamental role in protein folding, stability, and function. Abnormal glycosylation can lead to a range of severe clinical manifestations, including neurological and developmental disorders. Furthermore, ZIP8 is essential for the proper functioning of the immune system, as manganese plays a vital role in the activation of immune cells and the modulation of inflammatory responses. These findings highlight the need for a deeper understanding of the molecular mechanisms underlying ZIP8 function and regulation, which may contribute to the development of novel therapeutic strategies for patients with ZIP8-associated disorders.

2.2.2 ZIP8's function in manganese metabolism

To investigate the mechanisms and metabolism of ZIP8, researchers employ cell and animal models to understand the underlying processes. Cell models have been crucial in deciphering the metal transport activities of ZIP8, shedding light on the transport function and regulatory mechanisms. A seminal study demonstrated ZIP8's ability to transport Mn, Zn, and Fe in a *Xenopus laevis* oocyte expression system, establishing its role in metal ion homeostasis (32).

Cell models have also been used to explore ZIP8's potential involvement in various disease states. Research has shown that overexpressing ZIP8 in mouse fibroblast cells leads to morphological changes, F-actin reorganization, and increased cell proliferation and migration. In contrast, *ZIP8* knockout in chronic myelogenous leukemia cells results in increased cell-cell adhesion. Abnormal F-actin enrichment is observed in ZIP8 overexpressed lungs. The activation of transcription factors NF- κ B and Snail2 and their targets COL1A2 and E-cadherin is dependent on ZIP8 levels, suggesting that ZIP8 plays a critical role in regulating cell morphology and cytoskeleton via NF- κ B and Snail2 (95). Another study found that pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α), upregulated ZIP8 expression in HEK293 and A549 human cells, indicating a connection between ZIP8 and immune response (96). Immunofluorescence analysis has revealed ZIP8 expression in HEK293T cells to be primarily localized to the plasma membrane and, to a lesser extent, in early endosomes (32). Another study investigated ZIP8 localization in a human choroid plexus papilloma (HIBCPP) cell line, finding it enriched at the apical side of the cell monolayer (49)(97). Understanding the localization of each ZIP protein contributes to our knowledge of Mn transport across the HIBCPP BCB model, helping elucidate the mechanism of Mn homeostasis within the brain, Researchers have employed liver-specific knockout mice models (*Zip8*-LSKO) and inducible global knockout mice models (*Zip8*-iKO) to investigate ZIP8 function and localization in the liver (12). They observed notably reduced manganese levels in multiple organs and whole blood of both mouse models. In contrast, liver-specific overexpression of human ZIP8

(adeno-associated virus-ZIP8 [AAV-ZIP8]) in wild-type mice led to genetic variants at the ZIP8 locus with physiological traits (12).

In conclusion, the hepatic ZIP8 plays a vital role in regulating whole-body Mn homeostasis and Mn-dependent enzyme activity, potentially shedding light on the associations between genetic variants at the ZIP8 locus and various physiological traits. Despite these findings, it is essential to highlight that the function of intestinal ZIP8 is still not well understood. The intestine plays a crucial role in nutrient absorption, and understanding the function of intestinal ZIP8 could provide valuable insights into metal ion transport and homeostasis within this context. Further research is needed to clarify the role of intestinal ZIP8 and its impact on overall organism health and function.

Chapter 3: Methodology

3.1 Animals

All mice were housed in the University of Arizona's laboratory animal facility and the procedures for animal experiments were approved by the Institutional Animal Care and Use Committee. At 3 weeks of age, the mice were split and collected at 9 weeks except *Zip8&UBC* mice. The mice were maintained on a NIH-31 irradiated traditional rodent diet (Teklad 7913; Envigo, Indianapolis, IN, USA). The mice room was set up with 12 hours of light/dark cycles at 21-22 °C. The transgenic mice expressing Cre recombinase under the intestine-specific villin promoter (Vil-Cre mice), and the widespread deletion ubiquitin C (UBC) promoter (UBC-Cre) were purchased from the Jackson Laboratory, while the *Zip14*^{-/-} was purchased from the Mutant Mouse Resource & Research Center. *Zip8 Flox* mice were purchased from the Taconic Biosciences.

The *Zip8 Flox/Flox* mice were crossed with Vil-Cre mice to generate *Zip8 Flox/-&Vil-Cre+/-* mice. These mice were then bred with mice of the same genotype to produce *Zip8 Flox/Flox&Vil-Cre+/-* (*Zip8*-ISKO) mice. The same procedure was followed for *Zip8 Flox/Flox&Alb-Cre+/-* (*Zip8*-LSKO) and *Zip8 Flox/Flox&UBC-Cre+/-* (*Zip8*GKO) mice. To generate black *ZIP14*^{-/-} mice, the *Zip14*^{-/-} mice were bred with black background mice for 10 generations. *Zip8*-ISKO mice were crossed with black *Zip14*^{-/-} mice to generate *Zip8*-ISKO&*Zip14*^{-/-} double knockout mice. To induce widespread deletion of *Zip8*, the *Zip8 Flox/Flox&UBC+/-* mice were given tamoxifen once per day starting at 4 weeks of age and received an intraperitoneal injection (IP injection) for 5 days. The mice tissues were collected at 10 weeks of age. Tamoxifen was dissolved in corn oil at 20mg/ml and administered at a dose of 20ul per gram of mouse body weight.

The mice were anesthetized with ketamine/xylazine and sacrificed at 9 or 10 weeks of age. For blood collection, the blood was directly drawn from the heart using a syringe via cardiac puncture into EDTA-containing tubes and frozen and immediately stored in liquid nitrogen. After blood collection, the mice tissues were collected, rinsed in cold 1X PBS, dried on a clean paper towel, and stored in liquid nitrogen and -80 ° C freezer.

3.2 Genotyping

All mouse tails were collected at 2 to 3 weeks old and genotyped using the Mouse Direct PCR Kit (Bimake, Houston, TX, USA) with the following primers: *Zip14* wildtype forward 5' GGCTTTCAAGTGTGGGGCTTTC 3', *Zip14* wildtype reverse 5' AGAATCCAAAACCTCCCGCCC 3'

Zip14 knockout forward 5'GGCGGCGAATGGGCTG 3', *Zip14* knockout reverse 5' CAGGATCACACCATTGAGGCC 3', *Zip8 Flox* forward 5'CAGGGTTTCTGTGTAACAGG 3', *Zip8 Flox* reverse 5' AGTGTACAGGCTCCAGCTACC 3', *Vil-Cre* forward 5'CGACGGCCACTGCTCTCAC 3', *Vil-Cre* reverse 5'AGGCAAATTTGGTGTACGG 3', *UBC-Cre* transgene forward 5' GACGTCACCCGTTCTGTTG 3', *UBC-Cre*

transgene reverse 5' AGGCAAATTTGGTGTACGG 3', *UBC-Cre* internal positive control forward 5' CTAGGCCACAGAATTGAAAGATCT 3', *UBC-Cre* internal positive control reverse 5' GTAGGTGGAAATTCTAGCATCATCC 3'.

The thermocycle was set up as follows: an initial denaturation step at 94 °C for 300 seconds, followed by 4 cycles of denaturation at 94 °C for 30 seconds, annealing at 64 °C for 30 seconds, and extension at 72 °C for 60 seconds. This was followed by another 4 cycles of denaturation at 94 °C for 30 seconds, annealing at 61 °C for 30 seconds, and extension at 72 °C for 60 seconds. Next, 4 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 60 seconds were performed. Finally, 22 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 60 seconds were performed. The final extension step was performed at 72 °C for 2 minutes, followed by cooling at 4 °C overnight. Following PCR, agarose gel electrophoresis containing 1-2% agarose gel was performed.

3.3 Metal analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

For cell culture samples, cell pellets were lysed using 0.5M NaOH and sonicated on ice. The lysate was mixed with 70% HNO₃, incubated at room temperature overnight and heated at 80°C for 4 hours, and cooled to room temperature. The sample was then adjusted to a 3% HNO₃ solution using M.Q. water and sent to the University of Arizona's Arizona Laboratory for Emerging Contaminants (ALEC) for ICP-MS analysis.

For the mice samples, tissues were weighed in metal-free tubes and heated in an oven until a consistent dry weight was reached at two different time points. Then, 70% HNO₃ was added to the samples, incubated at room temperature overnight, and heated at 80°C for 6 hours. For blood samples, 50ul of blood was added to concentrated HNO₃ and incubated at room temperature overnight. The next day, it was heated at 80°C for 4 hours. After cooling to room temperature, the sample was centrifuged and diluted with M.Q. water to a 3% HNO₃ solution. The sample was then sent to ALEC for ICP-MS analysis.

3.4 Culture of Caco2 cell line

The WT and ZIP14 knockout Caco-2 cells were purchased from Ubigen through a contract. Caco2 cells were grown in DMEM with 10% FBS and 1% Penicillin/Aspirin at 37°C and 5% CO₂. For the Biotinylation experiment, Caco2 WT cells were grown for 22-23 days in a 6-well Transwell plate with media changes and TEER measurements every other day. On the final day, cell membrane isolation was performed using EZ-link Sulfo-NHS-SS-Biotin Sulfosuccinimidyl 2-(Biotinamido) Ethyl-1,3' Dithiopropionate from Thermo (24), while the rest of the samples were analyzed for protein expression using Western blot.

In manganese uptake experiments, 1x10⁶ cells were grown in 6-well plates for 48 hours. After washing with 1XPBS Ca⁺/Mg⁺ twice and aspirating the wells, cells were treated with manganese citrate at different concentrations and times as described. In the dose-dependent experiment, the cells were treated with 0.1 uM or 0.5 uM manganese citrate for 3 hours at 37 C with pH 7.4 uptake medium

(DMEM, 10% pyruvate, 1mM PIPES). In the time-dependent experiment, cells were treated with 0.5 μ M manganese for 1 or 3 hours, and manganese citrate was added after 2 hours in the 1 hour experiment. Following treatment, cells were washed with ice-cold PBS with 1mM EDTA three times before analysis by ICPMS.

