REGULATORY MECHANISMS OF *SLC39A4* (*ZIP4*) AND *SLC39A5* (*ZIP5*) IN THE ADAPTIVE RESPONSE TO ZINC AVAILABILITY

by

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Dedication

Previously, I dedicated my Master's thesis to my parents. I would now like to dedicate the work herein to my grandparents, none of whom were able to finish high school. I will never forget the stories of struggling to have just basic commodities in life. My Grandma Jones raised six children of her own, raised one of her granddaughters, and took care of her mother later in life. My Grandpa Jones served honorably in the Korean and Vietnam wars, survived as a prisoner-of-war, and retired as Sergeant Major. My Grandma Weaver raised four children of her own, helped raise several of her grand-children, and earned her graduate equivalence degree later in life. My Grandpa Weaver served honorably in World War II and earned his graduate equivalence degree later in life. I thank all of you for teaching us the importance of a good education. From all of you, I have learned the value of the basic things in life—hard work and perseverance.

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always encouraging Yi and me to do our best. With love and gratitude to all of you, 拙谢!

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Abstract

Zinc is an essential micronutrient. Zinc deficiency results in severe dermatitis, immune dysfunction, diarrhea, mental retardation, and a failure to thrive. Zinc toxicity can result in irreversible nerve damage or severe pancreatitis. Thus, the ability to regulate proper zinc concentrations within the cell and throughout the body is essential for survival. Two families of zinc transporters mediate the proper levels of zinc within mammals through their cumulative actions. The *Slc30a* (*ZnT*) transporter family exports zinc from the cytosol; whereas, the *Slc39a* (*Zrt-Irt-like protein, Zip*) transporter family imports zinc into the cytosol. Mutations in several members of these transporter families give rise to distinct diseases. One such disease, acrodermatitis enteropathica, is a rare autosomal-recessive disease that is a result of inadequate zinc uptake from the diet due to mutations in the *Slc39a4* (*Zip4*) gene. Loss of proper ZIP4 function ultimately results in death if untreated. Previous studies have shown that *Zip4* and a closely related paralog, *Zip5*, are regulated by inverse mechanisms. The goals of this dissertation have been to determine the molecular mechanisms that regulate *Zip4* and *Zip5* in the adaptive response to zinc availability and how *Zip4* impacts development. In this dissertation, I defend three specific aims. In the **first specific aim, I evaluate the hypothesis** that *Zip4* is essential for the normal growth and development of mammals. To address this aim**,** a *Zip4* knockout mouse model was employed. Loss of *Zip4* resulted in embryonic lethality at the egg cylinder stage, prior to organogenesis. Heterozygosity had a negative association with eye, heart, and brain development and resulted in hypersensitivity to zinc

deficiency. Excess zinc failed to rescue the lethal phenotype but ameliorated some of the heterozygous effects. In the **second specific aim, I evaluate the hypothesis** that *Zip4* and *Zip5* are post-transcriptionally regulated, inversely and dynamically in response to zinc availability. To address this aim, wild type mice were fed zinc adequate or zinc deficient diets. Some of the zinc deficient mice were repleted with zinc by oral gavage. Tissues known to express *Zip4* and *Zip5* were examined for expression of their mRNA levels, protein levels, and protein localization with time in response to changes in zinc availability. *Zip4* and *Zip5* had rapid and reciprocal regulation in response to zinc availability that was coordinated in multiple tissue types. *Zip4* expression was regulated primarily by stability of the mRNA and protein: both accumulate during zinc deficiency; ZIP4 protein is rapidly internalized from the apical membrane of enterocytes and visceral endoderm then degraded in response to zinc repletion. *Zip4* mRNA levels return to normal within 24 hours. *Zip5* expression is apparently regulated by a translational stall mechanism during zinc deficiency. The *Zip5* mRNA levels do not change with zinc availability and always remain polysome-associated; proteasome or lysosome inhibitor cocktails fail to restore ZIP5 protein levels. However, zinc repletion leads to the return of ZIP5 protein on the basolateral membrane of enterocytes, visceral endoderm, and pancreatic acinar cells. In the third **specific aim, I evaluate the hypothesis** that *Zip5* is regulated by a rapid post-transcriptional mechanism mediated by the 3' untranslated region of the mRNA in response to zinc availability. To address this aim, *in vivo* and *in vitro* techniques were utilized. Several regulatory miRNAs were identified that are predicted to target

the *Zip5* mRNA in an accessible region of the well-conserved 3' UTR that is predicted to form a stable stem-loop structure. These miRNAs are polysome-associated in tissues known to regulate *Zip5*. These miRNAs are detected predominantly in precursor form, implying additional regulatory mechanisms. Further work is necessary to demonstrate a functional link between these predicted regulators and *Zip5* expression. Altogether, this dissertation reveals that *Zip4* and *Zip5* are both regulated by intricate post-transcriptional mechanisms in response to zinc availability and that *Zip4* is essential for development and proper zinc homeostasis.

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List of Abbreviations

ActD = actinomycin D

AE = acrodermatitis enteropathica

bp = base pair

CDF = cation diffusion facilitator

CHX = cycloheximide

Dapi = 4’-6-Diamidino-2-phenylindole (fluorescent counterstain)

EDS = Ehlers-Danlos syndrome

Est nt. = estimated nucleotide length

EtBr = ethidium bromide

FBS = fetal bovine serum

Gav = gavage

h = hour

H&E = hematoxylin and eosin (counterstains)

HA tag = hemagglutinin antigen from influenza virus

IP = immunoprecipitation

IU = international unit(s)

Kb = kilobase

KO = knockout

Luc = luciferase

Mb = megabase

MEF = mouse embryo fibroblast

miRNA = microRNA

miR = miRNA

MRI = magnetic resonance imaging

MT = metallothionein

MTF-1 = metal-response element binding transcription factor 1

Mut = mutant

NAD = nicotinamide adenine dinucleotide

nt = nucleotide

PBS = phosphate buffered saline

***Slc30a* = solute carrier 30a superfamily**

***Slc39a* = solute carrier 39a superfamily**

UTR = untranslated region

VYS = visceral yolk sac

WT = wild type

Zap1 = zinc-activated protein 1

ZIP = zrt-irt-like protein (1-14 in mammals)

Zn = zinc

ZnA = zinc adequate

ZnD = zinc deficient

ZnT = zinc transporter (1-10 in mammals)

Zrt = zinc-regulated transporter (1-3 in yeast)

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Chapter 1

Background and Significance

I. Zinc and Human Health

Zinc is an essential micronutrient that is readily available in protein-rich foods such as red meats, shellfish, nuts, fish, and poultry. Zinc deficiency can be due to nutritional or genetic factors. Zinc deficiency impacts a plethora of human health problems, such as neonatal growth, birth weight, cardiac function, eye development, brain development and cognition (Fraker et al., 2000; Hurley, 1981b; Hurley, 1981a; Prasad, 1998a; Prasad, 1993).

The detrimental effects of maternal zinc deficiency have been known for some time, especially the effects on brain development and stature (Hurley and Shrader, 1975; Hurley, 1981b; Jankowski et al., 1995; Sever, 1981; Sever and Emanuel, 1973). Genetic factors can also contribute to zinc deficiency in the newborn including maternal mutations that give rise to zinc-deficient milk, as demonstrated by the lethal milk mutant mouse (Ackland and Mercer, 1992; Danks, 1985; Lee et al., 1992; Piletz and Ganschow, 1978; Piletz et al., 1987) or transient zinc deficiency in humans (Chowanadisai et al., 2006). The details of the preceding disease processes as well as the following descriptions will be discussed in more depth later, including the relevant gene names. The following descriptions will serve as an overview of the importance of zinc transporters in zinc homeostasis. In zebrafish, repression of a zinc importer using morpholinos resulted in disruption of the epithelial-to-mesenchymal

transition at the gastrula stage and prevented subsequent development (Yamashita et al., 2004). In *Drosophila*, disruption of another zinc importer resulted in loss of proper germ cell migration (Mathews et al., 2005; Moore et al., 1998). Moreover, other human zinc transporters are upregulated in various cancers and their expression profiles may correlate with tumor aggressiveness (Kagara et al., 2007; Kasper et al., 2005; Taylor et al., 2003; Li et al., 2007; Chowanadisai et al., 2008; Taylor et al., 2004a; Zhao et al., 2007). Additionally, mutations in other zinc importers can give rise to either characteristic symptoms and clinical sequelae called acrodermatitis enteropathica (1975; Agarwal and Vaishnava, 1969; Anabwani et al., 1985; Graves et al., 1980; Klein, 1974; Lorincz, 1967; Tompkins and Livingood, 1969) or a subtype of the connective-tissue disorder called Ehlers-Danlos syndrome, affecting stature and craniofacial development (Fukada et al., 2008).

The diversity of the dysfunctions seen in zinc deficiency, either nutritionally or through genetic mutation, reveals the diversity of requirements for zinc in biology. Many genes are essential for the dynamic maintenance of zinc homeostasis. Their varied functions and associated pathologies will be described in detail later.

As an overview of this dissertation, I will first review how zinc is utilized in the cell in various biochemical coordination sites. I will then provide detail regarding the regulation of zinc homeostasis from bacteria to mammals and some evolutionary context to understand the different adaptations. From this review of relevant literature, I will build the framework necessary to understand the primary research reported in subsequent chapters. The primary data contained herein are taken from

three published manuscripts and a partial data set of, as yet, unpublished results. The contributions of others to these data sets are listed in the methods section. In conjunction with other published results, these data begin to reveal some intricate mechanisms involved in the regulation of zinc homeostasis. Finally, to summarize, a published co-written review of trace metals and their essential roles in development will provide a context for the roles of zinc in biology as compared to iron and copper.

II. Zinc in Biology

A. Zinc Coordination and Utilization

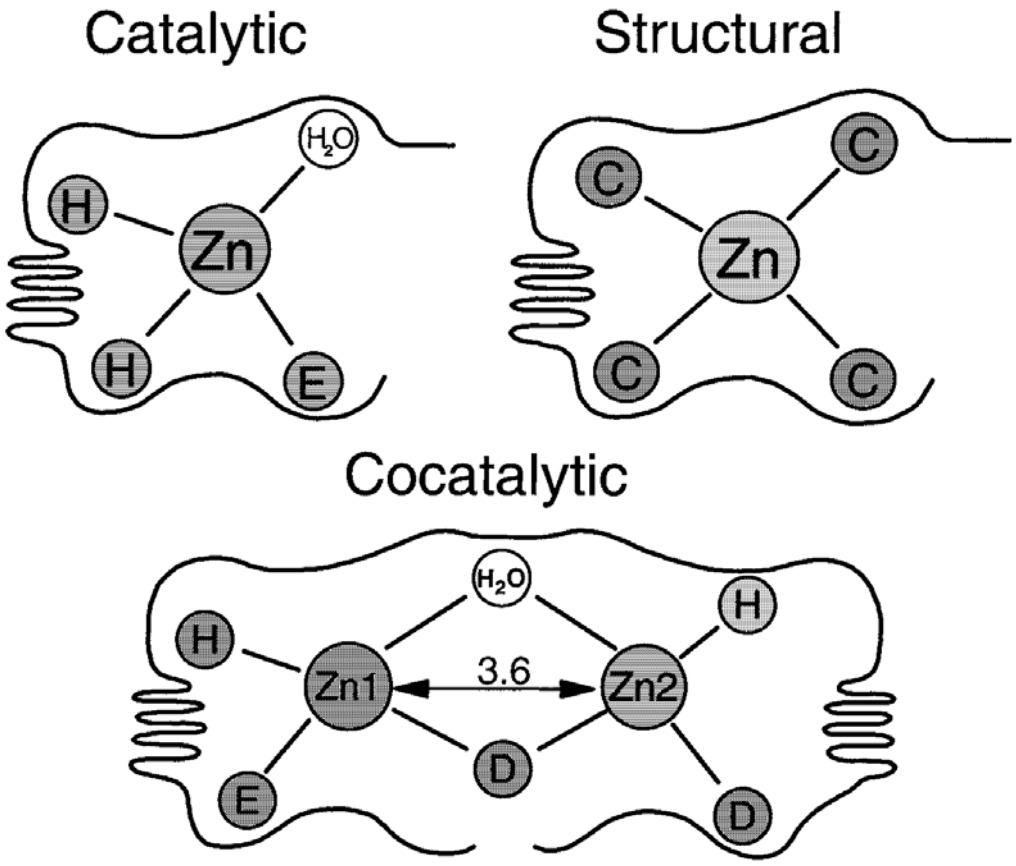
Zinc is the only metal found in all enzyme classes (Auld, 2001; Vallee and Auld, 1990) which reflects the versatility of this metal. Zinc has an atomic mass of 65.39 Daltons and belongs to the group 12 metals along with cadmium and mercury. Zinc preferentially loses its 2 s electrons resulting in a stable 2+ oxidation state whilst maintaining fully occupied d-orbitals (d10 electronic configuration). Zinc readily forms tetrahedral to octahedral coordination sites in biological systems (Koutmos et al., 2008; Auld, 2001). Based on these criteria, zinc is not a transition metal like iron and copper, which display redox chemistry and multiple d-orbital configurations that give rise to their geometry-dependent ligand field stabilization energies (Auld, 2001; Koutmos et al., 2008; M.Nic, 1997). The ability of zinc to function as a Lewis acid, coupled to a lack of redox chemistry and geometry-independent ligand strength, make zinc an ideal metal for structural and catalytic purposes in proteins (Koutmos et al., 2008).

Zinc is an essential micronutrient in all life forms (Auld, 2001) and is the second most abundant heavy metal found in the body, following iron. Zinc deficiency leads to developmental and cognitive diseases whereas zinc excess leads to toxicity. Therefore, regulatory systems are necessary to ensure that proper levels of zinc are maintained in the cell.

Zinc serves both structural and catalytic roles in proteins and functions in one of three major ways: catalytic, structural, and co-catalytic centers (Auld, 2001), as shown in Figure 1.1. Proteins typically have picomolar affinities for zinc (Auld, 2001).

Catalytic zinc centers usually have three amino acid ligands within proteins, typically histidine but also glutamate, and always have water or hydroxide as a fourth (sometimes fifth) ligand (Auld, 2001). Catalytic zinc sites facilitate the ionization, polarization, or displacement of water. In doing so, zinc can move away from the carboxy oxygen of glutamate (Koutmos et al., 2008; Vallee and Auld, 1990), which may reflect a transient dissociation (Auld, 2001; Koutmos et al., 2008). The reaction mechanism may also involve the expansion of the coordination sphere to add a fifth ligand, with subsequent ionization or displacement of water (Auld, 2001). The distances between the amino-acid ligands are either less than seven residues or greater than 100 (Auld, 2001). Some exceptions of 20 amino-acid spacers exist, but these exceptions also involve an interaction with an electron carrier cofactor such as NAD in the alcohol dehydrogenase family (Auld, 2001). Other representative enzyme

Figure 1.1: Overview of zinc coordination sites of characterized proteins. This cartoon depicts the types of ligand interactions that coordinate zinc. This cartoon cannot represent the dynamic nature of zinc coordination sites, such as the transient expansion of the coordination sphere (5 ligands) that can occur during catalysis, and for which good physical evidence supports in some reaction mechanisms. This figure indicates the amino acids H (histidine), C (cysteine), E (glutamate), D (aspartate) and the zinc sites 1 and 2 (Zn1 and Zn2, respectively) as well as water. Note that no details of the spacer distances between ligands are provided and, for the cocatalytic site, the interatomic distance of 3.6 Å is shown. Also, no delineation of the water species is given but it may exist either polarized by an additional amino-acid base, ionized to hydroxide anion, or displaced transiently by a nucleophilic substitution in the reaction mechanism. Reprinted by permission from Dr. David Auld, *Biometals*: 14(3): 271-313 (Copyright 2001 Springer Science and Business Media); See license in Appendix IV: List of license agreements for copyrighted materials.


Catalytic
Structural
H2O
H
Zn
H
E
C
C
Zn
C
C
Cocatalytic
H2O
H
H
Zn1
3.6
Zn2
D
E
D

families containing catalytic zinc centers include carbonic anhydrases, matrix-metalloproteases, carboxypeptidases (Auld, 2001).

Structural zinc centers usually have four protein ligands and no water (Auld, 2001). Cysteine is the most common amino-acid ligand found in these sites, followed by histidine (Auld, 2001). The distances between these amino-acid ligands is generally short with the entire set accommodated in usually less than 40 residues, but range from 15 to over 200 (Auld, 2001). Zinc-finger motifs of 20 to 30 amino-acid residues fall into the structural coordination category.

The zinc-finger motif is ubiquitously found in eukaryotes. Three percent of *Caenorhabditis elegans* protein sequences encode this motif (Clarke and Berg, 1998). A higher percentage of proteins in *Homo sapiens* containing the zinc-finger motif along with a dramatic expansion in the number of repeats per protein make the zinc-finger motif the most abundant motif in the human genome (Venter et al., 2001). In contrast, this motif is very rare in bacterial genomes, with only a single motif per protein and may have entered the prokaryotae domain by lateral gene transfer from aquatic eukaryotes (Clarke and Berg, 1998; Bouhouche et al., 2000).

Various forms of zinc-finger motifs differ primarily by the combinations of cysteine and histidine in the zinc-finger that coordinate the zinc. Nonetheless, all zinc-fingers bind zinc in a tetrahedral coordination (Auld, 2001; Berg, 1990). The *Xenopus* TFIIIA and the *Drosophila* Krüpple-type fingers have the classically described C_2H_2 zinc-finger motif, with two cysteine and two histidine zinc ligands. This motif is present in many eukaryotic transcription factors (Berg, 1990). The

classic TFIIIA/ Krüpple-type finger forms an ordered subdomain consisting of an α-helix and two antiparallel β-strands coordinated by zinc. Proteins such as steroid nuclear receptors have zinc-fingers in their DNA-binding domains with only cysteine ligands (C_2C_2) but do not form the same independent subdomain as the classic zinc-fingers (Kumar and Thompson, 1999; Berg, 1990); rather the DNA-binding domains of nuclear receptors contain two zinc-binding sites that vaguely resemble the classic zinc-fingers.

In addition to the differences with the classic zinc-finger subdomain, the two nuclear receptor zinc-fingers are structurally distinct from each other, with the first finger functioning in DNA sequence discrimination by the so-called P-box residues and the second finger functioning in receptor dimerization as well as other non-specific DNA interactions by the so-called D-box residues (Kumar and Thompson, 1999). In addition to the zinc-finger domains, there exists RING fingers (Borden, 1998) and LIM domains (Feuerstein et al., 1994; Sánchez-García and Rabbitts, 1994) that coordinate zinc and are involved in protein-protein interactions. The LIM or double zinc-finger motif is essentially one to three repeats of a double zinc finger motif containing a signature two-residue linker between the finger doublets (Feuerstein et al., 1994). Other, non-zinc-finger motif structural coordination proteins include, among others, alcohol dehydrogenases and protein kinase family members such as human Raf-1 (Auld, 2001).

Co-catalytic zinc centers are typified by either two or three zinc atoms closely coordinated to each other and to a catalytic zinc center (Auld, 2001). In the co-

catalytic centers, at least two zinc atoms are bridged by an aspartate (less frequently a glutamate) residue and sometimes a water molecule (Auld, 2001). The remaining two to three ligands are supplied primarily by histidines, aspartates or glutamates, but never cysteines (Auld, 2001). The co-catalytic sites are unique among the coordination types in that two of the zinc atoms are bridged by one residue (or water) with an interatomic distance dependent on the residue.

Protein-protein interfaces can also coordinate zinc (Auld, 2001). These sites have ligand coordination that resembles either catalytic or structural intrapeptide coordination sites discussed above (Auld, 2001) and will not be discussed further here.

A very unique zinc coordination type is found in metallothionein proteins that have very high affinities for multiple zinc ions and function as transient storage sites for zinc in the cytosol. These are small cysteine-rich proteins with multiple invariant cysteine ligands that altogether coordinate up to seven zinc ions. Metallothioneins will be discussed in more detail later (*vide infra*: Overview of Zinc Homeostasis in Mammals).

B. Overview of Zinc Homeostasis in Bacteria

All cells have transport and regulatory systems to maintain the required level of zinc as growth or developmental processes demand. These dynamic mechanisms ensure zinc homeostasis. Though, certainly these mechanisms are still areas of active investigation.

Bacterial cells can rapidly adapt to environmental conditions, including changes in available zinc. Pathogenic bacterial species have evolved zinc uptake transporters to aid in virulence when zinc is limiting (*exempli gratia* the lack of available zinc within the host's circulatory system); whilst zinc-tolerant bacterial species have evolved efficient zinc export transporters to prevent toxicity as discussed below. Less is known about zinc uptake and export during zinc replete but non-toxic conditions.

Figure 1.2 provides an overview of the transport systems in gram negative bacteria such as *Escherichia coli*. In general, less is known about zinc transport in gram positive bacteria. During normal zinc availability in *E. coli*, Pit likely functions as an inorganic phosphate cotransporter for zinc import (and perhaps export) (Beard et al., 2000). CitM in the gram positive *Bacillus subtilis* may cotransport zinc and citrate but with promiscuity as this transporter also transports magnesium, nickel, manganese, and cobalt (Krom et al., 2000).

The ability to adapt to zinc deficiency is essential to survival and is governed by high affinity zinc uptake systems. To begin, the zinc uptake regulator (Zur) belongs to the Fur (ferric uptake regulator) family of metal regulatory transcription factors in bacteria (Patzer and Hantke, 1998; Bagg and Neilands, 1987; Hantke, 1981). Zur functions as a repressor to the Znu operon in all gram negative and some gram positive bacteria (*exempli gratia Bacillus*; whereas, AdcR repressor regulates the operon analogous to Znu in *Streptococcus* gram positive bacteria (Panina et al., 2003). How Zur senses zinc is unknown (Lee and Helmann, 2007); nonetheless,

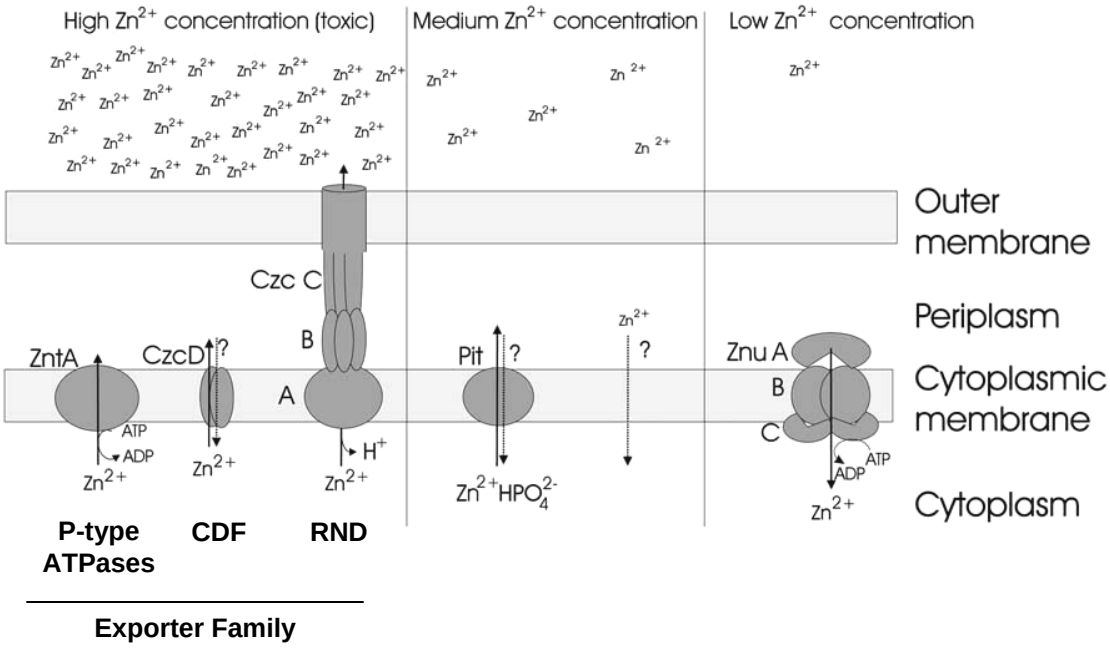


Figure 1.2: Overview of gram-negative bacterial zinc transport systems. Note that the nomenclature shown is only for gram-negative bacteria and should not be confused with gram-positive bacteria that may have identically named but unrelated genes. In this cartoon, the major, characterized bacterial transporters involved in zinc import and export are depicted. Note, the primary purpose of this figure is to indicate the location of the relevant transporters and should not be considered to represent the stoichiometry of the active transport forms nor can this static cartoon represent dynamic processes. Reprinted and modified by permission from Dr. Klaus Hantke, *Biometals*: 14(3): 239-249 (Copyright 2001 Springer Science and Business Media); See license in Appendix IV: List of license agreements for copyrighted materials.

zinc deficiency relieves Zur repressor function, which then allows transcription of the high affinity zinc uptake system ZnuABC (Hantke, 2005). In addition, relieving Zur repressor function allows transcription of the paralogs of ribosomal proteins L31, L33, L36, and S14 that do not contain a zinc ribbon motif as their normally expressed counterparts do (Panina et al., 2003; Makarova et al., 2001). During zinc deficiency, the basal ribosomal subunit proteins containing the zinc ribbon motifs are degraded and are replaced by the non-zinc-ribbon-containing paralogs (Shin et al., 2007; Panina et al., 2003). This mechanism might provide a rudimentary zinc storage mechanism for adaptation during zinc deficiency (Panina et al., 2003; Shin et al., 2007).

ZnuA functions as a periplasmic zinc binding protein and serves as the metal specificity determinant for this high affinity transport system (Hantke, 2005; Patzer and Hantke, 1998). Amongst most bacterial species with this transport system, zinc specificity seems to be governed by a region of ZnuA rich in histidine, aspartate, and glutamate residues, but this is not entirely accepted in the field as some transporters containing these signature residues may preferentially transport manganese (Hantke, 2005; Patzer and Hantke, 1998).

Moreover, the subcellular location of protein folding affects the affinity of some proteins for metals (Tottey et al., 2008). The manganese and copper periplasmic binding proteins MncA and CucA, respectively, both bind copper preferentially *in vitro* (Tottey et al., 2008). However, once MncA binds manganese, it does not exchange these ions for copper (Tottey et al., 2008). Moreover, unlike

CucA which is exported by the Sec pathway, MncA is exported by the Tat pathway, which allows MncA to fold in the cytoplasm that has no freely available copper or zinc but does contain available manganese (Tottey et al., 2008). Thus, once in the periplasm, the preference of MncA for manganese has been pre-programmed, so to speak (Tottey et al., 2008).

Returning back to ZnuABC transport, after zinc-bound periplasmic ZnuA docks, ZnuB functions as a transmembrane permease spanning the inner membrane and ZnuC functions as the ATPase engine in the cytoplasm (Hantke, 2001a). Another bacterial zinc transporter, ZupT has been identified that belongs to the Zrt-Irt-like protein (ZIP) family (to be discussed more appropriately below; *vide infra*: Overview of Zinc Homeostasis in Yeast) (Grass et al., 2002). Mutation of ZupT alone yields no phenotype. However, when the ZnuABC system is inactivated, loss of ZupT activity renders the bacteria more sensitive to zinc chelators, thus supporting its role as a low affinity zinc transporter in bacteria (Grass et al., 2002).

Mechanisms of zinc uptake in bacteria are less clear than those for export. In gram negative bacteria, the mechanism of transport of zinc across the outer membrane into the periplasm and vice-versa is unknown since the proteins regulated by the Znu operon only transport zinc from the periplasm across the inner membrane into the cytoplasm (Hantke, 2001a). In gram positive bacteria, the layer of teichoic acids on the surface of the bacteria can bind divalent cations and is thought to function like a cation exchanger (Hantke, 2001a).

Both gram-negative and positive bacteria take up iron via the production of siderophores. These are chelating peptides not encoded in the genome which essentially solubilize and chelate extracellular ferric iron (Hofte et al., 1993). The siderophore/iron chelate is then taken up by the cell. Excess zinc has been shown to upregulate the production of siderophore synthesis regardless of available iron levels and this may represent a connection between the regulation of the two metals (Hofte et al., 1993; Rossbach et al., 2000) or may represent competitive uptake.

Three major zinc export systems exist in gram-negative bacteria: the RND proton antiporters, the P-type ATPases, and the cation diffusion facilitator (CDF) exporters that function to remove excess zinc. Each will be considered in turn below. The RND (resistance, nodulation, and division protein family) type zinc exporters function as H^+-antiporters (Hantke, 2001a) and are composed of three major proteins. CzcA transports zinc out of the cytoplasm. CzcC transports zinc out of the periplasm into the extracellular space. CzcB is thought to connect the two transporters and thereby protect the periplasm from exposure to zinc (or other heavy metals) by making a direct conduit from the cytoplasm to the extracellular space. The *czc* genes encoding the RND-type exporter are regulated by a two-component signaling system, namely CzcSensor/CzcRegulator which is zinc-responsive (Grosse et al., 1999). Since the two-component signaling system responds to high extracellular but not intracellular zinc and results in the extrusion of zinc may indicate that at least some zinc uptake is not well regulated at least under zinc excess conditions. Moreover, the

RND type exporters are induced when zinc is in vast excess; whereas, the CDF type exporters function when zinc is replete (Anton et al., 1999).

P-type ATPases such as ZntA in *E. coli* utilize the power of ATP hydrolysis in the export of primarily lead or cadmium but also excess zinc across a concentration gradient in the form of a cysteine or glutathione thiolate conjugate (Sharma et al., 2000). ZntR is both a positive and negative transcriptional regulator of *zntA* (Noll and Lutsenko, 2000). Prior to metal-induced activation, ZntR is a *zntA* repressor; following metal activation, ZntR enhances RNA polymerase binding to the *zntA* promoter (Outten et al., 1999). Presence of the ZntA transporter increases the tolerance of toxic lead and cadmium concentrations nearly 500-fold but only modestly increases the tolerance of toxic zinc concentrations (≈ three-fold) (Yoon and Silver, 1991). This suggests there are other zinc-dedicated exporters, such as the RND and CDF-type exporters (Hantke, 2001a).

The ZntA and ZntR proteins of *E. coli* are unrelated to the identically named proteins in the gram-positive *Staphylococcus aureus.* The gram-positive ZntA is, instead, orthologous to the CzcD CDF gene (not to be confused with the previously mentioned CzcABC system, above) in gram negative species (Hantke, 2001b). These inconsistencies are due to difficulties in coordinating nomenclature among the different fields of bacterial research (Hantke, 2001a). To further the confusion, a genetic system analogous to the gram-negative ZntA exists in the gram-positive *Staphylococus* species termed CadA and CadC but these genes also exist in some gram-negative species due to horizontal gene transfer (Hantke, 2001a).

Cation diffusion facilitator (CDF) members, YiiP and ZitB in *E. coli* and CzcD in *Ralstonia metallidurans,* function to export excess zinc from the cytoplasm to the periplasm (Hantke, 2001a; Lu and Fu, 2007). A very recent report of the YiiP crystal structure (Lu and Fu, 2007) delivers the greatest insight so far into the mechanism of zinc transporter function. YiiP functions as a homodimer (Lu and Fu, 2007) whereas the mammalian CDF members function as homo- or heterodimers (Kambe et al., 2002; Suzuki et al., 2005b; Suzuki et al., 2005a). Each monomer of YiiP consists of six transmembrane domains and a cytoplasmic domain that itself consists of alternating α-helices and β-sheets in an αββαβ configuration (Lu and Fu, 2007). Remarkably, transmembrane domain five is shorter than the other transmembrane domains yielding solvent-accessible extracellular (periplasmic) and intracellular membrane cavities near two zinc centers in the crystal structure (Lu and Fu, 2007).

The YiiP cytoplasmic domain resembles the structure but not sequence of the copper metallochaperone Hah1 fold (Lu and Fu, 2007). YiiP thus conforms to the previously described observation that all metallochaperones and their cognate targets contain a common metallochaperone-like fold (Rosenzweig and O'Halloran, 2000). The authors of the YiiP crystal structure study proposed that this metallochaperone-like fold in YiiP serves as the target domain for a predicted, as yet unidentified, cytoplasmic zinc chaperone (Lu and Fu, 2007). These findings may deliver insights into mammalian CDF members as well since they share up to 30% sequence identity with YiiP (Lu and Fu, 2007). Once the zinc is bound to the cytoplasmic domain of a

YiiP monomer, dimerization occurs resulting in the translocation of zinc across the membrane to the extracellular (periplasmic) cavity (Lu and Fu, 2007). It is not known how the periplasmic zinc is extruded to the extracellular environment.

Higher eukaryotes, unlike most prokaryotes, can also chelate excess zinc via metallothioneins. However, several bacterial genomes have been found to encode a metallothionein-like cysteine-rich protein (Blindauer et al., 2002). *Synechococcus* PCC7942 encodes SmtA; whereas *Anabaena* PCC7120*, Pseudomonas aeruginosa*, *Pseudomonas putida,* and *E. coli* all encode BmtA-like proteins (Blindauer et al., 2002). The *E. coli* BmtA-like protein binds one zinc atom whereas the remainder each bind four zinc atoms (Blindauer et al., 2002). The metallothioneins will be discussed further, below (*vide infra*: Overview of Zinc Homeostasis in Higher Eukaryotes).

C. Overview of Zinc Homeostasis in Yeast

Lower eukaryotes, such as yeast, must solve the same major problems associated with maintaining zinc homeostasis as do bacteria: principally the intake of sufficient zinc and the export of excess zinc. Yeast, however, must add another aspect to zinc homeostasis, the storage of excess zinc for later use. The average yeast is approximately ten times larger than the average bacterium. Larger eukaryotic cells must be able to store zinc for future use since the nutrient requirements of large cells often exceed what they can immediately absorb from the environment. As discussed previously, bacteria may employ a crude zinc storage mechanism by cannibalizing subunits of the ribosome to free a fraction of cellular zinc. Yeast employ a more

efficient storage method as will be discussed. It should be noted that most of the yeast genetic work for zinc homeostasis has been performed in the budding yeast *Saccharomyces cerevisiae* with a few notable studies performed in the fission yeast *Schizosaccharomyces pombe*. To understand regulation of yeast zinc transporters, the transcriptional regulator Zap1p must first be considered.

Zinc-activated Protein 1 (Zap1p) (Transcription Factor)

In *S. cerevisiae*, the zinc-responsive transcriptional regulator, Zap1p, has seven classic, C_2H_2 zinc-fingers and two activation domains (Gaither and Eide, 2001a). Two of the zinc-fingers are nested in the second activation domain whereas the remaining five zinc-fingers comprise the DNA-binding/zinc-responsive domain (Gaither and Eide, 2001a). The canonical Zap1p, zinc-responsive cis-element is an 11 base-pair sequence ACCYYNAAGGT (Bird et al., 2000; Gaither and Eide, 2001a). The zinc sensor function of Zap1p and subsequent transcriptional activation seem to rely on two low affinity zinc fingers in activation domain two. During zinc replete states, the fingers bind zinc and apparently inactivate the transactivation domain through stable intramolecular interactions; whereas, loss of zinc binding, thereby activates Zap1p (Bird et al., 2003). Disruption of the two zinc fingers, by mutation, results in a constitutively active form of Zap1p (Bird et al., 2003). The Zap1 regulon consists of several dozen genes directly or indirectly involved in zinc homeostasis: including zinc transporters and detoxifiers of oxidative stress (Wu et al., 2008; Wu et al., 2007). Zap1p has a biphasic response to zinc deficiency with a differential gene-regulatory response to mild and severe zinc limitation (Wu et al.,

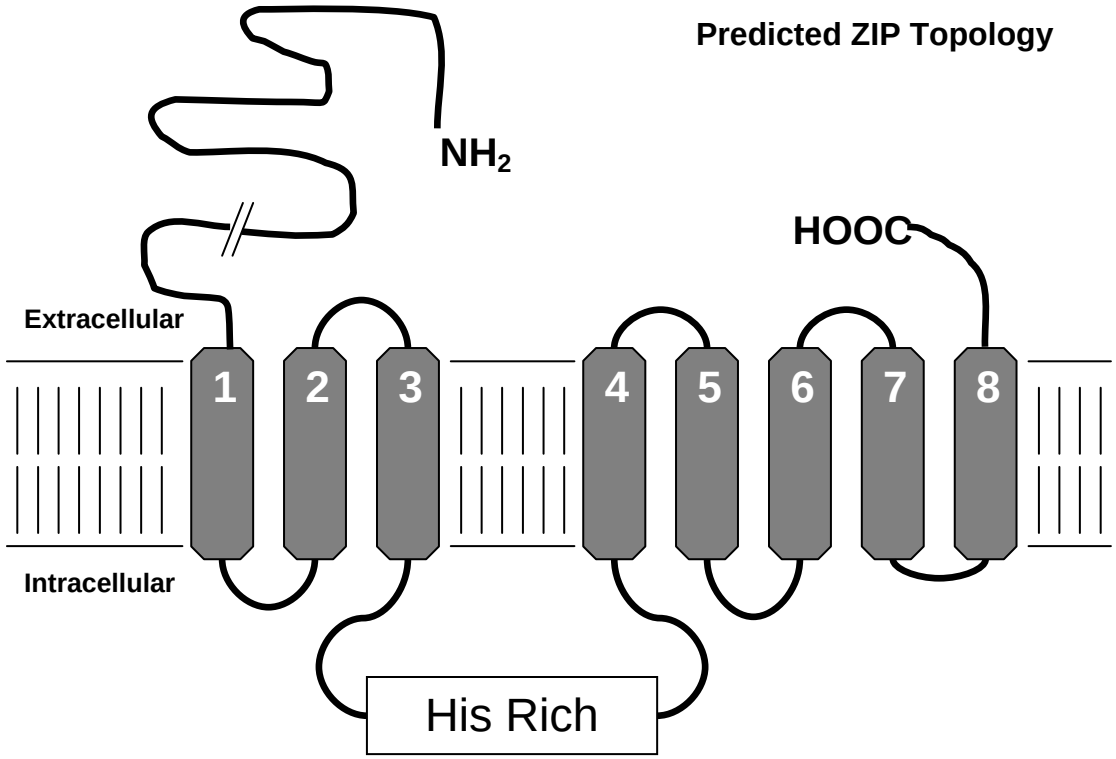
2008). The system does not apparently exist in the fission yeast, *S. pombe*, as no identifiable Zap1 homolog exists.

Yeast Zinc Transporters

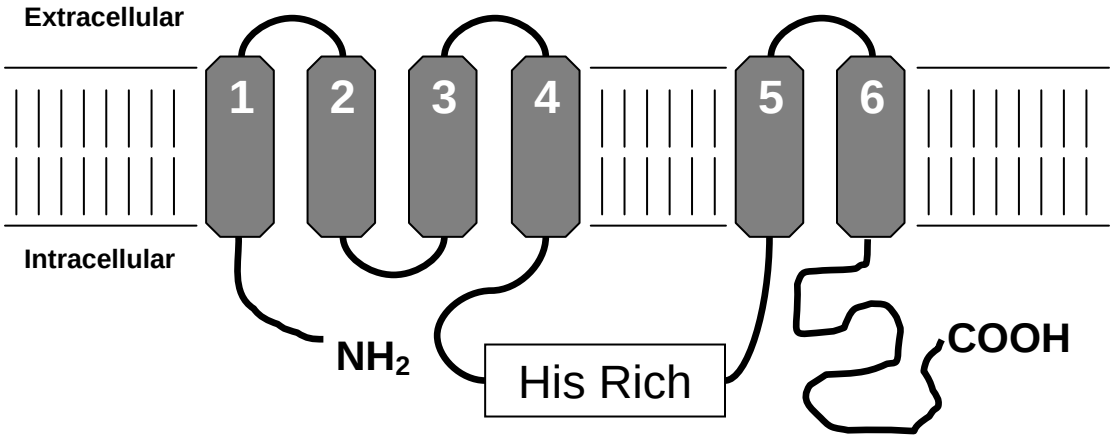
The budding yeast and fission yeast encode members of the Zrt-Irt-like Protein (ZIP) (Slc39a) and CDF (Slc30a) families. These families of transporters have members in all life forms and are thus thought to have originated early in evolution (Gaither and Eide, 2001a) since the last common ancestor for all extant species lived about four billion years ago (Hedges, 2002). Bacterial members of the CDF family were previously discussed. The yeast members of the CDF family will be discussed more below. The ZIP proteins derive their name and distinction by having homology to the yeast Zrt zinc transporters and the plant Irt iron transporters. The Zrt and Irt transporters have sequence homology and a conserved predicted topology (see Figure 1.3) of eight transmembrane domains and extracytoplasmic amino- and carboxy-termini. Additionally, conserved histidine residues (often with charged residues and glycine) in transmembrane domains four and five in conjunction with a highly variable histidine-rich intracellular loop between transmembrane domains three and four are thought to be essential for zinc transport (Gaither and Eide, 2001a; Guerinot, 2000a). Not much is known about the bacterial members of the ZIP family (ZupT was mentioned above; *vide ante*: Overview of Zinc Homeostasis in Bacteria) but the ZIPs play a preeminent role in zinc homeostasis in eukaryotes and aspects of their regulation is a major focus of this dissertation.

Figure 1.3: Predicted Topology of ZIP and CDF Family Zinc Transporters. This cartoon outlines the predicted topology for both zinc uptake (ZIP) and export (CDF; ZnT) transporters. The // marks for ZIPs indicate the variability in length and composition of the amino-terminus. These topology predictions are based on the various prediction algorithms such as TMpred, TopPred, and TMHMM. Links to each of these can be found on the publicly available server: http://ca.expasy.org/tools/ . The ZIP transporters are predicted to have eight transmembrane domains with extracellular amino- and carboxy-termini. The amino-termini of several ZIPs have N-linked glycosylation sites (not indicated). CDF transporters are predicted to have six transmembrane domains (12 for ZnT5) with intracellular amino- and carboxy-termini. These gross topologies are only intended to aid the reader in spatial orientation and obviously do not necessarily represent actual physical orientations.

Predicted ZIP Topology



Predicted CDF Topology



Based on homology, ZIPs are divided into four subfamilies: Liv-1 subfamily, gufA subfamily, ZIP subfamily I, and ZIP subfamily II (Gaither and Eide, 2001a). Yeast Zrt1 and Zrt2 belong to ZIP subfamily I. Yeast Zrt3 belongs to the gufA subfamily along with mostly predicted bacterial members. Most of the mammalian ZIPs belong to the Liv-1 subfamily except for ZIPs1-3 that belong to ZIP subfamily II.

As seen in Figure 1.4, most of the yeast (and mammalian) ZIP family members are thought to increase the total cytoplasmic concentration of zinc either from outside the cell or from within an intracellular organelle (Gaither and Eide, 2001a). This idea is based on ^{65}Zn uptake studies of transfected cells and the regulation patterns for several of the characterized ZIPs, as will be discussed here and later.

The mechanism of action for the ZIPs has been elusive. The yeast ZIPs may be energy-dependent permeases. It was stated that zinc-limited yeast starved for glucose one hour had no zinc uptake (Zhao and Eide, 1996a). Despite this claim, these authors have never published these data. Even if true, this result does not delineate direct from indirect effects since yeast are known to respond within minutes to glucose starvation at the transcriptional, translational, and post-translational levels including rapid changes in several signaling pathways that could in principle regulate transporter activity (Gorner et al., 2002). Thus, there is no direct demonstration of the energy dependence for ZIP transport activity. The only solid evidence regarding mechanism of ZIP transport has been the observation that certain members of the ZIP

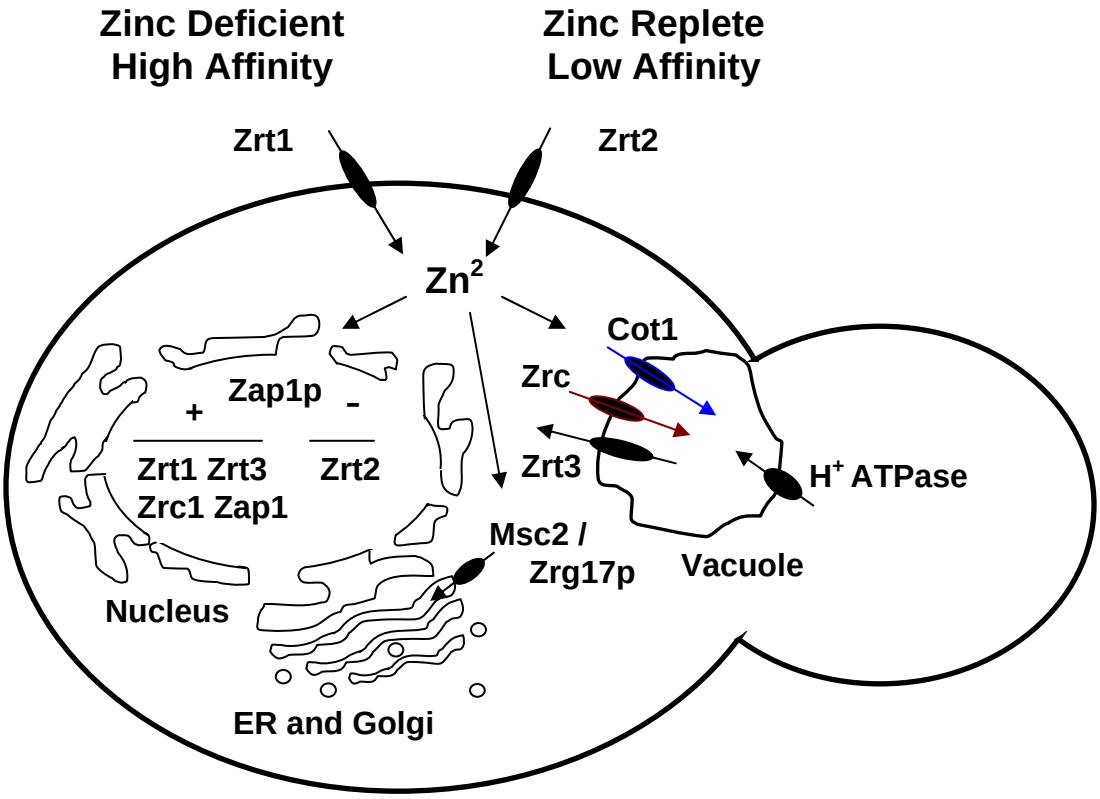


Figure 1.4: Overview of zinc homeostasis in the budding yeast *Saccharomyces cerevisiae*. This cartoon outlines the major organelles for which specific zinc uptake and export transporters or regulators have been identified. As indicated, depending on environmental availability, zinc uptake into yeast occurs through either the low affinity Zrt2 transporter or the high affinity Zrt1 transporter that is upregulated during zinc deficient conditions. Only the major transporters are indicated for the Zap1 regulon and this represents a much abbreviated list. The transcriptional responses to zinc-activated Zap1p are indicated as + (enhanced transcription) and – (repressed transcription). The dynamic processes occurring in the yeast cell with changes in zinc availability cannot be adequately depicted in this static cartoon.

family function as, or are stimulated by, symport of bicarbonate ion {Gaither, 2000 10236 /id}{Girijashanker, 2008 12773 /id;Liu, 2008 12741 /id}. More will be said, later, regarding the dearth of information on ZIP function and regulation.

