CHARACTERIZATION OF ZIP11 WITHIN THE MURINE GASTROINTESTINAL TRACT

By

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To my husband, Tanner Martin and my sisters, Britni Ruppert and Kelsey Nimmer
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>4</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>7</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>8</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>10</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>12</td>
</tr>
<tr>
<td><strong>1 INTRODUCTION</strong></td>
<td>14</td>
</tr>
<tr>
<td>Zinc and Zinc Transporters</td>
<td>14</td>
</tr>
<tr>
<td>Gastric Functionality and Signaling</td>
<td>16</td>
</tr>
<tr>
<td>Colonic Functionality and Signaling</td>
<td>18</td>
</tr>
<tr>
<td>The Role of Zinc in Gastric and Colonic Tissues</td>
<td>20</td>
</tr>
<tr>
<td><strong>2 MATERIALS AND METHODS</strong></td>
<td>27</td>
</tr>
<tr>
<td>Animals</td>
<td>27</td>
</tr>
<tr>
<td>Husbandry, Dietary Treatments, and LPS Administration</td>
<td>27</td>
</tr>
<tr>
<td>Genotyping</td>
<td>28</td>
</tr>
<tr>
<td>Tissue Collection</td>
<td>31</td>
</tr>
<tr>
<td>Measurement of Serum and Tissue Zinc Concentrations</td>
<td>32</td>
</tr>
<tr>
<td>RNA Isolation</td>
<td>32</td>
</tr>
<tr>
<td>Zinc Transporter Antibodies</td>
<td>34</td>
</tr>
<tr>
<td>Immunoblotting</td>
<td>34</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>36</td>
</tr>
<tr>
<td>Immunoperoxidase Staining</td>
<td>36</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>37</td>
</tr>
<tr>
<td>Isolation and Analysis of Serum microRNA</td>
<td>38</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>39</td>
</tr>
<tr>
<td><strong>3 ZIP11 KNOCK-OUT MOUSE MODEL CHARACTERIZATION</strong></td>
<td>42</td>
</tr>
<tr>
<td>Introductory Remarks</td>
<td>42</td>
</tr>
<tr>
<td>Results</td>
<td>45</td>
</tr>
<tr>
<td>Understanding the Zip11 Knockout Model</td>
<td>45</td>
</tr>
<tr>
<td>A Significant Decrease in the Zip11 RNA Expression in KO Mice</td>
<td>49</td>
</tr>
<tr>
<td>No Change in ZIP11 Protein Expression in KO Mice</td>
<td>50</td>
</tr>
<tr>
<td>Dietary Zinc Did Not Affect the Functionality of KO Mice</td>
<td>51</td>
</tr>
<tr>
<td>Discussion</td>
<td>53</td>
</tr>
</tbody>
</table>
4 EFFECT OF DIETARY ZINC ON EXPRESSION OF ZIP11 AND OTHER ZINC TRANSPORTERS WITHIN THE GASTROINTESTINAL TRACT ....................... 63

Introductory Remarks .................................................................................................................. 63

Results ....................................................................................................................................... 65

Features of the Zip11 Gene and the Predicted ZIP11 Topology .............................................. 65
Zip11 RNA and Protein Expression Tissue Distribution ......................................................... 65
Tissue Zinc Concentrations of Stomach, Intestines, and Colon ............................................... 67
Zip11 mRNA Decreases During Zinc Deficiency ................................................................. 68
ZIP11 Protein Expression Is Variable Among Tissues During Zinc Deficiency ......................... 69
ZIP11 Localizes to the Nuclei in the Gastric Tissue .............................................................. 71
ZIP11 Localizes to the Colonic Epithelial Cells ..................................................................... 71
ZIP4 Increases in the Colon During Zinc Deficiency ............................................................. 72
Zip5 Increases in the Stomach and Colon During Zinc Supplementation ............................... 73
Several microRNAs Increase During Zinc Deficiency .......................................................... 73

Discussion ................................................................................................................................ 74

5 CHARACTERIZATION OF ZIP11, ZIP4, AND ZIP5 EXPRESSION IN THE MURINE COLON DURING ACUTE INFLAMMATION ......................................................... 97

Introductory Remarks ................................................................................................................ 97

Results ....................................................................................................................................... 99

Colonic Tissue Zinc Decreases After an LPS Challenge .......................................................... 99
Colonic Metallothionein mRNA Expression Increases in Response to LPS ......................... 99
Zip11 Colonic mRNA Expression Decreases in Response to LPS ........................................ 99
Zip4 Colonic Expression Increases in Response to LPS ........................................................ 100
Zip5 Colonic Expression Decreases in Response to LPS ....................................................... 100

Discussion ................................................................................................................................ 100

6 CONCLUSIONS AND FUTURE DIRECTION ........................................................................ 108

LIST OF REFERENCES .............................................................................................................. 110

BIOGRAPHICAL SKETCH .......................................................................................................... 117
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td></td>
</tr>
<tr>
<td>Zip Transporter Primer/Probe Sequences</td>
<td>40</td>
</tr>
<tr>
<td>2-2</td>
<td></td>
</tr>
<tr>
<td>ZnT Transporter Primer/Probe Sequences</td>
<td>40</td>
</tr>
<tr>
<td>2-3</td>
<td></td>
</tr>
<tr>
<td>Other Primer/Probe Sequences</td>
<td>40</td>
</tr>
<tr>
<td>2-4</td>
<td></td>
</tr>
<tr>
<td>ZIP Transporter Antibody Sequences</td>
<td>41</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>3-1</td>
<td>Diagrams of the mouse <em>Zip11</em> gene, genetrap, and alternative slicing</td>
</tr>
<tr>
<td>3-2</td>
<td>Genotyping tail sample DNA</td>
</tr>
<tr>
<td>3-3</td>
<td>Confirming genotypes using different primer sets</td>
</tr>
<tr>
<td>3-4</td>
<td>Knock-out Zip11 transcript expression compared to wild-type.</td>
</tr>
<tr>
<td>3-5</td>
<td>Wild-type versus knock-out ZIP11 tissue distribution.</td>
</tr>
<tr>
<td>3-6</td>
<td>Plasma and tissue zinc concentrations of KO mice on dietary restriction</td>
</tr>
<tr>
<td>3-7</td>
<td>Effects of zinc depletion and supplementation on zinc-related gene transcripts in the stomach and colon of the KO mice</td>
</tr>
<tr>
<td>4-1</td>
<td>Features of the murine <em>Zip11</em> gene</td>
</tr>
<tr>
<td>4-2</td>
<td>Tissue distribution of the <em>Zip11</em> gene transcript in C57BL/6 mice.</td>
</tr>
<tr>
<td>4-3</td>
<td>ZIP11 protein tissue distribution in the murine model</td>
</tr>
<tr>
<td>4-4</td>
<td>Wild-type tissue zinc concentrations during ZnA, ZnD, or ZnS dietary conditions</td>
</tr>
<tr>
<td>4-5</td>
<td>Effects of dietary zinc depletion and repletion (ZnR) on serum zinc and GI tract MT expression.</td>
</tr>
<tr>
<td>4-6</td>
<td>Effects of dietary zinc depletion and repletion on the <em>Zip11</em> mRNA throughout the GI tract.</td>
</tr>
<tr>
<td>4-7</td>
<td>Effects of dietary zinc depletion and repletion on the murine ZIP11 protein expression in the stomach</td>
</tr>
<tr>
<td>4-8</td>
<td>Effects of dietary zinc depletion and repletion on the murine ZIP11 protein expression in the colon</td>
</tr>
<tr>
<td>4-9</td>
<td>Effects of dietary zinc depletion and repletion on the murine ZIP11 protein expression in the cecum and small intestine</td>
</tr>
<tr>
<td>4-10</td>
<td>Visualizing the murine ZIP11 protein with immunoperoxidase staining in the stomach.</td>
</tr>
<tr>
<td>4-11</td>
<td>Visualizing the murine ZIP11 protein with immunoperoxidase staining in the colon and cecum.</td>
</tr>
</tbody>
</table>
4-12 Immunofluorescence imaging of ZIP11 in the murine stomach. ......................... 92
4-13 Immunofluorescence imaging of ZIP11 in the murine colon. .......................... 93
4-14 The effect of ZnD or ZnR on ZIP4 expression in murine GI tract.................... 94
4-15 The effect of ZnS and ZnR on Zip5 expression in the murine GI tract. .......... 95
4-16 Identification of serum miRNAs responsive to dietary zinc deficiency in mice using a qPCR-based array.. ................................................................. 96

5-1 The effects of a ZnA or ZnS diet on murine colonic tissue zinc concentrations and MT mRNA expression after LPS administration.. ................................. 104
5-2 The effect of LPS administration on colonic Zip11 expression when mice were fed either a ZnA or ZnS diet................................................................. 105
5-3 The effect ZnA and ZnS diets have on murine Zip4 expression in the colon after LPS administration.. ................................................................. 106
5-4 The effect of LPS administration on Zip5 colonic expression when mice were either fed a ZnA or ZnS diet. ................................................................. 107
LIST OF ABBREVIATIONS

AAS  Atomic Absorption Spectrophotometry
AE   Acrodermatits Enteropathica
cDNA Complementary Deoxyribonucleic Acid
$C_T$ Threshold Cycle
DNA  Deoxyribonucleic Acid
ES   Embryonic Stem Cell
GI   Gastrointestinal Tract
HET  Heterozygous
HOM  Homozygous Null
IF   Immunofluorescence
IgG  Immunoglobulin G
IHC  Immunohistochemistry
ISC  Intestinal Stem Cell
KO   Knock-Out
LPS  Lipopolysaccharide
miRNA MicroRNA
MRE  Metal Response Element
mRNA Messenger Ribonucleic Acid
MT   Metallothionein
MTF-1 Metal Response Element Binding Transcription Factor – 1
NTC  No Template Control
PBS  Phosphate Buffered Saline
qPCR Quantitative Polymerase Chain Reaction
RNA  Ribonucleic Acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBP</td>
<td>Tata Binding Protein</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TV</td>
<td>Tubulovesicle</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
</tr>
<tr>
<td>ZIP</td>
<td>Zrt-Irt-like Zinc Transporter Superfamily</td>
</tr>
<tr>
<td>Zip11</td>
<td>Zip11 gene</td>
</tr>
<tr>
<td>Zip11</td>
<td>Zip11 mRNA</td>
</tr>
<tr>
<td>ZIP11</td>
<td>Zip11 protein</td>
</tr>
<tr>
<td>ZnA</td>
<td>Zinc Adequate</td>
</tr>
<tr>
<td>ZnD</td>
<td>Zinc Deficient</td>
</tr>
<tr>
<td>ZnR</td>
<td>Zinc Repletion</td>
</tr>
<tr>
<td>ZnS</td>
<td>Zinc Supplementation</td>
</tr>
<tr>
<td>ZnT</td>
<td>Zinc Transporter</td>
</tr>
</tbody>
</table>
CHARACTERIZATION OF ZIP11 WITHIN THE MURINE GASTROINTESTINAL TRACT

By
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Zinc transporters have been characterized to further understand the absorption and metabolism of dietary zinc. Two transporter families have been identified: the ZnT and the Zrt-Irt-like (Zip) transporters. A previously uncharacterized Zip transporter, Zip11, was found through microarray analysis to be highly expressed in the murine stomach, cecum, and colon. The objective was to characterize Zip11 within the murine gastrointestinal tract and to determine if dietary zinc and inflammation regulated Zip11. Through qPCR and western blot analysis, Zip11 was shown to be down-regulated during zinc deficiency in murine stomach tissue but appeared to be unaffected in the large and small intestine. Immunohistochemistry (IHC) revealed high ZIP11 expression in the nucleus of gastric and colonic epithelial cells, while some staining was seen in the cytoplasm of these cells. IHC analysis of the colon also revealed an increase in ZIP11 staining during zinc deficiency. Since inflammation can affect the colon, Zip11 expression was analyzed during acute inflammation. A lipopolysaccharide (LPS) murine model was utilized to create acute inflammatory responses for that purpose. ZIP11 protein increased after LPS administration. A Zip11 knock-out murine model was
obtained to further analyze functional roles of Zip11. Due to the genetrap used, a hypomorphic allele was created, resulting in no change to ZIP11 protein expression. The high expression of this specific zinc transporter in the stomach and colon relates to recent observations related to functions of zinc in these organs. For example, zinc deficiency is known to damage gastric mucosa, and total gastric acidity increases with supplementation. Research has also shown zinc accumulation in the parietal cell when the proton pump was inhibited, thus linking acid output and cellular zinc levels. The colon absorbs fractional zinc during healthy states; however, when zinc absorption is compromised during malnutrition, the colon appears to have an increased efficiency for absorption. In conclusion, Zip11 is highly expressed within the murine stomach and large intestine and appears to be partially regulated by zinc deficiency within these tissues. Zip11 may play a role in zinc homeostasis within these tissues, helping to maintain mucosal integrity and function.
CHAPTER 1
INTRODUCTION

Zinc and Zinc Transporters

Essential nutrition in living organisms requires the balance of many macronutrients and micronutrients. One particular micronutrient, zinc, has been shown to be required for proper growth and development in living organisms. This trace mineral is found throughout the human body, but the tissues with the high percentages are skeletal muscle and bone (57% and 29% of total body zinc, respectively) (13, 38). Zinc can play roles in three different biological functions: catalytic, structural, and regulatory. More than 300 enzymes require zinc for proper functionality, and a loss of function is observed when zinc is removed (13). Some examples of zinc metalloenzymes are carbonic anhydrase, alkaline phosphatase, RNA polymerases, and reverse transcriptase. Zinc is also required for structural stability of proteins, binding to such motifs as the zinc finger DNA-binding domains (38, 70). Newer evidence in the zinc field has also shown that zinc may influence cell signaling pathways involving STAT3, CREB, and protein kinases and phosphatases (12, 26).

An inducible metalloprotein known as metallothionein (MT) acts as a metal detoxifier and a zinc exchanger. Changes in dietary zinc concentration will influence MT. For example, during zinc deficiency, MT RNA expression will be significantly decreased in the kidney and liver. Zinc and other metals stabilize MT from degradation. Metal response element(s) (MRE) have been identified on the promoter region of the MT gene and several other genes. A protein that binds this MRE region, known as metal regulatory transcription factor (MTF-1), senses the intracellular zinc concentration, binds up Zn, and travels into the nucleus where it will recognize the MRE on the MT
gene and several other genes (38, 70). Zinc may also play regulatory roles involving "receptor-mediated signal transduction" (38), as with T-cell activation or regulation of insulin-like growth factor. With the high abundance of zinc in the central nervous system (CNS), several regulatory mechanisms must be in place to ensure zinc homeostasis. Both the regulation of MT and zinc transporters need to be working properly to ensure zinc does not accumulate in the CNS.

Several zinc transporter proteins have been identified and characterized over the years, and currently two families divide up these transporters. The ZnT family (Slc30A) is comprised of ten members, whose main objective is lowering the intracellular zinc concentration, whether by sequestering zinc into organelles or the efflux of zinc from cells. Fourteen members have been placed into the Zrt-Irt-like protein (ZIP) family (Slc39A), and their main function is to increase the intracellular zinc concentration of cells by transporting zinc into the cytoplasm from outside of the cell or from the organelles. The ZnT transporter family of proteins is predicted to have six transmembrane domains, while the Zip transporter protein family is predicted to contain eight transmembrane domains. Both zinc families contain a histidine-rich loop that is thought to contain a metal binding site (12, 19). These transporters not only facilitate the flux of zinc, but may also other elements such as iron, cadmium, and manganese (29, 49). Several zinc transporters have been characterized, and much is known about their cellular location, what nutrients they are shuttling, and if they play a role in zinc-related nutritional issues. The first zinc transporter to be discovered was ZnT1, and its expression has been shown to be distributed within several tissues. ZnT1 may play a role in zinc transfer from the enterocytes, recycling zinc from the kidney, and has also
been shown, with a knock-out (KO) model, to be essential for the transfer of maternal zinc to the embryo (5). $ZnT5$ has also been shown to have varying responses due to zinc supplementation and restriction, depending on the cell type being analyzed (44), and through a $ZnT5$ KO model that showed reduced growth and decreased bone density in mice (31), this transporter has been shown to be vital to zinc homeostasis.

