FUNCTION AND REGULATION OF INTESTINAL ZINC TRANSPORTER ZIP14

By

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To my parents, Gregory and Josephine Guthrie
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<tr>
<td>AAS</td>
<td>Atomic Absorption Spectroscopy</td>
</tr>
<tr>
<td>AE</td>
<td>Acrodermatitis Enteropathica</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens Junction</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>CDF</td>
<td>Cation Diffusion Facilitator</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts Per Minute</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element Binding Protein</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole Dihydrochloride</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran Sodium Sulfate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescent Isothiocyanate</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GUK</td>
<td>Guanylate Kinase</td>
</tr>
<tr>
<td>HR</td>
<td>Hour</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>IEC</td>
<td>Intestinal Epithelial Cells</td>
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<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL-1</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional Adhesion Molecule</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-like Factor 4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-darby Canine Kidney cells</td>
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<tr>
<td>MRE</td>
<td>Metal Response Element</td>
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<tr>
<td>MTF-1</td>
<td>Metal Response Element Binding Transcription Factor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDZ</td>
<td>Post Synaptic Density Protein, Drosophila Disc Large Tumor Suppressor, and Zonula Occludens-1 Protein</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate Carboxykinase</td>
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<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
</tr>
<tr>
<td>PNS</td>
<td>Post Nuclear Supernatant</td>
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<tr>
<td>PP1</td>
<td>Protein Phosphatase 1</td>
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<tr>
<td>PP2A</td>
<td>Protein Phosphatase 2 A</td>
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<tr>
<td>PTH1R</td>
<td>Parathyroid Hormone 1 Receptor</td>
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<tr>
<td>PTP1B</td>
<td>Protein Tyrosine Phosphatase 1 B</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>SC</td>
<td>Subcutaneous</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<td>SH3</td>
<td>SRC Homology 3</td>
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<tr>
<td>siRNA</td>
<td>Small Interfering Ribonucleic Acid</td>
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<tr>
<td>SNAP</td>
<td>s-Nitroso-N-Acetylpenicillamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
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</tr>
<tr>
<td>SRC</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial Electrical Resistance</td>
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<tr>
<td>TJ</td>
<td>Tight Junction</td>
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<tr>
<td>TNAP</td>
<td>Tissue Non-Specific Alkaline Phosphatases</td>
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<tr>
<td>TNF</td>
<td>Tissue Necrosis Factor</td>
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<tr>
<td>TPEN</td>
<td>1,2-Ethanediamine, N,N,N',N'-Tetrakis(2-Pyridinylmethyl)</td>
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<tr>
<td>ZIP</td>
<td>Zrt, Irt-like Protein</td>
</tr>
<tr>
<td>ZNT</td>
<td>Zinc Transporter</td>
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<td>ZO</td>
<td>Zonula Occludens</td>
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Abstract of Dissertation Presented to the Graduate School of the University Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

FUNCTION AND REGULATION OF INTESTINAL ZINC TRANSPORTER ZIP14

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Major: Nutritional Sciences

ZIP14 is a transporter that increases the intracellular labile pool of zinc. ZIP14 has a high level of expression in the gastrointestinal (GI) tract. Loss of the gene is not embryonically lethal, suggesting Zip14 functions in specialized, non-essential zinc transport in the intestine. ZIP14 is responsive to endotoxin-induced inflammation and intestinal permeability increases in response to inflammation and zinc supplementation decreases intestinal permeability. Whether the effect on intestinal permeability is dependent upon a zinc transporter is unknown. Based on these observations, the hypothesis of this research is that ZIP14 is responsible for zinc homeostasis in both basal and inflammation-mediated conditions which helps maintain intestinal function. In vivo experiments were performed with wild type mice (Zip14+/+) and a knockout mouse model of Zip14 (Zip14−/−). Western blots of extracts from isolated intestinal segments show ZIP14 protein distribution is highest in the duodenum and decreasing towards the colon. Isolated intestinal cytoplasmic and membrane fractions were used to establish that ZIP14 is localized to the basolateral membrane. Intestinal uptake studies using 65Zn administered orally by gavage or subcutaneously (SC) were performed. 65Zn was higher in the Zip14−/− compared to Zip14+/+ genotype in the intestine, following SC
injections, but not gavage. To determine an effect of endotoxin on intestinal ZIP14, LPS was injected intraperitoneally. ZIP14 transcript levels did not change within 18 hr, after the LPS treatment, but protein levels decreased at 3- and 6-hr. This response is opposite to results observed with liver ZIP14. Additionally, $^{65}$Zn studies with SC injection and gavage showed no difference in zinc transport between $Zip14^{+/+}$ and $Zip14^{-/-}$ in response to LPS. Lastly, $Zip14^{-/-}$ mice following a gavage of FITC-dextran, a fluorescent permeability marker, have higher plasma FITC-dextran compared to $Zip14^{+/+}$ mice. This increased intestinal permeability coincides with decreased expression of the tight junction proteins claudin-1 and occludin in the $Zip14^{-/-}$ mice. Furthermore, threonine phosphorylation of occludin, which is necessary for tight junction assembly, is decreased in $Zip14^{-/-}$ mice. In conclusion, ZIP14 is a basolateral zinc transporter that does not increase expression in response to LPS, but is necessary for the maintenance of intestinal barrier function via tight junction proteins.
Zinc Homeostasis

Zinc was first recognized as a biologically important micromineral in the fungus *aspergillus niger* in 1869 (1). The first reported role of zinc's essentiality in mammals for growth was observed in rats in 1933 (2). It was not until 1961 that an association of zinc deficiency with impaired growth was observed in humans (3) and not confirmed until 1963 (4-7). Zinc is now known to be required for the function of over 50 enzymes and numerous other proteins (8). The role of zinc has been categorized into three distinct biological roles: structural, catalytic, and regulatory (9). Zinc deficiency symptoms include retardation in growth and sexual development in males, as well as, impairments in gut, brain, and immune function (10). In zinc intake excess (150 mg/day to 2 g/day), a chronic zinc toxicity occurs and effects include anemia, hypocupremia, and immune suppression (11-15). Acute toxicity at high intakes of zinc (2.5 g/L) result in a variety of gastrointestinal disturbances (16). However, in healthy individuals, zinc levels are maintained in an appropriate range even with a varied level of zinc intake to prevent the onset of either deficiency or toxicity. To achieve this, a coordinated effort between zinc influx and efflux transporters at key sites of zinc incorporation must occur.

An observation made in the original examination of middle eastern dwarfs, in the 1960’s, who were severely zinc deficient, is that they rarely survived past the age of 25 due to complications with infection and inflammation (10). Furthermore, a hallmark of inflammation of the intestinal tissue is increased intestinal permeability mediated through impairment of the apical proteins that regulate paracellular flux. Collectively, these proteins are referred to as tight junction (TJ) proteins. The maintenance of the
intestinal barrier may be dependent on zinc (17, 18). ZIP14 is a recently identified zinc transporter that has high transcript abundance in the small intestine (19, 20). The functional role ZIP14 has in the intestine is unknown, but it is one of a limited number of transporters that is responsive to inflammation (19). The connection between intestinal zinc transport, intestinal inflammation, and TJ regulation may be linked through Zip14 and will be discussed further in this review.

**Intestinal Zinc Transport**

The transport of zinc into the intestine exhibits both passive, non-saturable and saturable kinetics (21). The intestine serves as the axis of dietary zinc uptake regulation. Transport across either the apical or basolateral membrane of the enterocyte is achieved via 2 main families of zinc transporters: Zrt-, Irt-like proteins (ZIP) and cation diffusion facilitator (CDF) zinc transporters (ZnT). ZIP transporters increase intracellular labile zinc by pulling zinc in from extracellular sites, or pulling zinc out of organelles and vesicles within the cell. The predicted structure of many ZIP family members is eight transmembrane domains with a histidine rich region between transmembrane domain III and IV, which is believed to facilitate zinc ion binding (22). ZnT transporters function to decrease the intracellular labile zinc concentration via export into extracellular sites or import into organelles and vesicles. The ZnT family typically has six transmembrane domains with a conserved histidine-rich region within the intracellular loop between transmembranes IV and V (22). The main focus of this section will be to review the primary transporters that are currently known in the literature to be present on the plasma membranes of the intestine which include ZIP4, ZIP5, ZnT1, and ZnT5.
The transporter for zinc across the apical membrane is ZIP4 (SLC39A4) (23). During experimental dietary zinc deficiency in mice, ZIP4 increases abundance at the apical membrane and rapidly decreases abundance when dietary zinc is replete (24, 25). One study originally suggested that, in response to low cellular zinc, ZIP4 transcript was stabilized rather than transcriptionally induced (26). However, a study in 2009 linked the transcription factor kruppel-like factor 4 (KLF4) to transcriptional upregulation in zinc deficiency (27). This latter finding has since been verified in lung Zip4 transcript regulation as well (28). Post-translational modification to ZIP4 in high zinc conditions leads to rapid endocytosis and degradation (29, 30). A histidine-rich cluster present in the cytoplasmic loop between transmembrane III and transmembrane IV of the human Zip4 protein is the target for ubiquitination in zinc replete conditions leading to protein degradation (30). In humans, a mutation in this gene leads to Acrodermatitis EnteroPATHICA (AE), an autosomal recessive disorder that leads to impaired growth, skin abnormalities, neurological issues, and impaired immune function (31, 32). AE can be overcome by supplemental administration of zinc (33). In mice, deletion of Zip4 is embryonically lethal. Recently a tamoxifen-inducible intestine-specific Zip4<sup>−/−</sup> mouse model has been created. At onset of loss-of-function, the mice display a rapid decline in health with wasting and death within nine days of knockout. ZIP5 protein declines and MT-1 protein markedly increases as villus integrity rapidly declines, tissue zinc decreases, and crypt architecture is disrupted (34). Administration of supplemental zinc does alleviate some of the effects of the loss of the gene. This study highlights the importance of intestinal ZIP4 expression for maintenance of intestinal zinc absorption from the lumen.
ZIP5, like ZIP4, has a high tissue-specific distribution in the intestine (25). ZIP5 localizes to the basolateral membrane of cells (25, 35). Neither dietary zinc supplementation, nor zinc restriction, alter the transcriptional expression of Zip5 (24). Rather, Zip5 transcript is retained in polysomes during zinc deficiency poised for rapid translation when cellular zinc concentrations become too high (26). The 3’UTR of the Zip5 transcript forms a stable stem loop structure to which polysomes are capable of binding (36). ZIP5 protein localization also rapidly responds to changes in zinc content of the cell. A high zinc concentration leads to increased protein abundance along the basolateral membrane, conceivably to move high zinc concentration from the circulation through the enterocyte into the lumen (35). Zinc deficiency leads to decreased expression of ZIP5 along the basolateral membrane by endocytosis (25, 26). The mechanism regulating the rapid decrease in protein localization during zinc deficiency in ZIP5 has yet to be elucidated. There currently exists no Zip5−/− model to study the overall impact of the gene on zinc homeostasis, yet it is known that ZIP5 does transport zinc preferentially over other divalent metal cations (35).

ZnT1 was the first zinc transporter identified in a mammalian species (37). This transporter was shown to have a ubiquitous tissue expression, but the relative distribution and localization of ZnT1 was found to be high in the basolateral membrane of the small intestine (38, 39). Intestinal ZnT1 is responsive to high dietary zinc at both the transcript and protein level with increased expression (38, 40-43). Znt-1 transcription rate is regulated via the metal response element-binding transcription factor (MTF-1) that binds to metal response elements (MRE) present in the promoter of ZnT-1 (37, 44, 45). MTF-1 is a zinc-sensing transcription factor that binds zinc as a
ligand and is therefore active in high zinc concentrations (46). ZnT1 is an essential zinc transporter as loss of the gene in a mouse KO model is embryonically lethal at gestation day nine (47). Supplemental zinc administration to the developing embryos is insufficient to rescue the phenotype of these mice. Despite the importance of the zinc transport capacity of ZnT1 in development, the upregulation of ZnT1 in high zinc conditions in a piglet model was unable to prevent excess zinc accumulation in the enterocyte after 24-hr of administration (48). This result may be due to the short term effect of the treatment and a longer adaptive response to high zinc via additional upregulation of ZnT1 may be necessary.

ZnT5 was simultaneously identified by two research groups in 2002 (49, 50). Original analysis of the sequence of ZnT5 led to discrepancies in the number of transmembrane domains. Kambe et al. determined there were 15 transmembrane domains (49). Cragg et al. determined there were only 12 transmembrane domains (50). There has not been further clarification of this issue, yet both putative transmembrane estimates are far greater than the 6 transmembrane domains observed in ZnT1 to ZnT4. ZnT5 has a wide tissue distribution determined by Northern blot with a high level of expression in pancreatic β-cells (49). Original immunofluorescence of confluent Caco-2 cells showed localization of ZnT5 to the apical membrane of the enterocyte (50). In subsequent studies, it was determined that ZnT5 has two major splice variants that have been shown to localize to different membranes of the cell targeted by differing C-terminal regions expressed by the variants (51, 52). Splice variant A shows strong localization to the Golgi (51). Splice variant B is present throughout the cell, but shows localization to the apical membrane as well. A unique
property of ZnT5 splice variant B is that in vitro research suggests it is a bi-directional zinc transporter that can assist in both luminal uptake of zinc and cytoplasmic efflux of zinc into the lumen (53). Of note, this observation has never been confirmed in vivo. Research into the regulation of ZnT5 by cellular zinc status suggests that transcriptional activity of the gene is reduced by high cellular zinc conditions, but the transcript stability is increased to balance total transcript abundance (51). Znt5−/− male mice have a decreased life span compared to female KO or wildtype mice with a 60% mortality rate by 40 weeks of age (54). These mice are susceptible to spontaneous death due to bradyarrhythmias. Developmentally, body weight is markedly lower in the KO mice; bone formation is altered leading to hunchback and osteopenia. The study did not look into the effect of supplemental zinc to rescue the effects of loss of gene function.

