# TRANSPORT AND REGULATION OF HEPATIC ZINC

By

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To my mother and grandfather

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| | |
|---|---|
| AP-1 | Activator protein-1 |
| AE | Acrodermatits enteropathica |
| ChIP | Chromatin Immunoprecipitation |
| DTPA | Diethylene triamine pentaacetic acid |
| EDS | Ehlers–Danlos syndrome |
| EST | Expressed sequence tag |
| GFP | Green fusion protein |
| HIF-1α | Hypoxia inducible factor-1 alpha |
| hnRNA | Heterogeneous nuclear RNA |
| IF | Immunofluorescence |
| IL-1β | Interleukin-1 beta |
| IL-6 | Interleukin-6 |
| iNOS | Inducible nitric oxide synthase |
| LPS | Lipopolysaccharide |
| MRE | Metal response element |
| mRNA | Messenger RNA |
| MT | Metallothionein |
| MTF-1 | Metal response element binding transcription factor-1 |
| NO | Nitric oxide |
| PI3K | Phosphatidylinositol-3-kinase |
| Pol II | RNA Polymerase II |
| qPCR | Quantitaive polymerase chain reaction |
| SNAP | s-nitroso n-acetyl pennicillamine |
| SNP | Single nucleotide polymorphism |

| | |
|---|---|
| TBP | TATA box-binding protein |
| TGN | Tran-golgi network |
| TMD | Transmembrane domain |
| TNAP | Tissue non-specific alkaline phosphatase |
| TPEN | N,N,N’,N’-Tetrakis-(2-pyridylmethyl)- Ethylenediamine |
| WT | Wild-type |
| ZIP | Zrt-Irt-like zinc transporter superfamily |
| ZnA | Zinc adequate |
| ZnD | Zinc deficient |
| ZnT | Zinc transporter |

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The anemia of chronic disease is driven by inflammatory cytokines. These cytokines also regulate genes that produce hypozincemia and hepatic zinc accumulation. In the sterile abscess model of inflammation, up-regulation of the zinc transporter, Zip14, by IL-6 is the mechanism responsible for the hypozincemia. However, experiments with IL-6 knockout mice show that LPS regulates Zip14 expression by a mechanism that is partially independent of IL-6. The LPS-induced model of sepsis may occur by a mechanism signaled by nitric oxide (NO) as a secondary messenger. Therefore, it was hypothesized that NO can modulate Zip14 expression during LPS-induced inflammation. To address this hypothesis, primary hepatocytes from wild-type mice were treated with the nitric oxide donor s-nitroso n-acetyl penicillamine (SNAP). After treatment with SNAP, Zip14 steady-state mRNA levels displayed a biphasic response with a maximal increase after 8 h, and a concomitant increase in the transcriptional activity of the gene. ChIP analysis documented the kinetics of AP-1 and Pol II association with the Zip14 promoter after NO exposure, indicating a role of AP-1 in transcription of Zip14. A more physiologic approach was then taken to investigate NO regulation by stimulating primary murine hepatocytes with IL-1β, an LPS-induced proinflammatory cytokine. IL-1β is a potent activator of inducible nitric oxide synthase (iNOS) and NO production. In support of our hypothesis, IL-1β treatment

led to a three-fold increase in Zip14 mRNA and enhanced zinc transport as measured by FluoZin3-AM fluorescence in wild-type, but not iNOS-/- hepatocytes. These data suggest that signaling pathways activated by NO are factors in the up-regulation of Zip14 that in turn mediates hepatic zinc accumulation and hypozincemia during inflammation and sepsis.

On the other hand, NO causes a decrease in hepatic Zip10 expression. Recently, Zip10 expression was shown to increase in vivo in the absence of metal-responsive transcription factor-1 (MTF-1). Therefore, a goal was set to determine if zinc and/ or NO could regulate hepatic Zip10, and if this regulation occurs via MTF-1. To answer these questions, both in vivo and in vitro methods were used. First, primary mouse hepatocytes were incubated with increasing amounts of zinc. A dose-dependant decrease in Zip10 mRNA expression was observed, with an apparent 10-fold decrease within 3 h after zinc addition. Similar results were observed with NO. Mice were fed a zinc-deficient diet (<1ppm) for 21 d. Interestingly, hepatic Zip10 mRNA increased five fold, and a concomitant increase in ZIP10 protein expression was also observed. The mechanism of Zip10 down-regulation by zinc and/ or NO was elucidated by using siRNA to knockdown MTF-1 expression in AML12 cells. Neither zinc nor NO could suppress Zip10 expression in the absence of MTF-1. Therefore, under these conditions MTF-1 is acting as a transcriptional repressor. These results suggest that hepatic Zip10 expression is negatively regulated by zinc through MTF-1, and Zip10 may be important for hepatic zinc uptake during deficiency.

In summary, the data presented here show that the liver controls zinc uptake under stress by either up- or down-regulating certain Zip transporters.

# CHAPTER 1
# INTRODUCTION

## Zinc as an Essential Nutrient

In 1869, the first evidence for zinc as an essential trace element was realized through studies of the growth of *Aspergillus niger* (Raulin, 1869). This finding was later confirmed by Steinberg, through systematic addition of zinc to culture medium (Steinberg, 1919). The next step towards defining zinc as essential was determined 7 years later, by growth of higher green plants (Sommer, 1926). Shortly after that discovery, the first indication of the necessity of zinc for mammalian species was found in rats (Todd et al., 1934). By the 1950's the importance of zinc in animal development was made clear by further studies involving parakeratosis in swine (Tucker, 1955), and the growth of chickens (O'Dell, 1958). In humans, a syndrome of iron deficiency, hepatosplenomegaly, short stature, and hypogonadism was identified in young Iranian men and boys (Prasad et al., 1961). Shortly thereafter these clinical manifestations were found to be responsive to zinc supplementation, identifying the first incidence of zinc deficiency in man (Sanstead et al., 1967).

## Zinc Deficiency

The symptoms of zinc deficiency include retarted growth, depressed immune function, skin lesions, depressed appetite, skeletal abnormalitites, and impaired reproductive ability. Human zinc deficiency is characterized as a type II nutritional deficiency in which growth is impaired without a reduction in tissue zinc concentrations (Golden, 1989). It has been suggested that a small pool of exchangeable zinc exists that help maintains tissue zinc. Results of stable zinc isotope studies support the concept of a labile, exchangeable zinc pool (Miller, et al., 1994). This pool may be critical to maintaining various cellular processes. In rodent models of zinc deficiency, plasma zinc drops rapidly when zinc is removed from the diet. At the same time,

regulatory changes occur in zinc transporter proteins and the amount of metallothionein-bound zinc decreases (reviewed in Liuzzi and Cousins, 2004; Liuzzi, et al., 2004). Clinical signs of zinc deficiency occur only when deficiency is severe enough to overcome homeostatic mechanisms that supply the various body pools necessary for zinc-dependent cellular functions. Zinc transporter proteins are critical for maintaining whole-body zinc levels. Mutations in the Zip4 gene leads to zinc malabsorption and cause acrodermatitis enteropathica (AE) which is characterized by impaired immunity, skin leasions, and an increased susceptibility to infections (Kury et al., 2002; Mills, 1989).

Although the exact mechanism causing impaired immunity by zinc deficiency is unknown, clinical studies analyzing total parenteral nutrition (TPN) solutions containing less than adequate zinc revealed a decrease in natural killer cell activity (Cousins, 1996). Furthermore, adequate zinc status may be necessary for the control and production of certain immune regulators, such as INF$\gamma$, IL-1, IL-2, IL-6, and TNF$\alpha$ (Ibs, 2003; Prasad, 2003).

## Absorption and Metabolism

Absorption of zinc begins in the small intestine, but may also occur in the colon. The jejunum and duodenum are the primary areas of absorption (Cousins, 1989). Luminal zinc perfusion studies indicate that the jejunum has the highest zinc absorption rate (Lee et al., 1989). Various factors play a role in the absorption process, including digestion of food, of zinc chelation by certain factors (e.g., phytates) and intestinal transit time.

Zinc absorption is maintained homeostatically by balancing dietary zinc absorption and endogenous secretions through adaptive regulation programmed by the dietary zinc supply (Cousins, 2006). The current Dietary Reference Intake (Institute of Medicine of the National Academies, Washington, DC) recommendations for Zn intake by humans are based on metabolic

assessments that measure this balance (Dietary Reference Intake). The intestine is paramount to maintaining that balance. The intestine retains absorptive capacity programmed by previous dietary intake when isolated from other systemic factors including pancreatic secretions (Hoadley et al., 1987). Analysis of true absorption indicates that low zinc intake increases the efficiency of zinc absorption, irrespective of endogenous zinc (Ziegler, 1989; Lee, 1993).

**Zinc Transporters**

Two classes of mammalian zinc transporters exist. The first is the ZnT family, which act to decrease intracellular zinc levels by transporting zinc from the cytoplasm to the lumen of organelles, or the extracellular space. The second group of proteins is the ZIP (Zrt-, Irt-like Protein) family, which is named after the yeast Zrt1 protein and the *Arabidopsis* Irt1 protein. The signature of the ZIP family is that, without any known exceptions, these proteins are responsible for increasing intracellular zinc levels by either transporting the metal from the extracellular space, or organellar lumen into the cytoplasm.

**The ZnT (SLC30) Family**

More than 100 members of the SLC30 family are found in organisms at all phylogenetic levels. The ZnT famly is divided into three subfamilies. Subfamily I contains mainly prokaryotic members, whereas subfamilies II and III contain eukaryotic and prokaryotic members in a similar ratio (reviewed in Liuzzi and Cousins, 2004). Most ZnT proteins have six transmembrane domains (TMDs) and are predicted to have cytoplasmic amino and carboxy termini. In addition, a classic characteristic of ZnT proteins is the long histidine-rich loop between TMDs IV and V, (HX)n (n = 3 to 6), which could represent a metal-binding domain. Highly amphiphatic TMDs I, II, and V are well conserved (reviewed in Liuzzi and Cousins, 2004).

**ZnT1 (SLC30A1)**

The first mammalian zinc transporter to be discovered was *ZnT1* (*Slc30a1*), which was mapped to chromosome 1 in both humans and mice (Palmiter and Findley, 1995). ZnT1 was identified by isolation from a rat kidney cDNA expression library by complementation of a mutated, zinc-sensitive BHK cell line. ZnT1 displays a ubiquitous tissue distribution, however it is more highly expressed in tissues involved in zinc acquisition, recycling, or transfer, such as the small intestine, renal tubular epithelium, and placenta (Reviewed in Liuzzi and Cousins, 2004). When neuronal cells were transfected with rZnT1 cDNA, the protein localized primarily to the plasma membrane with some punctate staining throughout the cell (Kim et al., 2000). In vivo immunolocalization studies indicate that, in growing male rats, ZnT1 is increasingly abundant along basolateral membranes of enterocytes where it may participate in zinc transfer into the circulation (Reviewed in Liuzzi and Cousins, 2004). Abundant expression of ZnT1 was also found on the basolateral surface in cells lining the thick ascending and distal convoluted tubules of the kidney (Cousins and McMahon, 2000). This localization of ZnT1 indicates that it may play a role in recovery of zinc from the glomerular filtrate. ZnT1 also localizes to the villous yolk sac membrane, suggesting that ZnT1 participates in zinc transport between maternal supplies and the fetus (Cousins and McMahon, 2000; Langmade et al., 2000; Liuzzi et al., 2003). Interestingly, homozygous targeted knockout of *ZnT1* leads to early embryonic lethality in the mouse, indicating that ZnT1 serves an essential function of transporting maternal zinc into the embryonic environment during the egg cylinder stage of development, and further suggests that ZnT1 plays a role in zinc homeostasis in adult mice (Andrews et al., 2004).

ZnT1 expression can be influenced differentially by the dietary zinc supply. Rats fed a diet deficient in zinc (<1 ppm) showed decreased ZnT1 mRNA expression, whereas rats fed a diet high in zinc (180 ppm) exhibited increased ZnT1 mRNA abundance (Liuzzi et al., 2003). These

findings indicate a metal responsive mode of regulation for the *ZnT1* gene. Indeed, in vitro DNA-binding assays demonstrated that mouse MTF-1 can bind avidly to two metal-response element sequences found in the ZnT1 promoter. Using mouse embryo fibroblasts with homozygous deletions of the MTF-1 gene, it was shown that this transcription factor is essential for basal as well as metal (zinc and cadmium) regulation of the ZnT1 gene in these cells. In vivo, ZnT1 mRNA was abundant in the midgestation visceral yolk sac and placenta. Dietary zinc deficiency during pregnancy leads to down-regulation of ZnT1 levels in the visceral yolk sac, but has little effect on the mRNA in the placenta. Homozygous knockout of the MTF-1 gene in mice also leads to a reduction in ZnT1 mRNA levels in the visceral yolk sac, suggesting that MTF-1 mediates the response of *ZnT1* to zinc in the visceral yolk sac (Langmade et al., 2000).