3.5 Western blotting analysis

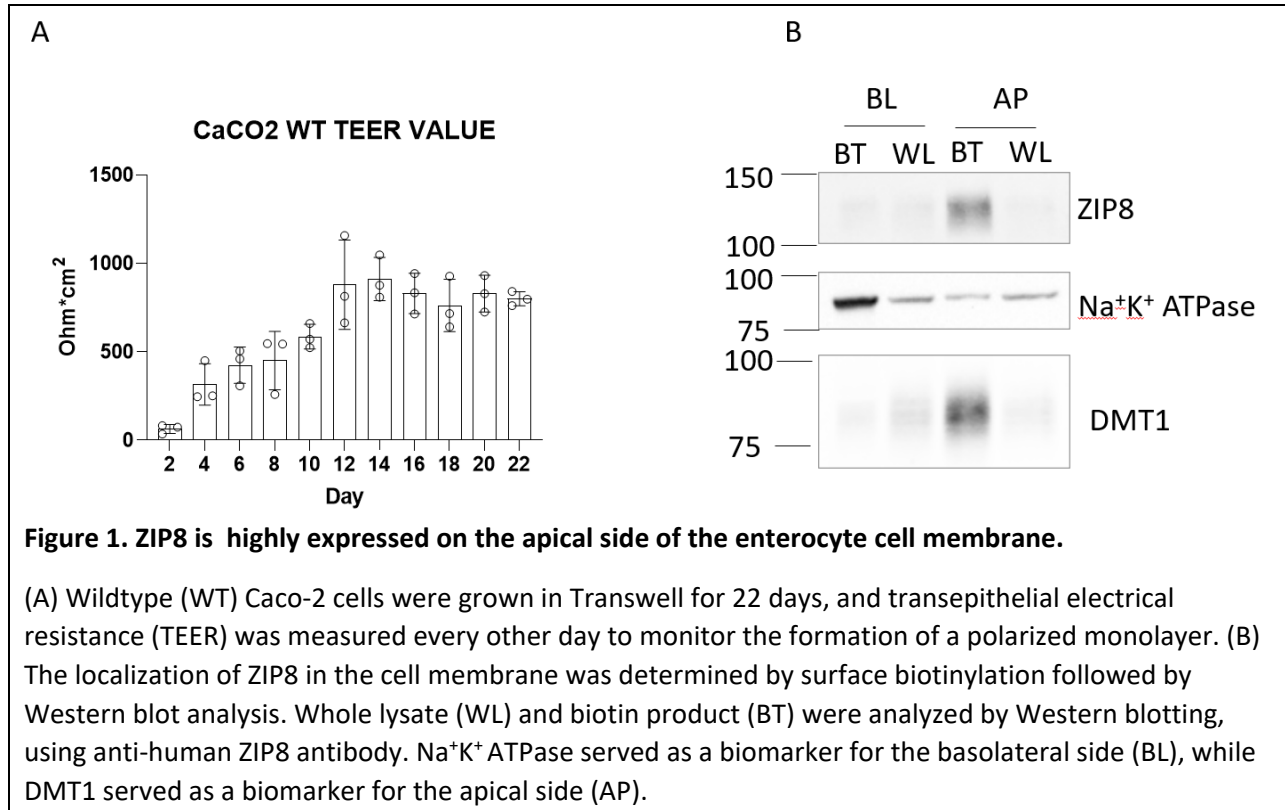
Cells and mouse samples were first lysed with NETT buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% Triton X-100, and 1 \times protease inhibitor mixture (Bimake, Houston, TX), pH 7.4) on ice, and the supernatant was collected after removing the nuclei by centrifugation at 10,000 \times g at 4 $^{\circ}$ C for 10 minutes. The protein concentration of the lysate was measured using the RC DCTM protein assay (Bio-Rad), and then mixed with 6 \times Laemmli buffer and incubated at 37C for 30 minutes. The proteins were separated electrophoretically on an SDS/10% polyacrylamide gel and transferred to nitrocellulose membranes (GVS, Sanford, ME). To block unspecific binding sites, 5% (w/v) nonfat dry milk in TBST (10 mM Tris/HCl, 150 mM NaCl, 0.1% (v/v), 1 ml of Tween 20, pH 7.5) was used. Primary antibodies (rabbit anti-mZIP8 (1:1000), rabbit anti-hZIP8 (1:3000), rabbit anti-mZIP14 (1:1000), rabbit anti-hZIP14 (1:1000)) were added and incubated overnight at 4C. The membrane was washed four times with 1X TBST (5 minutes each) and then incubated with HRP-conjugated goat anti-rabbit secondary antibodies (1:3000) at room temperature for 1 hour. The membrane was washed twice with 1X TBST and twice 1X TBS (5 minutes each), and then developed using enhanced chemiluminescence (SuperSignal West Pico, Thermo Fisher Scientific) and the ChemiDocTM MP Imaging System (Bio-Rad). To ensure equivalent loading, the blots were probed again with HRP-conjugated antibodies against β -ACTIN (1:20,000) and/or GAPDH (1:20,000). For detecting proteins with different molecular weights, the membrane was stripped using Restore PLUS Western blotting stripping buffer (Thermo Fisher Scientific) for 10 minutes, then blocked for 1 hour with blocking buffer, and incubated with another antibody. Mouse anti-Na⁺K⁺-ATPase (1:3000) and anti-DMT1 (1:5000), followed by HRP-conjugated secondary antibodies (1:3000), were used as loading controls for plasma membrane proteins.

3.6 Statistical Analysis

For mice ICPMS result, unpaired t-test was used to analyze the significant differences between the experiment and control groups. Differences in metal levels and protein levels among Caco2 uptake experiment lysate was analyzed using Two-way ANOVA. A *p*-value of less than 0.05 was considered statistically significant with * representing *p* < 0.05, ** representing *p* < 0.01, and *** representing *p* < 0.001. The analysis of data was carried out utilizing PRISM 8 software (GraphPad, La Jolla, CA, USA)

Chapter 4: Result

4.1 ZIP8 is localized to the apical membrane in Caco-2 cells



To determine the localization of ZIP8 in enterocytes, we employed the human colon cell line Caco-2 cells, which is a widely used model for studying intestinal absorption processes. We cultured these cells in Transwell plates for 22 days to facilitate the formation of a polarized monolayer, mimicking the characteristics of the human intestinal epithelium (Fig 1. A). To isolate the cell membrane, we conducted a cell surface biotinylation experiment. This technique allows for the selective labeling of cell surface proteins with biotin, facilitating their isolation and subsequent analysis. Following the isolation of biotinylated proteins, we performed Western blot analysis to detect the presence of ZIP8 in the membrane fractions. Our results demonstrated that ZIP8 is highly expressed in the apical side of the cell membrane in Caco-2 cells (Fig 1. B, SupFig.1). This finding confirms the localization of ZIP8 at the apical side of the intestinal epithelial cells, in line with our hypothesis. By determining the specific location of ZIP8 within the human intestinal epithelial cells, we can better understand its role in manganese absorption and, in turn, how it contributes to the regulation of systemic manganese homeostasis.

4.2 ZIP8 is a manganese importer in Caco-2 cells

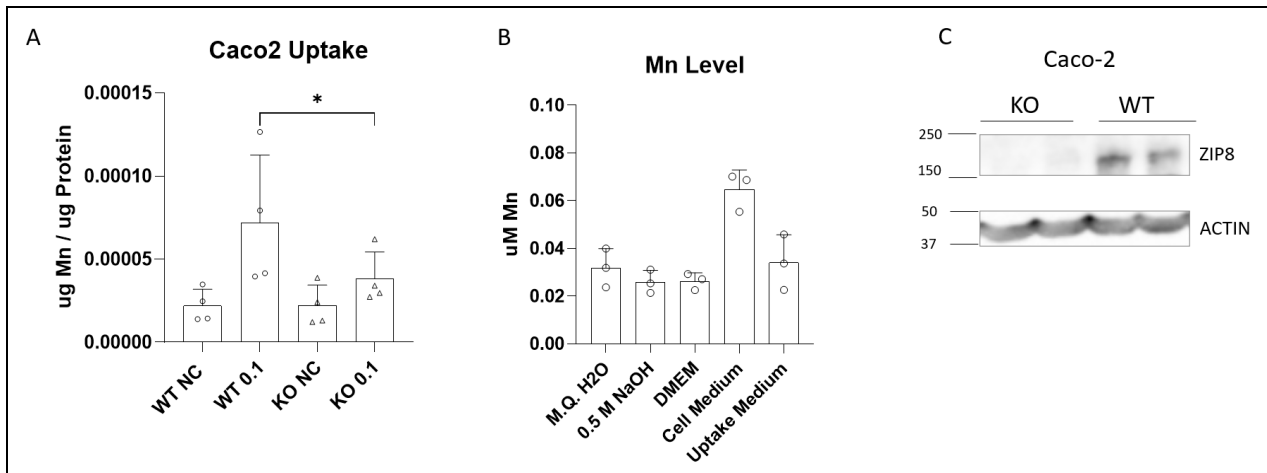
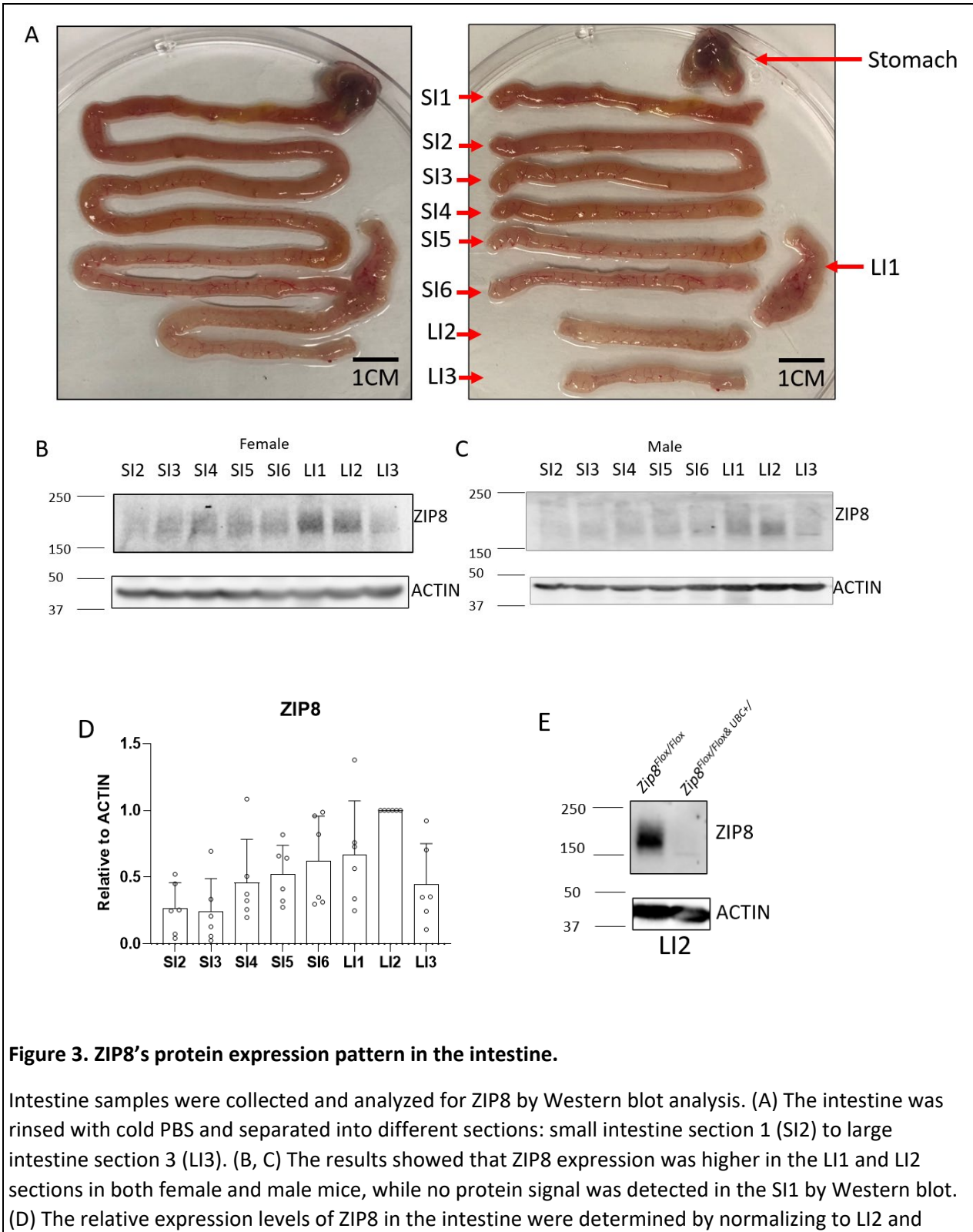


Figure 2. ZIP8 KO in Caco-2 cells decreased cellular manganese accumulation.

Caco-2 wildtype (WT) and ZIP8 knock-out (KO) cells were grown in 10 cm dishes for 2 days or in 6-well plates for 48 hours. (A) Both WT and KO cells were treated with 0.1 μM MnCl_2 for 3 hours, while negative control (NC) were treated with water for 3 hours. (B) Manganese level of each solutions were used in the Mn uptake experiment. (C) The Western blot analysis confirmed the expression of human-ZIP8 protein at a molecular weight of 150 to 250 kDa, ACTIN was used for loading control.

Upon identifying ZIP8 expression on the apical side of Caco-2 cells, we sought to investigate the functional role of ZIP8 in manganese transport, specifically whether it serves as an importer or exporter of manganese. To do so, we designed a manganese uptake experiment using wild-type Caco-2 cells (Caco-2-WT) and ZIP8 knockout cells (ZIP8KO) as our experimental models (Fig. 2C, Sup Fig. 2). We seeded both Caco-2-WT and ZIP8KO cells in 6-well plates and allowed them to grow for 48 hours to establish a confluent monolayer. Following this incubation period, we treated the cells with a 0.1 μM manganese chloride solution for 3 hours to expose them to manganese ions and allow for potential uptake. After the treatment, we collected cell lysates and subjected them to analysis using inductively coupled plasma mass spectrometry (ICP-MS), a highly sensitive and accurate method for measuring trace element concentrations in biological samples. Under baseline conditions, we did not detect any significant differences in manganese levels between Caco-2-WT and ZIP8KO cells (Fig. 2A). Consequently, we introduced 0.1 μM manganese into the cells to investigate potential differences between the WT and ZIP8KO cells after manganese accumulation. We used ICPMS to measure manganese levels in all solutions. The cellular medium contained approximately 0.07 μM manganese (Fig. 2B). To prevent manganese toxicity, we opted to add 0.1 μM manganese for the uptake experiment, a concentration 1.5 times higher than that in the cell medium. Subsequently, we discovered that manganese-treated ZIP8KO cells displayed lower intracellular manganese levels compared to Caco-2-WT cells (Fig. 2A). This finding indicates that the lack of ZIP8 leads to decreased manganese absorption in the Caco-2 cell model. Our findings imply that ZIP8 likely functions as a manganese importer in the human intestinal epithelial cells, contributing to the regulation of systemic manganese homeostasis.