Maintenance of normal cellular zinc concentrations in altered dietary zinc status is conceivably the main function of these transporters in the intestine. However, systemic inflammation is known to influence zinc homeostasis. Presumably this is mediated via one or more zinc transporters. In a study looking at both sterile abscess turpentine injection or lipopolysaccharide (LPS) intraperitoneal (IP) administration, Znt1 transcription was reduced in the liver following LPS injection and Znt5 transcript increased following turpentine injection (19). There was no observation on the altered transcript expression in the intestine with this study. The functional role of modified transporter expression would be informative in fully understanding the pathophysiology that occurs from intestinal inflammation.
Intestinal Tight Junction Proteins

Structure, Localization, and Function

The intestine is comprised of enterocytes that have semi-permeable membranes to facilitate the uptake of nutrients. The intestinal epithelium needs to be capable of transporting these nutrients while maintaining a physical barrier against other luminal contents and bacteria to which the apical membrane is continuously exposed. In part, this barrier is circumvented through specific transport proteins for amino acids, sugars, and ions through the transcellular pathway (55, 56). However, additional transport between cell-cell junctions occurs for multiple solutes that displays a non-specific selectivity through the paracellular pathway. The proximity of the cells at cell-cell junctions regulate solute and ion flux through the paracellular pathway. In order to facilitate the barrier at the cell-cell sites of contact, a complex of proteins between cells form a modifiable junction to seal the intercellular space from luminal content. Currently, there are greater than 38 identified integral membrane proteins associated with junctional permeability (57). These proteins are divided into 3 complexes: TJ (TJ), adherens junction (AJ), and desmosome (58). TJ complexes are proximal to the apical membrane and confer the relevant effect on selective paracellular permeability. The complex is a highly organized system containing the transmembrane proteins: claudins, junctional adhesion molecule (JAM), occludin, and tricellulin; cytoplasmic scaffolding protein zonula occludes (ZO); and an actomyosin anchoring protein (Figure 1-1).

The claudins were first discovered in 1998 (59). To date, there are 24 known members of the claudin family of proteins (60). Claudins all contain four transmembrane domains, two extracellular loops, one intracellular loop and N- and C-termini in the cytoplasmic compartment (59). Claudins are relatively small proteins (~24
kDa) that form 10 nm membrane particles when assembled (59, 61). *In vitro* studies with differentiated Caco-2 cells, have determined the pore radius formed is ~4.6 Å (62). Distribution and expression of claudins, within separate tissues, as well as, along the length of the gastrointestinal (GI) tract differ (63, 64). Claudins control the permeability of the paracellular pathway by creating charge-selective channels (65). It appears that individual claudins exert specific effects on the permeability of the TJ with claudins-1, -3, -4, -5, and -8 decreasing permeability and claudins-2 and -7 increasing permeability (66-72). Therefore, this variance in claudin expression and distribution is likely to play a large role in the relative permeability of tissues expressing different members of the family (73, 74). Given these differences, it is not surprising that claudin expression differs by family member under experimentally induced conditions. In animal models, LPS treatment decreases the expression of claudin-1(75, 76). Proinflammatory cytokines in various intestinal cell models have led to increased expression of claudin-2 (77-80). Both of these changes are associated with increased intestinal permeability (76, 77). In mouse models, the gene deletion of claudin-1 is lethal at 1-day postpartum due to transepidermal water loss (81). The murine knockout model of claudin-2 did not prove to be lethal, however these animals displayed an impairment in transepithileal reabsorption of Na\(^+\) (82). The necessity for claudins at the TJ and their proper functioning are integral to the normal functionality of the TJ barrier.

Occludin was the first protein of the TJ complex identified in 1993 in chickens (83). Unlike the claudins, there is only one occludin protein that is associated with TJ formation. This protein contains four transmembrane domains and both N- and C-termini extend into the cytoplasm. The C-terminal domain of occludin is necessary for
attachment to the cytoplasmic protein ZO-1, which helps with trafficking of occludin to the plasma membrane (84). Knockdown experiments with siRNA suggest occludin is responsible for regulation of large macromolecule flux across the TJ (85). However, occludin knockout mice appear to have functioning TJs with no change in the flux of macromolecules, mannitol or small ions, e.g. Na⁺ (86). The mice do show impaired growth, inflammation of the gastric epithelium, impaired mating capacity, and calcification in the brain (87). Therefore, the function of occludin at the TJ does appear to be more complex than what is known for the claudins. A direct effect of TJ permeability may not be noticeable with the loss of the gene, but the overt phenotypic effects seen from the loss of the gene suggest strongly it still exerts a function. Similar to what is seen with the claudins, occludin expression is altered by inflammatory states. Treatment by LPS in animal models can reduce protein abundance of occludin (75, 88). Additionally, the administration of cytokines interferon-γ (IFN-γ), interleukin 1 beta (IL-1β), or tumor necrosis factor alpha (TNF-α) to cell culture models also results in decreased occludin expression (89-91).

The third subfamily of transmembrane protein components of the TJ are members of the JAM family. The JAM proteins are members of the immunoglobulin (Ig) superfamily that contain one transmembrane domain, a cytoplasmic C-terminal domain, and two extracellular domains (92, 93). This family contains four members designated JAM-1, -2, -3, and -4 (also referred to as JAM-A, -B, -C, and -4). JAM-4 differs from the other three members in the PSD-95/Discs-large/ZO-1 (PDZ) binding domain that interacts with cytoplasmic anchoring proteins (94). JAMs display a wide tissue distribution, but JAM-1, JAM-3, and JAM-4 have been determined to be in the intestine,
whereas JAM-2 has strong localization in endothelial tissue (92-96). However, JAM-3 is not associated with the TJ, but rather is localized to the basolateral side of the enterocyte with the desmosomes (97). Unique to the other TJ proteins, JAM proteins are expressed on leukocytes and platelets as well (98, 99). In addition to junctional function, JAM-1 can bind leukocyte integrin α-Lβ2 mediating leukocyte transmigration (100). In the original study identifying the function of JAM-1 in TJ formation, JAM-1 transfected CHO cells had decreased permeability as determined by fluorescent isothiocyanate (FITC) -dextran passage across transwell plates (93). In intestinal T84 cells, antibodies targeted to the extracellular domain of JAM-1 resulted in impaired TJ assembly and lowered transepithileal electrical resistance (TEER) values (101). In functional studies of JAM-1 utilizing a knockout mouse model, intestinal permeability was shown to be increased and the effect of dextran sodium sulfate (DSS) administration to the Jam-1−/− mice greatly exacerbated permeability relative to wild type DSS-treated mice (96, 102). In in vitro experiments in T84 cells, exposure to IFN-γ or IFN-γ and TNF-α led to disassembly and internalization of JAM-1(89, 103). Research does not suggest that JAM-1, or the other JAM proteins, have any selectivity towards molecules or ions that pass through the paracellular pore, but rather have an overall effect on assembly at the TJ.

The final transmembrane protein associated with the TJ is tricellulin. Tricellulin was not discovered to be associated with the TJ until 2005. This membrane protein is unique in that it forms junctions between the contact points of three adjoining cells, rather than the junction between two adjacent cells (104). This protein has four transmembrane domains, two extracellular loops, one intracellular loop and both N- and
C-terminus extend into the cytoplasm. Tricellulin exhibits high expression levels in the small intestine, kidney, and lung. Unlike the other transmembrane TJ proteins that bind to the cytoplasmic protein ZO-1, which assists with assembly and anchoring, tricellulin does not require ZO-1 for assembly (105). Yet, tricellulin recruitment and assembly is dependent upon the TJ protein occludin. There is limited research specific to the function of tricellulin in the intestine. However, in in vitro models of overexpression utilizing canine kidney MDCK cell line, tricellulin did not show a significant increase in TEER, or in inhibition of transport of ions, Na⁺ or Cl⁻, but did decrease the flux of macromolecules ≥ 900 Da (106).

The main cytoplasmic proteins that the transmembrane TJ proteins bind to for assembly and scaffolding are members of the ZO family. Three ZO proteins, designated ZO-1, -2, and -3 have been identified (107-109). ZO proteins have multiple binding motifs along the N-terminal region including three PDZ-binding domains, one Src homology-3 (SH3) domain and a region of homology to guanylate kinase (GUK) (108). The ZO proteins bind directly to the C-terminal domains of the transmembrane proteins through the multiple domains present along their N-terminus. Claudins bind to the PDZ-1 domain of both ZO-1 and ZO-2. Claudin assembly is contingent upon initial migration of ZO-1 to the site of TJ formation (110). Occludin interacts with GUK domain of ZO-1 and loss of the full length occludin C-terminus greatly impairs assembly and strength of interaction (84, 111). JAM-1 binds to a PDZ domain, suggested to be domain 2, and in a cell model appears to recruit ZO-1 which in turn recruits occludin (112). Generation of Zo-1⁻/⁻ mice is embryonically lethal with impaired growth by 8.5 days-post fertilization and apoptosis in the neural tube at day 9.5 (113). Administration
of LPS to mice causes a decrease in ZO-1 at the TJ and an increase in the TritonX-100 soluble fraction of ZO-1. TritonX-100 soluble fractions represent the protein content that is not complexed in association with the cytoskeletal matrix (76). However, in the Caco-2 cell model, the direct administration of IL1-1β failed to alter ZO-1 expression even though occludin levels decreased (90). This result may suggest that the regulation of ZO-1 in response to endotoxin involves a different signaling pathway than occludin.

Whereas overall protein abundance of the TJ proteins alters their permeability a second level of regulation is also present. The assembly and disassembly of the TJ proteins, as well as the regulation of pore size, is mediated through post-translational modifications. Myosin light chain (MLC) is the cytoskeletal structure to which ZO-1 is bound. Phosphorylation of this protein structure leads to conformation changes that cause contraction and impairment of the barrier function. Phosphorylation of MLC can occur through either MLC kinase (MLCK) or through Rho-associated kinase (ROCK) (114). Inhibition of MLCK prior to administration of LPS can inhibit the contraction of the actomyosin cytoskeleton thereby decreasing the effect of endotoxin induced intestinal permeability (115). Assembly of the TJ proteins claudin-1, ZO-1 and occludin also appear to be dependent on post-translational modifications. Two members of the serine/threonine protein kinase C (PKC), η and ζ, have been shown to modify all three proteins (116-119). Dephosphorylation of these proteins has also been observed to occur by protein phosphatase 2A (PP2A) (119). Depletion of PP2A leads to accelerated assembly of TJs (119, 120). Occludin also appears to be dephosphorylated by protein phosphatase 1 (PP1) with preferential targeting of serine residues, whereas PP2A dephosphorylates threonine residues (120).
**Zinc and Tight Junctions**

Over the past two decades a role for zinc in the modification of TJ permeability has been established. Zinc deficiency in inflammatory diseases in humans has been correlated with impairment of barrier function. In children with either acute or persistent diarrhea, 5 mg/kg bodyweight/day of zinc for 2 weeks decreased intestinal permeability, as determined by mannitol/lactulose excretion, and those children who presented with hypozincemia prior to supplementation showed even greater improvements in TJ barrier function (121). Crohn’s disease commonly is associated with reduced zinc levels and increased permeability (122, 123). Again, supplemental zinc treatment, as assessed by excreted mannitol/lactulose ratios, improved TJ barrier function (124). Close genetic relatives of Crohn’s disease patients also have impaired barrier function. This observation suggests that barrier dysfunction in this disease may be the cause of the onset of inflammation rather than a result of inflammation and tissue damage (123). Currently, there are no studies that have examined close relatives of Crohn's patients for their zinc status as well. However, it would be interesting to see if this population also exhibits lower zinc status and whether supplementation of zinc could prevent the onset of the disease in these individuals. In animal models of inflammatory bowel disease, a benefit of zinc supplementation has been observed. In experimental models of colitis induced in rats, a 7-day administration of supplemental zinc decreased the number of open TJs visualized under electron microscopy (125). In experimentally induced protein malnutrition in guinea pigs, zinc supplementation of a low protein diet restored TJ barrier function to that of the control diet (18). In weanling pigs, which have a high rate of mortality from diarrhea, supplementation with a diet containing either zinc oxide or tetrabasic zinc chloride (2000 mg/kg) for 14 days had decreased mannitol
absorption. Both transcript and protein abundance of occludin and ZO-1 were significantly increased in the supplemental groups relative to control (126). Mice fed a chronic alcohol diet for 4 weeks developed zinc deficiency, increased serum endotoxin and serum FITC-dextran, with decreased protein expression of ZO-1 and altered localization of ZO-1 and occludin (127). These results taken together make a strong case for the role of zinc in modifying intestinal barrier function, yet in these studies the mechanism of barrier improvement could be an indirect effect of zinc, possibly through modifying inflammation at the site and thereby altering cytokines or increasing reactive oxygen species (ROS) and their pro-apoptotic effects on intestinal tissue (127-129).

Cell culture studies suggest a direct role of zinc in the regulation of TJ barrier function. Caco-2 cells have impaired TEER, an indicator of open TJs, when grown in zinc-free media for 18 days. A significant decrease in TEER relative to control does not occur until day 6. In addition, occludin, ZO-1, E-cadherin, β-catenin, F-Actin and β-tubulin all exhibit impaired assembly (130). When differentiated Caco-2 cells are made zinc deficient with the intracellular zinc chelator, TPEN, for 24 hrs. decreased protein expression of claudin-1, occludin, and ZO-1 was observed. The decreased expression was dose-dependent (0, 2, 3, and 4 μM TPEN) with 4 μM TPEN leading to the greatest decrease in protein abundance. In parallel with the dose dependent decrease in protein expression was decreased TEER and increased FITC-dextran flux (127). In a study by Wang et al., zinc supplementation (100 μM) to Caco-2 cells resulted in an increase in TEER relative to standard media. However, mannitol flux increased in response to zinc supplementation, contrary to what would be expected with increased TEER. In addition, protein expression of claudin-2 and claudin-7 decreased in the supplemented group.
Increased expression of claudin-2 is associated with decreased barrier function and increased permeability, so improved TEER would be expected (67). Claudin-7 is both apically and basolaterally localized along the intestine and loss of expression is associated with upregulation of the inflammatory response and matrix metalloproteinases, and epithelial damage (132). Claudin-7 downregulated expression may explain the increased flux of mannitol, as pore size and ion selectivity for solute permeability differs between claudins (65, 133). It should also be noted that treatments lasted for one week and a significant increase in lactate dehydrogenase leakage in the supplemented group was observed bringing into question the potential for zinc toxicity to the cells. Occludin and ZO-1 protein abundance and assembly at the TJ appear to be the most sensitive to zinc status among the TJ proteins. Though, both claudin-1 and claudin-2 have also shown altered protein abundance and assembly in a number of studies as well. Together, these studies all suggest a strong role for zinc in the maintenance and proper function of the TJ mediated through the proteins that directly comprise the TJ complex.