In yeast, Zrt1 functions as a high affinity zinc uptake transporter {Zhao, 1996 12990 /id}{Dainty, 2008 12992 /id} making it the first characterized zinc uptake transporter and the first reported member of the ZIP family. The second member of the ZIP family to be reported was the Irt1 transporter from *Arabidopsis thaliana*. Irt1 transports iron, primarily, and will not be considered further (Eide et al., 1996). In the budding yeast, *S. cerevisiae*, Zrt1 is upregulated by Zap1p in response to zinc deficiency (Zhao and Eide, 1997). The Zrt1 promoter contains a canonical zinc-responsive element targeted by Zap1p (discussed above). No Zap1 homolog has been identified in the fission yeast *S. pombe*, yet the fission yeast is still able to upregulate a Zrt1 ortholog (termed *zrt1*$^{+}$ or just *zrt1*) in response to zinc deficiency (Dainty et al., 2008). In fact, the fission yeast also seems to have a zinc regulon with zinc-responsive genes orthologous to the budding yeast (Dainty et al., 2008). Thus, these effects are presumably mediated by another, as yet uncharacterized, zinc-responsive transcription factor or perhaps through post-transcriptional mechanisms.

Following zinc repletion, Zrt1 is rapidly down-regulated at the transcriptional level to prevent production of more protein and at post-translational level to inactivate the transporter at the plasma membrane and thereby prevent zinc toxicity (Gitan et al., 1998). Regarding the post-translational inactivation, upon zinc repletion, Zrt1 at the plasma membrane is mono-ubiquitinated in the large cytoplasmic loop, then

endocytosed, and degraded in the vacuole (Gitan et al., 1998; Gitan and Eide, 2000). Later, the ubiquitination site was identified as lysine 195 and a so-called metal response domain comprising residues 205-211 of Zrt1 (DATSMDV) independent of the ubiquitination site was found to be essential for ubiquitination, internalization, and subsequent degradation; mutation of this sequence had no effect on zinc uptake activity whereas mutation in this region (especially the underlined residues) prevented ubiquitination in response to zinc and could thus serve a solely regulatory role (Gitan et al., 2003). These results give the first glimpse of rapid post-transcriptional regulatory mechanisms of ZIPs. Upon switching the growth media to zinc replete conditions, the authors showed a rapid loss of the intact Zrt1 but did not consider accumulation of degradation/processed products.

In yeast, there are two low-affinity zinc transporters, Zrt2 (Zhao and Eide, 1996b) and Fet4 (Waters and Eide, 2002). Zrt2 is a zinc-specific transporter that is upregulated when zinc is readily available and down-regulated by Zap1p during zinc deficiency (Bird et al., 2004). This transcriptional repression during zinc deficiency occurs through a non-canonical ZRE that overlaps the transcriptional start site of Zrt2; in this way, Zap1p only binds appreciably to this cis-element when it is highly active (during zinc deficiency) and thereby blocks transcription, presumably to allow more Zrt1 to be placed on the plasma membrane. Fet4, also regulated by Zap1p, functions at the plasma membrane as a promiscuous metal transporter with low affinity for several metals and *fet4*Δ mutants are viable (Dix et al., 1994; Dix et al.,

1997). *S. pombe* was also found to encode a Fet4 but not a Zrt2 homolog (Dainty et al., 2008).

In contrast to bacteria, all eukaryotic cells have organelles. With regard to zinc homeostasis, this allows the advantage of zinc storage and subsequent mobilization. In the budding yeast, the vacuole, which is analogous to the vertebrate lysosome, serves as a zinc storage site and the release of zinc during zinc deficiency is conferred by the Zap1p-regulated vacuolar transporter Zrt3 (MacDiarmid et al., 2000; MacDiarmid et al., 2002).

Zrt3 is a member of the gufA subfamily of ZIP transporters (Gaither and Eide, 2001a). This subfamily contains the least number of characterized transporters. Opposing the action of Zrt3 is the CDF member, Zrc1, which functions as a proton antiport to transport zinc into the vacuole for storage (Gaither and Eide, 2001a). A net proton gradient is diffused by the action of the vacuolar proton-ATPase. Thus, ATP indirectly powers the storage of zinc in the vacuole. A recent study showed that the selectivity for zinc by Zrc1 is governed, surprisingly, through an asparagine residue in the second transmembrane domain (Lin et al., 2008). They found that mutation of this residue to an isoleucine altered the selectivity of this transporter to prefer iron over zinc (Lin et al., 2008). This residue is conserved in other yeast and some bacterial CDF members but not mammalian members. It should be noted that Zrt3 and Zrc1 orthologs do exist in *S. pombe* but they do not appear to be regulated at the transcript level as in *S. cerevisiae* (Dainty et al., 2008). The fission yeast ortholog

of Zrc1, Zhf1, transports excess zinc from the cytoplasm into the endoplasmic reticulum (Borrelly et al., 2002).

In the yeast, the CDF family members Msc2 and Zrg17 are thought to provide zinc to the secretory pathway for the proper folding of zinc-dependent enzymes (Ellis et al., 2004; Ellis et al., 2005). Moreover, Zrg17 is upregulated during zinc deficiency supporting a role as an adaptive mechanism for the secretory pathway when zinc is scarce (Lyons et al., 2000). Transport mechanisms of zinc into and out of other organelles are even less well understood in the yeast. Iron is known to be transported into the mitochondrion by mitochondrial ferric transporters (MFTs) 1 and 2 but these do not transport zinc (Li and Kaplan, 1997). Moreover, to date, the author is unaware of any characterized zinc exporters functioning at the plasma membrane in the yeast.

The yeast vacuole might take on another role, the detoxification of excess zinc. This notion has been proposed by others and is supported by the observations that during zinc deficiency, Zrc1 is normally upregulated and *Zrc1*Δ mutants do not adapt to a shift from zinc deficient media to zinc replete media and are prone to die, thereafter (MacDiarmid et al., 2003). In this way, the yeast vacuole may take on a preeminent role in zinc homeostasis: to supply zinc during deficient states and prevent toxicity upon zinc repletion. Higher eukaryotes can detoxify and store zinc using the small cysteine-rich metallothioneins. In contrast, lower eukaryotic metallothioneins principally bind copper with the notable exception of Zym1 from fission yeast which binds zinc (Borrelly et al., 2002). These differences might

suggest a greater necessity for copper binding proteins than zinc binding proteins, likely a result of the high redox capacity of copper.

In conclusion, the budding yeast and fission yeast do have some notable differences in their regulators of zinc homeostasis as mentioned. At first glance, this may seem surprising. However, the two yeasts apparently diverged 1 billion years ago (Hedges, 2002), which further supports the ancient origins of ZIP and CDF family members and their essential activities. The author would speculate that, most probably, the regulatory mechanisms of the ZIP orthologs and CDF orthologs diverged as the species diverged. However, the orthologous transporters were conserved. The conservation implies that the essential functions of the transporters prevented much divergence of their structures and chemistries (thus, sequences) necessary to fulfill those functions.

D. Overview of Zinc Homeostasis in Higher Eukaryotes

Thus far, we have only considered single cell organisms. As we will now see, multicellular organisms must also procure sufficient zinc from the environment and extrude excess zinc. For yeast, cellular storage of zinc was solved by the vacuole, which allowed for rapid mobilization of zinc per cellular demands. However, homeostatic regulation of zinc to this point has not dealt with the problem of intercellular zinc distribution. This can present quite a challenge since very small invertebrates such as the nematode *Caenorhabditis elegans* are more than 100 times larger than the average yeast cell whilst larger vertebrates such as mammals are on the order of thousands to tens-of thousands of times larger than the simple nematode

and contain hard tissues and complex nervous systems. Thus the requirement becomes apparent to adequately distribute zinc from the site of absorption to the sites of utilization and storage with very fine accuracy.

These problems of scale and complexity may explain the expansion of mammalian ZIPs, ZnTs (the vertebrate CDF family members), and metallothioneins with the requirement to distribute zinc within and amongst different cell types, tissues, and organs at different times in development or as cellular processes demand. The intercellular distribution of zinc becomes a priority in large vertebrates, in contrast to the preceding model organisms. Moreover, the intracellular mobilization of zinc also becomes more complex with specialized cell types such as neurons, measuring up to a meter in length. These distances vastly exceed the diffusion-limited distribution of zinc and must employ specialized compartmentalization and regulation to fulfill transient cellular requirements.

The molecular biology of zinc homeostasis in higher eukaryotes has relied heavily on the model organisms of developmental biology including flies, zebrafish, and rodents. Cell culture strategies have also proven to be both valuable and treacherous in the understanding of zinc homeostasis and those key studies as well as caveats will be discussed. Throughout this section, various systems will be discussed and specified as necessary but major emphasis will be placed on mammalian systems since these are most germane to understanding human disease.

Metal Response Element Binding Transcription Factor 1

MTF1 is a transcriptional regulator with both activator and repressor functions which is responsive to zinc, cadmium, hypoxia, and oxidative stress (Laity and Andrews, 2007; Andrews, 2001). In contrast to Zap1p, MTF1 resides in the cytoplasm during zinc replete conditions. Following exposure to elevated zinc, MTF1 translocates into the nucleus and alters the transcriptional rates of an array of genes including the zinc chelators metallothioneins 1 and 2 (I and II in older literature), the zinc exporter ZnT1, γGCS heavy chain, and the hsp70 member BiP, among many others (Langmade et al., 2000; Andrews, 2001; Lichtlen et al., 2001). MTF1-responsive genes contain one or more metal-response elements (MREs) consisting of the core consensus sequence TGCRCNC in either orientation (Andrews, 2001). The zinc sensor function of MTF1 has been the focus of intense research and has recently been, in part, illuminated.

MTF1 has several structural domains, including an amino-terminal domain followed by six classical C_2H_2 zinc-finger domains (= "the zinc-finger domain"), an acidic region, a proline-rich region, a serine/threonine-rich region, and a carboxy-domain (Andrews, 2001). The acidic, proline-rich, and serine/threonine-rich regions constitute the three transactivation domains of MTF1 (Laity and Andrews, 2007; Andrews, 2001). The acidic, proline-rich, and serine/threonine-rich regions are important for transcriptional activity, as their deletion resulted in a loss of transcriptional activity (Radtke et al., 1995). The acidic domain is necessary for the recruitment of the histone acetyltransferase p300 but the acidic domain does not form

any regular secondary structure with or without zinc as determined by NMR (Li et al., 2008). The p300 function was necessary for induction of *MT1* but not *ZnT1* following zinc activation (Li et al., 2008). These data argue for synergistic or cooperative interactions of a multiprotein complex in transcriptional activation of certain MTF1- regulated genes. Substitution of the three transactivation domains with the viral VP16 acidic transactivation domain retained transcriptional activity but became more active in the absence of zinc, thereby losing some zinc-responsiveness (Radtke et al., 1995). Human MTF1 has a greater dynamic range of induction (about 10-fold) than mouse MTF1 (about 3-fold) following zinc-activation (Radtke et al., 1995) and that a chimeric mouse MTF1 containing the human MTF1 acidic domain gains the heightened response to zinc (Muller et al., 1995). This suggests that one or more of the intact MTF1 transactivation domains, probably the acidic domain, and especially so for hMTF1, contributes at least partially to zinc-responsiveness.

More clarification of the MTF1 zinc sensor function has emerged; wherein, the authors of a recent study focused on the zinc-finger domain (Li et al., 2006). Previous studies showed that the reversible DNA-binding activity of MTF-1 in cells required the addition of zinc to the media; the zinc-sensitive domain was mapped to the zinc-finger domain (Dalton et al., 1997). Another study found that the zinc fingers were functionally heterogeneous where fingers 5 and 6 were dispensable but finger 1 was essential in metal responsiveness (Bittel et al., 2000). All zinc-regulated MTF1 orthologs, from *Fugu* to humans, have 92% or greater protein sequence identity in the zinc finger domain (Andrews, 2001). Put another way, less than 8% of

the ancestral vertebrate MTF1 zinc-finger domain has diverged across the extant species of vertebrates, which share a most recent common ancestor over 400 million years ago (Hedges, 2002). The remainder of MTF1 has much less conservation. Although highly conserved amongst all vertebrate orthologs, several of the linker peptides are highly divergent from the canonical linker sequence, TGEK/RP (Laity and Andrews, 2007). Linkers 4-5 and 5-6 (the sequences between zinc-fingers 4-5 and 5-6, respectively) were canonical (Li et al., 2006). When the authors mutated the linker 1-2 peptide to the canonical sequence, all zinc-responsiveness was lost, resulting in a constitutively nuclear and transcriptionally active MTF1 (Li et al., 2006). In addition, similarly changing the short linker 3-4 peptide TGKT resulted in reduced DNA binding and zinc responsiveness (Li et al., 2006). In contrast, mutation of the linker 2-3 TKEKP had no measurable effect (Li et al., 2006). In this way, these non-canonical peptide linkers seem to dictate the affinity for zinc in this domain and thereby allow MTF1 to gauge free zinc concentrations (Li et al., 2006). The critical function of the MTF1 zinc-finger domain in the regulation of zinc homeostasis in vertebrates explains the remarkable sequence identity and conservation of the MTF1 zinc-finger domain throughout evolution.

Intracellular Storage of Zinc in Vertebrates: Metallothioneins

Higher eukaryotes store zinc in the form of protein complexes, with zinc ions conjugated to cysteine ligands in metallothioneins. These complexes are labile and can serve to rapidly supply zinc. As mentioned above, certain of the metallothioneins (MTs) are regulated in response to zinc (and other metals/stressors) by MTF1 (Geiser

et al., 1993). The mouse genome encodes four *MTs* whereas the human genome encodes eleven (Andrews, 2001). The paralogs have differences in expression patterns and conceivably function. In the mouse, MT-1 and MT-2 both aid in the adaptive response to zinc deficiency, especially during maternal zinc deficiency (Andrews and Geiser, 1999). The brain-specific MT isoform, MT-3, has been shown to be important in neuronal function (El, I et al., 2006). MT-3 functions in a multiprotein complex with HSP 70, HSP 84, and creatine kinase (and perhaps others) and may be associated with various neuropathologies (El, I et al., 2006). The *MT-4* gene is located 20 kb upstream of the *MT-3* gene in both the mouse and human (Quaife et al., 1994). *MT-4* is expressed in the differentiated, cornified layers of stratified squamous epithelia; whereas, *MT-1* is expressed in the actively proliferating cells of the stratified epithelia implying differentiation-dependent isoform-switching (Quaife et al., 1994). Yet, none of the MTs are essential genes in the absence of heavy metal stress.

All of the MTs are small proteins (less than 14 kDa) and consist of a cysteine-rich sequence (*exempli gratia*, 20 cysteine residues out of 60 total residues for mouse MT1) that can bind up to seven zinc ions in two zinc clusters. The MTs have exceptionally strong affinities for zinc. The seven zinc ions bound to MT-2 have a stability constant in the 10^{13} M^{-1} range (Jacob et al., 1998) yet these proteins can surrender zinc during deficient conditions. Paradoxically, MTs can exist in the apoform *in vivo* (Jacob et al., 1998; Yang et al., 2001). Therefore, the transfer of zinc from MTs to acceptor proteins cannot be explained by a two-component system.

Rather, the redox state of cysteine ligands in MTs can mobilize zinc under oxidizing conditions, thereby making zinc transfer bidirectional; thus, MTs can store and release zinc (Maret and Vallee, 1998; Jacob et al., 1998). The presence of glutathione disulfide alone increases the rate of zinc transfer; whereas, glutathione alone represses zinc transfer from MT, yet in conjunction with glutathione disulfide, they enhance the rate of zinc transfer and the total number of zinc ions released (Jiang et al., 1998). Moreover, the stability constant stated above is not equally divided amongst the zinc ions and thiol/zinc cluster rearrangements can provide some driving force for additional release of zinc. Furthermore, intracellular ligands, such as citrate and perhaps many different inorganic metabolites, can aid in the transfer of zinc to lower affinity enzymes during oxidative stress (Jacob et al., 1998).

Essentially, MTs function as biologically active pools of zinc. MTs serve to compartmentalize available zinc during normal states, detoxify heavy metals such as cadmium and mercury, and provide zinc during zinc deficiency (Abe et al., 2000; Breen et al., 1995; Bucio et al., 1995; Bylander et al., 1994; Chan and Cherian, 1992; Chellman et al., 1985; Conrad et al., 1997; Dalton et al., 1996a; De et al., 1990; De et al., 1991; Klaassen et al., 1999). There has been some controversy regarding the interactions of certain of the MTs with ATP and glutathione (Maret et al., 2002; Zangger et al., 2000). Nonetheless, MTs are more than zinc chelators; they function as a dynamic reservoir to efficiently compartmentalize zinc in a cell-type specific manner.

Vertebrate CDF members, the ZnTs (Slc30a family)

Cells are able to rid excess zinc through the action of the previously mentioned CDF family members. However, multicellular organisms also employ CDF members, the ZnTs (solute carrier 30a family; *slc30a* family), to other ends, namely the redistribution of zinc both within the cell and throughout the body. Mammals have ten *Slc30a* genes. The redistribution of zinc is achieved in conjunction with opposing transporters, the ZIPs, to be discussed below.

The ZnTs typically consist of six transmembrane domains (1, 2, and 5 most highly conserved), a histidine-rich internal loop between transmembrane domains 4 and 5, and a long carboxy-terminal domain in the cytoplasm (topology as described previously for the CDF member YiiP in bacteria) (Palmiter and Huang, 2004; Lu and Fu, 2007). An exception to this topology is ZnT5 with 12 to 15 predicted transmembrane domains (Jackson et al., 2007; Valentine et al., 2007; Ford, 2004) (Palmiter and Huang, 2004).

ZnT1 was the first characterized zinc transporter (thus, the name Zn Transporter 1) identified in a cDNA screen for resistance to zinc toxicity (Palmiter and Findley, 1995). ZnT1 is in part transcriptionally activated by MTF1 (Langmade et al., 2000). ZnT1 is thought to be the primary transporter through which higher eukaryotic cells excrete excess zinc at the plasma membrane, especially in the gut and embryonic visceral endoderm (Palmiter and Huang, 2004). This gene is essential in mice (Andrews et al., 2004; Palmiter and Huang, 2004).

In mammals, the ZnTs function to export excess zinc from the cytoplasm to the extracellular compartment (as stated for ZnT1) or into intracellular organelles and vesicles either in the *trans*-Golgi network (ZnTs5-7), general secretory granules (ZnTs 4 and 5), endosomes (ZnTs2 and 4), pancreatic β-cell-specific insulin-containing secretory vesicles (ZnTs 5 and 8), or synaptic vesicles (ZnT3 and perhaps ZnT8 which has been detected in brain and liver (Palmiter and Huang, 2004)). As mentioned previously, the ZnTs function as homo- or hetero-dimers as does the bacterial YiiP (Kambe et al., 2002; Suzuki et al., 2005b; Suzuki et al., 2005a). Interestingly, several of the ZnT members are essential in the mouse, ZnTs1, 4, and 5. The effects of genetic ablation will be described later (*vide infra*: Chapter 6, The Genetics of Essential Metal Homeostasis during Development). Based on the structural information recently obtained for the bacterial CDF member, YiiP, more structure/function studies should emerge for the vertebrate members of this transport family.

Mammalian ZIP Members (Slc39a family)

Mammals have 14 ZIP paralogs (the *Slc39a* family), which are primarily zinc importers (increase cytosolic zinc). Yet, evidence suggests that certain of the mammalian ZIPs may also serve essential roles in iron metabolism, such as ZIP2 during development (Peters et al., 2007) and ZIP14 in various cells (Liuzzi et al., 2006) and possibly manganese metabolism, such as ZIP8 and ZIP14 which may transport both zinc and manganese (He et al., 2006) (Liu et al., 2008; Girijashanker et al., 2008). Unlike other metals such as copper and iron, no cases of zinc overload as

a result of an inborn error in zinc metabolism have been reported. Wilson's disease and the various hemochromatoses in humans are the result of heritable mutations that result in copper and iron overload, respectively. All zinc overload cases reported are the result of environmental exposure, such as metal fume fever, brass-founder's ague, or zinc shakes which result from exposure to zinc oxide fumes.

ZIPs 1, 2, and 3 were first identified in *A. thaliana* (Grotz et al., 1998). ZIPs1 and 3 of *A. thaliana* are upregulated during zinc deficient conditions in the roots (Grotz et al., 1998). Additionally, the *A. thaliana* ZIP4 gene is induced during zinc deficiency but was localized to chloroplasts (Grotz et al., 1998). The *A. thaliana* ZIPs are clustered mostly in ZIP subfamily I; whereas, the mammalian ZIPs 1-3 are clustered in ZIP subfamily II (Gaither and Eide, 2001a). However, mammalian ZIP4 and most of the remaining mammalian ZIPs are clustered in the Liv-1 subfamily (Gaither and Eide, 2001a).

A systematic study of mammalian ZIPs1-3 shows similarity in gene structure (only 4 exons each) and a highly conserved sequence in transmembrane domain four (HSVFEGLAV/LGLQ) (Dufner-Beattie et al., 2003a). The proteins are all similar in size: 309 to 324 amino-acids in the primary sequences and are highly conserved. The mouse genome also contains a ZIP1 pseudogene without introns but with a partial polyA tract and accumulated mismatches and codon deletions (Dufner-Beattie et al., 2003a).

Mammalian ZIPs1-3 have tissue and cell-type specific expression patterns. ZIP1 is ubiquitously expressed at a low level in the lamina propria of mucosal linings;

ZIP3 is most highly expressed in intestine, visceral yolk sac, and testis; and ZIP2 is most highly expressed in skin, liver, ovary, visceral yolk sac, and dendritic cells (Dufner-Beattie et al., 2003a; Peters et al., 2007). These transporters appear to be selective for zinc over other heavy metals (Dufner-Beattie et al., 2003a). No changes were found in the mRNA or protein levels for these transporters with zinc deficiency. Expression of HA-tagged ZIP1 and ZIP3 in transient transfection assay reveal predominantly intracellular localization when zinc is replete and relocalization to the plasma membrane when zinc is deficient (Wang et al., 2004a). Thus, these transporters are regulated at the post-translational level. Double knockout of *Zips 1* and *3* (Dufner-Beattie et al., 2006), knockout of *Zip2* (Peters et al., 2007), and triple knockout of *Zips1-3* (which constitutes all of the subfamily II ZIPs in mammals) (Kambe et al., 2008) in mice is not lethal and causes no overt phenotype. However, these genes are necessary for the adaptation to zinc deficiency during pregnancy and their absence results in an increase in abnormal morphogenesis during maternal zinc deficiency, compared to wild type controls (Dufner-Beattie et al., 2006; Kambe et al., 2008). These studies suggest that these transporters play very cell-specific roles in zinc homeostasis but not primary roles in the acquisition of dietary zinc especially during zinc replete states (Dufner-Beattie et al., 2003a).

The importance of the ZIPs to mammalian zinc homeostasis is exemplified by two rare, autosomal recessive diseases, acrodermatitis enteropathica (AE) and a sub-type of Ehlers-Danlos syndrome (EDS, discussed below). AE has insidious onset upon weaning, resulting in death by 18 months of age if untreated. Several clinical

features are required for a diagnosis of AE including psoriasiform hyperplasia, low serum zinc, diarrhea, and responsiveness to zinc replacement therapy (Maverakis et al., 2007). The AE disease allele was mapped to chromosomal position 8q24.3 and the authors of those studies suggested *Slc39a4* (*hZip4*) as the candidate disease gene based on homology to other identified zinc transporters (Wang et al., 2001; Kury et al., 2002). Pedigree analysis identified mutations in the *Slc39a4* (human *Zip4*) gene in eight families afflicted with AE (Kury et al., 2002) and more familial mutations continue to be characterized (Meftah et al., 2006; Jensen et al., 2008). Human *Slc39a4* (ZIP4 hereafter) encodes a protein of 647 amino-acids, and a variety of disease alleles gives rise to AE including missense mutations, nonsense mutations, deletions, insertions and splice site mutations thus impacting the severity of the disease in humans (Kury et al., 2002).

Breastfed infants are protected from developing AE symptoms until weaning. However, bovine milk fails to provide protection (Eckhert et al., 1977). Using gel filtration analysis, zinc in bovine milk co-migrates with a high molecular weight fraction; whereas, zinc in human milk co-migrates with a low molecular weight fraction, suggesting that zinc availability is greater in human milk (Eckhert et al., 1977). An alternate route of zinc uptake beyond ZIP4 (Eckhert et al., 1977) must explain the efficacy of zinc replacement therapy with pharmacological doses.

Alternate route for dietary uptake of zinc likely exists since reports have been made of patients homozygous for a predicted null allele or patients who are compound heterozygotes for two, different null alleles and thus unable to retain any

zinc transport activity by ZIP4 (Meftah et al., 2006). However, the alternate route is less than compensatory in action since these patients have severe manifestations of AE unless provided with high levels of zinc. Thus, this alternate route likely does not represent a *bona fide* redundancy for ZIP4 function and may be the result of residual zinc transport by other metal transporters, such as the divalent metal transporter 1 (DMT1) inorganic ferrous iron transporter which has been shown to transport other metal cations such as nickel, cadmium, lead, cobalt, manganese, and zinc with varying selectivity (Gunshin et al., 2001; Gunshin et al., 1997) thus leading to its generic name. Alternatively, other ZIP transporters such as ZIP1 may provide some protection, which is difficult to prove since an antibody specific for endogenous ZIP1 is not available, and thus its cellular localization has yet to be proven, *in vivo*.

Consistent with previous findings in humans (Kury et al., 2002), Northern blot analysis showed a tissue-limited expression pattern for *Zip4* in mouse with highest levels detected in proximal small intestine, distal small intestine, and visceral yolk sac, and lower levels detected in stomach and liver (Dufner-Beattie et al., 2003b). The mouse *Zip4* mRNA has an imprecise transcriptional start site (Dufner-Beattie et al., 2003b) and human and mouse *Zip4* genes each give rise to a low abundance shorter variant of the protein reflecting alternate transcriptional start sites in intron 1. In humans, the amino-terminal 64 amino-acids are replaced with a 39 amino-acid peptide encoded in the first intron (Kury et al., 2002). In mouse, the amino terminal 64 amino-acids are replaced with a 16 amino-acid peptide also encoded in the first intron (Dufner-Beattie et al., 2003b). The importance of these rare variants, if any, is

unclear. The mZIP4 protein selectivity transports zinc over other heavy metals and has saturable uptake kinetics under the transient transfection assay conditions employed (Dufner-Beattie et al., 2003b).

In a subsequent study, the authors examined six known *Zip4* missense mutations that give rise to AE (Wang et al., 2004c). These were P200L in the amino-terminus, G340D in transmembrane domain one, L382P and G384R in transmembrane domain three, G539R in transmembrane domain five, and G643R in transmembrane domain eight. Expression constructs with the wild-type and various mutant cDNAs included an HA epitope tag at the carboxy terminus to facilitate detection by Western blot and by extracellular HA-antibody detection. The mutations studied were categorized into one of two general types: 1) mutants that misslocalize, do not accumulate at the cell surface, and have defective N-linked glycosylation and 2) mutants that do accumulate at the cell surface but have decreased zinc uptake activity (Wang et al., 2004c). The effects of the first class suggest that these mutations destabilize the protein and result in improper folding followed by ER-assisted degradation (Wang et al., 2004c). In contrast, the effects of the second class suggest that these mutant proteins lose zinc-responsiveness since they directly traffic to the plasma membrane (unlike wild-type ZIP4) but have very diminished zinc uptake (Wang et al., 2004c).

In a related study, the roles of both ZIP4 and the closely related paralog, ZIP5 in zinc homeostasis and how they adapt to zinc availability were examined. Tissues from outbred CD-1 mice, including pregnant dams that were fed either zinc deficient

diet starting on day eight of pregnancy (d1 = day of vaginal plug) or a control diet with normal levels of zinc, were examined. In response to zinc deficiency, *Zip4* mRNA was upregulated in the proximal small intestine of the dam by two days of zinc deficient diet and intensely by five days of zinc deficient diet (Dufner-Beattie et al., 2004). Moreover, a noticeable induction of *Zip4* mRNA in the visceral yolk sac by five days of zinc deficient diet was reported (Dufner-Beattie et al., 2004). Intraperitoneal injection of zinc to zinc-deficient dams results in the down-regulation of *Zip4* mRNA by 8 hours and back to zinc-adequate levels after 24 hours (Dufner-Beattie et al., 2003b). **The mechanisms of the mRNA down-regulation are unknown and are a focus of this dissertation.** Furthermore, by five days of zinc deficient diet, ZIP4 protein is strongly induced and localized to the apical membranes of the visceral yolk sac and maternal intestine (Dufner-Beattie et al., 2004). Intraperitoneal injection of zinc to zinc-deficient dams results in a loss of ZIP4 immunostaining by 9 hours in the visceral yolk sac (Dufner-Beattie et al., 2003b). **The mechanisms of the protein down regulation are unknown and are a focus of this dissertation.** Interestingly, ZIP4 immunostaining colocalizes with insulin immunostaining, but nowhere else in the pancreas, and is not affected by zinc availability. Thus, in the pancreas, *Zip4* expression is constitutively restricted to β-cells (Dufner-Beattie et al., 2004). Other, very recent findings regarding the regulation of *Zip4* will be considered in the final summary discussion (Kambe and Andrews, 2009).

Zip5 is a closely related paralog to *Zip4* (Dufner-Beattie et al., 2004). Several *Zip5* mRNAs were identified; yet, they all give rise to the same protein sequence and only differ by the length of the 5' untranslated region (Dufner-Beattie et al., 2004). ZIP4 and ZIP5 proteins share 36% similarity overall and almost 50% in the carboxy half of the protein (Dufner-Beattie et al., 2004). In contrast to *Zip4*, under the reported experimental conditions, no change in *Zip5* mRNA level and apparently no change in protein level were detected, but a loss of ZIP5 immunostaining on the basolateral membranes of intestine, visceral yolk sac, and pancreatic acinar cells was noted during zinc deficiency (Dufner-Beattie et al., 2004). It was thus concluded that the ZIP5 epitope may become masked by a post-translational modification during zinc deficiency but that the level of the protein remains constant (Dufner-Beattie et al., 2004). **This conclusion was re-evaluated in the work herein.**

ZIP5 protein localizes predominately to basolateral membranes in transfected polarized cells as well as in the intestinal epithelium, visceral endoderm, and pancreatic acinar cells when zinc is replete (Dufner-Beattie et al., 2004; Wang et al., 2004b). In contrast, ZIP4 protein is predominately apical in transfected polarized cells and the epithelia of the zinc deficient intestine and visceral endoderm (Dufner-Beattie et al., 2004; Wang et al., 2004b). From these collective data, a model emerged whereby during zinc deficient states, when ZIP5 is not localized to the basolateral membrane, ZIP4 is localized at the apical membrane and serves to increase cytosolic zinc from the digesta for net bodily accumulation of zinc (Dufner-Beattie et al., 2004; Wang et al., 2004b). In contrast, during zinc adequate states,

when ZIP4 is not detectable, ZIP5 is localized at the basolateral membrane and may serve to increase cytosolic zinc from the blood for subsequent elimination via a ZnT transporter (Dufner-Beattie et al., 2004; Wang et al., 2004b).

The above reports strongly imply that *Zip4* and *Zip5* have multiple modes of dynamic regulation in response to zinc availability, the mechanisms of which are not known. The established role of *Zip4* in disease makes a strong case for the significance of these studies to clarify the molecular mechanisms of ZIP regulation and their activity *in vivo*. **The work in this dissertation will focus largely on the molecular mechanisms involved in regulating *Zip4* and *Zip5* in the adaptive response to zinc availability and how *Zip4* impacts development**. Work regarding the molecular regulation and function of ZIPs has continued to be forthcoming and some studies were published concurrently or after the work herein. These reports will be considered in the final Discussion section.

After considering the first two mammalian members of the Liv-1 subfamily, ZIP4 and ZIP5, the remainder of the subfamily will now be considered. For many of these members, little if anything is known about regulation or function. ZIP6 is the mammalian Liv-1 ortholog that was found in zebrafish to be essential for proper epithelial-mesenchymal transition during the gastrula stage (Yamashita et al., 2004). In zebrafish, Liv-1 was shown to be a downstream target of STAT3; loss of Liv-1 prevented proper nuclear localization and function of the zinc-finger protein Snail which is necessary for normal epithelial-mesenchymal transition (Yamashita et al., 2004). ZIP6 was found to be upregulated in breast and other cancers (Taylor et al.,

2003; Taylor et al., 2004b; Taylor et al., 2007). Similar to Liv-1, ZIP6 was found to be necessary for the proper regulation of Snail and Slug through the p44/42 MAPK pathway (Zhao et al., 2007). ZIP6 was also found to have a potential role in the maturation of a subclass of antigen presenting cells, dendritic cells (Kitamura et al., 2006). Overexpression of ZIP6 or addition of exogenous zinc resulted in a decline of MHC class II molecules expressed at the cell surface of dendritic cells; whereas, chelation of zinc resulted in enhanced MHC class II molecules expressed at the cell surface of dendritic cells (Kitamura et al., 2006). These observations implicate ZIP6 in normal development and immune function.

The role of zinc alone in signal transduction is only emerging. In addition to the probable role of zinc in STAT signal transduction implicated by Liv-1, the role of zinc in other signal transduction was shown by cross-linking the IgE receptor, resulting in a wave of zinc released from the endoplasmic reticulum (Yamasaki et al., 2007b). This wave was dependent on calcium and activation of the MAPK/ERK signaling pathways (Yamasaki et al., 2007b). The wave of zinc may affect the activity levels of zinc-dependent phosphatases and transcription factors and thereby serve as a second messenger in signal transduction (Yamasaki et al., 2007b).

Increased cytosolic zinc by ZIP7 affects aberrant growth factor signal transduction in breast cancer cells (Taylor et al., 2008). ZIP7 was found to be localized to the *trans*-Golgi network during normal or zinc deficient conditions (Huang et al., 2005). The addition of excess zinc resulted in a loss of ZIP7 protein but no change in mRNA levels (Huang et al., 2005). Thus ZIP7 seems to function as

a zinc retrieval mechanism for the cell during deficient or basal conditions by returning zinc from the secretory pathway back to the cytoplasm. Based on these results, ZIP7 appears to be regulated post-translationally. There has been no more information on this regulation published.

In another study, researchers set out to define the gene causative to cadmium sensitivity. Prior to the study, the researchers knew that some inbred strains of mice have more or less sensitivity to cadmium as measured by testicular necrosis following dietary exposure and an almost 5 Mb region of mouse chromosome 3 contained the cadmium sensitivity locus, *Cdm*, resulting in the autosomal recessive trait (Dalton et al., 2005). By interbreeding the resistant and sensitive strains with subsequent backcrossing to the resistant strain, an extensive analysis of polymorphic microsatellite markers narrowed the *Cdm* locus to an 880 Kb segment containing three putative genes, including the mouse *Zip8* gene (Dalton et al., 2005). Further characterization allowed the unequivocal identification of *Zip8* as the cadmium sensitivity gene (Dalton et al., 2005). The *ZIP8* mRNAs were found to be identical amongst the sensitive and resistant strains but that the accumulation of the mRNA differed in endothelial cells of the testis perhaps through an as yet unidentified alteration of transcriptional regulatory sequences (Dalton et al., 2005). The toxic effects of cadmium on the testis and renal proximal tubules were shown to be *Zip8* gene-dosage dependent via transgenic mice; the cadmium resistant mice strain was made cadmium sensitive with the integration of three additional copies of the *Zip8* gene carried on a bacterial artificial chromosome (Wang et al., 2007). The tissue-

specific expression pattern of the transgenic *Zip8* mice was identical to the non-transgenic control mice, thereby supporting the role of *Zip8* in cadmium sensitivity (Wang et al., 2007).

In a later study, the same group provided evidence that ZIP8 functions as a zinc and/or manganese/bicarbonate symporter in endothelial cells of the lung, kidney, liver, and testis and that cadmium competes with manganese and zinc for transport during toxic exposure (He et al., 2006) (Liu et al., 2008). The observation that the uptake of zinc and manganese require bicarbonate ion was the first indication of zinc transport mechanism by ZIPs. Very recently, ZIP8 was also found to localize to the plasma membrane during zinc deficiency and internalize when zinc was replete suggesting a role for post-translational regulation (Liu et al., 2008). The latter results follow the findings reported herein.

No published results for ZIPs 9, 11, and 12 exist at the time of this dissertation, but the field is ever-growing and should be expected to reveal interesting roles for the uncharacterized ZIP transporters in zinc (or other metal) homeostasis soon. *Zip10* is negatively regulated by MTF1 via an intronic set of MREs (Zheng et al., 2008) and thus is the only ZIP thus far where there is identifiable induction regulation at the transcriptional level, other than cell-specific transcriptional expression regulation (even though *Zip8* mRNA abundance does have sensitive-allele versus resistant-allele differences and thus potential transcriptional differences).

Very recently, ZIP13 was found to be required for normal connective tissue development and maintenance; absence of ZIP13 resulted in a subtype of Ehlers-

Danlos syndrome (EDS) (Fukada et al., 2008). *Zip13* knock-out mice develop growth retardation, craniofacial abnormalities, and connective tissue malformations including a loss of dermal and corneal stromal collagen formation (Fukada et al., 2008). Moreover, these findings compelled the researchers to examine patients who were presenting all of the classical features of EDS but were wild-type for all known EDS-related genes. The researchers found, through single nucleotide polymorphism arrays and microsatellite mapping, a region of heterozygosity in the parents of two homozygous siblings; this region contained human *Slc39a13* (*Zip13*) (Fukada et al., 2008). The SNP contained in the sibling patients resulted in the point mutation G74D altering a glycine conserved in the transmembrane domain two of ZIP13 in all vertebrates (Fukada et al., 2008). Furthermore, using microarray analyses of osteoblasts and chondrocytes from *Zip13* knock-out mice, researchers found that only members of the BMP/TGF-β signaling pathway were disrupted in those cell lineages (Fukada et al., 2008). Further work showed that Smad proteins did not translocate to the nucleus upon phosphorylation by the BMP-activated receptor complex thus altering the signal transduction (Fukada et al., 2008). Thus, ZIP13 seems to function in a very tissue-specific way to provide adequate amounts of zinc to connective tissues and thereby allow normal cellular processes, including signal transduction, to function.

ZIP14 was previously reported to be a ferrous iron transporter when transfected into HEK 293 cells, insect Sf 9 cells, and AML12 mouse hepatoma cells (Liuzzi et al., 2006). The report was later challenged by the finding that *Zip14* and

Zip8 were likely the result of a gene duplication event when vertebrates diverged (Girijashanker et al., 2008). The later finding showed that ZIP14 had very similar function as ZIP8 and also conferred cadmium sensitivity but had selectivity for zinc and manganese under physiological conditions (Girijashanker et al., 2008). Both ZIP8 and ZIP14 had similar electroneutral uptake of zinc/bicarbonate complexes in *Xenopus laevis* oocytes (Girijashanker et al., 2008). *Zip14* was upregulated at the mRNA and protein levels in the liver by exposure to lipopolysaccharide or turpentine (Liuzzi et al., 2005). The *Zip14* upregulation effects required the interleukin 6 signaling pathway as the effects were quite reduced in *IL6* knockout mice (Liuzzi et al., 2005).

III. Concluding Remarks for Introduction

Metal homeostasis is still poorly understood but the field is developing rapidly. The seemingly contradictory results aforementioned only reinforce that little about metal homeostasis in general is yet known with great certainty. Most notably, the cell culture studies repeatedly show that some effects seem to be cell-type specific (other examples will discussed in the final summary section). Ultimately, despite the difficulty of *in vivo* studies, they are essential to quell discrepancies. Studies of the regulation and mechanistic action of ZIP transporters are still few in number and many principal questions remain: 1) How do ZIPs translocate zinc across the membrane; 2) What is the driving force of transport; 3) Do the activity levels of ZIPs change by posttranslational modifications or protein-protein interactions; 4) What is

the structure of ZIPs beyond their predicted topology; 5) Do ZIPs function as multimers as evidence suggests for the CDF family members; 6) How are the ZIPs regulated in response to changes in zinc availability; and 7) What is the functional explanation for the expansion of ZIP paralogs in mammals? Answers to some of these questions have recently begun to emerge. Of course, these questions will take the efforts of many researchers and some time to be solved.

Certainly, the preceding descriptions of the mechanisms employed to achieve zinc homeostasis for the representative phyla are incomplete and future research will add to the complexity and subtlety of these important transport and regulatory processes. Specifically, beyond MTF1, the explanation of how cells sense zinc availability needs further clarification. From the aforementioned observations and the work to be shown herein, most mammalian ZIP transporters apparently are primarily regulated through post-transcriptional mechanisms. Yet, how the diverse, zinc-responsive post-transcriptional regulatory processes are coordinated is unknown. Yet, the data presented herein show that they are coordinated. In addition, ZIP transporters seem to be required for normal development. Loss of function in some of the ZIPs has been shown to result in abnormal development. The role of *Zip4* in development will be presented herein.

IV. Goals and Specific Aims of This Dissertation

The ability of life forms to adapt to zinc availability has been exemplified by the complex networks of gene products in the preceding descriptions. As discussed,

the ubiquity of zinc utilization in biological systems is no less than remarkable. Therefore, the impact of zinc deficiency/dysregulation on human health cannot be overstated. As such, the genetic and molecular pathways that govern proper zinc homeostasis are paramount to understand; however, much time and effort from many researchers will be necessary to uncover all of these mechanisms. **Thus, the overall goal of my research** has been built upon previous findings and has focused largely on the molecular mechanisms involved in regulating *Zip4* and *Zip5* in the adaptive response to zinc availability and how *Zip4* impacts development. In presenting this dissertation, I will defend three specific aims.

In **specific aim 1, I evaluated the hypothesis** that *Zip4* is essential for the normal growth and development of mammals. **To address this aim,** a *Zip4* knockout mouse model was employed. **The purpose of this study** was to determine the effects of loss-of-*Zip4*-function in development. Based on the clinical findings in AE patients, I predicted that normal *Zip4* function would be required for the maintenance of zinc homeostasis and for the overall health of the animal.

In **specific aim 2, I evaluated the hypothesis** that *Zip4* and *Zip5* are post-transcriptionally regulated inversely and dynamically in response to zinc availability. **To address this aim,** wild type mice were fed zinc adequate or zinc deficient diets as previously described (Dufner-Beattie et al., 2003b; Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006). Some of the zinc deficient mice were repleted with zinc by oral gavage. Tissues known to express *Zip4* and *Zip5* were examined for expression of their mRNA levels, protein levels, and protein localization with time in

response to changes in zinc availability. **The purpose of this study** was to determine at what level *Zip4* and *Zip5* are regulated in response to zinc availability. Based on previous findings that ZIP4 and ZIP5 proteins were localized to the apical and the basolateral membranes, respectively, in various cell types with zinc deficient and zinc adequate conditions, respectively, I predicted that *Zip4* and *Zip5* would have reciprocal regulation in response to zinc availability.

In **specific aim 3, I evaluated the hypothesis** that *Zip5* is regulated by a rapid post-transcriptional mechanism mediated by the 3' untranslated region of the mRNA in response to zinc availability. This aim evolved with time as the other two aims were met. **To address this aim,** *in vivo* and *in vitro* techniques were utilized. The *in vivo* approach addressed physiologically relevant cellular regulation whilst the *in vitro* techniques were used to dissect those mechanisms using defined conditions. **The purpose of this study** was to determine at what level *Zip5* expression is regulated. Based on the findings in the second aim, I predicted that *Zip5* is regulated by a translational stall mechanism.

Chapter 2

Experimental Methods

I. Animal Care and Dietary Zinc Manipulation

Experiments involving mice were performed in accordance with the guidelines from the National Institutes of Health for the care and use of animals and were approved by the Institutional Animal Care and Use Committee. Mouse diets were purchased from Dyets Inc. (Dyets.com) and were identical except for zinc levels, which were as follows: the zinc-deficient diet = 1 ppm zinc plus deionized water supplied in a zinc-free acid-washed bottle; the zinc-adequate diet = 30 ppm zinc with normal water; and the zinc-excess diet consisted of the zinc-adequate diet plus 250 ppm zinc added to otherwise normal drinking water.

The effect of zinc deficiency during pregnancy on morphogenesis and growth of the embryo was monitored as described in detail previously (Dufner-Beattie et al., 2003b; Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006). Maintenance and dietary studies of the ZIP4 colony and CD-1 wild-type mice for experiments reported herein were performed by the author of this dissertation except as noted.