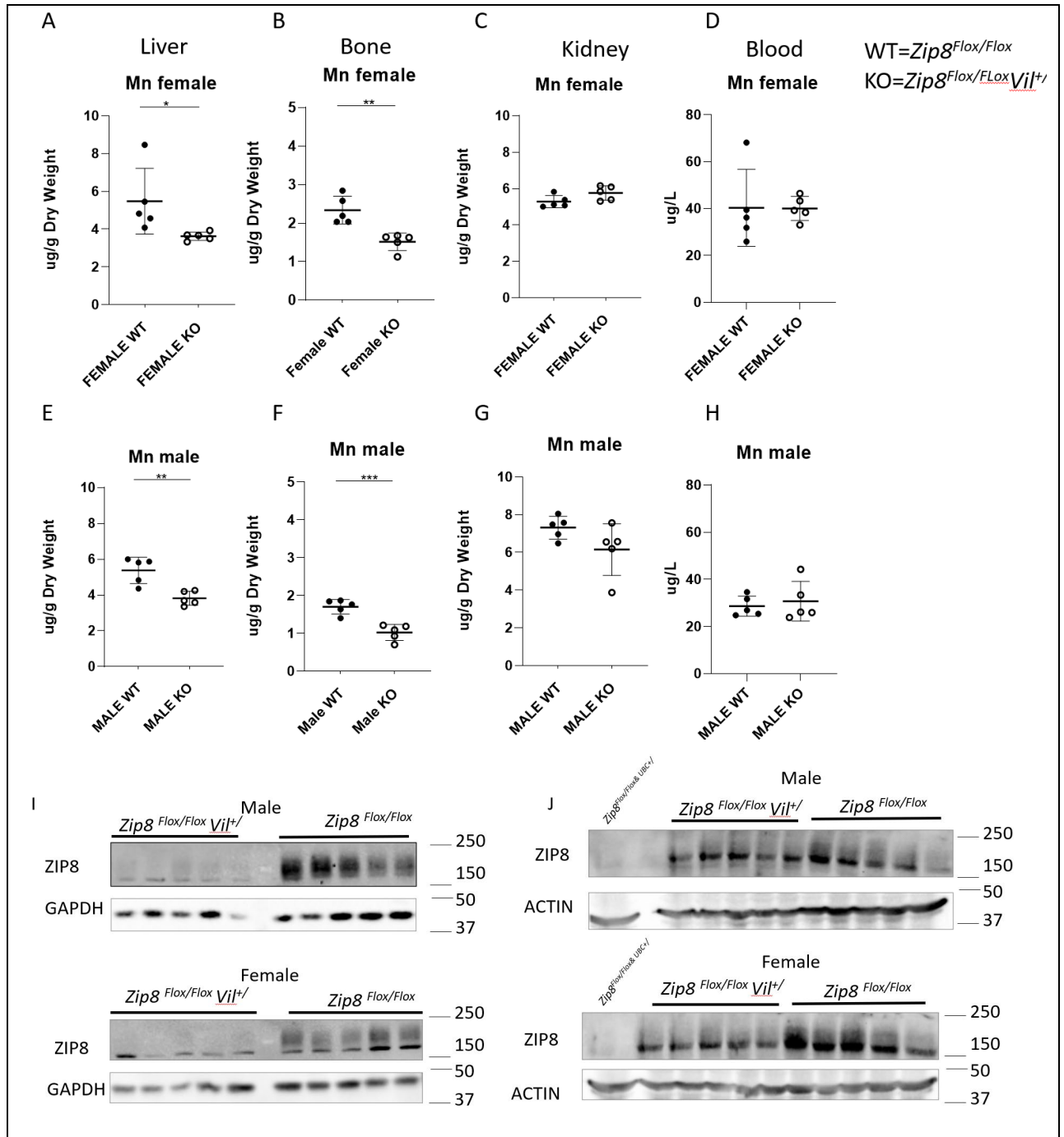
4.3 ZIP8 is highly expressed in the large intestine



expressed as a ratio of ZIP8 to β -Actin (n=6). (E) The Western blot analysis confirmed that the specific band detected at a molecular weight of 150 to 250 kDa represents ZIP8.

ZIP8 has been identified as a critical regulator of manganese transport in cell membranes, potentially impacting overall manganese homeostasis within the body. To further explore the distribution of this membrane protein in the intestinal tract, we collected mouse intestine samples and performed a comprehensive analysis using western blotting. In this analysis, the mouse ZIP8 antibodies were detected at 150 kDa in the western blot (Fig. 3E), allowing us to determine the specific regions within the intestine where ZIP8 expression is. Through our analysis, we discovered that ZIP8 expression was present in various sections of the intestine, spanning from the second segment of the small intestine (SI2) to the third segment of the large intestine (LI3) (Fig. 3B, C). The ZIP8 protein expression was unable to detect in the intestine using Western blot, specifically in section 1, the duodenum (Supplementary Fig. 3E, F). By. The duodenum is the part of the small intestine closest to the stomach, characterized by its low pH values, ranging from approximately 4.74 (fasted) to 4.87 (fed) (97). These acidic conditions may hinder the detection of ZIP8 protein expression in this region. Interestingly, ZIP8 expression was not detected in the first segment of the small intestine (SI1) using western blotting (Supplementary Fig. 3E, F). By comparing the relative expression of ZIP8 to β -Actin, a commonly used loading control, we found that ZIP8 expression was especially pronounced in the first and second segments of the large intestine (LI1 and LI2) (Fig. 3D, Supplementary Fig. 3-4). These findings underscore the importance of intestinal ZIP8 in the regulation of systemic manganese homeostasis, as the protein's distribution throughout the intestinal tract suggests a significant role in the absorption and transport of manganese.

4.4 *Zip8* intestine-specific knock out mice (*Zip8*-ISKO) have decreased manganese levels in the liver and bone



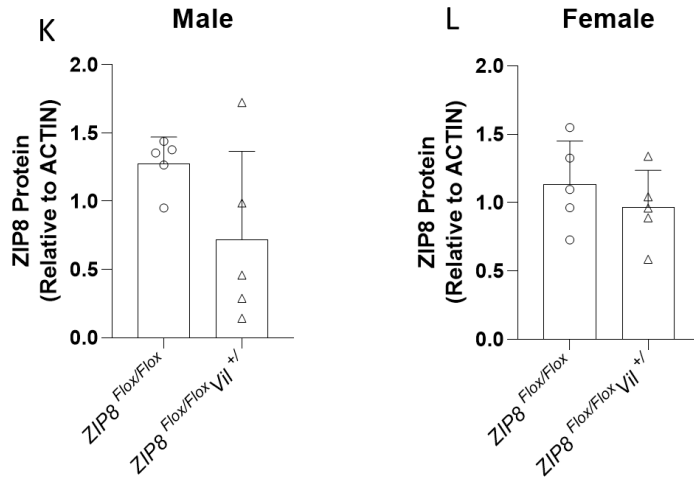


Figure 4. *Zip8* intestine-specific knock out (*Zip8*-ISKO, *Zip8*^{Flox/Flox Vil^{+/-}}) decreased manganese levels in the liver and bone.

Levels of manganese in the liver, bone, kidney, and blood were measured in 9-week-old female (panels A to D, n=5) and male (panels E to F, n=5) *Zip8*^{Flox/Flox} and *Zip8*^{Flox/Flox Vil^{+/-}} mice. Statistical analysis was performed using an unpaired t-test, with * indicating $p < 0.05$, ** indicating $p < 0.01$, and *** indicating $p < 0.001$. The Western blot analysis in panels (I) confirmed the deletion of intestinal ZIP8 in the *Zip8*-ISKO mice, while GAPDH was used as loading control. We also confirmed that intestinal inactivation of ZIP8 does not alter ZIP8 in the liver (J). ZIP8 expression in the liver was quantified relative to ACTIN in both male (K) and female (L) mice.

To elucidate the role of ZIP8 in intestinal manganese absorption, we generated intestine-specific *Zip8* knockout (*Zip8*-ISKO) mice by crossing *Zip8 Flox* mice with *Vil-Cre* mice. These mice were subsequently sacrificed at 9 weeks of age, and various tissues, including the kidney, blood, liver, and bones, were collected for inductively coupled plasma mass spectrometry (ICP-MS) analysis to measure manganese levels. To ensure successful generation of *Zip8*-ISKO mice, we first confirmed protein expression using western blotting. The results demonstrated that ZIP8 expression was successfully knocked down in the intestines of *Zip8*-ISKO mice (Fig. 4I, Sup Fig. 6A), while *Zip8* expression in the liver remained unchanged (Fig. 4J, Sup Fig. 6C). This observation provides evidence that the targeted deletion of *Zip8* was specific to the intestinal tissue, thus allowing us to investigate the role of *Zip8* in enterocyte manganese absorption. Following the confirmation of *Zip8* knockout, we proceeded to measure manganese levels in the kidney, blood, liver, and bones using ICP-MS (Fig. 4 A-H). Our analysis revealed that both male and female *Zip8*-ISKO mice displayed significantly decreased manganese levels in the bones and liver when compared to their wild-type counterparts (Fig. 4 A, B, E, F). This result suggests that ZIP8 functions as a manganese importer in enterocytes, playing a crucial role in maintaining systemic manganese homeostasis. The generation and characterization of *Zip8*-ISKO mice have allowed us to gain valuable insights into the physiological role of ZIP8 in intestinal manganese absorption. By demonstrating a marked decrease in manganese levels in key tissues of *Zip8*-ISKO mice, our findings support the hypothesis that ZIP8 functions as a manganese importer in enterocytes.

4.5 *Zip8*-ISKO&*Zip14*^{-/-} double knock out mice (DKO) have decreased manganese levels in the brain and blood compared with *Zip14*^{-/-} mice

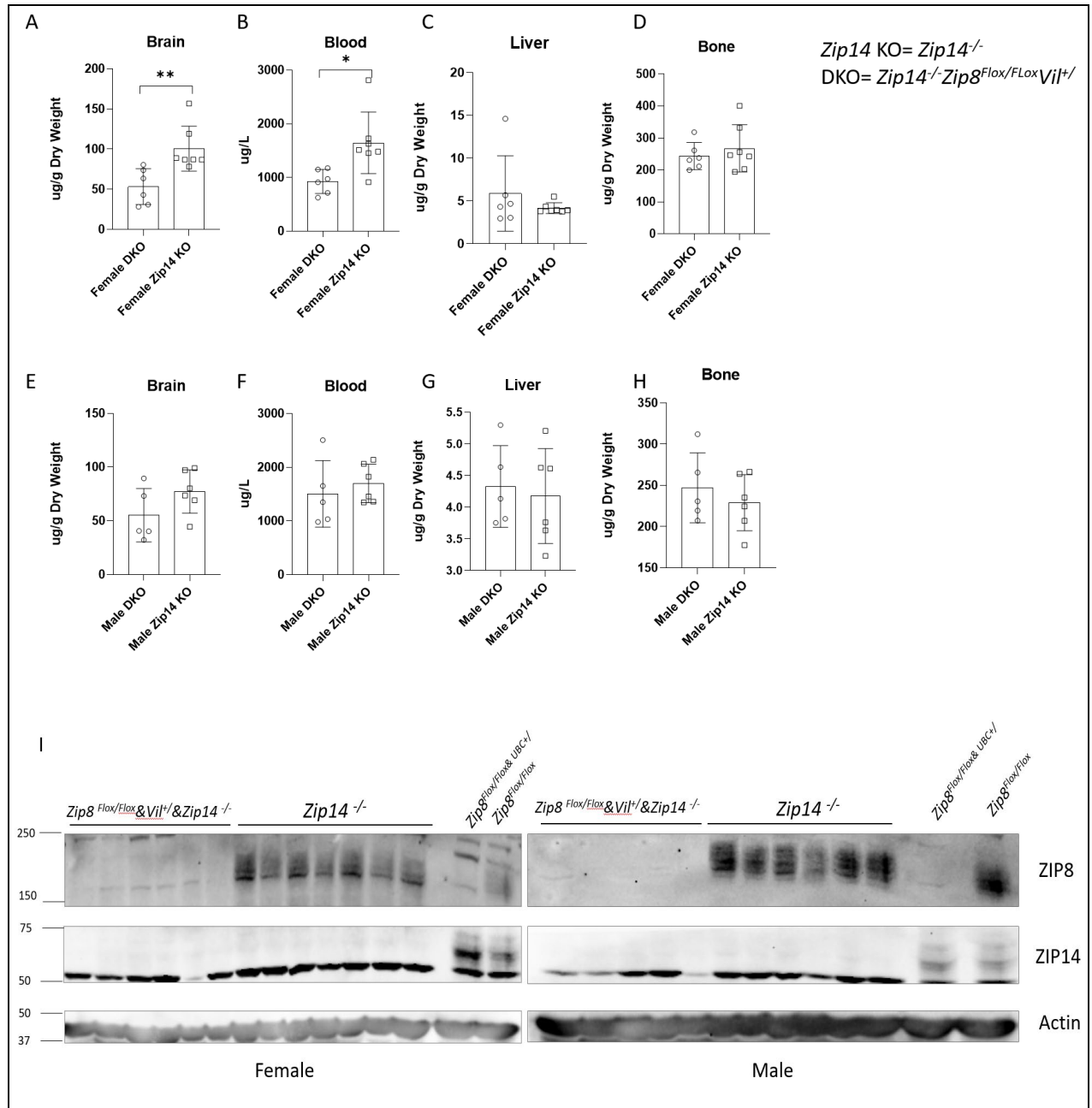


Figure 5. Deletion of *Zip8* in the intestine ameliorate manganese loading in *Zip14*^{-/-} female mice.