Many of these studies looking at the role of zinc in TJ assembly and function did not determine any mechanistic way in which zinc can have a direct effect. In in vitro studies with Caco-2 cells, supplemental zinc oxide can reduce the transcriptional expression of inflammatory cytokines, including induced TNF-α (134). As mentioned above, TNF-α alters barrier expression in animal models. Also, zinc is known to inhibit phosphatase activity, in particular, PP2A which regulates the dephosphorylation of occludin (135-139). So, it is possible that either through indirect regulation of cytokines or direct alteration of protein phosphorylation, zinc can modulate the TJ barrier.
Zip14 Function and Gene Regulation

ZIP14 is a recently characterized member of the LZT (LIV-1 subfamily of ZIP zinc transporters) subfamily of zinc transporters (140). The transcript for mature ZIP14 contains 9 exons, yet two variants exist that contain either exon 4A or 4B. The proteins encoded from these alternatively spliced transcripts both contain 489 amino acids, but ZIP14A is 53,574 Da and ZIP14B is 53,962 Da (20). Consensus sequence analysis of ZIP14 shows that it is most closely related to ZIP8, yet it does not contain a highly conserved motif in its transmembrane domain V, HEXPHEXGD, that contains histidines believed critical for zinc transport among the SLC39 subfamily (20, 140). Instead, ZIP14 contains a glutamic acid substitution in place of a conserved histidine residue, EEXPHEXGD. Transient transfection of a ZIP14 expression vector in Chinese hamster ovary cells was able to increase the intracellular zinc concentration, indicating that even with the glutamic acid substitution, ZIP14 is still capable of zinc transport (140). The alteration in binding sequence may affect the diversity of cation substrates that ZIP14 is capable of transporting. In fact, several cations have been identified as substrate for ZIP14 transport including non-transferrin bound iron (NTBI), cadmium, copper, and manganese (20, 141). ZIP14B has an 8 times higher affinity for Cd$^{2+}$ and 4-times higher affinity for Mn$^{+}$ than does ZIP14A. Both variants selectively transport Zn$^{2+}$ over Cd$^{2+}$. A more recent publication looking into the transport activities of ZIP14 in the Xenopus oocyte model did show that Zn$^{2+}$ and Cd$^{2+}$ are preferentially transported in the presence of competing divalent cations (142). This finding is unique, as there is still limited data on the main substrates for most of the SLC39 subfamily of transporters (143).
There are several reports on the tissue distribution of ZIP14. Taylor et al. utilized a multi-tissue expression array and hybridized human cDNA to a ZIP14 specific probe and found the liver, pancreas, fetal liver, thyroid gland, heart, and fetal heart to be contain the highest expression of ZIP14 (140). Tominaga et al. performed qualitative PCR using a Zip14 primer on tissues from C57Bl/6 mice and found the liver to contain the greatest transcript abundance followed by kidney, stromal-vascular cells, heart, brain, and adipocytes (144). Liuzzi et al. performed qPCR on RNA samples from CD-1 mice on select tissues and observed Zip14 from highest to lowest expression in the duodenum, followed by jejenum, liver, heart, kidney, spleen, and pancreas (141). Girijashanker et al. examined copy number of the individual transcript variants, Zip14a and Zip14b, in select tissues in C57Bl/6 mice and found the liver had the highest abundance in both transcripts, followed by the duodenum in both transcripts, and a differential expression of the transcripts in lung, kidney, testis, and brain (20).

The functional relevance of ZIP14 in zinc homeostasis is in the process of being elucidated. It was originally shown that Zip14 is unresponsive to either a zinc adequate, or zinc deficient diet in the small intestine of CD-1 mice (24). However, in the liver of CD-1 mice administered IP of either turpentine or lipopolysaccharide (LPS), Zip14 transcript increased 3.1- and 3.7-fold, respectively (19). In addition to Zip14, metallothionen (Mt-1), an MTF-1 regulated gene, increased 37- and 32-fold, respectively, with these treatments. Serum zinc levels decrease in the inflammatory response and approximately a 50% reduction in serum zinc was observed in the treated mice (145). Immunohistochemistry showed the localization ZIP14 to the plasma membrane in response to LPS treatment in the liver, which could involve ZIP14 in an
interaction with serum zinc (19). Taken together, these data strongly suggest ZIP14 plays a major role in the hypozincemia observed in inflammation.

ZIP14 responsiveness to LPS and turpentine is observed in wild type mice, but the response to turpentine in IL-6−/− mice is lost. With the treatment of LPS in the IL-6−/− model, there is a strong reduction in Zip14 expression, but the effect is not completely lost (19). LPS increases the expression of IL-1β, which is the upstream enhancer of IL-6 activity. However, IL-1β also activates iNOS. To further explore this, Lichten et al. utilized iNOS−/− and isolated primary hepatocytes for IL-1β treatments (146). In wild type hepatocytes, IL-1β increased ZIP14 mRNA, but the increase was lost in iNOS−/− cells. ZIP14 mRNA expression could be increased in the iNOS−/− mice when they were treated with S-nitroso N-acetyl penicillamine (SNAP), a nitric oxide (NO) donor. Immunofluorescence detection of increased ZIP14 protein levels and increased intracellular zinc following SNAP treatment further confirmed the mechanism.

Recently, with the generation of a Zip14−/− mouse model a better understanding of the systemic role of this gene has been able to be elucidated. In female Zip14−/− mice, no changes in basal serum zinc or tissue zinc were observed (147). Orally administered 65Zn showed total body % 65Zn absorption and liver 65Zn uptake was reduced. There appears to be transporter compensation for the loss of Zip14 in the liver with Zip4 and Znt1 significantly decreased and Zip7, Zip8, Znt6, and Znt8 significantly increased in transcript abundance. In response to LPS, Zip14−/− mice do not experience serum hypozincemia and have decreased circulating IL-6 levels. Within the liver following LPS, the Zip14−/− mice fail to accumulate tissue zinc unlike WT mice.
Phenotypically, Zip14−/− mice have stunted growth and shortened bones (148). This phenotype was linked to impaired chondrocyte differentiation and targeted specifically at alterations in signaling pathway involved in the process. Chondrocyte differentiation is linked to signaling through activation of the parathyroid 1 receptor (PTH1R) G-protein coupled receptor. Observations into the activity of downstream targets of this pathway showed increased cAMP activity which led to decreased phosphorylation of cAMP-response element binding protein (CREB) and transcriptional activation of c-fos. This altered pathway was linked to enhanced phosphodiesterase (PDE) activity. It was determined that zinc either directly, or indirectly, inhibits the activity of PDE and loss of Zip14 abrogated this inhibition. In addition to altered bone morphology, the mice also display altered elevated fatty acid synthase expression and and gluconeogenesis with elevated adiposity (147, 148). Phosphoenolpyruvate carboxykinase (PEPCK) and serum glucose levels are both decreased in Zip14−/−. These mice also have elevated tyrosine phosphorylation of the insulin receptor. The downstream signaling target of insulin receptor, Akt, is also hyperphosphorylated in the Zip14−/− phenotype (147). It is possible since zinc can directly inhibit phosphatase activity loss of Zip14 can lead to increased phosphorylation (135, 136, 139). ZIP14 was experimentally shown to be capable of inhibiting protein tyrosine phosphatase 1 b (PTP1B) activity by overexpression in liver AML12 cells (138). In vivo observations with Zip14−/− mice produced through the hepatocyte growth factor signaling cascade and PTP1B (149).

It is interesting to speculate on ZIP14 function in the intestine. The gastrointestinal tract turnover rate of protein is one of the highest among all tissues (150). Zinc
deficiency can cause a decrease in gut-associated lymphoid tissue (151). The response to microbial infection by the intestine is decreased in zinc deficiency (152). Proteins associated with the formation of TJ decrease in abundance in zinc deficiency (130). The combination of rapid turnover, sensitivity of immune mediators, and function of intestinal barrier proteins to zinc in the intestine all suggest that an effective transport of zinc to the intestine would be important to protect against infection. The activity of ZIP14 in the intestine may be a modulator of the zinc supply to enterocytes during infection and inflammation. This role will need to be examined. Also, intestinal localization of ZIP14 is not known. ZIP14 may take zinc from dietary sources at the lumen, via an apical localization. ZIP14 may also further contribute to the hypozincemia of inflammation and intestinal defense, via basolateral localization.

These observations have led to the hypothesis that ZIP14 is a zinc transporter that is responsible for zinc homeostasis in both basal and inflammation-mediated conditions which helps maintain intestinal function. The specific aims that will address this are to: (1) characterize ZIP14 distribution and localization in the GI tract, (2) identify the contribution of ZIP14 to zinc transport in the GI tract, (3) determine the ZIP14 responsiveness to LPS in the GI tract and the role that it provides in intestinal zinc homeostasis, (4) establish if ZIP14 modifies intestinal barrier function, and (5) identify altered signaling pathways in the ZIP14 KO phenotype.
CHAPTER 2
MATERIALS AND METHODS

Cell Culture

Caco-2 cells (ATCC), a human colorectal adenocarcinoma cell line, were cultured in Dulbecco’s modified Eagles Medium (DMEM) supplemented with 20% fetal bovine serum (FBS, Sigma), 2 mM glutamine (Sigma), and penicillin/streptomycin (Sigma). Media was changed every 2 days in culturing cells. Cells were trypsinized (0.025%, Sigma) at 80% confluence and plated at $2.2 \times 10^6$ cells/well in 10 cm plates. For some experiments, Caco-2 cells were cultured in 12-well Transwell® plates for 21-days post-confluence as they are known to spontaneously differentiate into an enterocyte phenotype with fully formed TJs.

Animals and Treatments

Young adult (8-12 weeks) male C57BL/6 strain mice were purchased from Charles River Breeding Laboratories. Zip14$^{+/+}$ and Zip14$^{+/+}$ mice were purchased form Mutant Mouse Regional Resource Center (MMRRC), University of California, Davis. A targeted mutation in Zip14 gene (exons 3 through 5) was generated in strain 129/SvEvBrd-derived embryonic stem cells. The chimeric mice were bred to C57BL/6 mice to generate F1 Zip14$^{+/+}$ mice. Zip14$^{+/+}$ mice were obtained through further breeding of founder Zip14$^{+/+}$ mice in the Genetic and Cancer Research Complex (GCRC) facility, University of Florida. Mice were given free access to tap water and received commercial diets (Harlan Teklad-7912-030811M, 60 mg ZnO/kg) with a 12 hr light/dark cycle. Protocols were approved by the University of Florida Institutional Animal Care and Use Committee. The design of experiments in this dissertation project was composed of a single dietary treatment with three treatment interventions,
lipopolysaccharide injection, FITC-dextran gavage, and antibiotic administration in drinking water. During IP experiments with phosphate buffered saline (PBS) or LPS, and for the day of sacrifice, mice were administered isoflurane (Baxter) anesthesia. For LPS treatments, mice received a 500 µL IP injection with a 27-gauge needle of PBS or LPS (2 mg/kg) at time points 3-, 6-, and 18-hr prior to sacrifice via cardiac puncture. For FITC-dextran permeability experiments, mice were fasted overnight prior to FITC-dextran gavage (600 mg/kg; 250µL). In the antibiotic study, 1 mg/mL neomycin and 0.5 mg/mL ampicillin were added to the drinking water for 1 month. Mice were then sacrificed in the same manner as LPS treatments.

**RNA Extraction and Quantitative Real-Time PCR**

For animal studies, intestinal tissue was excised, perfused with ice-cold PBS, cut open longitudinally and mucosa was scraped using a glass microscope slide. Mucosal scraping was immediately placed in RNAlater (Ambion). For RNA extraction, tissues were centrifuged for 5 minutes at 10,000 x g and RNAlater aspirated off. TriReagent (Ambion) was added to tissue and homogenized by Polytron. Caco-2 cells were washed of media two times with PBS following treatment and then TriReagent was added to the cell culture well. Cells were homogenized through multiple passages through a pipette tip. RNA was purified from one mL of TriReagent homogenized cell and tissues according to the TriReagent protocol. Total RNA was treated with Turbo DNase (Ambion) to remove genomic DNA contamination. Two µg total RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Oligonucleotide primers and Taqman probes for target genes were designed using Primer Express® software (Applied Biosystems) from sequences obtained through GenBank. For screens of TJ genes (Table 2-1) and TJ signaling
genes (Table 2-2) Sybr green was used. Melt curves were performed for each primer sets designed for Sybr green use to confirm target transcript specificity. For screens of Zip (Table 2-3) and ZnT (Table 2-4) transporters Taqman probes were used. To normalize data, either a universal 18S or TATA binding protein was used (Table 2-5). Values were obtained via the relative standard curve method. qPCR was run on a StepOne Plus thermal cycler (Applied Biosystems) using either TaqMan Fast Universal PCR Master Mix (Applied Biosystems) or Fast Sybr Green PCR Master Mix (Applied Biosystems).

**Western Analysis and Polyclonal Antibodies**

Antibodies designed within the lab were synthesized and conjugated, and injected into rabbits. An extra N-terminal cysteine is added to facilitate conjugation. The sequences for the in-lab designed antibodies are as follows: mouse anti-ZIP14-CSNELDGKAPGTD, mouse anti-ZIP2-, and mouse anti-ZNT6-. Human anti-Zip14 was purchased (HPA016508-100µL, Sigma Aldrich). All other antibodies were purchased from commercial vendors: anti-early endosome antigen-1 (EEA1, 3288P, Cell signaling); anti-SGLT1 (ab14686, Abcam); anti-Na+/K ATPase (sc-21712, Santa Cruz Biotechnology); anti-ACTB (A2066-.5ML, Sigma Aldrich); anti-CLDN1 (ab15098, Abcam); anti-OCLN (sc-5562, Santa Cruz Biotechnology); anti-phosphorylated threonine (8781S, Cell Signaling); anti-RAB11 (5589P, Cell Signaling); and anti-PRKCζ (sc-216, Santa Cruz Biotechnology).

For western blots of intestinal proteins, mucosa of intestinal segments was scraped and washed with PBS and prepared by homogenization with a Polytron in 50mM Tris Buffer (pH 7.4) containing 1% Triton X-100, 1x HALT™ protease inhibitor cocktail (Pierce), 1x HALT™ phosphatase cocktail (Pierce), and 1 mM PMSF.
Cytoplasmic and nuclear fractions were extracted using NE-PER extraction system (Pierce) according to manufacturer’s protocol. Proteins were separated by electrophoresis on 10% SDS-PAGE and then transferred onto nitrocellulose membranes. Blots were incubated with target primary antibodies (generally 2 µg/mL). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as the secondary antibody. Immunoreactive bands were visualized using the Protein Simple Fluorchem E imager. For protein band intensity quantification, images were imported into AlphaView SA image analysis software.