$ZIP4$ is most well known for its mutation that causes the disease acrodermatitis enteropathica (AE) (44, 72). $ZIP4$ can be found highly expressed in the gastrointestinal tract (GI), including the three regions of the small intestine, and to a lesser extent in the stomach and colon (18). The $ZIP4$ mutation causes zinc deficiency within these AE patients by affecting the absorption of zinc by the intestines. Those suffering from AE, however, can overcome the deficiency by zinc supplementation (44), suggesting other zinc transporters must exist within the intestine. $Zip5$, unlike the expression of $Zip4$ on the apical membrane, is expressed on the basolateral membrane of the enterocyte. During zinc repletion $ZIP5$ localizes to the basolateral surface, but during deficiency it is internalized and degraded in enterocytes (44, 64, 71). $Zip14$ is another zinc transporter found to be important in zinc homeostasis in the liver (7) and it is also expressed within the small intestine, where it could potentially play a role during inflammation (11).

**Gastric Functionality and Signaling**

Even though the stomach may not be a major site for nutrient absorption, this organ is a secretory tissue that has many important functions to prepare nutrients to be absorbed in the small intestine. Within the three major regions of the stomach (nonglandular, body, and pylorus), the gastric mucosa harbors the secretion glands and gastric pits. The different gastric secretions consist of hydrochloric acid, pepsinogen, bicarbonate, gastrin, intrinsic factor, mucus, and water, and these products can be
involved in several functions of the stomach, including endocrine and motility functions (35, 40, 65). The gastric glands of the upper portion of the stomach contain four cell types found in different regions of the gland. These cell types consist of the chief cells, parietal cells, enterochromaffin-like (ECL) cells and neck cells, moving from the base towards the opening of the gland, respectively. Cells found within the pyloric region of the stomach are known as G cells and D cells. These cells secrete either gastrin or somatostatin, respectively, and these secretions are also involved in acid secretion of parietal cells (65). All of the cells within the stomach have specific functions to maintain a protective mucosal barrier, control the pH, or aid with digestion.

The parietal cell has many ion transporters and channels located on the apical and basolateral membranes. These transporters are important to maintain the proper ion balance within the parietal cell. When acid secretion occurs, the intracellular lumen of a parietal cell is depleted of chloride ions, and bicarbonate ions begin to accumulate (40, 65). Transporters such as H,K-ATPase, Slc26a9, and possibly NHE3 localize to the apical surface; while, the Slc4a2, NHE1,2,4; Na, K-ATPase, and possibly NKCC1 localize to the basolateral surface (40). Many factors stimulate or repress the secretion of acid from the parietal cell. Gastrin, histamine, and acetylcholine (ACh) are the major stimulators of acid secretion. Gastrin will either act on the parietal cell directly, binding to the CCK\textsubscript{B} receptors, increasing intracellular Ca\textsuperscript{2+} or indirectly, binding to the ECL cells. The ECL cell releases histamine after gastrin activation or neural stimulation by pituitary adenylate cyclase-activating polypeptide (PACAP) (40, 61, 65). Histamine will then induce the cAMP-mediated PKA pathway by binding to the H\textsubscript{2} receptors on the basolateral surface. Lastly, ACh is released by the vagus nerve due to another neural
stimulation and binds to the M₃ receptor. ACh binding also stimulates an increase in intracellular Ca²⁺ (40, 61). The stimulation of the parietal cell by increasing intracellular calcium and cAMP causes the inactive tubulovesicle (TV) to move to the apical membrane. The TVs store the H,K-ATPase proton pumps, and upon stimulation TVs are trafficked to the apical membrane where acid can now be secreted out of the parietal cell (40, 61, 79). The stomach has a complex network of mechanisms involved in creating a toxic environment for harmful bacteria, maintaining proper pH for digestion, and beginning the process of breaking down nutrients. Without these networks working properly, digestion can be severely altered, the integrity of the mucosal layers will be disrupted, and issues such as acid reflux may occur.

**Colonic Functionality and Signaling**

The large intestine consists of the cecum, colon, rectum and anus, but for this section the colon will be covered more in depth. The colon’s major role involves the absorption of water, Na⁺ and other minerals, and vitamins, such as Vitamin K. The colon is the last region for these molecules to be recaptured before excretion. Unlike the small intestine, the colon does not have villi present at the luminal surface, rather colonic crypts are present. The cells present in the colonic crypt are similar to those found in the intestinal mucosa; however, paneth cells have not been detected in the lower regions of the colonic crypts. A region of intestinal stem-cells (ISCs) has been identified at the base of the crypt, containing crypt base columnar cells (CBCCs) and +4 stem cells. These ISCs will start to differentiate into secretory goblet and enteroendocrine cells or enterocyte absorptive cells as they move up the colonic crypt (51). These cells are responsible for the secretory and absorptive properties of the colon.
The transport of NaCl in the colon is through electroneutral absorption of ions. The cell surface exchangers, Na/H and Cl/HCO$_3^-$ are located on the apical membrane, as in the stomach, with two exceptions being the NHE1 and a Cl/HCO$_3^-$ exchanger located on the basolateral membrane. Evidence has shown that these exchangers can be affected by the cystic fibrosis transmembrane conductance regulator (CFTR), and mutations in this gene can have a detrimental effect on fluid absorption in the colon. Another pathway for Na$^+$ uptake is also found in the distal colon and is known as the epithelial Na$^+$ channel (ENaC). This channel is localized to the apical membrane, and the Na$^+$ absorption coincides with Cl$^-$ absorption through a Cl$^-$ channel or paracellular diffusion. These ions exit through the Na/K-ATPase pump, Cl$^-$ channels, or the Cl/HNO$_3^-$ exchanger located on the basolateral membrane (22).

Along with the absorption of electrolytes, the colon’s secretory pathway is also important for maintaining fluid balance. The secretory pathway is thought to assist the transport of mucus out of the crypts, while retaining the hydration of the mucus. This balance of secretion and absorption is critical for maintaining barrier function and avoiding secretory diarrhea in conjunction with a large loss of electrolytes (22). Secretory channels & transporters found within the colon consist of the Na$^+$ 2Cl$^-$ K$^+$ cotransporter type 1 (NKCC1), Cl$^-$ and K channels, and a variety of pathways that are involved in secreting bicarbonate. The NKCC1 transporter is found on the basolateral membrane of colonocytes, and research with NKCC1 knock-out mice showed Cl$^-$ secretion reduction in the colon. The Cl$^-$ channels involved in secretion consist of the CFTR protein (apical membrane), Ca-activated Cl$^-$ channels, and in some animals basolateral Cl$^-$ channels, though this channel has not been confirmed in humans (22).
As with the chloride channels, potassium channels are also located on both the apical and basolateral membranes. Two important regulators of ion secretion in the colon are the cAMP and cGMP-dependent secretions. Concentration changes of cAMP and cGMP can both result in Cl⁻ flux; depending on the protein they are activating (22). Lastly, a calcium-sensing receptor (CaSR) has been identified in the colon on both the apical and basolateral membranes. This receptor is important for altering fluid secretion by detecting changes in calcium levels, thus reacting by increasing protein kinase C activity, which in turn augments the breakdown of cAMP or cGMP. The destruction of cAMP or cGMP will cause a decrease in fluid secretion. By increasing calcium delivery to the receptor, CaSR could be a potential therapeutic target during disease states (IBD, diarrhea, etc) to help alter secretion and reabsorption of electrolytes (22).

In recent years, the scientific community has seen a significant rise in the number of publications studying the effects of commensal microbiota on gut health and the health of an individual. The colon contains the highest concentration, $10^{12}$ cells/gram of wet weight, of gut microbes compared to the other regions of the GI tract, such as the stomach which harbors around $10^1$ cells/gram of wet weight (37, 62). Several factors can vary the amount, type, or location of the commensal bacteria in the host, such as age, diet, antibiotic usage, or environmental exposures (62). Colonic zinc concentration could be an important factor when examining commensal microbiota health and diseases that affect this microbial community.

**The Role of Zinc in Gastric and Colonic Tissues**

Zinc metabolism and regulation is found throughout various tissues such as ZIP4 in the enterocytes (18, 46), ZnT2 present in pancreatic acinar cells (25, 46), and Zip14 in the liver (48). The stomach does not appear to be a site of zinc absorption (36), but
zinc regulation within the gastric mucosa has been shown to be important during the beginning of digestion. The accumulation of zinc into the tubulovesicle (TV) compartments of the parietal cells may be coupled with the proton pump on the TV’s membrane and possibly regulated by the secretion of HCl from the parietal cell. When the H,K-ATPase pump is inhibited, the concentration of Zn$^{2+}$ increases within the cytoplasm of the cell due to immobilization into the TV compartment. Along with the work mentioned above, researchers from Dr. David Soybel’s lab were able to show that zinc may be regulating the back diffusion of hydrogen ions from the TVs (24, 54). Zinc could thus be an important factor in tubulovesicle maturation and maintaining the integrity of a mature parietal cell.

Studies looking at dietary zinc status have revealed findings that may also show the importance of zinc for acid secretion. Stomachs of zinc deficient rats show signs of gastric damage when compared to control rats, and these deficient rats also had a decrease in acidic output. Some explanations for this decrease in acid could be the reduction of carbonic anhydrase activity, or an increase of hydrogen ions being reabsorbed by the eroded gastric mucosa. Carbonic anhydrase, a zinc metalloenzyme, is activated by zinc and is required for carbonic acid production from water and carbon dioxide in the parietal cells (56).

The effect of zinc supplementation has also been analyzed within rat stomachs by Yamaguchi et al. Zinc sulfate provided in water or as an intraperitoneal (ip) injection resulted in an increase in acid secretion. In the ip injected rats, the pylorus was ligated one hour after the zinc was administered and total gastric acidity was measured 3 hours after ligation. The total gastric acidity increased when 3 µg of zinc/100 g of body weight
up to 50 µg of zinc/100 g of body weight was ip administered; however, total acidity reached a maximum at 50 µg Zn/100 g of body weight (77). Yamaguchi et al. also wanted to determine the effects of known stimulators of acid secretion (histamine, gastrin, ACC) on the total acidity of gastric secretion after rats were injected with zinc (77). Rats were pretreated with an ip injection of zinc and 1 hour later were injected with histamine, tetragastrin (a peptide fragment of gastrin), and ACC. The combination of histamine or tetragastrin injections after ip zinc administration produced a significant increase in acid secretion when compared to injection of zinc alone; however, the combination of ACC and zinc did not produce a change in gastric acidity. Further studies showed that total acidity of gastric secretion was significantly decreased when zinc and an ACC inhibitor were both administered; however, when ACC was administered along with zinc and the inhibitor, gastric acid secretion levels returned to increased levels seen in ACC stimulated control rats. ACC could be an underlying factor of the zinc stimulating mechanism of acid secretion (77). This previous study did not examine the integrity of the gastric mucosa, but other research has shown that a disruption in the level of zinc within a parietal cell results in decreased cell viability (39).

Monochloramine is an oxidant produced by bacterial and host interactions within the stomach. This oxidant has been shown to disrupt calcium homeostasis and mucosal integrity. Monochloramine has also been shown to negatively influence the intracellular levels of zinc within a parietal cell, and this increase in intracellular zinc reduces cell viability (39). If monochloramine disrupts calcium homeostasis then this oxidant could also be interrupting the stimulation of the H,K-ATPase transporter by calcium. As mentioned above, if this transporter of HCl is blocked, the concentration of
zinc within the cytoplasm increases causing a decrease in cell viability. Maintaining zinc homeostasis within the gastric mucosa and parietal cells appears to be an important factor in normal gastric functions. Since zinc could be affecting gastric mucosa integrity and acid secretion, zinc transporter proteins may be involved in regulating the zinc concentrations to maintain these functions. Using IHC, ZnT4 and ZnT5 were visualized within the cytoplasm of the murine parietal cell. ZnT6 was detected within the murine chief cells, while ZnT7 was expressed within the murine neck cells (79). These transporters could be involved in sequestering zinc into the cytoplasm to aid in gastric secretions.

\(^{65}\text{Zn}\) has been used for decades to study zinc absorption by the gastrointestinal tract (14, 55). Of particular note is a study where rats were fed a zinc deficient diet two days prior to measuring zinc absorption. This approach was used so the zinc concentration within the gut would not interfere with the specific activity of the \(^{65}\text{Zn}\) dose administered (14). \(^{65}\text{Zn}\) was injected into the different regions of the small intestine and large intestine of fasted rats. \(^{65}\text{Zn}\) was measured daily with a whole body gamma counter. \(^{65}\text{Zn}\) retained by each tissue and the contribution to overall zinc absorption by each section were also measured. These isotope studies showed that zinc absorption decreases moving down the small intestine with the duodenum contributing to the highest absorption and the large intestine, including the cecum and colon revealing minimal zinc absorption (14, 64). The large intestine has been shown to be incapable of absorbing sufficient zinc to meet requirements for proper growth in rats (28). Hara et al. also analyzed how zinc absorption in the large intestine was affected by a gastric acid inhibitor (omeprazole), along with removing the large intestine. Their results indicated
that the large intestine has increased efficiency to absorb zinc when omeprazole was provided to the rats. Rats with their cecum and colon removed, showed a decrease in zinc absorption, and the zinc absorption compensation seen in control groups, with their large intestines intact, was lost due to the removal of the large intestine (28). Though the studies mentioned above reported the colon to have little zinc absorptive properties, Nevah et al. were able to show that the colon could play a larger role in zinc absorption in rats. Zinc absorption studies were completed by injecting $^{65}$Zn into ligated colonic segments (55). Uptake, mucosal retention, and absorption were calculated after the tissue and luminal $^{65}$Zn content was measured by a gamma spectrometer. This group was able to show that the colon could absorb 14% of the $^{65}$Zn injected into the ligated colon within 15 minutes (55).

Changes in the large intestine can be attributable to differences in species, age, or the commensal bacterial population which could be causing variations among research studies. The large intestine may have a low efficiency for zinc absorption in healthy individuals; however, the large intestine can be a site of zinc absorption when gastric acid secretion or small intestinal zinc absorption is impaired. Another key factor in examining zinc absorption with $^{65}$Zn is the amount of time a dose is in contact with a particular segment; i.e. the transit time of each segment will affect results (14). Taking transit time into consideration, the colon could be a significant contributor to zinc absorption when colonic transit time is low, such as during constipation. The colon has the potential to be a large contributor to zinc absorption during different disease states where gastric acid production is altered, small intestinal zinc absorption is decreased, or during times when transit time is significantly altered.
Expression of only a few Zip and ZnT transporters has been characterized within the colon. ZIP4 localizes to the apical membrane, while ZIP5 localizes to the basolateral membrane of the colonic epithelial cells (16, 71). Zip4 expression in the colon, as with the small intestine, increases during ZnD, while Zip5 does not seem to be altered by ZnD. ZnT6 and ZnT1 are also expressed within the cecum and colon, and could be involved in zinc import (44, 64, 79). Other ZnT transporters shown to be strongly expressed in the colon were ZnT4 and ZnT7, which both showed expression in the epithelial and goblet cells (44, 79). Some of these zinc transporters may be involved with importing zinc into the colonic cells and others may be involved in trafficking zinc throughout the colonic cells. Endogenous zinc may play a role in regulating Cl⁻ secretion from the colonic cells. Physiological levels of zinc (~100 µM) have been shown to inhibit Cl⁻ secretion (64), which could be detrimental for the system’s ability to respond to toxins or pathogenic bacteria. Endogenous zinc secretion by the colon is not well understood. More research is required to understand how differing zinc concentrations can affect mucus secretion, pH, the microbial community, and mucosal integrity.

With all of the information discussed above, obtaining some preliminary pilot study data, and the availability of a knock-out mouse model, a hypothesis was formed that Zip11 is highly expressed within the murine gastrointestinal tract and influences zinc metabolism within the GI tract, mainly in the stomach and colon. To investigate this hypothesis, three specific aims were proposed in this dissertation project:

- Examine the phenotypic effects of the Zip11⁻/⁻ mutation and analyze zinc metabolism within this mouse model.
• Characterize expression of Zip11 and other zinc transporters in the gastrointestinal tract, particularly the stomach and colon, and selected other organs during control or dietary zinc studies.

• Characterize expression of Zip11 and other zinc transporters in colon during acute inflammation.
CHAPTER 2
MATERIALS AND METHODS

Animals

A random genetrap insertion in the Zip11 gene (between exons 4 and 5) was created by Bay Genomics MMRRC in the mouse line 129P2/OlaHsd, derived from embryonic stem cells. This clone was sent to UC Davis, University of California, where chimeras were generated. The chimeras were bred with C57BL/6 mice to generate F1 Zip11+/− mice. Two heterozygous breeding pairs were obtained and further breeding was completed at the Genetic and Cancer Research Complex (GCRC), University of Florida. Once a knock-out line had been bred from heterozygous mice, confirmed by genotyping, knock-out breeders were placed together to provide a sufficient number of knock-out animals for the experiments. The phenotype produced by the specific knock-out line did not lead to lethality.