The double knock-out (DKO) mice were generated by crossing *Zip8* intestine-specific knock-out (*Zip8*-ISKO) mice with *Zip14* knock-out mice (*Zip8*-ISKO&*Zip14*^{-/-} mice). Levels of manganese in the brain, blood, liver, and bone were measured in 9-week-old female (panels A to D, n=6~7) and male (panels E to

F, n=5~6) mice. Statistical analysis was performed using an unpaired t-test, with * indicating $p < 0.05$, ** indicating $p < 0.01$, and *** indicating $p < 0.001$. The Western blot analysis in panel (I) confirmed the inactivation of intestinal ZIP8 in the DKO mice.

In an attempt to further investigate the ZIP8 in manganese homeostasis, we generated a double knockout (DKO) mouse model by crossing *Zip8*-ISKO mice with *Zip14*^{-/-} mice. Loss of ZIP14 function have been previously reported to result in manganese overload in the brain and blood in mice (23, 84). Similar to the *Zip8*-ISKO mice, these DKO mice were sacrificed at 9 weeks of age, and various tissues were collected for ICP-MS manganese level measurements (Fig. 5A-H) and western blot analysis (Fig. 5I, Sup Fig. 7). The creation of this DKO mouse model allowed us to explore the combined effects of *Zip8* and *Zip14* deletion on systemic manganese homeostasis. Our research determined that in female DKO mice, both brain and blood manganese levels were significantly lower compared to control mice (Fig. 5A, B). In male DKO mice, average manganese concentrations in the brain and blood also decreased relative to control mice (Fig 5 E, F). These findings suggest that the absence of ZIP8 in the intestine reduces the body's manganese load in a setting marked by manganese overload due to the *Zip14* mutation. This evidence strongly supports the conclusion that ZIP8 deletion in Caco-2 cells leads to diminished manganese absorption. Moreover, the creation of the DKO mouse model has facilitated a deeper insight into the intricate relationship between ZIP8 and ZIP14 in regulating manganese balance. Our results from the DKO mouse model, particularly in female mice, emphasize the significant role of ZIP8 in controlling intestinal manganese absorption. In situations of manganese overload due to Zip14 deficiency, the loss of ZIP8 function seems to alleviate the excessive manganese load on the body in female mice, suggesting that further investigation is required to fully understand the sex-specific differences. This observation cautiously highlights the potential therapeutic value of targeting ZIP8 in conditions related to manganese imbalance, taking into account the varying impact across sexes.

Chapter 5: Discussion

The present study aimed to elucidate the role of intestinal ZIP8 in manganese homeostasis regulation. To investigate the function and localization of ZIP8, we utilized both human cell models and mouse models for our experiments. Caco-2 cell cultures serve as an essential model system for understanding the dynamics of nutrient transport in the intestinal epithelium. By employing genetically modified Caco-2 cell lines, researchers can gain valuable insights into metal ion uptake and transport mechanisms.

We cultured Caco-2 cells until they formed a polarized monolayer and collected the cell membrane using biotinylation. Subsequently, we analyzed the location of ZIP8 through Western blot analysis. Transepithelial electrical resistance (TEER) measurements were utilized to verify the formation of a Caco-2 cell monolayer in the Transwell plate. Over a period of 2-3 weeks, Caco-2 cells develop a dense, spontaneously differentiated layer of polarized enterocytes and a monolayer of columnar cells. These layers are interconnected by tight junction protein complexes, which restrict the diffusion of substances across the barrier. The Caco-2 monolayer typically exhibits a TEER ranging from 500-1100 $\Omega\cdot\text{cm}^2$ (98, 99).

Through a biotinylation experiment, we confirmed the localization of ZIP8 on the apical side of Caco-2 cells. This groundbreaking discovery marks the first experimental evidence demonstrating the localization of ZIP8 in enterocytes. Mouse intestines were also used for immunohistochemistry and immunofluorescence to determine the localization of ZIP8 in mice. However, we were unable to obtain definitive results for ZIP8 localization in mouse villi. This could be attributed to the antibody's inefficacy, or the signal for "ZIP8" may be a nonspecific band, similar to the western blot results (Sup Fig.3,4).

Then, we detected the metal level between Caco-2 WT and ZIP8 knockout cell to confirm the manganese changed. The cell lysate inductively coupled plasma mass spectrometry (ICP-MS) results revealed lower manganese levels in the ZIP8 knockout Caco-2 cell lysate following the manganese experiment. This finding suggests that ZIP8 could function as a manganese importer on the apical side of enterocytes. The 0.1 μM MnCl_2 concentration was 1.5 times greater than that of the cell medium and 2.5 times greater than the uptake medium (Fig. 2B). The normal range for manganese levels in the blood is between 4 and 15 ng/mL (0.073 to 0.273 μM) according to the Agency for Toxic Substances and Disease Registry (ATSDR) (100). Our prior radioactive manganese 54 experiment demonstrated that using 0.1 μM manganese is a safe level to prevent cell death {Scheiber, 2019 #3}. This experiment provides evidence supporting the hypothesis that ZIP8 is the one of primary transporter responsible for mediating manganese uptake in enterocytes. Further research is necessary to elucidate the precise mechanisms by which ZIP8 regulates manganese uptake and transport in enterocytes. Examining the interactions between ZIP8 and other transporters, as well as the modulation of ZIP8 expression under various physiological and pathological conditions, will provide valuable insights into its role in maintaining manganese homeostasis.

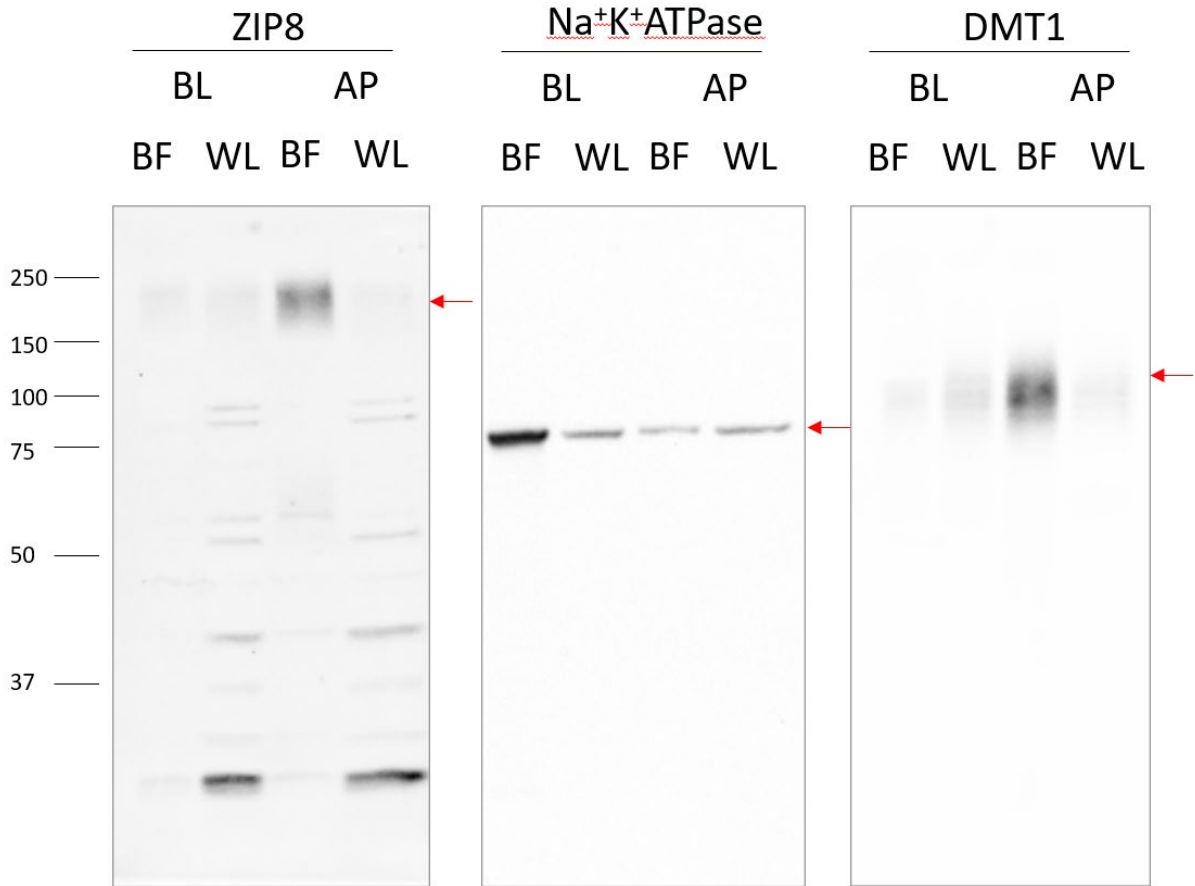
We continued to investigate ZIP8 expression in mouse intestines by dividing the intestines into distinct sections and identifying ZIP8 expression in all areas except for the small intestine section 1 (SI1), which corresponds to the duodenum (Supplementary Fig. 3E, F). One potential reason for this absence might be the lack of ZIP8 expression in SI1. However, a research group has detected both mRNA and protein levels of ZIP8 in the human duodenum {Balusikova, 2022 #148}, and another study group detected the mRNA levels of ZIP8 in the mouse duodenum {Girijashanker, 2008 #147}. Consequently, our inability to detect protein expression in SI1 could be due to technical limitations. Next, we generated mouse models to examine ZIP8's function in vivo. Our results present compelling evidence that ZIP8 plays a vital role in regulating dietary manganese absorption in vivo. Intestine-specific *Zip8* knockout mice exhibit reduced manganese levels in the liver and bone, suggesting that enterocyte-specific knockout of *Zip8* leads to manganese deficiency. However, these mice do not show decreased blood manganese concentrations at nine weeks of age, likely because other manganese transporters continue to regulate manganese homeostasis, and the control system remains functional (101). Manganese may be mobilized from storage tissues, such as the liver and bones, to maintain manganese levels in other tissues, including the kidney. These results are similar to other *Zip8* mice resulting in liver-specific knock out mice and global knock out mice which are decreased the manganese absorption in the body (12).