**Immunohistochemistry, Immunocytochemistry and Fluorescence Microscopy**

Tissue sections from the intestine (jejunum), from WT and Zip14−/− mice were fresh frozen in optimal cutting temperature (OCT) compound (VWR), embedded in paraffin, cut as 5 µm sections, and mounted. Sections were incubated with 10 µg/ml of anti-rabbit anti-claudin-1 primary antibody (Abcam), followed by addition of anti-rabbit IgG-Alexa 594 conjugate (Molecular Probes). Negative controls were incubated with antibody to which antigenic peptide had been incubated before exposure to tissue (147). Nuclei counterstaining was performed with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). All fluorescent visualization was done at the UF Core Microscopy Facilities by confocal microscopy.

**Villus and Crypt Isolation**

The small intestine of three WT mice were washed with ice-cold PBS, everted on a glass Pasteur pipette, and cut into 2-3 mm length segments. The segments were placed in ice-cold Hank’s balanced salt solution (low Mg/Ca content) containing 0.5 mM dithiothreitol (DTT) and place in 4°C for 5 min under constant stirring. Segments were then passed through a 250 µm strainer and placed 150 mL of ice-cold chelating buffer.
(27 mM trisodium citrate, 5 mM Na2HPO4, 96 mM NaCl, 8 mM KH2PO4, 1.5 mM KCl, 0.5 mM DTT, 55 mM D-sorbitol, and 44 mM sucrose) for 20 min at 4ºC under constant stirring (153). Segments were passed through the same strainer and the supernatants containing IECs were labeled as villus fraction wash (Vw). Segments were transferred to 50 mL conical tube containing 20 mL of ice-cold chelating buffer. These tubes were placed on a Nutator in 4ºC for 5 min. Segments were then passed through the same strainer and the supernatants were collected. These collected fractions were considered enriched in villus cells (VFF). This process was repeated 10 times. Segments were then incubated in 100 mL of chelating buffer at 4ºC for 10 min under constant stirring. The segments were strained and the supernatant discarded. The segments were then placed in 50 mL conical tubes as described above and underwent 10 more 5 min washes. Supernatant collected from these washes was considered the crypt enriched fractions (CF). Confirmation of villus and crypt isolation was determined by alkaline phosphatase activity of each fraction with the p-nitrophenyl phosphate assay (Invitrogen). The fractions with the highest alkaline phosphatase activity were considered villus-enriched and low alkaline phosphatase activity fractions were considered crypt-enriched. Crypt enrichment was confirmed by observation of fraction under light-field microscopy. For western blots, several fractions of villus or crypt were pooled (153).

**Endosome Isolation**

The isolation of crude endosomes using the method used has been described by Stasky et al. previously (154). For animal studies, WT or Zip14<–/–> mice were sacrificed and the duodenum and jejunum excised. Mucosa was removed, via scraping, and washed three times in ice-cold PBS 3 times. The tissue was then resuspended in a
hypotonic homogenization buffer (3 mM imidazole pH 7.4, 250 mM sucrose) and incubated for 15 min. The cells were centrifuged at 1300 x g for 10 min. Cells were resuspended in fresh homogenization buffer, containing 0.03 mM cyclohexamide, 1 mM PMSF, and HALT™ protease inhibitor cocktail, and lysed by 5 passages in a dounce homogenizer. Lysed cells were centrifuged at 2000 x g for 10 min. The supernatant was collected and considered to represent the post nuclear supernatant (PNS). The PNS was mixed with a 3 mM imidazole (pH 7.4)/62% sucrose solution at a ratio of 1:1.2 to bring the sample concentration to 40.6% sucrose. The high sucrose sample was then layered (5 mL) on the bottom of a SW40 ultracentrifuge tube. Layered on top of this sample was a 3 mM imidazole/35% sucrose solution (4 mL), followed by a 3 mM imidazole/8.6% sucrose solution (3 mL). The sucrose gradient samples were centrifuged for 3 hr at 100,000 x g. A white band that is visible at the interphase between the 35% sucrose and 8.6% sucrose differential gradient containing the crude endosomal fraction was removed with a Pasteur pipette. This fraction was diluted 1:1 in 3 mM imidazole containing 1 mM EDTA to reduce the sucrose content. The diluted fraction was centrifuged for 1 hr at 100,000 x g to form a pellet. The pellet was solubilized in 3 mM imidazole containing 1 mM EDTA and used for western blotting.

**Endosomal Intracellular Labile Zinc Concentration**

Endosome fractions were isolated as described above in the endosome isolation section. Following endosome pelleting, protein was suspended in 200 µL PBS. Samples were sonicated at level two for 10 seconds two times. FluoZin-3AM (1 mM) was added to reach a concentration of 5 µM. Samples were incubated at 37ºC for one hr, then 95 µL was pipetted into 96-well black clear bottom plates (Costar). Fluorescence was read on a SpectraMax M5 microplate reader (Molecular Devices) at
Ex/Em 495/516 nm. Normalization of values was done by quantification of protein content with BCA protein assay kit (Pierce).

**Serum Zinc Concentrations**

Whole blood was collected via cardiac puncture from WT and Zip14\(-/-\) mice either treated with PBS or LPS. Blood was allowed to clot in silicon dioxide coated tubes (Capiject, Terumo Medical Corp, Elkton, MD) for 30 min on ice. Blood was centrifuged for 10 min at 3000 x g in 4ºC. Serum was collected and flash frozen or used directly in atomic absorption spectroscopy (AAS) to determine the zinc concentration.

**Tissue Zinc Concentrations**

A proximal section of jejunum was removed from WT and Zip14\(-/-\) mice and washed with PBS. The section was then placed in a pre-weighed 1.5 mL tube and then weighed again. The difference in weight was used to determine total tissue segment weight. The tissue segment was then placed in a glass test tube that was washed in HNO\(_3\). To the tissue, 1 mL of HNO\(_3\) was added. The tube was placed in a dilute bead bath heated to 90ºC for 2 hr, and then allowed to cool overnight. To determine zinc content, AAS was performed on 200 µL of HNO\(_3\) tissue digest diluted in 800 µL H\(_2\)O. Zinc was calculated as Zn/mg weight tissue weight.

**Transfections**

For luciferase assay experiments, Caco-2 cells were seeded at 1.1 x 10\(^5\) cells/well in 48-well plates. Firefly luciferase vector (300 ng/well, Promega) construct containing the experimental promoter, or empty vector, and pSV-β-Galactosidase (200 ng/well, Promega), functioning as a normalizing reporter, was transfected with Polyjet™. Twenty-four hr following transfection, cells are administered experimental treatments. Following treatments, cells were lysed with passive lysis buffer (Promega) for 30 mins at
room temperature on a table top shaking plate. Following lysis, 20 µL of cell supernatant was transferred to an opaque white 96-well plate. A reaction solution containing 94 mM Tris PO₄ pH 7.8, 11 mM MgCl₂, 2 mM ATP, 1 mM D-luciferin (Goldbio), and 1x Tropix Galacton Plus substrate (Invitrogen) was added at a volume of 80 µL to the cell lysis solution, to initiate luciferase enzyme activity, from an automated injection system on a luminometer (Dynex MLX96). The luminometer was set to read for 15 sec/well with a 5 sec integration following injection. The plate was then left to sit for one hr to allow the luciferase reaction to reach completion, and then 100 µL of Tropix Light Emission Accelerator II (Invitrogen) was added to initiate the β-galactosidase luminescence reaction.

**Expression Vectors and Luciferase Reporter Constructs**

Zip14 promoter deletion constructs were generated from genomic DNA isolated from AML12 cells. A universal reverse primer located +60 bp downstream of the transcription start site and containing a HindIII restriction enzyme recognition site was used for all amplifications (AAGCTTTACTGAGTGGGTGCGTCC). Four deletion constructs with a KPN restriction enzyme recognition site were generated with forward primers as follows: -1500 bp upstream (GGTACCGGCCAGAAGAGTCTGAGTGG), -1000 bp upstream (GGTACCAGCTGAGTTCTTCCTGCCAC), -500 bp upstream (GGTACCTTCTTTGTTGGCTGGGT), -100 bp upstream (GGTACCAGGGTGGCCAGGGTAACG). Constructs were inserted into the pGL4-Basic luciferase vector (Promega).

**Apical Membrane Isolation Biotinylation Assay**

Intestinal tissue excised from mice was washed with ice-cold PBS containing 1 x protease inhibitors (HALT, Pierce), 1 x phosphatase inhibitors (HALT, Pierce) and 1 mM
PMSF. The intestine was everted using a glass Pasteur pipette and then incubated in 10 ml of PBS containing Sulfo-NHS-SS-Biotin (Thermo Scientific) at a final concentration of 0.48 mg/ml for two hr at 4ºC. Samples were washed three times in ice-cold PBS and intestinal epithelial cells (IECs) were isolated by mucosal scraping with a microscope slide. IECs were collected by centrifugation at 400 x g for 5 min at 4ºC, and then washed three times in ice-cold PBS. Total protein was extracted by incubation in non-denaturing lysis buffer containing 20 mM Tris HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 x protease inhibitors, 1 x phosphatase inhibitors and 1 mM PMSF at 4ºC for 1 hr with gentle agitation, followed by centrifugation at 16,000 x g for 20 min at 4ºC to remove insoluble material.

Biotinylated proteins were affinity purified using Streptavidin-sepharose bead conjugate (3419S, Cell Signaling). The sepharose bead slurry (10 µL) was combined with 200 µL of total cell lysis from biotinylated intestinal segments (1 mg/mL). Samples are incubated in the bead slurry overnight at 4ºC with gentle agitation. The following day, samples are centrifuged at 14,000 x g in a chilled centrifuge and washed five times in non-denaturing lysis buffer containing inhibitors for proteases and phosphatases. The bead pellet was then suspended in 25 µL of SDS loading buffer and heated at 95ºC for 5 min. Following incubation, the beads were centrifuged at 14,000 x g for 1 min and the SDS loading buffer fraction was used for western blotting (modified protocol from (155)).

**FITC-dextran Permeability Assay**

This protocol was adapted from Wang et al. for the use in a cell culture model system (156). In animal model experiments, WT and Zip14−/− mice were fasted overnight. In the morning, mice were administered 600 mg/kg bodyweight FITC-dextran
via gavage (250 µL). One hr following gavage the mice were sacrificed. Blood was collected via cardiac puncture and placed in K2-EDTA coated tubes for plasma collection. Plasma samples were diluted 2-fold with PBS and FITC-dextran content determined by fluorescence (Ex/Em 485/530). For cell culture experiments, Caco-2 cells were plated in 12-well Transwell plates and Zip14 was knocked down by siRNA transfection as described below in siRNA section. Three days post-transfection, Caco-2 cells were administered either media or media containing 0.5 ng/mL IL-1β for 12 hr. Following the 12 hr time point, the bottom well compartment medium was replaced with phenol-free HBSS (low Mg and Ca) and 100 µL (10 mg/mL) FITC-dextran was added to the top well compartment. HBSS was collected after 1 hr and FITC-dextran fluorescence was quantified.

**siRNA**

Caco-2 cells were plated in 12-well Transwell plates at 6x10^4 cells/well. Shortly after cells were plated, 109 µL of siRNA/transfection reagent complex was added to each well. The complex consisted of siRNA, (Dharmacon) either Zip14 targeting or scrambled non-targeting (50 nM/well) and was transfected using 9 µl/well HyPerFect transfection reagent (Qiagen) according to manufacturer protocol. Cells were incubated with siRNA for 72 hr prior to experimental procedures.

**65Zn Administration**

WT and Zip14/− mice were administered 65Zn to determine both endogenous transport and excretion of zinc and zinc uptake. For endogenous transport and excretion, mice were administered by SC injection of 65Zn (3 µCi) for 3 hr. Mice were sacrificed by cardiac puncture and the entire length of the small intestine and colon was excised. The contents of the intestine were washed out with 18 mL of PBS/EDTA metal
chelating buffer and collected in 50 mL conical tubes. The contents were kept overnight at 4°C. The following day, the contents were homogenized and a 1 mL aliquot was used to determine counts per minute (cpm) of sample. The total length of excised intestine was weighed and placed in a counting tube to determine cpm. For zinc uptake experiments, $^{65}$Zn (2 μCi) was administered via gavage. Three hr following gavage, mice were sacrificed via cardiac puncture and the entire length of the intestine was excised. The intestine was washed with metal chelating buffer, then weighed and placed in a counting tube to determine intestinal tissue cpm. Cpm was determined using the Cobra II Auto Gamma Counter (Packard). Values from experiment were expressed as percent cpm of sample to total cpm of dose administered.

Statistics

All statistical analyses were done using SigmaStat (Systat Software Inc). For experiments with a single control and experimental group, Students t-test was used. For experiments containing a single control and multiple experimental groups, one-way ANOVA followed by Tukey’s post-hoc test for all pairwise comparisons among means was used. Significance was considered to be a value of P<0.05.
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| ZnT2 | Forward 5'-CCGACCAGCACAAGAC-3'  
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Probe 5'-FAM-CAGGCTGACCACCTCCTG-3' |
| ZnT5 | Forward 5'-CTGCTCGGCTTTGTCATG-3'  
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Probe: 5'-FAM-CGCCGTGCCTACTACCGATTGG-BHQ1-3' |
| II-6 | Forward: 5'-CCAGAACCGCTATGAACTCCT-3'  
Reverse: 5'-CACCCAGCATCAGTCCCCAGA-3'  
Probe: 5'-FAM-TCTGCAAGAGACTTCCATCCAGTTG-BHQ1-3' |
| Cxcl1| Forward: 5'-CACCCAAACCGAAGTGTATAGC-3'  
Reverse: 5'-AATTCTGCTAAACGGAGCTTT-3' |
| IL2 R| Forward: 5'-CAAGTCCTGCAGGCATGTAC-3'  
Reverse: 5'-CTGGGACAAGGGGCACAAGTGT-3' |
| Selp | Forward: 5'-GGGGCTCCTCCTCCAGATGCT-3'  
Reverse: 5'-GCTGAACCGAGTCATGG-3' |
CHAPTER 3
CHARACTERIZATION OF INTESTINAL ZIP14 EXPRESSION AND DISTRIBUTION

Introductory Remarks

The intestine is the axis of dietary uptake and is integral in systemic zinc balance through its capacity to excrete excess zinc. Several zinc transporters have been already identified that play important roles in both processes. The presence of an additional plasma membrane-localized ZIP transporter is of interest as it may represent either a specialized function within intestinal zinc transport, or act in secondary supportive function for other transporters. As loss of ZIP4 from the apical membrane of the intestine is fatal to mice it is unlikely that ZIP14, if present at the apical membrane, would be involved in major dietary zinc uptake (34, 157). Alternatively, ZIP14 may function in specialized zinc transport. The presence of the basolateral transporter ZIP5 regulates the uptake of zinc into the intestine from systemic circulation and is likely a mediator of zinc excretion during high systemic zinc (26). The absolute necessity for this gene is not known, so ZIP14 if localized at the basolateral membrane may also have role in clearing high systemic zinc.