Husbandry, Dietary Treatments, and LPS Administration

Mice were given free access to tap water and received commercial rodent diets (Harlan Teklad-7912) with 12 hour light and dark cycle. Wild-type, heterozygous, and knock-out mice generated from the founder mice were all used at different times for different experiments during this dissertation project. Both male and female young adult (8 – 12 weeks) mice were used at different points of this research. Different dietary treatments were used. The murine purified diet consisted of the AIN76 diet with egg white protein and either low zinc (ZnD, <1 mg Zn/kg diet), adequate zinc (ZnA, 30 mg Zn/kg diet), or high zinc (180 mg Zn/kg diet) (46, 53). When mice were fed the purified zinc diets, they were placed in hanging stainless steel cages and received free access to Mill-Q® water and food. Mice were acclimated to the hanging wire cages for one
week while receiving free access to water and ZnA food. After acclimation, mice were switched to new hanging wire cages and provided the appropriate experimental diet and water. Mice were placed on the varying zinc diets from 1 – 3 weeks depending on the dietary experiment. Weights and food intake were measured weekly. After dietary experiments, mice were anesthetized using Isoflurane and sacrificed by cardiac puncture and exsanguination. Blood and tissues (stomach, small intestine, large intestine, spleen, pancreas, liver, kidney and brain) were immediately excised for later analyses. Wild-type mice from another in-house knock-out mouse model (Zip14−/−), with a C57BL6 background, were also used in a separate dietary experiment and lipopolysaccharide (LPS) experiments. For LPS experiments, mice were placed on ZnA or high zinc (ZnS) diets for one week. An intraperitoneal (ip) injection of LPS (2 mg/kg) was administered to the mice. The mice were anesthetized and sacrificed 18 hours post injection. Blood and tissues were collected for later analyses. Protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

**Genotyping**

Genomic DNA was extracted from mouse tail samples using the prepGEM tissue kit (ZyGEM). After tips were cut in half with a razor blade and placed in a 0.2 mL PCR tube, 89 µL of DNA-free water, 10 µL of 10x Buffer GOLD, and 1 µL of prepGEM enzyme were added to the tube. Samples were vortexed and centrifuged briefly. Next, the samples were placed in a thermal cycler and incubated at 75°C for 15 minutes and 95°C for 5 minutes. The DNA-containing supernatants were collected and concentrations were measured by optical density at 260 nm (Nanodrop®). The samples were diluted to 100 ng/µL and used as templates in the polymerase chain reaction (PCR). Two primer/probe sets were used in a multiplex PCR reaction. The first set was
designed to amplify a region of the LacZ gene which was inserted into the knock-out
genetrap (sense primer 5’-ATCAGGATATGTGGCGGATGA-3’, anti-sense 5’-
TGATTGTGTAGTCGGTTATGCA-3’, and probe 5’-FAM-
CGCCCCACGCGATGGTAACAG-BHQ-3’). The second set was designed to amplify a
region that contained a housekeeping gene, UC-329 (sense primer 5’-
GTCATCAAGTGAGAAAGACATCC-3’, anti-sense primer 5’-
CATCATGAATTAGATAAGCCCATT-3’, and probe 5’-HEX-
CTCCTGGCTGCCTGGC-BHQ-3’). The final concentration of primers and
probes in the reaction mixture was 1400 nM and 390 nM, respectively. Master mix and
template DNA (100 ng/µL) were placed into each well on a 96-well plate. The
genotyping assay used a StepOnePlus sequence detection (Applied Biosystems) using
the comparative C_T method for analysis. This method required triplicate wells for the
comparative C_T calculations performed by the ABI instrument’s software. Previously
genotyped samples were used to verify assay data produced by unknown samples.
The C_T values for the triplicates were averaged, and the ΔC_T of each sample was
calculated by subtracting the normalizing gene C_T (C_TNORM) from the gene of interest C_T
(C_TGOI). For this genotyping experiment, UC329 was used as the normalizing gene and
LacZ was the gene of interest. The calculation for genotyping the Zip11 knock-out line
was as follows: ΔC_T = LacZ C_TGOI – UC329 C_TNORM. The wild-type (WT) always had a
large ΔC_T, ranging from 3 – 18, when compared to the heterozygous (HET) mice and
the homozygous (HOM) null mice. The HOM and HET mice had very low or
sometimes negative ΔC_T values because of the LacZ gene amplifying early in the
cycles. Depending on the previously genotyped controls, the HOM null ΔC_T could range
from -0.6 – -2.2, while the HET $\Delta C_T$ values could range from -1.0 – 1.0. Genotyping results were confirmed by running PCR with a specific primer set (sense primer 5’-TGTTCCTGCCCCTAGCACCT-3’ and anti-sense 5’-CCTCCACCTGCAAAACTCG) that would amplify a genetrap region only found in the HOM null and HET genotypes. Through a series of PCRs, different forward primers were used to find a region in the intron between exons 4 and 5 that would amplify with the genetrap reverse primer. The final concentration of each primer was 0.5 µM, and these primers were added to the Phusion High-Fidelity PCR master mix with GC buffer (NEB) which was diluted in PCR water to a concentration of 1X. Each well contained 19 µL of master mix and 1 µL of template DNA. Reaction mixtures were incubated at 98°C for 30 seconds, amplified for 35 cycles at 98°C for 10s, 58°C for 15s, and 72°C for 30s, and lastly a final extension step at 72°C for 7 minutes. Four µL of 6X Orange DNA loading dye (Thermo) was added to each sample before placement into the gel well. Five µL of the O’GeneRuler 1kb DNA ladder was added to a well to help determine the size of the amplified product. No template control samples, using PCR water as the template, were also run to show there was no contamination. The PCR samples were run on a 1% agarose gel made of agarose, 1X TAE, and ethidium bromide (EtBr, for visualization) for 45 minutes at 75 volts. UV light was used to visualize the PCR products on the gel, using a FluorChem E Imager (Protein Simple).

The Zip11 transcript was also analyzed in the WT and HOM samples. The cDNA samples of several tissues were used in a similar experiment to the genotyping PCR. The primer set used in this experiment was the Zip11 RNA sequences mentioned in Table 2-1. The Phusion master mix protocol mentioned above was the same one used.
for this experiment. The Zip11 forward and reverse primers were diluted to a working solution of 3 µM and further diluted in the master mix to a final concentration of 0.5 µM. The cycling instructions were also the same as mentioned above for the genotyping PCR. 4 µL of 6X Orange DNA loading dye was added to each PCR product and these products along with the O’GeneRuler 50 bp DNA marker were run on a 3% agarose gel (agarose, 0.5X TBE, 1 µL EtBr) for 45 – 60 minutes at 75V. The gel was exposed to UV light (FluorChem E Imager) to visualize the PCR products. Following the same protocol for the Phusion master mix and PCR run method, presence of the genetrap was confirmed in cDNA samples by using a primer set (sense primer 5’-CAAGGTACAGCTCCGTGGT-3’ and anti-sense primer 5’-GACAGTATCGGCCTCAGGAAGATCG-3’), provided by BayGenomics, that would amplify a region (~620 bp) only in KO and HET mice. The only change to the run method provided previously was the annealing temperature being set to 60°C. The PCR products were run on a 1% agarose gel (agarose, 1X TAE, 1 µL EtBr) for 45 minutes at 75V. The gel was exposed to a UV light as mentioned above for visualization.

**Tissue Collection**

For this dissertation research several tissues were collected from different parts of the mice. After blood was collected, tissues (stomach, small intestine, large intestine, spleen, pancreas, liver, kidney and brain) were removed, and depending on the later analyses, were either placed in RNA later (Ambion), flash frozen with liquid nitrogen or fixed in 10% formalin (Fisher). Specifically, concerning the GI tract, the stomach, small intestine, cecum, and colon were flushed with ice cold 1X phosphate buffered saline (PBS), containing 1X Halt Protease Inhibitor (Pierce) solution, several times to remove
food particles and feces. Pieces of each tissue were carefully cut and placed into the appropriate container for further analysis of RNA and protein.

**Measurement of Serum and Tissue Zinc Concentrations**

Blood was collected by cardiac puncture with a 25 gauge needle (Becton Dickinson). Each blood sample was placed in a CAPIJECT capillary blood collection tube (Terumo) and inverted several times before being placed on ice for one hour. The samples were centrifuged at 2,000 x g for 10 minutes at 4°C to allow complete separation of the serum from the red blood cells. Serum samples were carefully removed and diluted 1:5 in Milli-Q® water. Several different tissue samples were collected and digested in 1 – 2 mL of HNO₃ for 2 hours at 90°C. Acid digested samples were diluted in 1:3 Milli-Q® water. Serum and tissue zinc concentrations were measured by flame atomic absorption spectrophotometry (AAS). Values for tissue zinc were normalized to the wet tissue weight.

**RNA Isolation**

Tissues were excised from mice and immediately placed in RNAlater® (Ambion). Tissues were subsequently removed from RNAlater® and homogenized (Brinkmann Polytron Instruments) in TRI Reagent® (Molecular Research Center). The homogenate was allowed to rest at room temperature to allow complete disassociation. Chloroform (200 μL) was then added to 1 mL of the TRI Reagent solution. The solution was shaken for 15 seconds and allowed to incubate for 2-3 minutes at room temperature. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The upper clear phase was carefully removed and placed in a new labeled tube. A 100% isopropanol solution (500 μl) was added to each tube with the aqueous phase. Mixtures were vortexed and allowed to incubate for 10 minutes at room temperature. The samples were centrifuged
at 12,000 x g for 10 minutes at 4°C. The pellets, formed after centrifugation, were washed two times with 1 mL of 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol wash was discarded after each spin. After the second spin, the ethanol was removed and pellets were allowed to air dry. Once the ethanol was completely evaporated, 30 – 50 µL of PCR water, depending on pellet size, was added to each tube. Tubes were placed in a 37°C water bath for 10 minutes to allow pellet to dissolve in the water.

To remove any residual DNA contamination, all RNA samples were treated with TURBO DNase reagents (Ambion) as described by the manufacturer. Total RNA concentrations were measured using a Nanodrop spectrophotometer (Thermo Scientific). For one-step PCR, DNase treated samples were diluted to 10 ng/µL. The primer and probe sequences are shown in Table 2-1, 2-2, and 2-3. All primers and probes were used at concentrations of 900 nm and 250 nm, respectively. Master mix (11 µL) and 1 µL of diluted RNA were combined for the PCR reaction. Standards were created using a serial dilution from the stock RNA. Reaction mixtures were incubated at 48°C for 30 minutes followed by 95°C for 15 minutes and amplification of 45 cycles at 95°C for 15 seconds, and then 60°C for 60 seconds. For two-step qPCR, RNA samples were first converted to cDNA following the High Capacity cDNA Reverse Transcription protocol (Applied Biosystems). cDNA samples were diluted from the stock at a 1:15 dilution in PCR water. Standards were created using the stock cDNA by making a serial dilution. Reaction mixtures were incubated at 95°C for 20 seconds and amplification of 45 cycles at 95°C for 1 second and 60°C for 20 seconds. Fluorescence was measured
with a StepOnePlus Real Time PCR System (Applied Biosystems). Relative quantitation for assays used 18S or TATA binding protein (TBP) mRNA as normalizers.

**Zinc Transporter Antibodies**

Peptides listed on Table 2-4 were used to generate polyclonal antibodies against ZIP5 and ZIP11. Each peptide was synthesized (Biosynthesis) with an additional N-terminal cysteine to facilitate conjugation to maleimide-activated keyhole limpet hemocyanin (Pierce). The conjugated peptides were used to produce polyclonal antibodies in rabbits (Cocalico Biologicals) and IgG fractions were affinity purified (Pierce).

**Immunoblotting**

Several tissues were flash frozen in liquid nitrogen immediately after collection. Tissues were triturated, using the Polytron® homogenizer, on ice in a Tris-Triton buffer (1M Tris-HCl, pH 7.4, 1% Triton X-100 (Sigma), 1X Halt Protease Inhibitor). The homogenates were incubated in 4°C on a nutator for one hour. After incubation, samples were centrifuged at 12,000 x g for 20 minutes at 4°C. The supernatant was collected from tubes, leaving the pellet behind. Protein samples were aliquoted to reduce the number of freeze/thaw cycles. Total cell lysate protein concentrations were measured by a RC/DC assay kit (BioRad), following the manufacturer’s protocol. The only deviation to the RC/DC protocol was reducing the amount of reagents used by 1/4. For example, the protocol says to use 100 μL of standards and samples, but 25 μL of standards and samples were used. Depending on protein abundance 25 – 50 μg of total protein was denatured in a 6X sample loading buffer (350 mM Tris, pH 6.8, 600 mM dithiothreitol (DTT), 10% sodium dodecyl sulfate (SDS), 0.0012% Bromophenol blue and 30% Glycerol) for 15 minutes at 37°C. Denatured proteins were separated by
10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a Protran nitrocellulose membrane (Whatman) overnight at 30 volts. Protein transfer was confirmed by Ponceau Red staining (0.25% Ponceau stain red, 40% Methanol and 15% Glacial acetic acid). Ponceau stained membranes were washed with TBS-T (Tris-Buffered Saline, containing 0.05% Tween-20) to remove any traces of the Ponceau stain. After washing, membranes were blocked in 5% nonfat dry milk in TBS-T for one hour on an orbital shaker at room temperature. Incubation times and primary antibody concentrations were different between the housekeeping protein, tubulin (Abcam) and the zinc transporter proteins ZIP4 (Thermo Scientific), ZIP5 (in-house), and ZIP11 (ProSci or in-house). The ProSci ZIP11 antibody bound to a protein at ~25 kD and 70kD. The in-house ZIP11 antibody bound to a protein at 35 kD (the predicted size of the ZIP11 protein). Tubulin was diluted 1:5,000 in 5% nonfat dry milk in TBS-T and incubated for one hour at room temperature on an orbital shaker. ZIP4, ZIP5, and ZIP11 were individually diluted to a final concentration of 2 μg/mL in 5% non-fat dry milk in TBS-T. The milk/antibody solution also contained 0.02% sodium azide. The membranes were incubated in this milk/antibody solution overnight on a nutator at 4°C. To test the specificity of an antibody, primary antibodies were pre-incubated with the specific peptides used as the antigen when creating the antibody in the rabbits. After primary antibody incubation, membranes were washed four times with TBS-T for five minutes. Next, the membranes were incubated with an anti-rabbit secondary antibody (GE Healthcare) for 45 minutes to one hour. Membranes incubated with the tubulin primary antibody were incubated with the secondary antibody conjugated to horseradish peroxidase at a 1:10,000 dilution in 5% nonfat dry milk in TBS-T.
Membranes incubated with the Zip transporter primary antibodies were incubated with the secondary antibody conjugated to horseradish peroxidase at a 1:5,000 dilution in 5% nonfat dry milk in TBS-T. Following secondary antibody incubation, membranes were washed two times with TBS-T and two times with 1X TBS. Immunoreactivity was visualized by enhanced chemiluminescence (Thermo Scientific) and a FluorChem E Imager.

**Immunohistochemistry**

Stomach and colon tissue pieces were collected and placed in 10% formalin for 24 hours at room temperature. Tissues were carefully removed from the formalin and placed in cold 1X PBS. Samples were placed in a 4°C cooler until ready for paraffin embedding. A mouse tissue array slide (US Biomax) was also purchased to examine ZIP11 staining in several tissue slices. The same protocol mentioned below was used with the tissue array slide. Slides were deparaffinized by immersing them 2X in 100% xylene for 5 minutes, 3X in 100% ethanol for 3 minutes, 2X in 95% ethanol for 3 minutes, 70% ethanol for 3 minutes, and lastly tap water for 5 minutes. Next, antigen retrieval was performed by immersing slides in a 95°C, 10 mM sodium citrate solution (pH 6.0) for 10 – 12 minutes. After antigen retrieval, slides were washed in tap water.

Two different IHC methods were used during this research, an immunoperoxidase staining technique and immunofluorescence.