II. *Slc39a4* (*Zip4*) Targeting Vector Construction

Construction of the *ZIP4* targeting vector was the work of Dr. Jodi Dufner-Beattie and was performed as follows. A bacterial artificial chromosome that

contained the *Zip4* gene from the 129Sl/Sv mouse strain was obtained from Research Genetics (invitrogen.com) and sequenced by Bruce Roe (University of Oklahoma).

The 7.3 kilobase (Kb) upstream arm of the targeting vector spanned from a HindIII site located approximately 7.1 kb upstream of exon one and extended downstream to the *Zip4* start codon. A BamHI site that had been introduced immediately downstream of the start codon by PCR was fused in-frame with the coding region of the *EGFP* cDNA from the vector pEGFPKT1loxneo (kind gift from Mario Capecchi, University of Utah). A *loxP*-flanked *MC1-Neo* cassette was immediately downstream of the *EGFP* cDNA in this vector. The 3.7 kb downstream arm was subcloned downstream of this *MC1-Neo* cassette and extended from a BsrBI site located within intron one to an AfeI site within exon 11. This targeting construct was inserted into the HpaI and PmeI sites in 4317G9 (kind gift from Richard Palmiter (University of Washington)) which are flanked by the negative selection markers, herpes simplex virus thymidine kinase and diphtheria toxin.

III. Targeted Disruption of the *Slc39a4* (*Zip4*) Gene in Mouse Embryonic Stem Cells

The targeting vector was linearized within the vector backbone with AscI and electroporated into mouse CJ7 embryonic stem (ES) cells by the KUMC transgenic facility (directed by Wenhao Xu). Selected colonies were screened by Southern blot hybridization and karyotyped, as described in detail previously (Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006). Homologous recombination of the targeting

vector into the endogenous locus results in the insertion of an XhoI site, allowing the targeted and wild-type alleles to be distinguished via Southern blot hybridization using both 5' and 3' flanking probes.

IV. Generation of *Slc39a4* (*Zip4*)-knockout Mice

Breeding of chimeras to establish agouti lines was the work of Jim Geiser. Chimeric mice were generated by microinjection of two independent *Zip4*$^{+/-}$ ES cell clones into d4 C57BL/6 blastocysts, followed by transfer to pseudopregnant CD-1 foster mothers by the KUMC transgenic facility. Resulting chimeric mice were crossed with C57BL/6 females (Harlan, Indianapolis, IN). Germline transmission was confirmed by PCR from tail DNA of agouti offspring and results from the PCR screen were initially confirmed by Southern blot analysis. Agouti offspring heterozygous for the *Zip4*-knockout allele were mated with commercially available (B6-FVB) mice that express Cre recombinase driven by the adenovirus *EIIa* promoter (JAX.org) to remove the *loxP*-flanked *MC1-Neo* cassette. PCR was used to verify complete excision of the *MC1-Neo* cassette in the *Zip4*$^{+/-}$ x *EIIa-Cre* F1 progeny with the following primers: ZIP4 Ins Sense (anneals to a site upstream of the first LoxP site) 5'- GACGAGTTCTTCTGAGGGGATCG- 3', mZIP4 Sense (anneals to a site downstream of the endogenous start codon; this site is lost in the targeted allele) 5'-CACAGGGACTTGTGTTGGCTCTG-3', and mZIP4 Antisense (anneals downstream in both reactions) 5'-GAATGGAGCTGTGTGTCCCAGG-3'. All mice to this point were heterozygous. A 375 bp amplimer indicated a wild-type allele and

served as a PCR positive control. The absence or presence of a 297 bp neo-primed amplimer indicated the excision or retention of the *MC1-Neo* cassette, respectively. In this instance, Cre recombinase is expressed in most tissues by the adenovirus *EIIa* promoter. Conceivably, a mouse could show excision of *MC1-Neo* by PCR of a tail sample but have residual germline retention of Neo and thus transmit Neo to the next generation. However, this becomes highly improbable after several generations. More than two generations of a negative *MC1-Neo* result assured no transmission. Nonetheless, Neo screening continued for multiple generations.

V. PCR-based Genotyping of *ZIP4* Heterozygous Crosses

Mice from heterozygous crosses were genotyped by PCR analysis using the following three primers: *GFP* Sense 5'-TCACTGCATTCTAGTTGTGGTTTGTCC-3', *mZIP4* Sense 5'-CACAGGGACTTGTGTTGGCTCTG-3', and *mZIP4* Antisense 5'-GAATGGAGCTGTGTGTCCCAGG-3'. One antisense primer was common to both the wild-type and targeted alleles; a second primer was specific to the wild-type allele; the third primer anneals downstream of the *EGFP* open reading frame. The wild-type reaction produces a 375 bp amplimer (same as above); whereas, the knock-out reaction produces a 277 bp amplimer. This strategy allowed wild-type, knock-out, and heterozygous mice to be easily distinguished in a single reaction.

Based on the above breeding scheme, the genetic background of these mice is mixed 129SI/SV: C57BL/6: FVB. Multiple heterozygous *Zip4*-knockout mice in which the *MC1-Neo* cassette had been completely removed were used to create the

working colony of heterozygous knockout mice. Two separate lines of *Zip4*-knockout mice were generated from separate ES clones and were never interbred. Both displayed the same homozygous lethal phenotype. Therefore, the studies reported herein focused on the C4 cell line derived *Zip4*-knockout mice.

VI. Blastocyst Collection

Blastocysts were collected and manipulated by the transgenic facility at KUMC. *Zip4*$^{+/-}$ females between the age of three and four weeks were superovulated by intraperitoneal injection of 5 IU PMSG (sigma.com) followed 47 h later by the injection of 5 IU human chorionic gonadotropin (sigma.com). Females were then paired with wild-type males for mating overnight. On day 4 (day 1 = day of plug) of pregnancy, females were euthanized and their uteri were excised and placed in 3 ml of HEPES-buffered M2 medium (Specialtymedia.com). Uteri were flushed with this medium and blastocysts and morulae were collected and transferred to a glass depression slide in sterile PBS for imaging. Those embryos that had not yet developed to the blastocyst stage were cultured in KSOM medium (Specialtymedia.com) in a humidified atmosphere of 5% CO_2 until the blastocyst stage was reached before imaging by microscopy, as described below.

VII. Microscopy for *ZIP4* Conceptuses and Extraembryonic Tissues

Fluorescence imaging and immunodetection of *EGFP* were performed as described in detail previously (Dufner-Beattie et al., 2004; Dufner-Beattie et al.,

2005; Dufner-Beattie et al., 2006). EGFP fluorescence was detected directly using a Leica MZFLIII dissecting microscope. Dr. Andrews gave instruction and helped in the dissection of murine conceptuses from implantation sites. All conceptuses were genotyped by carefully removing a piece of visceral yolk sac and subjecting the lysate to PCR genotyping (primer strategy detailed above). *Zip4* expression was analyzed in frozen sections at different stages in development by using an anti-GFP antibody to enhance signal with a subsequent biotinylated secondary antibody and tertiary streptavidinQDot655 detection reagents (invitrogen.com). Antibody binding was detected using a Texas Red cube in a DM4000B Leica fluorescence microscope coupled with a DC500 digital camera (Leica.com). Red fluorescence indicated antibody binding whereas DAPI staining revealed nuclei with blue fluorescence. Phase contrast microscopy was also performed using the same microscope. Images were captured with Adobe Photoshop software.

VIII. Scanning by High Field Magnetic Resonance Imaging (MRI)

MRI was performed in collaboration with the Hoglund Brain Imaging center at KUMC. High field magnetic resonance imaging in axial and sagittal planes was performed on one wild-type and three weaned *Zip4* $^{+/-}$ mice post-mortem using a 9.4 T horizontal Varian scanner (Varian.com) with 31-cm bore, 400 mT/m gradient coil set and 38 mm inner diameter radio frequency volume coil, as described previously (Bilgen et al., 2005; Loganathan et al., 2006). The imaging parameters for the axial scans were TR = 4000 ms, TE = 12 ms, image matrix = 128 x 128, field-of-view = 20

mm x 20 mm, slice thickness = 1 mm, number of averages = 2 and number of slices = 30. The corresponding parameters for the sagittal scans were the same except image matrix = 256 x 128, field-of-view = 55 mm x 20 mm and slice thickness = 0.5 mm.

IX. RNA Extraction and Northern Blot Hybridization

The highest quality chemicals and reagents available were used throughout all experimentation (*exempli gratia*, molecular grade reagents were used whenever available). Care was taken to ensure the proper storage, expiration, and disposal of all chemicals and reagents. RNA was isolated using Trizol method (invitrogen.com). In brief, tissues were collected in ice-cold 1X PBS, quickly blotted dry, and snap frozen in liquid nitrogen. After degassing in dry ice, tubes containing tissues were securely closed and stored at -80˚C for later use. Frozen tissues were pulverized using a pestle and mortar that were pre-equilibrated in liquid nitrogen; the powder was used immediately or stored at -80˚C for later use.

For every 100 mg of frozen or fresh tissue, 1 ml of Trizol was added and the sample was homogenized with a Kinematica Polytron. Samples were shaken at room temperature for 30 minutes to ensure nucleoprotein dissociation from nucleic acids. Chloroform was added (0.2 ml per 1 ml of Trizol) and samples were shaken vigorously for 2 minutes, allowed to sit for another 2 minutes, and centrifuged to separate the phases. The aqueous phase was transferred to a fresh tube and isopropanol was added (0.5 ml per 1 ml of Trizol) and the samples were shaken and allowed to sit at room temperature for 15 minutes. RNA was then precipitated with a

high-speed spin. The pellets were washed with 70% ethanol and allowed to airdry before being resuspended in an adequate volume (50-100 µl per 100 mg tissue) of nuclease-free water (without DEPC). Samples were then assessed for concentration and quality by absorbance readings at 260 nm and 280 nm.

Total RNA (3 to 6 µg per well) was size-fractionated by agarose-formaldehyde gel electrophoresis, transferred and UV cross-linked to Zeta-probe membrane (Biorad). With modification from previously described, Northern blot membranes were hybridized at 68˚C in a formamide-based hybridization solution (50%[v/v] deionized formamide, 5X SSPE, 5X Denhardt's solution, 0.5%[w/v] SDS, 0.02 mg/ml denatured herring sperm DNA, and QS deionized, nuclease-free water) for 24-72 hours and washed under stringent conditions (Dalton et al., 1996b). Hybrids were detected by autoradiography at -80˚C with Kodak Biomax film (Kodak.com). Duplicate gels were stained with acridine orange to ensure equivalent loading and integrity of total RNA.

Probes for mouse *MT-1* and *Zip1* mRNAs were as described (Dufner-Beattie et al., 2003a; Dufner-Beattie et al., 2004). Riboprobes for *Zip4* and *Zip5* mRNAs were designed by scanning the mRNAs for optimal GC content regions. A 328 nucleotide probe for *Zip4* was subcloned with the primers: Sense 5'-CTTGCTGGATCATGTCATT-3' and Antisense 5'- TTCAGATAGCCCATACACA -3' and TA cloned into pGEMT vector. A 219 nucleotide probe for *Zip5* was subcloned with the primers: Sense 5'-GTGGCCTCTTCCTGCTCTT-3' and Antisense 5'-GAGGGGCTAGAGATGGTGA-3' and TA cloned into pGEMT vector.

Clones with the correct orientation were selected, linearized with NcoI, and used as templates to produce antisense riboprobes with Sp6 RNA polymerase. All probes were used at 2 x 10^6 cpm/ml of hybridization solution.

X. miRNA Northern Blot Hybridization

This technique is essentially a modification of the Northern blot hybridization technique and is based on the published method of miRNA detection by Northern blot (Varallyay et al., 2008; Varallyay et al., 2007). Total RNA (2 to 4 µg per well) was size-fractionated on 12.5% Acrylamide / 8.3M urea / 1X TBE gels. Decade RNA markers were labeled and cleaved according to the manufacturer's protocol (ambion.com). These were run with the samples to measure the size of hybridization bands. Prior to loading, mini-gels were pre-run for 1-2 hours at 250V. After loading, gels were run at 250V until the loading dye reached the bottom of the gel. They were subsequently stained with ethidium bromide to ensure loading, destained, and transferred to zeta-probe overnight with 20X SSC. Ethidium bromide staining was found to have no effect on hybridization signal. UV-crosslinked membranes were blocked for 1 hour and hybridized in formamide-based solution detailed above.

Locked-nucleic acid probes were ordered from Exiqon (exiqon.com) and end-labeled thereafter with γ^{32}P-ATP (6 µl nuclease-free water, 1 µl 10X polynucleotide kinase buffer (New England Biolabs), 1 µl probe (diluted to 2.5 pmol/µl), 1 µl high specific activity γ^{32}P-ATP (PerkinElmer), and1 µl T4 kinase were reacted at 37˚C for 1 hour). End-labeling of probes was confirmed by dot-blotting dilutions of labeled

probe and negative control (no template) on Zeta-probe. These dot-blots were UV-crosslinked and washed twice in 0.1X SSC at 68˚C. Dried membranes were exposed to film at -80˚C for 30 minutes. All probes were found to be efficiently labeled to high specific activity. Following synthesis, probes were diluted into 100 µl of hybridization buffer, denatured at 95˚C for 1 minute, snap-chilled in an ice-slurry for 2 minutes, then added to the blocked membranes and allowed to hybridize for 24-72 hours at 50˚C. Following hybridization, membranes were rinsed then washed twice with pre-warmed 2X SSC / 0.1% [w/v] SDS for 15 minutes. Dried membranes were exposed to film at -80˚C. These temperature and wash conditions were stringent for the < 25mer probes.

XI. Cell Culture

Cells were maintained at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units penicillin/ml, and 100 µg streptomycin/ml. To generate zinc-deficient conditions in culture, heat-inactivated fetal bovine serum (FBS) was treated with Chelex 100 resin, as described (Kim et al., 2004a). Essential metals except zinc were repleted by the DMEM which was adjusted to 10% Chelex-treated FBS.

Stock solutions of 10 mg/ml actinomycin D (Sigma-Aldrich, St. Louis, MO) and 10 mg/ml cycloheximide (Sigma-Aldrich) were prepared in methanol and 100% ethanol, respectively.

Mouse 266-6 cells were obtained from the American Type Culture Collection and cultured according to their suggested protocol and the intestinal cell line mIC-c12 (Bens et al., 1996) was a generous gift from Dr. A. Vandewalle (Paris, France) and was cultured as they described. These two cell lines were assayed for expression of the *Zip4* gene, but were not used in further experiments.

XII. Nuclear Run-on Assay

Nuclear Run-on assays were the work of Dr. Jodi Dufner-Beattie. Run-on assays were performed according to previously published methods (McKnight and Palmiter, 1979; Derman et al., 1981). Nuclei were purified from cultured Hepa cells, as well as the proximal small intestine from pregnant dams as described (Derman et al., 1981). Mice were fed the ZnA or ZnD diet for 1 day beginning on d8 of pregnancy (Dufner-Beattie et al., 2003b; Dufner-Beattie et al., 2004). Nuclei were labeled and RNA isolated as described (McKnight and Palmiter, 1979).

DNA was denatured and then immobilized on nylon membranes by slot blotting, as described (Dufner-Beattie et al., 2001). The cDNA (3 µg) for mouse *MT-I*, *β-actin* and *GAPDH* or the genomic mouse *Zip4* gene (Dufner-Beattie et al., 2003b) were denatured in 10 mM NaOH, neutralized by dilution into 6X SSC (350 µl total volume) and loaded via slot blot onto a well on strips of nylon membrane. Each well was rinsed with 500 µl of 6X SSC and the filter was exposed to UV light to crosslink the DNA.

Labeled run-on transcripts were hybridized (2.5 x 10^6 cpm/ml hybrid buffer) (Church and Gilbert, 1984) at 65°C for 36 h with membrane strips containing immobilized DNA. After hybridization, the membranes were washed successively as follows: 5 min at 25°C in 2X SSC; 30 min at 25°C in 2X SSC containing 10 μg/ml RNase A; 10 min at 65°C in 2X SSC, 0.1% SDS; 10 min at 65°C in 0.1X SSC, 0.1 % SDS. Hybrids were detected by autoradiography and quantified by radioanalytic analysis using ImageQuant software version 5.0 on a Typhoon 9410 phosphorimager.

XIII. Immunohistochemistry and Immunofluorescence

These methods were essentially as described in detail previously (Dufner-Beattie et al., 2003b; Dufner-Beattie et al., 2004), with the following modifications. Affinity-purified rabbit antiserum against ZIP4 and ZIP5 peptides were described previously (Wang et al., 2002; Dufner-Beattie et al., 2004). The ZIP4 peptide sequence was AEETPELLNPETRRL. The ZIP5 peptide sequence was ASEPEVQGQRENRQS. Antiserum against duodenal cytochrome b (DCytb) was previously described and kindly provided by Dr. Andrew McKie (King's College London) (McKie et al., 2001). Cell culture supernatant containing rat monoclonal antibody against the epithelial marker 4.8H1 was previously described (De Lisle and Ziemer, 2000) and kindly provided by Dr. Robert De Lisle (University of Kansas Medical Center). Tissue sections were fixed in freshly made 4% paraformaldehyde in 1X PBS for 20 minutes at room temperature then taken through sucrose series and snap frozen in OCT. Frozen blocks were sectioned then thawed in the presence of

freshly made 50 mM lysine in 1X PBS to quench endogenous fluorescence. Slides were then permeabilized with 0.1% Triton-X-100 / 0.1% Tween 20 in 1X PBS for 20 minutes at room temperature. Slides later subjected to peroxidase conjugate were next incubated in 1% hydrogen peroxide for 10 minutes at room temperature to inactivate endogenous peroxidase activity. Slides were then blocked with 10% normal goat serum in 1X PBS for 1 hour at room temperature. Immunohistochemistry for DCytb, ZIP4, and ZIP5 was performed using Zymed Histostain-SP kits (zymed.com) and DAB staining. Immunofluorescence for ZIP4, ZIP5, and 4.8H1 was performed at 1:500, 1:300, and 1:2 dilutions of primary antibodies, respectively, in 10% normal goat serum in 1X PBS for 2-4 hours at room temperature. Next slides were incubated with biotinylated affinity-purified goat anti-rabbit secondary antibody (1:500 dilution in 10% normal goat serum in 1X PBS for 1 hour at room temperature) (or Alexa Fluor conjugated secondary antibody for 4.8H1) and QDot 655 streptavidin conjugate (1:500 dilution in 1X PBS for 1 hour at room temperature) (invitrogen.com) to detect antibody binding sites.

Antigen retrieval was necessary to visualize ZIP5 in tissue sections. Slides were incubated in 10 mM sodium citrate, pH 6.0 using a PickCell 2100 retriever (antigenretriever.com) according to recommendations of the manufacturer. This step was performed after thawing the slides in 1X PBS and prior to lysine quenching. For ZIP5 visualization in the pancreas and intestine, tyramide signal amplification (perkinelmer.com) was used to enhance the specific fluorescence signal. To achieve this, slides were incubated with primary and secondary antibodies then with

streptavidin-conjugated horseradish peroxidase. Next, biotinylated tyramide substrate was carefully added to avoid motion. Observing strict incubation times, slides were rapidly quenched with excess 1X PBS washes then incubated with the QDot655 conjugate and visualized. Sections were mounted with 90% glycerol containing DAPI for subsequent imaging using a Leica DM 4000B fluorescence microscope and Adobe Photoshop image capture software.

XIV. Sucrose Density Gradient Fractionation of Polysomes and RT-PCR Detection of *Slc39a4* (*Zip4*) and *Slc39a5* (*Zip5*) Transcript Distribution

Visceral yolk sacs were collected on d14 of pregnancy from dams fed the ZnD and ZnA diet, as described in detail above. Freshly collected tissues were homogenized in 40 mM Hepes (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 100 μg/ml cycloheximide, and 400 U/ml of RNAse Block (stratagene.com). The homogenate was centrifuged at 10,000 x g at 4°C for 30 min. The supernatant was adjusted to 0.05% sodium deoxycholate, 2% Triton-X-100 and an aliquot was collected for extraction of total input RNA. For some experiments, polysomes were collected as a pellet and resuspended in Trizol for subsequent extraction. The cognate soluble fractions were also Trizol extracted. The soluble and pellet fractions were resuspended in equivalent volumes of nuclease-free water. Volume equivalents were run under denaturing conditions in 12.5% acrylamide/ 8.3M urea/ 1X TBE gels as detailed for miRNA Northern blot analysis. For other experiments, polysomes were separated on a linear 10-50% sucrose gradient by centrifugation at 40,000 x g at 4°C

for 2.5 h. Gradients were collected from the top and fractions were monitored for absorbance at 254 nm. Twenty-six fractions were collected into Trizol, *E. coli* rRNA (16S and 23S; roche-applied-science.com) was added to make the total RNA amount in each fraction approximately equivalent. RNA was isolated, dissolved in 5 mM EDTA (pH 8.0) and precipitated with 3 volumes of 4 M ammonium acetate (pH 7.0) at 0°C for 3 h to remove any trace DNA. The precipitate was collected by centrifugation, redissolved and ethanol precipitated. RNA was collected by centrifugation, dried under vacuum and dissolved in 22 µl of RNAse-free water. Volume equivalents of the fractions were then used for comparison by RT-PCR.

Reverse transcription was performed using StrataScript reverse transcriptase according to manufacturer's instruction (stratagene.com) with random primers. A sample with no reverse transcriptase added was used as negative control for the subsequent PCR. Samples were amplified by PCR for 35 cycles using Taq polymerase (NEB.com) and the following primers: Zip4 416 bp amplimer, sense primer 5'-GCCTGGTCTTGACTGCCTTGCT-3', antisense primer 5'-AGGCTCCACTTAGCTGCTGCTG-3'; Zip5 524 bp amplimer, sense primer 5'-TGGGCCCAGCTGAACAGGAACA-3', antisense primer 5'-GTGCTGGAGCTGGGGTTTTGA-3'. PCR products were resolved on 2% agarose-TAE gels containing 0.5 µg/ml ethidium bromide and imaged on an AlphaInnotech FluorChem 8900.

XV. Visceral Yolk Sac Explant Culture and Immunoprecipitation of ZIP5

Pregnant dams were fed ZnA or ZnD diet as described above. Visceral yolk sacs (VYS) (n = 5 per treatment) were collected on d14 into prewarmed DMEM, blotted to remove excess medium and transferred to prewarmed DMEM containing: 10% FBS; 10% Chelex-treated FBS with DMSO vehicle; 10% Chelex-treated FBS plus 100 μM $ZnCl_2$, 10% Chelex-treated FBS containing 50 nM epoxomicin (calbiochem.com), or 100 nM bafilomycin A1 (calbiochem.com) or 1 μM epoxomicin plus 50 μM MG132 (sigma.com) and 50 μM claso-lactacystin β-lactone (sigma.com) or 1 μM epoxomicin and 50 μM claso-lactacystin β-lactone (sigma.com). VYS (1 per 2 ml medium) were incubated at 37˚C for the indicated times and snap frozen in liquid nitrogen.

Samples were thawed by sonication (5 VYS per ml) in ice-cold lysis buffer containing phosphatase and protease inhibitors (20 mM Tris-base (pH 7.4), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 5 mM Na_3VO_4, and complete protease inhibitor cocktail (roche-applied-science.com), and membranes were prepared as described below. Membranes were resuspended in 300 μl of immunoprecipitation buffer (50 mM Tris base, pH 8.0, 150 mM NaCl, 1% NP-40, 0.2% SDS, 0.5% sodium deoxycholate, and Roche protease inhibitor cocktail) by sonication. An equivalent amount of total protein from each sample was used for Western blot analysis of ZIP4 and ZIP1, as described below.

For each immunoprecipitation (IP) reaction, 1 mg of VYS protein was adjusted to 0.5 ml with IP buffer, precleared with EZview red protein A affinity gel (sigma-aldrich.com), and then immunoprecipitated using normal rabbit IgG or ZIP5 antibody overnight at 4˚C. Samples were transferred to fresh tubes and incubated with EZview red protein A affinity gel at 4˚C for 4 hours to capture immune complexes. Immune complexes were collected by centrifugation and washed 5 times with 10 bed volumes of IP buffer per wash. ZIP5 protein was eluted from each complex with 10 μg of ZIP5 antigenic peptide, described previously (Dufner-Beattie et al., 2004), into a small volume of IP buffer at room temperature. A portion of the eluted volume was carefully transferred to a fresh tube containing SDS-sample buffer and freshly prepared DTT and heated at 42˚C for 30 min. Samples were analyzed using an 8% polyacrylamide gel and transferred to PVDF membranes and analyzed by Western blot detection as described below.

XVI. Preparation of Membrane Proteins and Western Blot Analysis

Conditions for protein solubility proved essential in the study of ZIP proteins by Western blot. Inadequate solubilization of multipass membrane proteins leads to their aggregation and precipitation out of solution. Moreover, for ZIP5 the use of freshly prepared extract proved to be essential in order to prevent its loss with time/low temperature from solution. Previous solubilization of ZIP5 proved to be inadequate (Dufner-Beattie et al., 2004; Huang et al., 2006). Better conditions of solubility were achieved as follows. Powdered tissue (100 mg) was homogenized in

1 ml of ice-cold lysis buffer (20 mM Tris-base (pH 7.4), 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 5 mM Na_3VO_4, and complete protease inhibitor cocktail (roche-applied-science.com) used at twice the concentration suggested for protease-rich tissues) using a Polytron homogenizer. Homogenates were then centrifuged at 500 X g for 10 min at 4 ˚C to pellet insoluble material. The clarified supernatant was collected and centrifuged at 100,000 X g for 45 min at 4˚C to obtain the membrane pellet. The high-speed supernatant was discarded. The membrane pellet was rinsed in wash buffer (150 mM NaCl, 5 mM phosphate buffer (pH 7.0), 1 mM DTT, 1 mM PMSF, 2 mM NaF, 5 mM Na_3VO_4, and complete protease inhibitor cocktail as above). Pellets were recollected by centrifugation at 20,000 X g for 15 min at 4˚C. The membrane pellet was then resuspended and solubilized in 100-200 µl modified RIPA with deoxycholate (150 mM NaCl, 50 mM Tris-base (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 2 mM NaF, 5 mM Na_3VO_4, and complete protease inhibitor cocktail as above) via sonication. The resuspension was centrifuged finally at 20,000 X g to eliminate any remaining insoluble material. The final supernatant was then collected and assayed for protein concentration via a BCA protein assay kit (piercenet.com).

We have found that incubation of membrane preparations at 37-42˚C in SDS loading buffer for 30 minutes works well to solubilize ZIP proteins and avoid aggregate formation (data not shown). Membrane proteins (20 to 25 µg) were resolved on 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride

membranes. Membranes were blocked overnight and then incubated with primary antibody in blocking solution at the appropriate dilution (ZIP4 antiserum 1:800, ZIP5 antiserum 1:600, and ZIP1 antiserum 1:800) for 2 h at room temperature. After extensive washing, membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and the blot was developed using ECL Plus reagent (amershambiosciences.com) according to manufacturer's instructions and detected using Kodak BioMax MS film (kodak.com).

XVII. Computational Prediction of miRNAs and RNA Secondary Structure

The following algorithms are publicly available and continuously updated. All commonly used RNA secondary structure prediction algorithms essentially are derived from mFold. The author of mFold allows public access and downloads from the following URL: http://mfold.bioinfo.rpi.edu/zukerm/ . Three validated algorithms that predict miRNA-mRNA target pairs can be found at the following URLs along with links to pilot and other representative publications: miRanda (miRBase) http://microrna.sanger.ac.uk/ , TargetScan http://www.targetscan.org/ , and PicTar http://pictar.mdc-berlin.de/ . Several servers run the mFold and more updated algorithms.

XVIII. Luciferase Reporter Constructs and Assay Method

The firefly luciferase coding sequence was subcloned immediately downstream of the human CMV core promoter in the pcDNA3.1 Hygro(+) vector

(Promega) by PCR cloning with an NheI site and a synthetic Sp6 RNA polymerase promoter at the 5' end and a BamHI site at the 3' end. The full-length wild-type *Zip5* 3' untranslated region (UTR) and a mutant 3' UTR (sequence detailed in Chapter 5) were generated by annealing synthetic oligos with engineered NotI sites at their 5' ends and ApaI sites at their 3' ends. These UTRs were subcloned immediately downstream of the luciferase coding sequence following a stop codon. Renilla luciferase was subcloned into pcDNA3.1 Hygro(+) containing the bovine growth hormone 3' UTR with an NheI site and a synthetic Sp6 RNA polymerase promoter at the 5' end and BamHI at the 3' end. The Renilla luciferase construct served as a positive control in transfection assays.

Cells were seeded at a density of $5X10^4$ cells per ml in 24 well plates. They were transfected with Lipofectamine2000 (invitrogen.com) at 1:1 or 1:2 ratios of DNA to Lipofectamine2000 depending on the cell type. Cells were transfected with 40 ng each of firefly and renilla luciferase reporters per well with either 800 ng total of miRNA expression vectors or empty EGFP expression vector. This low titration of reporter constructs was found to provide a large dynamic range of measurable luciferase activities without reaching saturation for either reporter. EGFP signal served as a qualitative measure of comparable transfection efficiency amongst treatments; whereas, all firefly luciferase values were normalized to renilla luciferase values and expressed as relative light units (RLUs). After experimentation, cells were lysed at room temperature with 1X passive lysis buffer for 30 minutes and assayed

immediately or after storage of lysates at -80˚C according to the manufacturer's specifications (Promega.com).

Chapter 3

The mouse acrodermatitis enteropathica gene *Slc39a4* (*Zip4*) is essential for early development and heterozygosity causes hypersensitivity to zinc deficiency

Citation of Chapter Publication

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Contributions to the Chapter

I generated or contributed to all data and figures except for panels 3.1A and 3.1B (design of the targeting construct by J.D.B.). Dr. Andrews, Dr. Dufner-Beattie and I all made substantial intellectual contribution to the design, interpretation, and report of experiments. Jim Geiser did most of the breeding for the data in Figure 3.8. Dr. Bilgen assisted in magnetic resonance imaging of the mice in Figure 3.7. Dr. Larson and Dr. Xu directed the electroporation of the targeting construct into mouse embryonic stem cells, injections of those cells into blastocysts, and subsequent introduction of blastocysts into recipient dams. Eileen Roach aided in the production of graphics.

Specific Aim and Hypothesis

Chapter 3 will address specific aim 1 and will evaluate the hypothesis that *Zip4* is essential for the normal growth and development of mammals. The purpose of this study is to determine the effects of loss-of-*Zip4*-function on development.

I. Abstract

The human *Zip4* gene (*Slc39a4*) is mutated in the rare recessive genetic disorder of zinc metabolism acrodermatitis enteropathica, but the physiological functions of *Zip4* are not well understood. Herein we demonstrate that homozygous *Zip4*-knockout mouse embryos die during early morphogenesis and heterozygous offspring are significantly underrepresented. At midgestation, an array of developmental defects including exencephalia, anophthalmia, and severe growth retardation were noted in heterozygous embryos and at weaning many (63/280) heterozygous offspring were hydrocephalic, growth retarded and missing one or both eyes. Maternal dietary zinc deficiency during pregnancy exacerbated these effects whereas zinc excess ameliorated these effects and protected embryonic development of heterozygotes but failed to rescue homozygous embryos. Heterozygous *Zip4* embryos were not underrepresented in litters from wild-type mothers, but were ≈ 10-times more likely to develop abnormally than were their wild-type littermates during zinc deficiency. Thus, embryonic and maternal *Zip4* gene expression are both critical for proper zinc homeostasis. These studies suggest that heterozygous mutations in the

acrodermatitis gene *Zip4* may be associated with a wider range of developmental defects than was previously appreciated, particularly when dietary zinc is limiting.

II. Introduction

A deficiency of the essential metal zinc can cause growth retardation, immune system dysfunction, male hypogonadism, skin lesions, and neurological disorders in humans (Prasad, 1998b; Prasad, 1983; Prasad, 2003). Maternal zinc deficiency impairs embryonic, fetal and post-natal development (Hurley and Swenerton, 1966; Hurley and Shrader, 1975; Hurley et al., 1971). In humans, the rare autosomal recessive disorder acrodermatitis enteropathica (AE) is thought to be caused by the inability to absorb sufficient intestinal zinc (Moynahan, 1974). Symptoms of severe nutritional zinc deficiency often develop soon after birth in bottle fed infants or after weaning from breast milk (Prasad, 1995). However, zinc supplementation reverses many of the symptoms of this otherwise lethal disease.

For over 30 years, little was known about the genetic cause of AE. The gene responsible for AE was mapped to human chromosomal region 8q24.3, and in 2002 was shown to be a member of the solute carrier 39A superfamily (*Slc39a*) (Wang et al., 2002; Kury et al., 2002), historically named the ZIP family (Guerinot, 2000b). The AE gene is *Zip4* (*Slc39a4*) and much of what we understand about *Zip4* regulation and function comes from recent studies of mice and transfected cells in culture (Dufner-Beattie et al., 2003b; Wang et al., 2004c; Kim et al., 2004a; Dufner-Beattie et al., 2004; Mao et al., 2007). The mouse and human ZIP4 proteins are well

conserved, and of the multiple amino acid changes due to point mutations found in AE patients (Kury et al., 2002; Wang et al., 2002; Meftah et al., 2006; Nakano et al., 2003; Lehnert et al., 2006), almost all occur at residues that are also conserved in the mouse protein (Dufner-Beattie et al., 2003b). Other *Zip4* mutations in AE patients involve premature termination codons, insertions, deletions or rearrangements of the gene that are likely to cause a complete loss of function, although this has not been directly demonstrated. Mouse ZIP4 functions as a zinc transporter in transfected cells, and although several of the amino acid substitutions found in AE patients appear to abolish its activity, others may only diminish its zinc transporter activity (Dufner-Beattie et al., 2003b; Wang et al., 2004c).

Zip4 gene expression is most active in mouse tissues involved in nutrient uptake, such as the intestine and embryonic visceral yolk sac, and the abundance of *Zip4* mRNA and the cellular localization and turn-over of this protein are regulated by zinc availability in these tissues (Dufner-Beattie et al., 2003b; Kim et al., 2004a; Dufner-Beattie et al., 2004; Mao et al., 2007). During periods of zinc deficiency, *Zip4* mRNA is induced and the encoded protein accumulates at the apical surfaces of enterocytes and visceral endoderm cells (Dufner-Beattie et al., 2003b; Dufner-Beattie et al., 2004). In contrast, zinc repletion can cause a slow loss of this mRNA and the rapid internalization and degradation of ZIP4 *in vivo* (Weaver et al., 2007) and in transfected cells *in vitro* (Kim et al., 2004a; Mao et al., 2007). This dynamic post-transcriptional control of ZIP4 in response to zinc suggests that it plays an important

role in zinc homeostasis, consistent with the finding that this gene is mutated in humans with AE.

To directly test this hypothesis, we created mice in which the *Zip4* gene was knocked out. This gene was shown to be essential for development of the early post-implantation mouse embryo, likely due to its function in the visceral endoderm. Remarkably, *Zip4* heterozygosity was found to exert pleiotropic effects on embryogenesis when dietary zinc was adequate, and to cause hypersensitivity to the teratogenic and embryotoxic effects of maternal dietary zinc deficiency during pregnancy. Both maternal and embryonic *Zip4* genes were found to play critical roles in zinc homeostasis during pregnancy. These studies further suggest the possibility that loss-of-function of a single human *Zip4* allele may be associated with dramatically increased sensitivity to dietary zinc deficiency and the risk of abnormal embryonic development.

III. Results

A. Generation of *Slc39a4* (*Zip4*)-knockout mice

Mice with a targeted disruption of the *Zip4* gene were generated by homologous recombination in embryonic stem (ES) cells. Two ES cell clones (C4 and D4) were selected to create chimeras and derive separate knockout lines that have yielded similar phenotypes. The targeting construct fused the initiator methionine codon of *Zip4* with the open reading frame of the enhanced green fluorescent protein (EGFP) reporter followed by several stop codons. This disrupted the protein coding

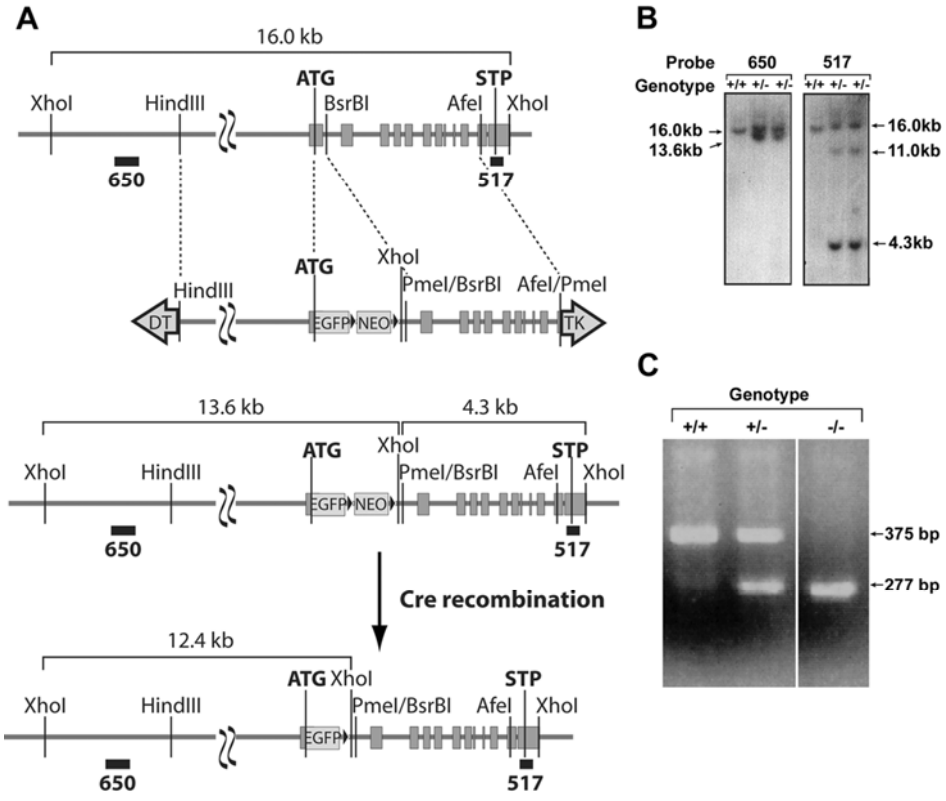
sequence of *Zip4* and deleted the remaining codons in exon 1. The remainder of the gene was not altered (see Figure 3.1A). This allowed for *EGFP* expression that was driven by the *Zip4* promoter in targeted cells. A *loxP*-flanked *Neo* cassette located downstream of the *EGFP* cDNA was used for positive selection, and flanking thymidine kinase and diphtheria toxin cassettes were used for negative selection.

Homologous recombinants were identified by Southern blot analysis of XhoI-digested genomic DNA (see Figure 3.1B), and the *MC1-Neo* cassette was then deleted by breeding heterozygous *Zip4* -knockout mice with commercially available transgenic mice that ubiquitously express Cre recombinase from early in development. Successful Cre-mediated excision was confirmed by PCR, and a PCR screen was designed to allow rapid *Zip4* genotyping of mice (see Figure 3.1C). Multiple heterozygous *Zip4*-knockout mice, in which excision of the *MC1-Neo* Cassette was complete, were crossed to generate working colonies of wild-type and heterozygous *Zip4*-knockouts for experimentation.

B. Homozygous *Slc39a4* (*Zip4*)-knockout mice die in utero during early development when expression of Zip4 is restricted to the visceral endoderm

Crossing $Zip4^{+/-}$ mice failed to yield homozygous offspring in mice raised on the zinc-adequate diet, and at weaning the heterozygous pups were also significantly underrepresented in the population (see Figure 3.2). At midgestation (d9), visceral yolk sacs and placentae from homozygous implantation sites could be identified (see Figure 3.3), and were represented in the expected (25%) frequency (see Figure 3.2).

Figure 3.1: Targeted disruption of the *Slc39a4* (*Zip4*) gene. Part A: Structure of the wild-type *Zip4* allele (**top**); 12 exons (**dark gray boxes**); translation start (**ATG**) and stop **(STP**), and restriction sites are indicated. Locations of the 650 bp 5'-flanking probe and the 517 bp 3'-flanking probe are noted. The targeting vector (**upper middle**); ***EGFP*** reporter fused in-frame to the *Zip4* start codon; the positive selection marker for neomycin (**NEO**), and the negative selection markers diphtheria toxin (**DT**) and herpes simplex virus thymidine kinase (**TK**). Structure of the targeted allele before (**lower middle**) and after (**bottom**) Cre-mediated excision of the *MC1-Neo* cassette is shown. **Part B:** Genomic DNA from wild-type (+/+) and two heterozygous *Zip4*-knockout (+/-) ES cells was digested with Xho I and Spe I and analyzed by Southern blotting using the 5'-flanking probe or the 3'-flanking probe, as indicated. **Part C:** Genomic DNA from *Zip4* wild-type (+/+), heterozygous knockout (+/-) and homozygous knockout (-/-) embryonic visceral yolk sacs was PCR-amplified to distinguish wild-type from mutant *Zip4* alleles. Amplification of the wild-type allele produced a 375 bp fragment, whereas amplification of the mutant allele produced a 277 bp fragment.


A
16.0 kb
ATG
STP
XhoI
HindIII
BsrBI
AfeI
XhoI
650
517
HindIII
ATG
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PmeI/BsrBI
AfeI/PmeI
DT
EGFP
NEO
TK
13.6 kb
4.3 kb
ATG
XhoI
STP
XhoI
HindIII
PmeI/BsrBI
AfeI
XhoI
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NEO
650
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Cre recombination
12.4 kb
ATG XhoI
STP
XhoI
HindIII
PmeI/BsrBI
AfeI
XhoI
EGFP
650
517
B
Probe
650
517
Genotype
+/+ +/- +/-
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+/-
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375 bp
277 bp

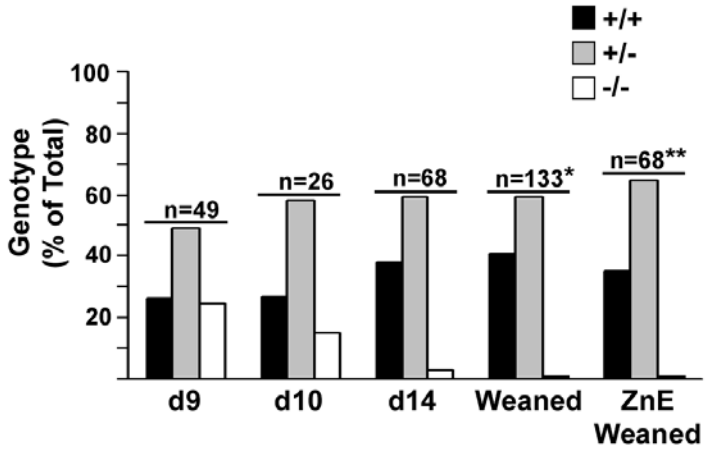


Figure 3.2: Genotypes of embryos and offspring derived by crossing heterozygous *Slc39a4* (*Zip4*)-knockout mice. *Zip4*$^{+/-}$ knockout mice were mated and conceptuses, embryos or fetuses were collected on the indicated days of pregnancy (**d9, d10 or d14**) and postnatal mice were collected at weaning (**Weaned**), and all individuals were genotyped. These mice were fed a zinc-adequate diet. *Note that about 40% of the total heterozygous offspring died or were morphologically abnormal. At weaning 22% were abnormal but are included in this graph. **In this experiment, pregnant mice (d1) were also provided excess zinc (**ZnE**) in the drinking water and all offspring were genotyped at weaning. The numbers in parentheses above the bars indicate the number of individuals genotyped at each time point. Data are expressed as the percentage of each genotype of the total (**n**).

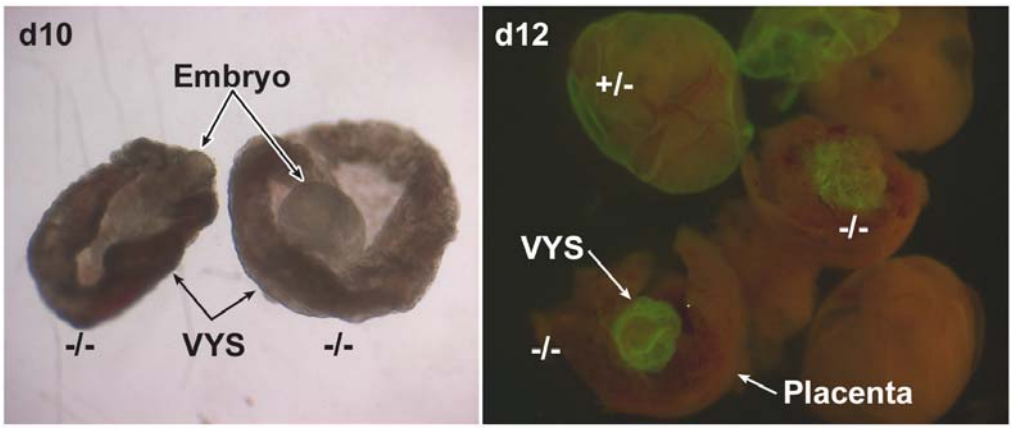


Figure 3.3: Phenotype of early mouse embryos homozygous for the *Slc39a4* (*Zip4*)-knockout allele. Heterozygous *Zip4*$^{+/-}$-knockout mice were crossed (d1 = vaginal plug) and implantation sites were harvested on d10 or d12 of pregnancy. Embryos within the visceral yolk sac (**VYS**) were photographed using a dissecting microscope (d10), and EGFP, expressed from the *Zip4*-knock-in allele(s), was detected by fluorescence on d12. Genotypes were determined by PCR of the *Zip4* gene. Homozygous (**-/-**) knockout embryos were completely malformed by d10 and only the visceral yolk sac remained on d12. *EGFP* expression was only detected in the visceral yolk sac endoderm.