**ZnT2 (SLC30A2)**

Similar to ZnT1, A cDNA encoding the second zinc transporter (ZnT-2) was isolated from a rat kidney cDNA expression library by complementation of a zinc-sensitive BHK cell line (Palmiter et al., 1996). However, unlike ZnT1, which is in the plasma membrane and lowers cellular zinc by stimulating zinc efflux, ZnT2 is localized on vesicles and allows the zinc-sensitive BHK cells to accumulate zinc to levels that are much higher than non-transformed cells can tolerate. ZnT2 mRNA has been detected in specific tissues of rodents: small intestine, kidney, placenta, pancreas, testis, seminal vesicles, and mammary gland (reviewed in Liuzzi and Cousins, 2004).

ZnT2 is up-regulated in the small intestine by high dietary Zn intake, during very late-stage gestation, and early lactation in maternal and fetal tissues (Liuzzi et al., 2003). Similarly, the lateral lobes of the prostate have a very high Zn content which correlates with high ZnT2 expression (Iguchi, 2002). The association of ZnT2 with high cellular Zn concentrations and its

vesicular localization in the acinar cells of the pancreas suggests that this transporter may function through an exocytotic pathway, before incorporation of Zn into secreted pancreatic proteins to control endogenous losses (Liuzzi et al., 2004).

The role of ZnT2 in maintenance of zinc homeostasis is not entirely known, however its production in the mammary gland, and concurrent decrease in abundance with milk zinc concentration suggests a role for this transporter in mammary gland zinc metabolism. Interestingly, while the abundance of ZnT2 at the basolateral membrane appears to remain constant, the expression of ZnT2 at the apical membrane of the mammary gland decreases through lactation (Kelleher and Lonnerdal, 2003). These results are consistent with ZnT2 relocalizing to an intracellular compartment of mammary epithelial cells during exposure to physiogically high levels of zinc, to possibly sequester excess cellular zinc (Kelleher and lonnerdal, 2003; Palmiter, 1996). Furthermore, a study investigating transient neonatal zinc deficiency in two breast-fed infants as a consequence of reduced zinc secretion into breast milk, identified a His to Arg mutation at amino acid residue 59 of ZnT2 in the mothers linking this gene mutation to infant zinc deficiency (Chowanadisai, 2006).

**ZnT3 (SLC30A3)**

ZnT3 protein is predicted to have six transmembrane domains and shares 52% amino acid identity with ZnT2, with the homology extending throughout the two sequences. The ZnT3 gene was identified and subsequently cloned by screening of a mouse λ library through homology with ZnT2 cDNA (Palmiter, 1996). ZnT3 mRNA is most abundant in the hippocampus and cortex of the mouse brain. The ZnT3 protein is detected immunologically in the mossy fibers, where zinc-containing vesicles are most abundant.

The mammalian brain contains an abundant amount of zinc, with 5-15% concentrated in synaptic vesicles in a subset of glutamatergic neurons (Haug, 1967; Perez-Clausell and Danscher, 1985; Frederickson and Moncrief, 1994). Zinc is also particularly abundant in the hippocampus. Homozygous disruption of murine *ZnT3* (*ZnT3*$^{-/-}$) decreases the amount of detectable zinc in these regions of the brain (Cole, 1999). Timm stain is used for histochemical detection of zinc, and this method revealed the reduction in zinc content corresponds exclusively to zinc packaged into synaptic vesicles (Frederickson, 1989). An intermediate level of both ZnT3 protein and histochemically reactive zinc was found in *ZnT3* heterozygotes (*ZnT3*$^{+/-}$) when compared to WT and *ZnT3*$^{-/-}$ mice, demonstrating that the amount of zinc in synaptic vesicles is limited by the abundance of ZnT3. These results suggest that zinc is taken up into synaptic vesicles through ZnT3 located at the vesicle membrane.

**ZnT4 (SLC30A4)**

A disorder of zinc deficiency was identified in mouse pups unable to survive infancy nursing on milk of mice homozygous for the autosomal-recessive mutation, lethal milk (*lm*) (Piletz and Ganschow, 1978). The major effect of the *lm* mutation is the production of Zn-deficient milk (Ackland and Mercer, 1992). The gene responsible for the lm phenotype was later identified as *ZnT4* by positional cloning (Huang and Gitschier, 1997). Confirmation of the zinc transport function of ZnT4 was achieved by complementation of the yeast *ZRC1* mutant. A single C to T point mutation at base 934 leads to a nonsense mutation and premature translation termination of ZnT4. This mutation leads to an approximately 50% reduction in milk of *lm* animals (Ackland and Mercer, 1992; Lee, 1992). However, because milk from lm mice is not completely void of zinc, and maternal zinc supplementation is able to rescue the lethal milk phenotype, other zinc transporters may be active, such as the aforementioned ZnT2. Indeed, analysis of *hZnT4* gene expression in a mammary gland disorder leading to reduced zinc

secretion into human milk indicated that ZnT4 was not responsible for post-natal zinc deficiency (Michalczyk et al., 2003).

ZnT4 is not only found in mammary tissue, but it is also abundantly expressed in the mouse brain and intestinal epithelial cells (Huang and Gitschier, 1997; Liuzzi, 2001; Murgia, 1999). ZnT4 localizes to intracellular vesicles (Huang, 1997; Kelleher, 2002; Murgia, 1999). The endogenous ZnT4 was detected in the Golgi apparatus as well as in the vesicular compartment in rat normal kidney (NRK) cells, whereas transfection of a Myc-tagged version of ZnT4 into Caco-2 cells revealed expression in an endosomal compartment (Huang, 1997; Murgia C 1999). Expression of *ZnT4* appears to be independent of zinc status (Liuzzi et al., 2001). However, an increased extracellular zinc concentration induces trafficking of ZnT4 from trans-golgi network (TGN) to the cytoplasmic vesicular compartment in cultured NRK cells (Huang, 1997).

Overall, ZnT4 appears to facilitate entry of zinc into secretory vesicles of certain glands (mammary and submaxillary) and thereby allows secretion of zinc by these exocrine glands (Reviewed in Palmiter and Huang, 2004).

**ZnT5 (SLC30A5)**

A database search of DNA sequences homologous to yeast ZRC1 allowed identification of ZnT5. Human ZnT5 cDNA encodes a 765-amino acid protein with 15 predicted membrane-spanning domains (Kambe, 2002). ZnT5 was ubiquitously expressed in all tested human tissues, but was most abundantly expressed in insulin-containing beta cells that contain zinc at the highest level in the body (Kambe et al., 2002). Another report, published at almost the same time, reported the cloning of a human zinc transporter expressed at the apical membrane of the Caco-2 human small intestinal cell line model designated hZTL1 (human ZnT-Like transporter 1) (Cragg et al., 2002). However, hZTL1 was subsequently identified as hZnT5.

An intriguing aspect of ZnT5 function is the observation that this transporter interacts with ZnT6 to form a complex that can transport zinc into the secretory pathway (Ellis et al., 2005). Additionally, ZnT5 and ZnT6 are both located in the trans-Golgi network of mammalian cells (Kambe et al., 2002; Huang, 2002), and are expressed in many of the same tissues (Seve et al., 2004). The formation of ZnT5/ZnT6 hetero-oligomeric complexes is considered to be essential for their functions, because both genes need to be expressed to activate tissue-nonspecific alkaline phosphatase (TNAP) (Suzuki et al., 2005).

Regulation of *ZnT5* is complex; two major transcripts of *ZnT5* were identified by Northern blotting, and comparison of the two published sequences shows that they differ at both the 5' and 3' ends, with variant B being a shorter transcript (Kambe et al., 2002; Cragg et al., 2002). Alignment of both sequences with the human genome reveals that they are splice variants of the SLC30A5 gene, incorporating different exons at the 5' and 3' ends (Jackson et al., 2007). Variant B was localized to the plasma membrane, where evidence for bidirectional function (Valentine et al., 2007) indicates possible roles in both the uptake and efflux of zinc. Both increased and reduced expression of *ZnT5* has been found in response to zinc, including increased expression in Caco-2 cells exposed to 100 μM zinc (Cragg et al., 2002), decreased expression in human intestinal mucosa in response to zinc supplementation (Cragg et al., 2005), and reduced expression in the mouse placenta in response to both a zinc-restricted and zinc-supplemented diet (Helston et al., 2006). Therefore, two modes of regulation appear to exist for *ZnT5*, transcriptional repression and increased mRNA stability (reviewed in Jackson et al., 2008).

The importance of ZnT5 to zinc homeostasis is emphasized by deletion of *ZnT5* in mice, which leads to poor growth, abnormal bone development, weight loss, and male-specific cardiac arrhythmias (Inoue et al., 2002).

**ZnT6 (SLC30A6)**

Search of the EST databases with the amino acid sequence of mouse ZnT4 revealed the zinc transporter homolog ZnT6 (Huang, L. et al 2002). Overexpression of ZnT6 in both wild-type yeast and mutants that are deficient in cytoplasmic zinc causes growth inhibition, but this inhibition is abolished in mutant cells with high cytoplasmic zinc. ZnT6 may function in transporting cytoplasmic zinc into the Golgi apparatus as well as the vesicular compartment. ZnT5 and ZnT6 are both localized to the TGN (Kambe et al., 2002; Huang, 2002), and functionally interact to activate TNAP (Ellis et al., 2005; Suzuki et al., 2005).

ZnT6 mRNA was found in the liver, brain, kidney, and small intestine. Intriguingly, the protein was only detected in the brain and lung suggesting that a post-transcriptional mechanism may play a role in tissue-specific expression of the ZnT6 protein (Huang, L. et al 2002).

**ZnT7 (SLC30A7)**

ZnT7 was identified by homology to the amino acid sequence of ZnT1 in the EST databases (Kirschke and Huang, 2003). The Znt7 gene is expressed in many mouse tissues including liver, kidney, spleen, heart, brain, small intestine, and lung, with abundant expression in small intestine and liver and less expression in the heart. However, expression of ZnT7 protein is limited to the tissues of lung and small intestine with abundant expression in the proximal segment (duodenum and part of jejunum) of the small intestine (Kirschke and Huang, 2003). When over-expressed in chinese hamster ovary cells (CHO), ZnT7 leads to zinc accumulation in the Golgi apparatus. ZnT7 localizes to a vesicular compartment seemingly different from that of ZnT2, ZnT3, ZnT4, ZnT5 or ZnT6 in the hBRIE 380, WI-38, and transiently transfected NRK cells suggesting that ZnT7 may also be involved in transporting zinc into a unique vesicular compartment. Disruption of *ZnT7* in DT40 cells results in a 20%

decrease in TNAP activity (Suzuki et al., 2005). Therefore, there seems to be at least partial dependence of TNAP activity on ZnT7.

*Znt7* knockout mice display a zinc-deficient phenotype that is unresponsive to dietary zinc supplementation (Huang et al., 2007). Furthermore, these mice demonstrate poor growth and have decreased body fat composition. This, suggesting that ZnT7 plays a critical role in maintaining cellular zinc homeostasis, and may be involved in the regulation of body composition.

**ZnT8 (SLC30A8)**

Insulin-secreting ß-cells, located in the islets of Langerhans of the pancreas, accumulate very high amounts of zinc (Zalewski et al., 1994). Insulin is thought to be stored inside secretory vesicles as a solid hexamer bound with two $Zn^{2+}$ ions per hexamer (Emdin et al., 1980). Insulin is then released by exocytosis in response to external stimuli, such as elevated glucose concentrations. When exocytosis of insulin occurs, insulin granules fuse with the ß-cell plasma membrane, releasing insulin as well as zinc, into the circulation (Qian et al., 2000). Interestingly, a complex relationship between zinc and both type 1 and type 2 diabetes arises because of several complications of diabetes may be mediated through oxidative stress, which is amplified in part by zinc deficiency (reviewed in Chausmer, 1998).