The inflammatory response is a necessary biological function for organismal survival. The decrease of serum zinc and iron has been postulated to serve as a protective mechanism against blood borne pathogens that may utilize these minerals for their own metabolic processes (158, 159). It is also likely that the decrease is attributed to the shunting of minerals to the liver for the production of acute phase proteins (9). ZIP14 has been implicated in this process, as it is highly responsive to upregulation from the endotoxin i.e., LPS (19). The response in the liver has been shown to correspond with the marked increase in zinc concentration and loss of Zip14 abrogates
the increase in liver zinc content normally observed in endotoxemia (147). Also, the decrease in serum zinc from LPS administration is lost in Zip14<sup>−/−</sup> mice.

The upregulation of the gene is not entirely limited to the liver, as muscle, adipose, and pancreas expression also increases (147). The intestine is an organ that is intimately involved in the immune response to endotoxemia as it is the site of ingress for a majority of pathogens. The expression of Zip14 is very high in the intestine, yet based on prior results, LPS exposure for 18 hr does not lead to an elevation in Zip14 transcript (147). The relevance this result has on the way Zip14 is utilized in the intestine is likely very important. Since little is known on the localization of Zip14 in the intestine, this response may reflect a function of the intestine to either decrease dietary uptake of zinc during inflammation, or to decrease systemic uptake of zinc so it can be shunted towards the liver.

Previous research has implicated ZIP14 in the regulation of multiple signaling pathways within various tissues. In chondrocytes, ZIP14 is necessary for c-fos mediated proliferation which is lost by decreased phosphodiesterase activity towards cAMP (148). In the liver, ZIP14 is implicated in c-Met phosphorylation during liver regeneration through inhibition of PTP1B (138). Also, within the liver, phosphorylation of the insulin receptor is increased with the loss of Zip14 (147). Taken together, it appears ZIP14 has a strong influence on signaling pathways that may be unique to each given tissue. The clarification of which pathways ZIP14 regulates in the intestine will be instrumental in developing a complete understanding of ZIP14 function.

With these observations, the purpose of the research reported in this chapter is to characterize ZIP14 in the intestine. This focus will include the localization of ZIP14 and
its effect on basal zinc transport. In addition, the response of ZIP14 to LPS administration and what changes in zinc transport arise from this response are described. Finally, signaling pathways that are mediated by ZIP14 in the intestine are reported.

**Results**

**Zip14 is Localized to the Basolateral Membrane**

The abundance of Zip14 at both the transcript and protein level is highest in the duodenum and decreases along the G.I. tract to display fairly minimal expression in the colon (Figure 3-1A,B). The values between transcript and protein are in very good agreement with one another. Expression of ZIP14 from the crypt to the villus increases in abundance. Fraction enrichment in villus and crypt were confirmed by alkaline phosphatase activity (Figure 3-1C).

The fractionation of cellular compartments into cytoplasmic and membrane show no presence of ZIP14 in the cytoplasm (Figure 3-2a). Na/K\(^+\) ATPase a basolateral membrane marker and SGLT1 an apical membrane marker confirm membrane fraction isolation. Separation of apical and basolateral membrane fractions display a strong enrichment of ZIP14 in the basolateral fraction in both mouse small intestine (Figure 3-2B) and Caco-2 cells (Figure 3-2C). The markers Na/K\(^+\) ATPase and SGLT1 again confirm the purity of the isolated fractions with no appearance of the proteins in the apical and basolateral lanes, respectively. The presence of ZIP14 in the same protein fractions with the basolateral marker Na/K\(^+\) ATPase confirm ZIP14 localization.

**ZIP14 Transports Zinc into the Intracellular Compartment though Endosomes**

The effect of Zip14\(^{-/-}\) on zinc transport was observed in jejunal tissue. A significant increase in total zinc content (P<0.004) is present in the Zip14\(^{-/-}\) mice as determined by
AAS (Figure 3-3A). A qPCR screen of all known ZIP and ZnT transporters in WT and Zip14−/− mice only showed significant change between the genotype in Zip2 (P<0.05) and ZnT6 (P<0.05) with the transcript levels of Zip12, ZnT3, and ZnT8 being undetectable in the jejunum (Figure 3-3B,C). Confirmation of altered expression of Zip2 and ZnT6 was performed by western blot. However, neither gene had an observable difference in protein content between WT and Zip14−/− mice.

To understand the transport mechanism that could lead to tissue zinc accumulation in the absence of any observable change in zinc transporter expression, 65Zn was administered by both SC injection (3 µCi) and gavage (2µCi). Following SC injection, a significant increase in % tissue uptake of intestinal 65Zn was found in the Zip14−/− mice (Figure 3-4B); however, there was no change in % luminal secretion in either genotype (Figure 3-4A). There were no observable differences in 65Zn % tissue uptake in the intestine (Figure 3-4C).

In order to explain the accumulation of zinc in the tissue with no change in zinc secretion, experiments were directed toward looking for trapped intracellular zinc. Crude endosomes were isolated using a sucrose gradient protocol (Figure 3-5). ZIP14 was found associated with endosomes isolated from mouse small intestine (Figure 3-5B). Isolation of endosomes was confirmed with the presence of EEA1, and early endosome marker and RAB11, a late endosomal marker (Figure 3-5B), and the absence of the plasma membrane marker, GPR39 (Figure 3-5B) which is not associated with endosomes (160). The zinc content of endosomes in Zip14−/− mice was found 5-fold higher than in WT mice by fluorescent assay with an intracellular zinc fluorophore, Fluozin-3AM (Figure 3-5D).
ZIP14 Response to LPS Administration in the Intestine Differs From Other Organs

Administration of LPS was used to examine the response of Zip14 to proinflammatory mediators. Response of mice to LPS injection was confirmed at three time points 3-, 6-, and 18 hr with a sharp decrease in serum zinc levels in the LPS treatment group (Figure 3-6A) and a transient increase in Il-6 transcript in the intestine at 3 hr, then a return to basal levels by 6- and 18-hr (Figure 3-6B). Transcript expression of Zip14 failed to respond to treatment at any time points (Figure 3-6C). Protein levels of ZIP14, determined by western blot, decreased following LPS injection at both 3- and 6-hr time points (Figure 3-6D). By 18 hr of LPS treatment, ZIP14 expression was back to the basal level of expression. To further understand this lack of response at the transcriptional level of Zip14 in response to LPS, Caco-2 cells were treated with media or media containing 0.05 ng/mL Il-1β at time points 1-, 3-, 6- and 9-hr. The dosage effectiveness of Il-1β was confirmed by a >15-fold increase in IL-6 transcripts at 3-hr, which moderately declined to time point 9-hr, but still maintained 5-fold expression above control cells (Figure 3-7A). ZIP14 transcript increased approximately 3-fold at 3-hr and maintained expression levels through the 9-hr time point, yet the increase was observed in both the media treated and the 0.5 ng/mL IL-1β treated groups (Figure 3-7B).

To further clarify whether this was a transcriptionally mediated effect directed by the ZIP14 promoter in Caco-2 cells, mouse promoter deletion constructs of Zip14 were transfected and treated with medium or 0.5 ng/mL IL-1β for 24 hr. Deletion constructs for regions -1500, -1000, and -500 bp upstream of the TSS of Zip14 showed high levels of transactivation by the addition of both media and IL-1β (Figure 3-7C). This response was lost in the deletion construct with the minimal -100 bp upstream Zip14 promoter.
The treatment with IL-1β did not lead to an increased transactivation of any of the promoter deletion constructs over what is observed in the media only treatment. There was a trend for decreased transactivation by IL-1β in -1500, -1000, and -500 bp promoter constructs.

The transcription of Zip14 appears to be highly sensitive to cellular change as observed with transcriptional activity of the promoter region in transfected Caco-2 cells. The lack of response from in vivo Zip14 transcript following LPS administration in mice suggests that even minute external stimuli may also have an effect on the transcriptional activity of ZIP14 in vivo as well. As microbiota in the lumen can interact with the intestine an experiment to examine the level of ZIP14 in the absence of potential microbial stimulants was performed. Antibiotic administration of 1 mg/mL neomycin and 0.5 mg/mL ampicillin for one month led to a decrease in ZIP14 expression treatment group compared to untreated mice (Figure 3-7D).

Administration of LPS to WT and Zip14−/− mice for 18 hr resulted in a significant increase in Zip4 transcript in WT PBS vs WT LPS (P<0.001) and in WT LPS vs Zip14−/− LPS (P<0.005). Zip5 transcript decreased significantly in WT PBS vs. WT LPS (P<0.001), but the difference between WT LPS vs Zip14−/− LPS did not reach significance (P<0.078) (Figure 3-8A). In both WT and Zip14−/−, Zip8 shows an increase in expression in the LPS treatment groups. This trend is not significantly different (P<0.08) from PBS treatment (Figure 3-8A). Only one ZnT transporter, ZnT10, significantly decreased in WT PBS vs WT LPS (P<0.001), but no difference between WT LPS and Zip14−/− was observed (Figure 3-8B).
The transport of systemic zinc into the intestine as determined by SC injection of $^{65}\text{Zn}$ Luminal secretion of $^{65}\text{Zn}$ was almost completely lost in the LPS treated groups (P<0.001), but displayed no difference between WT and $\text{Zip14}^{-/-}$ within the LPS treatment (Figure 3-9A). There was a decrease in total % tissue uptake/retention with the administration of LPS (Figure 3-9B). The content of $^{65}\text{Zn}$ was still significantly higher in the $\text{Zip14}^{-/-}$ LPS compared to WT LPS treated groups (P<0.05). A trend was seen within the WT groups between PBS and LPS treatment, but the difference did not reach significance. Percent intestinal tissue uptake following gavage differed significantly between PBS and LPS groups (P<0.001), but did not show any difference in genotypes within the LPS treatment groups (Figure 3-9C).

**The NFκB Pathway is Altered by Loss of Zip14**

A PCR array containing primers for various signal transduction pathways was used with pooled jejunum cDNA samples from male WT or $\text{Zip14}^{-/-}$ mice. Analysis of the PCR results showed 3 genes from the array that had a >4-fold change in expression from WT and $\text{Zip14}^{-/-}$. The genes chemokine ligand 1 (Cxcl1), e-selectin protein (Selp), and interleukin 2 (IL2) were shown to be decreased in the $\text{Zip14}^{-/-}$. All three genes are associated with the NFκB signaling pathway. qPCR was run to verify these results. Cxcl1 (P<0.001) and IL-2 (P<0.01) were confirmed significantly changed (Figure 3-10). Selp failed to show a significant change between genotypes.

**Discussion**

The characterization of ZIP14 in the intestinal tract and its response to LPS was examined in this section. Dietary zinc uptake along the intestinal tract in animal models is highest at the jejunum and duodenum and decreases towards the colon. Transcript abundance of the intestinal zinc exporter ZnT1 is consistent with those transport
observations giving a strong implication in the role of zinc transporters and the uptake of dietary zinc (38). In this report, ZIP14 protein abundance along the intestinal tract displays the same segment-specific distribution.

To further understand the role ZIP14 plays in the transport of zinc at the site of the intestine, localization at the polarized epithelia was also examined. A previous report has shown that loss of Zip14 leads to total decreased zinc absorption and a trend for decreased liver zinc uptake, but with no change in serum zinc levels (147). These observations in female mice led to the speculation that ZIP14 may be an apically localized transporter to account for absorption changes. The loss of Zip4, an apically bound zinc transporter, leads to embryonic lethality in mouse models and is therefore already known to be the main zinc importer of dietary zinc at the apical membrane (157). Furthermore, ZIP4 intestine-specific presence is necessary for survival unless large doses of supplemental zinc are administered (34). Dietary zinc uptake is known to proceed through both transcellular pathways and paracellular pathways. Since humans with mutations in Zip4 develop zinc malabsorption it is unlikely the paracellular pathway would be capable of enough zinc uptake to maintain systemic zinc concentrations (21, 33). Hence, ZIP14 could plausibly function as a second apical zinc importer that functions in support of ZIP4.

To conclusively establish the localization in enterocytes, fractionation approaches to isolate total membrane from cytoplasmic proteins and an apical biotinylation protocol that allows for isolation of apical membrane from the basolateral membrane and cytoplasmic fractions were performed. The basolateral fraction contained ZIP14 as determined by western blot and confirmed by colocalization with Na/K⁺ ATPase. This
result with the mouse model was confirmed in the human colonic cell line, Caco-2. A basolateral localization of ZIP14 does change the implications of the purpose of this transporter. The only known ZIP transporter at the basolateral membrane is ZIP5. A ZIP5/− mouse model has never been generated and the absolute necessity of this transporter is unknown. The importance for two basolateral transporters to increase enterocyte uptake of zinc is unclear. The two potential functions of basolateral zinc import is to either increase enterocyte zinc content in the absence of dietary zinc intake, or to increase the loss of zinc through passage to the lumen via efflux in conditions of high systemic zinc. From limited data available, it was shown that ZIP5 expression along the basolateral membrane in isolated cells is highest in culture conditions of high zinc concentration. This increased expression would suggest that it is primarily responsible for increased zinc loss through pulling systemic zinc into the enterocyte for luminal efflux (25, 35). Therefore, expression of ZIP14 may be to function in basal homeostasis of zinc, as it already has a high level of transcript expression along the intestine, and is unresponsive to dietary zinc conditions (19, 20, 24).