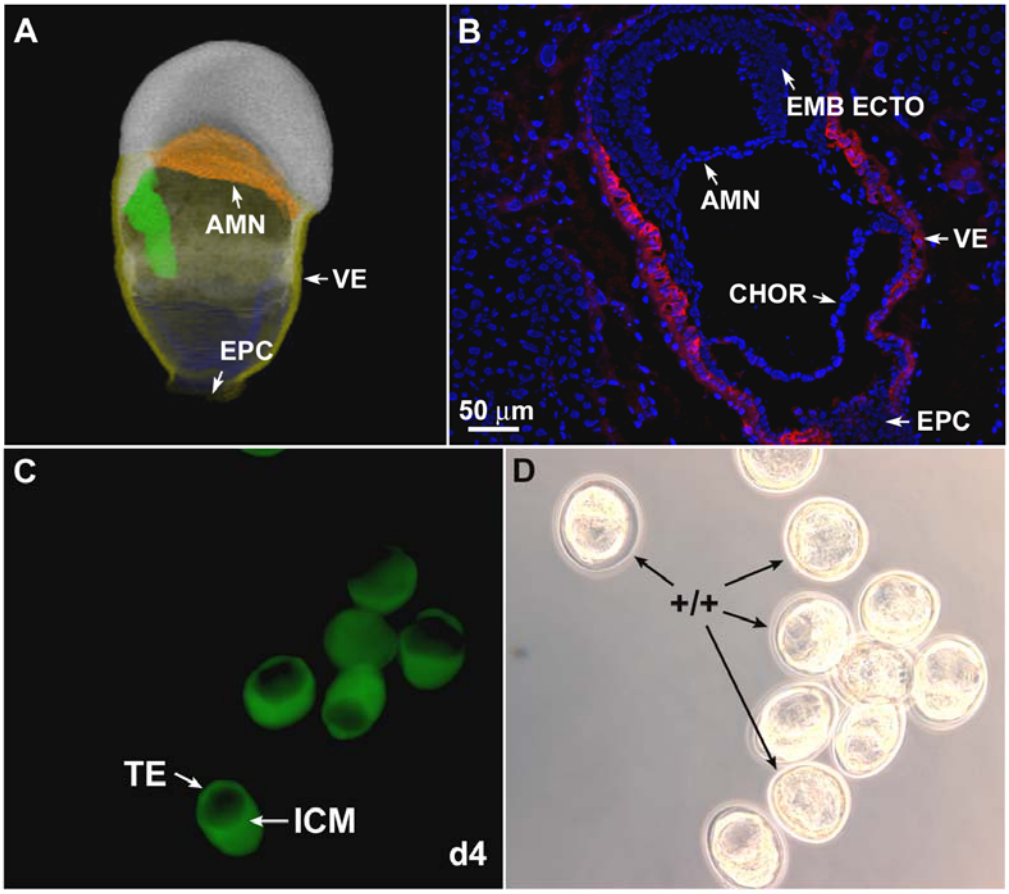
However, none of these embryos progressed past the egg cylinder stage, and by d10 only a residual mass of embryonic cells remained (see Figure 3.3).

In the homozygous and heterozygous implantation sites, only the visceral yolk sac actively expressed *EGFP* under control of the *Zip4* promoter (see Figure 3.3). *EGFP* expression was not detected in the placenta or in the embryo proper. This result is consistent with and extends earlier studies of expression of the endogenous *Zip4* gene (Dufner-Beattie et al., 2003b; Dufner-Beattie et al., 2004). Expression of *EGFP* was restricted to the visceral yolk sac, and examination of d8 implantation sites using an antibody against EGFP to increase sensitivity and specificity revealed expression only in the visceral endoderm at the time when morphogenesis of the homozygous embryo fails (see Figure 3.4). However, *EGFP* expression was detectable in the inner cell mass cells as well as in the trophectoderm of d4 blastocysts before differentiation of the visceral endoderm occurs (see Figure 3.4C).

C. *Slc39a4* (*Zip4*) haploinsufficiency causes hypersensitivity to zinc deficiency

As mentioned above, the $Zip4^{+/-}$ offspring were underrepresented at weaning and loss of these offspring apparently occurs after midgestation in mice fed the zinc adequate diet (see Figure 3.2). Examination of one litter of embryos at d10 revealed a wide range of abnormalities (see Figure 3.5). In most litters there were more apparently normal embryos and fetuses than there were abnormal ones. However, compared with their wild-type littermates, the $Zip4^{+/-}$ embryos often varied greatly in

Figure 3.4: Detection of EGFP expressed from the *Slc39a4* (*Zip4*)-knockin allele in a d8 mouse embryo and d4 blastocyst. Part A: A diagrammatic view of the d8 mouse embryo indicating the amnion (**AMN**), visceral endoderm (**VE**), and ectoplacental cone (**EPC**). This is the egg cylinder stage of development. **Part B**: Expression of the *Zip4* gene was detected using an antibody specific for EGFP which was expressed from the *Zip4* knock-in allele. Cryosections of d8 mouse embryos obtained by crossing $Zip4^{+/-}$-knockout mice were prepared, and EGFP was detected by immunofluorescence. Sections from wild-type embryos showed no immunostaining for EGFP (not shown). EGFP was restricted to the visceral endoderm (**VE**). The chorion (**CHOR**) and embryonic ectoderm (**EMB ECTO**), AMN and EPC are shown, but only the VE contained detectable EGFP. **Part C**: $Zip4^{+/-}$-knockout females were bred with wild-type males and blastocysts were harvested on d4 of pregnancy. EGFP fluorescence was detected. **TE**, trophectoderm; **ICM**, inner cell mass. **Part D:** Phase contrast image showing all of the blastocysts in the field of view in Part C. Based on the lack of EGFP fluorescence four wild-type (+/+) embryos are indicated, whereas six *Zip4*-knockout embryos were fluorescent.


A
AMN
VE
EPC
B
EMB ECTO
AMN
VE
CHOR
50 μm
EPC
C
TE
ICM
d4
D
+/+

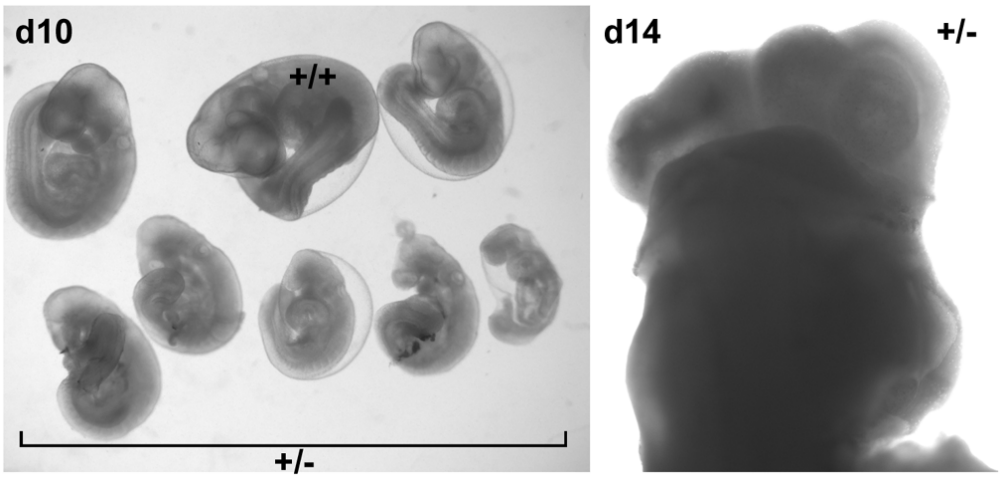


Figure 3.5: Pleiotropic phenotypes associated with haploinsufficiency of *Slc39a4* (*Zip4*) in developing mouse embryos. Heterozygous *Zip4*-knockout (+/-) mice were crossed and embryos were harvested on d10 or d14 of pregnancy. Wild-type and heterozygous Zip4 embryos were separated based on EGFP fluorescence in the VYS and photographed. The genotype of each embryo was then determined by PCR. One wild-type (+/+) embryo is indicated (d10). All the other embryos shown were heterozygous (+/-) for the *Zip4*-knockout allele (d10 and d14). Note the highly variable phenotype of the heterozygous embryos on d10 and the d14 embryo with exencephalia.

size and morphology. Many were smaller and some were severely growth retarded and exhibited abnormal cranio-facial development. Remarkably, embryos with exencephalia were occasionally found at midgestation (see Figure 3.5). These morphological abnormalities are reminiscent of the teratology of maternal zinc deficiency (Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006), and were never seen in wild-type embryos in these litters.

After parturition, a few heterozygous offspring died before weaning and about 22% (63/280) of those that survived past weaning were also morphologically abnormal. Thus, about 40% of the heterozygous post-implantation embryos were ultimately abnormal. An extreme example of an abnormal but weaned heterozygous *Zip4*-knockout mouse is shown in Figure 3.6. These mice were often much smaller and had a domed head. High field magnetic resonance imaging (MRI) of several weaned $Zip4^{+/-}$ mice revealed striking results (see Figure 3.7). These three heterozygous mice had no eyes or only one eye and showed various degrees of hydrocephalus. This was verified by examination of paraffin sections from the brain from an affected $Zip4^{+/-}$ mouse after perfusion fixation (see Figure 3.7). In addition, abnormal thickening of the ventricular septum and wall of the left ventricle (see Figure 3.7), and skeletal abnormalities were present in some of these affected $Zip4^{+/-}$ mice. These abnormalities were never seen among the hundreds of wild-type offspring we examined.

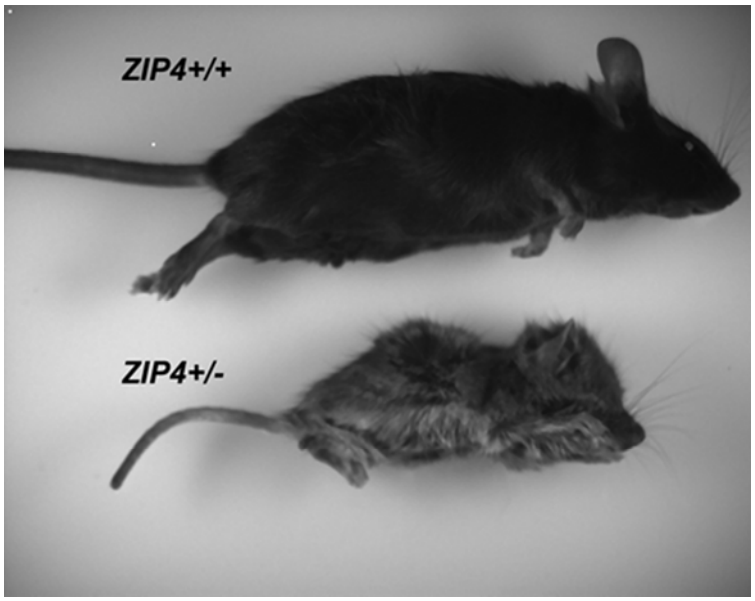
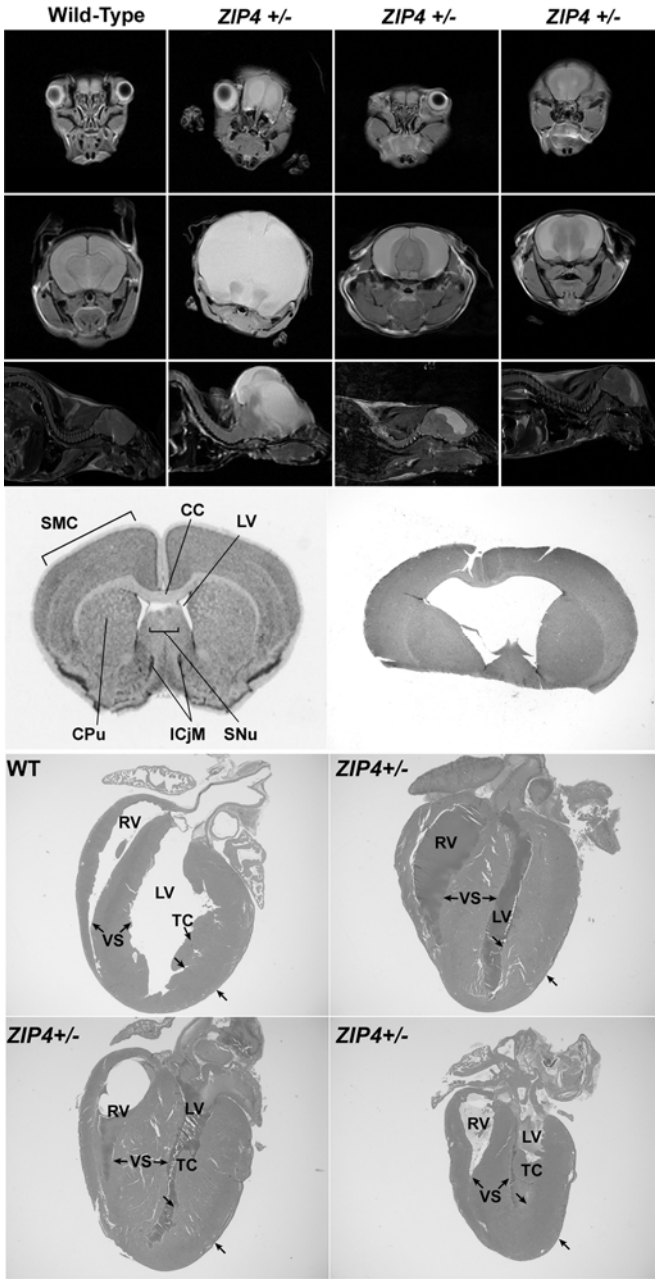


Figure 3.6: Gross morphology of a wild-type and a *Slc39a4* (*Zip4*$^{+/-}$) heterozygous-knockout mouse just after weaning. A wild-type (+/+) and a severely abnormal *Zip4* heterozygous (+/-) mouse just after weaning were photographed and then analyzed by high field magnetic resonance imaging at the Hoglund Brain Imaging Center at our institution (see first two columns in the top set of panels in Figure 7 below).

Figure 3.7: Pleiotropic phenotypes associated with *Slc39a4* (*Zip4*) haploinsufficiency in weaned mice. Wild-type mice and *Zip4*$^{+/-}$-knockout mice (***Zip4***$^{+/-}$) that were obviously abnormal were examined using high field MRI (**top collection of three rows**). **Top row:** Axial sections through the face at the plane of the eyes. **Second row**: Axial sections through the midbrain region. **Third row:** Sagittal sections near the midline region. White areas in the brain indicate water accumulation. Images are not displayed proportional to the size of the mice. **Middle two panels**: Left: Histology of the normal mouse brain at Bregma 1.18 to 0.86 mm. **SMC**, Sensory Motor Cortex; **CC**, Corpus Callosum; **LV**, Lateral Ventricle; **CPu**, Caudate Putamen; **ICjM**, Island of Calleja (major); **SNu,** Septal Nuclei. Right: Paraffin section of an affected *Zip4*$^{+/-}$ brain stained with toluidine blue confirmed hydrocephalus. **Bottom set of four panels**: Sagittal section from near the center of the mouse heart stained with H & E. Histology of wild-type (**WT**) and three *Zip* $^{+/-}$ hearts from affected weaned mice (not those show in MRI panels). **RV**, right ventricle; **LV**, left ventricle; **VS**, ventricular septum; **TC**, Trabeculae Carneae. Arrows indicate the relative thickness of the ventricular septum and the outer wall of the left ventricle. The heart on the bottom right was smaller than the others.


Wild-Type
ZIP4 +/-
ZIP4 +/-
ZIP4 +/-
SMC
CC
LV
CPu
ICjM
SNu
WT
RV
LV
TC
VS
ZIP4+/-
RV
VS
LV
ZIP4+/-
RV
LV
VS
TC
ZIP4+/-
RV
LV
TC
VS

The above results were all obtained with mice fed a zinc-adequate diet. Also including excess zinc (250 ppm $ZnCl_2$) in the drinking water and feeding the zinc-adequate diet from d1 of pregnancy until weaning, protected development of many heterozygous *Zip4* embryos, but failed to rescue the homozygous *Zip4*- knockout embryos (see Figure 3.2). At weaning, wild-type and heterozygous pups were present at the expected Mendelian frequency, and only 1 heterozygous pup (3%) displayed the abnormal eye phenotype.

To further test the hypothesis that the pleiotropic teratogenic and embryotoxic effects of *Zip4* heterozygosity reflect impaired zinc homeostasis during embryogenesis, $Zip4^{+/-}$ mice were mated and pregnant females were fed a zinc-deficient diet beginning on d8 of pregnancy. Embryos were examined on d14 and genotyped by PCR (see Figure 3.8). $Zip4^{+/-}$ embryos were significantly underrepresented in the population (21 wild-type and 23 heterozygous), and about 50% of were lost by d14. About 2/3 of the remaining $Zip4^{+/-}$ embryos were morphologically abnormal, with the majority (61%) being classified as severely abnormal. In contrast, 71% of the wild-type embryos in these same litters appeared morphologically normal and only a small percentage (4.7%) was severely abnormal (see Figure 3.8). Thus, maternal zinc status modulates the detrimental effects of embryonic *Zip4* heterozygosity on development, and heterozygous embryos are hypersensitive to zinc deficiency.

The contributions of maternal *Zip4* gene expression to the teratogenic and embryotoxic effects of *Zip4* heterozygosity were examined. Wild-type females were

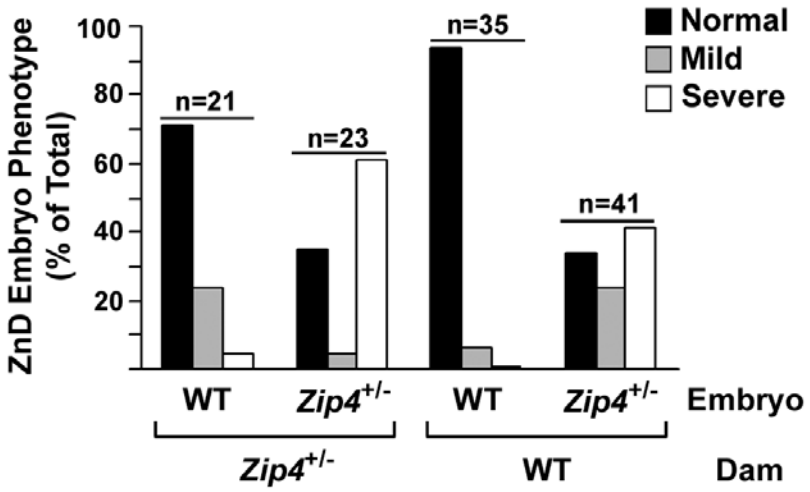


Figure 3.8: Effects of dietary zinc-deficiency during pregnancy, in relation to embryonic and maternal *Slc39a4* (*ZIP4*)-knockout genotype, on the development of wild-type and heterozygous embryos. *Zip4*$^{+/-}$-knockout females (**Zip4**$^{+/-}$) or wild-type (**WT**) females (**Dam**) were crossed with *Zip4*$^{+/-}$-knockout males and pregnant females were fed a zinc-deficient diet beginning on d8. Embryos were examined on d14 of pregnancy and scored as severely abnormal if they were dramatically growth retarded and displayed multiple morphological abnormalities, or mildly abnormal if they exhibited delayed limb development and retarded growth. Embryos were individually genotyped and the total number **(n)** in each genotype is shown above. Morphological data (**ZnD Embryo Phenotypes**) are expressed as the percentage of embryos in each genotype of the total number examined (**n**).

mated with *Zip4*$^{+/-}$ males and the relationship between genotype and phenotype of the d14 embryos in response to maternal dietary zinc deficiency was determined. Remarkably, *Zip4*$^{+/-}$ embryos in these litters were not underrepresented at d14 (see Figure 3.8), nor were *Zip4*$^{+/-}$ offspring underrepresented (44 *Zip4* $^{+/-}$ and 42 wild-type) at weaning when dietary zinc is adequate. However, 18% (8 pups) of those weaned pups also display the same types of abnormalities and had failed to thrive. Maternal zinc deficiency also profoundly affected the development of *Zip4*$^{+/-}$ embryos in wild-type females. About 2/3 of the heterozygous embryos were morphologically abnormal, and 41% were severely abnormal in dams fed a zinc-deficient diet. In contrast, 94% of the wild-type embryos were normal and none were severely abnormal in these same litters (see Figure 3.8). Among the abnormal *Zip4* $^{+/-}$ embryos, 7 of the 8 embryos had one or no eyes, and 4 had exencephalia at d14.

These results establish that maternal *Zip4* heterozygosity sensitizes the developing embryo (wild-type and *Zip4*$^{+/-}$) to the deleterious effects of zinc deficiency during pregnancy. Furthermore, in combination with embryonic *Zip4* heterozygosity, maternal *Zip4* heterozygosity dramatically increases the chances of abnormal development and diminishes the chances of survival of the embryo to parturition, and these effects are exacerbated by dietary zinc deficiency during pregnancy. Finally, these results suggest that the abnormal phenotypes noted at weaning in *Zip4* heterozygous offspring reflect the diminished function of the embryonic *Zip4* gene.

IV. Discussion

The human recessive genetic disorder AE is caused by mutations in the *Slc39a4* (*Zip4*) gene, and the studies of *Zip4*-knockout mice reported herein confirm that this zinc transporter plays a pivotal role in zinc homeostasis in the mammalian embryo and newborn. Some patients with AE are homozygous for mutations in the *Zip4* gene that likely lead to a complete loss of function of this protein. For example, patients homozygous for a point mutation, a 5 bp deletion or 53 bp insertion that each introduces a premature stop codon within the coding region of *Zip4* mRNA have been described (Kury et al., 2002; Meftah et al., 2006; Nakano et al., 2003). These mutations would cause the synthesis of severely truncated peptides, although that has not been proven to occur. A complete loss of function was mimicked in these *Zip4*-knockout mice. In mice, a complete loss of function of ZIP4 was embryonic lethal. Similarly, in humans, the apparently complete loss of ZIP4 function is lethal but at a later stage (postnatal) unless zinc is supplemented in the diet. Therefore, the early post-implantation death of homozygous *Zip4*-knockout mouse embryos was an unexpected finding. Human AE patients generally present symptoms after birth, and they often benefit greatly by zinc therapy (Sandstrom et al., 1994). This suggests the existence of secondary zinc uptake systems that can compensate for the loss of ZIP4 function in the newborn human. However, we were unable to rescue the embryonic lethality caused by the complete loss of ZIP4 function in mice by providing excess zinc orally and/or by intra-peritoneal injection to the mother, suggesting the lack of an adequate backup system for zinc uptake through the mother and into the embryonic

environment at this critical stage of development. In addition to the essential function of *Zip4*, the copper transporter *Ctr1* (Lee et al., 2001) and the zinc efflux transporter *ZnT1* (Andrews et al., 2004) are also essential genes at this stage of mouse development. Taken together, these studies suggest that morphogenesis of the mouse embryo requires the development of functional systems to maintain homeostasis of essential metals at the egg cylinder stage. It is interesting to note that the *fear-of-intimacy* gene in *Drosophila* encodes a LIV-1 family zinc transporter that is essential during early development of the fly (Mathews et al., 2005). Similarly, in zebrafish, a member of the ZIP superfamily (*zLIV-1*) is essential during early morphogenesis (Yamashita et al., 2004). *Zip4* is the first example of a mammalian *Zip* gene shown to serve an essential function during embryogenesis and the most active expression of the *Zip4* gene is restricted to the visceral endoderm, although this gene was detectably expressed in all cells in the blastocyst. The visceral endoderm cells are the third cell type to differentiate from the inner cell mass and on d7.5 they surround the extra-embryonic region of the developing conceptus.

The pleiotropic effects of *Zip4* heterozygosity on embryonic development in mice were profound, and both embryonic and maternal *Zip4* genes were implicated in controlling sensitivity to zinc deficiency. Some AE patients are homozygous or compound-heterozygous for *Zip4* mutations that may result in hypomorphic alleles. For example, patients homozygous for the P200L mutation have been reported (Lehnert et al., 2006; Kury et al., 2002), and this mutation diminishes but does not eliminate the zinc transport activity of ZIP4 in transfected cell assays (Wang et al.,

2004c). Although, to our knowledge there are no published studies showing that *Zip4* heterozygosity in humans is associated with effects on embryonic development, our studies suggest that dietary zinc availability as well as gene dosage may modulate the severity and type of symptoms associated with AE mutations. It is interesting to note that gene dosage for *Atp7a*, the Menke's disease gene, determines the sensitivity of the developing zebrafish notochord to copper deficiency (Mendelsohn et al., 2006). Similarly, a transient neonatal zinc deficiency during breast feeding has been associated with heterozygosity in the maternal *ZnT2* gene in women (Chowanadisai et al., 2006). Zinc nutritional status, interacting with mutations in human *Zip4* may be associated with a wide range of developmental abnormalities.

Neurogenesis appears to be remarkably sensitive to zinc deficiency in heterozygous *Zip4*-knockout embryos. Exencephalia, hydrocephalus, anophthalmia and anopia all occurred in these animals under normal dietary zinc conditions. Remarkably, many of these abnormalities have been previously associated with zinc deficiency in the rat (Hurley and Swenerton, 1966; Hurley et al., 1971; Sandstead et al., 1975; Rogers and Hurley, 1987), and zinc is essential for brain development and function in monkeys as well (Sandstead, 2003). It is thought that diminished DNA and RNA synthesis in neurons during zinc deficiency may cause abnormal neurogenesis (Terhune and Sandstead, 1972; Sandstead and Rinaldi, 1969). The retina is particularly rich in zinc which is concentrated in photoreceptors and retinal pigment epithelial cells (Ugarte and Osborne, 2001) where it may function as a neuromodulator (Chappell and Redenti, 2001; Redenti and Chappell, 2004).

Depletion of intracellular zinc induces caspase-dependent apoptosis in retinal cells (Hyun et al., 2000).

The severity and penetrance of these abnormalities in these mice may reflect interactions of *Zip4* with genetic modifiers. Inbred C57BL/6 mice have a high rate (up to 12%) of spontaneous microphthalmia and ocular infections (see Jaxmice.jax.org/library/notes/463aHTML) that is exacerbated by alcohol (Smith et al., 1994). Ocular phenotypes such as abnormal retina and optic nerve have been reported in *p53*-knockout mice bred onto the C57BL/6 background but not onto the 129/SvJ background (Ikeda et al., 1999). However, true anophthalmia and anopia as seen in these *Zip4* heterozygous mice are unusual in C57BL strains of mice (Smith et al., 1994). Furthermore, these mice are on a mixed genetic background (129/SvJ: C57BL/6: B6-FVB), and none of these neuro- or ocular developmental abnormalities were seen during examination of several hundred wild-type mice from the same litters. Thus, the pleiotropic phenotypes observed here reflect the loss of function of a *Zip4* allele, and there is no evidence that this exacerbates an inherent susceptibility to malformations in this genetic background. It is intriguing to note that acrodermatitis enteropathica has been associated with LDL deficiency (Wallis et al., 1974), and many of the same phenotypes reported here in *Zip4*-knockout mice were also noted in *apo B*-knockout mice (Huang et al., 1995). Homozygous *apo B*-knockout embryos die before d9 of gestation, and heterozygous embryos showed increased intrauterine death and abnormal neurogenesis. Incomplete neurotube closure and hydrocephalus were noted in some *apo B*-knockout mice, but no eye phenotypes were reported, and

many of the male mice were infertile (Huang et al., 1995). Whether ZIP4 influences LDL metabolism in mice remains to be examined.

Chapter 4

Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse *Slc39a4* and *Slc39a5* zinc transporters (*Zip4 and Zip5*)

Citation of Chapter Publication

Weaver B.P., Dufner-Beattie J., Kambe T., Andrews G.K. 2007. Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse *Slc39a4* and *Slc39a5* zinc transporters (*Zip4* and *Zip5*). *Biol Chem.* 388 (12): 1301-1312. PMID: 18020946. Helmut Sies, Editor-in-Chief *Biol Chem.*

Contributions to the Chapter

I generated all data and figures except for panel 4.1A (J.D.B.), panel 4.1C (J.D.B.), Figure 4.2 (J.D.B.), panel 4.5D (T.K.), and the cartoon summary Figure 4.9 (G.K.A.). Dr. Andrews and I both made substantial intellectual contribution to the design, interpretation, and report of experiments. Dr. Dufner-Beattie and Dr. Kambe contributed to the design, interpretation, and report of their relevant contributions. Eileen Roach aided in the production of graphics.

Specific Aim and Hypothesis

Chapter 4 will address specific aim 2 and will evaluate the hypothesis that *Zip4* and *Zip5* are post-transcriptionally regulated inversely and dynamically in response to zinc availability. The purpose of this study is to determine at what level *Zip4* and *Zip5* are regulated in response to zinc availability.

I. Abstract

Dietary zinc deficiency in mice is accompanied by enhanced expression of the zinc uptake transporter *Slc39a4* (*Zip4*) and repressed expression of *Slc39a5* (*Zip5*) in tissues which regulate zinc homeostasis (intestine, pancreas and visceral yolk sac). Herein, mechanisms controlling this differential expression were investigated. The induction of *Zip4* mRNA during zinc deficiency, and its repression in response to zinc repletion were found to reflect changes in *Zip4* mRNA stability and not changes in the relative rate of transcription of this gene. During zinc deficiency, ZIP4 protein levels are increased and this protein is localized on the apical membranes. Administration of an oral gavage of zinc caused ZIP4 internalization and degradation in enterocytes and visceral endoderm cells. Similarly, ZIP4 is induced by zinc deficiency in cultured mouse Hepa cells and is rapidly degraded in response to added zinc. *Zip5* mRNA abundance does not change in response to zinc, but the translation of this mRNA was found to be zinc-responsive. During zinc deficiency, *Zip5* mRNA remains associated with polysomes while the protein is internalized and degraded in

enterocytes, acinar cells and endoderm cells. After zinc-gavage ZIP5 is rapidly re-synthesized and targeted to the basolateral membranes of these cell-types.

II. Introduction

The storage, efflux and uptake of zinc are regulated in response to zinc availability. In mammals fed a diet with adequate zinc, this metal is absorbed by intestinal enterocytes, but excess zinc is released through the pancreas and back through the small intestine (King et al., 2000; Hambidge and Krebs, 2001). Under zinc-deficient conditions, zinc absorption by the small intestine increases, and zinc release from the pancreas and small intestine decreases (Hambidge and Krebs, 2001). During embryonic development of the mouse, the visceral endoderm of the yolk sac plays an important role in zinc homeostasis and also undergoes molecular adaptation to changes in maternal zinc status similar to those seen in the maternal intestine (Dufner-Beattie et al., 2004; Dufner-Beattie et al., 2007). We are exploring the mechanisms behind this adaptive response.

Two superfamilies of mammalian zinc transporters have been identified (Palmiter and Huang, 2004; Taylor and Nicholson, 2003; Guerinot, 2000b). Solute carrier 30A (*Slc30a*) family members, named ZnTs, function in zinc efflux and compartmentalization (Palmiter and Huang, 2004), whereas the *Slc39a* family members, named ZIPs, function in the uptake of zinc and other metals into the cytoplasm. In mice and humans, 14 members of the ZIP family have been identified (Guerinot, 2000b; Taylor and Nicholson, 2003). These proteins have eight predicted

transmembrane domains and the ZIP superfamily can be subdivided into four subfamilies based on structural homology (Taylor and Nicholson, 2003). In mammals, most of the ZIP proteins fall into one of two subfamilies named subfamily II (3 members) and LIV-1 (9 members). Studies of knockout mice revealed that the members of subfamily II are particularly important when dietary zinc becomes limiting (Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006; Peters et al., 2007). Less is known about the physiological functions of most LIV-1 family members. However, in humans, the importance of *Zip4* is exemplified by the rare autosomal recessive disease, acrodermatitis enteropathica (AE), which is caused by mutations in the *Zip4* gene (Wang et al., 2002; Kury et al., 2002). This disease can be fatal if untreated by the administration of excess dietary zinc (Maverakis et al., 2007). The mouse *Zip4* gene is essential early during development of the post-implantation embryo and heterozygosity hypersensitizes mice to the effects of dietary zinc deficiency (Dufner-Beattie et al., 2007). Although the physiological functions of ZIP5 are unknown, it is a closely related member of the LIV-1 subfamily that can also function as a zinc transporter (Dufner-Beattie et al., 2003b; Wang et al., 2004b; Dufner-Beattie et al., 2004) and which is also expressed in tissues involved in zinc homeostasis (intestine, pancreas, visceral endoderm).

Our previous studies of ZIP4 and ZIP5 led to the suggestion that these proteins serve antagonistic functions in zinc homeostasis (Dufner-Beattie et al., 2004). ZIP4 being important for zinc acquisition from the diet (or mother), whereas ZIP5 functions to remove excess zinc from the blood when zinc is replete. *Zip4*

mRNA abundance is increased in the adult and neonatal intestine and embryonic visceral yolk sac during periods of dietary zinc deficiency (Dufner-Beattie et al., 2003b; Huang et al., 2006) leading to the intense localization of ZIP4 protein on the apical membranes of enterocytes and visceral endoderm cells, and increased amounts of this protein (Huang et al., 2006). Studies of transfected cells revealed that zinc can stimulate the endocytosis and degradation of ZIP4 (Kim et al., 2004a; Mao et al., 2007), and ZIP4 is removed from the apical surfaces of enterocytes and endoderm cells several hours after an injection of zinc into the peritoneum of zinc deficient mice (Dufner-Beattie et al., 2003b). Our recent studies of mouse ZIP5 revealed that this protein is localized on basolateral membranes of intestinal enterocytes, visceral endoderm cells and pancreatic acinar cells when dietary zinc is not limiting (Dufner-Beattie et al., 2004), but in contrast to ZIP4, this protein is removed from these basolateral membranes of each of these cell-types during periods of dietary zinc deficiency. These changes in cellular localization were not accompanied by changes in *Zip5* mRNA abundance (Dufner-Beattie et al., 2004).

The molecular mechanisms underlying the complex regulation of these zinc transporters in these specific cell-types in response to dietary zinc is intriguing, yet poorly understood. Herein, we extended our previous studies of *Zip4* and *Zip5* regulation *in vivo* to include analyses of the effects of oral zinc repletion in zinc deficient mice and cultured cells and studies of the mechanisms involved in controlling these genes and proteins. These studies reveal that *Zip4* and *Zip5* expression is fine-tuned by several novel post-transcriptional, translational and post-

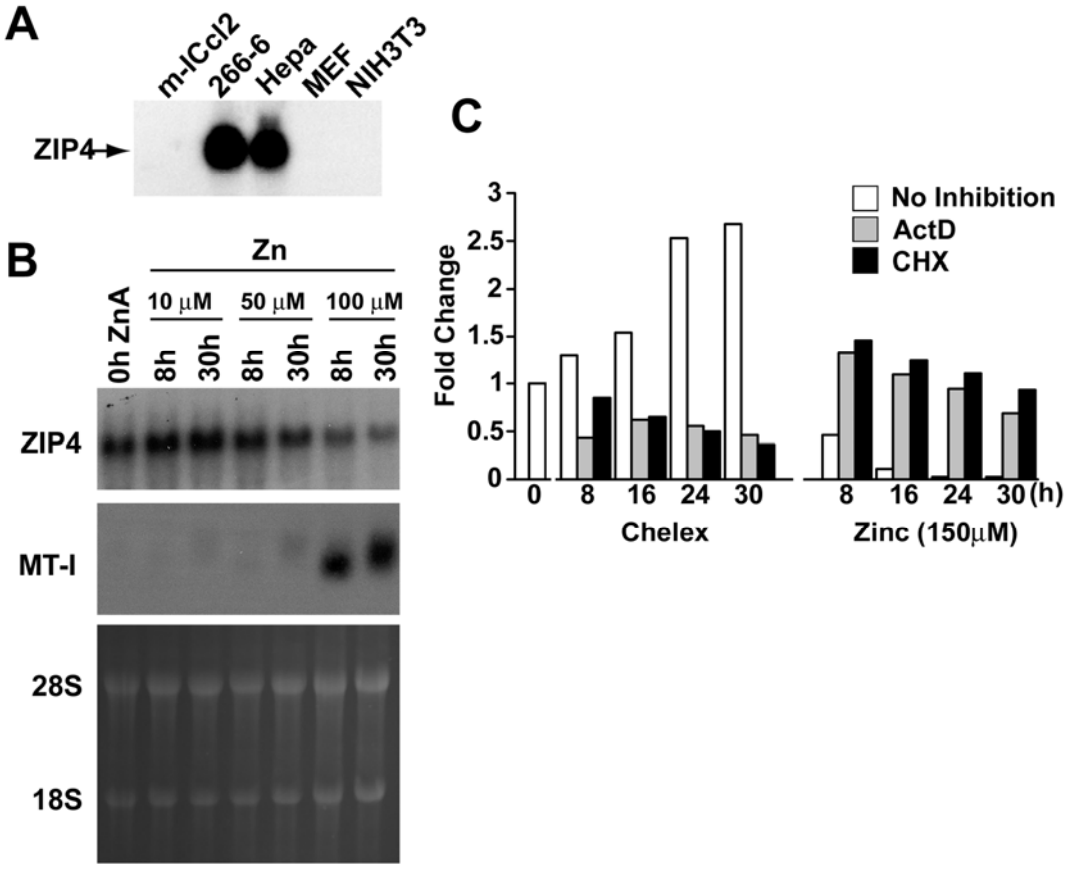
translational mechanisms that each sense available zinc and reciprocally regulate these zinc transporters often within the same cell-types and coordinately within the animal.

III. Results

A. Zinc regulation of *Slc39a4* (*Zip4*) mRNA abundance requires new protein and RNA synthesis

Several mouse cell lines were examined to determine if they express and regulate the *Zip4* gene in response to zinc. *Zip4* mRNA was readily detectable by Northern blot hybridization of total RNA from mouse Hepa cells and the acinar-derived cell line 266-6, but not from mouse embryo fibroblasts (MEF), NIH3T3 cells or the mouse intestinal crypt-like cell line mIC-c12 (see Figure 4.1A). In mouse Hepa cells, but not in 266-6 cells, zinc concentration in the culture medium modulated *Zip4* mRNA abundance (see Figure 4.1B and C) and as shown below, ZIP4 protein abundance in a manner that mimicked the regulation of *Zip4* in mice (shown later in Figure 4.5D). Therefore, we used these cells as a model system in which to study regulation of the endogenous *Zip4* gene. In the presence of 10% FBS in the culture medium, the addition of 100 to 150 μM zinc maximally repressed *Zip4* mRNA (Dufner-Beattie et al., 2003b) and maximally induced *metallothioneins-1* (*MT-1*) mRNA in Hepa cells (see Figure 4.1B and C). Expression of the mouse *MT-1* gene is regulated by the zinc-sensing transcription factor MTF-1 (Andrews, 2001) and

Figure 4.1: Effects of RNA and Protein Synthesis Inhibitors on Zinc Regulation of *Zip4* mRNA Abundance. **Part A:** Northern blot detection of *Zip4* mRNA in various mouse cell lines. Total RNA from the indicated mouse cell lines was fractionated by formaldehyde-agarose gel electrophoresis and transferred to nylon membranes. Membranes were hybridized with a mouse *Zip4* cDNA probe and hybrids were detected by autoradiography (Dufner-Beattie et al., 2003b). **Part B:** Northern blot detection of *Zip4* mRNA in mouse Hepa cells incubated for the indicated times (**0h ZnA = 0 time**) in culture medium containing 10% FBS plus the indicated concentration of zinc (Zn). Total RNA was blotted and membranes hybridized with *Zip4* and *MT-1* probes. Duplicate gels were stained with acridine orange (28S and 18S rRNAs are indicated) as a control for RNA integrity and loading. **Part C:** Quantitation of Northern blots of *Zip4* mRNA in mouse Hepa cells incubated for the indicated times in culture medium containing 10% Chelex-treated FBS (**Chelex**) plus or minus 150 μM $ZnSO_4$ (**Zinc**), and plus or minus 10 μg/ml of actinomycin D (**ActD**), cycloheximide (**CHX**) or no inhibitor (**No Inhibition**). The first unfilled bar (left) represents the untreated control (fold change = 1) to which all other samples are compared. Total RNA was fractionated by electrophoresis, transferred to membranes and hybridized with the *Zip4* probe and quantified by densitometry. Duplicate gels were stained with acridine orange as a control for RNA integrity and loading (not shown). Values (fold change) are expressed as the ratio of the experimental value to the 0 time untreated value.


A
m-ICcl2
266-6
Hepa
MEF
NIH3T3
ZIP4
B
Zn
0h ZnA
10 μM
50 μM
100 μM
8h
30h
8h
30h
8h
30h
ZIP4
MT-I
28S
18S
C
No Inhibition
ActD
CHX
Fold Change
3
2.5
2
1.5
1
0.5
0
0
8
16
24
30
Chelex
8
16
24
30 (h)
Zinc (150μM)

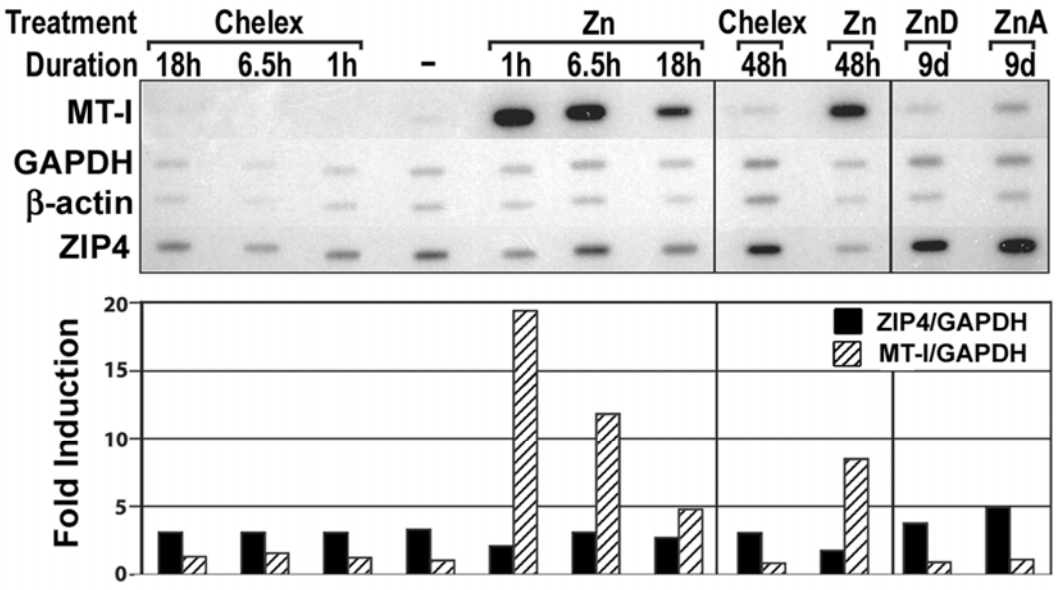
MT-1 functions to chelate excess zinc and provide a biologically important pool of labile zinc when zinc becomes limiting (Dalton et al., 1996c).

The addition of 150 μM zinc to the culture medium reduced *Zip4* mRNA abundance to low levels by 16 h (see Figure 4.1C, Zinc). In contrast, *Zip4* mRNA abundance increased in these cells cultured in medium containing 10% Chelex-treated FBS. This increase was clearly detectable by 8 h and was ≈ 3-fold greater than control levels by 30 h of incubation (see Figure 4.1C, Chelex). Similarly, incubation of these cells with the zinc chelator TPEN induced *Zip4* mRNA (data not shown). The inclusion of actinomycin D or cycloheximide in the culture medium blocked the induction as well as repression of *Zip4* mRNA by zinc (see Figure 4.1C). Similar results were obtained using α-amanitin to inhibit mRNA synthesis (data not shown).

B. Zinc does not regulate transcription of the *Zip4* gene

An *in vitro* nuclear run-on transcription assay was employed to directly examine the effects of zinc on *Zip4* gene transcription (see Figure 4.2). The effects of zinc on the relative rate of transcription of the *MT-1* gene was monitored as a positive control for zinc induction, and the rates of transcription of the *GAPDH* and *β-actin* genes were monitored as controls for house-keeping gene expression. When normalized to the transcription rate for the housekeeping genes, the relative rate of transcription of the *Zip4* gene in Hepa cells incubated in medium containing 150 μM zinc or 10% Chelex-treated FBS remained constant for up to 48 h, long after *Zip4* mRNA levels had increased. In contrast, the rate of *MT-1* gene transcription was rapidly and dramatically induced in response to zinc.

Figure 4.2: Effects of zinc on the relative rate of transcription of the *Zip4* gene. A nuclear run-on assay was employed to measure the relative rate of transcription of the mouse *Zip4*, *MT-1*, *GAPDH* and *β-actin* genes in Hepa cells and the mouse intestine (**far right two lanes**). Hepa cells were incubated for the indicated times (**lane marked (-) = 0 time**) in culture medium containing 10% Chelex-treated FBS (**Chelex**) or in this medium also containing exogenous zinc (**150 μM $ZnSO_4$**). The proximal small intestine (duodenum) was collected from pregnant mice (5 per group) fed a zinc adequate (**ZnA**) or zinc deficient (**ZnD**) diet for 24 h beginning on d8 of pregnancy, as described previously (Dufner-Beattie et al., 2003b; Dufner-Beattie et al., 2004). Nuclei isolated from cells and intestines were incubated *in vitro* in buffer containing [α-^{32}P]UTP to label run-on transcripts. RNAs were isolated and equal counts of labeled run-on transcripts were hybridized to the indicated DNAs immobilized on nylon membrane strips. The cDNAs for mouse *MT-1*, *GAPDH* and *β-actin* and *Zip4* genomic DNA were immobilized on the filter. The amount of labeled RNA hybridized was detected by autoradiography (**top panel**) and quantified by phosphorimaging. Values for *Zip4* and *MT-1* hybrids are normalized to those of *GAPDH* and are expressed as fold-induction relative to the 0 time values (**bottom panel**).