**Immunoperoxidase Staining**

For this procedure, the Vectastain Elite ABC (Vector Laboratories) protocol was followed. Briefly, slides were immersed in 3% hydrogen peroxide diluted in methanol for 10 minutes to block any endogenous peroxidase activity. During incubation periods, slides were placed in a humidified chamber. After a wash, tissues were incubated with
normal goat blocking serum provided, for 20 – 30 minutes. Tissues were next incubated overnight at 4°C with the ZIP11 primary antibody (ProSci, 2.5 µg/mL) diluted in the blocking serum. To test the specificity of the antibody, the primary antibody solution was pre-incubated with the peptide, used as the antigen. Tissues were washed with 1X PBS and incubated with the biotinylated secondary antibody provided, for 30 minutes at room temperature. After another wash, tissues were incubated at room temperature with the Vectastain Elite ABC Reagent for 30 minutes. Tissues were washed to remove ABC reagent, and then incubated with a peroxidase substrate solution (ImmPACT DAB, Vector Laboratories) for 1 – 3 minutes, depending on tissue expression of ZIP11. The substrate was rinsed off with tap water and to ensure the tissues did not dry out during the last steps, water droplets were carefully placed onto each tissue piece. Lastly, the slides were stained with hematoxylin (Santa Cruz) to visualize the nuclei. Slides were then passed through the deparaffinization solutions in reverse order, starting with 70% ethanol and ending with 100% xylene, to dehydrate the tissues. After coverslips were mounted over tissue sections with Cytoseal™ 60 (Thermo), staining was viewed with a microscope (Carl Zeiss, Axiovert S100).

**Immunofluorescence**

As mentioned above, a humidified chamber was used during incubation periods. After deparaffinization, tissues were incubated with a 3% BSA (bovine serum albumin) in TBS-T (1X TBS, 0.05% Tween-20) blocking buffer to prevent nonspecific binding and to reduce background interference. After blocking, tissues were incubated overnight in 4°C with a mixture of the in-house ZIP11 (10 µg/mL), produced from rabbits and H,K-ATPase (1:2000, Novus), produced from mice, primary antibodies diluted in 3% BSA. Slides were rinsed before incubation at room temperature with a mixture of secondary
antibodies in 3% BSA: donkey, anti-rabbit IgG, labeled with AlexaFluor 594 (1:300, Invitrogen) and goat, anti-mouse IgG, labeled with AlexFluor 488 (1:400, Invitrogen). After slides were rinsed, they were incubated at room temperature with DAPI (4',6-diamidino-2-phenylindole, 1:200, Molecular Probes) for 30 minutes. Samples were visualized on a Laser Scanning Confocal Fluorescent Microscope (Leica TCS-SP2) and a Spinning Disk Confocal Fluorescent Microscope (Olympus IX2-DSU) at the Cell and Tissue Core Facility within the McKnight Brain Institute at the University of Florida.

**Isolation and Analysis of Serum microRNA**

Serum RNA was isolated from the mice fed either a zinc adequate (ZnA) or a zinc deficient (ZnD) diet using Qiagen’s miRNeasy Mini Kit, following the manufacturer’s protocol. Synthetic C. elegans miRNA (Syn-cel-miR-39, Qiagen) was added to pooled serum samples as a normalizer during analyses (42). Total RNA concentrations were measured with the Nanodrop spectrophotometry. Reverse transcription (RT) of the miRNA in the total RNA sample was amplified using the miScript II RT Kit (Qiagen), following the manufacturer’s protocol. HiSpec buffer, miScript nucleic acid mix, miScript reverse transcriptase mix and RNase-free water made up the RT master mix. The reaction mixture consisted of 11 µL of master mix and 9 µL (~160 ng) of template RNA. Reaction mixtures were incubated for 60 minutes at 37°C and then at 95°C for 5 minutes to inactivate the transcriptase. The cDNA mixtures were diluted by adding 200 µL of RNase-free water to each 20 µL reaction mixture. These diluted mixtures were separated into two 110 µL aliquots and stored at -20°C. Using the mouse serum and plasma miRNA PCR array (Qiagen) and a SYBR Green miScript PCR kit (Qiagen), real-time PCR was performed with the serum miRNA cDNA samples, using the manufacturer’s protocol. Briefly, the reaction mixture consisted of 1,375 µL of 2X
Quantitect SYBR Green PCR master mix, 275 µL of 10X miScript Universal Primer, 1000 µL of RNase-free water, and 100 µL (0.5 – 1 ng) of diluted template cDNA. The reaction mixture (25 µL) was placed into wells of the PCR array plate. Reaction mixtures were incubated at 95°C for 15 minutes and amplification of 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. A melting curve was generated to ensure that one product was amplified during the reactions. Data from PCR runs were analyzed using miScript miRNA software provided by Qiagen.

**Statistical Analysis**

Data are presented as the means ± SD. Significant changes between groups were analyzed by unpaired, two-tail Student’s t-test. Statistical significance was set to p<0.05.
### Table 2-1. Zip Transporter Primer/Probe Sequences

<table>
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<th>Transporter</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
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<tr>
<td>Zip4</td>
<td>5’-CTCTGCAGCTGGCACCACCA-3’</td>
<td>5’-CACCAAGTCTGAACGAGAGCTTT-3’</td>
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<td>5’-CCACATCAGCGGTCAAGGAA3’</td>
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<td>5’-TGAGGTGGTGGTTAGTCTAGTGA-3’</td>
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### Table 2-2. ZnT Transporter Primer/Probe Sequences

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<tr>
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<td>ZnT6</td>
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<td>5’-GCCAGCAGAAGATCAATCAG-3’</td>
<td>5’-FAM-TGTCAGGACATCCAATCCGT-3’</td>
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### Table 2-3. Other Primer/Probe Sequences

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<td>5’-GATCCGAGGGCCTCCTACTAAC-3’</td>
<td>5’-CGCCGTCGCTACTACGATTGG-3’</td>
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<tr>
<td>TBP</td>
<td>5’-TCTCCCGTGTCGCTATT-3’</td>
<td>5’-GGTCTTACACACCCATAGAAA-3’</td>
<td>5’-FAM-TCTCCGCCGTCGCCCAGATCAATCATCA-BHQ1-3’</td>
</tr>
<tr>
<td>MT</td>
<td>5’-GCTGTGCCTGATGTGCA-3’</td>
<td>5’-AGGAAAGCCTGGGTTGGT-3’</td>
<td>5’-FAM-AGCGCTGCCACCACGTGAAATAGATCGTAC-3’</td>
</tr>
</tbody>
</table>
Table 2-4. ZIP Transporter Antibody Sequences

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZIP5</td>
<td>CRNKRD LGEPNPD</td>
</tr>
<tr>
<td>ZIP11</td>
<td>CPALMKKS DPRDPTS</td>
</tr>
</tbody>
</table>
CHAPTER 3
ZIP11 KNOCK-OUT MOUSE MODEL CHARACTERIZATION

Introductory Remarks

Knock-out mice are created by two different strategies, one being a gene targeting method and the other, a gene trapping method. Knock-out mouse models are important when studying the functionality of a specific gene. The gene targeting method is also known as homologous recombination. This method introduces a targeting vector into an embryonic stem (ES) cell that has an identical existing gene sequence present. The vector will insert itself both upstream and downstream of the existing gene, and the ES cell’s own repair system will recognize the identical sequences and remove the existing gene and replace it with the artificial DNA sequence, found in the target vector (1). The artificial DNA is inactive and will cause the gene function to be knocked-out. A conditional KO mouse model has also been created to knock-out gene function in a specific tissue or organ. This model utilizes a Cre-lox system which uses the Cre recombinase enzyme and loxP sites in the DNA for a specific deletion in the target tissue. Usually two genetically altered mouse lines are used for this method, one line containing loxP sites flanking the target gene, and the second containing the Cre recombinase in a specific tissue. The breeding of these two mice will produce offspring that will inherit both genes. The DNA piece bordered by the loxP sites will be removed and inactivated only in tissues that express the Cre transgene (41).

The second conventional KO strategy is the gene trapping method. This method uses a piece of artificial DNA with a reporter gene to be randomly inserted into the intron of any gene in an ES cell. The reporter genes within the vectors are used to identify cell lines where the gene has been successfully interrupted. Gene trapping
vectors usually have an important piece of the transcriptional component removed, which could be the enhancer, promoter, or polyadenylation (polyA) signal (1, 66). The vector will be transcriptionally activated only when inserted into the endogenous gene (1). A genetrap vector can contain its own promoter and require the endogenous enhancer to drive the transcription of the artificial DNA. The second type of gene trap vector involves a vector containing a splice acceptor sequence, followed by a reporter gene, and ending with a polyA signal. The vector is inserted downstream of the endogenous promoter, allowing for the expression of the reporter gene fused to the upstream exons. The third gene trap vector involves a vector with its own promoter and a splice donor sequence, inserting itself upstream of the endogenous polyadenylation site (1, 66). The reporter gene vector will be transcribed and fused to the downstream exons. Gene trap vectors inserted near the 5’ end of a gene are more likely to inactivate the gene than insertions near the middle or 3’ end of the gene. Another concern with the random gene trap insertion method is the possibility of the gene trap inactivating multiple genes or genes coded on the opposite strand also being inactivated by the insertion. Lastly, another disadvantage of using random genetrap vectors is the possibility of alternative slicing producing hypomorphic alleles (66), in which the altered gene products are found to have a decreased level of activity.

Several zinc transporter genes have been knocked-out in order to study their functionality in the mouse. ZnT1^{-/-} and Zip4^{-/-} mouse models both produce an embryonic lethal mutation in mice (5, 17, 44). Heterozygous ZnT1 mice developed normally, but female embryos did not develop properly if the dam was zinc deficient (5). The ZnT1 KO model revealed that ZnT1 was an important protein involved in the transport of
maternal zinc to the embryo (44). Though the Zip4 KO model is embryonic lethal, the mutation is not lethal to AE patients that have lost ZIP4 function. The AE patients’ symptoms can be relieved by zinc supplementation (44). Zn deficiency affected the embryonic development in Zip4 heterozygotes, while Zn supplementation helped protect the heterozygous embryo, but did not aid in preventing lethality in Zip4 homozygous null mice (17). An obvious difference between species occurs with the loss of function of ZIP4, with humans having another zinc transport system available to provide zinc to the embryo.

The Zip1 KO model does not show any phenotypic differences when zinc levels are adequate; however, during ZnD the Zip1 KO negatively affects embryonic development. The double Zip1/Zip3 KO model revealed that both of these proteins are essential for proper embryonic development during times of zinc deficiency (15, 44). After ZnT3 was knocked down in the mouse model, the amount of labile zinc within certain areas of the brain was decreased (44). Understanding zinc transporter functionality in the brain is important considering that zinc cannot diffuse across the blood-brain barrier. Both the ZnT5 and ZnT7 KO models have revealed poor growth in mice. Using a genetrap method to knock-out the function of ZnT5 resulted in irregular bone development, due to the key involvement of ZnT5 in osteoblast maturation (31, 44). The genetrap method was also used to create ZnT7−/− mice, which resulted in irregular zinc absorption in the gut and a decrease in body-fat composition (30, 44). Investigators have also found abnormalities with the bone, teeth, and connective tissue with the Zip13 KO mouse model (20, 44). A recent investigation, by Beker et al, of a Zip14−/− mouse model has revealed new information into key roles Zip14 may be playing.
in intestinal zinc absorption and glycolysis in the liver. The Zip14<sup>−/−</sup> mice had an increase in body fat, lower blood glucose, and higher insulin levels when compared to the wild-type controls. The Zip14 knock-out model also revealed an increase in iron-regulated genes, such as DMT1, hepcidin, and ferritin, in the liver (7). These key findings elucidate further information into the complex roles Zip14 plays in zinc, iron, and glucose homeostasis.

Knock-out models are an important aspect of zinc transport study because they provide an in vivo system to analyze the functionality of a specific gene. Not every knockout model will provide phenotypic differences; however, when a KO model is made available, each will be able to shed new light on that specific zinc transporter. A KO model could reveal functionality results about Zip11. Therefore, the aim of this chapter was to examine the phenotypic effects of the Zip11<sup>−/−</sup> mutation and analyze zinc metabolism within this mouse model.

**Results**

**Understanding the Zip11 Knockout Model**

A pGT0lxf genetrap construct (RRJ574) was created by the Bay Genomics MMRRC in the mouse embryonic stem (ES) cell line 129P2/OlaHsd. The construct of the genetrap consisted of a splice acceptor (SA) sequence upstream of a β-geo reporter gene (fusion of β-galactosidase and neomycin transferase) and a polyadenylation (pA) sequence downstream of the reporter gene. This construct was randomly inserted into an intron region of the genomic DNA within the ES cell. Confirmation of the genetrap insertion was completed by Bay Genomics. The confirmed clone was sent to UC Davis, where chimeras were generated by injecting the ES cell line into C57BL/6 host
blastocysts. The chimeras were bred with C57BL/6 mice to generate heterozygous breeders. Working in collaboration with Dr. Fudi Wang (Zhejiang University, China), our lab obtained two pairs of heterozygous breeders from UC Davis.

The offspring from the heterozygous breeding pairs were genotyped using tail clippings obtained shortly after weaning. Since the genetrap was inserted randomly, conventional genotyping could not be used since primers around the genetrap were not available. Originally the genetrap was identified to be located between exons 3 and 5 using the UCSC Genome Browser. Using this computer program, the location of the genetrap was identified on the antisense strand of chromosome 11 at 113244853 – 113650079. The 5’ RACE Sequence provided by the International Gene Trap Consortium (IGTC), was blasted against the mRNA sequence of both isoforms of mouse Zip11, using NCBI blast. The alignment of the blasted sequences stopped at the end of exon 4, which meant the genetrap was located in the large intron between exons 4 and 5. This intron has an approximate size of 59,000 base pairs which was determined using the blasted sequences and the base pair numbers provided by NCBI. Diagrams of the mouse Zip11 gene, the genetrap, and the transcripts produced by alternative splicing can be found in Figure 3-1A. The positioning of the random genetrap is unfavorable, due to the genetrap insertion in the middle of the gene, rather than towards the first and second exons of the gene. A genetrap insertion towards the 3’ end of a gene is not preferred because a functioning protein could be translated from the fusion protein. Another issue arising with this genetrap is the alternative splicing activity that occurs within the Zip11 gene between exon 5 and 6, forming two isoforms. Issues arising with alternative splicing and a randomly inserted genetrap can produce
hypomorphic alleles, where in some instances full-functioning transcript and the mutant transcript would be produced (Figure 3-1B).

Genotyping the offspring of the heterozygous breeding pairs was completed using a multiplex, comparative C_T method. The protocol, followed for genotyping these mice, is discussed in the methods and materials section of this dissertation. The data collected from the comparative C_T PCR run provides numerical data for each sample’s ∆C_T and averages the sample triplicates into a ∆C_T mean. The LacZ gene is found within the genetrap and is considered the gene of interest. An ultraconserved gene, UC329, was used as the normalizing gene for genotyping. A calculation (LacZ C_{TGOI} – UC329 C_{TNORM}) was used to determine the ∆C_T of each sample. The wild-type samples did not express LacZ, so the C_T for the LacZ was usually 35 – 45, which indicates no LacZ is present for amplification. For samples with no LacZ amplification, the cycle number, 45, was arbitrarily set as the LacZ C_T. After the ∆C_T means were determined for the unknown samples, they were sorted from smallest to largest, along with the previously genotyped samples for WT, HET, and HOM null. The values were also analyzed as a graph (Figure 3-2A) to better represent the data. The WT samples had large ∆C_T means, ranging from 3 – 18, when compared to the HET or HOM null mice. The HOM null and HET mice had very low or sometimes negative ∆C_T values because of the LacZ gene amplifying early in the cycles. Depending on the previously genotyped controls the HOM ∆C_T could range from -0.6 – -2.2, while the HET ∆C_T values could range from -1.0 – +1.0. The ranges overlap due to the control samples determining which samples were considered HET or HOM null. The LacZ gene was either present
as one copy (HET) or two copies (HOM null), so the ΔC_T mean values for the HET mice were usually half of the value for HOM null mice.

To ensure that the comparative C_T method was providing accurate genotypes, I also ran PCRs with a primer set that would amplify a region in the genetrap DNA only found in the HOM null and HET mice. I needed to run these PCRs because our RNA and protein data from the KO model (to be discussed below) were not showing the results that would be expected from a KO. The PCR products were run on an agarose gel and amplified regions were visualized using UV light. The amplified regions appeared as a bright band (~2.2 kb) for the HOM samples and a fairly faint, almost undetectable band for the HET samples. The WT and no template control (NTC) samples did not have any amplification (Figure 3-2B). Another confirmation used to analyze the HOM null mice was using cDNA from several tissues of both the WT and KO mice. For this experiment, I used a primer set that would amplify a region of the Zip11 gene towards the 3’ end of the gene. The size of the amplified Zip11 transcript region was approximately 98 bp. Primers were constructed towards the 3’ region to ensure this region had been removed during the genetrap splicing. Unfortunately, the results from these PCR experiments revealed that the Zip11 transcript was still being amplified in all tissues evaluated from the KO model (Figure 3-3A). After seeing the unexpected Zip11 amplification in all tissues from WT or KO mice, another primer set was used to amplify a region (~620 bp) in the tissue cDNA from exon 2 to the β-gal portion of the genetrap. The tissue samples used for analysis were from a male and female mouse. This amplified region should only appear in the KO tissues, which was confirmed in Figure 3-3B. The faint band appearing in the WT female cecum tissue was
not expected, but the stomach and liver tissues were from the same female mouse and both WT samples show no amplification.