To address whether ZIP14 regulates basal zinc homeostasis in the intestine, tissue zinc content and ZIP and ZnT transporter expression was examined when Zip14 was deleted. Surprisingly, zinc content of intestinal tissue of Zip14/− was higher than that in WT controls. This observation could not be explained by altered zinc transporter compensation, as no significant changes at the protein level were observed in either ZIP or ZnT proteins. The functional activity of the transporters was not observed, so this effect cannot be ruled out as a possibility. Tissue ⁶⁵Zn uptake following SC injection also was higher in the Zip14/− intestine relative to the WT control. There were no
observed additional losses of zinc in the luminal contents though, which suggests the higher zinc content in the intestine is not being excreted into the lumen. A similar result was observed in a model of intestinal copper transport. \textit{Ctr1}^{-/-} led to accumulation of 8 times the Cu content in isolated IECs relative to controls (161). It was suggested that this could be due to an accumulation of vesicular Cu. Another research group utilizing a Zip14 overexpression system in HepG2 cells observed a 50% co-localization of 3xFLAG-tagged Zip14 with early endosome antigen (EEA1) and lysosome-associated membrane protein 1 (LAMP1), both markers of endosomes (162). However, that study was unable to present any \textit{in vivo} data to confirm the likelihood of endosomal compartmentalization of ZIP14.

In the current study, isolation of crude endosomes in WT mice confirmed the presence of ZIP14 in endosomal vesicles. The utilization of the zinc fluorphore, Fluozin-3AM, confirmed a 5-fold increase in endosomal zinc in \textit{Zip14}^{-/-} mice compared to control. The presence of ZIP14 in an endosomal fraction is of interest as vesicular zinc release affects regulatory mechanisms of signaling pathways associated with immune response (163, 164). If ZIP5 function is primarily associated with regulating homeostatic levels of zinc through monitoring systemic zinc levels, then it is possible that ZIP14 functions in a more specialized manner to regulate signaling events in the intestine.

ZIP14 transcript and protein abundances increased in response to LPS in the liver are associated with the hypozincemia of inflammation (19, 147). Additional tissues in response to LPS that show an increase in Zip14 transcript abundance include the muscle, white adipose tissue and pancreas (147). In the intestine, treatment with LPS
did not lead to a comparable increase in Zip14 transcripts. Additionally, ZIP14 protein levels decreased after acute LPS administration. This result is contrary to what was expected based on observation with other tissues. A unique characteristic of the intestinal epithelium that contrasts with that of other tissues is that it is constantly exposed to luminal contents and the resident microbiota within the lumen. Toll-like receptor (TLR)-2 and TLR-4 are active in the intestine in the presence of commensal bacteria as well as pathogenic bacteria. Loss of these receptors and their activation by microbiota leads to increased mortality and impaired capacity to repair tissue damage (165, 166). The major ligand for TLR-4 is LPS and therefore the pathway of ZIP14 activation is likely present to a degree in basal conditions of the intestine. In addition, dendritic cells, cells involved in the adaptive immune response, sample contents of the lumen including commensal and pathogenic bacteria (167, 168). The function of this sampling may be to activate the innate immune pathway as a means to enhance the mucosal barrier from pathogens. With the constant exposure to this environment, mechanisms involved in intestinal Zip14 transcriptional regulation, via LPS, may be fully activated. In the current study, results in the Caco-2 cell model for both endogenous Zip14 transcript expression and trans-activation of Zip14 promoter show increased expression after administration of complete medium containing serum that is not enhanced further by addition of IL-1β. The administration of IL-1β is known to induce Zip14 transcript expression in hepatocytes (146). These results may suggest that the transcriptional regulation of Zip14 in response to exposure to circulating cytokines may be very sensitive in the intestine and lead to a chronic elevation of Zip14 expression not observed in other tissues. To test this effect, the antibiotics neomycin and ampicillin
were administered for one month to WT mice. The antibiotic treatment led to a decrease in ZIP14 expression. This response may reflect a decrease in activation of the Zip14 promoter through a decrease in the microbiota present to activate the dendritic cell immune response signaling and TLR4 signaling. However, the effects of antibiotics on intestinal homeostasis have been well documented and include the possibility that increased apoptosis may explain the decrease in ZIP14 (166).

The effect of LPS on multiple Zip and ZnT transporters has been observed before in the liver (19, 147), but the effect of LPS on transporters within the intestine has not. In this chapter, there was an increase in the apical transporter Zip4 and a decrease in the basolateral transporter Zip5. As previously discussed, ZIP4 expression increases in response to low zinc status to increase uptake of dietary zinc (24, 169). Zip5 transcripts during low zinc status are normally bound to polysomes poised for transcription, but there is not a reduction in transcript abundance (26). A recent paper suggests that Zip5 regulation at the polysome is mediated though mircoRNA (miR) stabilization of the transcripts in part achieved through the interaction with miR-193a (36). Generally, miRs are believed to decrease transcript abundance through degradation, but some experimental evidence suggests they are capable of increasing transcript stability (170). The expression of mir-193a decreases in the liver in response to LPS administration (171). It is possible then that the decrease in Zip5 transcript is due to decreased expression of mir-193a which leads to impaired stability at the polysome.

The response of Zip4 and Zip5 does appear to mimic dietary zinc deficiency and it is plausible that the intestine experiences a localized zinc deficiency during the hypozincemia of inflammation. This effect is supported by the results from injection of
$^{65}\text{Zn}$ in this study. A decrease in total $^{65}\text{Zn}$ absorbed in the WT and $\text{Zip14}^{-/-}$ mice was observed. In addition, $^{65}\text{Zn}$ secretion into the lumen was undetectable following LPS in both genotypes. These observations, in addition to the decrease in ZIP14 abundance after LPS, suggest that systemic zinc uptake is shunted away from the intestine during inflammation.

ZnT10 expression was also shown to be decreased in both genotypes following LPS administration. There is very little known about this transporter, but its response to LPS in the intestine is interesting for a number of reasons. Expression of ZnT10 is highest in the intestine among all adult tissues. Some research in an in vitro FLAG-tagged system suggests it is localized at the Golgi, but can migrate to the plasma membrane to increase zinc efflux (172). However, this activity was not examined in intestinal cells, so it is unclear whether this would be related to transport activity at the basolateral or apical membrane. Interestingly, DNA methylation of ZnT10 has been associated with human colorectal cancer (173). Given that this gene appears responsive to inflammation, a role linking its regulation and the progression of colon cancer development would be an interesting line of research.

Zinc regulation of the immune response is a tightly linked phenomenon (174). The loss of ZIP14 results in decreased secretion of serum Il-6 and upregulation of the feedback inhibitor to NFkB, SOCS3 (147). In this chapter, a signal transduction array looking at multiple pathways was utilized to determine if loss of Zip14 was of any direct impact in the intestine. The result of this array analysis was the significant decrease in two transcripts, Il-2 and Cxcl1. These two genes are part of the NFkB pathway and further confirm the role of zinc and Zip14 in modulating immune function. IL-2 plays a
pivotal role in T-cell activation and zinc contributes to both expression of IL-2 and the high affinity IL-2 receptors expressed on lymphocytes (175). The large content of immune cells within the intestine could relate to the IL-2 mRNA abundance found using the array. Enterocytes themselves may not express IL-2, but lamina propria contain mononuclear cells that do produce this cytokine (176). Intestinal cells express IL-2 receptor and cell viability is improved by IL-2 mediated signaling of transforming growth factor beta 1 (TGF-β1) (177). Cxcl1 is an inflammatory chemokine that is a downstream target of NFκB transcriptional regulation (178). There is less data available on the relationship between Cxcl1 and zinc. In keratinocytes, zinc deficiency was associated with elevated Cxcl1 expression (179). This result is in contradiction to the observed result in the current study, but the impact of gut immune cells may need to be accounted for.

In conclusion, results from this section have confirmed that ZIP14 is a basolateral zinc transporter. These results also suggest that ZIP14 may function to release zinc from vesicles that have undergone endocytosis based on the presence of ZIP14 in endosomes and the high level of zinc accumulated in endosomes when the Zip14 gene is deleted. The association of zinc-containing vesicles and signal transduction pathway regulation in enterocytes fits well with the already observed effects of Zip14 on signaling pathways (138, 139, 147, 148, 164).
Figure 3-1. Expression of Zip14 in intestinal segments. The entire length of the intestine was excised and divided into 4 individual segments (duodenum, jejunum, ileum, and colon). Crypt and villus cell fractions were isolated as outlined in Materials and Methods. (A) mRNA was quantified using qPCR and Zip14 values normalized to TBP. (B) Alkaline phosphatase activity values of isolated villus and crypt fractions normalized to protein concentration of sample. Samples VF2-VF5 were pooled for villus fraction and samples CF1-CF3 were pooled for crypt fraction (VW, villus wash; VF, villus fraction; CF, crypt fraction). (C) Protein (45 µg) was resolved on 10% SDS-PAGE and examined with anti-mZIP14 (4 µg/mL) and anti-β-actin (ACTB, 1:2000). Data are expressed as means ± S.D., n=3 mice.
Figure 3-2. Membrane localization of ZIP14 within enterocytes and Caco-2 cells. (A) Jejunal tissue mucosal scrapings were collected and cytoplasmic and total membrane was isolated using a NE-PER tissue reagents then fractions were subjected to western blot analysis with anti-mZIP14 (4 µg/mL), anti-Na/K+ ATPase (2 µg/mL, basolateral marker), or anti-SGLT1 (2 µg/mL, apical marker). (B) Total small intestine from mice were subjected to apical surface biotinylation and purified using streptavidin-conjugated sepharose beads. The pellet fraction (apical) and supernatant (basolateral/cytoplasmic) were analyzed as in A. (C) Caco-2 cells, grown to 21-day post-confluence on Transwell plates, were subjected to biotinylation in the upper well (apical compartment) and fractionated and analyzed as in part B with anti-hZIP14 (1 µg/mL), n=6 pooled samples.
Figure 3-3. Zip14-/− mice have increased intestinal zinc concentration. WT and KO jejunal segments were isolated from mice. Tissue zinc was assessed from total tissue while mRNA and protein was assessed from mucosal scrapings of jejunum. (A) Tissue samples were digested in HNO₃ and then zinc concentration was determined by AAS. Values were normalized to tissue wet weight, n=10 mice. (B,C) Global analysis of Zip and ZnT transcripts within jejunal mucosal scrapings from WT and Zip14-/− mice using qPCR. Data was normalized to TBP, n=5 mice. (D,E) Protein abundance of ZIP and ZnT transporters found significant in B and C were analyzed by western blots using 10% SDS-PAGE (anti-ZIP2, 2 µg/mL; anti-ZIP6, 2 µg/mL). ACTB was used as a loading control for western blots. Data expressed as mean ± S.D. Statistics calculated for fold change in expression between genotypes of each gene.
Figure 3-4. Zinc absorption and intestinal uptake is altered in Zip14<sup>−/−</sup> mice. Fasted mice received either 3 µCi of <sup>65</sup>Zn by subcutaneous injection or 2 µCi of <sup>65</sup>Zn by gavage and were sacrificed 3 hr later. (A,B) Percent luminal uptake and tissue uptake of <sup>65</sup>Zn from injection were determined after excision of the total length of the small intestine and perfusion of the lumen with 18 mL of PBS/EDTA zinc chelating buffer. Collected wash solution was the luminal fraction. Radioactivity of the luminal content or total tissue content was divided into total cpm counts from the dose and expressed as a percentage. (C) Percent uptake for tissue zinc from gavage was calculated as outlined in A,B. Data expressed as mean ± S.D., n=5 mice.
Figure 3-5. Endosomal fractions accumulate zinc with deletion of Zip14. Endosomes were isolated by sucrose gradient centrifugation and analyzed for ZIP14 presence and intracellular zinc concentration. (A) Schematic of sucrose gradient for crude endosome isolation and photo of representative sample following ultracentrifugation. White interphase band contains crude endosomes. (B) Western blot from endosome fraction from mucosal scrapings of small intestine confirming presence of ZIP14 protein. Total tissue protein (TP) and crude endosome (CE) fraction protein lanes were loaded and blotted for positive control early endosome marker EEA1 (2 µg/mL) and late endosome marker RAB11 (2 µg/mL) and negative control membrane marker GPR39 (2 µg/mL). (C) Intracellular labile zinc content of the isolated endosome as determined through incubation with the intracellular zinc-chelating fluorophore Fluozin-3AM. Data are expressed as relative fluorescence. Values normalized to the endosomal protein concentration. Data expressed as mean ± S.D., n=3 mice.
Figure 3-6. Response of intestinal ZIP14 to LPS exposure. WT mice were given intraperitoneal injections of LPS (2 mg/kg) at time points 3-, 6-, and 18-hr. Serum was collected via cardiac puncture and jejunal mucosal scrapings were collected at each time point for total RNA and protein isolation. (A) Serum zinc was analyzed by AAS. (B,C) IL-6 and Zip14 mRNA levels were measured by qPCR. TBP was used to normalize mRNA data. (D) ZIP14 abundance was measured by western analysis using 10% SDS-PAGE. Antibody concentrations were the same as listed in Figure 3-3. Molecular masses of ZIP14 and ACTB were 240 and 43 kDa, respectively. Numerical data are expressed as mean ± S.D., n=3 mice.
Figure 3-7. Intestinal ZIP14 is highly sensitive to IL-1β and neomycin. (A,B) Response of IL-6 and Zip14 mRNA in Caco-2 cells exposed to medium with or without 0.05 ng/mL IL-1β for, respectively, using qPCR. (C) Caco-2 cells were transfected with promoter deletion constructs of mouse Zip14. Cells were treated with medium as in A, B. Values expressed as relative luciferase units and normalized to the internal control expression plasmid SV-β-Gal. (D) Western blot of intestinal ZIP14 from WT mice treated with antibiotics (Ab, 1 mg/mL neomycin, 0.5 mg/mL ampicillin) in drinking water (+) or regular drinking water (-) for one month. Data expressed as mean ± S.D., n=3 mice.
Figure 3-8. Effect of LPS treatment on jejunal Zip and ZnT transporter mRNAs in WT and Zip14−/− mice. Mice were treated with LPS for 18 hr prior to sacrifice and then RNA from jejunal segments was isolated for qPCR. (A) Zip transporters. (B) ZnT transporters. Values are expressed as Zip14−/− transcript level over WT transcript levels and normalized to TBP. Data expressed as mean ± S.D., n=5 mice.
Figure 3-9. Zinc absorption is not altered by LPS in Zip14-/- mice. Fasted mice received either injection of either PBS or LPS (2 mg/kg). Then 15 hr later either 3 µCi of 65Zn by subcutaneous injection or 2 µCi of 65Zn by gavage and were sacrificed 3 hr later. (A,B) Percent luminal uptake and tissue uptake of 65Zn from injection were determined by excision of total length of the small intestine and perfusion of the lumen with 18 mL of PBS/EDTA zinc chelating buffer. Collected wash solution was luminal fraction. Radioactivity of the luminal content or total tissue content was divided into total cpm counts from the dose and expressed as a percentage. (C) Percent uptake for tissue zinc from gavage was calculated as outlined in A,B. Data expressed as mean ± S.D., n=5 mice.
Figure 3-10. *Zip14*−/− leads to altered expression of genes associated with the NFκB signaling pathway. (A) Pooled samples (n=3) of jejunal cDNA from WT and *Zip14*−/− mice were used in a signal transduction pathway array analysis. Samples outside the purple lines denote fold-change results greater than 4-fold (Test sample, *Zip14*−/−; Control Sample, WT). (B) Transcripts with greater than 4-fold change in *Zip14*−/− were confirmed by qPCR of individual samples and normalized to 18S. Data expressed as mean ± S.D., n=3 mice.
CHAPTER 4
ZIP14 MEDIATES INTESTINAL PERMEABILITY THROUGH REGULATION OF TJ PROTEINS

Introductory Remarks

The main function of the G.I. tract is the uptake of nutrients to maintain metabolic processes within the body. There is also the requirement to keep unwanted pathogens and molecules out of the systemic circulation. This function is in part achieved by transcellular transport mediated by high affinity transmembrane proteins that display selectivity for amino acids, carbohydrates, vitamins, and minerals. However, the secondary system of uptake for H₂O and a number of solutes and ions takes place at the junctional regions between adjacent enterocytes. This paracellular pathway, unlike the transcellular pathway, is regulated by a series of proteins that control the proximity of cell to cell junctions, and hence the “tightness” of the junctions. These proteins are collectively referred to as tight junction (TJ) proteins.