Treatment
Chelex
Zn
Chelex
Zn
ZnD
ZnA
Duration
18h
6.5h
1h
–
1h
6.5h
18h
48h
48h
9d
9d
MT-I
GAPDH
β-actin
ZIP4
Fold Induction
20
15
10
5
0
ZIP4/GAPDH
MT-I/GAPDH

Transcription run-on assays were also performed using nuclei isolated from the mouse intestine obtained from pregnant mice fed a zinc-adequate (ZnA) or zinc-deficient (ZnD) deficient diet (see Figure 4.2; 9d samples). In this experiment, pregnant mice were fed the ZnA or ZnD diet beginning on d8. Our previous studies showed that *Zip4* mRNA is detectably increased in the dam's intestine within 24 h under these experimental conditions (Dufner-Beattie et al., 2004). Therefore, the relative rate of transcription of *Zip4* was determined 24 h after initiation of the ZnD diet. No difference in the transcription rate was noted between the ZnA and ZnD samples.

C. Zinc reciprocally regulates the cellular localization of Slc39a4 (ZIP4) and Slc39a5 (ZIP5) proteins in the absence of changes in mRNA abundance

To further examine the reciprocal regulation of ZIP4 and ZIP5 by dietary zinc, mice were fed the ZnD diet and then re-administered zinc by oral gavage. *Zip4* and *Zip5* mRNA levels (see Figure 4.3) and cellular localization (see Figure 4.4) were monitored within the first few hours thereafter. Newly weaned mice grow rapidly and double in size within two or three weeks after weaning when fed the ZnA diet, but they fail to grow well when fed the ZnD diet and within 10 days they display overt signs of zinc deficiency such as dermatitis, as well as stunted growth (Dufner-Beattie et al., 2005). Zinc demands also increase during pregnancy coincident with the rapid growth and differentiation of the embryo. Embryos in pregnant mice fed a ZnD diet beginning on d8 of pregnancy have a greatly increased risk of abnormal development (Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006). As noted

Figure 4.3: Effects of zinc repletion on *Zip4* and *Zip5* mRNA abundance in the proximal small intestine (duodenum) and visceral yolk sac. Northern blot detection of *Zip4*, *MT-1*, *Zip5* and *Zip1* mRNAs in the maternal (**Dam**) proximal small intestine (duodenum) and the fetal **VYS** is shown. Pregnant mice were fed a zinc adequate (**ZnA**) or zinc deficient (**ZnD**) diet for 6 days beginning on d8 of pregnancy (ending d14). The zinc deficient mice were then given an oral gavage of zinc or saline (**6C**), and tissues were harvested at the indicated times thereafter. Two independent groups of mice, labeled **6(1)** and **6(2)** were analyzed for the 6 h zinc-gavage time point. Specific transcripts were detected using total RNA, as described in the legend to Figure 1. *Zip1* mRNA served as a good loading control since it has equivalent expression amongst many tissues and is not zinc regulated.

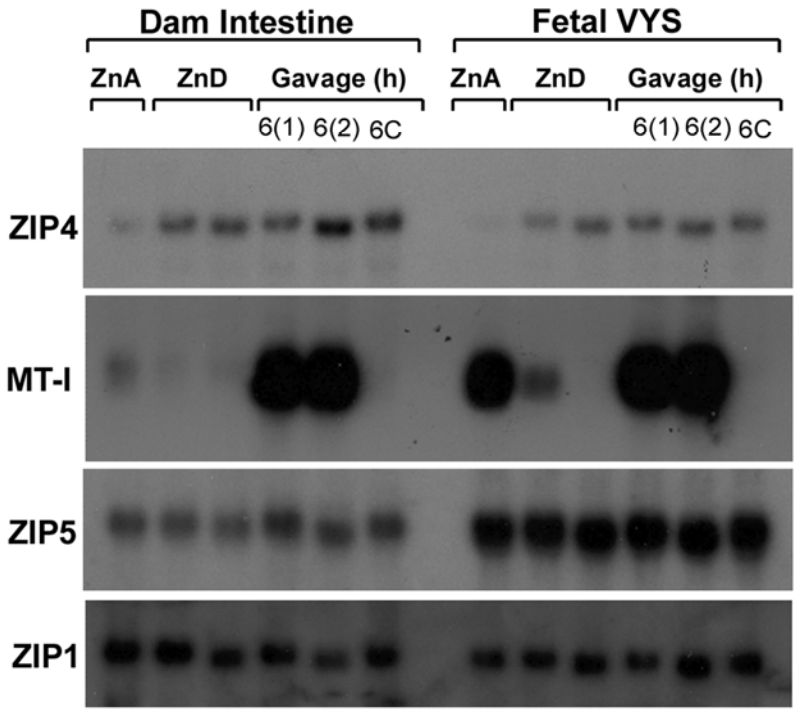
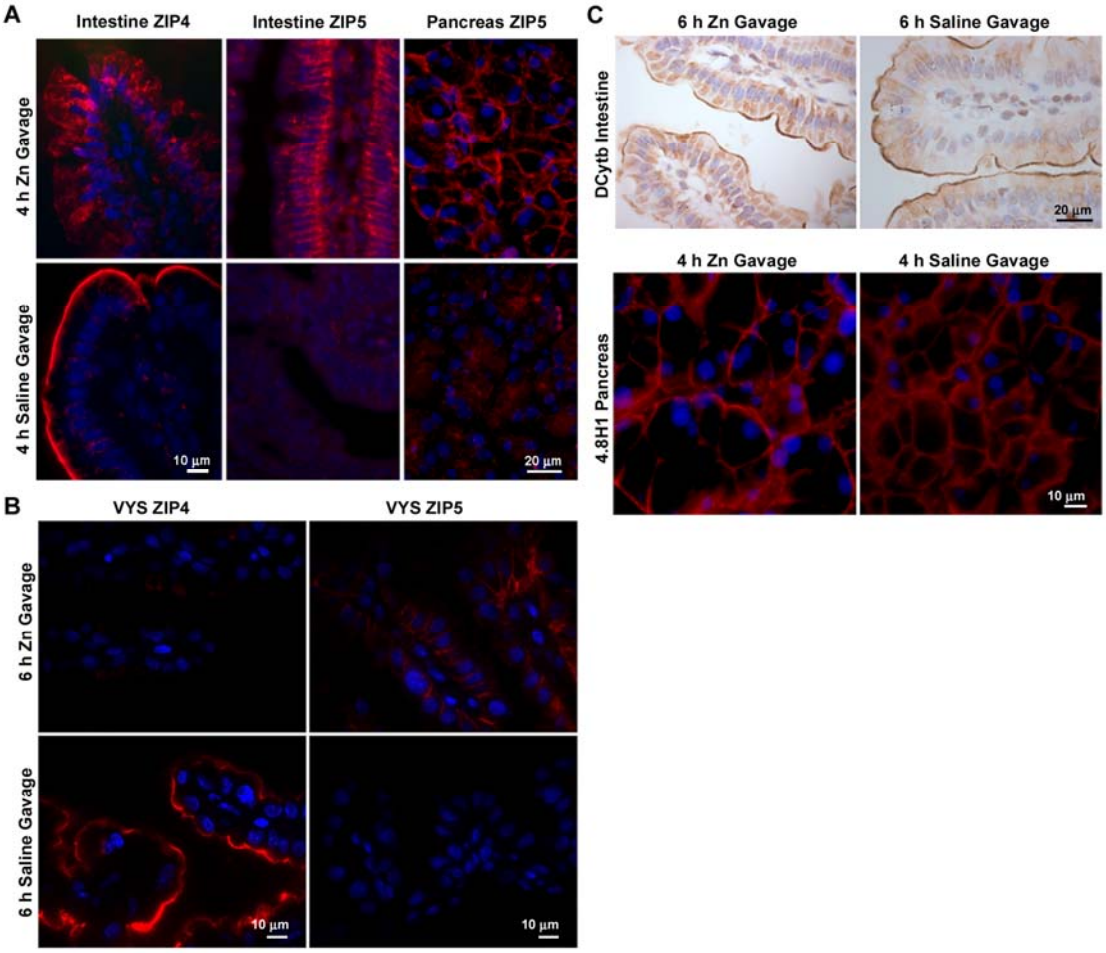

Dam Intestine
Fetal VYS
ZnA
ZnD
Gavage (h)
ZnA
ZnD
Gavage (h)
6(1) 6(2) 6C
6(1) 6(2) 6C
ZIP4
MT-I
ZIP5
ZIP1

Figure 4.4: Effects of zinc repletion on the cellular localization of ZIP4 and ZIP5 proteins in the proximal small intestine (duodenum) and pancreas. Two zinc deficiency models were used. Weanling mice were fed a zinc deficient diet for 10 days beginning on the day of weaning. Pregnant mice were fed a zinc deficient diet for 6 days beginning on d8 of pregnancy. On day 10 for weanlings and d14 for pregnant dams, the zinc deficient mice were given an oral gavage of zinc (100 μmoles $ZnCl_2$/kg body weight) or saline, and tissues were harvested at the indicated times thereafter and fixed with paraformaldehyde in PBS. Cryosections of the indicated fixed tissues were prepared and ZIP4 and ZIP5 (Parts A, B), or the indicated control proteins (Part C), were detected by immunofluorescence or immunohistochemistry. Part A: Immunofluorescence localization of ZIP4 and ZIP5 in the proximal small intestine (duodenum) and pancreas. Zinc deficient weanling mice were given an oral gavage of zinc or saline for the indicated times before harvest. Red: Sites of antibody binding; blue: Nuclei. Part B: Immunofluorescence localization of ZIP4 and ZIP5 in the VYS. Zinc deficient pregnant mice were given an oral gavage of zinc or saline, and fetal VYS was harvested 6 h later. Part C: Immunohistochemical localization of the iron-regulated ferric reductase DCytb in the proximal small intestine and immunofluorescence localization of the basolateral membrane protein 4.8H1 in the pancreas from zinc deficient weanling mice given an oral gavage of zinc or saline for the indicated time. Brown deposits: Sites of antibody binding. Sections were counterstained with hematoxylin. The specificity of each of the antibodies used in this figure has been previously demonstrated (McKie et al., 2001).


A
Intestine ZIP4
Intestine ZIP5
Pancreas ZIP5
4 h Zn Gavage
4 h Saline Gavage
10 μm
20 μm
B
VYS ZIP4
VYS ZIP5
6 h Zn Gavage
6 h Saline Gavage
10 μm
10 μm
C
6 h Zn Gavage
6 h Saline Gavage
DCytb Intestine
20 μm
4 h Zn Gavage
4 h Saline Gavage
4.8H1 Pancreas
10 μm

previously (Dufner-Beattie et al., 2003b; Dufner-Beattie et al., 2004), *Zip4* mRNA abundance was significantly increased in the maternal intestine and fetal VYS in mice fed the ZnD diet while *Zip5* and *Zip1* mRNA abundance were unaltered (see Figure 4.3). In a previous study we noted that *Zip4* mRNA levels in the VYS decline by 9 h after an intraperitoneal injection of zinc; earlier time points were not examined in that study. In both of the above experimental models of dietary zinc deficiency (newly weaned mice and pregnant mice) we found that an oral gavage of zinc, equivalent to the amount of zinc that might be eaten in one day by consuming the ZnA diet, did not cause a change in the abundance of *Zip4*, *Zip5* or *Zip1* mRNAs within 6 h in the intestine or VYS (see Figure 4.3). However, *MT-1* mRNA was dramatically induced by a zinc gavage but not a saline gavage.

The rapid effects of zinc repletion on the localization of ZIP4 and ZIP5 were examined herein. ZIP4 was found to be removed from the apical membrane and internalized in enterocytes and visceral endoderm cells (see Figure 4.4A and B) while ZIP5 was relocalized to the basolateral membrane of enterocytes, visceral endoderm cells (see Figure 4.4A and B) and pancreatic acinar cells (see Figure 4.4A) within a few (4 to 6 h) hours of administering an oral gavage of zinc. Administration of an oral gavage of saline did not elicit these changes. As an another important control for the specificity of these changes in membrane localization it was noted that zinc deficiency did not alter the apical localization of the iron-regulated duodenal cytochrome b (DCytb) on enterocytes nor the basolateral localization of the pancreatic protein 4.8H1 (see Figure 4.4C). This control is lacking in all previously

reported studies of ZIP4 and ZIP5 trafficking, but serves to confirm the specificity of the effects of zinc.

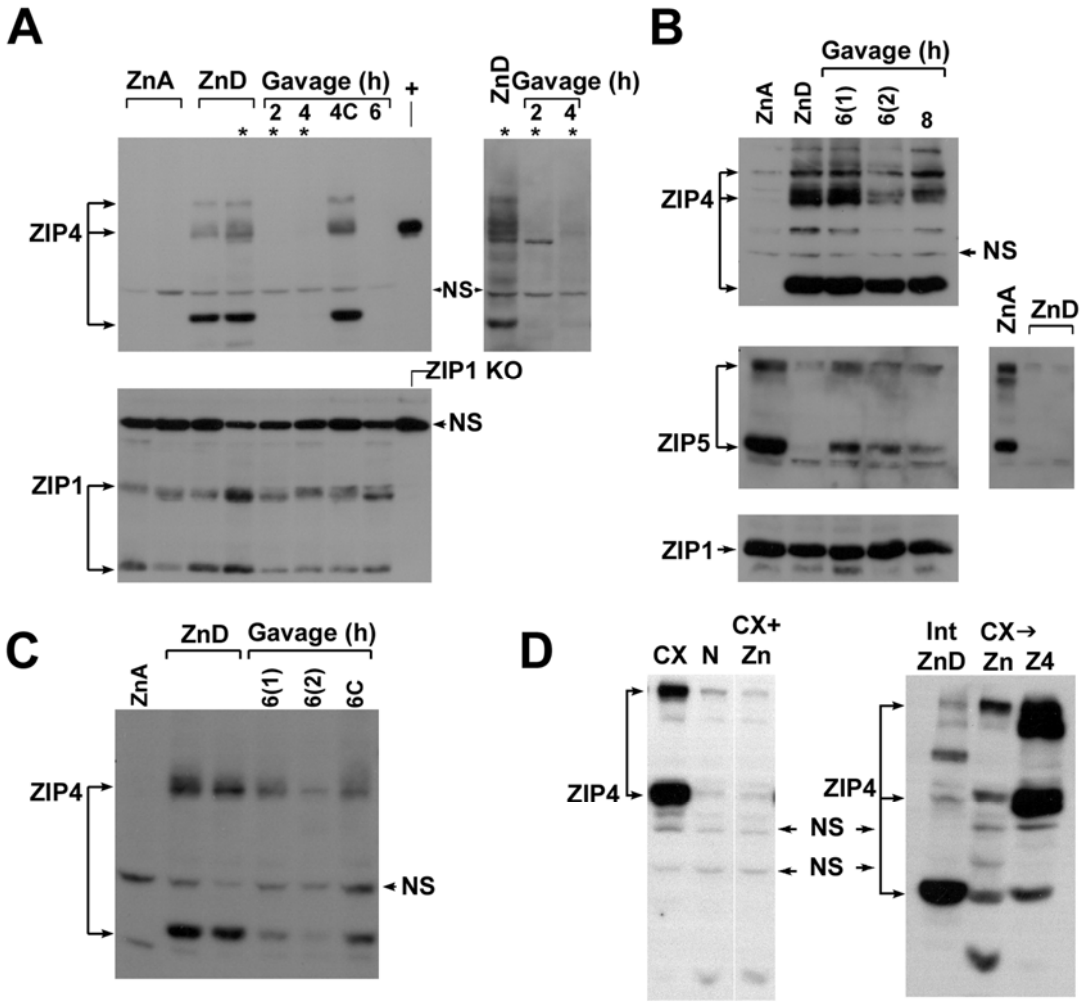
D. Zinc can regulate the turn-over of ZIP4 and ZIP5 proteins

Western blot analysis was used to examine ZIP4 and ZIP5 proteins in response to zinc. ZIP4 protein was not detected in membranes prepared from the intestine (see Figure 4.5A and C) or VYS (see Figure 4.5B) taken from mice fed the ZnA diet. In contrast, this protein was abundant in the membranes prepared from mice fed the ZnD diet. Multiple species of ZIP4 were detected which may reflect glycosylated forms of the protein (Kim et al., 2004a). In addition, an ≈ 37 kDa immunoreactive band was detected in the ZnD samples. The identity of this peptide remains to be determined, but it appears to be ZIP4 specific and is present in the intestine and VYS only in zinc deficient mice (see Figure 4.5A).

An oral gavage of zinc resulted in the rapid loss of ZIP4 immunoreactivity in Western blots of intestinal membrane preparations (see Figure 4.5A and C). By 2 h after the zinc gavage, little ZIP4 remained in the intestine membrane preparations from zinc deficient newly weaned mice. In contrast, ZIP1 abundance was unaffected by dietary zinc or by an oral gavage of zinc. ZIP1 served as a good loading control for these membrane preparations. Western blot analysis of the maternal intestine from pregnant mice fed the ZnD diet and then given an oral gavage of zinc also revealed a significant loss of ZIP4 immunoreactivity (see Figure 4.5C). Although this reduction was not as dramatic as that noted in the intestine of newly weaned mice, it was significant and reproducible. In contrast, Western blot analysis of membrane

Figure 4.5: Effects of zinc on ZIP4 and ZIP5 protein abundance in the proximal small intestine (duodenum), visceral yolk sac and cultured mouse Hepa cells. Part A: Western blot detection of ZIP4 (**top panels**) and ZIP1 (**bottom panel**) in membrane proteins from the proximal small intestine of mice fed a zinc adequate (**ZnA**) or zinc deficient (**ZnD**) diet for 10 days beginning on the day of weaning and then given an oral **gavage** of zinc or saline (**4C**), as detailed in the legend to Figure 4.4. Proximal small intestine was harvested at the indicated times after gavage (**2, 4 or 6 h**). Three prominent immunoreactive ZIP4 bands (~150 kDa; ~73 kDa and 37 kDa) are indicated by arrows. **Top panel; + ,** ZIP4 expressed in transfected cells (~73 kDa). **NS**, non-specific band. **Top right** panel; a longer exposure of lanes marked (*****) in the **top left** panel. **Bottom panel;** *Zip1* KO, membrane proteins from intestine of homozygous *ZIP1*- knockout mice (Dufner-Beattie et al., 2006). **NS**, non-specific band. **Parts B and C:** Detection of ZIP4, ZIP5 and ZIP1 in VYS (**Part B**) or maternal small intestine (**Part C**). Pregnant mice were fed a **ZnA** or **ZnD** diet beginning on d8 of pregnancy. On d14, zinc deficient mice were given an oral gavage of zinc or saline (**6C**) and VYS and maternal proximal small intestine were harvested at the indicated times (**6 and 8 h**) thereafter. Two separate experiments (n = 5 each) are shown (**6(1) and 6(2)**). **Part B** right panel shows a ZIP5 Western blot of ZnA VYS and two additional and separate ZnD samples. Only the specific ~35 kDa band for ZIP1 is shown. **Part D**: Detection of ZIP4 in Hepa cells or zinc-deficient intestine (**Int ZnD**). Hepa cells were cultured in medium containing either 10% normal FBS (**N**), 10% Chelex-treated FBS (**CX**), 10% Chelex-treated FBS to

which 20 μM $ZnSO_4$ had been added prior to culture (**CX + Zn**) or 10% Chelex-treated FBS to which 20 μM $ZnSO_4$ had been added for the last 2 hours of the overnight culture (**CX → N**). In the **right panel**, **Z4** refers to Hepa cells that were transfected with a ZIP4 expression vector.


A
ZnA
ZnD
Gavage (h)
2
4
4C
6
+
ZnD
Gavage (h)
2
4
ZIP4
NS
ZIP1 KO
NS
ZIP1
B
Gavage (h)
ZnA
ZnD
6(1)
6(2)
8
ZIP4
NS
ZnA
ZnD
ZIP5
ZIP1
C
ZnD
Gavage (h)
ZnA
6(1)
6(2)
6C
ZIP4
NS
D
CX
N
CX+ Zn
ZIP4
NS
NS
Int ZnD
CX→ Zn
Z4
ZIP4
NS
NS

proteins from the VYS revealed moderately reduced abundance of ZIP4 by 8 h after the zinc gavage (see Figure 4.5B). Taken together, the immunolocalization (see Figure 4.4) and Western blot analyses (see Figure 4.5) suggest that zinc stimulates the endocytosis of ZIP4 *in vivo* in enterocytes and visceral endoderm cells, as was previously noted in transfected cultured cells (Kim et al., 2004a), and that endocytosis can, but may not necessarily, lead to rapid degradation of the protein.

Hepa cells upregulated ZIP4 protein when cultured in zinc-deficient medium (see Figure 4.5D, left panel). This induction was blocked when zinc-deficient medium was supplemented with zinc during the incubation (see Figure 4.5D, left panel). Similar to intestine, repletion of zinc to zinc-deficient Hepa cells resulted in the rapid loss of ZIP4 immunoreactivity (see Figure 4.5D, right panel).

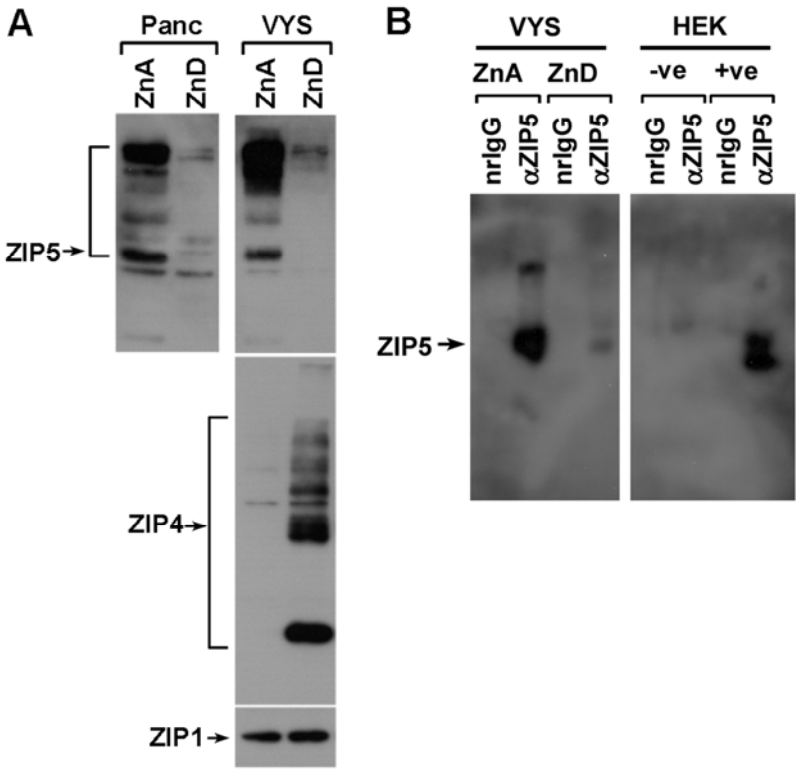
ZIP5 could not be detected by Western blot analysis of intestine membrane preparations, but this protein was readily detectable in membrane preparations from the VYS and from the pancreas. ZIP5 was detected in VYS membranes from mice fed the ZnA diet, but was dramatically and reproducibly reduced or absent in VYS membranes from mice fed the ZnD diet (see Figure 4.5B). However, ZIP5 was rapidly induced, in response to an oral gavage of zinc, in the embryonic VYS (see Figure 4.5). In a previous report, we concluded that ZIP5 protein levels do not change in the VYS as a result of zinc deficiency (Dufner-Beattie et al., 2004). We reanalyzed the original data and discovered that we were misled by a non-specific band in those Western blots. Apparently ZIP5 protein aggregated in those samples leaving a non-specific band at the approximate molecular weight expected for ZIP5

which confounded our interpretation. Herein, we repeated the results in the VYS multiple times (see Figure 4.5B and Figure 4.6) and then extended these studies to include analyses of the pancreas. During zinc deficiency, ZIP5 was degraded in the maternal pancreas as well as in the VYS (see Figure 4.6A; note the induction of ZIP4 in VYS collected from those dams). To further validate these findings we immunoprecipitated ZIP5 from the VYS and examined the precipitates by Western blot analysis. This approach increased the level of sensitivity of detection and reduced background (see Figure 4.6B). The results confirm the conclusion that ZIP5 is dramatically reduced or absent in the VYS during zinc deficiency.

E. ZIP5 protein synthesis is controlled by a zinc-regulated translational mechanism

The rapid accumulation of ZIP5 synthesized from preexisting mRNA could reflect the recruitment of this mRNA back into actively translating polysomes in response to zinc, the activation of translation of stalled polysomal mRNA or the inhibition of rapid turn-over of this protein. To begin to address these possibilities, we first determined whether the association of *Zip5* mRNA with polysomes was altered during zinc deficiency (see Figure 4.7). Visceral yolk sac polysomes (membrane bound and free) were prepared from mice fed the ZnA or ZnD diet during pregnancy (d8 to d14). Polysomes were fractionated by sucrose density gradient sedimentation and the distribution of *Zip4* and *Zip5* mRNAs in the gradient was determined by RT-PCR (see Figure 4.7B). The results revealed that *Zip5* mRNA is predominantly associated with the polysome fractions regardless of dietary zinc

Figure 4.6: Effect of zinc deficiency on ZIP5 protein abundance in the pancreas and visceral yolk sac. Part A: Western blot detection of ZIP5 (**top panels**), ZIP4 (**middle panel**) and ZIP1 (**bottom panel**) in membrane proteins prepared from pancreas (**top left**) and VYS (**top right**) from pregnant dams fed a zinc adequate (**ZnA**) or zinc deficient (**ZnD**) diet for 6 days beginning on d8 of pregnancy. Note that ZIP4 and ZIP1 are not expressed in pancreatic acinar cells. **Part B:** Immunoprecipitation and Western blot detection of ZIP5 in VYS and HEK 293 cells transfected with empty vector (**-ve**) or with (**+ve**) a ZIP5 expression vector. Visceral yolk sac and HEK 293 cells were sonicated in IP buffer and samples were precipitated overnight using normal rabbit IgG (**nrIgG**) or ZIP5 antiserum (**αZIP5**). Immune complexes were subjected to Western blot analysis using ZIP5 antiserum.


A
Panc
VYS
ZnA
ZnD
ZnA
ZnD
ZIP5
ZIP4
ZIP1
B
VYS
HEK
ZnA
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nrIgG
αZIP5
ZIP5

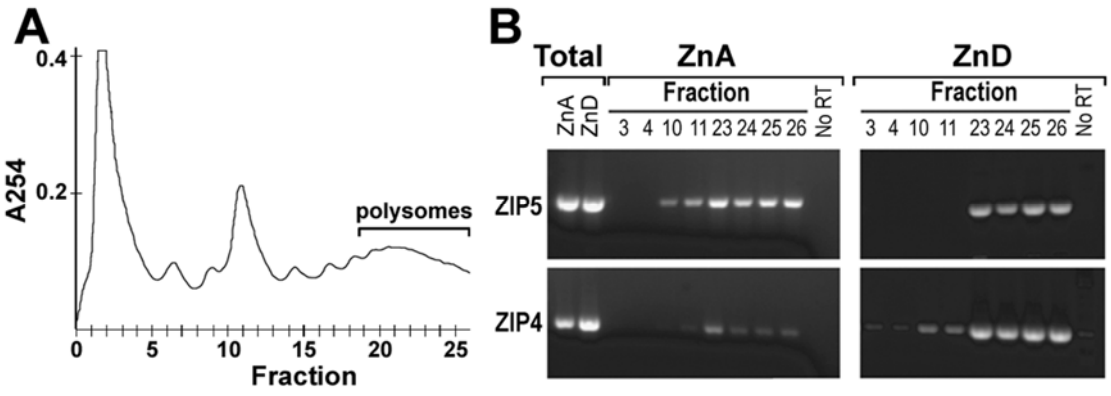


Figure 4.7: Effects of dietary zinc on the association of *Zip4* and *Zip5* mRNAs with polysomes. Fetal VYS was harvested on d14 from dams fed the **ZnA** or the **ZnD** diet beginning on d8 of pregnancy. Membrane bound and free ribosomes were solubilized and resolved on 10-50% linear sucrose gradients. **Part A:** Fractions from the sucrose gradient were monitored for absorbance at 254 nm (**A254**) beginning at the top of the gradient (**fraction 0**). Fractions 0-5 contained the smallest particles, whereas fraction 6, 9 and 11 contained 40S ribosomal subunits, 60S ribosomal subunits and 80S monosomes, respectively. Fractions 20-26 contained polysomes. **Part B**: RNA was isolated from the indicated fractions and amplified by RT-PCR using primers specific for *Zip4* and *Zip5* mRNAs. **Total ZnA** and **ZnD**: RT-PCR products from total extract RNA isolated before sucrose gradient fractionation. **No RT**: Negative controls where the reverse transcriptase enzyme was omitted.

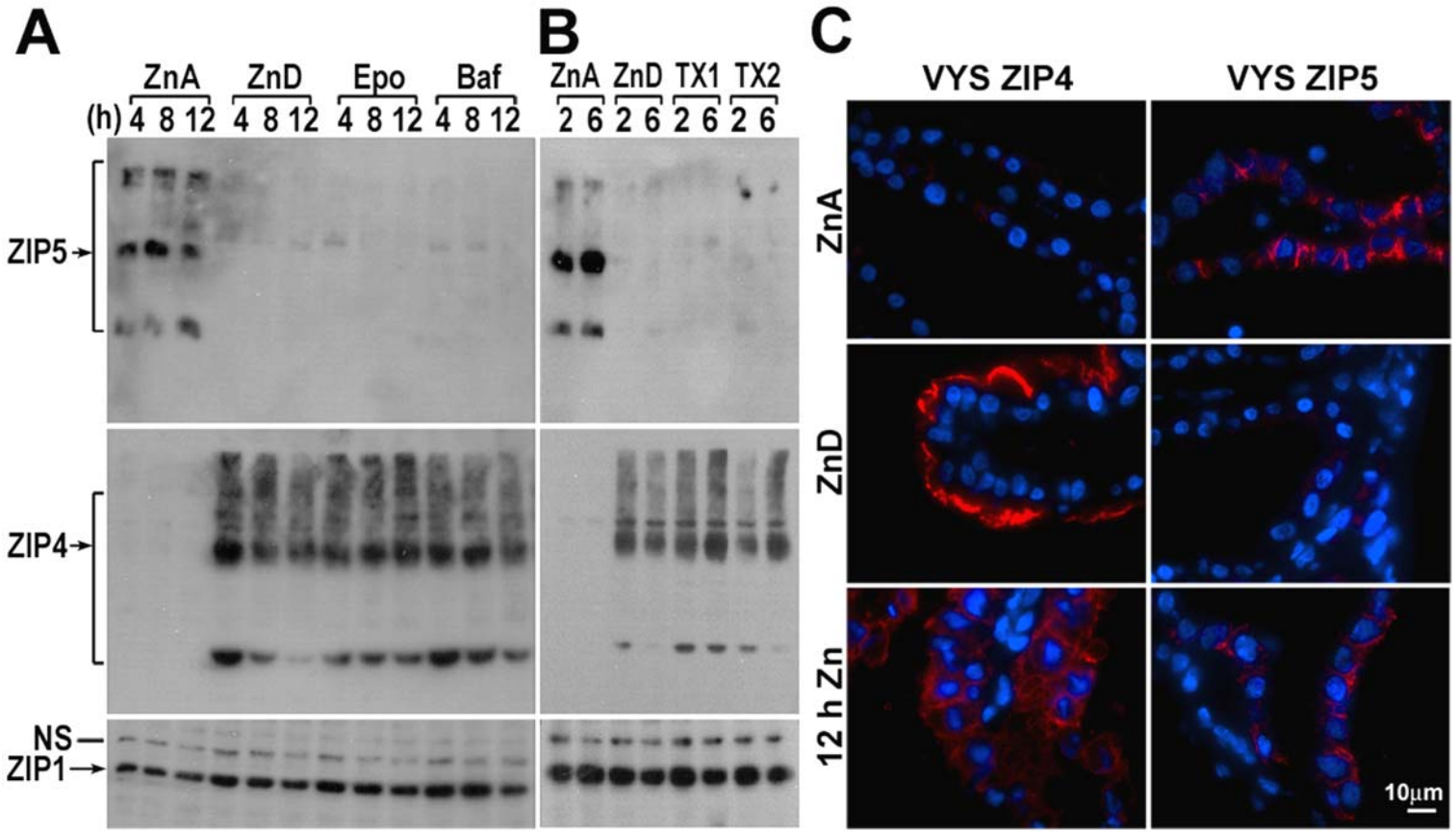
status. *Zip5* mRNA apparently does not appear to cycle on and off polysomes in response to zinc.

ZIP5 protein is underrepresented in total methionine and cysteine residues which, coupled with its relative low abundance, precluded unequivocal detection of the pulse-labeled protein. However, the effects of the cell-permeable proteasomal inhibitors, epoxomicin, MG132 and clasto-lactacystin β-lactone, and the specific lysosomal inhibitor, bafilomycin A1 on ZIP5 protein abundance were examined in VYS explant cultures. VYS harvested from pregnant mice fed the ZnD diet, were incubated in medium containing lysosomal inhibitor, or proteasome inhibitors either individually (see Figure 4.8A) or in cocktails with or without MG132 (see Figure 4.8B). The latter inhibitor has been reported to transiently stall translation (Cowan and Morley, 2004). Protein synthesis was monitored in these explant cultures and no difference in total ^{35}S-methionine and cysteine incorporation was found (not shown). IP analysis of ZIP5 in membrane preparations revealed that neither the proteasomal nor lysosomal inhibitors caused the accumulation of ZIP5 protein in the VYS, even after 12 h of incubation (see Figure 4.8, Parts A & B). In contrast, the inclusion of exogenous zinc in the VYS explant culture resulted in the internalization of ZIP4 and the reappearance of ZIP5 on basolateral membranes by 12 h (see Figure 4.8C).

These results suggest that ZIP5 is not being synthesized and rapidly degraded in the VYS during zinc deficiency, but instead are consistent with the concept that zinc deficiency causes the translation of *Zip5* mRNA to stall on the polysomes and

Figure 4.8: Effects of proteasome and lysosome pathway inhibitors or zinc on ZIP5 and ZIP4 protein abundance and localization in the visceral yolk sac explant cultures. VYS were harvested from d14 fetuses carried in pregnant females that had been fed a zinc adequate diet or a zinc deficient diet beginning on d8 of pregnancy. VYS were then cultured *in vitro* under the conditions described below. **Part A:** VYS from zinc-adequate dams were incubated in zinc-adequate medium (DMEM containing 10% FBS; **ZnA**). VYS from zinc-deficient dams were incubated in zinc-deficient medium (DMEM containing 10% Chelex-treated FBS with DMSO vehicle; **ZnD**), or ZnD medium also containing 50 nM synthetic epoxomicin (**Epo**) or 100 nM bafilomycin A1 (**Baf**) for the indicated times (up to 12 h). ZIP5 was immunoprecipitated (**top left panel**) and detected as described in the legend to Figure 6B. An equal amount of total membrane protein from each sample was examined by Western blot analysis for ZIP4 (**middle panels**) and ZIP1 as a loading control (**bottom panels**). **Part B:** VYS from zinc-adequate dams were incubated in **ZnA** medium. VYS from zinc-deficient dams were incubated in **ZnD** medium plus or minus a mixture of 1 μM epoxomicin, 50 μM MG132 and 50 μM clasto-lactacystin β-lactone (**TX1**) or 1 μM epoxomicin and 50 μM clasto-lactacystin β-lactone (**TX2**) for the indicated times. ZIP5, ZIP4 and ZIP1 detected by Western blot analysis as described in Part A. **Part C:** VYS from zinc-adequate dams were incubated for 12 h in **ZnA** medium, as above. VYS from zinc-deficient dams were incubated for 12 h in **ZnD** medium plus or minus 100 μM $ZnCl_2$ (**12 h Zn**). VYS were fixed and

processed, and ZIP4 and ZIP5 were detected by immunofluorescence as described in the legend to Figure 4. **Blue,** nuclei; **red**, sites of antibody binding.


A
ZnA
ZnD
Epo
Baf
(h) 4 8 12 4 8 12 4 8 12 4 8 12
ZIP5
ZIP4
NS
ZIP1
B
ZnA ZnD TX1 TX2
2 6 2 6 2 6 2 6
C
VYS ZIP4
VYS ZIP5
ZnA
ZnD
12 h Zn
10μm

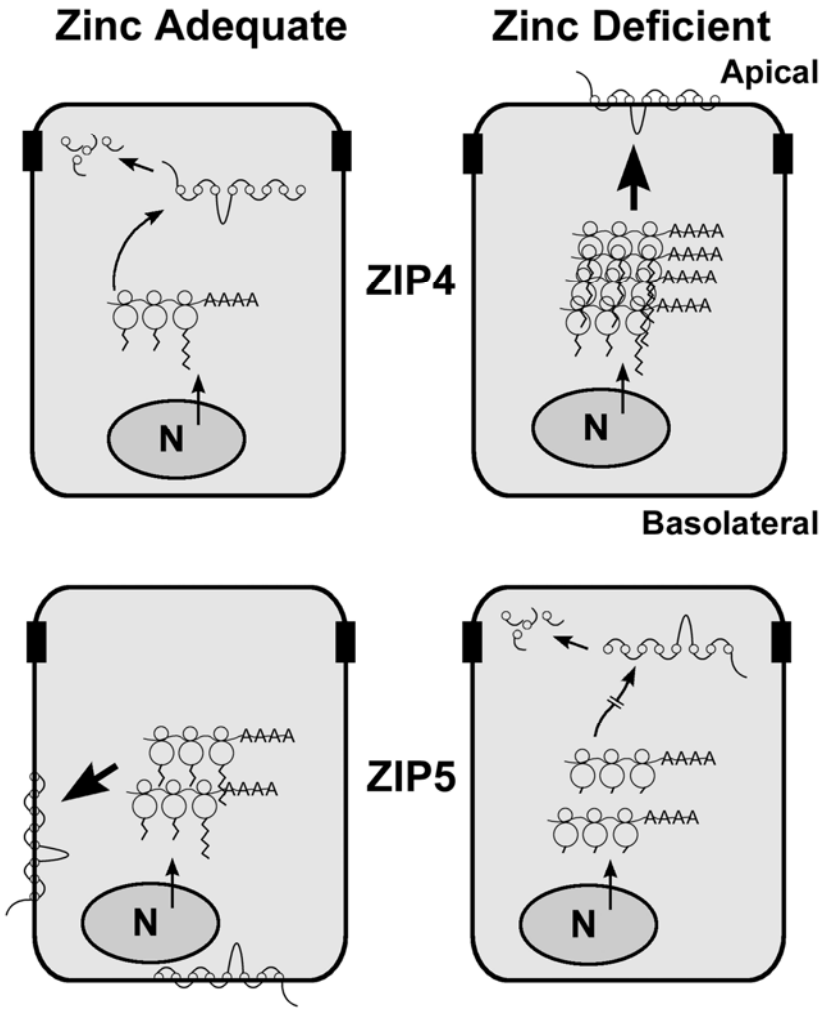
that a zinc-sensing mechanism reactivates its translation when zinc is repleted. This zinc-sensing mechanism appears to be cell autonomous in the VYS endoderm.

IV. Discussion

How ZIP4 and ZIP5 proteins are upregulated and downregulated, respectively, in response to zinc deficiency (Dufner-Beattie et al., 2004), in the same cell-types and coordinately in several organs is unknown (Cousins et al., 2006). Herein, we have continued to address these issues. The results of this study reveal that several novel zinc-sensing pathways orchestrate these changes via diverse post-transcriptional, translational and post-translational mechanisms *in vivo* (see Figure 4.9) Studies of ZIP4 in transfected cells suggested that zinc regulates the trafficking of this protein (Kim et al., 2004a; Wang et al., 2004c) and a recent study identified a histidine-rich region of the human ZIP4 protein that is essential for its ubiquitination and degradation following exposure to a high concentration of zinc (Mao et al., 2007). Nothing is known about the trafficking of ZIP5 in transfected cells although it is targeted to the basolateral membrane in polarized cells (Wang et al., 2004b). We extended these studies *in vivo* to examine the physiological relevance of these findings in transfected cells. It is of paramount importance to understand how these zinc transporters are regulated *in vivo* although these studies are technically challenging.

Figure 4.9: Cartoon summarizing mechanisms by which ZIP4 and ZIP5 are reciprocally regulated by zinc in the same cell. The mouse *Zip4* and *Zip5* genes are actively expressed in the intestine and fetal visceral yolk sac. *Zip5* is also actively expressed in pancreatic acinar cells. This cartoon depicts the polarized intestinal enterocyte. **N**, nucleus. Polysomal mRNAs are indicated as **lines ending in AAAA and bound by three ribosomes** with the protein tails extending from the ribosomes. ZIP4 and ZIP5 proteins are indicated as **lines connected by 8 circles** representing the 8 transmembrane domains. The degraded proteins are indicated as **individual circles and lines** inside the cells. Zinc apparently does not alter the rate of transcription of either *Zip4* (**top**) or *Zip5* (**bottom**), as indicated by the **arrows on the nuclei** in each panel. **Large bold arrows** indicate the most active expression of each of these zinc transporters. **Top left**: When zinc is adequate, *Zip4* mRNA is unstable and ZIP4 protein is not detected on the apical membrane but is degraded. **Top right**: When zinc becomes deficient, *Zip4* mRNA is stabilized, leading to increased accumulation of *Zip4* mRNA and ZIP4 protein and the localization of ZIP4 at the apical membrane. Repletion of zinc causes the rapid endocytosis and ultimate degradation of ZIP4 and destabilization of *Zip4* mRNA. **Bottom left**: *Zip5* mRNA abundance remains unchanged in response to zinc. When zinc is adequate, *Zip5* mRNA is translated and ZIP5 protein accumulates in the basolateral membrane. **Bottom right:** When zinc becomes deficient, *Zip5* mRNA translation is arrested (**hatched arrow**) but this mRNA remains associated with polysomes. ZIP5 protein is removed from the

membrane and degraded. Repletion of zinc causes the rapid resynthesis and relocalization of this protein to the basolateral membrane.


Zinc Adequate
Zinc Deficient
Apical
ZIP4
AAAA
N
Basolateral
ZIP5

We previously reported that *Zip4* mRNA abundance increases *in vivo* in enterocytes and visceral endoderm cells during periods of zinc deficiency (Dufner-Beattie et al., 2003b). This regulation can be mimicked in cultured mouse Hepa cells that express *Zip4* by manipulating zinc in the culture medium. This finding strongly supports the concept that changes in zinc availability directly regulate *Zip4* expression. However, the induction of *Zip4* mRNA occurs relatively slowly in response to zinc deficiency *in vivo* and *in vitro* and is not accompanied by an increase in the relative rate of *Zip4* transcription, but rather reflects increased mRNA stability. It is interesting to note that the *Zip4* gene has no metal response element, no identifiable TATA box or Inr sequence in the proximal promoter region, and it has two transcription start sites producing an A form and a smaller, much less abundant B form of the mRNA (Dufner-Beattie et al., 2003b). In addition, 5' end mapping experiments suggest that the transcription start site of the abundant and zinc inducible A form of *Zip4* mRNA is also imprecise (J. Dufner-Beattie, unpublished results). Taken together these results are most consistent with the concept that the *Zip4* gene is not inducible but its tissue-specific expression is tightly controlled as is the stability of its mRNA.

How zinc regulates the stability of *Zip4* mRNA remains to be determined. These studies suggest the possibility that zinc may repress the expression of a protein that specifically stabilizes this mRNA. But, it was also noted that excess zinc destabilizes *Zip4* mRNA in a process that requires new protein synthesis. Zinc may reciprocally regulate genes that stabilize and those that destabilize *Zip4* mRNA. This

mRNA does not contain an AU-rich sequence in the 3'-untranslated region (UTR) which would be typical of an mRNA with a very short half life (Wilkie et al., 2003). Details of the zinc-sensing mechanism(s) that regulate(s) *Zip4* mRNA abundance remain to be delineated.

ZIP4 protein localization and turn-over were also shown to be regulated by zinc availability *in vivo*. This was particularly obvious in the intestines of zinc deficient mice given a bolus of zinc directly into the stomach. Previous findings of transfected cells showed internalization of ZIP4 in response zinc (Kim et al., 2004a). We now report that zinc repletion *in vivo* caused the rapid endocytosis of ZIP4 and degradation of ZIP4 in the intestine. During the course of our studies, it was reported that human ZIP4 in transfected cells can be internalized and degraded in response zinc and it was noted that the extent of degradation was influenced by the zinc concentration and involved both proteasomal and lysosomal degradation pathways (Mao et al., 2007). Our studies are consistent with the concept that internalization and subsequent degradation can both be regulated by zinc. ZIP4 in the intestine of newly weaned mice was rapidly internalized and degraded in response to a bolus of oral zinc, whereas ZIP4 in the visceral yolk sac was internalized but not necessarily degraded. This may reflect a dose-response for zinc. The concentration of zinc in the gut after an oral gavage of zinc would be expected to be significantly greater than that seen by the embryonic VYS after a maternal zinc gavage. Perhaps ZIP4 can be recycled to the membrane under some physiological conditions. As an important tool for the future study of ZIP4 protein regulation, we have also shown that Hepa cells

regulate ZIP4 protein in a manner similar to that seen *in vivo* with its upregulation during zinc deficiency and rapid degradation following zinc repletion.

The synthesis and targeting of ZIP5 to basolateral membranes *in vivo* are both modulated by zinc. This was documented to occur in the VYS and pancreas by Western blot analysis, but we assume this also occurs in the intestine, based on the lack of a significant immunofluorescence signal for this protein from zinc-deficient mice and the rapid reappearance of the signal after zinc-repletion. Remarkably, zinc stimulated the rapid accumulation of this protein in the absence of changes in mRNA. This represents the first demonstration of translational control of a zinc transporter in response to zinc. Translational control mechanisms are known to be operative in iron homeostasis. The regulation of the translation of ferritin and ferroportin 1 by iron (McKie et al., 2000; Crichton et al., 2002) and the arrest of ceruloplasmin translation in macrophages exposed to IFN-γ (Mazumder et al., 2005) reflect changes in the initiation of translation and release of the nontranslating mRNAs from polysomes. *Zip5* mRNA remains associated with polysomes during zinc-deficiency and inhibitors of the proteasome and lysosome did not result in the re-accumulation of ZIP5 protein in visceral yolk sac explant cultures. Taken together, these data support a translational stall mechanism for ZIP5 when dietary zinc is limiting. When phosphorylated, the fragile X mental retardation protein associates with polysomes that appear to be stalled in the act of translation (Ceman et al., 2003), and recent studies have suggested important roles for miRNAs in this (Jin et al., 2004) and other

translational control mechanisms (Kim et al., 2004b). Further studies are required to delineate the mechanisms which control ZIP5 translation.