In 2004, the islets of Langerhans–specific zinc transporter, ZnT8, was identified in ß-cells, and shown to facilitate the accumulation of zinc from the cytoplasm into intracellular vesicles (Chimienti et al., 2004). Moreover, a ZnT8-EGFP fusion protein was colocalized with insulin secretory granules in the rat insulin-secreting INS-1 cell line, suggesting ZnT8 may be a major component for providing zinc to insulin maturation and/or storage processes in insulin-secreting pancreatic ß-cells. Three years later, the importance of *ZnT8* in the etiology of diabetes became clear when the *ZnT8* gene was first associated with a novel risk locus for type II diabetes (Sladek

et al., 2007; Saxena et al., 2007; Scott et al., 2007; Zeggini et al., 2007). By genotyping of 921 metabolically characterized German subjects for candidate single nucleotide polymorphisms (SNPs) of SLC30A8, the SNP rs13266634 associated with reduced insulin secretion stimulated by orally or intravenously administered glucose, but not with insulin resistance (Staiger et al., 2007). Furthermore, the major non-synonymous SNP at Arg325-encoding C-allele confers a minor risk (odds ratio 1.07–1.18) of disease. In non-diabetic subjects with a family history of type 2 diabetes, the C-allele was associated with increased circulating proinsulin-to-insulin ratio (Kirchkoff et al., 2008), and decreased insulin responses in intravenous glucose tolerance tests (Boesgaard et al., 2008), indicating a dominant effect on insulin secretion, β-cell mass, or both.

In addition, by utilizing microarray expression profiling of human and rodent pancreas and islet cells and screening with radioimmunoprecipitation assays using new-onset type 1 diabetes and prediabetic sera, ZnT8 was identified as a major autoantigen in human type I diabetes (Wenzlau et al., 2007). However, autoantibodies to ZnT8 in human type 1 diabetic patients show little cross-reactivity to other human Zn transporters or even to mouse ZnT8, which is 82% identical in sequence. Interestingly, the amino acid encoded by a common polymorphism in human ZnT8 at aa325 (either Arg or Trp) is a key determinant of two of the three major conformational epitopes in the protein (Wenzlau et al., 2008). The autoantibody responses to the ZnT8 Arg- and Trp-restricted isoepitopes segregated with the alleles encoding the respective variant amino acids, which indicates that humoral type 1 diabetes autoimmunity to ZnT8 is directed against self and not nonself epitope determinants (Wenzlau et al., 2008). Recently, mutagenesis of mZnT8 Q324 to arginine (equivalent to R325 in the human protein) allowed for reactivity with human autoimmune sera, which further indicates that the hZnT8 epitope is critically dependent upon the arginine residue at position 325 (Wenzlau et al., 2008).

Finally, the implications of hZnT8 identification as an autoantigen for type I diabetes are far reaching, and show that hZnT8 autoantibodies can be used as an additional and independent predictive marker for type I diabetes (Wenzlau et al., 2008).

**ZnT9 (SLC30A9)**

The *ZnT9* gene was originally isolated from human embryonic lung cells (Sim and Chow, 1999). The 569 amino acid protein has a putative cation efflux motif, a DNA excision repair motif, and a nuclear receptor interaction sequence. Although the protein is predicted to have 6 transmembrane domains, it associates with cytosol and nuclear fractions, not membranes (Sim and Chow, 1999). To date, there have no studies assessing the function of this protein.

**ZnT10 (SLC30A10)**

Utilizing DNA sequence homology with *ZnT1, ZnT10* was found (Seve et al., 2004). From EST analysis results, ZnT10 had a restricted expression profile to the fetal liver and fetal brain. This is the first ZnT predicted to have a fetal restricted expression. It is therefore possible to speculate that ZnT10 plays an important role in zinc homeostasis during fetal development. However, no studies have been conducted to analyze the function of ZnT10.

**The ZIP Family (SLC39)**

The human SLC39 (Slc39 nomenclature for the mouse) transporters are members of the ZIP family of metal ion transporters (Eng et al., 1998; Gaither and Eide, 2001). The first members to be identified were Zrt1 and Zrt2, the primary zinc uptake transporters in the yeast *Saccharomyces cerevisiae*, and Irt the major iron uptake transporter in roots of *Arabidopsis thaliana,* hence the designation ZIP for Zrt, Irt-like protein (reviewed in Eide, 2004). Most ZIP proteins have eight predicted transmembrane domains (TMDs) and similar predicted membrane topologies with the N- and C-termini of the protein located along the extracellular surface of the membrane. Many members also have a long loop region located between TMDs 3 and 4, and a

histidine-rich domain with the sequence (HX)n where n generally ranges from 3 to 5. Due to their sequence conservation and amphipathic nature, TMDs IV and V are predicted to form a cavity through which metals may pass (Eide, 2006).

**Zip1 (SLC39A1)**

Zip1/ ZIRTL was identified through homology with the *Arabidopsis thaliana* ZIP1 transporter, and is expressed in a wide variety of tissues and cell types (Lioumi et al., 1999; Gaither, 2001). Zip1 is localized to different areas of the cell in a cell-type specific manner. In K562 cells, hZip1 localizes to the plasma membrane where it allows energy-independent zinc uptake (Gaither, 2001). In cell types such as COS-7 or PC3, hZip1 is localized mainly in the endoplasmic reticulum (Milon et al., 2001). Transport of zinc into K562 cells by hZip1 was indistinguishable from endogenous uptake, and could be abolished by antisense RNA directed against hZip1 indicating a requirement for Zip1-mediated zinc transport in these cells (Gaither LA and Eide DJ 2001). Moreover, saturable $^{65}$Zn uptake kinetics correlate with increased Zip1 mRNA abundance after exposure to prolactin and testosterone (reviewed in Liuzzi and Cousins, 2004; Costello et al., 1999).

The murine orthologue, mZip1, is present in all tissues except for the pancreas, and the abundance of Zip1 mRNA is not regulated by dietary zinc in the intestine or visceral endoderm, tissues involved in nutrient absorption (Dufner-Beattie et al., 2003). Furthermore, studies of transfected cells revealed that Zip1 is mainly present in intracellular organelles in cells cultured in zinc adequate medium but is recruited to the cell surface when zinc is limiting, suggesting a post-transcriptional regulatory mechanism (Wang et al., 2004). Recently, a protein chimera of Zip1 was created demonstrating that a di-leucine sorting signal of ZIP1 was required and sufficient for endocytosis of the protein (Huang and Kirschke, 2007).

Additionally, homozygous knockout of mZip1 produces no phenotype when dietary zinc intake is normal, but can adversely effect embryo survival during pregnancy when intake of zinc is limiting (Dufner-Beattie et al., 2006).

**Zip2 (SLC39A2)**

The human zinc transporter, hZip2, was identified by similarity of the protein coding sequence to zinc transporters characterized in fungi and plants (Gaither and Eide, 2000). Similarly, the murine orthologue mZip2 was identified by sequence similarity with hZip2 (Dufner-Beattie et al., 2003). Expression of hZip2 is low and appears to be limited to the prostate, uterus, cervical epithelium, optic nerve, and monocytes (reviewed in Liuzzi and Cousins, 2004). Expression of mZip2 also appears to be tissue-restricted, with the highest levels detected in the skin, liver, ovary, and visceral yolk sac (Dufner-Beattie et al., 2003). Both hZip2 and mZip2, when transfected into K562 or HEK293 cells, allow zinc uptake activity (Gaither and Eide, 2000; Dufner-Beattie et al., 2003).

While these orthologues share 78% sequence similarity, they appear to be regulated differently and in a cell-type/ tissue specific manner. Treatment of the THP-1 monocytic cell line, or human peripheral blood mononuclear cells with TPEN, a cell-permeable zinc chelator, resulted in a large increase in hZip2 mRNA levels, suggesting zinc regulated expression (Cao et al., 2001). However, mZip2 was unresponsive to dietary zinc restriction in the intestine and visceral yolk sac (Dufner-Beattie et al., 2003). $Zip2^{-/-}$ mice demonstrate no overt phenotype, but are however more sensitive to dietary zinc deficiency during pregnancy (Peters et al., 2007).

**Zip3 (SLC39A3)**

Similar to hZip1 and hZip2, hZip3 was identified by comparison of fungal and plant ZIPs with mammalian ESTs (Gaither and Eide, 2000). The mouse orthologue, mZip3, was again identified through protein homology with its human counterpart (Dufner-Beattie et al., 2003).

Low levels of Zip3 expression can be detected in many tissues, with the highest levels in the testes (Dufner-Beattie et al., 2003). Additionally, Zip3 mRNA is not regulated by dietary zinc in the intestine or visceral endoderm, tissues involved in nutrient absorption. Zip3 is capable of zinc uptake when transfected into HEK293 cells. However, zinc uptake could be inhibited by various metals suggesting ZIP3 mediated metal transport is not specific for zinc (Dufner-Beattie et al., 2003). Cell transfection of Zip3 revealed the presence of the protein in intracellular organelles in zinc-replete medium, but recruitment to the cell surface when zinc is limiting (Wang et al., 2004).

Although mammary epithelial cells were shown to have a requirement for Zip3-mediated zinc import (Kelleher and Lonnerdal, 2005), mice lacking the Zip3 transporter exhibited no obvious phenotypic abnormalities under normal growth conditions and were only slightly more susceptible to the effects of dietary zinc deficiency (Dufner-Beattie, et al., 2005). These findings are somewhat disappointing considering a substantial amount of $Zn^{2+}$ is transferred by the mammary gland from the maternal circulation into milk, supplying zinc to the suckling neonate. However creation of ZIP1, ZIP3 double-knockout mice showed that these proteins were essential for normal embryo development during zinc deficiency (Dufner-Beattie et al., 2006). Moreover, the Zip1, Zip2, and Zip3 triple-knockout mouse, was indistinguishable from its WT littermates when zinc was adequate, but displayed a similar zinc deficiency-sensitive phenotype (Kambe et al., 2008).

**Zip4 (SLC39A4)**

Zinc deficiency leads to growth retardation, immune-system dysfunction, alopecia, severe dermatitis, diarrhea, and, occasionally, mental disorders. This pathophysiology is seen in the rare, autosomal recessively inherited disease of intestinal zinc malabsorption, acrodermatitis enteropathica (AE). The genetic origin of the disease was a telomeric region of 3.5 cM on

chromosome 8q24.3, and identified as the AE susceptibility gene region (Wang et al., 2001). Through screening of potential gene targets, a genomic sequence predicted to produce a protein with the capability of zinc binding, and shown to be homologous to other Zip proteins, was identified and named hZip4 (Wang et al., 2002). Abundant expression of hZIP4 was identified in tissues involved in zinc absorption/ reabsorption, such as the small intestine, stomach, colon, as well as in kidney (Wang et al., 2002). Several mutations in hZip4 ranging from missense mutations, splicing defects, and transcription-inactivating upstream deletions were discovered in patients with AE (Wang et al., 2002; Küry S. et al., 2002). Although hZip4 mutations are critical to the etiology of AE, they can be overcome through dietary zinc supplementation (Wang et al., 2002). Therefore, other mechanisms of intestinal zinc absorption must be present.

The study of the murine orthologue, mZip4, has provided most of the information regarding the structure, function, and regulation of Zip4 (reviewed in Andrews, 2008). The mouse and human Zip4 proteins are well conserved, and share 76% homology (Dufner-Beattie et al., 2003). While ZIP4 functions as a zinc transporter in transfected cells, several AE mutations appear to abolish its activity by causing retention in the endoplasmic reticulum, and others apparently diminish its zinc uptake activity (Wang et al., 2004).

Expression of Zip4 seems to be regulated by both transcriptional and post-transcriptional mechanisms in response to zinc availability. The abundance of Zip4 mRNA, cellular localization, and turnover of this protein are regulated by zinc availability in the intestine and visceral yolk sac (Dufner-Beattie et al., 2003; Liuzzi et al., 2004; Kim et al., 2004; Dufner-Beattie et al., 2004; Mao et al., 2007; Weaver et al., 2007). By using RNA and protein synthesis inhibitors and run-on transcription assays, increased expression of Zip4 during zinc deficiency was shown to be due to stabilization of Zip4 mRNA, not transcription (Weaver et al., 2007).

Recently however, the transcription factor Krüppel-like Factor 4 (KLF4), which is induced during zinc restriction, was associated with transcriptional upregulation of ZIP4 (Liuzzi et al., 2009). During dietary zinc deficiency, ZIP4 localizes to the apical membranes of enterocytes in the intestine and visceral endoderm cells in the embryonic visceral yolk sac (Dufner-Beattie et al., 2007; Liuzzi et al., 2004; Dufner-Beattie et al., 2004). However, zinc repletion was reported to cause mRNA degradation and rapid endocytosis of ZIP4 (Weaver et al., 2007). Interestingly, a histidine-rich region within the large intracellular loop between putative transmembrane domains III and IV may play a role in the response of ZIP4 to zinc by regulating endocytosis and ubiquitination (Mao et al., 2007). Moreover, dietary zinc restriction affects proteolytic processing of the protein, resulting in removal of the extracellular amino-terminal ectodomain, and leaves a 37 kDa peptide of ZIP4 as the primary protein found (Weaver et al., 2007; Kambe and Andrews, 2009). Furthermore, certain AE mutations can inhibit this cleavage, suggesting an important role of proteolytic cleavage in regulation of Zip4 (Kambe and Andrews, 2009).