The KO mice, whether male or female, did not have any phenotypic differences compared to the WT mice. Mouse weights were consistent among the genotypes. No apparent difference was seen with percentages of KO versus WT offspring. Since this genetrap seems to not have knocked-out the Zip11 gene, a new knock-out line would need to be established to evaluate phenotypes in mice without Zip11.

**A Significant Decrease in the Zip11 RNA Expression in KO Mice**

Once mice were genotyped and confirmed to be HOM null mice, we started breeding a KO line. WT mice that were offspring of the HET breeders were separated and bred together to have a WT line to use as controls for experiments. Tissue from WT and KO mice was extracted and processed to isolate RNA. PCR results revealed a 50-80% decrease in most tissues (Figure 3-4A). The C\textsubscript{T} values for the mice ranged from approximately 28 – 30. The WT mice had C\textsubscript{T} values on the lower side of the range, while the KO mice amplified around cycle 30. The C\textsubscript{T} differences revealed about a 2-fold difference between the WT and KO mice. The duodenum did not have a significant decrease due to the combination of male and female data. This tissue was the only tissue to have a significant difference in Zip11 mRNA expression between male and female mice. The stomach tissue of WT and KO mice was divided into three regions (non-glandular, body, and pylorus) and the Zip11 mRNA expression of each region was analyzed. The three regions were looked at individually since they each have different cell types and function. As with the other tissue Zip11 mRNA expression, a 50% decrease in Zip11 expression was seen in the KO tissue versus WT tissue in the
non-glandular and body sections, but not the pylorus section (Figure 3-4B). If the \textit{Zip11} gene had been properly knocked-out, the results would have shown us little to no \textit{Zip11} RNA expression in all tissues. These results revealed that the genetrap was knocking down some of the \textit{Zip11} gene transcripts with mutant transcripts, but full-functioning transcripts were still being produced with the genetrap present. With the reduction in mRNA expression but not a complete loss of expression, the genetrap represents a hypomorphic rather than a null allele (66, 75).

\textbf{No Change in ZIP11 Protein Expression in KO Mice}

After seeing the significant reduction in the expression of \textit{Zip11} mRNA in the KO mice compared to the WT mice, a decrease in the protein should be the expected result. Expression of ZIP11, based on western analysis data, did not consistently show a decrease in any of the tissues evaluated (Figure 3-5). The ProSci ZIP11 antibody bound to a protein expressed at 25 kD, and no change in the ZIP11 expression was seen within the stomach or cecum (Figure 3-5A). ZIP11 expression was also visualized using an in-house ZIP11 antibody. This antibody bound to a protein with a size of 35 kD. The commercial ZIP11 antibody (Pro-Sci) bound to a protein at a size lower than the expected 35 kD size. This Pro-Sci antibody was used at the beginning of this dissertation research, but after issues with the KO mouse model, an in-house ZIP11 antibody was isolated and purified and used for the remaining research sections. The colon tissue appeared to have lower ZIP11 expression, but the expression was inconsistent (Figure 3-5B). With the ZIP11 expression prominent in all protein samples, the results again indicate the genetrap is producing hypomorphic alleles (75). These hypomorphic alleles can produce both the full-functioning \textit{Zip11} transcript and the
truncated fusion transcript, which would translate into a functioning ZIP11 protein and the non-functional fusion protein, respectively.

**Dietary Zinc Did Not Affect the Functionality of KO Mice**

A dietary study with ZnA, ZnD, and ZnS diets was set-up to analyze if varying zinc concentrations in diets would affect the functionality of the KO mice. Each dietary group had 9 – 10 mice. The groups were broken down further by genotype (WT and KO n = 4 – 5) and sex (male n = 2 – 3 and female n = 1 – 2). The mice were acclimated in hanging wire cages for 1 week and changed to the experimental diets for 3 weeks. Mouse weight & food intake fluctuated as expected (46, 53). No phenotypic differences were seen between the genotypes on each diet. Female mice seemed to fluctuate with weight gain/loss more than the male mice, but no significant changes were noted. To confirm the efficiency of the dietary study, plasma samples were collected from the blood samples. No significant changes in the plasma zinc concentrations were seen between the KO and WT samples, so data from the WT and KO plasma zinc concentrations were combined to analyze concentrations between diets. The plasma samples from ZnD samples showed a significant decrease in zinc concentration, while the ZnS diet resulted in no change compared to the ZnA, which has been seen previously in zinc restricted dietary studies (52) (Figure 3-6A). Several tissue samples were excised to analyze the tissue zinc concentrations. The KO tissue samples did not have significant zinc concentration differences when comparing the different diet effects (Figure 3-6B). The duodenum was the only tissue to show a significant difference in zinc concentration during ZnD. No significant changes were seen between the WT and KO samples; however the stomach, colon, and liver of KO mice samples had a trend of
lower zinc concentrations when compared to the WT from mice that were on the ZnA diet.

The KO stomach and colon tissues were analyzed to determine further the effects of zinc depletion and supplementation on zinc-related gene transcripts. The zinc scavenger metallothionein (MT) can differ in expression depending on available metals in tissue and blood. MT expression has been previously described to decrease during periods of zinc deficiency, while expression increases during periods of high zinc (12, 59). The KO stomach and colon tissue MT expression decreased significantly during ZnD, which is another confirmation that the deficient diet worked properly (Figure 3-7). The MT expression increased noticeably in ZnS samples when compared to the ZnA samples in the colon; however, the change was not found to be significant. The stomach tissue also showed an increase in MT during ZnS but was not significant. When comparing the KO relative MT mRNA data to the WT data, the colon MT values had no significant changes noted between the KO and WT samples; however, the knock-out stomach ZnD samples appeared to respond better than the WT ZnD samples with a significant decrease in relative MT mRNA. This significant decrease of MT would suggest a lower level of certain metals (cadmium, copper, etc) in the stomach; however, zinc concentration did not decrease in the stomach during zinc deficiency. Other metal concentrations, such as copper or cadmium, may be affected in the stomach during zinc deficiency in this KO mouse model.

Zip4 expression in the colon also responded as previously described (46), by increasing significantly during ZnD and having no change during ZnS when compared to ZnA (Figure 3-7B). Expression of Zip11mRNA in the stomach and colon of KO mice
was unresponsive to ZnD, while ZnS caused an increase in expression within the colon, expressing a significantly higher expression in the ZnS group versus the ZnA group (Figure 3-7B). Together these results proved that the Zip11 genetrap did not knock-out the gene correctly, creating a hypomorph allele. Since the gene was not knocked-out, KO data from the dietary study did not appear to have any significant changes when compared to WT data.

**Discussion**

The usage of a random genetrap insertion in the mouse model used for this dissertation research proved to be unfavorable, by creating a hypomorphic allele. Hypomorphic allele production by a genetrap has been previously discussed (66, 75). Briefly, since the genetrap vector inserts into an intron of the gene, alternative splicing can be affected, resulting in altered levels of the gene to be knocked-out. In the case of the Zip11 knock-out model, the mice were genotyped as null mice (Zip11<sup>−/−</sup>) because they had two copies of the LacZ gene; however, when RNA was transcribed from the DNA, alternative splicing may have created both wild-type and knock-out transcripts, resulting in the failure to observe the Zip11 transcript and protein to be knocked-out in the null samples. Some animals that have a hypomorphic allele still reveal differences between the knock-out and wild-type animals, such as in the research by Wilson et al (75). Even though their genetrap did not knock-out the activity of inositol 1,3,4-trisphosphate 5/6-kinase (ITPK1), expression of the protein was decreased when compared to the WT or HET mice. Mice with the hypomorphic allele had an increase of neural tube defects when compared to the WT (75), which meant that the decrease in the enzyme affected the KO mouse even though the gene was not completely knocked-
The knock-out model unfortunately did not show any signs of being affected by a partial knocked-out allele. The breeders produce offspring at the same rate as the WT, along with the KO mice having similar weights and eating patterns. The remaining Zip and ZnT transporters were also analyzed to determine if mRNA expression levels were different between the WT and KD animals. Of the other thirteen Zip transporters, only Zip4 mRNA showed an increase expression in the KD animal when compared to WT levels in the murine stomach (data not shown). An increase in Zip1 mRNA expression in KD samples compared to WT was only seen in the murine colon (data not shown). The ZnT transporter mRNA expression did not show any changes when comparing KD and WT samples (data not shown).

When knock-out models are used in research, the hope is that the genetrap worked correctly. The results from the comparative Cₜ genotyping were consistently providing evidence of WT, HET, and HOM null mice. The transcript results revealed a 50-80% decrease in Zip11 mRNA, which was the first evidence that the genetrap did not work properly. Since the genetrap is suppose to produce a fusion transcript (66), Zip11 transcript should not have been detected in the HOM null mice. The primer/probe set used for Zip11 was specifically designed to be located at the end of the transcript in exon 10. The genetrap should have produced a fusion transcript that only contained exons 1 – 4. Using commercial ZIP11 antibodies and a previously made in-house ZIP11 antibody, the protein data never consistently showed that the ZIP11 activity was reduced or lost. Once a new ZIP11 antibody was made from a peptide, specifically designed to be located after the genetrap, the hope was to see protein results consistent with the transcript results. The HOM null mice only showed a slight decrease
in ZIP11 protein in the colon, which was not consistent with the stomach protein expression results. Since the transcript showed decreased expression and the protein had varying expression among tissues, the genetrap was considered a hypomorph (66, 75). As other research with KO mice has shown (7, 17), the genetrap should cause a complete loss of Zip11 transcript and protein expression; however, since this particular KO model used a random genetrap insertion, the unfavorable outcomes that could happen, did happen. The purpose of this aim was to examine the phenotypic effects of the Zip11\(^{-}\) mutation and analyze zinc metabolism within this mouse model, and the results concluded that the Zip11 gene was not knocked-out properly and the mice did not show any differing phenotypes or gene activity. Research with the KO mice was discontinued, and further research was only completed with the WT mice.
Figure 3-1. Diagrams of the mouse *Zip11* gene, genetrap, and alternative splicing. (A) The *Zip11* gene including the introns and exons, along with an expanded view of the random genetrap insertion between exons 4 and 5. Alternative mRNA splicing of the *Zip11* genetrap produces a full-functioning WT transcript (B) and a *Zip11*-βgal fusion transcript (C). The Λ represents splicing events.
Figure 3-2. Genotyping tail sample DNA. (A) The sample data are graphically represented by their ΔC_T means. The darkest bars represent the HOM null samples, the gray bars the HET samples, and the light gray the WT samples. Values shown are means ± SE (n=3 wells per sample). (B) The amplified region of the Zip11 gene with the genetrap present. Bands highlighted by the white box represent the 2.2 kb region present in the HOM Null DNA. This amplified region is not present in the WT and NTC samples.
Figure 3-3. Confirming genotypes using different primer sets. (A) The 98 bp band represents a region in the Zip11 mRNA in exon 10. Both the WT and KO samples revealed Zip11 transcript in all tissues presented, though the KO samples appeared to have less Zip11 expression. (B) The region represented by the band at ~620 bp was amplified using primer sequences specific for the KO samples. The reverse primer could only be found in the β-gal region of the genetrap vector and the forward primer was made from the BayGenomics sequence tag.
Figure 3-4. Knock-out Zip11 transcript expression compared to wild-type. The tissues were extracted and used for qPCR analysis of Zip11 expression. (A) Expression of KO compared to WT Zip11 mRNA in several tissues. (B) Zip11 mRNA expression of KO compared to WT in each region of the mouse stomach. Values were normalized to 18S and WT levels were set to 1. Data are expressed as ± SD (*=P<0.05, **=P<0.01, ***=P<0.001) (n=4 for each tissue sample).
Figure 3-5. Wild-type versus knock-out ZIP11 tissue distribution. Western blot analysis of ZIP11 expression. Tubulin was used a loading control (55kD). (A) The presence of ZIP11 (~25kD) in the stomach and cecum was visualized with the commercial ProSci antibody. (B) The presence of ZIP11 (35kD) in the stomach and colon was visualized with an in-house antibody.
Figure 3-6. Plasma and tissue zinc concentrations of KO mice on dietary restriction. Both plasma and tissue zinc concentrations were measured by AAS. Male and female mice were included in these analyses. (A) Measures of plasma zinc indicating the effectiveness of the diet for zinc depletion (n= 9–10). Plasma was diluted with Milli-Q® water. (B) Tissues were digested with HNO₃ and diluted with Milli-Q® water. Data are expressed as ± SD (*=P<0.05) (n=3–4).
Figure 3-7. Effects of zinc depletion and supplementation on zinc-related gene transcripts in the stomach and colon of the KO mice. Tissues were extracted and used for qPCR analysis. Values are normalized to 18S and ZnA levels were set to 1. Data are expressed as ± SD (*=P<0.05, **=P<0.01) (n=3–5). MT, Zip4, and Zip11 transcript expression in the KO stomach (A) and colon (B).
CHAPTER 4
EFFECT OF DIETARY ZINC ON EXPRESSION OF ZIP11 AND OTHER ZINC TRANSPORTERS WITHIN THE GASTROINTESTINAL TRACT

Introductory Remarks

The effect of dietary zinc on zinc transporter expression has been studied in rodents for years (18, 25, 43, 45). Zinc deficiency has been shown to affect the regulation of Zip4 (18, 46, 73), Zip1 and Zip3 (15), Zip10 (43), ZnT2 (45), and several other zinc transporters. Zinc repletion has also been shown to affect the regulation of some zinc transporters, such as Zip10 (43) and Zip5 (16, 71). Metallothioneins (MTs) are metal binding proteins that play roles in zinc homeostasis and protection against heavy metal damage. MTs can also be indicators for the effectiveness of a zinc deficient diet because in most tissues MT expression will be down-regulated during ZnD. The response of MT to zinc repletion or supplementation is the opposite of the deficient response. When there is an excess of zinc in the system, MT will be up-regulated by transcription factors that respond to zinc status (38, 70). With so many zinc transporters and other genes influenced by zinc status, studying the expression of Zip11 during dietary restriction is an important factor when characterizing this novel Zip transporter.

No structure, function, or regulatory information is available for the zinc transporter Zip11 (Slc39a11). Zip11 is part of the gufA subfamily of Zip transporters (44). Through a murine Gene Atlas (BioGPS) array analysis, Zip11 mRNA was found to be highly expressed in the stomach, large intestine, pancreas, and thioglycollate-stimulated peritoneal macrophages in mice. The human Gene Atlas array did not have any GI tract data, but revealed high expression in the pancreas and kidney. The Zip11
gene is well conserved across several species. The mouse Zip11 gene is found on the antisense strand of chromosome 11, while the human Zip11 gene is found on the antisense strand of chromosome 17. Since the Zip11 expression pattern revealed it to be highly expressed in the stomach and large intestine, the influence of dietary zinc intake needed to be conducted. The dietary studies in this section involved mice being placed on ZnA, ZnD, and ZnS diets for 2 weeks, or ZnD for 2 weeks, followed by 24 hours of the ZnS diet to replete zinc (ZnR) in some of the ZnD mice.

With the serum collected from this dietary study, another assay was completed to examine murine serum miRNAs. miRNAs are small non-protein-coding sequences that target complementary sequences found within the 3’-untranslated region (UTR). miRNAs have been found throughout different species, including plants, viruses, and animals (6). miRNAs have been shown to strongly influence the posttranscriptional regulation of gene expression. One miRNA can target hundreds of mRNA sequences, and vice versa a single mRNA sequence can be the target to several miRNAs. The ability for a single miRNA to regulate several mRNAs can develop a complex network of gene expression, which can in turn alter biological functions, such as cell-mediated death and proliferation, signaling cascades, and immune response (4). Different stages of cancer have been shown to have serum miRNAs up- or down-regulated, which has made these small sequences important in biomedical research, especially the fields looking into targeted therapeutic approaches (76).

Since no information can be found on Zip11, the aim of this chapter was to characterize Zip11 expression within gastrointestinal tract, particularly the stomach and
colon, and also examine a few select other zinc transporters in these tissues during dietary zinc studies.