In summary, these studies provide evidence that ZIP4 and ZIP5 are both dynamically regulated by several post-transcriptional, translational and post-translational mechanisms. Interestingly, distinct zinc-sensing pathways coordinately regulate these zinc transporters in such a way that ZIP4 is removed from the apical membrane and can be degraded while ZIP5 is re-synthesized and targeted to the basolateral membrane in response to increased zinc availability. In two specialized cell-types (enterocytes and visceral endoderm cells), these processes occur within the same cell. In addition, this coordinate regulation of ZIP5 extends to pancreatic acinar cells. Given the importance of ZIP4 in zinc homeostasis in humans and mice, our findings suggest that ZIP5 may also be a critically important zinc transporter, as evidenced by these intricate regulatory mechanisms. Our studies reveal for the first time the existence of several novel zinc-sensing mechanisms in mammals. Unraveling the details of these molecular mechanisms will require further investigation.

Chapter 5

Identification of miRNAs Predicted to Target the 3' Untranslated Region of the *Slc39a5* (*Zip5*) mRNA in Translational Regulation

Contributions to the Chapter

The results in this chapter were unpublished at the time this Dissertation was approved. I generated all data and figures. Dr. Andrews and I both made substantial intellectual contribution to the design, interpretation, and report of experiments.

Specific Aim and Hypothesis

Chapter 5 will address specific aim 3 and will evaluate the hypothesis that *Zip5* is regulated by a rapid post-transcriptional mechanism mediated by the 3' untranslated region of the mRNA in response to zinc availability. This aim evolved with time as the other two aims were met. The purpose of this study is to determine how *Zip5* translation is regulated.

I. Abstract

The *Slc39a5* (*Zip5*) gene is regulated by post-transcriptional mechanisms in response to changes in zinc availability. Specifically, the expression of the protein at the basolateral membranes of intestinal enterocytes, pancreatic acinar cells, and visceral endoderm is lost over several days of zinc deficiency but the levels of ZIP5 protein return rapidly after zinc repletion. However, the steady state level of *Zip5*

mRNA does not change. This mRNA remains polysome-associated and appears to be translationally arrested. In the current study, we identified two miRNAs that are predicted to target the *Zip5* mRNA in accessible regions of the 3' untranslated region (UTR). The target sites for these miRNAs are conserved across multiple mammalian species; the miRNAs are expressed in all tissues known to regulate *Zip5* expression, and the miRNAs are associated with the polysomes making them strong candidates for regulating *Zip5* translation. Interestingly, these miRNAs were found to occur predominantly in sizes greater than predicted for a mature miRNA. Together, these data support the hypothesis that miRNAs mediate a translational stall mechanism to regulate *Zip5* expression in response to zinc availability.

II. Introduction

The adaptive response to zinc involves dramatic changes in post-transcriptional regulation of the zinc transporters *Slc39a4* (*Zip4*) and *Slc39a5* (*Zip5*). Previously, we have shown that over several days of zinc deficiency *Zip4* mRNA is upregulated and ZIP4 protein accumulates on the apical surfaces of enterocytes and visceral endoderm in pregnant mice (Dufner-Beattie et al., 2004; Weaver et al., 2007) (some of these findings are reported, herein: see Chapter 4). Moreover, zinc repletion resulted in rapid, dramatic internalization and degradation of the protein (Weaver et al., 2007) and the reduction of *Zip4* mRNA levels (Dufner-Beattie et al., 2004).

We also discovered that zinc availability does not affect *Zip5* mRNA levels or their association with polysomes, but zinc deficiency leads to a loss of ZIP5 immunostaining from the basolateral membranes of intestinal enterocytes, pancreatic acinar cells, and visceral endoderm (Dufner-Beattie et al., 2004; Weaver et al., 2007) and a loss of the ZIP5 protein as monitored by Western blot (reported, herein: see Chapter 4) (Weaver et al., 2007). Moreover, we found that ZIP5 protein can be detected by immunofluorescence 12 hours after zinc repletion and it returns to normal levels by 24 hours (as reported, herein: see Chapter 4) (Weaver et al., 2007). Importantly, zinc alone, but not cocktails of proteasome or lysosome inhibitors without zinc, could cause ZIP5 levels to return to normal (as reported, herein: see Chapter 4) (Weaver et al., 2007). This phenomenon incited our current interest into how *Zip5* is regulated and implied that a novel, zinc-responsive translational stall mechanism may exist.

Several mechanisms regulating translational activity are known (Afonyushkin et al., 2005; Allard et al., 2005; Altuvia et al., 1998; Arrick et al., 1991; Ashizuka et al., 2002; Brengues et al., 2005; Ceman et al., 2003; Gray et al., 1996; Hess and Duncan, 1996; Jackson and Standart, 2007; Laggerbauer et al., 2001; Li et al., 2001; Muralidharan et al., 2007; Parker and Sheth, 2007). Many of these regulatory mechanisms function at the level of translation initiation prior to the assembly of 80S ribosomes on relevant mRNAs (Kapp and Lorsch, 2004; Kong et al., 2008). In the case of iron-responsive target mRNAs that possess iron-regulatory elements (IREs) in their 5'or 3' untranslated regions (UTRs), an iron sensor (either iron-regulatory

protein 1 or 2 (IRPs1 or 2)) binds to the IRE stem-loop during periods of iron deficiency when iron is lost from the Fe-S cluster (Walden et al., 2006). IRPs either block translation of target mRNAs by binding to the 5' UTR, such as with *ferritin heavy chain* and *light chain* mRNAs (Leibold and Munro, 1988; Leibold and Munro, 1988; Munro et al., 1988) or protect an mRNA from degradation by binding to the 3' UTR such as with *transferrin receptor* mRNA (Mullner et al., 1989; Mullner and Kuhn, 1988). In this way, diminished iron storage and enhanced iron acquisition, respectively, are coordinated during iron deficiency.

Some mRNAs are moved to structures called P-bodies for storage or degradation during environmental stress (Teixeira et al., 2005). This mechanism involves a block in translation initiation and is best detailed for the budding yeast, yet homologs of the requisite regulatory proteins have been identified in mammals (Parker and Sheth, 2007).

Another mechanism involves the fragile X mental retardation protein (FMRP) which oligomerizes, is phosphorylated, and associates with the polysomes in the act of translation (Laggerbauer et al., 2001; Li et al., 2001) (Ceman et al., 2003). This may require an interaction with miRNAs to yield translational stall (Jin et al., 2004). In addition, related proteins FXR1P and FXR2P are also RNA-binding proteins that may govern similar post-transcriptional control (Jin et al., 2004).

miRNA-mediated translational regulation has emerged as a widely distributed translational control mechanism (Friedman et al., 2008; Grimson et al., 2007; Vasudevan et al., 2007; Vasudevan and Steitz, 2007; Valencia-Sanchez et al., 2006).

miRNA expression patterns vary amongst tissues, developmental stages, and pathological conditions; thus, these small non-coding RNAs likely have roles in developmental and pathological processes (Ambros, 2004). In animals, miRNAs are small non-coding RNAs that are either dispersed throughout the genome as novel genes or are nested in the introns of host genes. Intergenic miRNAs are transcribed by RNA polymerase III, then capped and polyadenylated to generate pri-miRNAs (primary transcript miRNAs) (Denli et al., 2004). These are then processed by the RNAse Drosha whilst bound to the dsRNA-binding protein Pasha. The hairpin thereby formed is called a pre-miRNA (precursor miRNA) and is transported out of the nucleus in a RanGTP/exportin 5-mediated mechanism (Bohnsack et al., 2004; Yi et al., 2005; Yi et al., 2003) where it is processed further in the cytoplasm by Dicer into an ≈ 22 mer dsRNA with one strand selectively loaded onto one of four argonaute proteins (Pillai et al., 2004; Liu et al., 2004; Meister et al., 2004; Hutvagner et al., 2001).

In contrast to the intergenic miRNAs, intronically-encoded miRNAs are transcribed by RNA polymerase II as a consequence of the promoters of their parental genes (Ruby et al., 2007). Intronic miRNAs are then processed out of the parental mRNA with miRtron (intronic miRNA) lariat formation which is then debranched and folded into a pre-miRNA (Ruby et al., 2007). The resultant pre-miRNA bypasses the Drosha pathway for its formation but is still transported out of the nucleus and processed by Dicer as described above (Ruby et al., 2007).

Regardless of their origins, in animals, these miRNA/ribonucleoprotein complexes often function to affect mRNA translation and/or stability. These effects can result in translational stall (Wang et al., 2006; Valencia-Sanchez et al., 2006), with or without target mRNA degradation (Friedman et al., 2008; Grimson et al., 2007), or even translational stimulation (Vasudevan et al., 2007; Vasudevan and Steitz, 2007).

In this report, we set out to evaluate the hypothesis that *Zip5* is regulated by a rapid post-transcriptional mechanism mediated by the 3' untranslated region of the mRNA in response to zinc availability. Based on a tip from a collaborator (Dr. Christer Hogstrand, Kings College, London), we examined the possible involvement of miRNAs in regulating the translational activity of *Zip5* mRNA in response to zinc. We followed a rationale outlined in a recent review to experimentally validate predicted miRNA targets (Kuhn et al., 2008). This scheme requires the simultaneous satisfaction of four criteria: 1) computational prediction of miRNA-mRNA seed pairs, 2) ΔG analysis of the 3' UTR for the given mRNA to verify that miRNAs target accessible regions, 3) co-expression of both miRNA and mRNA *in vivo*, and 4) a functional assay to demonstrate regulation. In brief, we identified two miRNAs that are predicted by several algorithms to target putatively accessible and evolutionarily conserved regions of the *Slc39a5* (*Zip5*) 3' UTR. Intervening between these target sites, we also identified a region of the *Zip5* 3' UTR that is predicted to generate very stable stem loops reminiscent of the IRE in iron-responsive genes. The predicted miRNAs were found to be expressed in the intestine, visceral yolk sac, and pancreas

and were polysome-associated, suggesting a potential role in translational control of *Zip5*. We are in the process of developing an adequate functional assay to evaluate the link between these miRNAs and the predicted stem-loop structure within the 3' UTR and the translational stall of the *Zip5* mRNA in response to zinc deficiency.

III. Results

A. miRNAs predicted to target *Slc39a5* (*Zip5*)

In collaboration with Dr. Christer Hogstrand from King's College London, we identified a set of miRNAs that are predicted to target the *Slc39a5* (*Zip5*) mRNA. The first list of putative miRNAs was obtained via the publicly available miRanda (miRBase) algorithm. This rendered a list of approximately two dozen putative miRNAs that could potentially target the 3' UTR of *Zip5* mRNA. We next narrowed the possible candidates by utilizing two other previously validated algorithms that also predict miRNA-mRNA seed pairs, namely TargetScan (Friedman et al., 2008; Grimson et al., 2007) and PicTar (Grun et al., 2005; Krek et al., 2005; Lall et al., 2006). All three algorithms predict miRNA-mRNA interactions based on 5' miRNA seed sites in the 3' UTRs of mRNAs; however, their weighting and threshold values for other criteria including site conservation or combinatorial action differ and thus their outputs differ.

As shown in Figure 5.1, three miRNAs, mmu-miR-193, mmu-miR-328, and mmu-miR-137, were predicted by all three algorithms to target the 3' UTR of the

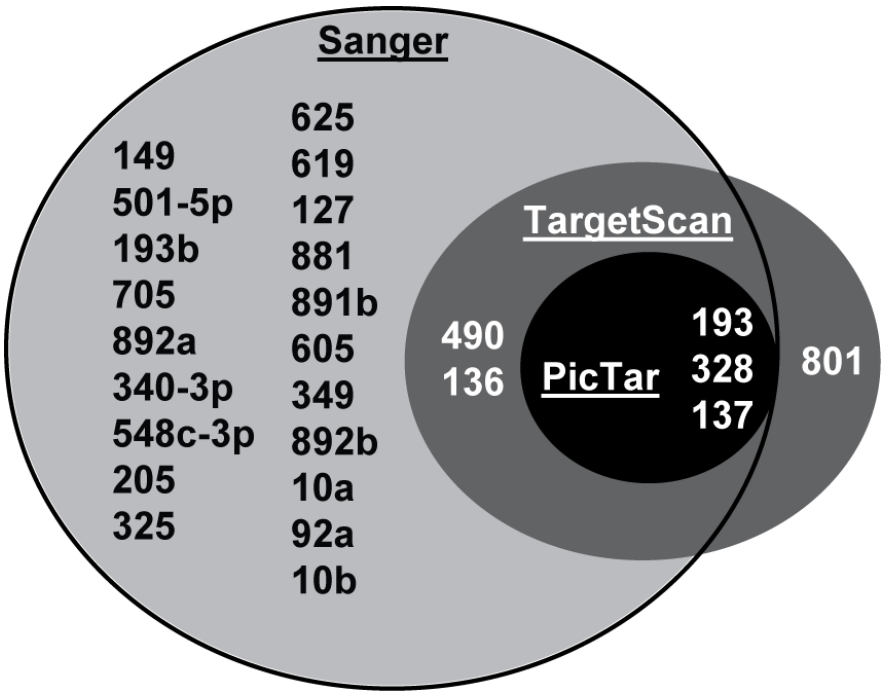


Figure 5.1: Venn-diagram of All Candidate miRNAs Predicted to Target *Slc39a5* (*Zip5*) by Three Algorithms. In this diagram, all shaded regions contained within the perimeter of another shaded region are a subset of the larger set. All miRNAs predicted only by the miRBase algorithm (**Sanger**) are listed in the light gray shaded region. All miRNAs predicted by **TargetScan** are listed in the dark gray shaded region. All miRNAs predicted by **PicTar** are listed in the black shaded region. miRNA-801 is predicted only by the TargetScan algorithm. Both miRNA-490 and miRNA-136 are predicted by Sanger and TargetScan to target the 3' untranslated region (UTR) of *Zip5* mRNA. Whereas, miRNA-193, miRNA-328, and miRNA-137 are predicted by all three algorithms to target the 3' UTR of *Zip5* mRNA.

Zip5 mRNA. For the sake of simplicity, these will be referred to hereafter as simply miR-193, miR-328, and miR-137, respectively with no "mir" versus "miR" distinction given to pre- or mature miRNAs, respectively. Upon examination of the data set, the reasons for this will become clearer. Very noteworthy, PicTar ranked *Zip5* as the highest probability target for miR-193 out of 208 possible targets and as the fourth highest probability target for miR-328 out of 147 targets. This same algorithm ranked *Zip5* as only the fifty-seventh highest probability target for miR-137 out of 468 possible targets. Based on the convergent prediction of the three algorithms, we concluded that one or more of these miRNAs is/are likely involved in the regulation of *Zip5* translation. These results satisfied the first criterion to validate miRNA targets.

B. miR-193 and miR-328 target putatively accessible regions of the 3' UTR of *Zip5* with predicted secondary structures

The majority of experimentally confirmed miRNA-mRNA interactions target putatively accessible regions in the 3' UTR of the mRNA of interest (Kuhn et al., 2008). Thus, the next criterion to consider was whether these predicted miRNAs target accessible regions of the 3' UTR in the *ZIP5* mRNA. First, a putative stem-loop structure representing the lowest free energy folded state for the 3' UTR of *Zip5* mRNA was generated using the publicly available mFold algorithm. As shown in Figures 5.2 and 5.3, miR-193 and miR-328 but not miR-137 target putatively accessible regions of the 3' UTR of *Zip5* mRNA.

Figure 5.2: Predicted Secondary Structure of the Mouse Full Length *Slc39a5* (*Zip5*) 3' Untranslated Region. RNA secondary structures such as this one are predicted by the publicly available mFold software. The 3' UTR sequence of *Zip5* can generate multiple stable stem-loop secondary structures depending on the length of sequence examined. This secondary structure is predicted to be the lowest free energy folding state from the full length 113 nucleotide sequence of the mouse *Zip5* 3' UTR; however, other mammalian species generate similar structures from their orthologous sequences. miRNA seed sites are indicated by bracketed arrows. Two putative seed sites each for **miR-193** and **miR-328** exist. The sites indicated on the left side are the upstream sites and the sites on the right side are the downstream sites. As indicated, the position of the **coding sequence** is left of the stem-loop (upstream) and the **polyA tail** is to the right of the stem-loop (downstream).

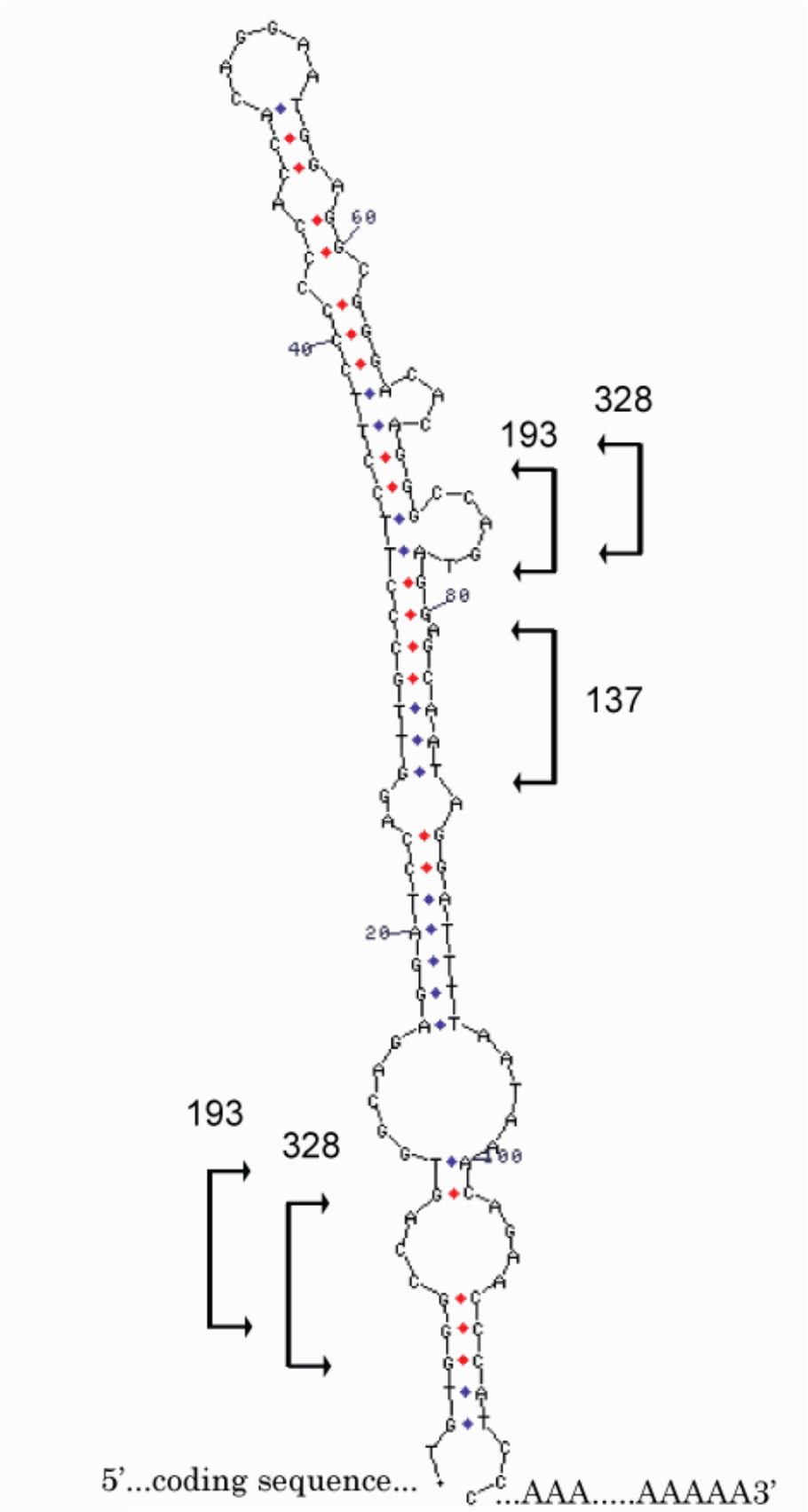
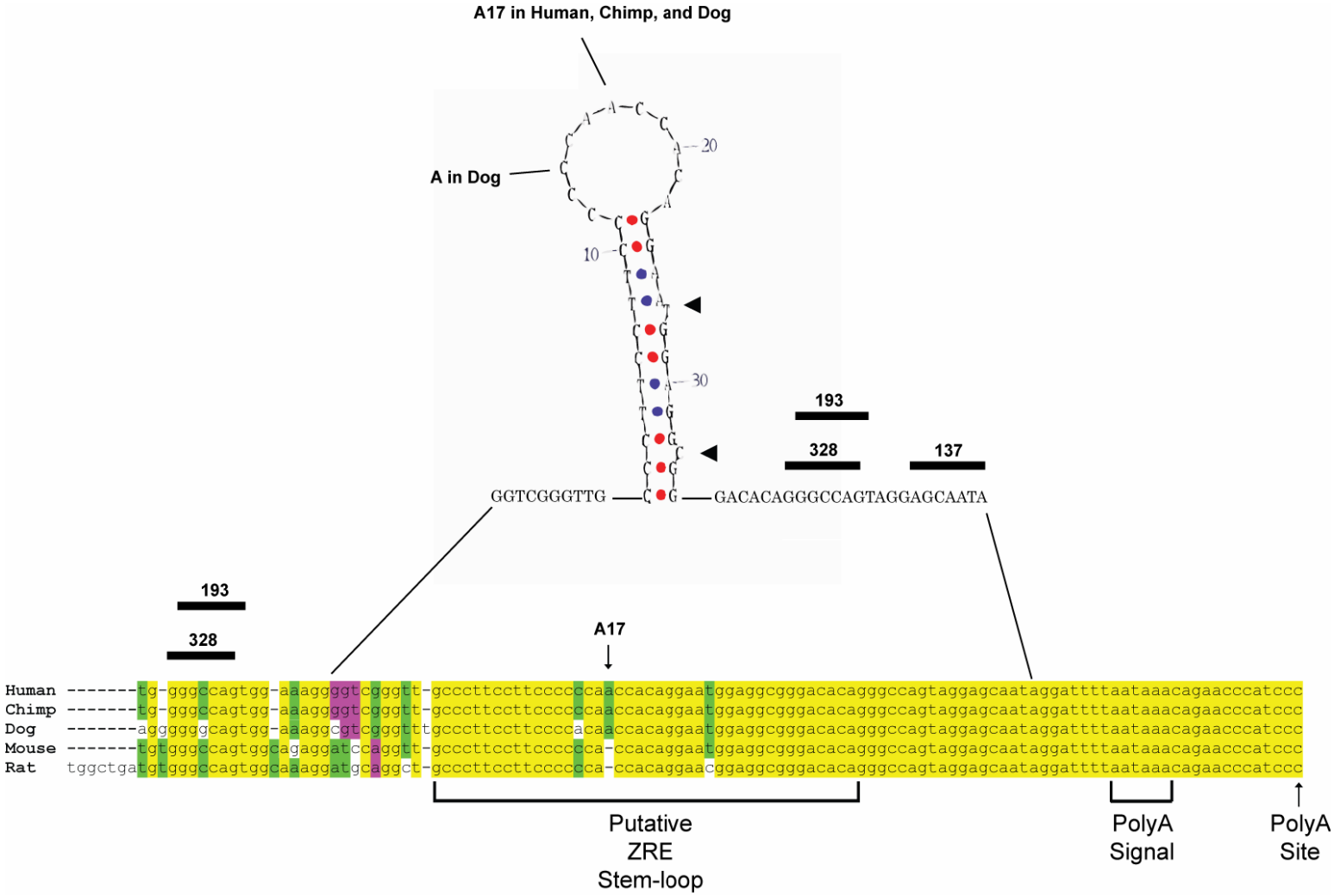

328
193
137
193
328
5'...coding sequence...
...AAA.....AAAAA3'

Figure 5.3: Alignment of *Slc39a5* (*Zip5*) Orthologs for Several Mammalian Species. The full length *Zip5* 3' untranslated regions (UTRs) for **Human, Chimp, Dog, Mouse,** and **Rat** were aligned and annotated with empirical information. The polyadenylation signal (**polyA signal**) and the polyadenylation site (**polyA site**) are indicated. Sequences highlighted in yellow are absolutely conserved amongst all species shown; whereas, the sequences in green and cyan are conserved for two or more species. Analysis of the *Zip5* 3' UTR sequence predicts several highly stable secondary structures (the full length structure is shown in Figure 5.2). A **putative zinc responsive element** (**ZRE**) **stem-loop** is indicated, that is highly conserved and is predicted to give rise to multiple stable stem-loop structures. One of several possible stable stem-loop structures is shown here with the human/chimpanzee sequence as input. This particular stem-loop structure is highly conserved with the indicated features. Humans and chimpanzees have an additional base in the terminal loop absent in the other species (**A17**). Two single nucleotide bulges are also conserved (indicated by **heavy arrow heads**). The first is a T at position 27 (it is a C only in the rat) and the second is a C at position 33 which is conserved for all of the species shown. By convention, miRNA seed sites are indicated with **black bars**.


A17 in Human, Chimp, and Dog
A in Dog
20
10
30
193
328
137
GGTCGGGTTG
GACACAGGGCCAGTAGGAGCAATA
193
328
A17
Human -------tg-gggccagtgg-aaagggtcgggtt-gcccttccttccccccaaccacaggaatggaggcgggacacagggccagtaggagcaataggattttaataaacagaacccatccc
Chimp -------tg-gggccagtgg-aaagggtcgggtt-gcccttccttccccccaaccacaggaatggaggcgggacacagggccagtaggagcaataggattttaataaacagaacccatccc
Dog -------aggggggcagtgg-aaaggcgtcgggttt-gcccttccttccccacaaccacaggaatggaggcgggacacagggccagtaggagcaataggattttaataaacagaacccatccc
Mouse -------tgtgggccagtggcagaggatccaggtt-gcccttccttccccccca-ccacaggaatggaggcgggacacagggccagtaggagcaataggattttaataaacagaacccatccc
Rat tggctgatgtgggccagtggcaaaggatgcaggct-gcccttccttccccccca-ccacaggaacggaggcgggacacagggccagtaggagcaataggattttaataaacagaacccatccc
Putative
ZRE
Stem-loop
PolyA
Signal
PolyA
Site

Remarkably, two seed-pairing sites for miR-193 and miR-328 which both overlap with a one nucleotide shift were detected. Moreover, both sets of sites are conserved across multiple mammalian species. The downstream sites (one overlapping seed site for each miR-328 and miR-193) were in an optimal location (> 15 nucleotides from the stop codon and toward the end of the 3' UTR). This has previously been shown to be a favorable context for the majority of miRNA seed sites (Grimson et al., 2007). The upstream sites had a less favorable location by prediction, positioned within 4-5 nucleotides downstream of the stop codon. Yet, this upstream position has a conserved sequence in humans, chimpanzees, rats, and mice.

Inspection of the top 20 mammalian target mRNAs for both miR-328 and miR-193 revealed that 4 and 5 targets, respectively, also had the other site in the given 3' UTR. Restating, ≈ 22.5% of the top 20 mammalian targets for each miRNA were also targets for the other miRNA (NF2 had six splicing variants and was therefore eliminated from the analysis so as not to skew the weighting; all of the NF2 variants had miR-328 sites but not miR-193 sites). Moreover, for all mRNA targets that had both miR-328 and miR-193 sites, less than half of those sites overlap. Since both seed sites are similar in sequence, this argues that the presence of retained overlapping seed sites for *Zip5* across multiple mammalian species likely persisted as a result of selective pressure in evolution. These results showed that miR-328 and miR-193 target evolutionarily-conserved seed sites in accessible regions of the thermodynamically-predicted lowest folding energy state for the *Zip5* 3' UTR and thereby satisfied the second criterion to validate miRNA targets.

Figure 5.3 demonstrates the high level of sequence conservation for the *Zip5* 3' UTR in mammals. As shown in Figures 5.2 and 5.3, the difference in predicted structure depends entirely on the length of sequence examined. Figure 5.2 shows a predicted structure for the full length 3' UTR sequence in the absence of the coding sequence. Figure 5.3 shows a predicted structure of a 35 nucleotide sequence. This predicted stable stem-loop (putative ZRE stem-loop) is highly conserved amongst all mammalian species shown and is reminiscent of the previously reported IRE structure (Ke et al., 1998).

C. Several different sizes of miR-193 and miR-328 transcripts are detected in mouse pancreas, visceral yolk sac, and intestine but miR-137 is not detected

The TaqMan quantitative RT-PCR method was initially used to determine the relative abundance of the three miRNAs of interest. We found this technique was unable to accurately quantify these miRNAs when compared to Northern blot hybridization. Moreover, Northern blot hybridization performed as previously described for miRNAs (Varallyay et al., 2008; Varallyay et al., 2007) revealed novel sizes of these miRNAs of interest. In this method, locked-nucleic acid probes were used due to their better mismatch discrimination and higher sensitivity than conventional DNA probes (Varallyay et al., 2008).

Tissues known to regulate *Zip5* (pancreas, intestine, and visceral yolk sac (VYS)) as well as two cell lines that do not express *Zip5* were examined for miR-328, miR-193, and miR-137 transcripts. As shown in Figures 5.4 and 5.5, miR-328 (upper

Figure 5.4: Northern Blot Analyses of miR-328, miR-193, and miR-137 Transcripts in Intestine, VYS, and Cell Lines. The intestine, VYS, and two cell lines were chosen for analyses by Northern blot hybridization. Pregnant dams were fed zinc adequate (**ZnA**) or zinc deficient (**ZnD**) diet beginning on day 8 of pregnancy (day 1 = day of plug). Visceral yolk sac (**VYS**) and intestine (**Int**) were dissected at day 14 and snap frozen in liquid nitrogen. **Hepa** and CaCo2 (**CaCo**) cells were cultured in **ZnA** (10% FBS in DMEM) or **ZnD** (10% chelex-treated FBS in DMEM) media for 48 hours and snap-frozen in liquid nitrogen. Total RNA (4 μg per well), extracted with Trizol according to recommendations of the manufacturer, was size-fractionated under denaturing conditions in pre-run 12.5% acrylamide gel and transferred overnight to Zetaprobe membranes. Membranes were UV-cross-linked, blocked, hybridized for 24-72 hours with γ^{32}P-end-labeled locked nucleic acid probes (exiqon.com), and then washed with stringent conditions. Membranes were exposed to film at -80°C. An RNA ladder was used to estimate the nucleotide length of detected bands (**Est. nt**). The nucleotide lengths of several marker bands are indicated. To ensure RNA quality and equal loading, a duplicate gel was stained with ethidium bromide (**EtBr**) and de-stained overnight (**bottom panel** overexposed to show resolution of smaller RNAs). Large RNA species remained at the top of the wells whilst smaller RNAs were resolved. The smallest bands visible in the EtBr stain correspond to > 55 nt. The bottom half of the gel is thus not shown.

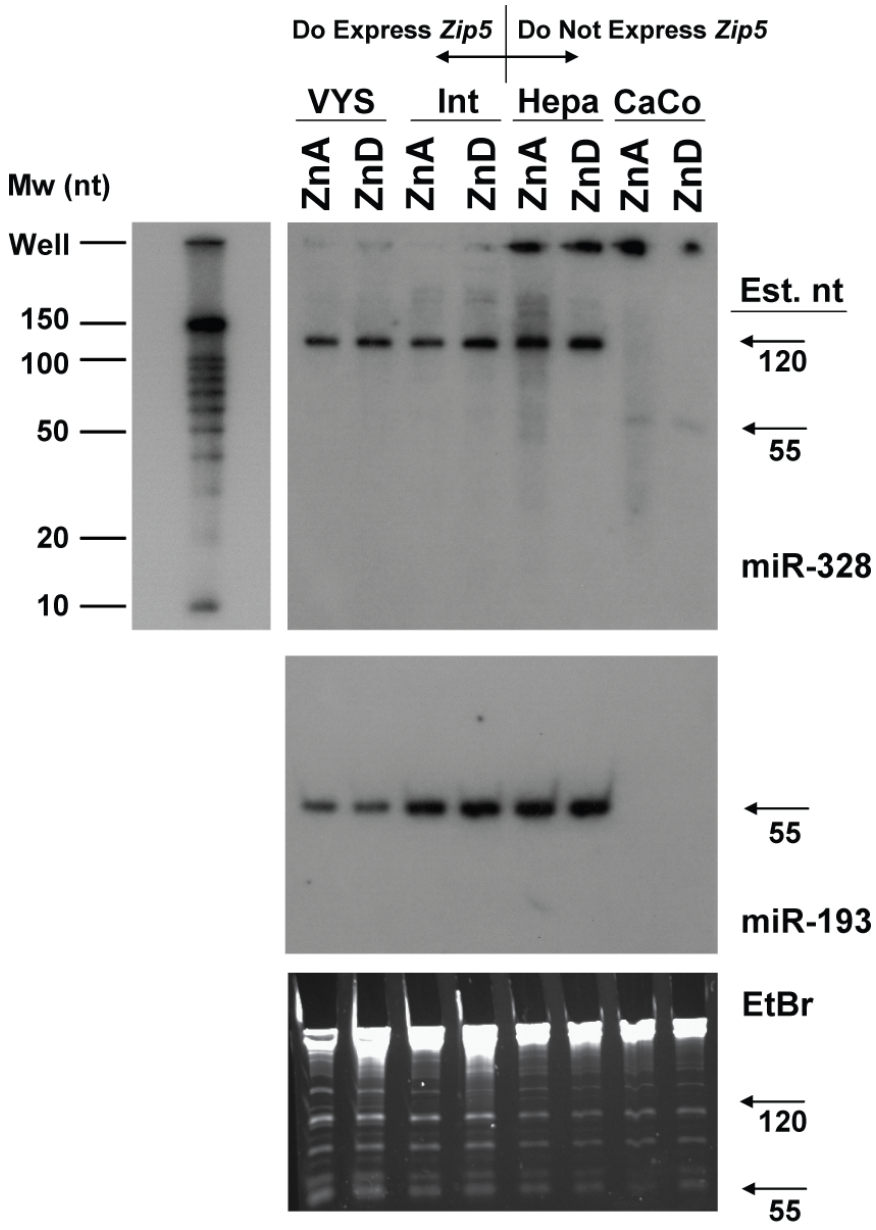
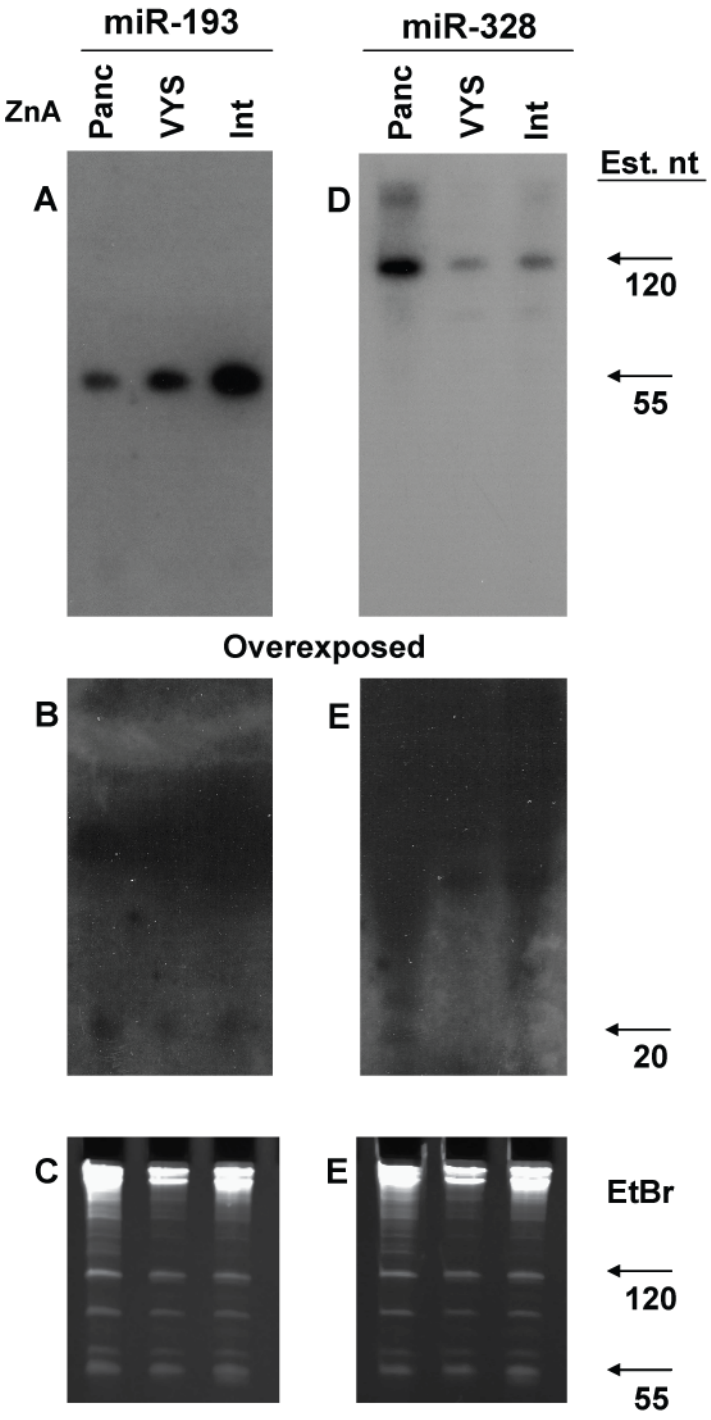

Do Express Zip5
Do Not Express Zip5
VYS
Int
Hepa
CaCo
ZnA
ZnD
ZnA
ZnD
ZnA
ZnD
ZnA
ZnD
Mw (nt)
Well
150
100
50
20
10
Est. nt
120
55
miR-328
55
miR-193
EtBr
120
55

Figure 5.5: Northern Blot Analyses of miR-328 and miR-193 Transcripts in Pancreas, VYS, and Intestine. The pancreas, VYS, and intestine were analyzed by Northern blot hybridization. Pregnant dams were fed zinc adequate (**ZnA**) or zinc deficient (**ZnD**) diet beginning on day 8 of pregnancy (day 1 = day of plug). Pancreas (**Panc**), Visceral yolk sac (**VYS**) and intestine (**Int**) were dissected at day 14 and snap frozen in liquid nitrogen. RNA was extracted and miRNA detected as described in the legend to Figure 5.4. **miR-193** (**panels A and B**) was predominantly detected at ≈ 55 nt with a minor band detected at ≈ 22 nt (see overexposed blot in **panel B**). **miR-328** (**panels D and E**) was predominantly detected at ≈ 120 nt with a minor band detected at ≈ 22 nt (see overexposed blot in **panel E**). To ensure RNA quality and equal loading, gels were stained with ethidium bromide (**EtBr**) and de-stained (**panels C and F**). The smallest bands visible in the EtBr stain correspond to > 55 nt. The bottom half of the gel is thus not shown.


miR-193
miR-328
ZnA
Panc
VYS
Int
Panc
VYS
Int
Est. nt
A
D
120
55
Overexposed
B
E
20
C
E
EtBr
120
55

panel) was detectable in VYS, intestine, pancreas, and Hepa cells with a major band detected at ≈ 120 nucleotides. CaCo2 cells had a faint band detected repeatedly at ≈ 55 nucleotides. miR-193 (middle panel) was detected in VYS, intestine, pancreas, and Hepa cells (with a major band detected at ≈ 55 nucleotides) but was not detected in CaCo2 cells. miR-193 levels differed by cell type but not by zinc availability. Not shown is the miR-137 blot that had no signal detected in any of the tissues or cells. Notably, the predominant species of miR-328 and miR-193 detected corresponded more closely to the precursor sizes and not the expected "mature" forms of ≈ 22 nucleotides. The predominant forms detected for miR-328 was about 120 nucleotides and for miR-193 about 55 nucleotides. Detection of the mature ≈ 22 mers is shown below.

Mature miR-328 is derived from intron 12 (short transcript) or 13 (long transcript) from the putative *elmo3* gene on mouse chromosome 8. The precursor hairpin (97 nucleotides) is predicted to include 12 bases of exon 13 (or 14). In the rat, the entire precursor is wholly intronic in *elmo3* (by prediction). Mouse *elmo3* was previously found to be expressed at moderate levels in many tissues (Keegan et al., 2005).

As shown in Figure 5.6, the miR-328 precursor is predicted to be 97 nucleotides in length; whereas, intron 12 (or 13) of mouse *elmo3* is predicted to be 108 nucleotides in length. Annotation of the putative *elmo3* gene in Ensembl predicts that the 97 nucleotide miR-328 precursor only partially overlaps the putative 108 nucleotide intron and overlaps exon 13 (or 14) in the mouse by 12 bases. However,

Figure 5.6: Precursor and Mature miRNA Sequences for miR-328 and miR-193. These **precursor hairpin and mature miRNA** ≈ 22 mers are taken from the publicly available miRBase website. The hairpins show distinctive stem-loop structures indicative of miRNA precursors. **miR-328** is derived from intron 12 (or 13) from either of two alternative transcripts of the putative *elmo3* gene. The predicted 97 nucleotide hairpin precursor is shown for miR-328. The predicted hairpin is thought to generate a mature miRNA corresponding to nucleotides 61-82 in the hairpin. **miR-193** is intergenic. The putative 66 nucleotide sequence hairpin precursor is shown for miR-193 and is predicted to generate both **major** and **minor** products from the same precursor hairpin—corresponding to nucleotides 41-62 and 7-28, respectively, in the hairpin. The LNA probe detects the major product (and the precursor).

the bases at positions 11 and 12 in the exon are an AG-dinucleotide that could function as a downstream in-frame splice acceptor and exclude this small non-coding RNA entirely from the coding sequence and thereby allow its removal as an intronic miRNA (miRtron). Conceivably, the putative 108 nucleotide intron plus the putative 12 nucleotide exon overlap add up to the observed 120 nucleotide product consistently detected by Northern blot hybridization.

As shown in Figure 5.6, the miR-193 precursor is predicted to be 66 nucleotides in length. However, we consistently detected a band ≈ 55 nucleotides in length. Perhaps nuclease trimming of the precursor could generate this predominant form. To the best of our knowledge, this is the first report for such novel forms of expression for miR-328 and miR-193. Nonetheless, these results reveal that miR-328 and miR-193 are co-expressed in pancreas, visceral yolk sac, and intestine, all of the tissues known to regulate *Zip5* and thereby satisfied the third criterion to validate miRNA targets.

D. Differential association of miR-193 and miR-328 with the polysome fraction

The predominant signals detected for miR-193 and miR-328 corresponded closely to pre-miRs in size and their steady-state levels did not change with zinc availability. We thus examined the association of these miRNAs with polysomes because *Zip5* mRNA remains polysome-associated during translational stalling (shown in Chapter 4). We reasoned that the mature miRNAs might be enriched in this fraction. Two crude fractions of RNAs were prepared from either the polysome

pellet (= the polysome fraction) or a high-speed soluble fraction (= the soluble fraction) from the VYS. As shown in Figure 5.7, differences were found in the extent of association of the two miRNAs with the soluble and polysomal fractions, but both were polysome associated regardless of zinc availability. miR-328 was predominantly polysome-associated whereas miR-193 was predominantly in the soluble fraction with a small amount polysome-associated. The fraction of miR-193 that was soluble appears to show zinc-dependent differences in the abundance of a 45 nucleotide band; however, this result needs to be confirmed.

The above results establish that these miRNAs can, in fact, associate with the polysome fraction (or some other high molecular weight fraction that co-sediments with polysomes) in the VYS. After overexposure of the blots, ≈ 22 mer forms of these miRNAs were detectable in the polysome fraction. Nonetheless, the predominant polysomal form was ≈ 120 nucleotides long for miR-328 and ≈ 55 nucleotides long for miR-193.

As shown in Figure 5.8, zinc availability had no significant effect on miR-328 or miR-193 abundance or size in either the soluble or polysomal fraction. An ≈ 40 nucleotide band was more readily visible for miR-193 in the soluble fraction but the relevance is unknown. Northern blot analysis of *Zip4* and *Zip5* mRNAs from the soluble and polysomal fractions revealed that these mRNAs were in the polysomal fraction, thus strengthening the conclusion that we had adequately enriched polysomes out of solution (data not shown). However, these data do not exclude the

Figure 5.7: Northern Blot Detection of miR-193 and miR-328 in Soluble and Polysomal Fractions from the Visceral Yolk Sac. Pregnant dams were fed zinc adequate (**ZnA**) or zinc deficient (**ZnD**) diet beginning on day 8 of pregnancy (day 1 = day of plug). Visceral yolk sacs (**VYS**) were dissected out of day 14 pregnant dams and snap frozen in liquid nitrogen. VYS were homogenized and processed to yield a high speed polysome pellet and a soluble fraction. RNA for the soluble (S**ol**) and polysomal (**Poly**) fractions was extracted with Trizol and resuspended in equivalent volumes. VYS were homogenized and processed to yield a high speed polysome (**Poly**) pellet and a soluble (**Sol**) fraction and RNA was extracted and miRNA detected as described in the legend to Figure 5.4. Right side panels in Figure 5.7 are **overexposed**. **miR-328** (**panels A and D**) was predominantly polysome-associated with a major band detected at ≈ 120 nt and a minor band detected at ≈ 22 nt (see overexposed blot in **panel D**). **miR-193** (**panel B**) was predominantly soluble but also had a polysome-associated fraction with a major band detected at ≈ 55 nt. A fainter ≈ 40 nt band was detected in the soluble fraction and a very faint ≈ 22 nt band was detected in the polysomal fraction (**panels E and F** are two independent blots of miR-193 shown to demonstrate the presence of a faint ≈ 22 nt band in the polysomal fraction). To ensure RNA quality, loading, and fractionation, gels were stained with ethidium bromide (**EtBr**) and de-stained (**panel C** overexposed to show resolution of smaller RNAs). Lower half of gel not shown.