In mice, homozygous knockout of ZIP4 is embryonic lethal (Dufner-Beattie et al., 2007). In humans however, complete loss of ZIP4 function, as in AE patients, is not lethal but left untreated postnatally results in morbidity that can be relieved with supplemental zinc (reviewd in Sandstrom et al., 1994). On the contrary, ZIP4 knockout embryos could not be saved by providing excess zinc orally and/or by intra-peritoneal injection to the mother (Dufner-Beattie et al., 2007). This difference between species could be due to the lack of an alternate transport system to supply zinc to the developing mouse embryo from the dam. Furthermore, heterozygous Zip4-knockout mouse embryos are more hypersensitive to zinc deficiency relative to their wild-type littermates, and display growth retardation and morphologic abnormalities (Dufner-Beattie et al., 2007).

**Zip5 (SLC39A5)**

Although several AE mutations in the Zip4 gene are likely to abolish the transport function of the Zip4 protein (Wang et al., 2002; Kury et al., 2002; Kury et al., 2003; Wang et al., 2004), the symptoms of AE can be alleviated through supplemental dietary zinc. Furthermore, some AE mutations fail to map to the same chromosomal region as SLC39A4 (Wang et al., 2002). Therefore, an additional ZIP protein was thought to be associated with the disease (Wang et al., 2002; reviewed in Eide, 2004), initially named "hORF1" and is now designated "Zip5."

The Zip4 and Zip5 proteins share 30% homology, and mouse and human Zip5 are very similar in sequence sharing 84% identity (Wang et al., 2004). Human Zip5 displays a similar pattern of tissue-specific expression as seen in mouse and human Zip4, with high expression in the liver, kidney, pancreas, and throughout the small intestine and colon (Wang et al., 2004). Unlike ZIP4, ZIP5 is localized to the basolateral surface of these cell types under zinc-replete conditions but is internalized during periods of dietary zinc deficiency (Dufner-Beattie et al., 2004). In transfected cells, mZIP5 does function in zinc uptake and is specific for zinc as a substrate (Wang et al., 2004). Zip5 mRNA abundance is irresponsive 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 (Weaver et al., 2007). Zinc-gavage induces rapid resynthesis of ZIP5, where it is then targeted to the basolateral membranes of these cell types. These results suggest that ZIP5 may oppose ZIP4, and may be involved in enterocyte sensing of body zinc status through serosal-to-mucosal transport of zinc (Dufner-Beattie et al., 2004; Wang et al., 2004).

**Zip6 (SLC39A6)**

ZIP6 (LIV-1) was identified as a novel gene whose expression is stimulated by estrogen treatment of MCF-7 and ZR-75 breast cancer cells (Manning et al., 1988). ZIP6 is known as the

founding member of the LZT (LIV-1 subfamily of ZIP zinc transporters) subfamily of ZIP transporters. The LIV-1 subfamily is a highly conserved group of eight transmembrane domain proteins that are mainly situated on the plasma membrane and transport zinc into cells. All nine of the LIV-1 family members contain the common histidine-rich domain between TMDs III and IV which is a hallmark of all ZIP proteins, as well as a unique, highly conserved putative metalloprotease motif (HEXPHEXGD), which closely resembles the active site motif of matrix metalloproteases, located in transmembrane domain V, and considerably increased histidine residues on the N-terminus and extracellular loop between TM II and III (Reviewed in Taylor et al., 2003; Liuzzi and Cousins, 2004; Taylor, 2007).

Functional analysis of cells transfected with ZIP6, indicate that this protein does act as a zinc importer that is localized to the plasma membrane of certain cell types (Taylor et al., 2003). Elevated expression of ZIP6 is observed in tissues sensitive to steroid hormones such as the placenta, mammary gland and prostate (Taylor et al., 2003). Furthermore, the association of abundant ZIP6 expression in HeLa and lung carcinoma cell lines, as well as in breast cancer cells with metastatic ability suggests a role for LIV-1 in breast cancer progression (Taylor et al., 2003; McClelland et al., 1998).

Recently, investigation of breast cancer specimens has substantiated an association of ZIP6 with estrogen receptors. These studies are fascinating in the fact that ZIP6 is considered a reliable marker of estrogen-receptor–positive cancers (Tozlu et al., 2006; Schneider et al., 2006) and moreover, that it is one of the genes used routinely to distinguish the luminal A type of clinical breast cancer (Chung et al., 2002; Perou et al., 2000; Reviewed in Taylor, 2007). Furthermore, the transcription factor STAT3 was shown to activate ZIP6 further implying a link to cancer development. Additionally, nuclear localization of the transcription factor Snail, which

plays a major role in the epithelial-to-mesenchymal transition (EMT) due to its ability to down regulate the expression of genes critical to cell adhesion, was dependent on ZIP6 expression (Yamashita et al., 2004).

Down regulation of ZIP6 after LPS exposure is associated with decreased intracellular zinc, increased surface expression of MHC class II molecules, and therefore maturation of splenic $CD11c^{+}$ dendritic cells (Kitamura et al., 2006). The addition of TPEN, a cell-permeable zinc chelator, increased the surface expression of MHC class II and costimulatory molecules on DCs, just as LPS did, and zinc supplementation or overexpression of ZIP6 inhibited the LPS-induced upregulation of MHC class II and costimulatory molecules. These results suggest that zinc homeostasis through regulation of zinc transporters, specifically ZIP6, is crucial to host immune response.

**Zip7 (SLC39A7)**

Originally identified through homology to the mouse KE4 gene, which has been mapped to the H2-K region of the mouse major histocompatability complex on chromosome 17(Ando et al., 1996), the human HKE4 gene was similarly mapped to the centromeric side of the HLA class II region of chromosome 6 (Ando et al., 1996). Subsequently, both human and mouse sequences were aligned with the ZIP family of proteins as unknown open reading frame sequences, and shown to exhibit similarity to the consensus sequence for ZIP transporters (Eng et al., 1998). HKE4 therefore is now known as ZIP7.

ZIP7 appears to be ubiquitously expressed (Taylor et al., 2004). Transfection of ZIP7 into cells causes an increase in intracellular zinc, as would be expected for a zinc importer (Taylor et al., 2004). However, ZIP7 localizes to the Golgi apparatus, not the plasma membrane, suggesting the increase in intracellular zinc is of vesicular origin (Taylor et al., 2004; Huang et al., 2005). Moreover, by using a mutant strain of yeast that was defective in the ZIP7

orthologue, zrt3, which controls release of stored zinc from vacuoles, complementation studies showed that ZIP7 was able to decrease the level of accumulated zinc in these yeast vacuoles and concomitantly increase the nuclear/cytoplasmic labile zinc level in the ZIP7-expressing zrt3 mutant (Huang et al., 2005). Additionally, while ZIP7 gene expression and protein localization remains unchanged by zinc status, the protein abundance of ZIP7 is repressed by supplemental zinc.

Another interesting aspect of ZIP7 function is the possibility that this protein may be involved in breast cancer progression (Taylor et al., 2008). Recent studies in the human breast cancer cell line MCF-7, and tamoxifen-resistant (TamR) MCF-7 cells indicate that ZIP7 is required for increasing intracellular zinc levels leading to activation of EGFR, Src and IGF-1R signalling molecules as well as increases in growth and invasion (Taylor et al., 2008), which are hallmarks of the aggressive phenotype of TamR cells (Reviewed in Taylor, 2008).

**Zip8 (SLC39A8)**

Resistance to Cd-induced testicular toxicity is a trait seen in a few inbred mouse strains (Lucis and Lucis, 1969). The resistance phenotype is autosomal-recessive, and the gene responsible for the trait was named *Cdm* (Taylor et al., 1973). Refinement of the *Cdm* gene locus (Dalton et al., 2000) allowed for identification of the *Cdm* gene as the eigth member of the ZIP family, Zip8 (Dalton et al., 2005).

Cd is presumably transported inadvertently into the vascular endothelial cells of the testis, resulting in increased cellular accumulation and toxicity (Dalton et al., 2005). Moreover, transgenic mice (BTZIP8-3) created with three copies of the 129/SvJ Slc39a8 gene inserted into the Cd-resistant C57BL/6J genome (already containing two copies of the Slc39a8 gene), showed that Cd treatment reversed Cd resistance (seen in nontransgenic littermates) to Cd sensitivity in BTZIP8-3 mice (Wang et al., 2007).

ZIP8 expression is found in lung, kidney, testis, liver, brain, small intestine, and the membrane fraction of mature RBCs (Wang et al., 2007; Ryu et al., 2008). MDCK cultures transfected with Zip8 reveal a plasma membrane localization for this transporter during zinc-deficient (ZnD) conditions, but internalization during zinc-adequate (ZnA) conditions (Wang, B et al 2007; Liu Z et al 2008). In contrast, no difference in plasma membrane bound ZIP8 could be detected in mature RBCs after dietary zinc deficiency (Ryu MS et al 2008). These may be cell type differences, or in vivo versus in vitro effects. ZIP8 functions as a divalent cation transporter for Mn, Zn, and Cd in mouse fetal fibroblast (MFF) cultures (Dalton et al., 2005; He et al., 2006). However, specificity of Zn transport could not be shown until studies of inhibition of Cd influx were conducted in ZIP8 cRNA-injected *Xenopus* oocytes (Liu et al., 2008). Additionally electrogenic experiments in *Xenopus* oocytes revealed that ZIP8-mediated divalent cation movement across the membrane occurs as the $Cd^{2+}/[HCO_3^-]_2$ and $Zn^{2+}/[HCO_3^-]_2$ electroneutral complexes (Liu et al., 2008).

Human Zip8 was originally named Bacillus calmette-guerin-induced gene in monocyte clone 103 (BIGM103), because the gene was induced in primary human monocytes following exposure to the Bacillus calmette-guerin cell wall skeleton (Begum et al., 2002). Interestingly, BIGM103 was not constitutively expressed, but could be induced by inflammatory mediators such as LPS and TNF-$\alpha$ in the lung. Furthermore, TNF-$\alpha$ stimulated Zip8 expression in primary human lung epithelia obtained from multiple human donors and BEAS-2B cell cultures (Besecker et al., 2008). In addition, TNF-$\alpha$ induced the expression of glycosylated ZIP8 that translocated to the plasma membrane and mitochondria, resulting in an increase in intracellular zinc content and cell survival. In contrast, Zip8 inhibition reduced cellular zinc content and

impaired mitochondrial function in response to TNF-α, resulting in greater cell death (Besecker et al., 2008).

**Zip9 (SLC39A9)**

The sequences of mouse and human Zip8 were identified by The National Institutes of Health Mammalian Gene Collection (MGC) Program to identify and sequence a cDNA clone containing a complete ORF for each human and mouse gene (Strausberg et al., 2002). Sequence homology places ZIP9 in the ZIP family, however it is the lone mammalian member of ZIP subfamily I (Taylor et al., 2007). There are no other descriptions of structure, function, or regulation of ZIP9 in the literature.

**Zip10 (SLC39A10)**

Metal-response element binding transcription factor 1 (MTF-1) is a zinc finger protein that recognizes short cis-acting DNA sequences, termed metal response elements (TGCRCNC), which are present in the promoters of metal-responsive genes (Searle et al., 1985). MTF-1 is conserved throughout evolution, with orthologs having been characterized in the mouse (Radtke et al., 1993), humans (Brugnera et al., 1994), Drosophila (Zhang et al., 2001) and fish (Chen et al., 2002). Homozygous disruption of the mouse MTF-1 gene results in lethal liver degeneration on day 14 of gestation (Wang et al., 2004). However, development of liver specific MTF-1 conditional knockout mice allowed for identification of a novel MTF-1 regulated gene, Zip10 (Wimmer et al., 2005).