In the double knockout (DKO) mouse model, the *Zip8* intestine-specific knockout mice were used to determine its effect on manganese contents of *Zip14*^{-/-} mice, an animal model for manganese overload. The female DKO mice displayed significantly decreased manganese levels in the brain and blood, supporting the notion that *Zip8*-ISKO can reduce manganese loading is under manganese overload conditions. This finding suggests that for future clinical studies of manganese overload patients, decreasing manganese intake may also reduce manganese accumulation in the brain and blood. However, no differences in manganese levels were observed in the liver and bones between *Zip14*^{-/-} and DKO male mice. Three potential explanations exist for this observation: firstly, *Zip8*-ISKO is not a complete knockout but rather a substantial knockdown. Some mice still exhibit ZIP8 antibody signals in the western blot results (Fig. 4 I,J, Fig. 5 I), suggesting that certain levels of ZIP8 may remain functional in manganese uptake. The second reason could be that other metal transporters, such as ZNT10, DMT1, or others, assist manganese uptake on the apical side of enterocytes. Investigating these metal transport proteins could be a future research direction to better understand manganese transport function and metabolism(43, 67). The third reason could be that there is sex-specific effect for manganese absorption. A study has investigated the sex-specific effects of manganese exposure on the gut microbiome in C57BL/6 mice (102). This study revealed that manganese exposure led to a significant change in the gut microbiome composition, with distinct alterations observed in male and female mice. Notably, the manganese ATP-binding cassette (ABC) transporters and multicopper oxidase genes encoding multicopper oxidase, which oxidizes Mn²⁺ to Mn³⁺, exhibited a significant increase in females, but a decrease in males. These findings suggest that the gut bacteria in male and female animals may have different capacities or responses to mediate Mn utilization and oxidation. The observed sex-specific differences emphasize the importance of considering sex as a factor when assessing the impact of manganese on gut microbiome dynamics and potential health consequences of such exposure. However, the exact relationship between the microbiome and manganese absorption remains unclear and warrants further investigation.

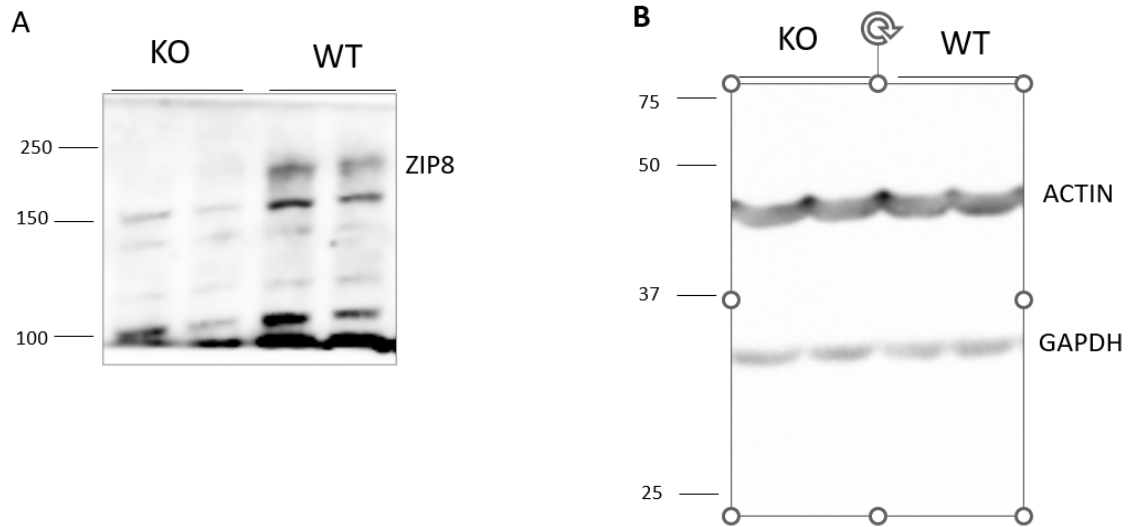
As manganese is an essential trace element, it is crucial to maintain proper homeostatic control of its levels within the body. Dysregulation of manganese metabolism can lead to various pathological conditions, including neurodegenerative diseases and developmental disorders. Therefore, a deeper understanding of the molecular mechanisms governing manganese uptake, transport, and excretion is vital for the development of targeted therapies and preventive measures for these disorders. In addition to its role in manganese transport, ZIP8 has been implicated in the transport of other divalent metal ions, such as iron, zinc, and cadmium(28, 92-94). Exploring the interplay between ZIP8 and other metal transporters, as well as the relative contributions of these proteins to the overall metal ion balance in the body, will be crucial for understanding the complexities of metal ion homeostasis. It is also important to consider the potential involvement of ZIP8 in the transport and homeostasis of other trace elements and metal ions. Although the current study focuses on manganese, the ability of ZIP8 to transport other metals suggests that it may have broader implications in trace element metabolism. Further research is needed to fully understand the role of ZIP8 in the homeostasis of other essential trace elements and to determine whether alterations in ZIP8 function can lead to imbalances in multiple trace elements.

In summary, we demonstrate that ZIP8 plays a role in mediating manganese uptake from the apical membrane of enterocytes. Furthermore, our *in vivo* data strongly support the idea that intestinal ZIP8 plays a significant role in maintaining systemic manganese homeostasis. A better understanding of ZIP8's function and its interactions with other potential manganese transporters will contribute to a more comprehensive understanding of manganese metabolism and may inform future strategies for managing manganese-related disorders.

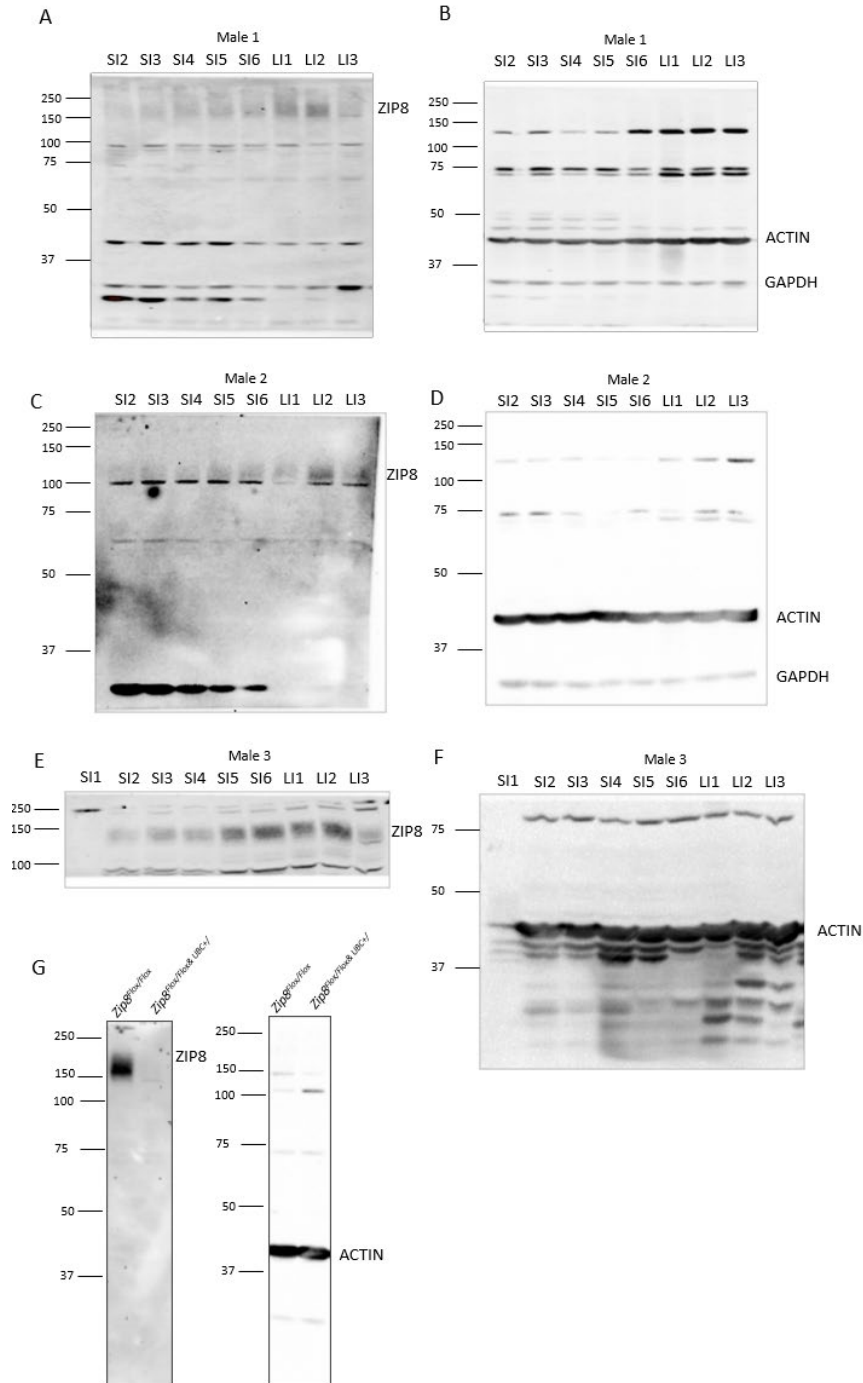
Chapter 6: Supplemental Figures



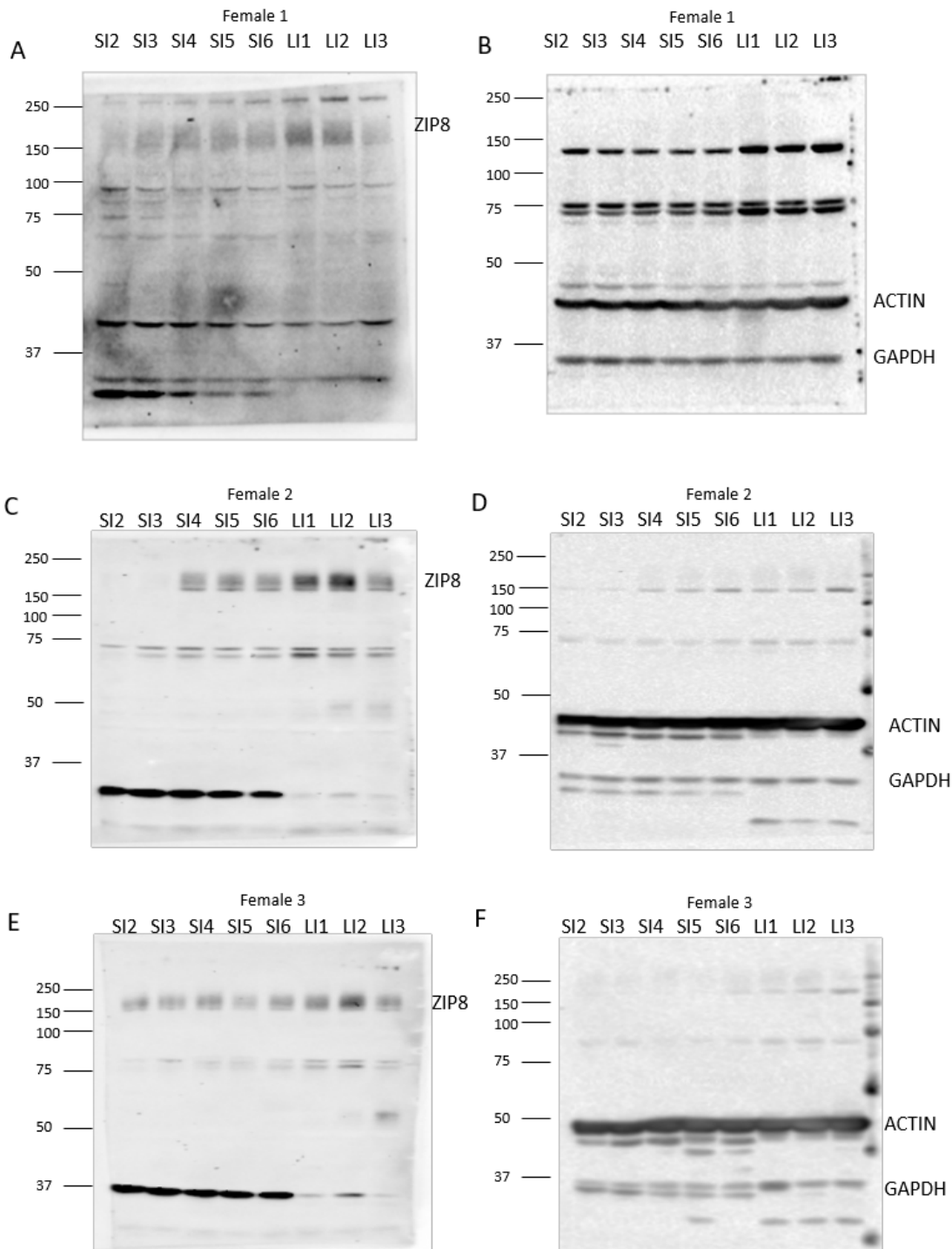
Supplemental Figure 1. ZIP8 is highly expressed in the apical side of the cell membrane. The localization of ZIP8 in the cell membrane was determined by surface biotinylation followed by Western blot analysis. Whole lysate (WL) and biotin product (BT) were analyzed by Western blot, using anti-human ZIP8 antibody. Na⁺ K⁺ ATPase served as a biomarker for the basolateral side (BL), while DMT1 served as a biomarker for the apical side (AP).