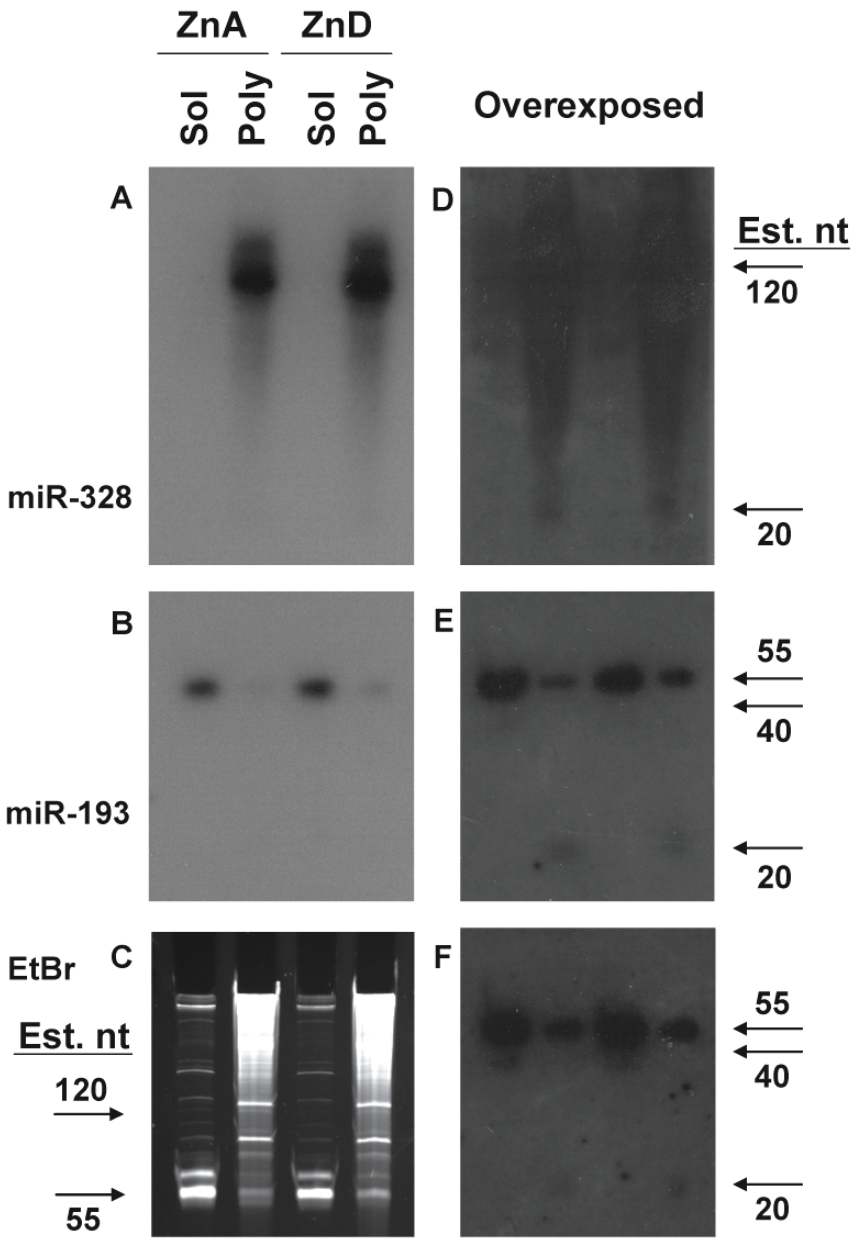
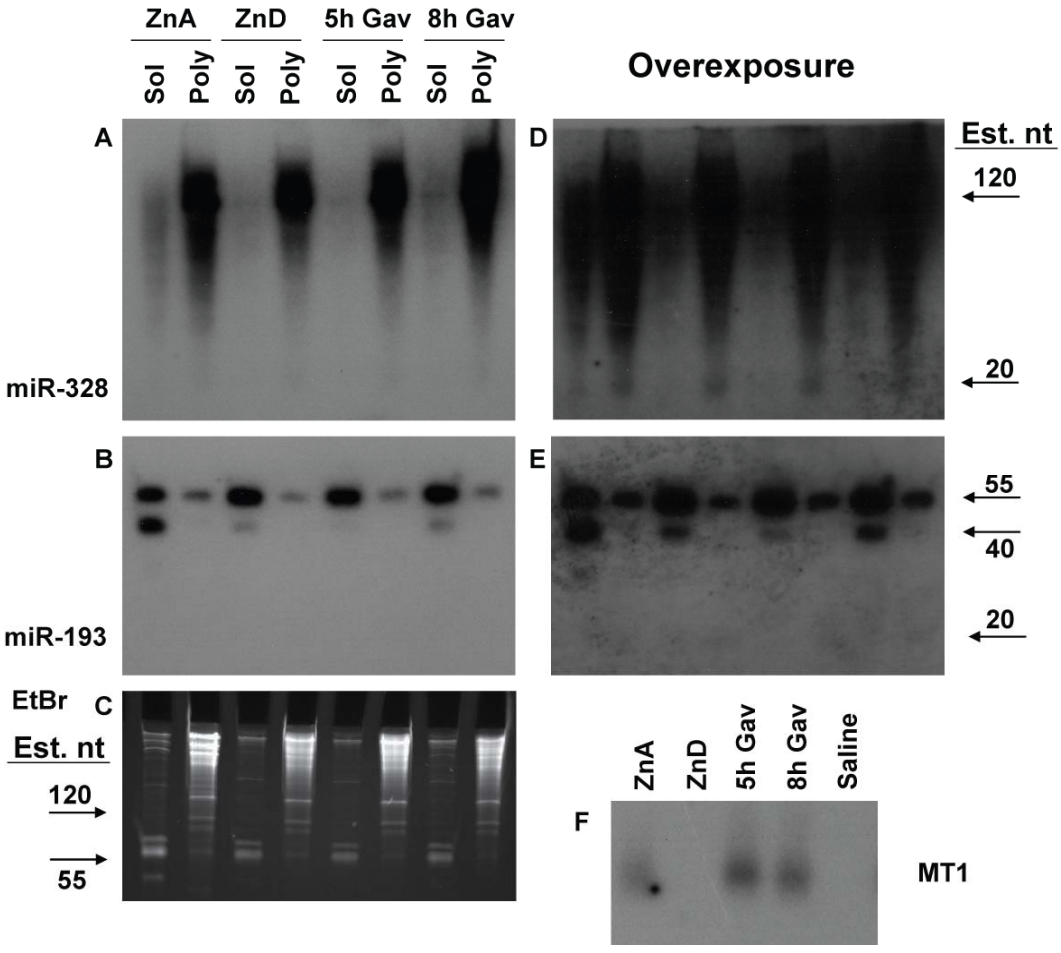

ZnA
ZnD
Sol
Poly
Sol
Poly
Overexposed
A
D
Est. nt
120
miR-328
20
B
E
55
40
miR-193
20
EtBr
C
F
Est. nt
120
55
55
40
20

Figure 5.8: Northern Blot Detection of miR-193 and miR-328 in Soluble and Polysomal Fractions from Visceral Yolk Sac with Zinc Gavage. As described in the legend to Figure 5.7, pregnant dams were fed zinc adequate (**ZnA**) or zinc deficient (**ZnD**) diet beginning on day 8 of pregnancy (day 1 = day of plug). Some of the ZnD dams were repleted with zinc by an oral gavage of $ZnCl_2$ (Weaver et al., 2007) and VYS were collected at 5 (**5h Gav**) or 8 (**8h Gav**) hours following gavage. VYS were homogenized and processed to yield a high speed polysome (**Poly**) pellet and a soluble (**Sol**) fraction and RNA was extracted and miRNA detected as described in the legend to Figure 5.4. **miR-328** (**panels A and D**) was predominantly polysome-associated with a major band detected at ≈ 120 nt and a minor band detected at ≈ 22 nt (**panel D**). **miR-193** (**panel B**) was mostly associated with the soluble fraction but a minor polysome-associated fraction with a prominent band at ≈ 55 nt was detected. A Northern blot for *metallothionein 1* (**MT1)** was used to monitor zinc effects in the VYS at 5 and 8 hours following gavage as previously described (Weaver et al., 2007) (**panel F**). In addition to oral gavage with zinc (**5h** and **8h Gav**), we gavaged some mice with normal saline (**Saline**) as a control for stress. Zinc gavage but not saline upregulated *MT1* indicating these mice responded to zinc repletion.


ZnA
ZnD
5h Gav
8h Gav
Sol
Poly
Sol
Poly
Sol
Poly
Sol
Poly
Overexposure
A
miR-328
D
Est. nt
120
20
B
miR-193
E
55
40
20
EtBr
C
Est. nt
120
55
ZnA
ZnD
5h Gav
8h Gav
Saline
F
MT1

possibility that these miRNAs may associate with so-called heavy miRNPs that can co-sediment with polysomes as shown before (Thermann and Hentze, 2007) (considered in the discussion section).

IV. Discussion

Previously, we have shown that *Zip5* is regulated rapidly in response to zinc availability (Dufner-Beattie et al., 2004; Weaver et al., 2007). In fact, ZIP5 protein is destabilized during zinc deficiency but rapidly reaccumulates on the basolateral membranes of intestinal enterocytes, visceral endoderm, and pancreatic acinar cells following zinc repletion (Weaver et al., 2007). These changes in the protein expression occur without changes in the level of mRNA which remains polysome-associated regardless of zinc availability. Moreover, our previous data (see Chapter 4) support a translational stall mechanism in the regulation of *Zip5* in response to zinc availability. Various mechanisms for translational regulation have been published and many involve sequences in the untranslated regions of the given mRNA.

We show herein that the *Zip5* 3' UTR is highly conserved across multiple mammalian species and that this UTR is predicted to generate several highly stable stem-loop structures. Moreover, as we show in this report, miR-328 and miR-193 are predicted to target the 3' UTR and may be involved in the regulation of *Zip5* expression in response to zinc availability. miR-328 and miR-193 are entirely or partially associated with the polysome fraction. miR-193 may show some zinc-

dependent differences. Interestingly, both of these miRNAs target very similar regions of the 3' UTR.

Recently, miR-398 in *Arabidopsis thaliana* was found to regulate both cytosolic *Cu/Zn superoxide dismutase* and *Cox5b-1* (a cytochrome c oxidase subunit) by the degradation of these mRNAs in response to copper limitation (Yamasaki et al., 2007a). In addition, the *plastocyanin* mRNA was similarly down-regulated by miR-397, miR-408, and miR-857 (bdel-Ghany and Pilon, 2008). Unlike mammals, miRNAs always target the degradation of their cognate mRNAs in *A. thaliana.* Nonetheless, these studies give precedent for the regulation of metal-responsive genes by post-transcriptional mechanisms involving miRNAs.

As a cautionary note, qRT-PCR as well as microarray techniques cannot distinguish precursor miRNAs from mature miRNAs. Moreover, both qRT-PCR and microarray analyses are highly prone to quantitative errors for miRNAs expressed at low levels (our personal experience for numerous miRNAs when subsequently assessed by Northern blot; data not shown). However, the Northern blot technique is the only method that allows visual discrimination of precursors from mature forms, since it includes a size fractionation step prior to blotting. Sadly, the current literature has a real dearth of studies using the Northern blot technique to measure miRNA levels. Many more studies rely entirely on qRT-PCR and microarray methods because of their high throughput capacity. The data herein show without question that the predominant forms of miR-328 and miR-193 correspond more closely in size

to the precursors than to the predicted mature forms. Moreover, Trizol has been used as the preferential RNA isolation method herein and has been shown to reliably capture ≈ 22 mers as detected by the validated Northern blot data already published by others (Varallyay et al., 2008; Varallyay et al., 2007) and consistent with our findings with Let-7b, miR-26a, and other miRNAs in unrelated studies (data not shown).

The canonical annotation of mature miRNAs as ≈ 22 mers has arisen out of historical precedent for early findings of small non-coding regulatory RNAs. In fact, all cloning strategies for mature miRNAs have the *a priori* parameter to enrich for RNAs < 24 nucleotides in length. This practice may have resulted in an inappropriate delimitation on the size variability of small regulatory non-coding RNAs. In support of this contention, Piwi-interacting RNAs (piRNAs) were discovered to function as slightly larger than classical miRNAs at ≈ 24-30 nucleotides in length but primarily serve to repress so-called selfish DNA elements in the germline, such as transposons (Aravin et al., 2008; Aravin et al., 2007; Aravin et al., 2003; Brennecke et al., 2008). This, of course, is not to say that classical ≈ 22 mer miRNAs do not have important regulatory functions. Rather, with time, miRNAs may be found to be more heterogeneous in size and function or that they themselves are subject to post-transcriptional regulation where they are processed differentially in response to stressors or stimuli.

In light of no dramatic zinc-dependent changes in expression or polysome association of the miRNAs shown in this study, it is interesting to note that miR-328 and miR-193 both have two seed sites that significantly overlap in the 3' UTR of *Zip5* mRNA. This compelled us to consider the possibility of antagonistic action between these two miRNAs especially considering that both miRNAs are available in polysome fractions. In this speculative scheme, the activity of protein factors in the miRNPs may change with availability of zinc and this activity cannot be visualized by measuring the steady-state levels of these miRNAs. Moreover, both miRNA precursors and predicted mature forms have favorable annealing energies which may allow for antisense regulation of each other. This latter possibility has no precedent in the literature. Future work will need to consider these possibilities.

Future work will also need to determine puromycin sensitivity of the *Zip5* mRNA/protein complex to distinguish *bona fide* polysomes in the state of translational stall from heavy miRNPs (pseudo-polysomes) that have been shown to co-sediment with polysomes (Thermann and Hentze, 2007). If puromycin treatment shifts *Zip5* mRNA to the subpolysome (soluble) fraction, then this will demonstrate that *Zip5* mRNA is in fact translationally stalled. It is highly unlikely that *Zip5* mRNA is not associated with polysomes since the polysome fractionation shown in Chapter 4 yielded similar distributions of the mRNA across the sucrose gradient in both zinc adequate and zinc deficient samples.

To demonstrate regulation of *Zip5* translation by miR-193 and miR-328, we made reporter constructs with the full length or a mutant version of the *Zip5* 3' UTR immediately following the firefly luciferase coding sequence as shown in Figure 5.9. All data were normalized to a co-transfected renilla luciferase construct with the bovine growth hormone 3' UTR. We have been unable to demonstrate repeatable zinc-responsive regulation. We have tried to develop other functional assays including use of reticulocyte lysates programmed with small quantities of capped and polyadenylated reporter and control mRNAs made *in vitro* with the mMESSAGE mMACHINE kit (ambion.com). Further work is required to confirm the feasibility and utility of these systems.

Very recently, a report shows that the choice of promoter for the transfected reporter cassette can affect whether an mRNA is silenced at a pre-initiation or a post-initiation step (Kong et al., 2008). With relevance to this research, the recent paper showed that constructs using the CMV promoter were regulated by a pre-initiation translational mechanism which resulted in the mRNA reporter being shunted to the subpolysomal (soluble) fraction. Those findings are relevant to this work since our previous data (shown in Chapter 4) demonstrated that *Zip5* mRNA is always polysome-associated and thus most likely regulated by a post-initiation translational mechanism. In this way, the difficulty in developing a cell-culture-based functional

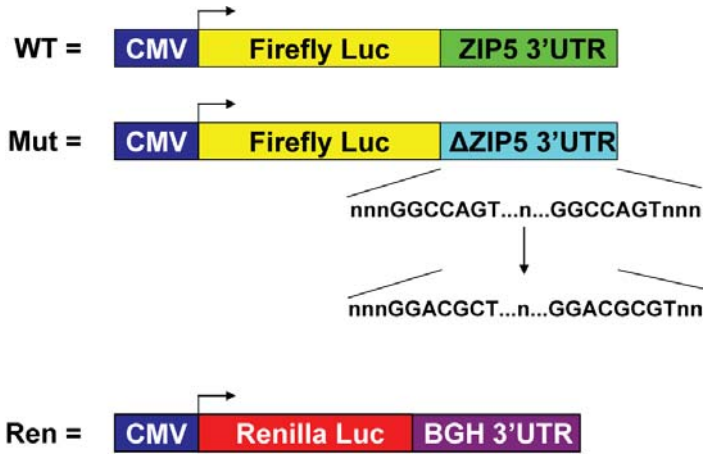


Figure 5.9: Expression Constructs Used in Transient Transfection Assays for Functional Assays. Expression constructs were made to test the ability of the *Slc39a5* 3' untranslated region (**ZIP5 3'UTR**) to confer zinc-responsive post-transcriptional regulation on luciferase reporter constructs. The full length wild type (**WT**) and mutated (**Mut, ΔZIP5 3'UTR**) versions of the *Zip5* 3' UTR were subcloned immediately downstream of the firefly luciferase (**Firefly Luc**) coding sequence including a stop codon. These fragments were placed downstream of the hCMV minimal promoter (**CMV**) in the pcDNA3.1 Hygro(+) vector (Promega). A construct utilizing renilla luciferase (**Renilla Luc**) had the bovine growth hormone 3' UTR (**BGH 3'UTR**) placed downstream (**Ren**) and was used to normalize values. Cells were transfected with low titrations of the luciferase constructs with or without miRNA expression vectors (expressing the mature or pre-miR sequences) or with or without pre-miRNAs directly.

assay may be explained, in part, by an inappropriate choice of promoter. Thus, future experimentation should consider promoter effects by employing alternate promoters. In addition, other convergent approaches will be used to determine the possible link between the miRNA species shown herein and the translational regulation of *Zip5*.

Nonetheless, these data demonstrate the existence of two miRNAs localized to the polysomes and expressed in tissues known to regulate *Zip5*, visceral yolk sac and intestine. Moreover, these miRNAs are conserved and predicted to target accessible regions of the *Zip5* 3' UTR. Remarkably, these miRNAs were found to predominantly accumulate as longer species than the expected mature forms.

Chapter 6

Review

The Genetics of Essential Metal Homeostasis during Development

Citation of Chapter Publication

Kambe T., Weaver B.P., Andrews G.K. 2008. The genetics of essential metal homeostasis during development. *Genesis*. 46(4): 214-228. Review. PMID: 18395838.

Contributions to the Chapter

I was primarily responsible for the sections “Regulation of Iron Homeostasis” and “Iron Homeostasis and Development” including Figure 6.2 and Table 6.2. All authors contributed concepts for discussion and approved the final version.

I. Abstract

The essential metals copper, zinc and iron play key roles in embryonic, fetal and postnatal development in higher eukaryotes. Recent advances in our understanding of the molecules involved in the intricate control of the homeostasis of these metals and the availability of natural mutations and targeted mutations in many of the genes involved have allowed for elucidation of the diverse roles of these metals during development. Evidence suggests that the ability of the embryo to control the

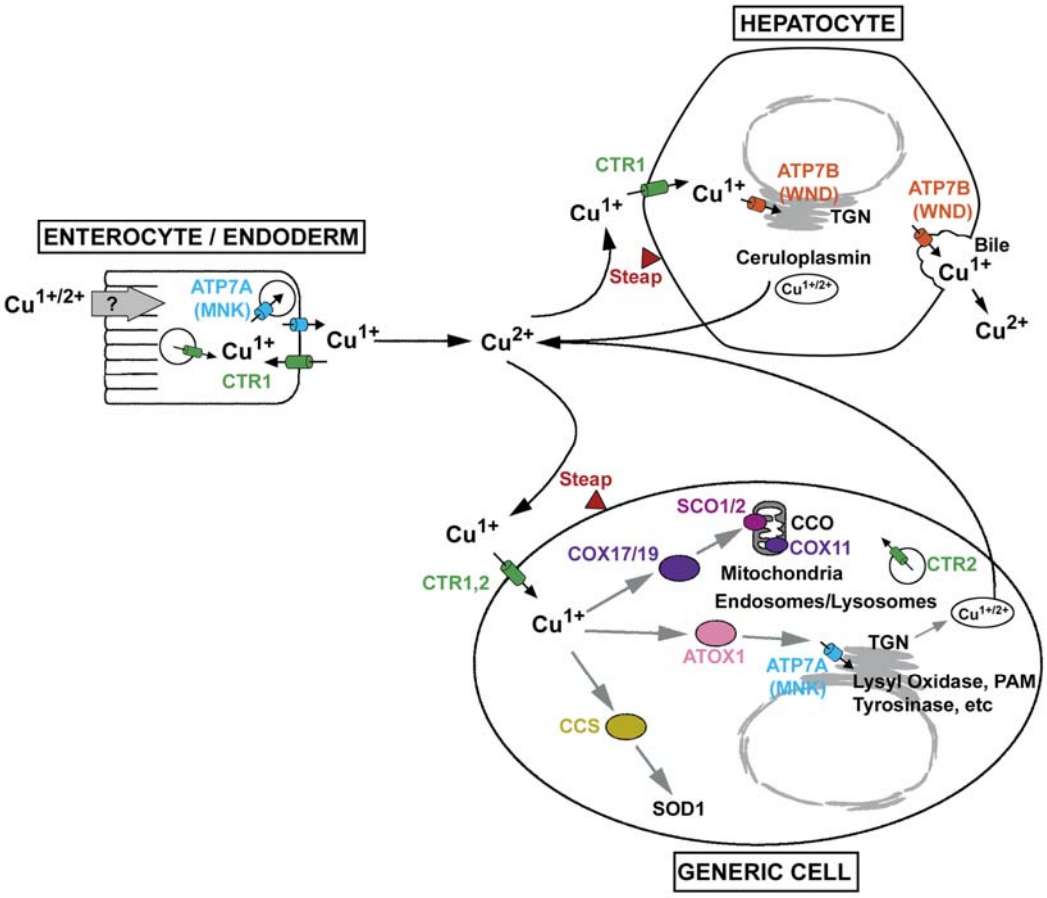
homeostasis of these metals becomes essential at the blastocyst stage and during early morphogenesis. However, these metals play unique roles throughout development and exert pleiotropic, metal-specific and often cell-specific effects on morphogenesis, growth and differentiation. Herein, we briefly review the major players known to be involved in the homeostasis of each of these essential metals and their known roles in development.

II. Regulation of Copper Homeostasis

Copper plays a critical role in metabolism as a cofactor for several cupric enzymes, including cytochrome *c* oxidase (CCO), lysyl oxidase, tyrosinase, copper/zinc superoxide dismutase (SOD1) and ferroxidases (Pena et al., 1999; Shim and Harris, 2003; Prohaska and Gybina, 2004). However, copper is a potentially toxic metal because of its ability to redox cycle and support fenton chemistry leading to the production of freely radicals (Halliwell and Gutteridge, 1984). Therefore, copper homeostasis is very tightly regulated (see Figure 6.1). Copper deficiency stimulates the absorption of copper by the intestine (Kuo et al., 2001) but the mechanisms of uptake of dietary copper are not understood (Maryon et al., 2007). CTR1 (Slc31a1), a high affinity, trimeric copper transport protein is essential for acquisition of dietary copper (Nose et al., 2006). CTR1 has been localized to basolateral and intracellular membranes of enterocytes in the adult (Kuo et al., 2006; Zimnicka et al., 2007) and thus does not appear to play a role in the uptake of dietary copper in the adult, although it has been localized to the apical surface of the

Figure 6.1: Overview of Copper Homeostasis. Copper is oxidized (**Cu^{2+}**) in the intestinal lumen and in the serum, but is reduced to **Cu^{1+}** before transport into cells. The **Steap** ferric/cupric reductases (Steaps 2, 3 and 4) are localized to endosomes/lysosomes and the plasma membrane and may be essential for the reduction of copper, as well as the reduction of iron (Ohgami et al., 2006). Copper is transported into **ENTEROCYTES/ENDODERM** cells by unknown mechanisms (endocytosis?). **CTR1** is essential for the acquisition of dietary copper but its function in the enterocyte is unknown. In many other cell types (**GENERIC CELL**), copper is taken up by **CTR1** localized to the plasma membrane and perhaps also by **CTR2** (Slc31a2). Copper taken up by **ENTEROCYTES/ENDODERM** is exported into portal blood/conceptus, respectively, by **ATP7A** (Menke's disease protein, **MNK**) which is localized to vesicles trafficking toward the basolateral membrane and to the basolateral membrane. Copper exported to portal blood is taken up into the liver, the primary organ that regulates copper homeostasis. In the **HEPATOCYTE**, **ATP7B** (Wilson's disease protein, **WND**) effluxes excess copper into the bile and puts copper into the *trans*-Golgi network (**TGN**) where it is loaded into **ceruloplasmin**, a ferroxidase that is the primary copper binding protein in serum. Inside the cell (**GENERIC CELL**), copper is distributed to cytoplasmic copper chaperones (**COX17/19, ATOX1, CCS**) which, in turn, deliver copper to mitochondrial inner membrane and ultimately cytochrome C oxidase (**CCO**) or to **ATP7A** in the **TGN**, and cytoplasmic **SOD1**, respectively. The assembly of copper into CCO is an active area of investigation. It is thought that copper in **COX17/19**

(and probably COX23/MTCP1) is thought to be first transferred to both **COX11** and **SCO1/2** and ultimately to **CCO.** **ATP7A** transports copper into the TGN and activates copper containing secretory and membrane-bound proteins (**lysyl oxidase, tyrosinase, peptidylglycine alpha-amidating monooxygenase (PAM)**). It should be noted that the cellular localization and abundance of **CTR1**, **ATP7A** and **ATP7B** are dynamically regulated by copper availability which is not reflected in this static cartoon.


HEPATOCYTE
ENTEROCYTE / ENDODERM
Cu1+/2+
?
ATP7A
(MNK)
Cu1+
CTR1
Cu1+
Cu2+
Steap
CTR1
Cu1+
ATP7B
(WND)
TGN
Ceruloplasmin
Cu1+/2+
ATP7B
(WND)
Bile
Cu1+
Cu2+
Steap
Cu1+
CTR1,2
SCO1/2
CCO
COX11
COX17/19
Mitochondria
CTR2
Endosomes/Lysosomes
Cu1+/2+
Cu1+
ATOX1
TGN
ATP7A
(MNK)
Lysyl Oxidase, PAM
Tyrosinase, etc
CCS
SOD1
GENERIC CELL

enterocyte in the suckling pup (Kuo et al., 2006). Intestine-specific *Ctr1*-knockout mice accumulate copper in the intestine (Nose et al., 2006). The subcellular localization and abundance of CTR1 are cell-type specific (Klomp et al., 2002; Kuo et al., 2006). Copper is exported from enterocytes into the blood stream via the ATP7A P-type ATPase transporter which is localized to the basolateral membrane and/or secretory pathway (Nyasae et al., 2007; Hamza et al., 2003). The importance of ATP7A in copper homeostasis is revealed by the fact that copper export from enterocytes is impaired in patients with Menke's disease (MNK) and in mice with mutations in the *Atp7a* gene (*mottled/brindled* mouse), resulting in a severe copper deficiency phenotype (Mercer, 1998). Copper homeostasis is further regulated by the liver which sequesters copper absorbed from the diet (Lutsenko et al., 2007). Hepatocytes are important for copper loading into ceruloplasmin, a ferroxidase, which is the primary copper-binding protein in serum, and excess copper is excreted into the bile (Lutsenko et al., 2007; Shim and Harris, 2003). Both of these processes are regulated by ATP7B P-type ATPase transporter. Patients with Wilson's disease (WND) have mutations in *Atp7b* resulting in the hyper-accumulation of copper in the liver because of impaired excretion of copper into the bile and secretory pathway (where copper is loaded into ceruloplasmin).

Copper deficiency in neonatal mice leads to increase levels of CTR1 in several organs (Kuo et al., 2006) whereas highly elevated copper stimulates its rapid endocytosis and degradation (Petris et al., 2003). In the mammary gland, prolactin increases the abundance and plasma membrane localization of CTR1 and ATP7A (Kelleher and

Lonnerdal, 2006a). Copper stimulates the trafficking of ATP7A and ATP7B proteins, which are localized to the *trans*-Golgi network (TGN) when copper concentrations are normal, and are trafficked to cytosolic vesicles as copper increases. ATP7A is further trafficked to the plasma membrane in response to extreme concentrations of copper whereas ATP7B apparently is not (Lutsenko et al., 2007; Kim and Petris, 2007).

Copper inside the cell is distributed to three major destinations: SOD1 in the cytosol, CCO in mitochondria, and cupric enzymes in the TGN (Prohaska and Gybina, 2004). Specific copper chaperones have essential functions in the distribution of intracellular copper (see Figure 6.1: GENERIC CELL). CCS, the copper chaperone for SOD delivers copper specifically to SOD1 and the copper chaperone ATOX1 interacts with ATP7A and ATP7B and delivers copper into the TGN. The assembly of CCO is a complex process that involves several copper chaperones (Cobine et al., 2006; Hamza and Gitlin, 2002; Horng et al., 2005; Sacconi et al., 2005). It is thought that COX17, and perhaps the structurally related COX19 and COX23/MTCP1 (Barros et al., 2004; Sacconi et al., 2005; Schoenfeld et al., 2005), transfer copper to the mitochondrial inner membrane. COX17 transfers copper to both COX11 and SCO1 which participate in the assembly of CCO (Horng et al., 2005; Cobine et al., 2006). Studies of Bedlington Terriers with copper toxicosis resembling WND lead to the identification of COMMD1 (MURR1) (Van de Sluis et al., 2002) which directly interacts with ATP7B and regulates it stability (Tao et al., 2003; de Bie et al., 2007). Regulation of the ubiquitin pathway may account

for many functions of the COMMD protein family (Maine and Burstein, 2007). It was recently discovered that XIAP (X-linked inhibitor of apoptosis) regulates COMMD1 protein levels via its ubiquitin E3 ligase activity and therefore functions as a modulator of copper homeostasis (Burstein et al., 2004). XIAP directly binds copper leading to a conformational change and causing decreased stability of the protein and loss of XIAP's caspase blocking activity (Mufti et al., 2007). This feedback regulation may be important for copper homeostasis.

III. Copper Homeostasis and Development

Copper is required for normal development (Keen et al., 1998; Madsen and Gitlin, 2007) and dietary copper deficiency during embryonic development can be teratogenic and embryotoxic. Mouse embryos with swollen hind brains and offspring with severe neurological impairment and organogenesis defects in multiple tissues (severe connective tissue abnormalities, skeletal defects, lung abnormalities, *et cetera*) are observed in offspring from copper deficient dams (Keen et al., 1998). The extent and timing of copper deficiency dictate the severity and tissue-specificity of the effects on the embryo, fetus and newborn.

Mutations in genes that control copper homeostasis accentuate the multiple roles of copper during embryogenesis and early development (see Table 6.1). This topic has been the subject of recent reviews (Shim and Harris, 2003; Madsen and Gitlin, 2007). *Crt1* and *Cox17* homozygous knock-out mice both die around E8.5-E10.5 suggesting that the acquisition of copper by the embryo becomes critical during

Table 6.1: Phenotypes of Animals with Mutations in Genes Critical for Copper Homeostasis during Development

Gene	Functions	Phenotypes	Reference
Atox1	Copper chaperone for ATP7A/B	Peri-natal lethal, severe growth retardation in null mouse	(Hamza *et alii* 2001)
Atp7a	Copper transporter into TGN	Fatal in early childhood in MNK disease,	(Mercer 1998)
		embryonic lethal, perinatal lethal or hypopigmentation in *mottled/brindled* mouse,	(Mercer 1998)
		impaired synaptogenesis and axonal targeting, cytoskeletal dysfunction in *mottled/brindled* mouse,	(Niciu *et alii*, 2007; El Meskini *et alii*, 2007)
		impaired notochord development in *calamity* zebrafish,	(Mendelsohn *et alii*, 2006)
		impaired early larval development and adult pigmentation in *Drosophila*	(Norgate *et alii*, 2006)
Atp7b	Copper transporter into TGN	Abnormal copper accumulation in the liver and CNS in WND patients,	(Van Hoof *et alii*, 1992)
		abnormal copper accumulation in the liver of Long-Evans Cinnamon rat,	(Muramatsu *et alii*, 1994)
		copper deficiency in breast milk in *toxic milk* and null mouse	(Buiakova *et alii*, 1999)
Commd1	Ubiquitin pathway regulation ATP7B stability	Copper toxicosis in Bedlington terrier	(van de Sluis *et alii*, 2002)
		Embryonic lethal (E9.5~E10.5) in null mouse, no placental vascularization	(van de Sluis *et alii*, 2007)
Cox17	Copper chaperone for CCO	Embryonic lethal (E8.5~E10) in null mouse	(Takahashi *et alii*, 2002)
Ctr1	Copper importer (cell signaling?)	Embryonic lethal (E8.5~E10.5) in null mouse, impaired development of neuroectoderm,	(Lee *et alii*, 2001; Kuo *et alii*, 2001)
		mesoderm arrest at larval stage in *Drosophila*	(Turski and Thiele, 2007)
Sco1/2	Copper chaperone for CCO	Fatal infantile cardioencephalomyopathy, CCO deficiency (*Sco2*),	(Papadopoulou *et alii*, 1999)
		neo-natal liver failure and encephalopathy (*Sco1*) in humans	(Valnot *et alii*, 2000)

gastrulation and mesoderm formation (Takahashi et al., 2002; Lee et al., 2001; Kuo et al., 2001). As discussed below, the acquisition of zinc and iron by the embryo also becomes essential at about this stage of mouse development. It is interesting to note, however, that CTR1 has recently been shown to play roles in coordinating morphogenesis and progenitor cell fate, and in regulating embryonic stem cell differentiation (Haremaki et al., 2007). In *Xenopus laevis*, CTR1 both promotes differentiation and inhibits the morphogenesis of mesoderm and neurectoderm, perhaps by activating the Ras-MAP kinase cascade (Haremaki et al., 2007). Similar concepts regarding roles of zinc in regulating signal transduction cascades have recently emerged (Beyersmann and Haase, 2001; Haase and Maret, 2005; Klein et al., 2002; Haase and Rink, 2007; Yamasaki et al., 2007b).

Mutations in *Atp7a* in *mottled/brindled* mouse strains lead to three distinct phenotypes which reflect differences in copper transport and trafficking functions of the ATP7A protein (Kim and Petris, 2007). An early embryonic lethal phenotype of *Atp7a* (mo11H) reflects a lack of copper delivery to the secretory pathway, whereas peri-natal lethality of *Atp7a* reflects decreased copper delivery to the secretory pathway and constitutive localization to the plasma membrane (Kim and Petris, 2007). ATP7A plays a role in brain development. It is abundant in axons of the neonatal mouse brain but absent from adult axons (El Meskini et al., 2005), and in the *mottled/brindled* mouse ATP7A deficiency in Purkinje cells and olfactory sensory neurons is associated with impaired synaptogenesis and axonal targeting (Niciu et al., 2007; El Meskini et al., 2007). In *Drosophila,* the orthologous copper transporter

DmATP7 is essential for early larval development and adult pigmentation (Norgate et al., 2006). In zebrafish, copper plays a key role in development of the notochord. Mutations in the ortholog of *Atp7a* (*calamity*) result in copper deficiency phenotypes, and *Atp7a* gene dosage determines the sensitivity to copper deficiency consistent with the concept that suboptimal copper can contribute to birth defects (Mendelsohn et al., 2006).

The functions of the newly identified COMMD1 and XIAP in copper homeostasis during development remain to be clarified, but current information suggests links between the control of apoptosis, hypoxia, copper homeostasis and the ubiquitin pathway. Although *Xiap* knockout mice display no overt phenotype (Harlin et al., 2001), cells from these mice contain reduced copper levels and modestly elevated levels of COMMD1 (Burstein et al., 2004). In contrast, *Commd1* knockout embryos die on E9.5-E10.5, similar to the timing of lethality of *Ctr1* and *Cox17* knockout embryos, as mentioned above (Van de Sluis et al., 2007), but *Commd1* knockout embryos display heightened expression of hypoxia-inducible factor-1 (HIF-1) target genes, due to increased stability of HIF-1, and an absence of placental vascularization (Van de Sluis et al., 2007). The role of copper homeostasis in these processes remains obscure.

The proper transfer of copper from the mother to the developing embryo and neonate is also of critical importance. *Toxic milk* mice and mice with targeted mutations in *Atp7b* produce copper deficient breast milk, leading to neurological abnormalities, growth retardation and death of the suckling pup (Buiakova et al.,

1999). Similarly, the conditional knockout of *Ctr1* in the neonatal intestine is lethal before weaning, but this phenotype can be ameliorated by copper administration (Nose et al., 2006).

The copper chaperone ATOX1 plays a critical role in peri-natal copper homeostasis (Hamza et al., 2001) and about half (45%) of homozygous *Atox1* knockout offspring die before weaning while surviving pups fail to grow, have loose skin, hypopigmentation and seizures. Maternal *Atox1* deficiency exacerbates the severity of the copper deficiency phenotype of homozygous offspring (Hamza et al., 2001). *Atox1* knockout cells accumulate excess intracellular copper due to impaired efflux, and ATOX1 plays an essential role in the copper-mediated trafficking of ATP7A (Hamza et al., 2003).

Mutations in *Sco1/Sco2* copper chaperone genes have been documented in humans. Mutations in *Sco2* may cause fatal infantile cardioencephalomyopathy and CCO deficiency (Papadopoulou et al., 1999), whereas mutations in *Sco1* are associated with neonatal-onset liver failure and encephalopathy (Valnot et al., 2000). It is interesting to note that these phenotypes differ from patients with other mutations that affect CCO assembly. This suggests that SCO1 and SCO2 may have functions in addition to CCO assembly.

Almost all of the copper in blood is bound to ceruloplasmin (CP) but mutations in the *cp* gene are not associated with defective copper metabolism, but rather have been associated with defective iron metabolism. In humans CP deficiency has been associated with late-onset retinal and basal ganglia degeneration

(Harris et al., 1995) and increased iron accumulation in the liver and other tissues. This role for CP in iron metabolism reflects its function as a copper-containing ferroxidase, and mice with CP deficiency have impaired intestinal uptake of iron (Cherukuri et al., 2005).

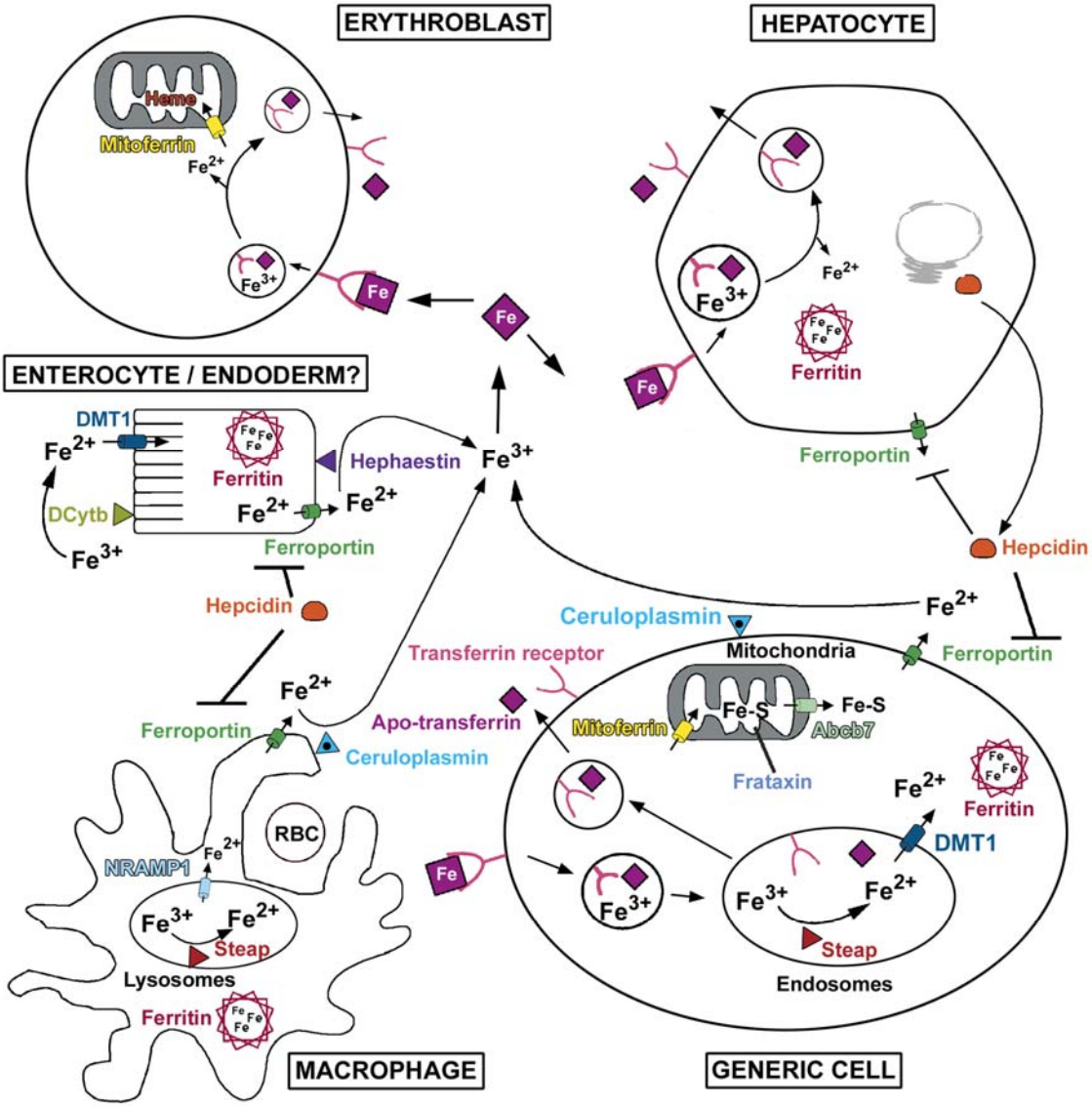
IV. Regulation of Iron Homeostasis

Iron is the most abundant transition metal and is utilized in both Fe-S cluster containing proteins and in proteins that contain porphyrin conjugates, primarily heme. Iron deficiency is the most common cause of anemia, and iron overload results in toxicity due to increased free radical formation. In the past decade significant advances have been made in our understanding of iron homeostasis and this subject has been recently reviewed in much greater detail than can be presented herein (DeDomenico et al., 2008; Andrews and Schmidt, 2007). A brief overview is presented below (see Figure 6.2).

Iron is regulated primarily at the level of absorption (Andrews and Schmidt, 2007; DeDomenico et al., 2008) (see Figure 6.2), and is lost from the body by bleeding and sloughing of the gut epithelium. Heme-conjugated iron is a major dietary source of iron, but a high-affinity heme transporter has not been identified (Qiu et al., 2006). Non-heme iron in the gut is reduced to Fe^{2+} by a ferric reductase(s) (Miret et al., 2003) and transported into the enterocyte by DMT1 (Slc11a2) (DeDomenico et al., 2008). Iron is transported out of the enterocyte into the circulation by ferroportin (Slc40a1) (Donovan et al., 2005) where it is oxidized to

Figure 6.2: Overview of Iron Homeostasis. Iron is oxidized ($\mathbf{Fe^{3+}}$) in the intestinal lumen and in the serum, but is reduced ($\mathbf{Fe^{2+}}$) before transport into cells. Iron ($\mathbf{Fe^{2+}}$) is transported into **ENTEROCYTES** by **DMT1** after extracellular reduction perhaps by **DCytb**. Iron uptake by visceral **ENDODERM** cells is apparently not dependent on DMT1 and could require the transferrin uptake system. Iron is transported out of cells by **Ferroportin** and is thought to be subsequently oxidized ($\mathbf{Fe^{3+}}$) by membrane bound **Hephaestin** and **Ceruloplasmin.** Iron, inflammation and hypoxia regulate **Hepcidin**, a polypeptide hormone made by the **HEPATOCYTE. Hepcidin** binds to **Ferroportin** resulting in degradation of the transporter. In the serum, $\mathbf{Fe^{3+}}$ binds to **Transferrin**. Developing **ERYTHROBLASTS** (heme is the major pool of iron) and many other cell types take up iron via the transferrin-mediated endocytic pathway (**see GENERIC CELL**). After binding to the **transferrin receptor,** the iron-transferrin complex is internalized and iron is released from transferrin in the acidified **endosome,** reduced by a **Steap** metalloreductase and then transported into the cytoplasm by **DMT1**. Apo-transferrin and the transferrin receptor are returned to circulation and plasma membrane, respectively, via the recycling endosome pathway. All non-erythroid cells store iron complexed with the ferritin heavy and light chains (**Ferritin**) which can store up to 4,000 atoms of iron per molecule. Cytoplasmic iron is transported into **mitochondria** by **mitoferrin** where it is loaded into **Fe-S** clusters in a process that requires **Frataxin**. A substrate essential for Fe-S assembly in the cytoplasm is transported out of the mitochondria by the ATP-binding cassette transporter **ABCB7 (see GENERIC CELL).** In the **erythroblast**, mitochondrial iron

is primarily utilized for **heme** synthesis. A major fraction of iron is recycled by the reticuloendothelial **MACROPHAGE** which phagocytoses aged red blood cells (**RBC**). The iron is solubilized in the **lysosomes,** reduced by a **Steap** metalloreductase and exported out of the lysosome by **NRAMP1**. Iron is released from the macrophage by ferroportin, subsequently reoxidized by **Ceruloplasmin** and conveyed again via transferrin through the systemic circulation.