Results

Features of the Zip11 Gene and the Predicted ZIP11 Topology

The murine Zip11 gene and coding sequence (CDS) information was obtained from the NCBI website (Figure 4-1). The gene consists of ten exons, nine of which are translated from the coding sequence. The Zip11 gene has two known isoforms with 21 base pairs removed during alternative slicing from the end of exon 5, forming isoform 2. Most ZIP protein structures have eight transmembrane domains, but the predicted protein structure of ZIP11 has six transmembrane domains with the N-terminus located in the extracellular surface. A histidine-rich loop is usually found in most Zip transporter proteins, but this area is not found in the ZIP11 protein. Isoform differences were analyzed by blasting Slc39a11 variant 1 and 2. The predicted size of the ZIP11 protein is 35 kD. The predicted topology of ZIP11 was analyzed using several websites that use specific transmembrane prediction software. Combining all of the results, my analysis predicts ZIP11 as having six transmembrane domains. This topology image was created with a depiction of where the variable region of the isoforms (red box), the in-house ZIP11 antibody sequence (green box), and the ProSci ZIP11 antibody sequence (blue box) are approximately located.

Zip11 RNA and Protein Expression Tissue Distribution

Since there are no data available on Zip11 besides the Gene Atlas array, the Zip11 expression in C57BL/6 mice was first examined under normal dietary conditions to analyze the tissue distribution. The Zip11 transcript was found to be highly expressed in murine stomach, cecum, and colon (Figure 4-2A). Figure 4-2A shows
Zip11 expression within several tissues, as the relative expression of Zip11 mRNA compared to 18S mRNA. The stomach was separated into three regions (non-glandular, body, and pylorus), and Zip11 expression was examined in these regions (Figure 4-2B). The body and pyloric expression values were compared to the non-glandular region values due to this region having the lowest expression in the stomach. Three to four male samples were used for each gastric region. This Zip11 mRNA expression pattern showed that the highest expression was in the glandular regions of the stomach.

The in-house ZIP11 antibody was used for western blot analysis with ZIP11 visualized at 35 kD, the predicted size of ZIP11 (Figure 4-3A). The Ponceau staining of the membrane was used as the loading control. The protein data revealed similar results to the transcript data with the stomach and large intestine having the highest expression of ZIP11 protein. In the small intestine ZIP11 protein expression followed the mRNA with the duodenum presenting the lowest ZIP11 expression, and expression increasing through the jejunum to the ileum. qPCR data for kidney tissue revealed much lower Zip11 mRNA expression when compared to the stomach or large intestine, but protein data revealed strong ZIP11 expression. The pancreas also showed high expression, while the spleen and brain did not have any ZIP11 protein. A mouse tissue array was purchased to enable visualization of ZIP11 protein in several tissues (Figure 4-3B). The stomach and colon tissue samples on the slide did not provide a good representation of the tissue, so the figure includes two tissues from C57BL/6 male mice. The ProSci ZIP11 antibody was used for detection of the protein in the stomach, colon, and the entire tissue array. The tissue array images were zoomed to 630X, while the
stomach and colon images are at 100X. The immunoperoxidase staining revealed ZIP11 (brown stain) expression in the stomach, small intestine, colon, pancreas, liver, and kidney. The nuclei of the different cell types are stained in blue. A small amount of staining was seen in the cerebellum and skeletal muscle, but at levels much lower than the other tissues. The spleen, as with the western blot analysis, showed no expression of ZIP11. The stomach reveals staining throughout the gastric cells, and the small intestine has staining throughout the villi with a stronger concentration at the apical membrane of the enterocytes. Biomax could not provide a verification of which small intestine section was used on the slide, but with the strong staining, the slice was most likely from the distal region. The staining of ZIP11 in the stomach and colon slices on the tissue array showed similar results to my tissue sections, but the slices used were not handled carefully while making the array slide, and appeared to be torn. The colonic epithelial cells appear to have the most ZIP11 staining, while the pancreas and liver seem to have staining throughout the cytoplasm. The kidney section was a slice of the medulla region with staining throughout the cells making up the collecting ducts. The staining in the brain section is in the white matter, with no staining in the granule cell layer.

**Tissue Zinc Concentrations of Stomach, Intestines, and Colon**

Tissues were collected from mice that had been on a ZnA, ZnD, or ZnS diet for 3 weeks. Tissue Zn concentrations were measured by AAS (Figure 4-4). A trend of lower tissue zinc during ZnD was recorded in the stomach, duodenum, colon, and liver. Only the colon of the ZnD mice had a significant decrease in colonic zinc concentration compared to those mice fed the ZnA diet. The stomach and duodenum both revealed
an increase in tissue zinc concentration during zinc supplementation when compared to the ZnD samples. The ZnS increase was not significantly higher than the ZnA, so these results show that ZnS does not result in significantly more zinc in the stomach and duodenum when compared to ZnA.

**Zip11 mRNA Decreases During Zinc Deficiency**

The effectiveness of the ZnD diet to decrease serum zinc or the ZnR diet to effectively return serum zinc levels back to the concentrations of that found in the ZnA samples was examined by measuring the zinc concentration by AAS (Figure 4-5A). Male mice were placed into the ZnA (n=5) or ZnD (n=10) groups. After 2 weeks on the ZnD diet, five ZnD mice were changed to a high zinc diet for 24 hours (ZnR). In the ZnR group, the serum zinc increased back to ZnA levels and actually increased significantly above the ZnA serum Zn concentrations (Figure 4-5A).

MT and Zip11 mRNA expression throughout of the GI tract was analyzed through qPCR of cDNA samples transcribed from the isolated RNA of each tissue. Only the stomach and ileum tissues revealed a significant down-regulation in MT during ZnD; however, each of the GI tissues analyzed showed a significant up-regulation of MT due to ZnR (Figure 4-5B). In stomach there was a significant decrease in Zip11 transcript in the ZnD and ZnR group when compared to the ZnA samples (Figure 4-6). With no change between the ZnD and ZnR Zip11 mRNA expression in the stomach, the 24 hour period of ZnR with a diet high in zinc appears to not be a long enough period to recover the Zip11 mRNA levels from the decrease produced by ZnD conditions. Of the three segments of the small intestine, the ZnR duodenum samples were the only samples to show a change in Zip11 mRNA expression with a significant decrease in expression.
The Zip11 transcript expression in the cecum significantly decreased in both the ZnD and ZnR samples when compared to ZnA samples. The colonic Zip11 mRNA expression was unaffected by ZnD or ZnR.

**ZIP11 Protein Expression Is Variable Among Tissues During Zinc Deficiency**

Using the in-house ZIP11 antibody, the ZIP11 protein was visualized at 35 kD, the predicted size of ZIP11. ZIP11 protein expression in the stomach was found to decrease slightly in ZnD samples, and the expression returned to ZnA levels after zinc repletion (Figure 4-7A). Relative densitometry was used to compare ZIP11 expression data from the blot, and ZIP11 was normalized to tubulin (Figure 4-7B). A peptide competition assay provided evidence of the specificity of the in-house ZIP11 antibody. ZIP11 was visualized at 35 kD, but when the peptide, used to make the ZIP11 antibody, was first incubated with the ZIP11 antibody, the antibody could no longer bind to the 35 kD protein, so a band was not visualized (Figure 4-7C). ZIP11 protein expression in the colon was found to have no change during ZnD or ZnR (Figure 4-8A), which follows the mRNA data that also showed no change at the transcript level. Even though there was not a significant response of ZIP11 to the dietary regimens, there was a trend of ZIP11 to increase during ZnD and ZnR in the colon. Relative densitometry was used to compare data provided in the immunoblot, and ZIP11 was normalized to tubulin (Figure 4-8B). A peptide competition assay was also completed with the colonic immunoblot to assess the specificity of the ZIP11 in-house antibody. As with the stomach blots, the 35 kD ZIP11 band was not visualized when the ZIP11 antibody was pre-incubated with the peptide (Figure 4-8C). ZIP11 was also analyzed with western blotting for the cecum (Figure 4-9A) during ZnA, ZnD, and ZnR conditions and the three sections of the small
intestine (duodenum, jejunum, and ileum) tissues (Figure 4-9B) during ZnA and ZnD conditions. The cecum ZIP11 expression did not change during zinc restriction or repletion. No significant changes in ZIP11 expression within the small intestine were seen. The small intestine ZIP11 expression, however, was unusual when comparing this result to the tissue distribution blot mentioned above. The duodenum and jejunum should have had lower ZIP11 expression than the ileum as seen previously in the ZIP11 protein distribution blot but all three tissue sections showed strong ZIP11 bands when comparing ZnA and ZnD samples.

ZIP11 expression in the colon and stomach were also visualized using immunoperoxidase staining. The tissue samples were either incubated with the in-house ZIP11 antibody or the antibody plus the peptide to test specificity of the antibody. The results did not show any difference in staining between the ZnA and ZnD stomach samples (Figure 4-10A), but there seems to be a difference in ZIP11 localization in the gastric mucosa. The darker staining, indicating the ZIP11 protein, in the lower regions of the gastric pits seems to partially migrate to the luminal side of the gastric mucosa during ZnD. Gastric ZIP11 IHC staining, from another dietary study that included a zinc repletion stage, showed similar patterns to the western blot data (Figure 4-10B). The strong ZIP11 staining seen in the ZnA stomach samples decreases in the ZnD samples, and the staining appears to return in the ZnR group, similar to the staining seen in ZnA samples. The similar ZIP11 migration pattern is also seen among the stomach samples with a darker staining in the lower gastric pits during ZnA, migrating to the luminal gastric surface during ZnD. The colon revealed distinct staining of ZIP11 within the finger-like projections, known as colonic crypts (Figure 4-11A). The staining appears to
increase during ZnD throughout the crypts, while the staining in the ZnR samples appears to increase more than the ZnA samples, but still less than the ZnD samples. The staining at the luminal surface suggests that ZIP11 is expressed in the epithelial cells of the colon. The cecum ZIP11 staining appears to follow the Zip11 mRNA data presented previously. The strong staining seen in the ZnA cecum samples decreases dramatically in the ZnD, and the 24 hour ZnR does not seem like enough time to result in up-regulation of ZIP11 in the cecum (Figure 4-11B). Like the stomach ZIP11 staining, the cecum ZIP11 staining appears throughout the cytoplasm of the epithelial cells, but does not have the distinct staining pattern seen at the surface of the colonic epithelial cells.

**ZIP11 Localizes to the Nuclei in the Gastric Tissue**

Since ZIP11 is highly expressed in the gastric mucosa, the next step was to see if ZIP11 was expressed on the membrane of the parietal cell, the acid secreting cell of the stomach using immunofluorescence. Using H,K-ATPase as a membrane marker for parietal cells, I found that in ZnA tissues, ZIP11 was not co-localized with the parietal cell marker; rather it was co-localized with DAPI, the nuclei marker (Figure 4-12). The white arrows indicate the co-localization of the DAPI and ZIP11 which appear as a pink color (Figure 4-12B). Extensive red fluorescent ZIP11 staining also appears to be located in the cytoplasm of the chief cells, found in the lower portions of the gastric glands (yellow arrows) (Figure 4-12A).

**ZIP11 Localizes to the Colonic Epithelial Cells**

After seeing the high expression of ZIP11 within the colon, the next step was to see if ZIP11 was localized to the membrane of colonic epithelial cells. β-catenin
antibody was used as a marker for the plasma membrane of colonic epithelial cells. Colonic tissue samples revealed a co-localization of ZIP11 to the colonic epithelial cells with fluorescence seen at the membranes and throughout the epithelial cells (Figure 4-13A). Samples also revealed ZIP11 (red) to be localized to epithelial cell nuclei (white arrows) (Figure 4-13B). Co-localization of ZIP11 with the nuclei marker (DAPI) was confirmed in other images not shown. ZnD colonic samples also showed an increase in ZIP11 fluorescence (Figure 4-13A) which indicates an up-regulation of ZIP11 during zinc deficiency in the colon as seen in the immunoperoxidase staining (Figure 4-11).

**ZIP4 Increases in the Colon During Zinc Deficiency**

In a pilot study looking at Zip11 expression in the stomach, Zip4 mRNA significantly increased in the stomach during ZnD (data not shown); however, in the latest dietary study Zip4 did not increase significantly during zinc deficiency in the stomach (Figure 4-14A). This result was unusual because during ZnR, Zip4 mRNA expression decreased significantly when compared to ZnA samples. A significant increase in colonic Zip4 transcripts was found in the ZnD samples compared to ZnA samples, and with ZnR, Zip4 expression significantly decreased to below ZnA levels (Figure 4-14A). Zip4 mRNA expression in the small intestine was analyzed to verify the up-regulation expected during zinc deficiency. As confirmed by this positive control, the duodenum, jejunum, and ileum all exhibited a significant up-regulation of Zip4 during ZnD, and the 24-hour zinc repletion attenuated the Zip4 response. The cecum Zip4 mRNA response was similar to that seen in the stomach, with the Zip4 expression only responding in the ZnR samples.
ZIP4 protein expression in the stomach did not exhibit any changes during zinc deficiency or repletion (Figure 4-14B). I did not expect to see significant changes in ZIP4 since the transcript levels did not change in ZnD samples and only had a minor decrease in ZnR samples. The ZIP4 protein expression in the colon increased significantly due to zinc deficiency (Figure 4-14C). Relative densitometry was used to compare immunoblot data (Figure 4-14D). Colonic ZIP4 expression was normalized to tubulin.

**Zip5 Increases in the Stomach and Colon During Zinc Supplementation**

The results obtained from this experiment revealed that in the stomach and colon of mice fed a zinc supplemental diet, Zip5 transcript is significantly up-regulated in the ZnS groups when compared to the ZnA groups (Figure 4-15A). Zip5 expression throughout the GI tract was also examined during zinc repletion. The 24-hour zinc repletion did not result in any changes in the Zip5 expression, except in the cecum. The Zip5 mRNA response in the cecum was unexpected but showed a significant down-regulation during ZnD and a return to ZnA expression levels, following ZnR (Figure 4-15B). ZIP5 protein expression in the murine stomach revealed a significant increase in both the ZnD and ZnR samples (Figure 4-15C). The colonic ZIP5 expression showed a significant increase only in the ZnR samples (Figure 4-15D). Both the gastric and colonic immunoblot data is represented graphically by relative densitometry with ZIP5 being normalized to tubulin (Figure 4-15E and F).

**Several microRNAs Increase During Zinc Deficiency**

Serum collected from several mice after two weeks on a zinc deficient diet was analyzed for expression of zinc-responsive miRNAs. The miScript miRNA PCR array
protocol developed by Qiagen was used to isolate and analyze serum miRNA. Among the 84 miRNAs measured, 19 had a fold change of greater than 2 when comparing the ZnD and ZnA samples. A scatter plot provided a graphical representation of the up-regulated miRNAs during zinc deficiency (Figure 4-16A). Among the 19 miRNAs up-regulated, 13 were of key interest due to the gastrointestinal cancers each were associated with. These 13 miRNAs were shown graphically as the fold change in the ZnD samples over the ZnA samples (Figure 4-16B). The miRNAs that were of interest to this study were miR-122-5p, miR-17-3p, miR-192-5p, miR-21a-5p, miR-221-3p, miR-29a-3p, miR-34a-5p, miR-31-5p, and miR-148a-39. One of the control genes on the PCR array, SNORD95, was also highly up-regulated during ZnD. Two miRNAs in each gastrointestinal cancer group had a fold change of greater than or equal to four during ZnD. The gastric cancer miRNAs, miR-21a-5p and miR-34a-5p, had a fold change of 4.6 and 15.1, respectively during ZnD. The colon cancer miRNAs, miR-221-3p and miR-29a-3p, each had a fold change around 4.0 during ZnD.