Unlike the previously described activation of another zinc transporter, ZnT-1 by MTF-1, Zip10 expression is suppressed by induction of MTF-1 (Wimmer et al., 2002). This was the first Zip gene identified as an MTF-1 target, and moreover Zip10 was the first gene repressed by metal induction of MTF-1. Of particular note is the location of the MRE in Zip10 (Fig. 2-1). The MRE identified by Wimmer et al., was located +17 bases downstream of the transcription

start site (TSS). Furthermore, an additional MRE was found upstream of the TSS in zebrafish that is not conserved in other species (Zeng et al., 2008). Zip10 expression in vivo is suppressed by zinc in both the gill and kidney of zebrafish. Analysis of drZip10 suggested that two transcripts are produced and regulated by two separate promoters located approximately 16kb apart. Interestingly, reporter gene studies utilizing the first promoter (associated with zebrafish gill) that contains 2 MREs flanking the TSS (upstream and downstream) were positively influenced by zinc. In contrast, the second promoter (present in the kidney) contains an additional MRE located in the first intron that was required to repress reporter gene activity (Zeng et al., 2008). These regulatory differences seen in vitro versus in vivo may be due to the physical inability of the promoters to interact through DNA looping in vitro (Zeng et al., 2008). However, other mechanisms are plausible (e.g., steric hinderance of Pol II transcription) and therefore need to be examined further.

Prior to identification of mZip10, a 40-kDa zinc transport protein in the rat renal brush border was purified (Kumar and Prasad, 1999). Later characterization of the protein along with its mRNA revealed sequence homology to known Zip proteins, and was identified as the rat orthologue of Zip10 (Kaler and Prasad, 2007). In transfected cells, rZip10 localizes to the plasma membrane where it transports zinc. In contrast to the zinc suppression of Zip10 expression in the mouse and zebrafish, rZip10 increases expression in response to supplemental zinc. Furthermore, rZip10 responds positively to thyroid hormone stimulation (Pawan et al., 2007). These results, along with the lack of MREs in the rZip10 promoter suggest that rZip10 is regulated in a different manner than the mouse, human, and zebrafish orthologues.

Another interesting aspect of Zip10 function is its possible role in metastatic breast cancer progression. Screening for ZIP10 mRNA expression in breast cancer samples suggested that

ZIP10 was significantly associated with the metastasis of breast cancer to the lymph node (Kagara et al., 2007). In addition, the expression of ZIP10 mRNA was higher in the invasive and metastatic breast cancer cell lines. Moreover, by using in vitro cell migration assays, knockdown of ZIP10, and concomitantly decreased intracellular zinc, inhibited the migratory activity of metastatic breast cancer cells. These findings demonstrate an intriguing role for zinc and ZIP10 in the migratory activity of highly metastatic breast cancer cells, and suggest ZIP10 (similar to ZIP6 for other forms of breast cancer) may be used as a possible marker for the metastatic phenotype of breast cancer and a novel drug target.

**Mouse** (*Mus musculus*)
**CCGAGCGGAGAGGAGATGCACACGGCACTCGAGTGTGAGGTA**
**Human** *(Homo sapiens)*
CACGATTTGGTGCAGCCGGGGTTTGGTA**CCGAGCGGAGAGGAGATGCACACGGCACTCG**
**AGTGTGAGGTA**
**Zebra fish** (*Danio rerio*)
GCAGTGGGCTCTGAGTTTTGTGCGGTGCGCAGCGTGAGTGTTTTAGTA**CCGAGCGGAGAGG**
**AGATGCACACGGCACTCGAGTGAGGTA**

Figure 2-1. Sequence alignment by BlastZ of ZIP10 5'-UTR beginning at the putative TSS in mouse, human, and zebrafish. The bold lettering indicates complete homology between all species; the MRE sites are underlined.

**Zip11 (SLC39A11)**

The Zip11 protein product is a member of the gufA subfamily of ZIP transporters, named after the *Myxococcus xanthus* gene, which has unknown function. No other structure, function, or regulatory information is available.

**Zip12 (SLC39A12)**

The schizophrenic brain seems to have a lower concentration of zinc than that of a normal brain (Kimura and Kumura, 1965). Screening of a schizophrenia susceptibility locus on chromosome 10p for proteins that may be involved in zinc transport, revealed Zip12. An

association was made between a missense homozygous mutation in Zip12, and frequency of schizophrenia development in a small group of patients (Bly, 2006). No other structure, function, or regulatory information is available.

**Zip13 (SLC39A13)**

Patients with Ehlers–Danlos syndrome type VI (MIM225400) display a phenotype of progressive kyphoscoliosis, hypermobility of joints, and hyperelasticity of skin combined with severe hypotonia of skeletal muscles (Beighton et al., 1998). The molecular defect in this kyphoscoliotic form of EDS is deficiency of lysyl hydroxylase (LH1 encoded by the PLOD1 gene), the enzyme responsible for conversion of certain lysyl residues in the triple–helical domains of collagen α-chains to hydroxylysine (Giunta et al., 2005). A disorder similar to EDS VI was recognized in six patients from two consanguineous families, however distinct phenotypic components such as short stature where identified as well (Giunta et al., 2008). This led to clinical characterization of the spondylocheiro dysplastic form of EDS (SCD-EDS). An SCD-EDS-linked region on chromosome 11 was isolated and searched for possible mutations among candidate genes. Genomic and cDNA sequencing of 26 candidates revealed only polymorphic mutations, except those in SLC39A13, in which all six patients were homozygous for the same 9 bp in-frame deletion in exon 4.

SLC39A13 encodes the previously uncharacterized zinc transporter ZIP13, a member of the Liv-1 subfamily of ZIP zinc transporters (Taylor, 2007). Phylogenetic analysis revealed a close homology between the Golgi associated Zip7 and Zip13. This led Guinta and others (2005), to suggest that the defect present in ZIP13 would cause an increase in the concentration of $Zn^{2+}$ in the ER and a competition with $Fe^{2+}$ for binding to lysyl hydroxylase, prolyl 4-hydroxylase, and prolyl 3-hydroxylase, thus impairing hydroxylation of lysyl and prolyl residues. This is entirely possible considering this deletion in Zip13 affects TMD III, which therefore may

hinder proper folding of the protein and thus impairs 3D conformation and function of the transporter.

Shortly after identification of the association of Zip13 with SCD-EDS, the homozygous Zip13 knockout mouse was characterized (Fukada et al., 2008). The phenotypic abnormalities associated with the $Zip13^{-/-}$ mice are reduced osteogenesis, abnormal cartilage development, reduced dentin and alveolar bone, abnormal craniofacial features, as well as decreased dermal and corneal stromal collagen. Of importance is the identified involvement of ZIP13 in BMP/TGF-β signaling pathways in connective tissue forming cells and in nuclear translocation of Smad proteins. Loss of Slc39a13 caused dysregulation of BMP/TGF-β -mediated gene expression including expression of *Runx2* and *Msx2,* genes critically involved in bone, tooth, and craniofacial development (Komori et al., 1997; Vainio et al., 1993; Satokata, 2000; Nie, 2006; Alappat et al., 2003). Smad proteins are phosphorylated downstream of BMP or TGF-β receptor complex, followed by nuclear translocation. Among the Smad proteins, all receptor-regulated Smad (R- Smad) and Smad4 possess a Zn-binding motif in the MH1 domain for their DNA binding (Chai et al., 2003). How ZIP13 affects these signaling pathways is unclear. However, ZIP13 was shown to localize to the Golgi apparatus, and $Zn^{2+}$ accumulation in the Golgi was increased in $Zip13^{-/-}$ cells, indicating that ZIP13 functions as a Zinc transporter allowing for efflux of $Zn^{2+}$ from the Golgi into the cytoplasm, where $Zn^{2+}$ may interact with Smad.

Overall, the phenotypic changes observed in SCD-EDS and $Zip13^{-/-}$ mice appear quite similar. Of particular importance, clinical observations such as dwarfism, delayed bone growth, and increased skin fragility are seen in cases of dietary zinc deficiency (Hambidge and Krebs, 2007). Therefore, further analysis of these zinc-regulated pathways involved in bone and connective tissue development need to be conducted.

**Zip14 (SLC39A14)**

The LZT subfamily members are distinguished from other ZIP transporter members by their consensus sequence HEXPHEXGD in TMD V (Taylor et al., 2003). Amino acid sequence analysis of human ZIP14 revealed a slightly altered motif, EEXPHEXGD, similar to that of ZIP8 (Taylor et al., 2005). Curiosity in this difference led to the first study of ZIP14 function. Zinc uptake experiments demonstrated that while the histidine residue in TMD V is critical for transport of zinc by other zinc transporters, the glutamic acid residue substitution in ZIP14 also allows zinc transport (Taylor et al., 2005). This was the first demonstration of zinc influx by a human LZT protein containing an altered signature motif.

The murine ortholog, mZip14, was identified by isolation of genes expressed in the mouse fibroblastic cell line 3T3-L1 (which mimic adipocyte hyperplasia) during the earliest stages of adipocyte differentiation some of which positively regulate differentiation (Imagawa et al., 1999; Nishizuka et al., 2002). Although ZIP14 expression was elevated during adipogenesis and was highly restricted to the differentiation state of 3T3-L1 cells, the exact role that ZIP14 plays in this process remains to be determined (Tominaga et al., 2005).

A very interesting function of ZIP14 lies in the response of this gene to inflammation. Hypoferremia and hypozincemia are among the classical changes observed across species during the acute phase response (Moshage, 1997). The exact reason for a decrease in serum levels of these minerals is unclear, but may be related to host defense by decreasing iron and zinc availability for pathogenic microorganisms (Jurado, 1997). In response to cytokine treatment and inflammation, zinc is redistributed among various tissues, particularly the liver (Cousins and Leinart, 1988). A common model of murine inflammation involves turpentine injection activating a known cytokine cascade of IL-6 and leptin mediated by IL-1β. IL-6 is the main proinflammatory cytokine regulating the response of acute-phase genes (Siewert, E., et al 2004).

Screening of all known ZnT and Zip transcritpts from the livers of WT and IL-$6^{-/-}$ mice injected with turpentine, led to identification of Zip14 as an acute phase gene (Liuzzi et al., 2005). These studies demonstrated a clear dependence of liver ZIP14 function on IL-6 production, whereby ZIP14 contributes to the hypozincemia of inflammation and infection. Furthermore, for the first time endogenous ZIP14 was localized to the plasma membrane of hepatocytes, where the abundance of the transporter was increased by IL-6.

Identification of Zip14 regulation by IL-6 led to speculation of other transport activities. The hypoferremia of inflammation is produced by IL-6 through induction of hepcidin synthesis in the liver (Nemeth et al., 2004a). The mechanism accounting for the reduction in serum iron is through the hepcidin-induced internalization and degredation of ferroportin-1 (*fpn1*)(Nemeth et al., 2004b). Because of this similarity in the clearance of both metals by IL-6, albeit by different mechanisms, was there a chance that ZIP14 also transported iron? To answer that question, iron metabolism needed to be examined more closely.

Homeostatic mechanisms tightly control the intestinal absorption, systemic transport, cellular uptake, storage, and cellular efflux of iron. Normally iron in plasma is bound to its transport protein transferrin (Tf). However, during iron overload, the iron-binding capacity of plasma Tf can be exceeded, resulting in the hepatic accumulation of non-Tf-bound iron (NTBI) (Hentze, MW et al 2004). The NTBI concentrations in the plasma of humans with hereditary hemochromatosis or β-thalassemia usually range from 0.4 to 20 μM (Cabantchik et al., 2005; Jacobs et al., 2005). Animal studies indicate that the liver is the major target of plasma NTBI (Craven et al., 1987). Therefore, the question was asked, could ZIP14 transport NTBI? In a study by Liuzzi et al., (2006), ZIP14 was indeed shown to mediate both zinc and NTBI uptake into hepatocytes.

Furthermore, patients with hereditary hemochromatosis have significant levels of NTBI in their serum (Sarkar, 1970; Chua et al., 2004). Mutation of a single base pair in the hereditary hemochromatosis gene (HFE) causes iron overload in the liver, as well as heart, pancreas, parathyroid and pituitary glands, leading to multiorgan dysfunction (Fletcher and Halliday, 2002; Hentze et al., 2004). Functional studies show hepatocytes from $Hfe^{-/-}$ knock-out mice can take up more NTBI (Chua, AC et al 2004) and more hepatic iron than wild-type mice (Zhou, 1998). The role of ZIP14 in HFE-mediated iron overload was examined in HepG2 cells (Gao et al., 2008). Interestingly, expression of HFE in HepG2 cells resulted in a lower abundance ZIP14, possibly by a post-transcriptional mechanism. Additionally, iron uptake was unaffected by HFE expression after Zip14 knockdown, implying that HFE has a direct effect on Zip14-mediated iron transport. Therefore, the reduction in NTBI uptake by HFE may be mediated by ZIP14 function.