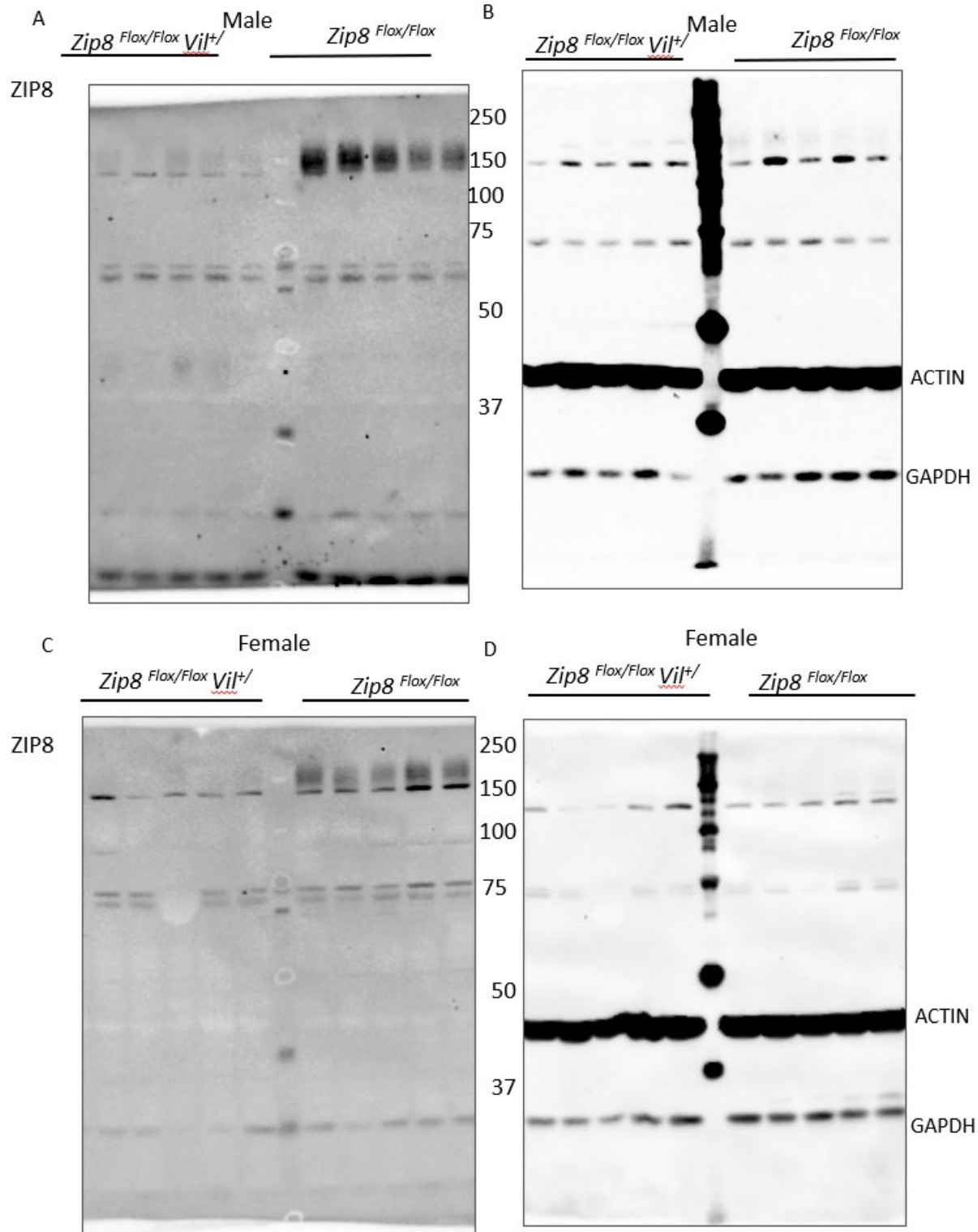
Supplemental Figure 2. ZIP8 expression in Caco-2 cells. The figure compares ZIP8 expression between wildtype (WT) and *ZIP8* knockout (KO) Caco-2 cells. Panel (A) presents the Western blot analysis, which demonstrates the presence of human-ZIP8 protein within the molecular weight range of 150-250 kDa in Caco-2 cells. Panel (B) shows the loading controls, with ACTIN and GAPDH used to ensure equal sample loading across the lanes.



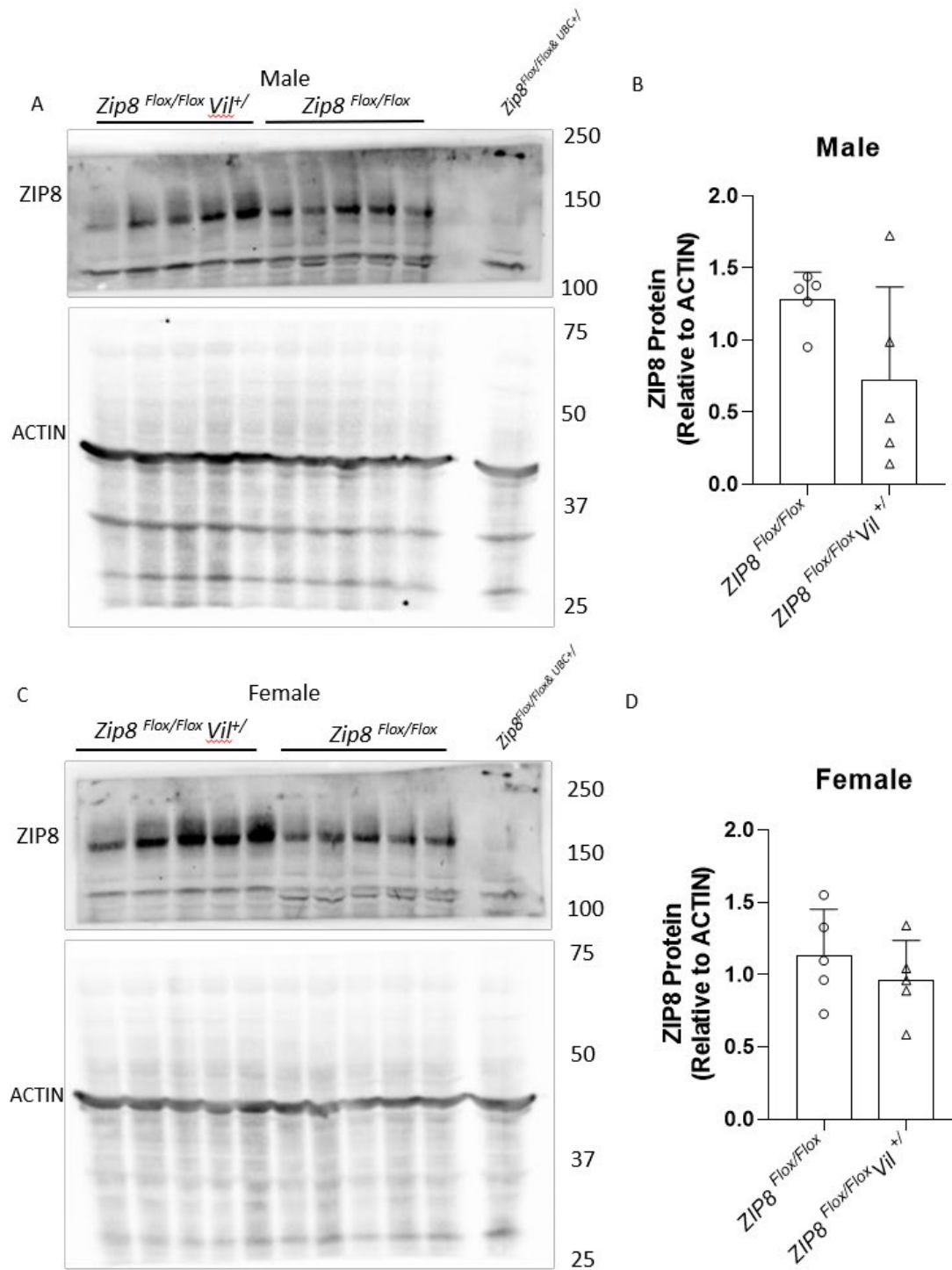
Supplemental Figure 3. ZIP8 expression levels in the small intestine (SI2) and large intestine (LI3) of wild-type male mice. The expression levels of ZIP8 were measured in the SI1 and LI3 sections of wild-type male mice using a method such as Western blot analysis. The results show the relative levels of ZIP8 expression in the different sections of the intestine (A-F). Panel (G) presents the Western blot analysis, which demonstrates the presence of mouse-ZIP8 protein within the molecular weight range of 150-250 kDa in LI2 in the mice. ACTIN and GAPDH were used to ensure equal sample loading across the lanes.



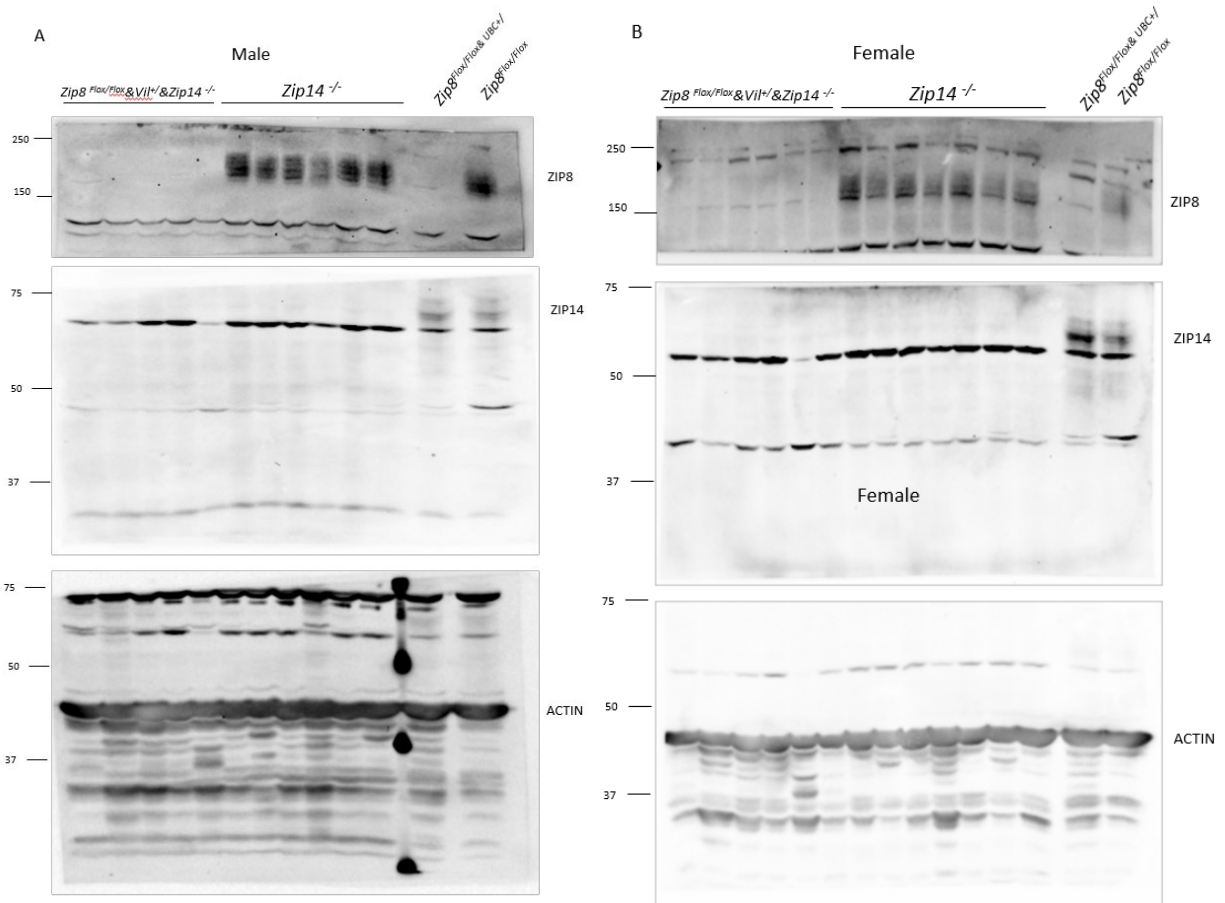
Supplemental Figure 4. ZIP8 expression levels in the small intestine (SI2) and large intestine (LI3) of wild-type female mice. The expression levels of ZIP8 were measured in the SI1 and LI3 sections of wild-type male mice using a method such as Western blot analysis. The results show the relative levels of ZIP8 expression in the different sections of the intestine(A-F). ACTIN and GAPDH were used to ensure equal sample loading across the lanes.