Discussion

Studying gene expression during zinc deficiency, supplementation or repletion can help open avenues for further research into the mechanism of nutritionally-regulated gene expression. Zinc deficiency still remains a problem among people in third world countries, so continued research on zinc regulated genes is necessary for the further development of biomarkers and disease-related therapies. Continued research of nutritionally-regulated zinc transporter genes is also necessary considering the increase in gastrointestinal-related diseases, such as irritable bowel disease (3).
The purpose of this chapter was to characterize Zip11 in the GI tract, and from the results Zip11 does show high expression in the gastric and large intestinal tissue, along with dietary zinc partially regulating the gene expression. The high expression of Zip11 in the stomach and large intestine was of particular interest because Zip transporters have not been thoroughly examined in the gastric tissues, and the large intestine appears to play a role in zinc absorption (28). Also, zinc has been shown to play a role in gastric acid secretion (54, 56, 77) and influencing colonic health (69). Though Zip11 is unlike Zip4 in that it is down-regulated by zinc deficiency, this Zip transporter may still be involved in zinc processing during normal or high zinc status. The stomach and colon have been shown to not have a strong influence in zinc absorption during normal, healthy states; however, the colon has been shown to absorb zinc when the small intestine has been compromised (28). Other genes that were found to be dysregulated in the stomach and colon during zinc deficiency and/or repletion were MT, Zip4, and Zip5. Both the stomach and ileum exhibited a significant down-regulation in MT mRNA due to ZnD, which shows that MT mRNA expression in these tissues responds to ZnD, as seen in the liver (48). The up-regulation of Zip4 mRNA in the small intestine confirmed the efficiency of the zinc deficient diet, and the response of Zip4, both mRNA and protein expression, in the colon showed that this tissue responds similar to small intestine. Hence, the colon could be an additional site of zinc absorption when the system is in a state of deficiency or malnutrition. ZIP5 protein expression in the stomach responds with an increase during zinc deficiency, and expression remains high after 24 hours of repletion, suggesting that this protein does not rapidly adjust to zinc repletion. This response is different than what has been reported previously in that
ZIP5 responds to zinc supplementation and not deficiency (16, 71). Colonic ZIP5 protein expression, however, did show similar results to those previously reported in the small intestine (16, 71) in that ZIP5 only responds to additional zinc, as seen in the repletion samples. This finding suggests a possible role for ZIP5 as a monitor of zinc status. Three ZnT transporters related to gastric tissue were also examined in the ZnA and ZnD gastric samples (data not shown). ZnT4, ZnT5, and ZnT6 all showed increases in mRNA expression with ZnT4 and ZnT5 showing significant up-regulation in ZnD samples (data not shown). Zinc deficiency does seem to play a regulatory role in zinc transporter expression, which could be important in maintaining proper acid secretion and mucosal integrity during disease states in the stomach.

Colonic microbiota composition of mice fed ZnA or ZnD diets were previously analyzed to determine if zinc intake differences could be detected. Using denaturing gradient gel electrophoresis analysis and 16S rRNA sequencing, Shore et al. (2010) were able to show a significant difference in the microbiota composition when comparing the ZnA and ZnD mice (unpublished data). Zinc absorption in the colon has been shown to be affected by transit time (14) and diseases resulting in impaired zinc absorption in the upper gut (28), such as advanced Crohn’s disease or gastroesophageal reflux disease (GERD). Research has also shown the beneficial affects zinc can have on stimulating gut repair and improving gut mucosa by demonstrating the effects zinc carnosine (ZnC, a commercially available health food product) has during in vitro and in vivo studies in cells, rats, mice and humans (50). ZnC can be found in health stores with claims to support healthy gastrointestinal activities. Mahmood et al. were able to show the stimulatory effects ZnC had on cell
proliferation and how this compound improved gastrointestinal injury in rats and mice. Human volunteers also showed improvement in gut permeability (injury), caused by commercial NSAIDs (50). Continued study of the effects of zinc and the roles of zinc transporters in the maintenance of the colonic mucosa and overall colon health will help advance the possibility that zinc could contribute to treatment of distal gut diseases.

The localization of ZIP11 to the nucleus of the parietal cell is of interest, considering that the parietal cell is the site of acid secretion in the stomach. Zinc has been shown to be important in the maturation of tubulovesicles within the parietal cell and may play a role in maintaining the integrity of the parietal cell (24). Zinc deficiency has also been shown to negatively affect the gastric mucosa and the secretion of acid (77). With the localization of ZIP11 to the nucleus of the gastric parietal cells and the decrease of ZIP11 protein expression seen in stomach tissue, ZIP11 could possibly play a role in gene regulation or zinc monitoring within the nucleus. With the strong staining of ZIP11 at the base of the gastric glands, where chief cells and Paneth cells are located, another possible role for this zinc transporter could be to influence zymogenic secretions. ZIP11 could also be involved in zinc regulation within the Paneth cells found in the lower region of the gastric glands. Strong staining in these stem cell granules indicated the ZIP11 protein to be present.

Localization of ZIP11 to the colonic epithelial cells indicates that ZIP11 may be involved in maintaining mucosal integrity within the colon or may possibly play a role in zinc sequestration during zinc deficiency. The co-localization of ZIP11 and β-catenin shows that ZIP11 is located throughout the epithelial cell, whether at the plasma membrane or cytoplasm. A strong fluorescence of ZIP11 is also seen within the nuclei.
of the colonic epithelial cells, and this localization was identified by a co-localization of ZIP11 and DAPI (nuclear marker). Colonic pH is also tightly regulated as seen in the stomach which could indicate ZIP11 playing a role in zinc homeostasis to help maintain the pH balance within these tissues. The localization of ZIP11 to the nuclei of gastric and colonic epithelial cells could indicate that this transporter plays a role in gene regulation or influencing zinc concentration within the nuclei.

A previous study looked at circulating miRNAs in human serum during acute zinc deficiency and repletion. Most of the miRNAs in the serum that responded to ZnD were down-regulated when compared to baseline levels (60). The study discussed in this dissertation aimed to find circulating miRNAs in mouse serum that were zinc responsive. The miRNA analysis provided a large scale PCR array approach that has not been previously used, comparing murine ZnD and ZnA serum. It was interesting to note that the miRNAs affected by murine zinc deficiency were only up-regulated, with no down-regulation present. Considering where zinc is absorbed and the effects zinc can have on the health of the gastric mucosa and intestinal integrity, the miRNA analysis revealed interesting results with 8 out of the 11 gastric cancer- and 3 out of the 8 colon cancer-related miRNAs on the array up-regulated during zinc deficiency. Another interesting result from this array was that miRNAs previously found to be expressed during liver cancer and injury, were up-regulated during zinc deficiency. Both miR-21a-5p and miR-31-5p have been studied previously and shown to be up-regulated in esophageal ZnD tissue (4). The affect of zinc deficiency on the progression of oral-esophageal squamous cell carcinoma (OSCC, ESCC) has been previously discussed (58). The rat studies completed by Alder et al. revealed that the ZnD esophagus had a
miRNA signature that was similar to the human ESCC or tongue SCC miRNA profiles, with miR-21 and miR-31 as the strongest up-regulated species (4). Something to point out is that the Alder et al. research looked at chronic zinc deficiency in specific tissues (4), while my murine miRNA data showed significant up-regulation of these two miRNAs in serum, after only 2 weeks of zinc deficiency, which shows that zinc deficiency can affect regulatory systems rather quickly.

The p53 tumor suppressor network is critical for regulating cellular responses to DNA damage and activation of cancer-related genes. Several miRNAs have been shown to be directly up-regulated by p53 after DNA damage. miR-34a is one of these miRNAs that has been shown to be regulated by the p53 tumor suppressor network and it has also been shown to be deleted in several human cancers (9). miR-34a has also been shown by Liuzzi et al. (2011) to be up-regulated in ZnD small intestine and thymus (unpublished data). The significant up-regulation of miR-34a in the serum of ZnD mice could be an indication of DNA damage caused by zinc deficiency. MiR-122 is a liver specific miRNA that has decreased expression during liver disease and cancer. This miRNA has been shown to be important in regulating hepatic and plasma iron levels. When miR-122 was inhibited in mice using an anti-miR compound, an up-regulation in some genes involved in iron homeostasis (Hamp, Hfe, Hjv, and Bmpr1a) was detected (8). This research by Castoldi, et al showed that hepatic miRNA-122 expression is essential in preventing iron deficiency in the plasma and liver (8). MiR-122 was also significantly up-regulated in ZnD murine serum, which indicates that iron homeostasis is affected during zinc deficiency. This miRNA up-regulation could be a defensive mechanism of the murine system in trying to prevent liver injury and altered iron
homeostasis caused by prolonged zinc deficiency. Further studies into how other dietary components (copper, iron, folate, etc) affect serum miRNAs in the mouse are needed to fully understand the complexity of miRNA expression in response to micronutrients.
Figure 4-1. Features of the murine Zip11 gene. (A) The intron-exon organization of murine Zip11 on chromosome 11 is shown, along with the Zip11 coding sequence (CDS). (B) Predicted topology for mouse ZIP11 showing six transmembrane-spanning regions. The red box indicates the variable region of the isoforms. The green box indicates the region of the in-house ZIP11 antibody sequence. The blue box indicates the region of the Pro-Sci ZIP11 sequence.
Figure 4-2. Tissue distribution of the Zip11 gene transcript in C57BL/6 mice. Tissues were extracted and used for qPCR analysis of Zip11 expression. (A) Zip11 mRNA tissue distribution in C57BL/6 mice. Values were normalized to 18S and pancreatic expression was set to 1. (B) Zip11 mRNA expression distribution in the 3 regions of the mouse stomach. Values were normalized to 18S and fundic expression was set to 1. Data are expressed as ± SD (*=P<0.001, **=P<0.0000001) (n=4–5 for each tissue sample).
Figure 4-3. ZIP11 protein tissue distribution in the murine model. Tissues were extracted and used for western blot analysis and immunoperoxidase staining. (A) ZIP11 was visualized at 35 kD by the in-house antibody. The Ponceau staining from the blot was used as the loading control. (B) ZIP11 was visualized using the ProSci ZIP11 antibody and an IHC technique. The stomach and colon tissues were extracted from C57BL/6 male mice, and the other tissues used for the IHC were located on a mouse tissue array slide. The tissues were visualized with a microscope, and the tissue array tissues were visualized with the 63X objective, while the stomach and colon are with the 10X. ZIP11 is stained brown and nuclei are stained blue.
Figure 4-4. Wild-type tissue zinc concentrations during ZnA, ZnD, or ZnS dietary conditions. Both male and female mice were used in this analysis. Tissues were digested with HNO3 and diluted with 3 volumes of Milli-Q® water. Tissue zinc concentrations were measured by atomic absorption spectrophotometry. Data are expressed as ± SD (*=P<0.05, **=P<0.01) (n= 4–5).
Figure 4-5. Effects of dietary zinc depletion and repletion (ZnR) on serum zinc and GI tract MT expression. (A) Measures of serum zinc indicating the effectiveness of the diet for zinc depletion and repletion. Zn concentration was measured by AAS. Tissues were extracted and used for qPCR analysis. (B) Relative MT transcript expression throughout the GI tract during ZnA, ZnD, and ZnR conditions. Values were normalized to 18S and the ZnA data was set to 1. All data are expressed as ± SD (*=P<0.05, **=P<0.01, ***=P<0.001, ‡=P<0.0001) (n= 3–5).
Figure 4-6. Effects of dietary zinc depletion and repletion on the Zip11 mRNA throughout the GI tract. Samples were analyzed using qPCR. Values were normalized to 18S and the ZnA data was set to 1. All data are expressed as ± SD (**=P<0.01, ‡=P<0.0001) (n=3–5).
Figure 4-7. Effects of dietary zinc depletion and repletion on the murine ZIP11 protein expression in the stomach. A representative western blot analysis and relative densitometry of the 35 kD protein, normalized to tubulin is provided. (A) Western analysis of total stomach lysate showing the decrease of ZIP11 during ZnD and the return of the ZIP11 expression upon ZnR. (B) Immunoblot data represented by densitometric values. (C) Immunoblot images providing evidence of the specificity of the in-house ZIP11 antibody by revealing no bands at 35 kD when the antibody and peptide were incubated together (red box). Tubulin is provided as a loading control.
Figure 4-8. Effects of dietary zinc depletion and repletion on the murine ZIP11 protein expression in the colon. A representative western blot analysis and relative densitometry of the 35 kD protein, normalized to tubulin is provided. (A) Western analysis of total colon lysate showing no change in ZIP11 during ZnD and ZnR. (B) Immunoblot data represented by densitometric values. (C) Immunoblot images providing evidence of the specificity of the in-house ZIP11 antibody by revealing no bands at 35 kD when the antibody and peptide were incubated together (red box). Tubulin is provided as a loading control.
Figure 4-9. Effects of dietary zinc depletion and repletion on the murine ZIP11 protein expression in the cecum and small intestine. Tissues were extracted and used for western blot analysis with the in-house ZIP11 antibody. (A) ZIP11 expression in the cecum during ZnA and ZnD conditions. ZIP11 and tubulin were visualized at 35 kD and 55 kD, respectively. Tubulin is shown as a loading control. (B) ZIP11 expression in the three sections of the small intestine during ZnA and ZnD conditions. ZIP11 was visualized at 35 kD and the Ponceau staining was used as the loading control.
Figure 4-10. Visualizing the murine ZIP11 protein with immunoperoxidase staining in the stomach. Tissues were fixed in 10% formalin, and mounted onto slides. The in-house ZIP11 antibody was incubated with murine tissues (b,c) and the specificity of the antibody was tested by pre-incubating with the peptide used to make the Ab (a). Samples were visualized with a microscope using 100X objective (a,b) or 630X (c) magnification. (A) The 630X images reveals the migration of ZIP11 during ZnD, where it is no longer located in the base of the gastric glands. (B) ZIP11 staining in stomach tissue of ZnA, ZnD, and ZnR mice. Less ZIP11 staining is seen in the ZnD samples with the staining returning to ZnA levels in the ZnR samples. ZIP11 is stained brown, and nuclei are stained blue. Strong staining in the chief cell region is indicated by blue arrows.
Figure 4-11. Visualizing the murine ZIP11 protein with immunoperoxidase staining in the colon and cecum. The in-house ZIP11 antibody was incubated with murine tissues (b,c) and the specificity of the antibody was tested by pre-incubating with the peptide used to make the Ab (a). Samples were visualized with a microscope using 100X (a,b) or 630X (c) magnification. (A) The colon reveals a distinct staining of ZIP11 on the luminal side of the mucosa. ZnD and ZnR samples appear to have more staining than ZnA samples, with the ZnD samples having staining throughout the mucosa. (B) The cecum shows strong ZIP11 staining in the ZnA samples with a decrease of staining appearing in the ZnD samples. The staining in ZnR samples does not appear to recover as seen in the stomach samples. ZIP11 is stained brown and nuclei are stained blue.
Figure 4-12. Immunofluorescence imaging of ZIP11 in the murine stomach. A tiled image of DAPI (nuclei marker), H-K-ATPase (parietal cell marker), ZIP11 (in-house antibody), and a merged image of all 3 fluorescing labels in the stomach tissue using the 63X objective, yellow arrows indicate ZIP11 staining in the chief cell region (A). White arrows indicate co-localization of ZIP11 and DAPI, shown in pink using the 63X objective with 4Z digital zoom (B). Images were taken using a Laser Scanning Confocal Fluorescent Microscope.
Figure 4-13. Immunofluorescence imaging of ZIP11 in the murine colon. (A) Images show ZIP11 (in-house antibody) in red, β-catenin, an epithelial cell marker in green, and a merged image overlapping ZIP11 and β-catenin. The merged image shows co-localization of ZIP11 to the colonic epithelial cells. (B) The merged image is increased in size to visualize the ZIP11 staining (red) in the epithelial cell nuclei (white arrows). Images were obtained using the 20X objective on a Scanning Disk Confocal Fluorescent Microscope.
Figure 4-14. The effect of ZnD or ZnR on ZIP4 expression in murine GI tract. (A) Zip4 transcript variation during ZnA, ZnD, or ZnR dietary conditions. Tissues were extracted and used for qPCR analysis of Zip4 mRNA expression. The small intestinal expression confirms the efficiency of the ZnD diet. Values were normalized to 18S and ZnA values were set to 1. Tissues were extracted and used for western blot analysis. ZIP4 expression in the stomach (B) and colon (C) was analyzed with the protein being visualized at ~40 kD. (D) Immunoblot data for colonic ZIP4 is represented by densitometric values. Values are expressed as ± SD (*=P<0.05, **=P<0.01, ‡=P<0.0001) (n=4–5).
Figure 4-15. The effect of ZnS and ZnR on Zip5 expression in the murine GI tract. Tissues were extracted and used for qPCR and immunoblot analysis of Zip5 mRNA expression. (A) Zip5 mRNA expression is up-regulated in the stomach and colon during ZnS. (B) Zip5 mRNA expression changes only in the cecum tissue with a decrease during ZnD. Values were normalized to 18S and the ZnA values were set to 1. (C) Stomach ZIP5 protein expression reveals an increase in ZnD and ZnR, while the ZIP5 colonic expression is increased in the ZnR (D). Tubulin is shown as a loading control for both immunoblots. Immunoblot data for stomach (E) and colon (F) is represented by densitometric values. Values are expressed as ± SD (*=P<0.05, **=P<0.001) (n=4–5).
Figure 4-16. Identification of serum miRNAs responsive to dietary zinc deficiency in mice using a qPCR-based array. Circulating miRNAs were isolated from pooled sera collected from mice after 2 weeks on the zinc deficient diet and were quantified using a pathway-focused PCR array, specifically designed to detect known miRNAs in mouse serum. (A) A scatter plot indicating miRNAs in the ZnD sample that had a fold change of above 2. Red = up-regulated miRNAs. (B) A graphical representation of the specific miRNAs of interest and those that had a fold change of above 2. Values were normalized to cel-miR-39 levels.
CHAPTER 5
CHARACTERIZATION OF ZIP11, ZIP4, AND ZIP5 EXPRESSION IN THE MURINE COLON DURING ACUTE INFLAMMATION

Introductory Remarks

Bacterial lipopolysaccharide (LPS) challenges have been used in animal models, over the years, to imitate an acute inflammatory response (2, 10, 48). Responses to an infection or inflammation could be fever, increases in several plasma proteins, such as C-reactive protein and ceruloplasmin, muscle loss (resulting in a negative nitrogen balance), hypozincemia, and many more systematic changes (21). The changes observed with acute-phase proteins during inflammatory conditions, can be caused by trauma, infection, and other stress-inducing situations. An interesting attribute of acute-phase proteins is that they do not all increase at the same time or concentrations in patients with the same disease (21). Inflammatory cytokines, such as IL-6, IL-1β, TNF-α, and INF-γ, are produced during early inflammation and will activate the production of acute-phase proteins. The variation in the production of cytokines by an individual and the responses modulated by these varying cytokines could be one explanation of the differences seen between patients (21). Plasma zinc levels decrease significantly during inflammation, and this drop is thought to be caused by the importation of zinc into major organs, mainly the liver (26). The pro-inflammatory cytokine, IL-6, that is the predominant stimulator of acute-phase proteins, has also been shown to up-regulate Zip14 (48). ZIP14 is thought to play a major role in the hypozincemia state seen during infection and inflammation.