The Zip14 gene is located on mouse chromosome 14, spanning base-pair position 61955763 to 62219851. Multiple studies and tissue array data show that the liver expresses the greatest amount of Zip14, followed by the intestine (Tominaga et al., 2005; Liuzzi et al., 2006; Girijashanker et al., 2008). At least two distinct mRNA transcripts have been identified (Liuzzi et al., 2005). The reference sequence, NM 144808, is a 3660 bp sequence containing the entire Zip14 coding sequence (CDS 262-1731). This message also contains a 1929 bp 3untranslated region (UTR). The second mRNA is a splice variant. This variant, BC021530 or ZIP14B, is a 2174 bp sequence also containing the complete coding sequence for Zip14 (CDS 262-1731). However, the open reading frames (ORFs) of the two transcripts are not perfect matches (67% similarity). The reference transcript, named ZIP14A, contains exon 4 located at bps 713-882, whereas the splice variant contains exon 3 at bps 713-882. Furthermore, the variant is missing the extended 3′-UTR that is contained in the reference sequence. Recently, the tissue distribution

and transport functions of the splice-variants were investigated (Girijashanker et al., 2008). In the C57BL/6J mouse ZIP14A expression is highest in liver, duodenum, kidney, and testis, whereas ZIP14B expression is highest in liver, duodenum, brain, and testis. Both variants were found to transport $Zn^{2+}$ in stably retroviral-infected mouse fetal fibroblast cultures and transiently transfected Madin-Darby canine kidney (MDCK) polarized epithelial cells. Transport of $Cd^{2+}$ was also demonstrated in a $HCO_3^-$-dependant manner. Similar to previous results (Tominaga et al., 2005; Liuzzi et al., 2005) membrane-bound ZIP14A and ZIP14B transporters localized to the apical surface of MDCK cells and are generally glycosylated. While transport activities and cellular localization of the variants are similar, it is unclear if there is functional significance to the presence of alternative Zip14 products. However, ZIP14A and ZIP14B may play tissue-specific roles in zinc transport. Furthermore, due to the critical nature of ZIP14 function during pathophysiologic conditions, it might lead one to speculate on the importance of this transporter during growth and development. While the only ZIP transporter shown to be critical for development was Zip4, Zip14 may also prove to be vital to this process.

## The Functional Roles of Zinc

### Interrelations of Zinc and Metallothionein

*In vivo*, zinc metabolism is controlled homeostatically through mechanisms related to absorption and excretion (via zinc transporters), and through intracellular proteins such as metallothionein. Metallothioneins are small (6-7 kDa), cysteine-rich heavy-metal-binding proteins, and can bind up to seven zinc atoms (reviewed in Davis and Cousins, 2000). Approximately 5 to 10% of the total cellular zinc is found complexed with metallothionein under normal physiological conditions (Andrews, 2001). Metallothioneins are the most abundant heavy metal-binding proteins in the body (reviewed in Andrews, 2000). Metallothioneins are thought to function in the homeostasis of zinc through involvement in zinc absorption, tissue

distribution, and protection against acute stress (Davis and Cousins, 2000). During periods of acute stress, there is a decrease in plasma zinc levels, along with an increase in tissue zinc, particularly in the liver. This reduction in plasma zinc is directly related to the changes in kinetics of zinc metabolism, which lead to increased binding of the metal to metallothionein (Dunn and Cousins, 1988). In support of a link among zinc, metallothionein, and regulation of zinc metabolism, results of experiments with endotoxin-challenged metallothionein null mice (MT KO) show no decrease in plasma zinc (Philcox et al., 1995). Therefore, metallothionein plays a crucial role in plasma zinc clearance and tissue zinc uptake.

Metallothionein KO mice lack functional expression of metallothionein, and allow zinc metabolism to be studied in the absence of metallothionein (Davis et al., 1998). Transgenic mice have been developed that have approximately 55 additional copies of the metallothionein-I gene in their genome (Palmiter et al., 1993). These transgenic mice provide a model for studying the effect of exaggerated metallothionein levels on zinc metabolism.

Metallothionein is transcriptionally regulated by zinc, through metal responsive elements in the promoter region of the gene (Andrews, 2000). However, the metallothionein gene is also transcriptionally regulated by glucocorticoid hormones and specific cytokines. This allows for the possibility that this metalloprotein plays a role in inflammatory and stress-related responses (reviewed in Davis and Cousins, 2000).

**Zinc and Metallothionein as Cellular Antioxidants**

The oxidation of many different cellular constituents is involved in the pathogenesis of an array of diseases (Gutteridge and Halliwell, 2000). Cellular oxidative stress occurs when the antioxidant defense system becomes overwhelmed. There are many different forms of oxidants, including reactive oxygen species ($H_2O_2$, $O_2^-$, and OH), reactive nitrogen species (NO and $ONOO^-$), and carbon centered radicals (e.g., $\bullet CCl_3$). These oxidative species can damage lipids,

proteins, and nucleic acids (Farber, 1994). When oxidative stressors are introduced into cells, specific cellular antioxidant defense mechanisms are present to quench or reduce the radical mediated damage that may occur. These defense mechanisms involve antioxidant nutrients such as tocopherols and ascorbate, as well as the cellular antioxidants glutathione and superoxide dismutase (Yu, 1994). If damage does occur, there are ways for the cell to repair itself. For instance, oxidized bases are removed from DNA to prevent further damage, oxidized lipid membranes are reduced by the action of glutathione peroxidase, and damaged proteins are committed to a proteasome degradation pathway. If these cellular defenses are overcome, and oxidative stress occurs in great amounts, the cells may not survive. Therefore, establishing a way to overcome oxidative stress is important for cell survival.

Supplemental zinc provides additional protection against certain radicals (Blain et al., 1998). Zinc is a potent inducer of metallothionein expression. Consequently, the antioxidant protection attributed to zinc may be due to metallothionein induction. Metallothionein expression is also induced in response to oxidative stress (reviewed in Davis and Cousins, 2000). Therefore, there is high correlation between increased metallothionein synthesis and protection from oxidative stress. However, this protection is not always observed. In studies using metallothionein knock out and metallothionein transgenic mice, carbon tetrachloride-induced hepatotoxicity was reduced initially by the presence of metallothionein in control compared to knock out mice (Davis et al., 2001). In contrast, metallothionein transgenic and wild-type mice did not differ in the levels of carbon tetrachloride-induced hepatotoxicity, despite a large degree of difference in hepatic metallothionein and zinc content. Further examination of zinc and metallothionein as cellular antioxidants in cells from this mouse model led to differing results. In two independent studies, overexpresssion of metallothionein was found to be ineffective at

increasing primary hepatocyte viability, and may actually be deleterious (Davis et al. unpublished data). These results are a remarkable contrast from the apparent protective effects of metallothionein observed with carbon tetrachloride treated rat hepatocytes (Schroeder and Cousins, 1990), and numerous other reports describing a protective effect of this protein (Thornalley and Vasek, 1985; Matsubara, 1987; Schwartz et al., 1995; Cai et al., 2000; Zhou et al., 2001).

**Metallothionein, Nitric Oxide, and Oxidative Stress**

During hepatic inflammation or endotoxemia, hepatocytes can respond to cytokine or bacterial LPS stimulation by activating the inducible form of nitric oxide synthase, which can generate a large amount of nitric oxide from arginine (Nüssler et al., 1993). This production of NO has been shown to be both hepatoprotective and cytotoxic (Wink and Mitchell, 1998). The paradoxical effects of nitric oxide may relate to its ability to interact with $O_2^-$ to form peroxynitrite, which is highly reactive and may account for part of nitric oxide-induced cytotoxicity (Fu et al., 2001). The susceptibility of cells to peroxynitrite and nitric oxide is primarily dependent upon their reducing capacity (thiol content) (Kim et al., 1999). Metallothionein has been shown, in vitro, to react directly with peroxynitrite to prevent lipoprotein and DNA damage caused by this reactive nitrogen species (Cai et al., 2000). However, the protection by metallothionein in this study was not dose dependent. Increasing levels of the protein did not provide further protection against DNA damage, or LDL oxidation. Nonetheless, metallothionein over expression has been documented to be protective against the NO donor S-Nitroso-N-acetylpenicillamine (SNAP)-induced killing of, and DNA single-strand breaks in NIH 3T3 cells (Schwarz et al., 1995).

Further in vitro studies have elucidated a link between the cellular redox state and metal ion homeostasis (Jacob et al., 1998; reviewed in Maret, 2000). The metal thiolate clusters of

metallothionein possess the unique ability to function as a redox unit, therefore the protein has the potential to be involved in a redox-sensitive signaling pathway (Pearce et al., 2000). Indeed, nitric oxide has been shown to S-nitrosylate metallothionein and release zinc from cultured pulmonary artery endothelial cells, as confirmed by fluorescence fusion-protein modified metallothionein that undergoes conformational changes in the presence of nitric oxide (Pearce et al., 2000). These alterations in cellular zinc homeostasis may lead to the protective effects of metallothionein against nitric oxide toxicity (Schwarz et al., 1995).

## Regulatory Roles of Zinc

### Metal Response Element Binding Transcription Factor-1 (MTF-1)

The transcription of the MT gene in response to heavy metal stress is regulated by the transcription factor MTF-1 through binding to the five copies of metal responsive elements (MREs) in the proximal promoter of metallothionein. MTF-1 contains six zinc fingers of the $C_2H_2$ type. These zinc fingers provide the sequence-specific interaction with DNA at the MRE consensus sequence 5′TGCRCNC 3′. MTF -1 is also involved in the response to oxidative stress (Dalton et al., 1997; Gunes et al., 1998), hypoxia (Green et al., 2001; Murphy et al., 1999), and amino acid deprivation (Adilakshmi and Laine, 2002). Cellular oxidative stress produced through the use of $H_2O_2$ and tert-butylhydroquinone, has been shown to increase MTF-1 binding to the MREs of the MT promoter (Dalton et al., 1997). MTF-1 is a cytoplasmic protein that has been shown to translocate to the nucleus under a variety of stress conditions, including heavy metal and oxidative stress (Saydam et al., 2001). Although MTF-1 translocation is necessary for transcription of responsive genes to occur, it is not sufficient. Further studies in hepatocytes have revealed that MTF-1 may not only be regulated post-transcriptionally under certain stress conditions, but also at the level of gene expression (Lichten et al. unpublished observations). These data suggest that MTF-1 is a central component of the cell's response to stress.

# CHAPTER 2
# MATERIALS AND METHODS

## Animals

The iNOS$^{-/-}$ and control strain C57BL/6 mice were purchased from Jackson Laboratory. The IL-6$^{-/-}$ mice, derived from Jackson Laboratory stocks, were a generous gift from Dr. Lyle Moldawer (Department of Surgery, University of Florida). The iNOS$^{-/-}$ mice were purchased from Jackson Laboratory. MT$^{-/-}$ mice were breed in-house from founder mice purchased from the Jackson Laboratory. The control mice for the MT$^{-/-}$ strain are 129S3/SvImJ mice. Six- to eight-week old male mice were used in all experiments. Mice were given free access to tap water and received commercial rodent diets [Harlan Teklad] with a 12 h light-dark cycle. Protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

## Hepatocyte Isolation and culture

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Isolation of hepatocytes began with an infusion of a calcium-free solution containing 140 mM NaCl, 7 mM KCl, and 10 mM HEPES buffer (pH 7.3), via the subhepatic inferior vena cava. Next, a solution containing 67 mM NaCl, 7 mM KCl, 5 mM $CaCl_2$, 100 mM Hepes buffer (pH 7.3), and 0.04% (w/v) collagenase (Sigma type IV collagenase, C-5138), at a flow rate of 7 mL/min for 14 min, was allowed to perfuse the liver. Upon completion of perfusion, the liver was excised rapidly, and transferred to 15 mL of the collagenase solution, and the liver cells were aseptically liberated. They were then passed through a 100 µm cell strainer, suspended in a buffered wash medium (William's Medium E + 10 mM Hepes, pH 7.3), and collected by centrifugation (50 x g for 4 min). Cells were then washed in the same buffer twice and the final cell pellet was resuspended in attachment medium (WME supplemented with 10% FBS (v/v),

100 nM insulin, 100 nM dexamethasone, 100 U/mL penicillin, and 100 mg/mL streptomycin). An aliquot of the final cell suspension was then removed, and placed into solution with Trypan Blue to assess cell viability. Only suspensions with > 95% viability were used in experiments. Cells were seeded at 2.5 x $10^5$ cells/ well in 12-well culture plates, 5 x $10^5$ cells/ well in 6-well culture plates, and 2 x $10^5$ cells/ chamber in 4-chamber glass microscopy slides. After plating, the cells are allowed to attach for 3 h (37°C, 5% $CO_2$). Following selective attachment of parenchymal cells, medium in each well was exchanged, and these culture conditions continued for 18-22 h.