ERYTHROBLAST
HEPATOCYTE
Heme
Mitoferrin
Fe2+
Fe3+
Fe
Ferritin
ENTEROCYTE / ENDODERM?
DMT1
DCytb
Hephaestin
Ferroportin
Hepcidin
Ceruloplasmin
Transferrin receptor
Apo-transferrin
Mitochondria
Fe-S
Abcb7
Frataxin
NRAMP1
RBC
Steap
Lysosomes
Endosomes
MACROPHAGE
GENERIC CELL

Fe^{3+} by hephaestin and perhaps ceruloplasmin (Miret et al., 2003). DMT1 is not essential for iron uptake in the fetus (Gunshin et al., 2005). In fact, alternate and as yet unidentified iron uptake mechanisms exist in hepatocytes, placenta and most other cells. Ferroportin, in contrast, plays a key role in iron export from the visceral endoderm of the yolk sac (Donovan et al., 2005). In the circulation, transferrin binds Fe^{3+} and transports it throughout the body.

Transferrin-receptor-mediated endocytosis is the major path by which all cells take up iron (DeDomenico et al., 2008). Receptor bound transferrin undergoes endocytosis, Fe^{3+} is released from transferrin in the acidified endosome, and apo-transferrin and its receptor are recycled back to circulation and plasma membrane, respectively (see Figure 6.2: GENERIC CELL). Transferrin receptor-1 is a high-affinity, ubiquitously expressed receptor; in contrast transferrin receptor-2 is expressed predominately in liver and its function is not known (Wallace et al., 2008). The released Fe^{3+} is reduced to Fe^{2+} by a member of the STEAP metalloreductase family (STEAP3 in the erythroblast) and transported out of the endosome by DMT1 (Ohgami et al., 2006). In all non-erythroid cells, any free iron is chelated by ferritin. Iron is transported into mitochondria by mitoferrin (Slc25a37) (Shaw et al., 2006) where it is loaded into Fe-S clusters in a frataxin-dependent process and used for heme synthesis in red blood cells (Muhlenhoff et al., 2002). Mitochondria also export a substrate essential for cytosolic Fe-S cluster assembly via the ATP-binding cassette transporter ABCB7 complex (Burke and Ardehali, 2007; Lill et al., 2006). The heme in red blood cells represents the largest pool of iron in the body. Heme iron is

recycled by macrophages of the reticuloendothelial system which phagocytose aged red blood cells (see Figure 6.2). Iron is transported out of the lysosome by NRAMP1 (Slc11a1) (Lam-Yuk-Tseung et al., 2006) and ultimately released from the macrophage by ferroportin as Fe^{2+} which is then oxidized by ceruloplasmin and bound again by transferrin (Andrews and Schmidt, 2007). Macrophages play an essential role in iron homeostasis.

Iron homeostasis is tightly controlled by several post-transcriptional mechanisms. In brief, the iron-regulatory proteins (IRP1 and IRP2) function as iron sensors that regulate iron metabolism genes as well as some genes with no direct link to iron metabolism. IRPs regulate the stability of transferrin receptor-1 mRNA and the translation of ferritin mRNA by binding to stem-loop structures termed iron-responsive elements (IRE) in the UTRs of these mRNAs. Transferrin receptor-2 mRNA lacks IREs and is not iron-regulated (Fleming et al., 2000). When iron is limiting, IRPs bind to the 5' UTR of ferritin mRNA and block its translation and bind to the 3' UTR of transferrin receptor-1 mRNA which is stabilized (DeDomenico et al., 2008). DMT1 and ferroportin mRNAs also contain IREs that are thought to be regulated by IRPs.

Ferroportin transporter activity is down-regulated by the binding of the peptide hormone hepcidin which causes phosphorylation of ferroportin and its subsequent internalization, ubiquitination and degradation (DeDomenico et al., 2008; Ganz, 2007). Hepcidin therefore inhibits dietary iron absorption and the efflux of recycled iron from macrophages. Hepcidin is secreted by hepatocytes in response to

several different stimuli (DeDomenico et al., 2007b). Hypoxia resulting from anemia or other causes, inflammatory cytokines and iron all regulate hepcidin secretion. Not all of the mechanisms of *hepcidin* (*hamp*) gene regulation are understood, but transcription of *hamp* is induced by Stat 3 in response to Il-6 (Wrighting and Andrews, 2006) and by bone morphogenic protein/Smad4 signaling in response to membrane-bound hemojuvelin (Babitt et al., 2006; Babitt et al., 2007; Silvestri et al., 2008). Hemojuvelin acts as a BMP co-receptor which facilitates BMP receptor activation (Babitt et al., 2006). The transcription factor HIF-1α represses *hamp* gene expression in response to hypoxia (Peyssonnaux et al., 2007). Regulation of ferroportin is important for proper maintenance of iron homeostasis.

V. Iron Homeostasis and Development

Hypochromic, microcytic anemias resulting from defects in iron metabolism genes leading to impaired development of red blood cells have provided insight into the mechanisms of homeostasis of this essential metal. Several rodent strains (see Table 6.2) have been described that have defective erythropoiesis such as hypotransferrinemic (Tf^{hpx}) mice, sex-linked anemia ($Heph^{sla}$) mice, *nm1054* (*Steap3*) mice, the Belgrade rat ($Slc11a2^{b}$), and microcytic anemia ($Slc11a2^{mk}$) mice (Touret et al., 2004; Ohgami et al., 2005; Andrews, 2000). Similarly, *Dmt1* (*Slc11a2*) knockout mice (Gunshin et al., 2005), the zebrafish mutants *Chianti* (*Tfr1a)* (Wingert et al., 2004), *Chardonnay* (*Dmt1*) (Donovan et al., 2002), and *Frascati* (*mitoferrin: Mfrn*) (Shaw et al., 2006), show erythroid maturation arrest. The zebrafish mutant

Table 6.2: Phenotypes of Animals with Mutations in Genes Critical for Iron Homeostasis during Development

Gene	Functions	Phenotype	Reference
		Hypochromic, microcytic anemia, defective erythropoiesis	
Dmt1	Iron transporter (*Slc11a2*)		
	in *Slc11a2*^{b} Belgrade rat		(Touret *et alii*, 2004)
	in *Slc11a2*^{Mk} Microcytic anemia mouse		(Touret *et alii*, 2004)
	in *Slc11a2* $^{-/-}$ null mouse		(Gunshin *et alii*, 2005)
	in zebrafish *Chardonnay*		(Donovan *et alii*, 2002)
Fpn	Iron efflux in zebrafish *Weisshergst*		(Donavan *et alii*, 2000)
Heph	Iron oxidase in Sex-linked anemia mouse		(reviewed by Andrews, 2000)
Mtfrn	Mitochondrial iron importer in zebrafish *Frascati* and null mouse ES cells		(Shaw *et alii*, 2006)
Steap3	Endosomal ferric/cupric reductase in null mouse and *nm1054* mouse		(Ohgami *et alii*, 2005)
Tf	Serum iron carrier in *Hpx* mouse		(reviewed by Andrews, 2000)
Tfr1	Transferrin receptor for iron uptake in zebrafish *Chianti*		(Wingert *et alii*, 2004)
		Impaired embryonic development	
Abcb7	Mitochondrial efflux of Fe-S substrate	Embryonic lethal ~E6.5 in null mouse, extraembryonic defect	(Pondarre *et alii*, 2006)
Fpn	Iron efflux	Embryonic lethal ~E7.5 in null mouse, visceral endoderm defect,	(Donovan *et alii*, 2005)
		hypomorphic allele, midgestation lethal, neurogenesis defects in *Flatiron* mouse	(Zohn *et alii*, 2007)
Fxn	Fe-S cluster assembly	Embryonic lethal ~E5-E7 in null mouse (early post-implantation)	(Cossee *et alii*, 2000)
Fth1	Iron storage	Embryonic lethal (~E3.5-E9.5) in null mouse, defective heart development	(Ferreira *et alii*, 2000)
Irp1+ Irp2	Post-transcriptional regulation	Embryonic lethal blastocyst stage in double null mouse, iron overload	(Smith *et alii*, 2004, 2006)
Tfr1	Endocytic uptake of iron in mouse	Embryonic lethal ~E12.5 in null mouse, impaired CNS and erythroid development	(Levy *et alii*, 1999)

weisshergst exhibits a defect in hemoglobin accumulation, due to mutations in the *Ferroportin* (*Fpn*) gene, which is also eventually lethal (Donovan et al., 2000).

Mutations in iron metabolism genes can profoundly affect embryonic, fetal and peri-natal development. Mice with targeted deletions of the *Abcb7* gene, which is essential for cytosolic Fe-S cluster assembly, die soon after implantation due to a defect in extra-embryonic tissues, but this gene is critical for the development of many cell-types and is mutated in X-linked sideroblastic anemia with ataxia (Pondarre et al., 2006). Frataxin (FXN) functions as a chaperone for mitochondrial (and extra-mitochondrial) Fe-S cluster biogenesis (Muhlenhoff et al., 2002; Martelli et al., 2007). Knockout of the mouse *Fxn* gene revealed that it is essential, and homozygous embryos also die a few days after implantation (Cossee et al., 2000). The mechanism of lethality may not be associated with iron accumulation as is seen in Friedreich's ataxia. It is interesting to note that the *Fxn* gene in *Arabidopsis thaliana* is essential at the globular stage of early development when the three major tissue types emerge (Vazzola et al., 2007). The *flatiron* (*ffe*) mutation in mice is due to a hypomorphic *Fpn* mutation that exerts a dominant negative function in heterozygous mice leading to iron loading in the liver, high serum ferritin and low transferrin iron saturation, whereas homozygous *ffe* embryos die at midgestation and have severe anemia (Zohn et al., 2007). In contrast, *Fpn*-knockout mice revealed that this iron export protein is essential for development of the early (≈ E7.5) embryo (see Table 6.2) (Donovan et al., 2005). Ferroportin is localized to the basolateral membrane of the visceral endoderm at this stage of development and conditional

knockout of *Fpn* in the visceral endoderm confirms its importance in those cells. Mice heterozygous for *Fpn* exhibit smaller cell volume and lower intracellular hemoglobin in both developing reticulocytes and mature erythrocytes in adults (Donovan et al., 2005).

Perturbations of intracellular iron storage and distribution can also negatively impact development. Knockout of the mouse *H-ferritin* (*Fth1*) gene is early embryonic lethal when homozygous (Ferreira et al., 2000) and L-ferritin cannot complement the loss of function of *Fth1*. This suggests that these isoforms of ferritin have unique functions and that the ability to store/chelate/distribute iron is essential early during mouse development. The expression pattern of *Fth1* suggests a prominent role in development of the heart (Ferreira et al., 2000). *Fth1* expression in the E9.5 mouse embryo is restricted to heart and CNS but later becomes more ubiquitous (Ferreira et al., 2000).

IRP1 and IRP2 regulate iron metabolism genes at the post-transcriptional level. Neither alone is essential for development. *IRP1* knockout mice misregulate iron metabolism in the kidney and brown fat, whereas *IRP2* knockout mice misregulate iron homeostasis in all tissues and develop adult-onset neurodegenerative disease (Smith et al., 2004). Mice with a single allele of *IRP1* and no functional *IRP2* genes develop a more severe form of neurodegeneration (Smith et al., 2004) whereas a complete loss of both *IPR1* and *IRP2* genes is embryonic lethal (Smith et al., 2006). These double-knockout blastocysts were discolored and morphologically abnormal

perhaps due to the over-expression of ferritin and they did not survive past E6.5 *in utero* (Smith et al., 2006).

Homozygous knockout of *transferrin receptor-1 (TfR1)* in mice revealed essential roles in erythropoiesis and in development of the central nervous system (Levy et al., 1999). Homozygous *Tfr1* knockout mice die *in utero* and are severely anemic with increased apoptosis in the neuroepithelium. Haploinsufficiency of *Tfr1* is associated with microcytic anemia and reduced iron stores. In contrast, *Tfr2* knockout mice are viable but conditional knockout of this gene in the adult liver leads to iron overload (Wallace et al., 2007; Wallace et al., 2004) similar to type 3 hemochromatosis in humans, suggesting a role for TFR2 as an iron-bound transferrin sensor (Johnson and Enns, 2004; Robb and Wessling-Resnick, 2004; Wallace et al., 2008).

VI. Regulation of Zinc Homeostasis

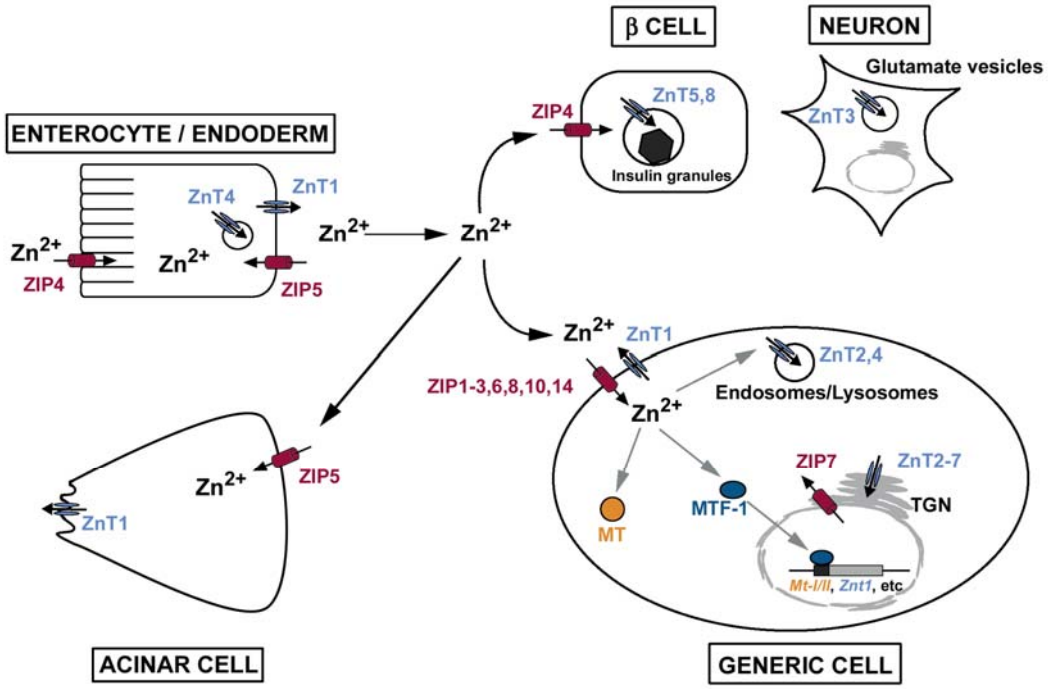
Zinc serves structural and/or catalytic roles in hundreds of peptides (Vallee and Auld, 1990; Berg and Shi, 1996; Krishna et al., 2003; Ravasi et al., 2003) and is the second most abundant essential metal. Therefore, when zinc is deficient numerous cellular processes are affected. Zinc deficiency during development is highly teratogenic and embryotoxic and the outcomes are highly variable depending on the extent and timing of the zinc deficiency. Zinc is also toxic when in excess. Therefore, the maintenance of zinc homeostasis is critical and multiple genes have evolved to modulate the storage (4 *Metallothionein* genes in mice), efflux (10 *ZnT*

genes) and uptake (14 *Zip* genes) of this metal in response to zinc availability (see Figure 6.3). In addition, recent studies suggest the possibility that zinc may be stored in and released from intracellular vesicular compartments (Devirgiliis et al., 2004; Yamasaki et al., 2007b). The zinc transporters of higher eukaryotes are often expressed in a tissue-specific manner and in specific cellular localizations. In addition, they can display specific changes in cellular localization and stability in response to zinc deficiency or excess (Kambe et al., 2004; Eide, 2006; Cousins et al., 2006). The diverse roles of these molecules in zinc homeostasis are only beginning to be understood and much remains to be determined.

When zinc is replete in the diet, it is absorbed from the gut by enterocytes in the small intestine, and excess zinc is released through the pancreas and small intestine, as well as in small amounts from the kidney (King et al., 2000; Hambidge and Krebs, 2001; McClain, 1990). However, under zinc- deficient conditions, zinc absorption by the small intestine increases, and release from the pancreas and small intestine decreases (Hambidge and Krebs, 2001). In the embryonic environment of the mouse, the visceral endoderm plays an important role in zinc homeostasis during peri-implantation development (Dufner-Beattie et al., 2007). How animals and cells adapt to zinc status and the roles of these zinc transporters (uptake and efflux) in the adaptive process are active areas of investigation.

Figure 6.3: Overview of Zinc Homeostasis. Twenty four different genes encode proteins that may be involved in the uptake (*Zip* genes) or efflux (*ZnT* genes) of this metal in a cell-specific, developmentally-regulated and zinc-regulated manner. The functions of many of these genes remain to be determined. Therefore, this cartoon provides only a superficial and static view of zinc homeostasis. **ZIP4** plays a critical role in the absorption of dietary zinc by **ENTEROCYTES/ENDODERM** cells when zinc is limiting, but other transporters must also play important roles. Zinc is thought to be exported into portal blood or into the conceptus by **ZnT1** localized on the basolateral membrane. Other ZnT proteins (*id est* **ZnT4**) also likely play a role in this process. **ZIP5** is localized to the basolateral membranes of enterocytes, endoderm cells and pancreatic acinar cells where it may serve to remove zinc from the blood when zinc is replete. In peripheral tissues (**GENERIC CELL**), zinc is probably taken up by various **ZIP** transporters localized on the plasma membrane. To date **ZIPs1, 2, 3, 6, 8, 10, and 14** have each been shown to have zinc transporter activity in transfection or oocyte injection studies, and most show tissue-specific patterns of expression. Inside the cell, free zinc levels are kept low and zinc can be bound to **MT** or transported into secretory vesicles, **endosomes/lysosomes** or zincosomes by **ZnT2** and **ZnT4**. Zinc activates the zinc-sensing transcription factor **MTF-1** which regulates transcription of the mouse ***Mt-I/II*** and ***ZnT1*** genes and represses expression of ***Zip10*** in an effort to control excess zinc. **ZnTs2-7** participate in the delivery of zinc into the secretory pathway, whereas **ZIP7** may transport zinc out of the *trans*-Golgi apparatus into the cytoplasm. **ZnT3** transports zinc into

glutamate containing vesicles in the brain whereas **ZnT8** transports zinc into pancreatic β-cell insulin secretory granules. ZIP4 is expressed in β-cells but its localization in those cells has not been reported.


β CELL
NEURON
Glutamate vesicles
ZnT5,8
ZIP4
ZnT3
Insulin granules
ENTEROCYTE / ENDODERM
ZnT4
ZnT1
Zn2+
Zn2+
Zn2+
Zn2+
ZIP4
ZIP5
Zn2+
ZnT1
ZnT2,4
ZIP1-3,6,8,10,14
Endosomes/Lysosomes
Zn2+
ZIP7
ZnT2-7
TGN
MTF-1
MT
Mt-I/II, Znt1, etc
Zn2+
ZIP5
ZnT1
ACINAR CELL
GENERIC CELL

VII. Metallothioneins and MTF-1 in Development

A portion of the total cellular zinc pool (≈ 5-15%) can be bound by the cysteine-rich metallothioneins (MT) (Coyle et al., 2002). In the mouse, there are four MT family members, and in humans there are eleven (Kagi, 1991). *Mt* genes (*Mt-I* and *Mt-II* in mice) are zinc-regulated by the transcription factor MTF-1 (Laity and Andrews, 2007); in the presence of high zinc their transcription rate is increased (see Figure 6.3). In contrast, under low zinc conditions, MT levels decrease due to a lower transcription rate and protein destabilization (Andrews, 1990). Although *Mt* genes are non-essential in mice, MTs appear to function as a "zinc buffer" that can provide a labile pool of zinc for use by other proteins when zinc is limiting (Krezel and Maret, 2007; Maret and Krezel, 2007). Deletion of *Mt-I* and *Mt-II* genes sensitizes mice to the teratogenic effects of zinc deficiency during pregnancy (Andrews and Geiser, 1999), whereas over-expression of *Mt-I* dramatically protects mice from these effects (Dalton et al., 1996b) (see Table 6.3).

The zinc-finger transcription factor MTF-1 regulates basal and metal-induced expression of mammalian *Mt* genes (Lichtlen and Schaffner, 2001). Deletion of the *Mtf-1* gene in mice is lethal at E14 due to degeneration of the liver (Günes et al., 1998). This appears to be a cell-autonomous effect (Wang et al., 2004d), but its relationship with zinc metabolism in the embryo is unclear. MTF-1 is also essential for basal expression and zinc regulation of *Mt* genes in the visceral endoderm of the

Table 6.3. Phenotypes of Animals with Mutations in Genes Critical for Zinc Homeostasis during Development

Gene	Functions	Phenotypes	Reference
Mt-I/II	Zinc buffer-pool	Increased embryo sensitivity to zinc deficiency in null mouse,	(Andrews and Geiser, 1999)
		increased resistance to zinc deficiency when over-expressed	(Dalton *et alii*, 1996)
Mtf-1	Zinc-sensing transcription factor	Embryonic lethal (E14) due to liver degeneration in null mouse	(Wang *et alii*, 2004)
Zip1	Zinc importer	Impaired embryonic adaptation to zinc deficiency in null mouse	(Dufner-Beattie *et alii*, 2006)
Zip2	Zinc importer	Impaired embryonic adaptation to zinc deficiency,	
		altered iron and calcium homeostasis in null mouse during zinc deficiency	(Peters *et alii*, 2007)
Zip3	Zinc importer	Impaired embryonic adaptation to zinc deficiency (cell type-specific) in null mouse	(Dufner-Beattie *et alii*, 2005)
Zip4	Zinc importer	Childhood lethal acrodermatitis enteropathica in humans,	(Maverakis *et alii*, 2007)
	(*Liv-1* subfamily member)	e mbryonic lethal (E8 ~ E9) in homozygous null mouse,	
		hypersensitivity to zinc deficiency in heterozygous null mice,	(Dufner-Beattie *et alii*, 2007)
		lethal trait A46 in bovine (*Zip4*?)	(Machen *et alii*, 1996)
Liv-1	Zinc importer	Impaired epithelial-mesenchymal transition in zebrafish gastrula	(Yamashita *et alii*, 2004)
FOI	Zinc importer (*Liv-1* like)	Impaired germ cell migration and gonad morphogenesis in *Drosophila*	(Moore *et alii*, 1998)
ZnT1	Zinc exporter	Embryonic lethal (E8 ~ E9), transporting maternal zinc into embryo	(Andrews *et alii*, 2004)
ZnT2	Vesicular zinc transporter	Dermatitis due to low zinc in breast milk (in human)	(Chowanadisai *et alii*, 2006)
ZnT4	Vesicular zinc transporter	*Lethal milk* mutation in mice, suckling pups die	(Huang and Gitschier, 1997)
ZnT5	Zinc transporter-Golgi	Growth retardation, osteopenia, male specific cardiac sudden death in null mouse	(Inoue *et alii*, 2002)

mouse embryo (Andrews et al., 2001), and this tissue appears to play a key role in the acquisition of essential metals during development, as is discussed below. Interestingly, MTF-1 regulates zinc-responsiveness of the *ZnT1* (a major zinc efflux transporter) gene (Langmade et al., 2000) and represses expression of the *Zip10* gene, a member of the zinc uptake transporter family whose function during development is unknown (Wimmer et al., 2005). These findings suggest that mammalian MTF-1 plays an important role in zinc homeostasis.

VIII. ZnT Family Zinc Transporters (Slc30a) in Development

Two superfamilies of mammalian zinc transporters have been identified that belong to the solute carrier (Slc)30a and the Slc39a families (Palmiter and Huang, 2004; Taylor and Nicholson, 2003; Guerinot, 2000b). Slc30a members, named ZnTs, function in zinc efflux and compartmentalization and are cation diffusion proteins (Palmiter and Huang, 2004). There are ten members of this family in mammals and several are known to have important functions during development. Only those are discussed herein (Seve et al., 2004) (see Table 6.3). Targeted deletion of *ZnT1* results in early embryonic lethality in homozygous knockout mice (Andrews et al., 2004). High levels of *ZnT1* mRNA are found in the maternal deciduum and in the visceral yolk sac surrounding the embryo, which suggests that this protein is involved in the transfer of maternal zinc into the uterine and embryonic environments (Andrews et al., 2004). ZnT1 is a ubiquitous zinc efflux transporter that can protect cells *in vitro* from zinc toxicity (Palmiter, 2004). The naturally-occurring *lethal milk* mouse

carries a nonsense mutation in the *ZnT4* gene (Huang and Gitschier, 1997). These mice produce zinc-deficient milk which, in turn, causes a lethal zinc deficiency in the nursing pups. A recent study found heterozygous mutations in the human *ZnT2* gene in women with low zinc concentrations in milk leading to transient zinc deficiency and dermatitis in the nursing child (Chowanadisai et al., 2006). Homozygous deletion of *ZnT5* results in poor growth, osteopenia, low body fat, muscle weakness, and male-specific cardiac death (Inoue et al., 2002). Complexes containing ZnT5 and 6, as well as complexes containing homo-oligomers of ZnT7 function to transport zinc into the secretory pathway which activates zinc-requiring enzymes such as alkaline phosphatase (Suzuki et al., 2005b; Suzuki et al., 2005a). Our understanding of this family of zinc transporters has advanced significantly in the past few years. In contrast, we know much less about zinc uptake transporters.

IX. ZIP Family Zinc Transporters (*Slc39a*) in Development

Members of the *Slc39a* family, named ZIPs, function in the uptake of zinc and other metals (Eng et al., 1998; Guerinot, 2000b; Taylor and Nicholson, 2003), and members of this family are found in all eukaryotes. In mice and humans there are 14 members of the ZIP family. The *Slc39a* superfamily can be subdivided into four subfamilies based on structural homology (Taylor and Nicholson, 2003). In mammals, most ZIP proteins fall into one of two subfamilies named subfamily II (3 members) and LIV-1 (9 members).

Members of subfamily II (ZIPs1-3) were the first to be described in mammals. They are well conserved, can function to transport zinc in transfection studies and are actively expressed in a highly cell-specific manner in mice (Gaither and Eide, 2001b; Dufner-Beattie et al., 2003a; Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006; Peters et al., 2007; Huang et al., 2006). Targeted deletion of mouse *ZIP1, 2* or *3* genes revealed that none of these genes is essential (Dufner-Beattie et al., 2006; Peters et al., 2007) and deletion of the entire subfamily also does not cause an obvious phenotype (Kambe et al., 2008). However, subfamily II knockout mice are dramatically more sensitive to the teratogenic effects of dietary zinc deficiency during pregnancy (Dufner-Beattie et al., 2005; Dufner-Beattie et al., 2006). The zinc deficient knockout embryos are severely growth retarded and have obvious craniofacial abnormalities and limb defects. ZIPs1, 2 and 3 appear to function specifically during adaptation to zinc deficiency consistent with the finding that these proteins are recruited to the surface in zinc deficient cells (Wang et al., 2004a).

Several members of the LIV-1 subfamily have interesting roles in early development. In humans, mutations in the *Zip4* gene cause the rare and fatal genetic disorder acrodermatitis enteropathica (Wang et al., 2002; Kury et al., 2002) which reflects an impaired ability of the intestine to absorb dietary zinc. The symptoms of this disease often appear after weaning in humans but can be ameliorated by dietary zinc supplementation (Maverakis et al., 2007). In contrast, the mouse *Zip4* gene is essential during early embryonic development (E7.5 to E9) and the homozygous knockout embryos fail to undergo morphogenesis (Dufner-Beattie et al., 2007).

Although expression of *Zip4* can be detected in the preimplantation mouse embryo, it is actively expressed only in the visceral endoderm on E7.5 when the embryo is at the egg cylinder stage of development.

Interestingly, *Zip4* heterozygosity is teratogenic and embryotoxic in mice. These effects are highly pleiotropic and include growth retardation, exencephalia, and cranio-facial abnormalities in midgestation embryos, as well as hydrocephalia, anopia (loss of eyes), heart and skeletal defects in a significant percentage of the offspring (Dufner-Beattie et al., 2007). These abnormalities are largely prevented by supplementation of dietary zinc. Later during pregnancy (E14), *Zip4* gene expression in the visceral endoderm is regulated by zinc availability (Dufner-Beattie et al., 2003b). During zinc deficiency, *Zip4* mRNA and protein stability are increased and ZIP4 is trafficked to the apical membrane. Repletion of zinc causes the rapid internalization and destabilization of ZIP4 in the visceral endoderm (Weaver et al., 2007). This pattern of regulation mimics that seen in the adult intestine where ZIP4 serves an essential function in the uptake of dietary zinc.

The functions of other mammalian ZIP family members in development remain to be explored, but it is interesting to note that the *fear-of-intimacy* gene in *Drosophila* encodes a LIV-1-like protein that can function as a zinc transporter and that is essential during early development of the fly (Moore et al., 1998; Mathews et al., 2005). This gene controls germ cell migration and gonad formation which suggests a key role for zinc in these processes. Similarly, morpholino knockdown of zebrafish *zLiv-1* revealed a function in epithelial-mesenchymal transition in the

gastrula (Yamashita et al., 2004). *zLiv-1* is a downstream target of STAT3, and is essential for the nuclear localization of the zinc-finger protein Snail, which regulates epithelial-mesenchymal transition. Remarkably, human ZIP6, the founding member of the LIV-1 family, plays a role in cell invasion by targeting the ERK1/2-Snail/Slug pathway (Zhao et al., 2007). In addition, ZIP10 has recently been shown to play a role in breast cancer invasive behavior (Kagara et al., 2007), and aberrant *Zip4* expression in pancreatic ductal carcinoma has been reported to correlate with metastatic potential (Li et al., 2007). *Zip4* expression in the pancreas is normally restricted to pancreatic β-cells (Dufner-Beattie et al., 2004). Thus, several members of the LIV-1 family may have fundamental functions in regulating cell migration and differentiation during early development.

X. Zinc Signaling and its Potential Role in Development

Zinc has been suggested to function as an intracellular second messenger as well as extracellular signaling molecule. These effects may, in turn, impact development of the embryo, although this concept has not yet been supported by direct evidence (Hansson, 1996; Kim et al., 2000; Beyersmann and Haase, 2001; Yamasaki et al., 2007b). Zinc influences signal transduction cascades in a cell-type and zinc concentration-dependent manner. For example, low micromolar concentrations of zinc inhibit hormone- or forskolin-stimulated cAMP production in neuroblastoma cells, perhaps by directly interacting with adenylate cyclase and altering the V_{max} of the enzyme (Klein et al., 2002). Similarly, zinc treatment can

inhibit cyclic nucleotide phosphodiesterase (PDE) activity and expression in monocytes, resulting in increased levels of cGMP, activation of protein kinase A and subsequent inhibition of Raf-1. This leads to altered NF-κB signaling and suppression of TNF-α production (vonBulow et al., 2007). Zinc also has insulinomimetic effects and apparently small increases in intracellular zinc can inhibit protein tyrosine phosphatase 1B activity in insulin and insulin-like growth factor signaling leading to increased phosphorylation of the receptor (Samet et al., 2003; Haase and Maret, 2005). Zinc also influences the EGF pathway and the NMDA receptor (cited in (Samet et al., 2003)). In *C. elegans*, *Cdf-1*, which encodes a protein functionally similar to vertebrate ZnT-1, was identified as a positive regulator of the Ras pathway, by promoting zinc efflux and relieving repression of Ras-mediated signaling (Bruinsma et al., 2002).

The release of intracellular zinc leads to the activation of 12-lipoxygenase and p38 mitogen-activated protein kinase (MAPK), which contributes to the toxicity of zinc in neurons and oligodendrocytes (Zhang et al., 2007). These authors also comment that extracellular signal-regulated kinase (ERK1/2) activation, caused by glutathione depletion during oxidative stress, is mediated by the intracellular release of zinc in HT22 cells (Zhang et al., 2007). A recent study of mast cells revealed that cross-linking IgE receptors induces the release of free zinc from the perinuclear area and ER, an effect dependent on calcium influx and MAPK activation, suggesting that zinc may function as a novel intracellular second messenger (Yamasaki et al., 2007b). Given the roles of extracellular and intracellular zinc in regulating cell signaling, it

seems likely that altering zinc homeostasis at a critical time in early development would have dramatic, pleiotropic effects on morphogenesis of the embryo.

XI. Essential Metal Interactions

Although most of the above discussion has dealt with these essential metals individually, it should be noted that there are significant interactions between essential metals and the status of one metal can affect that of another. For example, excess zinc induces copper deficiency which leads to diminished intestinal absorption of iron (Cherukuri et al., 2005) and decreased activity of ceruloplasmin, the copper containing ferroxidase, which in turn may destabilize ferroportin (DeDomenico et al., 2007a). Marginal zinc deficiency in pregnant rats leads to increased maternal ceruloplasmin and increased copper in the milk, perhaps by increasing the expression of *Ctr1*, *Atp7a* and *Atp7b* genes in the mammary gland, which in turn can cause signs of copper toxicity in the nursing pups (Kelleher and Lonnerdal, 2003). Analyses of all of the essential metal ions in the liver and embryos of *Zip2*-knockout mice revealed unique alterations in iron but not zinc levels (Peters et al., 2007). Zinc deficiency has been shown to cause an increase in iron in the liver, whereas zinc excess competes for iron uptake in the gut (Kordas and Stoltzfus, 2004; Yamaji et al., 2001; Kelleher and Lonnerdal, 2006b; Hurley et al., 1983), perhaps by altering the activity of iron transport in the intestine. *Zip2*-knockout mice fail to accumulate iron in the liver during zinc deficiency (Peters et al., 2007), but the mechanisms underlying this effect are unknown.

Zinc and calcium metabolism are also interconnected (Maret, 2001). A functional link has been proposed between L-type calcium channels and the zinc efflux transporter ZnT1 (Segal et al., 2004; Ohana et al., 2006), and extracellular zinc can trigger the release of calcium from intracellular pools in HT29 cells (Hershfinkel et al., 2001) and inhibit calcium influx in several cultured cell lines (Gore et al., 2004). A dramatic increase in calcium occurs in embryos from zinc-deficient *Zip2*-knockout mice (Peters et al., 2007). However, there is no evidence that any ZIP family member can transport calcium suggesting an indirect role of ZIP2 in calcium metabolism.

Chapter 7

Summary Discussion of Dissertation

Zinc is an essential micronutrient for all life. Zinc is utilized in thousands of proteins, peptides, and sub-domains and serves both catalytic and structural roles. Thus, the ability of all organisms to regulate the optimal level of zinc is also essential. Higher organisms, such as mammals, must be able to 1) distribute zinc to the correct subcellular location for biochemical purposes, 2) redistribute zinc throughout the body from sites of absorption and storage to sites of utilization, 3) increase the uptake of zinc during dietary deficient states, and 4) increase the efflux of zinc during dietary replete or excess states. Two families of zinc transporters, the *Slc30a* (*ZnT* or *CDF*) and the *Slc39a* (*Zip*) families, are essential for the regulation of zinc homeostasis. Disease states are caused by mutations in several members of the two transporter families. Therefore, the need to understand how these transporters function and are regulated is obvious to human health. One disease, acrodermatitis enteropathica (AE), is a rare autosomal recessive disease that results in mental retardation, characteristic dermatitis, diarrhea, cognitive deficits, and death if untreated (Agarwal and Vaishnava, 1969; Meftah et al., 2006; Prasad, 1995; Prasad, 1984). The AE gene was identified as *Slc39a4* (*Zip4*) (Kury et al., 2002; Wang et al., 2001). Previous work has shown that this gene encodes a zinc-selective transporter (Dufner-Beattie et al., 2003b).

Little about the function and regulation of ZIP transporters is known. Prior to the work reported herein, both ZIP4 and ZIP5 were suggested to be involved in the

adaptive response to zinc availability (Dufner-Beattie et al., 2004; Wang et al., 2004b). The ZIP4 transporter was found to accumulate at the apical membrane of enterocytes and visceral endoderm during zinc deficiency; whereas, the ZIP5 transporter was found to accumulate at the basolateral membrane of enterocytes, visceral endoderm, and pancreatic acini during zinc adequacy (Dufner-Beattie et al., 2004). The overall goal of my research has been built upon those previous findings. My research has focused thus on the molecular mechanisms involved in regulating *Zip4* and *Zip5* in the adaptive response to zinc availability and the role of *Zip4* in development.

In the introduction, I stated that I would defend three specific aims in this dissertation. **In the first specific aim,** I evaluated the hypothesis that *Zip4* is essential to the normal growth and development of mammals. Using a *Zip4* knockout mouse model, mice were studied through various stages of development. We found that *Zip4* was essential for development past the egg cylinder stage and that heterozygosity caused hypersensitivity to zinc deficiency in development. Excess zinc fed to pregnant dams was insufficient to rescue the knockout embryos. However, excess zinc did ameliorate the severity of developmental defects in the heterozygous offspring. As discussed in the review chapter, several other genes involved in metal homeostasis are also critical in early development, such as the copper transporter *Ctr1* (Lee et al., 2001), the zinc efflux transporter *ZnT1* (Andrews et al., 2004), and the iron efflux transporter *Fpn* (Donovan et al., 2005). These observations compel the notion that normal development requires intact systems to

maintain homeostasis of essential metals at the egg cylinder stage, when organogenesis initiates. Moreover, both the fly (*fear-of-intimacy* (Mathews et al., 2005)) and the zebrafish (*Liv-1* (Yamashita et al., 2004)) encode Liv-1 family zinc transporters that are essential during early morphogenesis. Potentially new roles for ZIPs have also been implicated with their involvement in signal transduction. This very new field of study is largely unexplored. As stated in the review, *Zip4* (a Liv-1 family member) is the first example of a mammalian *Zip* gene shown to serve an essential function during embryogenesis. Our data are the first demonstration that the ZIP4 protein is expressed very early in development (in the first cells forming the visceral endoderm). This early expression is coincident with the detrimental effects of its absence. I predicted that, based on the clinical findings in AE patients, normal *Zip4* function should be required for the maintenance of zinc homeostasis and for the overall health of the animal. Our findings support this prediction and demonstrate, moreover, that *Zip4* is required for organogenesis and that heterozygosity may also impact human health, especially for populations with zinc deficient diets. Humans have a heterozygous frequency for an AE disease allele of between 1 in 700 to 1 in 1,000 individuals. Considering the heterozygous frequency, the impact of *Zip4* mutations may have a larger impact on human health in developing nations than the otherwise rare autosomal recessive disease.

In the second specific aim, I evaluated the hypothesis that *Zip4* and *Zip5* are post-transcriptionally regulated inversely and dynamically in response to zinc availability. Wild type mice were fed zinc adequate or zinc deficient diets. Some of

the zinc deficient mice were repleted with zinc by oral gavage. Tissues known to express *Zip4* and *Zip5* were examined for expression of their mRNA levels, protein levels, and protein localization with time in response to changes in zinc availability. We found that both *Zip4* and *Zip5* were regulated by post-transcriptional mechanisms. Specifically, *Zip4* mRNA levels were found to be regulated by stability of the mRNA and not by transcriptional rate. The mechanisms of this regulation are entirely unexplored. Moreover, the ZIP4 protein was found to be stabilized by zinc deficiency and to accumulate on the apical surface of enterocytes and visceral endoderm. In contrast, zinc repletion resulted in the rapid internalization and degradation of the protein.

Concurrently published with our work, another study found that a histidine-rich region in the large intracellular loop of human ZIP4 was required for internalization and degradation of the human ZIP4 protein following zinc repletion (Mao et al., 2007). This is not true for the yeast Zrt1 transporter where only lysine 195 (and not a histidine-rich region) was necessary for ubiquitination. The authors of the concurrent ZIP4 study did not carefully examine the mouse and human sequences. Mouse ZIP4 does not contain several of the histidine residues that were found to be required for internalization of human ZIP4; yet the work herein clearly showed that mouse ZIP4 is internalized and degraded following zinc repletion. Moreover, that concurrent study relied entirely on transient transfection of HEK 293 cells which do not process ZIP4 similarly to *in vivo* ZIP4 (discussed below) and thus may not reflect the same regulation. In support of this, the authors of the concurrent ZIP4 study

required excessive amounts of zinc to yield an observable effect. The results reported herein represent the first demonstration of the very rapid post-translational down-regulation of the ZIP4 protein *in vivo* in response to zinc repletion. The mechanism of sensing zinc and subsequent internalization remains entirely unknown.

Subsequent to the findings herein, the ectodomain of ZIP4 was found to be removed from the protein whilst on the plasma membrane (Kambe and Andrews, 2009). In the work herein, we showed that ZIP4 was processed *in vivo* to generate an ≈ 37 kDa band and that Hepa cells recapitulated this same pattern (see Chapter 4). A subsequent investigator in the lab used Hepa cells to explore this observation further (Kambe and Andrews, 2009). Hepa cells expressing endogenous or transfected tagged ZIP4 constructs were examined for the processing of ZIP4 in response to zinc availability. Regulation of ZIP4 processing by Hepa cells was found to be exquisitely sensitive to zinc repletion (4 μM zinc yielded an effect; in contrast to the HEK study by the other group that required 200 μM zinc under similar culture conditions) (Kambe and Andrews, 2009). The exact cleavage site was not determined and requires future work but was found to be affected by several AE mutations in neighboring residues (Kambe and Andrews, 2009). As an important practical note, these findings demonstrate the importance of utilizing an appropriate cell model in order to study regulatory processes.

In contrast to our *Zip4* findings, *Zip5* mRNA levels were unresponsive to zinc availability and always remain polysome-associated. In contrast to a previous report (Dufner-Beattie et al., 2004), our data herein clearly show that the ZIP5 protein is lost

during zinc deficiency but rapidly reaccumulates on the basolateral membranes of enterocytes, visceral endoderm, and pancreatic acinar cells. Overall, the data support a translational stall mechanism for the regulation of *Zip5* expression. This represents the first indication of such regulation in zinc homeostasis. This stall mechanism has yet to be delineated. I predicted, based on previous findings that ZIP4 and ZIP5 proteins are localized to the apical and the basolateral membranes, respectively, in various cell types with zinc deficient and zinc adequate conditions, respectively, that *Zip4* and *Zip5* would be post-transcriptionally and reciprocally regulated in response to zinc availability. Our findings support this prediction and demonstrate, moreover, that *Zip4* and *Zip5* were rapidly and coordinately regulated in several tissues in response to zinc availability.

In the third specific aim, I evaluated the hypothesis that *Zip5* is regulated by a rapid post-transcriptional mechanism mediated by the 3' untranslated region of the mRNA in response to zinc availability. Using *in vivo* and *in vitro* techniques, regulation of *Zip5* expression was examined. We report for the first time the existence of a highly conserved stem-loop structure in the *Zip5* 3' UTR reminiscent of the previously reported IRE. The significance of this element in *Zip5* regulation remains to be tested. Two miRNAs were found to be co-expressed in tissues that express and regulate *Zip5*. miR-328 and miR-193 are predicted to target accessible regions of the *Zip5* 3' UTR flanking the identified stem-loop structure and both miRNAs were found to be associated with the polysome fraction of RNA. These miRNAs were detected at sizes closer in length to their precursor miRNAs than to

their expected mature forms. This is the first report showing the abundant accumulation of these miRNAs on the polysomes in these novel sizes. The steady state levels of these miRNAs and their associations with the polysome fraction were not significantly affected by zinc availability. However, these findings cannot rule out zinc-dependent changes in the activity of ribonucleoprotein complexes. I predicted, based on the findings in the second aim, that *Zip5* is regulated by a translational stall mechanism. Our findings, though incomplete, are still consistent with this prediction. Based on 1) the high level of target sequence conservation across multiple mammalian species, 2) the predicted accessibility of the target sites, and 3) the association of miR-328 and miR-193 with the polysome fraction, we conclude that one or both of these miRNAs are involved in a translational stall mechanism for *Zip5*. If true, this finding would represent the first demonstration of miRNAs involved in regulating zinc homeostasis. Further work is necessary to prove the function of these miRNAs and evaluate the potential role of zinc-regulated RNA binding protein(s) that target the *Zip5* 3' UTR.

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Appendices

Appendix I: Citations of Published Papers in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry and Molecular Biology

First Author / Joint-first Author

1) Dufner-Beattie J., **Weaver B.P.,** Geiser J., Bilgen M., Larson M., Xu W., Andrews G.K. **2007.** The mouse acrodermatitis enteropathica gene *Slc39a4* (*Zip4*) is essential for early development and heterozygosity causes hypersensitivity to zinc deficiency. *Hum Mol Genet.* 16(12): 1391-1399. PMID: 17483098

2) Weaver B.P., Dufner-Beattie J., Kambe T., Andrews G.K. **2007.** Novel zinc-responsive post-transcriptional mechanisms reciprocally regulate expression of the mouse *Slc39a4* and *Slc39a5* zinc transporters (*Zip4* and *Zip5*). *Biol Chem.* 388 (12): 1301-1312. PMID: 18020946

Second Author

1) Kambe T., **Weaver B.P.**, Andrews G.K. **2008.** The genetics of essential metal homeostasis during development. *Genesis*. 46(4): 214-228. Review. PMID: 18395838

Appendix II: Citations of Peer-reviewed Presentation/Poster Abstracts

Benjamin P. Weaver and Glen K. Andrews. Posttranscriptional Regulation of the Mouse Zinc Transporters ZIP4 and ZIP5. **American Society for Cell Biology.** December 2005, San Francisco, CA. **Poster.**

Benjamin P. Weaver and Glen K. Andrews. Rapid Posttranscriptional Regulation of the Mouse Zinc Transporters ZIP4 and ZIP5 following Zinc Repletion. **FASEB Trace Element Metabolism: Integrating Basic and Applied Research.** June 2006, Snowmass Village, CO. **Poster.**

Benjamin P. Weaver. Posttranscriptional Regulation of ZIP4 and ZIP5 as a Result of Zinc Availability. **Biomedical Research Training Program.** May 2006, University of Kansas Medical Center, Kansas City, KS. **Presentation.**

Benjamin P. Weaver and Glen K. Andrews. Rapid Posttranscriptional Regulation of the Mouse Zinc Transporters ZIP4 and ZIP5. **NIH Graduate Student Festival.** October 2007, Bethesda, MD. **Poster.**

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