Supplemental Figure 5: Confirmation of intestinal ZIP8 deletion in *Zip8*-ISKO mice. The Western blot analysis demonstrates the deletion of intestinal ZIP8 in *Zip8*-ISKO mice. GAPDH was used as a loading control to ensure equal sample loading across the lanes. Panel (A-B) represents male mice (n=5), and panel (C-D) represents female mice (n=5).



Supplemental Figure 6: Effect of intestinal ZIP8 inactivation on liver ZIP8 expression in *Zip8*-ISKO mice. The figure demonstrates that intestinal inactivation of ZIP8 does not alter ZIP8 expression in the liver. Liver ZIP8 expression is shown for both *Zip8*-ISKO and control mice in male (A) and female (C) mice (n=5). ZIP8 expression in the liver was quantified relative to ACTIN for both male (B) and female (D) mice.



Supplemental Figure 7: Confirmation of intestinal ZIP8 and ZIP14 inactivation in DKO mice. The Western blot analysis demonstrates the inactivation of both intestinal ZIP8 and ZIP14 in the double knockout (DKO) mice. Panel (A) represents male mice (n=5~6), and panel (B) represents female mice (n=6~7). ACTIN was used as a loading control to ensure equal sample loading across the lanes.

Chapter 7: Reference

1. Sutton DC, Kelly JJ. Manganese-54: fractional distribution in wheat and occurrence in other foods. *Nature*. 1966;209(5028):1081-3.
2. Pennington JA, Young BE. Total diet study nutritional elements, 1982-1989. *J Am Diet Assoc*. 1991;91(2):179-83.
3. Freeland-Graves JH, Turnlund JR. Deliberations and evaluations of the approaches, endpoints and paradigms for manganese and molybdenum dietary recommendations. *J Nutr*. 1996;126(9 Suppl):2435S-40S.
4. Trumbo P, Yates AA, Schlicker S, Poos M. Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *J Am Diet Assoc*. 2001;101(3):294-301.
5. Rahil-Khazen R, Bolann BJ, Myking A, Ulvik RJ. Multi-element analysis of trace element levels in human autopsy tissues by using inductively coupled atomic emission spectrometry technique (ICP-AES). *J Trace Elem Med Biol*. 2002;16(1):15-25.
6. Harris WR, Chen Y. Electron paramagnetic resonance and difference ultraviolet studies of Mn²⁺ binding to serum transferrin. *J Inorg Biochem*. 1994;54(1):1-19.
7. Bornhorst J, Wehe CA, Huwel S, Karst U, Galla HJ, Schwerdtle T. Impact of manganese on and transfer across blood-brain and blood-cerebrospinal fluid barrier in vitro. *J Biol Chem*. 2012;287(21):17140-51.
8. McCabe SM, Zhao N. The Potential Roles of Blood-Brain Barrier and Blood-Cerebrospinal Fluid Barrier in Maintaining Brain Manganese Homeostasis. *Nutrients*. 2021;13(6).
9. Wolff NA, Garrick MD, Zhao L, Garrick LM, Ghio AJ, Thevenod F. A role for divalent metal transporter (DMT1) in mitochondrial uptake of iron and manganese. *Sci Rep*. 2018;8(1):211.
10. Papavasiliou PS, Miller ST, Cotzias GC. Role of liver in regulating distribution and excretion of manganese. *Am J Physiol*. 1966;211(1):211-6.
11. Madejczyk MS, Boyer JL, Ballatori N. Hepatic uptake and biliary excretion of manganese in the little skate, *Leucoraja erinacea*. *Comp Biochem Physiol C Toxicol Pharmacol*. 2009;149(4):566-71.
12. Lin W, Vann DR, Doulias PT, Wang T, Landesberg G, Li X, et al. Hepatic metal ion transporter ZIP8 regulates manganese homeostasis and manganese-dependent enzyme activity. *J Clin Invest*. 2017;127(6):2407-17.
13. Chen P, Bornhorst J, Aschner M. Manganese metabolism in humans. *Front Biosci (Landmark Ed)*. 2018;23:1655-79.
14. Li C, Zhou HM. The role of manganese superoxide dismutase in inflammation defense. *Enzyme Res*. 2011;2011:387176.
15. Wan Y, Fang J, Wang Y, Sun J, Sun Y, Sun X, et al. Antibacterial Zeolite Imidazole Frameworks with Manganese Doping for Immunomodulation to Accelerate Infected Wound Healing. *Adv Healthc Mater*. 2021;10(22):e2101515.
16. Sidoryk-Wegrzynowicz M, Aschner M. Role of astrocytes in manganese mediated neurotoxicity. *BMC Pharmacol Toxicol*. 2013;14:23.
17. Studer JM, Schweer WP, Gabler NK, Ross JW. Functions of manganese in reproduction. *Anim Reprod Sci*. 2022;238:106924.
18. Gong JH, Lo K, Liu Q, Li J, Lai S, Shadyab AH, et al. Dietary Manganese, Plasma Markers of Inflammation, and the Development of Type 2 Diabetes in Postmenopausal Women: Findings From the Women's Health Initiative. *Diabetes Care*. 2020;43(6):1344-51.

19. Caldwell RW, Rodriguez PC, Toque HA, Narayanan SP, Caldwell RB. Arginase: A Multifaceted Enzyme Important in Health and Disease. *Physiol Rev.* 2018;98(2):641-65.
20. Holley AK, Dhar SK, Xu Y, St Clair DK. Manganese superoxide dismutase: beyond life and death. *zip14Amino Acids.* 2012;42(1):139-58.
21. Boim AGF, Wragg J, Canniatti-Brazaca SG, Alleoni LRF. Human intestinal Caco-2 cell line in vitro assay to evaluate the absorption of Cd, Cu, Mn and Zn from urban environmental matrices. *Environ Geochem Health.* 2020;42(2):601-15.
22. Santamaria AB. Manganese exposure, essentiality & toxicity. *Indian J Med Res.* 2008;128(4):484-500.
23. Aydemir TB, Kim MH, Kim J, Colon-Perez LM, Banan G, Mareci TH, et al. Metal Transporter Zip14 (Slc39a14) Deletion in Mice Increases Manganese Deposition and Produces Neurotoxic Signatures and Diminished Motor Activity. *J Neurosci.* 2017;37(25):5996-6006.
24. Scheiber IF, Wu Y, Morgan SE, Zhao N. The intestinal metal transporter ZIP14 maintains systemic manganese homeostasis. *J Biol Chem.* 2019;294(23):9147-60.
25. Long LL, Li XR, Huang ZK, Jiang YM, Fu SX, Zheng W. Relationship between changes in brain MRI and (1)H-MRS, severity of chronic liver damage, and recovery after liver transplantation. *Exp Biol Med (Maywood).* 2009;234(9):1075-85.
26. Santos D, Batoreu C, Mateus L, Marreilha Dos Santos AP, Aschner M. Manganese in human parenteral nutrition: considerations for toxicity and biomonitoring. *Neurotoxicology.* 2014;43:36-45.
27. Mergler D, Baldwin M, Belanger S, Larribe F, Beuter A, Bowler R, et al. Manganese neurotoxicity, a continuum of dysfunction: results from a community based study. *Neurotoxicology.* 1999;20(2-3):327-42.
28. Galvez-Peralta M, He L, Jorge-Nebert LF, Wang B, Miller ML, Eppert BL, et al. ZIP8 zinc transporter: indispensable role for both multiple-organ organogenesis and hematopoiesis in utero. *PLoS One.* 2012;7(5):e36055.
29. van Raaij SEG, Srari SKS, Swinkels DW, van Swelm RPL. Iron uptake by ZIP8 and ZIP14 in human proximal tubular epithelial cells. *Biometals.* 2019;32(2):211-26.
30. Himeno S, Yanagiya T, Fujishiro H. The role of zinc transporters in cadmium and manganese transport in mammalian cells. *Biochimie.* 2009;91(10):1218-22.
31. Park JH, Hoglebe M, Gruneberg M, DuChesne I, von der Heiden AL, Reunert J, et al. SLC39A8 Deficiency: A Disorder of Manganese Transport and Glycosylation. *Am J Hum Genet.* 2015;97(6):894-903.
32. Wang CY, Jenkitkasemwong S, Duarte S, Sparkman BK, Shawki A, Mackenzie B, et al. ZIP8 is an iron and zinc transporter whose cell-surface expression is up-regulated by cellular iron loading. *J Biol Chem.* 2012;287(41):34032-43.
33. Prohaska T, Irrgeher J, Benefield J, Böhlke JK, Chesson LA, Coplen TB, et al. Standard atomic weights of the elements 2021 (IUPAC Technical Report). *Pure and Applied Chemistry.* 2022;94(5):573-600.
34. Reaney SH, Kwik-Urbe CL, Smith DR. Manganese oxidation state and its implications for toxicity. *Chem Res Toxicol.* 2002;15(9):1119-26.
35. Finley JW. Manganese absorption and retention by young women is associated with serum ferritin concentration. *Am J Clin Nutr.* 1999;70(1):37-43.
36. Aschner JL, Aschner M. Nutritional aspects of manganese homeostasis. *Mol Aspects Med.* 2005;26(4-5):353-62.
37. Winslow JWW, Limesand KH, Zhao N. The Functions of ZIP8, ZIP14, and ZnT10 in the Regulation of Systemic Manganese Homeostasis. *Int J Mol Sci.* 2020;21(9).

38. Fujishiro H, Kambe T. Manganese transport in mammals by zinc transporter family proteins, ZNT and ZIP. *J Pharmacol Sci.* 2022;148(1):125-33.
39. Jenkitkasemwong S, Akinyode A, Paulus E, Weiskirchen R, Hojyo S, Fukada T, et al. SLC39A14 deficiency alters manganese homeostasis and excretion resulting in brain manganese accumulation and motor deficits in mice. *Proc Natl Acad Sci U S A.* 2018;115(8):E1769-E78.
40. Xin Y, Gao H, Wang J, Qiang Y, Imam MU, Li Y, et al. Manganese transporter Slc39a14 deficiency revealed its key role in maintaining manganese homeostasis in mice. *Cell Discov.* 2017;3:17025.
41. Floriańczyk B, Kaczmarczyk R, Osuchowski J, Trojanowski T. Metallothionein and manganese concentrations in brain tumors. *Journal of Pre-Clinical and Clinical Research.* 2007;1(1):89-91.
42. Culotta VC, Yang M, O'Halloran TV. Activation of superoxide dismutases: putting the metal to the pedal. *Biochim Biophys Acta.* 2006;1763(7):747-58.
43. Mercadante CJ, Prajapati M, Conboy HL, Dash ME, Herrera C, Pettiglio MA, et al. Manganese transporter Slc30a10 controls physiological manganese excretion and toxicity. *J Clin Invest.* 2019;129(12):5442-61.
44. Roth JA, Garrick MD. Iron interactions and other biological reactions mediating the physiological and toxic actions of manganese. *Biochem Pharmacol.* 2003;66(1):1-13.
45. Takeda A. Manganese action in brain function. *Brain Res Brain Res Rev.* 2003;41(1):79-87.
46. Aschner M, Aschner JL. Manganese neurotoxicity: cellular effects and blood-brain barrier transport. *Neurosci Biobehav Rev.* 1991;15(3):333-40.
47. Garrick MD, Kuo HC, Vargas F, Singleton S, Zhao L, Smith JJ, et al. Comparison of mammalian cell lines expressing distinct isoforms of divalent metal transporter 1 in a tetracycline-regulated fashion. *Biochem J.* 2006;398(3):539-46.
48. Steimle BL, Smith FM, Kosman DJ. The solute carriers ZIP8 and ZIP14 regulate manganese accumulation in brain microvascular endothelial cells and control brain manganese levels. *J Biol Chem.* 2019;294(50):19197-208.
49. Morgan SE, Schroten H, Ishikawa H, Zhao N. Localization of ZIP14 and ZIP8 in HIBCPP Cells. *Brain Sci.* 2020;10(8).
50. Taylor CA, Hutchens S, Liu C, Jursa T, Shawlot W, Aschner M, et al. SLC30A10 transporter in the digestive system regulates brain manganese under basal conditions while brain SLC30A10 protects against neurotoxicity. *J Biol Chem.* 2019;294(6):1860-76.
51. Takeda A, Devenyi A, Connor JR. Evidence for non-transferrin-mediated uptake and release of iron and manganese in glial cell cultures from hypotransferrinemic mice. *J Neurosci Res.* 1998;51(4):454-62.
52. Erikson KM, Shihabi ZK, Aschner JL, Aschner M. Manganese accumulates in iron-deficient rat brain regions in a heterogeneous fashion and is associated with neurochemical alterations. *Biol Trace Elem Res.* 2002;87(1-3):143-56.
53. Erikson KM, Aschner M. Manganese neurotoxicity and glutamate-GABA interaction. *Neurochem Int.* 2003;43(4-5):475-80.
54. Sidoryk-Wegrzynowicz M, Aschner M. Manganese toxicity in the central nervous system: the glutamine/glutamate-gamma-aminobutyric acid cycle. *J Intern Med.* 2013;273(5):466-77.
55. Chen P, Chakraborty S, Mukhopadhyay S, Lee E, Paoliello MM, Bowman AB, et al. Manganese homeostasis in the nervous system. *J Neurochem.* 2015;134(4):601-10.
56. Olanow CW. Manganese-induced parkinsonism and Parkinson's disease. *Ann N Y Acad Sci.* 2004;1012:209-23.
57. Guilarte TR, Gonzales KK. Manganese-Induced Parkinsonism Is Not Idiopathic Parkinson's Disease: Environmental and Genetic Evidence. *Toxicol Sci.* 2015;146(2):204-12.