**Cell culture**

AML12 mouse hepatocytes (American Type Culture Collection) were grown in DMEM/F-12 containing 10% (v/v) FBS, 40 ng/mL dexamethasone, and ITS (insulin, Tf, selenium) supplement (BD Biosciences). Medium also contained penicillin, streptomycin, and amphotericin B (Sigma). These hepatocytes were maintained at 37°C in 5% $CO_2$.

**Antibodies**

The following antibodies were purchased from Santa Cruz Biotechnology: c-Fos (K-25), sc-253 (K-25); RNA Pol II (polymerase II), sc-899; and normal rabbit IgG, sc-2027. The ZIP14, ZIP10 and MTF-1 antibodies were developed as previously described (Liuzzi et al., 2005). To confirm equivalent loading, Western membranes were stripped and re-probed with mouse monoclonal anti-tubulin clone B-5-1-2 (Sigma), followed by HRP-conjugated goat anti-mouse IgG (Zymed).

**Protein Isolation and Immunoblotting**

For whole cell protein extracts, untreated and treated adherent cells were washed with 1X PBS and collected in sample dilution buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue, 5% 2-mercaptoethanol) containing a 1X concentration of protease

and phosphatase inhibitors (Roche). For membrane extracts, tissue samples were homogenized immediately in cold buffer (20 mM Hepes, pH 7.4/1 mM EDTA/300 mM mannitol) containing a protease inhibitor mixture (Pierce) by using a Potter-Elvehjem homogenizer. A membrane protein preparation (pellet) was prepared by centrifugation (100,000 x g) after nuclei and debris were first removed by centrifugation at 1,000 x g. Protein concentrations were determined using the RC/DC assay (BioRad). For immunoblotting, 40 µg of protein/lane was loaded onto 10% Tris-HCl polyacrylamide gels, and then electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were stained with Poneau Red to ensure equal loading. Stained membranes were blocked with 5-10% blocking solution (5-10% (w/v) Carnation non-fat dry milk, 30 mM Tris-Base pH 7.5, 0.1% (v/v) Tween-20, and 200 mM NaCl) for 1 h at room temperature depending on the antibody to be used for probing. Each primary antibody was used at a dilution between 1:200 and 1:1000 (v/v) in 5% milk, and incubated with membranes overnight at 4ºC with rotation. The blots were washed 5 x 5 min in 5% milk solution and then incubated with the appropriate secondary antibody (rabbit, goat, etc.) conjugated to horseradish perioxidase at a 1:5,000 to 1:20,000 dilution (v/v) for 1-2 h at room temperature with rotation. The blots were then washed for 5 x 5 min in 5% blocking solution and 2 x 5 min in freshly made TBS/Tween (30 mM Tris-Base, 0.1% Tween-20, and 200 mM NaCl pH 7.5). The bound secondary antibody was detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and x-ray film.

**Immunohistochemistry / Immunocytochemistry of Mouse Liver and Hepatocytes**

Liver sections from mice that had been anesthetized with halothane and exsanguinated were fixed with 10% formalin in PBS, embedded in paraffin, cut as 5-µM sections, and mounted. Incubation with the affinity purified primary antibodies (10 µg/ml) was followed by addition of anti-rabbit IgG-Alexa 594 conjugate or anti-goat IgG-FITC conjugate (Molecular Probes). As

negative controls, the respective peptides that were used as the antigens were incubated with the primary antibody before exposure to the tissue section, as previously described (Liuzzi et al., 2005). Counterstaining of nuclei was performed with 4', 6-diamidino-2-phenylindole (DAPI).

**Immunohistology of Mouse Brain**

The mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and isoflurane and then perfused intracardially with 4% paraformaldehyde. After perfusion, the brain was fixed for 24 h in 4% paraformaldehyde and transferred to 70% ethanol until being embedded in paraffin. The entire brain was serially sectioned (4 μm sections), and every 10th section was stained with hematoxylin and eosin (H&E). Immunostaining of adjacent sections was performed according to a standard streptavidin-peroxidase procedure and as previously described (Embury et al., 2005). The slides were deparaffinized and antigen exposure was achieved using the Trilogy antigen retrieval system (Cell Marque, Hot Springs, AR). Suppression of endogenous peroxidase activity was performed with 3% $H_2O_2$ for 10 min. Nonspecific binding was blocked with 10% normal goat serum for 1 hour and application of streptavidin and biotin (Streptavidin/Biotin Blocking Kit; Vector, Burlingame, CA). Tissues were incubated overnight at 4°C with the affinity purified Zip10 primary antibody (1:1000 dilution) in 3% normal serum and 0.1% Triton-X 100. A rabbit anti-goat secondary biotinylated antibody (Vector, Burlingame, CA) in 1% normal serum was applied, followed by incubation with avidin-biotin-peroxidase, and visualized with the DAB chromagen (Vector, Burlingame, CA). Each brain was anatomically divided into four sections: forebrain, diencephalon, mesencephalon and caudal mesencephalon for identification pruposes.

**Zinc Uptake and NO Production by Hepatocytes**

$iNOS^{-/-}$ or C57BL/6 hepatocytes were incubated with 5μM FluoZin 3AM (Invitrogen), a cell permeable zinc fluorophore, in serum free medium for 30 min. These cells had been

incubated with 40μM zinc prior to addition of the fluorphore. Intracellular zinc accumulation was measured as previously described (Liuzzi et al., 2005). The Griess Reaction was used to determine the synthesis of NO by measuring the nitrite content of the culture supernatant (LeClaire et al., 1995). The absorbance was measured at 570 nm, and nitrite concentrations were calculated by comparison with a standard curve prepared using NaNO2.

**RNA Isolation and Quantitative PCR (qPCR)**

Total mRNA was isolated from all cell lines and tissues using the TriZol reagent (Invitrogen) and DNase treated to eliminate any trace amounts of DNA (Ambion). The RNA concentration was measured by optical density at 260 nm, and then all RNA samples were diluted in $H_2O$ to a final concentration of 10 ng/μL. To measure either the steady state mRNA or transcription activity of all genes, real-time quantitative PCR (qPCR) was performed using the iCycler (Bio-Rad) and either SYBR Green chemistry (ABI). For quantification of real-time PCR data, a relative standard curve method was used for each cell type or tissue analyzed. This type of standard was made independently for each cell line used. For individual RT-qPCR reactions, 5 μL of both standards and samples were run in duplicate wells to ensure accuracy. The master mix used for all RT-qPCR experiments consists of 12.5 μL 2 X SYBR Green PCR Master Mix (ABI 4309155), 0.125 μL MultiScribe Reverse Transcriptase (ABI 4311235), 0.125 μL RNase Inhibitor (ABI N808-0119), 1.25 μL sense primer (5 μM), 1.25 μL antisense primer (5 μM), and 4.75 μl H2O for a total volume of 20 μL. The final reaction volume per well is 25 μL. RNA reaction mixtures were incubated at 48°C for 30 min followed by 95ºC for 15 min and amplification of 40 cycles at 95ºC for 15 s, and then 60ºC for 60 s. Table 2-1 illustrates all the primer sets used. To establish that a single product was amplified during the reaction, melting curves were generated for each reaction by a stepwise increase of the temperature from 55 to 95ºC and measurements were taken at every degree change. Reactions were also run without

reverse transcriptase to ensure that there was no DNA amplification. The primers used for mRNA amplification are generally located inside one exon or amplify two adjacent exons. The primers used to measure transcription activity span an exon-intron junction and assay for hnRNA amplification (Lipson and Baserga 1989). To quantify all data, qPCR was done in duplicate with samples from at least three independent experiments and data are graphed as the means ± the standard error of the means.

**Chromatin Immunoprecipitation (ChIP)**

ChIP analysis by qPCR was performed according to a modified protocol of Upstate Biotechnology, Inc. For all experiments, cells were seeded into 150-mm dishes and grown for 36 h prior to any experimental treatments. AML12 cells were seeded at 1.5 x $10^7$/150-mm dish in complete DMEM F-12K 50/50 with 3 dishes per treatment condition. All cells were transferred to fresh medium 12 h before treatment. Cells were then treated with reagents and for the specific time period indicated in each figure. After treatment, protein-DNA was cross-linked by adding formaldehyde directly to the culture medium to a final concentration of 1% and then stopped 10 min later by adding 2 M glycine to a final concentration of 0.125 M. Cross-linked chromatin was sheared to 500-800 bp in length by sonication using a Sonic Dismembrator (Model 60, Fisher Scientific Co.) for five bursts of 40 s at power 10 with 2-min cooling on ice between each burst. Total sonicated chromatin was diluted into aliquots equivalent to approximately 1 x $10^7$ cells, and these extracts were incubated with 2-10 µg of primary antibody overnight at 4ºC. A rabbit anti-chicken IgG was used as the nonspecific antibody control. Either protein A- Sepharose beads (Amersham Biosciences) or protein G-Sepharose beads (Zymed) were used to precipitate the antibody-bound complexes. Beads were incubated in a blocking solution (3% bovine serum albumin, 0.05% sodium azide, and protease inhibitor in TE pH 8.0) as a 50:50 bead to solution slurry overnight at 4°C. After incubation, 60 µL of the bead slurry

was added to each chromatin-antibody aliquot and incubated at 4°C with rotation for 4 h. Antibody-bead complexes were pelleted and resuspended in a series of wash buffers, each incubated for a 5 min rotation at 4°C, in a volume of 1 mL. Wash buffers, in order of washes, were: low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.0); high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl pH 8.0); LiCl buffer (0.25 M LiCl, 1% NP 40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0); and TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8.0). After the final wash, antibody-bead complexes were resuspended in 65°C elution buffer (1% SDS, 0.1 M NaHCO3), and incubated at 37°C for 40 min with vigorous shaking in a bacterial incubator. The DNA fragments in the immunoprecipitated complex were released by reversing the cross-linking at 65°C for either 5 h or overnight and purified using a QIAquick PCR purification kit (Qiagen) and eluting all samples in 100 µL TE. Purified, immunoprecipitated DNA was routinely visualized by ethidium bromide staining after gel electrophoresis to ensure the average DNA fragment size was 500-800 bp.

To measure the amount of DNA precipitated by the ChIP procedure, quantitative PCR (qPCR) was performed using the iCycler (Bio-Rad) and SYBR Green chemistry. For quantification of qPCR data, a relative standard curve method was used. To generate a standard curve, total purified DNA from input ChIP extracts was pooled and diluted by serial dilutions to either one-fourth or one-half concentrations to give six tubes with final concentrations of 1/3, 1/9, 1/27, 1/81, 1/243, and 1/729 dilutions (all per 5 µL volume). For individual qPCR reactions, 5 µL of both standards and samples were run in duplicate wells to ensure accuracy. The master mix used for all qPCR experiments consisted of 12.5 µL 2 X SYBR Green PCR Master Mix (ABI 4309155), 1.25 µL sense primer (5 µM), 1.25 µL antisense primer (5 µM), and 5 µL H2O

(Ambion) for a total volume of 20 µL. The final reaction volume per well was 25 µL. qPCR reaction mixtures were incubated at 50°C for 2 min followed by 95ºC for 15 min and amplification of 40 cycles at 95ºC for 15 s, and then 60ºC for 60 s. Table 2-1 illustrates all the primers sets used for qPCR analysis of DNA fragments. To establish that a single product was amplified during the reaction, melting curves were generated for each reaction by a stepwise increase of the temperature from 55 to 95ºC and measurements were taken at every degree change. The results are expressed as the ratio to input DNA. Samples from at least three independent immunoprecipitations were analyzed, and the means ± SD between conditions were graphed.