The colon is an interesting organ to research during acute and chronic inflammatory states because of the challenges that can arise in the colon during
inflammation, and the degree of colonic damage sustained during inflammation. Another key player of colonic health during healthy or disease states is the microbiota population. Research is delving into the complexities of these colonic bacterial populations, and the effects this population can have on an individual (27, 62). Nutrition can play a role in the progression of already developed inflammatory problems within the colon, along with affecting the health of the colonic microbiome (23). An important relationship exists between the intestinal immune system and the commensal microbiota to ensure that the epithelial cells and the mucosal immune system recognize the difference between non-pathogenic and pathogenic agents (27). The intestinal immune response involves the recruitment and activation of lymphocytes and macrophages to the site of injury or pathogenic invasion. These activated cells stimulate the production of inflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα) or interleukin-1β (IL-1β), which will mediate the progression of the inflammatory response (67).

Zinc deficiency has been shown to exacerbate the effects of colitis in a rat model (34). Suwendi et al were able to show that zinc deficiency can alter TNFα regulation, which in turn affects the inflammatory response and disease progression in the dextran sodium sulfate (DSS) colitis model. The purpose of this chapter was to characterize expression of Zip11 and other zinc transporters in the colon during acute inflammation. To gain more insight into the colon and zinc transporter response to acute inflammation, colonic tissues were collected from mice fed either ZnA or ZnS for one week and challenged with LPS for 18 hours.
Results

Colonic Tissue Zinc Decreases After an LPS Challenge

After LPS administration, the LPS/ZnA samples had significantly lower colonic tissue zinc when compared to the control PBS group (Figure 5-1A). The decrease in tissue zinc after LPS injection was abolished when mice were fed a ZnS diet. Zinc transporters may possibly be increased in the ZnS group to increase zinc is available for uptake into the colon in the supplemented groups. After seeing the difference in the colonic zinc concentration, expression of select zinc transporters were analyzed to see if they could possibly explain this diet-dependent response to LPS.

Colonic Metallothionein mRNA Expression Increases in Response to LPS

MT mRNA was analyzed as a positive control due to its response to LPS. In the colonic tissue, MT mRNA expression was up-regulated after the LPS challenge in the ZnA group (Figure 5-1B), suggesting there may be more available zinc within the colon. Since tissue zinc results did reveal a significant decrease in the LPS/ZnA group, this response must be attributed to a direct response of LPS on MT expression. MT expression responds as expected in the ZnS group with an increase in expression, but was independent of LPS challenge.

Zip11 Colonic mRNA Expression Decreases in Response to LPS

The diets did not play a significant role in the expression of Zip11 mRNA after LPS administration. Zip11 mRNA expression was only affected by LPS, with a significant decrease in the LPS/ZnA group (Figure 5-2A). ZIP11 protein did not decrease in response to either diet or LPS (Figure 5-2B). The ZIP11 protein expression in the LPS-treated, ZnA samples appear to increase when compared to the PBS
samples, which is the opposite of the results from the RNA, but similar to the increase in ZIP11 protein seen in the colon during zinc deficiency.

**Zip4 Colonic Expression Increases in Response to LPS**

Zip4 transcript expression was significantly up-regulated by LPS in the ZnA group and this response was attenuated with zinc supplementation (Figure 5-3A). ZIP4 protein expression did not exhibit the response to LPS or diet as was seen at the transcript level (Figure 5-3B).

**Zip5 Colonic Expression Decreases in Response to LPS**

Zip5 transcript expression was significantly down-regulated after LPS administration in the ZnA group (Figure 5-4A). Zinc supplementation did not significantly change the response of Zip5 to the LPS. This down-regulated response is consistent with the notion suggesting that the ZIP5 transporter is being internalized and degraded, so as not to export zinc out of the blood during an LPS challenge. The ZnA/LPS samples appear to have a decrease in ZIP5 protein expression but not during ZnS conditions (Figure 5-4B). The Zip5 expression pattern at the transcript and protein levels, suggest that the Zip5 is being down-regulated by LPS.

**Discussion**

The purpose of this chapter was to characterize *Zip11* and a few other zinc transporters in the colon during acute inflammation. Results showed that LPS may regulate *Zip11* and *Zip4* genes within the murine colon. The decrease in colonic tissue zinc seen in the LPS/ZnA group was unusual considering the increase of *Zip4* and MT mRNA expression in this group. With the up-regulation of *Zip4* mRNA, one would think that tissue would also have an increase of zinc because the ZIP4 protein should also increase because of the increase in transcription; however, the ZIP4 protein levels only
revealed a slight increase in the LPS/ZnA samples. The time point of this LPS experiment (18 hours) could be playing a role in that the ZIP4 protein up-regulation due to LPS from increased Zip4 transcription may require more time. Even if there is an up-regulation of ZIP4 at a later time point that does not mean that there is zinc available in contents passing through the colon. The liver imports a high amount of zinc during LPS challenges, so Zip4 may be up-regulated due to the hypozincemia, but zinc may not be available to absorb in the colon. Zip4 mRNA was down-regulated by zinc supplementation as seen previously in the small bowel (74) and also similar to results mentioned above in the dietary chapter. MT has been previously shown to up-regulate in the liver due to LPS (48) and responded in the murine colon similarly with an increase in the LPS/ZnA samples. MT mRNA expression could also be up-regulated due to an increase in another metal, free radicals or ROS (68), since the decrease in tissue zinc should not result in an increase in MT mRNA expression. The time point used for this study (18 hours post LPS injection) may play a role in the zinc levels in the colonic tissue. The decrease in colonic zinc could be indicating that the additional zinc brought into the mucosa had already been exported into the blood stream or that most zinc had already been cleared from the serum and gastrointestinal tract by the time it reaches the colon, due to mechanisms involved with the hypozincemia state, resulting from the LPS challenge. The increase in tissue zinc seen in both the LPS and PBS, ZnS groups indicates the potential of other zinc or metal transporters importing zinc or a paracellular transport of residual zinc into the colon.

The expression of MT in normal colonic epithelial cells has been found to be significantly higher than expression in diseased colon sections from ulcerative colitis.
and Crohn’s disease patients (33). These opposing results to inflammation from my results could suggest a difference in regulation of MT during acute and chronic inflammatory states or a difference in MT regulation in humans. The data presented here comes from an acute inflammation experiment, so the results showing an increase of MT in the ZnA mice after LPS treatment follows the results shown previously by Liuzzi et al (48). As expected, MT expression increased in ZnS samples, regardless of LPS treatment. Further studies are needed to analyze the effects zinc supplementation can have on MT, during inflammation, particularly an influence on inflammatory mediators, free radicals, or ROS.

Since there appears to be some ZIP11 localization to the colonic epithelial cells, this pattern could suggest involvement of ZIP11 in maintaining colonic mucosal integrity during inflammation. In the LPS-treated/ZnA samples Zip11 mRNA expression is significantly down-regulated at the transcript level but shows an increase at the protein level. There could be a post-transcriptional modification increasing the translation of ZIP11 in the colon in response to the LPS challenge. The lower Zip11 mRNA expression could also be suggesting that there is a sufficient supply of ZIP11 protein, as seen by the increase of ZIP11 in the ZnA/LPS samples, and in turn the system is signaling to lower transcription, until more protein is required. These opposing results with transcription and translation could also mean that the Zip11 mRNA was up-regulated at an earlier time point after LPS administration, and at the 18 hour time point, the system is now seeing that increase in translation of ZIP11. This increase in ZIP11 protein in the colon during LPS does follow what was seen in the colon during zinc
deficiency. The ZIP11 protein could be more stable during hypozincemia states within the murine system.

The up-regulation of Zip4 in the colon of LPS-treated/ZnA mice shows a response similar to what was also shown in a zinc deficient state. This Zip4 response in the colon further confirms that LPS may create a zinc deficient state throughout several systems in a mouse. As expected with supplemented zinc, Zip4 expression was reduced in the ZnS samples; however, the protein expression failed to produce this decrease in expression. The expression of Zip5 mRNA was significantly decreased in the LPS-treated groups, regardless of which diet the mice were on. These results suggest some influence of LPS on Zip5 translocation. During deficiency, ZIP5 is internalized from the basolateral membrane in a model transfection system (16). Since this study is looking at the response to acute inflammation and ZIP5 is not different in any samples, I conclude that Zip5 mRNA is partially down-regulated by inflammatory mediators. The down-regulation may help to ensure that the colonic epithelial cells are not transporting zinc from the blood back into the cell for excretion in an effort to conserve zinc concentrations during inflammation.

Colonic tissue responds to LPS as is seen with zinc deficiency with an up-regulation of Zip4. Since the tissue was collected 18 hours after the LPS challenge, tissue zinc concentrations and ZIP11, ZIP4 and ZIP5 transporters may not have had sufficient time to respond to the proinflammatory conditions. Since genes such as MT, Zip4, and Zip11 in the colon are responding to LPS, further research needs to elucidate zinc-related mechanisms involved in the inflammatory response in the colon.
Figure 5-1. The effects of a ZnA or ZnS diet on murine colonic tissue zinc concentrations and MT mRNA expression after LPS administration. (A) Tissue samples were extracted and digested in HNO3. After the digestion, samples were diluted and measured using AAS. Values are expressed as ± SD (n=4). The effect LPS had on colonic zinc concentrations of animals fed a ZnA diet was diminished when mice were fed a ZnS diet. (B) Tissues were extracted and used for qPCR analysis. MT expression was significantly up-regulated in the ZnA group, while zinc supplementation increase MT in the PBS and LPS groups. Values were normalized to TBP and the ZnA/PBS values were set to 1. Values are expressed as ± SD (n=3–4).
Figure 5-2. The effect of LPS administration on colonic Zip11 expression when mice were fed either a ZnA or ZnS diet. Tissues were extracted and used for qPCR analysis and western blotting. (A) Zip11 mRNA expression was significantly down-regulated in the ZnA diet group when administered LPS; however, the high zinc diet did not influence the Zip11 expression pattern when compared to the ZnA group. Values were normalized to TBP and the ZnA/PBS values were set to 1. Values are expressed as ±SD (n=4–5). (B) ZIP11 expression did not significantly change at the protein level. Tubulin was visualized as a loading control.
Figure 5-3. The effect ZnA and ZnS diets have on murine Zip4 expression in the colon after LPS administration. Tissues were extracted and used for qPCR analysis or western blotting. (A) Zip4 expression was significantly up-regulated due to LPS in the ZnA samples. Zip4 expression was significantly down-regulated in the ZnS, with no effect of LPS exhibited. Values were normalized to TBP and the ZnA/PBS values were set to 1. Values are expressed as ± SD (n=4–5). (B) ZIP4 was visualized at ~40 kD, revealing an increase in expression in the LPS-treated, ZnA samples. The dark banding above the 40 kD band is overexposed tubulin that was visualized prior to ZIP4. Tubulin was visualized as a loading control.
Figure 5-4. The effect of LPS administration on Zip5 colonic expression when mice were either fed a ZnA or ZnS diet. Tissues were extracted and used for RT-analysis or western blotting. (A) LPS administration caused a significant down-regulation of Zip5 in the ZnA samples. The ZnS samples showed similar responses to LPS but were not significant. ZnS did not affect the regulation of Zip5 when LPS was administered. Values were normalized to TBP and the ZnA/PBS group was set to 1. Values are expressed as ± SD (n=4–5). (B) ZIP5 protein expression appears to be decreased in the ZnA/LPS samples when compared to the ZnA/PBS samples, while the ZnS samples do not appear to change. Tubulin was visualized as a loading control.
CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTION

The current study was conducted to characterize the novel zinc transporter, Zip11, also known as Slc39a11. Though the random genetrap model did not correctly create a Zip11 knock-out species, many informative results were discovered about this zinc transporter. Zip11 is ubiquitously expressed in several tissues of the C57BL/6 mouse, with the stomach, large intestine, pancreas, and kidney having the highest protein expression. The main points of this Zip11 research were focused on the stomach, a secretory organ, and the colon, an absorptive organ. Zip11 mRNA and protein expression in the stomach is down-regulated during zinc deficiency and is increased during ZnD at the protein level in the colon. Similar to the stomach response (data not shown), the colonic Zip11 mRNA expression is down-regulated after LPS treatment. There may be a mechanism involved that down-regulates Zip11 mRNA in these tissues when Zip4 expression increases. The expression patterns of ZIP11 seen during IHC were also interesting within the stomach and the colon, exhibiting some ZIP11 localization in the nucleus of the parietal cell, throughout the chief cells, within the nucleus of the colonic epithelial cells and partial localization to the membrane of the epithelial cells. ZIP11 could also be an important transporter to study further because of its localization to two pH-regulated tissues, the stomach and colon. The regulation of zinc homeostasis in pH-regulated tissues will be an important avenue to research further when it comes to understanding more about zinc transporters. Another zinc transporter, ZnT9, has also been mentioned to be expressed in the nucleus during mitosis (12). Another report mentioned a possible role of zinc transporters in the nucleus when $^{65}$Zn appeared in the nucleus of spleen cells after a 2 hour intragastric
Further research using isolated parietal cells could elucidate whether Zip11 is involved in zinc homeostasis in the nucleus. The colonic expression of ZIP11 is visualized on the apical surface of the epithelial cells, suggesting that ZIP11 may play a role in zinc transport in the colon. The localization of ZIP11 in the colon could also indicate this transporter being important for zinc homeostasis in order to maintain colonic mucosal integrity and function. If a new Zip11 knock-out mouse was created, zinc transport in the colon could be analyzed by $^{65}$Zn gavage.

The LPS model brought forth some interesting results with LPS regulating the mRNA of Zip11 (down), Zip4 (up), and Zip5 (up) in the colon. Further research could look into inflammatory-mediated transcription factors responsible for regulating these Zip transporters in the colon. Of interest, would also be looking into inflammatory colonic diseases of humans and analyze expression patterns of Zip11 and other zinc transporters.

The findings of this dissertation research are important for gaining new insight into a novel zinc transporter, Zip11 that has been previously uncharacterized. By providing the characterization of Zip11 and evidence of its regulation by zinc deficiency, supplementation, repletion and LPS treatment, future studies elucidating zinc’s role in the stomach and colon will need to consider Zip11 along with other zinc transporters. Zip11, along with other zinc transporters, particularly Zip4 and Zip5 have the potential to play important roles involving zinc homeostasis when elucidating more information concerning the zinc and acid output link within the stomach, and when determining zinc and zinc transporter roles in maintaining colonic mucosal integrity and function.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Alyssa Brooke Maki was born and raised in Hibbing, Minnesota. In 2005, Alyssa graduated with her Bachelors of Science from the University of West Florida, where she majored in molecular biology. In fall 2008, she was accepted into the Food Science and Human Nutrition department at University of Florida, where she began her graduate studies by entering the doctoral program for nutritional sciences, working under Dr. Cousins. She received her Ph.D. from the University of Florida in the summer of 2013.