58. Kwakye GF, Paoliello MM, Mukhopadhyay S, Bowman AB, Aschner M. Manganese-Induced Parkinsonism and Parkinson's Disease: Shared and Distinguishable Features. *Int J Environ Res Public Health*. 2015;12(7):7519-40.
59. Perl DP, Olanow CW. The neuropathology of manganese-induced Parkinsonism. *J Neuropathol Exp Neurol*. 2007;66(8):675-82.
60. Racette BA, McGee-Minnich L, Moerlein SM, Mink JW, Videen TO, Perlmutter JS. Welding-related parkinsonism: clinical features, treatment, and pathophysiology. *Neurology*. 2001;56(1):8-13.
61. Tuschl K, Meyer E, Valdivia LE, Zhao N, Dadswell C, Abdul-Sada A, et al. Mutations in SLC39A14 disrupt manganese homeostasis and cause childhood-onset parkinsonism-dystonia. *Nat Commun*. 2016;7:11601.
62. Clegg MS, Donovan SM, Monaco MH, Baly DL, Ensunsa JL, Keen CL. The influence of manganese deficiency on serum IGF-1 and IGF binding proteins in the male rat. *Proc Soc Exp Biol Med*. 1998;219(1):41-7.
63. Itokawa Y. [Trace elements in long-term total parenteral nutrition]. *Nihon Rinsho*. 1996;54(1):172-8.
64. Rico H, Gomez-Raso N, Revilla M, Hernandez ER, Seco C, Paez E, et al. Effects on bone loss of manganese alone or with copper supplement in ovariectomized rats. A morphometric and densitometric study. *Eur J Obstet Gynecol Reprod Biol*. 2000;90(1):97-101.
65. Rahnama M, Bloniarz J, Zareba S, Swiatkowski W. [Study of estrogen deficiency impact on manganese levels in teeth and mandible of rats after ovariectomy]. *Rocz Panstw Zakl Hig*. 2003;54(1):33-8.
66. Wang C, Zhu Y, Long H, Ou M, Zhao S. Relationship between blood manganese and bone mineral density and bone mineral content in adults: A population-based cross-sectional study. *PLoS One*. 2022;17(10):e0276551.
67. Shawki A, Anthony SR, Nose Y, Engevik MA, Niespodzany EJ, Barrientos T, et al. Intestinal DMT1 is critical for iron absorption in the mouse but is not required for the absorption of copper or manganese. *Am J Physiol Gastrointest Liver Physiol*. 2015;309(8):G635-47.
68. Davidsson L. Approaches to improve iron bioavailability from complementary foods. *J Nutr*. 2003;133(5 Suppl 1):1560S-2S.
69. Lopez CA, Skaar EP. The Impact of Dietary Transition Metals on Host-Bacterial Interactions. *Cell Host Microbe*. 2018;23(6):737-48.
70. Tinkov AA, Martins AC, Avila DS, Gritsenko VA, Skalny AV, Santamaria A, et al. Gut Microbiota as a Potential Player in Mn-Induced Neurotoxicity. *Biomolecules*. 2021;11(9).
71. Wang H, Yang F, Xin R, Cui D, He J, Zhang S, et al. The gut microbiota attenuate neuroinflammation in manganese exposure by inhibiting cerebral NLRP3 inflammasome. *Biomed Pharmacother*. 2020;129:110449.
72. Liu J, Zhang X, Ta X, Luo M, Chang X, Wang H. Fecal microbiome transplantation attenuates manganese-induced neurotoxicity through regulation of the apelin signaling pathway by inhibition of autophagy in mouse brain. *Ecotoxicol Environ Saf*. 2022;242:113925.
73. Ghaisas S, Maher J, Kanthasamy A. Gut microbiome in health and disease: Linking the microbiome-gut-brain axis and environmental factors in the pathogenesis of systemic and neurodegenerative diseases. *Pharmacol Ther*. 2016;158:52-62.
74. Boycott KM, Beaulieu CL, Kernohan KD, Gebriel OH, Mhanni A, Chudley AE, et al. Autosomal-Recessive Intellectual Disability with Cerebellar Atrophy Syndrome Caused by Mutation of the Manganese and Zinc Transporter Gene SLC39A8. *Am J Hum Genet*. 2015;97(6):886-93.

75. Riley LG, Cowley MJ, Gayevskiy V, Roscioli T, Thorburn DR, Prelog K, et al. A SLC39A8 variant causes manganese deficiency, and glycosylation and mitochondrial disorders. *J Inher Metab Dis*. 2017;40(2):261-9.
76. Choi EK, Nguyen TT, Gupta N, Iwase S, Seo YA. Functional analysis of SLC39A8 mutations and their implications for manganese deficiency and mitochondrial disorders. *Sci Rep*. 2018;8(1):3163.
77. Hardy IJ, Gillanders L, Hardy G. Is manganese an essential supplement for parenteral nutrition? *Curr Opin Clin Nutr Metab Care*. 2008;11(3):289-96.
78. Quadri M, Federico A, Zhao T, Breedveld GJ, Battisti C, Delnooz C, et al. Mutations in SLC30A10 cause parkinsonism and dystonia with hypermanganesemia, polycythemia, and chronic liver disease. *Am J Hum Genet*. 2012;90(3):467-77.
79. Tuschl K, Clayton PT, Gospe SM, Jr., Gulab S, Ibrahim S, Singhi P, et al. Syndrome of hepatic cirrhosis, dystonia, polycythemia, and hypermanganesemia caused by mutations in SLC30A10, a manganese transporter in man. *Am J Hum Genet*. 2012;90(3):457-66.
80. Sreedharan S, Stephansson O, Schioth HB, Fredriksson R. Long evolutionary conservation and considerable tissue specificity of several atypical solute carrier transporters. *Gene*. 2011;478(1-2):11-8.
81. Xia Z, Wei J, Li Y, Wang J, Li W, Wang K, et al. Zebrafish *slc30a10* deficiency revealed a novel compensatory mechanism of *Atp2c1* in maintaining manganese homeostasis. *PLoS Genet*. 2017;13(7):e1006892.
82. Tuschl K, Gregory A, Meyer E, Clayton PT, Hayflick SJ, Mills PB, et al. SLC39A14 Deficiency. In: Adam MP, Mirzaa GM, Pagon RA, Wallace SE, Bean LJH, Gripp KW, et al., editors. *GeneReviews*((R)). Seattle (WA)1993.
83. Rodan LH, Hauptman M, D'Gama AM, Qualls AE, Cao S, Tuschl K, et al. Novel founder intronic variant in SLC39A14 in two families causing Manganism and potential treatment strategies. *Mol Genet Metab*. 2018;124(2):161-7.
84. Wu Y, Wei G, Zhao N. Restriction of Manganese Intake Prevents the Onset of Brain Manganese Overload in *Zip14*(-/-) Mice. *Int J Mol Sci*. 2021;22(13).
85. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science*. 2013;339(6121):786-91.
86. Wang C, Guan Y, Lv M, Zhang R, Guo Z, Wei X, et al. Manganese Increases the Sensitivity of the cGAS-STING Pathway for Double-Stranded DNA and Is Required for the Host Defense against DNA Viruses. *Immunity*. 2018;48(4):675-87 e7.
87. Ram DR, Ilyukha V, Volkova T, Buzdin A, Tai A, Smirnova I, et al. Balance between short and long isoforms of cFLIP regulates Fas-mediated apoptosis in vivo. *Proc Natl Acad Sci U S A*. 2016;113(6):1606-11.
88. Larkin B, Ilyukha V, Sorokin M, Buzdin A, Vannier E, Poltorak A. Cutting Edge: Activation of STING in T Cells Induces Type I IFN Responses and Cell Death. *J Immunol*. 2017;199(2):397-402.
89. Sarhan J, Liu BC, Muendlein HI, Weindel CG, Smirnova I, Tang AY, et al. Constitutive interferon signaling maintains critical threshold of MLKL expression to license necroptosis. *Cell Death Differ*. 2019;26(2):332-47.
90. Zhao Z, Ma Z, Wang B, Guan Y, Su XD, Jiang Z. Mn(2+) Directly Activates cGAS and Structural Analysis Suggests Mn(2+) Induces a Noncanonical Catalytic Synthesis of 2'3'-cGAMP. *Cell Rep*. 2020;32(7):108053.
91. Lv M, Chen M, Zhang R, Zhang W, Wang C, Zhang Y, et al. Manganese is critical for antitumor immune responses via cGAS-STING and improves the efficacy of clinical immunotherapy. *Cell Res*. 2020;30(11):966-79.

92. He L, Girijashanker K, Dalton TP, Reed J, Li H, Soleimani M, et al. ZIP8, member of the solute-carrier-39 (SLC39) metal-transporter family: characterization of transporter properties. *Mol Pharmacol*. 2006;70(1):171-80.
93. Aydemir TB, Liuzzi JP, McClellan S, Cousins RJ. Zinc transporter ZIP8 (SLC39A8) and zinc influence IFN-gamma expression in activated human T cells. *J Leukoc Biol*. 2009;86(2):337-48.
94. McDermott JR, Geng X, Jiang L, Galvez-Peralta M, Chen F, Nebert DW, et al. Zinc- and bicarbonate-dependent ZIP8 transporter mediates selenite uptake. *Oncotarget*. 2016;7(23):35327-40.
95. Geng X, Liu L, Banes-Berceli A, Yang Z, Kang P, Shen J, et al. Role of ZIP8 in regulating cell morphology and NF-kappaB/Snail2 signaling. *Metallomics*. 2018;10(7):953-64.
96. Liu MJ, Bao S, Galvez-Peralta M, Pyle CJ, Rudawsky AC, Pavlovicz RE, et al. ZIP8 regulates host defense through zinc-mediated inhibition of NF-kappaB. *Cell Rep*. 2013;3(2):386-400.
97. McConnell EL, Basit AW, Murdan S. Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. *J Pharm Pharmacol*. 2008;60(1):63-70.
98. Hubatsch I, Ragnarsson EG, Artursson P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat Protoc*. 2007;2(9):2111-9.
99. Chen S, Einspanier R, Schoen J. Transepithelial electrical resistance (TEER): a functional parameter to monitor the quality of oviduct epithelial cells cultured on filter supports. *Histochem Cell Biol*. 2015;144(5):509-15.
100. Williams M, Todd GD, Roney N, Crawford J, Coles C, McClure PR, et al. Toxicological Profile for Manganese. Agency for Toxic Substances and Disease Registry (ATSDR) Toxicological Profiles. Atlanta (GA)2012.
101. Felber DM, Wu Y, Zhao N. Regulation of the Metal Transporters ZIP14 and ZnT10 by Manganese Intake in Mice. *Nutrients*. 2019;11(9).
102. Chi L, Gao B, Bian X, Tu P, Ru H, Lu K. Manganese-induced sex-specific gut microbiome perturbations in C57BL/6 mice. *Toxicol Appl Pharmacol*. 2017;331:142-53.