The TJ protein complex serves an integral function in the maintenance of health in the intestine, but systemically as well. Acute GI diseases, such as diarrhea, display impaired barrier function and the cause/effect relationship are topics of ongoing research (180). Additionally, chronic intestinal disease, such as Crohn’s disease, has is associated with impaired permeability. Research has also shown that close relatives of patients with Crohn’s disease have higher intestinal permeability than the normal population (123). There is also a growing body of research showing that neurological diseases such as multiple sclerosis, Alzheimer’s, Parkinson’s, and seizure may be related to increased gut permeability (181).
The administration of supplemental zinc has been shown to decrease intestinal permeability. For treatment of acute and chronic diarrhea, zinc decreases fluid loss and intestinal permeability (121, 182). Likewise, Crohn’s disease patients in remission who are given supplementation of zinc have lower incidence of relapse and decreased gut permeability (124). Zinc's effect on permeability in animal models has been observed to alter the expression of TJ proteins (126).

ZIP14 is a zinc transporter that, as of yet, has undefined functions in the intestine. In the previous chapter, ZIP14 abundance was shown to decrease in response to LPS administration. It is known that LPS administration causes an increase in tight junction permeability (76). With the knowledge that zinc plays a direct role in TJ assembly and function and that Zip14 is a zinc transporter that responds to LPS in a manner similar to when TJ permeability decreases, the possibility for ZIP14 involvement in TJ regulation is evident. The purpose of this chapter is to determine if ZIP14 does regulate intestinal barrier permeability through influence on TJ proteins.

Results

**Intestinal Permeability is altered with loss of Zip14**

A significant increase (P<0.001) in FITC-dextran permeability was seen in Zip14−/− mice following a one-hr gavage (Figure 4-1A). These results were recapitulated in the Caco-2 cell model. Knockdown of Zip14 led to a significant increase (P<0.05) in the amount of FITC-dextran that leaked from the apical to basolateral compartments in a transwell plate (Figure 4-1B). Following administration of 0.5 ng/mL IL-1β to the apical well for 12 hr, FITC-dextran leakage into the basolateral compartment was significantly higher (P<0.05) in the Zip14 knockdown cells compared to control knockdown cells.
PCR Array of TJ Signaling in WT and Zip14<sup>−/−</sup>

Primers were designed to screen for both TJ transcripts and TJ signaling gene transcripts. In Zip14<sup>−/−</sup> mice, significant upregulation of two transcripts, Mpp5 (P<0.001) and Smurf-1 (P<0.006) were observed (Figure 4-2A). In Zip14<sup>−/−</sup> mice and WT mice treated with LPS (2 mg/kg) for 6 hr there was a significant decrease in transcript expression of 6 genes in Zip14<sup>−/−</sup> mice: Arhegf2 (P<0.001); Magi3 (P<0.001); Mpp5 (P<0.006); Prkcζ (P<0.001); Cldn1 (P<0.001); Cldn2 (P<0.001) (Figure 4-2A,B)

TJ Protein Expression in LPS Treated Zip14<sup>−/−</sup> Mice

Select genes from the TJ signaling screen were confirmed at the RNA and protein level. Cldn1 expression in jejunal tissue at the transcript level was not different between WT and Zip14<sup>−/−</sup> in either PBS or LPS treatment. Both genotypes responded to LPS treatment in a similar manner with Cldn1 expression with WT (P<0.05) and Zip14<sup>−/−</sup> (P<0.008) mice being significantly decreased (Figure 4-3A). Western blot of CLDN1 showed a significant decrease in abundance (P<0.001) following LPS administration in WT mice (Figure 4-3B,C). In both PBS and LPS treatments the Zip14<sup>−/−</sup> mice exhibited much lower expression than the WT groups (Figure 4-3B,C). PBS treated Zip14<sup>−/−</sup> mice had very low abundance of CLDN1 (P<0.001) and LPS Zip14<sup>−/−</sup> treated mice had low abundance CLDN1 (P<0.007). In confocal microscopy IF of jejunal CLDN1, Zip14<sup>−/−</sup> show very low intensity at the tight junction normally characterized by a mesh pattern as seen in the WT mice IF image (Figure 4-3D). Unlike CLDN1, OCLN protein was markedly reduced in Zip14<sup>−/−</sup> mice following LPS administration, but Zip14<sup>−/−</sup> treated with PBS did not differ from WT mice treated with PBS (Figure 4-4B).
Decreased PRKζ Abundance Alters Phosphorylation of Occludin in Zip14 KO Mice Following LPS

The administration of LPS to Zip14−/− mice led to a significant decrease in expression of PRKζ relative to WT mice treated with LPS (P<0.008) and Zip14−/− mice treated with PBS (P<0.001) at the 6 hr time point (Figure 4-5A). PRKζ is a signaling protein associated with threonine phosphorylation of occludin (116). Protein content as assessed by western blot mirrored the regulation seen in transcript expression with PRKζ abundance significantly lower (P<0.003) in the Zip14−/− mice treated with LPS relative to Zip14−/− treated with PBS (Figure 4-5B,C). Immunoprecipitation with anti-mouse threonine was performed and PRKζ protein was probed for occludin. LPS administration in WT mice led to a reduction in occludin phosphorylation (Figure 4-5D). Further reduction in phosphorylation was seen in Zip14−/− in both PBS and LPS treated groups.

Discussion

Intestinal barrier function is necessary to maintain intestinal integrity and systemic health. Perturbation of barrier function is associated with a number of intestine-specific diseases, such as diarrhea and Crohn’s disease (123, 182). Barrier function has also been associated with neurologic diseases such as Parkinson’s disease (181). Understanding the mechanisms that play a role in regulating TJ function could be beneficial in helping with the treatment of these diseases. Zinc plays a relevant role in the pathophysiology of tight junction associated disease. Zinc deficiency in animal models is associated with impaired tight junction permeability. Improvement in TJ barrier function in diarrhea and Crohn’s disease is known to occur from zinc supplementation (121, 124, 182). Parkinson’s disease patients also are known to
exhibit functional zinc deficiency (183). In this chapter, zinc transport through ZIP14 is shown to play a role in the regulation of the TJ proteins.

Permeability assessment is a longstanding, non-invasive measure of TJ barrier function. In this study, two models of permeability were assessed. The results of FITC-dextran show that in basal in vivo conditions the Zip14−/− mice have two-fold higher permeability in the gut. In comparison, studies in mice utilizing a 10 mg/kg dose of LPS for 12 hr have a lower than two-fold increase (from ~45 µg/mL to 75 µg/mL) in FITC-dextran (76). These similar findings suggest the increase seen in this study is therefore of physiological relevance. A number of perturbations may occur from this increase in permeability that may have a systemic effect. Of consideration is the potential increase in circulating LPS. This assessment was done using a 4 kDa molecule of dextran which is indicative of small molecule leakage. LPS monomers can be as small as 10 kDa and increase in size and form larger lipid bilayer sheets (184, 185). In other models of enhanced permeability, low grade inflammation is also prevalent. For an in vitro comparison, a Caco-2 cell model was used to examine the effect of Zip14 knockdown on paracellular permeability following IL-1β administration. Previous research has already shown that IL-1β alone is capable of increasing paracellular flux in this cell model (90). The knockdown of Zip14 would be expected to lead to an enhanced permeability when compounded with inflammation. This effect was confirmed from the results from the Caco-2 cell model. Based on the previous literature looking into the role of zinc on TJ protein expression and assembly, it is likely that the increase in FITC-dextran flux in the Zip14−/− mice and Caco-2 cell model is due to a direct alteration in
TJs. The response of TJ proteins to the inflammatory response *in vivo* became the target of my study.

Loss of *Zip14<sup>−/−</sup>* leads to upregulation of the TJ associated genes membrane protein palmitoylated 5 (Mpp5) and Smad ubiquitylation regulatory factor-1 (Smurf-1). MPP5 (also referred to in the literature as Pals1) is a tight junction associated and adherens junction associated protein that links transmembrane proteins to cytoskeletal elements (186, 187). In intestinal cells, MPP5 forms a heterotetramer complex that is necessary for the appropriate assembly of occludin and ZO-3 to the apical compartment (188). The mechanism that would lead to upregulation of MPP5 is unclear as little is known on transcription factors that regulate the gene. It may be possible that the upregulation of the gene is due to feedback mechanisms that are present to restore assembly of tight junctions, but without the presence of known regulators for MPP5 this is merely speculation. Of interest, however, is that MPP5 depletion in T-cells has been shown to decrease activation of NFkB through direct depression of T-cell receptor-mediated activation (189). As the serum IL-6 response to LPS has been shown previously to be decreased in *Zip14<sup>−/−</sup>* mice, MPP5s upregulation suggests a compensation mechanism to increase the impaired cytokine response (147). In response to LPS, MPP5 steady-state transcript was lower in *Zip14<sup>−/−</sup>* mice compared to control. The mechanisms are not known for this, but the results do correspond with the observations made in Caco-2 cells with *Zip14* knockdown. The knockdown cells treated with LPS had the greatest level of permeability. More research would be necessary to reconcile the high expression of MPP5 in *Zip14<sup>−/−</sup>* mice in basal conditions and the large decrease in expression of MPP5 with LPS administration in *Zip14<sup>−/−</sup>* mice.
However, these observations need to take into account steady-state levels of expression of MPP5 compared to acute response to LPS, which may incorporate a number of regulatory proteins that modify expression of MPP5 transcriptional activity.

SMURF1 is an ubiquitin ligase that regulates the TJ through the ubiquitination of Ras-related small GTP-binding protein, RhoA (190). RhoA is necessary for TJ assembly and impaired function of RhoA leads to the disorganization of occludin, ZO-1, and actin (191). However, overexpression of RhoA also leads to the disorganization of the TJ (191). The expression of RhoA displays spatiotemporal dynamics, as assembly and recruitment of the TJ have higher levels of expression, which decrease with the accumulation of E-cadherin, and likely this event is driven by G1 cell cycle mediators (192, 193). Given that RhoA is a dynamic mediator of paracellular permeability, an increase in Smurf1 does fit with the observed increases in permeability in Zip14−/− mice within the context of altering the initial formation of junctions as cells migrate along the crypt to villus of the intestine. Smurf1 is transcriptionally regulated by TNF-α (194). As mentioned previously, low grade inflammation may result from the permeability observed in this study. It may be that local elevated tissue TNF-α could lead to the increased transcript of Smurf1. However, SMURF1 also works as a feedback inhibitor that reduces the activity of TLR4 by ubiquination of the adapter protein MyD88 (195). So, this gene is involved in feedback regulation of cytokines in addition to direct regulation of TJ assembly. This function is of interest given the depression of NFκB signaling genes observed in Zip14−/− mice as discussed in the previous chapter.

Some genes associated with TJ formation displayed an altered transcript expression in the Zip14−/− mice compared to WT mice only when exposed to LPS.
Rho/Rac guanine nucleotide exchange factor 2 (Arhgef2, Gef-H1), one of the altered genes, is a GDP-GTP exchange factor (GEF) for Rho activation (196). Transfer of GTP to Rho makes this protein functionally active. In kidney epithelial tubular cells activation of Arhgef2 by TNF-α leads to MLC phosphorylation, through activation of RhoA activity, and leads to increased permeability. Another gene decreased in LPS treatment of Zip14−/− compared to WT is membrane-associated gulanylate kinase-like protein (Magi-3). Like Arhegf2, it is capable of activating RhoA (197). However, this gene is also shown to co-localize with ZO-1 and cingulin at the TJ (198). Whether the interaction requires RhoA is not known. In the current study, the decrease in Arhgef2 and Magi-3 in Zip14−/− mice treated with LPS does go contrary to the results expected. If loss of ZIP14 does impair permeability, it would be assumed genes associated with increased permeability would be more highly expressed. This result may reflect either a time dependent effect of the LPS treatment on gene expression or as mentioned above RhoA displays dynamic spatiotemporal activity and activation. This dynamic response may mean that regulation of the gene, either by high expression or decreased expression alters the formation of the TJ as Jou et al. has previously suggested (191). In the particular case of Magi-3, the interaction with ZO-1 may be an independent function and the decreased expression could directly lead to impaired ZO-1 assembly.

CLDN1 was considered an especially interesting target from the results of the signaling array. The FITC-dextran permeability assay utilizes a 4 kDa molecule that represents small molecule transport. Previous research has strongly implicated CLDN1 as the regulator of small molecule transport through the paracellular pore (65). This suggests CLDN1 may be a major target that is altered by loss of ZIP14. At the
transcript level, Cldn1 was reduced in both LPS treatments with the Zip14\(^{+/-}\) expression the lowest. Protein abundance of CLDN1, however, was reduced in both PBS and LPS treatments in the Zip14\(^{+/-}\) mice. The decrease in CLDN1 in the Zip14\(^{+/-}\) mice was confirmed with IF. CLDN1 is a dynamically regulated transmembrane protein that is endocytosed in inflammatory conditions (199). The increased flux of LPS that may occur from permeability could lead to low chronic inflammation. This inflammation may lead to altered localization of CLDN1 away from the TJ. The protein is also targeted for proteasomal degradation via ubiquitination by E3 ubiquitin ligases such as LNX1p80 (200). As has been shown with the elevated expression of Smurf1, some ubiquitin ligases are upregulated in the Zip14\(^{+/-}\) in basal conditions. It would fit then, that transcript levels are not affected while protein abundance is decreased. The results of both IF and western blot for CLDN1 fit well with the observed increase in permeability. This protein may represent the main mediator of altered flux seen in the Zip14\(^{+/-}\) mice.

Another target that was focused on within the study was OCLN. Due to the fact that OCLN and CLDN1 comprise the major constituents of paracellular permeability control and there was modest, but not significant decrease in OCLN expression at the transcript level, protein content was also examined. OCLN protein abundance mirrors the results of the transcript expression, yet the loss of protein abundance from the Zip14\(^{+/-}\) mice treated with LPS is greater than that seen in transcript levels. Whereas occludin does not regulate the passage of small ions in paracellular transport it has been implicated in large macromolecule flux (85). This effect was not observed in the current study as only one size FITC-marker was utilized. However, to gain further insight into OLCN function in the Zip14\(^{+/-}\) mice, the mediator of OLCN phosphorylation,
PRKCζ was also examined (116). From the screen of TJ associated proteins, Prckζ transcript was significantly reduced in the Zip14−/− mice treated with LPS. Confirmation of protein abundance showed the same trend with a significant decrease in PRCKζ in Zip14−/− mice treated with LPS compared to PBS. Immunoprecipitation of phosphorylated threonine, the target of PRCKζ, and western blot with OCLN antibody showed that both PBS and LPS treated groups in Zip14−/− mice had decreased OCLN abundance. For PRKCζ to be active, the protein must be phosphorylated by phosphoinositide-dependent protein kinase-1 (PDK-1) (201). Inactivation of PRKCζ is mediated by dephosphorylation by PP2A (202). Zinc is capable of inactivating PP2A through a src-dependent pathway (203). It is possible then that loss of Zip14 can play a direct role on occludin phosphorylation through both altered expression of PRKCζ and by modification of PRCK activity through inhibition of PP2A. OCLN threonine phosphorylation can also be directly inhibited by the activity of PP2A (120). More research in to the activity of PP2A in the Zip14−/− mice would help to clarify if this phosphatase is involved in the regulation of both proteins specifically by this transporter.

Zinc deficiency has been implicated in the impairment of TJ barrier function in human disease studies and in animal disease models. In the previous chapter, ZIP14 was found associated with transport of zinc in vesicles. Vesicular zinc has been shown to play a direct role in the signaling of immune response-mediated pathways (163). Zinc deficiency impairs signaling of MAPK, PKC, cAMP, ERK pathways. PKC is implicated directly in the function of TJ assembly, as mentioned above, and the reduced activity of PKC from ZIP14 loss is most likely zinc-mediated. Experimentally induced zinc deficiency in cell culture models leads to the same change in TJ associated genes.
with reduced abundance of CLDN1 and OCLN (127, 130). Since ZIP14 has been implicated in the transport of other divalent metal cations, such as iron, it is possible the increased permeability is not directly zinc-mediated (141, 142). Iron deficiency in animal models, however, does not lead to increased permeability (204). Also, in children, iron supplementation can lead to increased intestinal permeability (205).

In conclusion, the function of ZIP14 in the intestine is, in part, related to the regulation of intestinal barrier function. This regulation occurs at the transcriptional level for some TJ associated genes, such as Arhgef2, Smurf1 and Mpp5. For other genes, like CLDN1, there is both a transcriptional regulatory pathway as well as an independent translational pathway. There are also post-translational mechanisms that operate on other proteins such as OCLN. The multiple levels of regulation highlight that the function of ZIP14 in the intestine is of particular importance for barrier maintenance. The overall effect of this regulation of Zip14 on TJ is illustrated in the Figure 4-6. Zip14 is necessary for the maintenance of the TJ junction in both basal and LPS induced inflammation through the orchestrated regulation of the TJ proteins, CLDN1 and OCLN, at the transcriptional, translational, and post-translational level.
Figure 4-1. Intestinal permeability increases with loss of Zip14. (A) WT and Zip14\(^{-/-}\) mice were fasted overnight, then administered 600 mg/kg FITC-dextran by gavage for one hr. Plasma was collected and fluorescence measured at Ex/Em 485/530. Data were normalized to mouse bodyweight and expressed as mean ± S.D., n=10 mice. (B) Caco-2 cells were seeded and transfected with control or Zip14 siRNA for 72 hrs in Transwell plates. Twelve hr prior to FITC-dextran assay cells were treated with either media or media containing 0.5 ng/mL IL-1\(\beta\). One hr prior to assay, FITC-dextran (10 mg/mL) was added to the apical well and HBSS (-Ca and Mg) added to the basolateral well. After one hr HBSS was collected and FITC-dextran fluorescence measured. Data expressed as mean ± S.D., n=6.
Figure 4-2. Effect of Zip14<sup>−/−</sup> and LPS challenge to TJ-associated gene expression. WT and Zip14<sup>−/−</sup> jejunal segments treated with LPS were isolated and a screen of transcripts for genes associated with TJ and signaling of TJ assembly was performed. (A) Transcript levels of TJ signaling genes. (B) Transcript expression of tight junction integral genes. Data expressed as mean ± S.D., n=3.
Figure 4-3. Claudin-1 expression is decreased in Zip14−/− mice. WT mice and Zip14−/− mice were treated with LPS (2 mg/kg) for 18 hr then the jejunum was excised and used for qPCR, western blot, and immunofluorescence analysis. (A) Cldn1 transcript expression normalized to TBP. (B) Representative western blot with anti-CLDN1 antibody (2 µg/mL) and (C) densitometry quantification of western blot values. (D) Confocal microscopy immunofluorescence of CLDN1 (10 µg/mL, shown in red) and DAPI (shown in blue) staining of nuclei. Data expressed as mean ± S.D., n=3.
Figure 4-4. Occludin expression is decreased in Zip14<sup>−/−</sup> mice when treated with LPS. WT and Zip14<sup>−/−</sup> were administered PBS or LPS (2 mg/kg) for 6 hr then the jejunum was excised qPCR and western blot analysis. (A) Ocln transcript expression normalized to TBP. (B) Western blot of anti-OCLN antibody (2 µg/mL). Data expressed as mean ± S.D., n=3.
Figure 4-5. Prkcz phosphorylation of occuldin decreases in $\text{Zip14}^{+/+}$ mice and expression decreases in LPS challenge. WT and $\text{Zip14}^{-/-}$ were treated with LPS (2 mg/kg) for 6 hrs and jejunal segments were isolated for qPCR and western blot analysis. (A) Transcript abundance of Prkzζ, normalized to TBP, and normalized to TBP, n=3 mice. (B) Representative western blot with anti-PRCKζ antibody (2 µg/mL). (C) Densitometry of Prkcz western blots normalized to ACTB. (D) Immunoprecipitation with anti-phospho-threonine antibody and western with anti-OCLN. Rabbit IgG heavy chain used as control. Data expressed as mean ± S.D., n=3 mice.
Figure 4-6. Diagram of ZIP14 mediated TJ function. (A) In WT mice, ZIP14 is localized to the basolateral membrane and takes up zinc via endocytosis. Vesicular zinc is then released in order to maintain expression of claudin-1 and occludin. Phosphorylation of occludin is also maintained. (B) Zip14 knockout leads to zinc accumulation in vesicles, loss of claudin-1 at the TJ, and impaired phosphorylation of occludin. The loss of TJ barrier function leads to increased paracellular movement of enteric bacteria into systemic circulation. (C) In WT mice exposed to LPS, expression of ZIP14 at the basolateral membrane decreases. Hypozincemia occurs from response to LPS in the serum leading to decreased total release of zinc from vesicles that lead to decreased claudin-1 and occludin at the TJ and decreased occludin phosphorylation. (D) In Zip14−/− mice treated with LPS, decreased serum zinc and loss of ZIP14 release of vesicular zinc leads to larger decreases in claudin-1 and occludin at the TJ with decreased occludin phosphorylation. This loss of TJs leads to a greater paracellular permeability.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Since the identification of ZIP14 in 2005, a number of studies have described its cation transport capability (19, 141, 142, 146). Recent studies have finally begun to look into the functional relevance of ZIP14-mediated zinc transport (138, 147, 148). The purpose of this dissertation was to add to that understanding by examining the role of ZIP14 in the intestine. The results of the research indicate that ZIP14 is a zinc transporter that is localized to the basolateral plasma membrane. Commonly, IF is the standard for establishing the cellular localization of a protein. However, the specificity of the antibody for its target protein used is a limitation of IF that can give false positive identification to a protein’s localization. The utilization of a direct method of apical biotinylation prior to western blotting was able to remove this limitation.

Examination of Zip14 deletion showed an increase in intestinal zinc concentration from systemic zinc. Since no change in Zip or Znt transporters was observed, it was hypothesized that zinc was trapped in the enterocytes from loss of ZIP14. Isolation of crude endosomal fractions confirmed that ZIP14 is present in endosomes and loss of ZIP14 led to accumulation of zinc within endosomes. The relevance of this is considerable given research that suggests vesicular zinc is involved in the regulation of signaling pathways (163, 164). Signaling pathways that regulate the immune response in particular within the intestine could be altered. This likelihood was recapitulated in this study with the observation of decreased expression of two genes associated with the NFkB pathway, Cxcl1 and Il-2.
At the onset of research into this project, the assumption was that ZIP14 responds to LPS in a manner observed in the liver, muscle, white adipose tissue, and pancreas (19, 138, 147). However, in this study it was determined that Zip14 transcriptional regulation in the intestine does not respond to LPS exposure. To explain this discrepancy between a lack of transcriptional response from LPS by intestinal Zip14 and the marked upregulation of Zip14 by LPS in other tissues, it was hypothesized that resident luminal microbiota may already regulate Zip14 expression. The intestinal immune cells and receptors that mediate the inflammatory response are exposed to the microbial environment of the lumen which may lead to high Zip14 transcriptional activation (165-168). Treatment of WT mice with antibiotics for one month led to a decrease in ZIP14. This decrease is believed to be from a loss of luminal microbiota activation of the Zip14 promoter through depressed cytokine signaling in the intestine.

This dissertation was the first research to connect a specific zinc transporter with TJ barrier function. The functional role of ZIP14 in both basal and LPS-induced inflammation is to regulate the TJ barrier. Loss of the gene increases intestinal barrier permeability. When challenged with LPS, the loss of ZIP14 has a greater impact on TJ protein expression and post-translational modifications that can lead to decreased barrier function. The mechanism of this function is not entirely clear but the role of zinc in regulating phosphorylation of proteins in signaling pathways may be involved. PP2A is a mediator of PRKζ-target phosphorylation that zinc is capable of directly inhibiting (203). The connection between TJ barrier impairment in zinc deficiency and deletion of Zip14 would suggest that it is possible the etiology of certain diseases that are known to exhibit barrier impairment and zinc deficiency may be due to the deficiency, rather than
be the cause of the deficiency. This observation has been already suggested by others for Crohn’s disease (123).

As this dissertation was focused on the characterization and function of ZIP14 in the intestine, there are a number of new research questions that have arisen from the findings. The formation of endosomes that contain ZIP14 is likely mediated by some metal-sensing receptor. The potential identification of a zinc-sensing receptor that undergoes endocytosis would be a novel discovery. GPR39 is a known zinc-sensing receptor that is expressed in the intestine, yet it is currently thought that it does not undergo endocytosis (206, 207). Further research into the targets of vesicular zinc would also lead to a better understanding of the signaling pathways that zinc regulates.

ZIP14 response to LPS in the intestine is still not entirely clear. The observation that antibiotics decrease the abundance of ZIP14 suggests that it may be regulated by luminal microbiota. This observation requires further scrutiny as other factors that may have caused this decrease, such as increased cell apoptosis. Also, the regulation of Zip14 at the promoter level by LPS requires further investigation. As of yet, there is not a definitive transcription factor identified to regulate Zip14 upregulation. With the identification of this/these transcription factor(s), it would be easier to determine in vivo if intestinal Zip14 is chronically activated.

The regulation of TJ barrier function by ZIP14 is also an open question. In this dissertation, the TJ proteins that are altered by loss of Zip14 were identified. Also, the post-transcriptional decrease in threonine phosphorylation of OCLN was also observed. The underlying mechanism that led to TJ barrier protein decreases is still unknown. It is likely ZIP14-mediated signaling pathways are leading to the altered TJ expression.
These pathways need to be analyzed at the protein level. In this dissertation, transcript changes in the genes for TJ signaling were analyzed, yet this gives little insight into their activity. A screen for protein abundance and phosphorylation status of the signaling proteins would fill some of the gaps in understanding how ZIP14 is implicated in TJ barrier function.

This study was able to combine analytic experimental techniques with basic molecular techniques to characterize ZIP14 in the intestines. The use of a mouse model for Zip14 deletion further strengthened the results of this study as it gives a strong indication of the pathways that ZIP14 is involved in regulating. This study also aimed to target aspect of ZIP14 function that have an impact on human health and disease. The identification of ZIP14 as a regulator of intestinal permeability is a novel observation that implicates the onset of disease as being caused by perturbation in zinc homeostasis, rather than, a symptom of zinc homeostasis resulting from diseases states. A full understanding of the role of ZIP14 in altering TJ proteins and permeability has not been reached in this study. There still requires further research to understand if zinc is altering the transcriptional, post-transcriptional, translational, and post-translational processing of the TJ proteins. A further examination into target TJ proteins such as CLDN1 and OCLN at all 4 levels of regulation would help to clarify ZIP14 function. This study, however, does present a good foundation on top of which these further experiments can be done.
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BIOGRAPHICAL SKETCH

Gregory Guthrie was born in New Orleans, Louisiana. In 2005, he received his Bachelor of Science degree in Dietetics from Louisiana State University. He then continued his education in the field of clinical nutrition earning a Master of Science in Human Resources with a concentration in clinical nutrition in 2007 from the University of Louisiana, Lafayette. In the winter of 2008, Gregory moved to Gainesville, Florida and began work as a lab technician in the lab of Dr. Neil Shay. He entered the Doctor of Philosophy (Ph.D.) degree program in the Food Science and Human Nutrition (FSHN) department of the University of Florida in the summer of 2008. In the summer of 2010, Gregory moved to the lab of Dr. Robert Cousins in the FSHN department and changed his degree concentration to Ph.D. in Nutritional Sciences. In the fall semester of 2013, Gregory completed his Ph.D.