**Promoter Construction, Mutagenesis, and Nested Deletions**

The 0.5 kb and 5 Kb promoter fragments were amplified from mouse genomic DNA by using nested PCR: 0.5 Kb fragment (round 1) sense primer 5'-AAGGGATCCAAGAACAGGCA-3' and antisense primer, 5'-ACCCCCCGCAGACGAGC-3´, (round 2) sense primer 5'-TAATTGATCACTCCGAAACT-3', antisense 5'-CGGGGTTTTATAGTT-3'. The round 2 primers were created with an NheI linker sense, and HindIII antisense. For the 5 Kb promoter fragment the primers were: (round 1) sense primer 5'-CTGTTTTTATGGCTCACCTAACC-3' and antisense primer, 5'-AACCTGGATAGCCTACAATCCTG-3´, (round 2) sense primer 5'-AGACTAAAGTGAATATCACCCGC-3' and antisense 5'-TTCTAAGTACTACAGATGGCCTACAGT-3'. The round 2 primers of the 5 Kb promoter fragment were created with an SacI linker sense, and XhoI antisense. A 2156 bp fragment of the Zip14 promoter was created by nested PCR: (round 1) sense primer 5'-CATACTCACTATGGAGCTGAGCTG-3' and antisense primer 5'-TCCGTCCTCACCTGAAGTC-3', (round 2) sense primer 5'-

GCTAGCCACCACCCAAGTGGTAGCAT-3' and antisense primer 5'-GCTAGCCACCACCCAAGTGGTAGCAT-3'. The round 2 primers for the 2.1 Kb Zip14 promoter fragment were created with an NheI linker sense, and XhoI antisense. The PCR fragments were then cloned into the pGemT-Easy plasmid using T/A cloning and further inserted into pGL3-basic plasmid (Promega), immediately upstream of the luciferase gene. Plasmid constructs were confirmed by DNA sequencing using GLprimer2 sense 5'-CTTTATGTTTTTGGCGTCTTCCA-3' and RVprimer3 antisense 5'-CTAGCAAAATAGGCTGTCCC-3'. The Zip10pGL3 mutant construct containing the 0.5 Kb fragment was created using site-directed mutagenesis (quick change II, Startagene). The oligonucleotides used to generate the mutation of the MRE site were 5'-GTACCGAGCGGAGAGGAGAGGCCTACGGCACTCG-3' and 5'-CGAGTGCCGTAGGCCTCTCCTCTCCGCTCGGTAC-3'. This mutation introduced a novel StuI restriction site that was used for quick screening of mutated clones. Further confirmation of mutations and integrity of promoter fragments was performed by DNA sequencing.

Nested deletions of the Zip14 promoter construct were performed by using the Erase-a-Base System (Promega). Oligos were designed with 5' phosphorylated flanking MluI sites for insertion into the Zip14pGL3 construct: pGL3NdeIF 5'-CGCGTCATATGGATATCA-3' and pGL3NdeIR 5'-CGCGTGATATCCATATGA-3'. The oligos were annealed by using a thermocycler with one cycle at 95°C for 5 min, followed by 70 cycles at 95°C (-1°C/ cycle) for 1 min each cycle, and the held at 4°C for 10 min. The pGL3Zip14 construct was next digested with MluI and subsequently dephosphorylated using 5 units of Antarctic phosphatase (New England Biolabs) for 15 min at 37°C. The annealed oligos were the inserted into the pGL3Zip14 construct. The interior of the newly insterted oligos contained NdeI and EcoRV restriction sites

for 5' digestion by ExoIII nuclease. 300u ExoIII was then added to duplicate tubes containing 5µg of pGL3Zip14 plasmid and incubated at 37°C. Aliquots were removed at 30 s intervals, and digestion was checked on 1% agarose gels.

**Transfection and Luciferase Assay**

AML12 cells were seeded at $1x10^5$ cells/ well in 24-well plates. Transfection began 12h after seeding with 15 nM (final concentration) of siRNA for mMTF-1 (Smart Pool, Dharmacon) using HiPerFect transfection reagent (Qiagen), and was carried out for 48 h. For the luciferase assays, AML-12 cells were seeded on 12-well plates and transfected with 1 µg pGL3 plasmid and 0.001 µg pRL-TK plasmid (Promega), as an internal control, using Effectene reagent (Qiagen). After a 48 h incubation, the cell medium was replaced by medium with or without 100 µM ZnSO4. After 24 h incubation, cells were washed with PBS and lyzed by 500 µl Passive Lysis Buffer (Promega) per well. Luciferease activities were measured with a Dual-GloTM Luciferase Assay System (Promega) in a GloMaxTM 20/20 Luminometry System (Promega) by following the manufacturer's protocol. The raw values of firefly luciferase were normalized to renilla luciferase that transfected concurrently in all the assays to correct for differences in transfection efficiency. The promoter activity assays were measured in triplicate in each experiment and shown as fold change relative to pGL3-Basic under either condition. At least three sets of independent experiments were performed for each set of constructs.

**Statistical Analysis**

Data are presented as the means ± S.D. or S.E.M. and were analyzed by two-way ANOVA. Bonferroni's post-test was used for multiple comparisons. Statistical significance was set at $p < 0.05$.

Table 2-1. Real-Time qPCR Primer Sets

| Primer Pair | Gene Region | Primer Sequence |
|---|---|---|
| Zip14 hnRNA | Exon5-Intron5 Junction | Sense: 5´-TCCTGGTGGTTGCCTTGC-3´<br>Antisense: 5´-AGAGGAAACCGTACCCCCATA-3´ |
| Zip14 mRNA | Exon 10 | Sense: 5´-GTAAACCTTGAGCTGCACTTAGC-3´<br>Antisense: 5´-TGCAGCCGCTTCATGGT-3´ |
| Zip14 promoter 1 | c-Fos -486 | Sense: 5´-TGGTTGGCTGGGGTAGGCAAA-3´<br>Antisense: 5´-TCGCTCCTGAGGGAGAGTGCC-3´ |
| Zip14 promoter 2 | TSS | Sense: 5´-TTGGCCAGGGTAACGACGCT-3´<br>Antisense: 5´-CATGCCCGGCCATATACCCT-3´ |
| Zip10 mRNA | Exon 10 | Sense: 5´-TGGCTTACATAGGAATGCTCATAGG-3´<br>Antisense: 5´-TGCGAAGATCCAGAGTGTGATG-3´ |
| Zip10 promoter | TSS | Sense: 5´-GAATACACGACTGGGTGCAG-3´<br>Antisense: 5-TGCAAACGATGGCGATGAT-3´ |
| Zip10 ChIP DS 1 | Exon2 | Sense: 5´-GCTGATGATAAACACCTGCATGA-3´<br>Antisense: 5´-TGCAAACGATGGCGATGAT-3´ |
| Zip10 ChIP DS 2 | Exon10 | Sense: 5´-CAGCTTGCCTCTGTTCCTTGT-3´<br>Antisense: 5´-TGCAGGCCACTGGATTCTC-3´ |
| *Mt* promoter | TSS | Sense: 5´-TCCTGCTCCACCGGTAAGAC-3´<br>Antisense: 5´-GCGGTCCCAACTTGGTATTCT-3´ |
| *Mt* mRNA | Exon2 | Sense: 5'-GCTGTGCCTGATGTGACGAA-3'<br>Antisense: 5'-AGGAAGACGCTGGGTTGGT-3' |
| *Mt* hnRNA | Intron2-Exon2 junction | Sense: 5'-CCTCCCTCATGCTGTCTTCT-3'<br>Antisense: 5'-CCAAGGTGTCCCAACTCACT-3' |
| *ZnT1* mRNA | Exon1 | Sense: 5´-CACGACTTACCCATTGCTCAAG-3´<br>Antisense: 5´-CTTTCACCAAGTGTTTGATATCGATT-3´ |
| *ZnT1* hnRNA | Exon1-Intron1 Junction | Sense: 5´-GACCAGGAGGAGACCAACAC-3´<br>Antisense: 5´-CACCCCAAACCCAACCAC-3´ |

# CHAPTER 3
# NITRIC OXIDE CONTRIBUTES TO THE UP-REGULATION AND FUNCTIONAL ACTIVITY OF THE ZINC TRANSPORTER ZIP14 IN MURINE HEPATOCYTES

## Introduction

Zinc is essential for numerous catalytic, structural, and regulatory roles in cells (King and Cousins 2005). Thus, organisms require specific and efficient transport mechanisms to maintain cellular zinc homeostasis. The movement of zinc into and out of cells, and subcellular organelles is mediated by zinc transport proteins. Specifically, the Zip (Zrt/Irt-like) family of transport proteins include members which mediate the zinc uptake into cells (Eide 2006). This family includes Zip1-8, and Zip14 which all have functional zinc transport activities in mammals (Chimenov and Kerppola 2001; Cousins and Leinart 1988; Liuzzi and Cousins 2004). In rodent models of inflammation, plasma concentrations of zinc are transiently decreased (Cousins et al., 2006). In response to cytokine treatment, zinc is redistributed among various tissues, particularly the liver (Cousins and Leinart 1988). This redistribution is accompanied by an increase of hepatic metallothionein-bound zinc. However, the mechanism of this redistribution, and the role and physiologic significance of hypozincemia in response to inflammation is not well understood. These events chronologically are similar to dysregulated iron metabolism referred to as the anemia of chronic disease (Weiss 2002).

We have recently identified Zip14 (Slc39a14) as a zinc transport protein involved in hepatic zinc uptake during murine models of inflammation (Liuzzi et al., 2005). In the sterile abscess model of inflammation, up-regulation of Zip14 is the mechanism responsible for hypozincemia. In this model, experiments with IL-$6^{-/-}$ mice demonstrated that steady-state mRNA levels and transport activities of Zip14 are dependent upon the production of IL-6. LPS-stimulated hypozincemia does follow changes in plasma cytokine levels (Gaetke et al., 1997). However, companion experiments with IL-$6^{-/-}$ mice suggest that LPS regulates Zip14 expression

via a mechanism that is partially independent of IL-6. IL-1β, an LPS-induced proinflammatory cytokine, is a potent activator of iNOS (inducible nitric oxide synthase) and NO production (Geller et al., 1995). Consequently, the LPS-induced increase in Zip14 expression may occur by a mechanism signaled by NO (nitric oxide) as a secondary messenger.

NO has demonstrated the ability to both up-regulate and down-regulate the expression of genes through various mechanisms. In iron homeostasis, NO activates IRP1 (iron regulatory protein) through interaction with its Fe-S center, thus regulating iron influx and storage through binding of IRP1 to IREs (iron responsive elements) on mRNAs of TfR (transferrin receptor) and ferritin (Kim and Ponka 2002; Pantopoulos 2004; Paradkar and Roth 2006). Zinc-finger proteins are another major target of NO regulation. Nitrosylation of zinc-thiolate clusters leads to transient impairment of the DNA-binding activities of some zinc-finger transcription factors such as Sp1, p53, NFκB, and AP-1 (reviewed in Kronke 2003)). However, activation of these same transcription factors by NO has also been observed (Blanchette et al., 2007; Fukumura et al., 2006; Salazar-Montes et al., 2006; Schlieper et al., 2007).

The purpose of the present experiments was to determine if NO mediates the up-regulation Zip14 expression, and how this regulation occurs. The results show that IL-1B induction of Zip14 is fully prevented in hepatocytes from iNOS$^{-/-}$ vs. WT mice. Augmentation of cellular NO levels with an NO donor, however, produced full induction of Zip14 in the iNOS$^{-/-}$ hepatocytes. Furthermore, NO augmented the transcriptional activity of Zip14 and this up-regulation led to an increase in intracellular labile zinc as detected by fluorescence. Finally, chromatin immuoprecipitation analysis shows NO increases binding of the transcription factor AP-1 to the Zip14 promoter.

## Results

### Induction of Zip14 Expression in Mouse Hepatocytes by IL-1β is NO Dependent

LPS stimulates synthesis of IL6, TNFα, and IL-1β (Faggioni et al., 1998). Of the proinflammatory mediators, IL-1β signals the production of NO. Since we found that LPS induces hepatic Zip14 expression in mice (Liuzzi et al., 2005), I have examined which of these cytokines regulates Zip14 in primary mouse hepatocytes. I exposed primary hepatocytes from WT (C57BL/ 6) mice to 100u/ml of IL-1β. After 8 h of treatment, IL-1β caused an approximate two-fold increase in relative Zip14 mRNA levels (Fig. 3-1A). However, with primary hepatocytes from $iNOS^{-/-}$ mice, IL-1β was unable to stimulate an increase in Zip14 expression. When hepatocytes from the WT mice were incubated with 100u/ml of IL-1β for up to 16 h, the iNOS mRNA increased to a level seven-fold higher than untreated cells (Fig. 3-1B). Increased iNOS expression corresponded with a major increase in nitrite production upon treatment with IL-1β (Fig. 3-1C). As further proof of an NO-mediated response, 100μM SNAP was added to $iNOS^{-/-}$ hepatocytes, and caused a significant two-fold increase in Zip14 expression (Fig. 3-1A). In contrast, no differences in Zip14 mRNA levels were observed between hepatocytes from WT and $MT^{-/-}$ mice (Fig. 3-1D), indicating that NO increased Zip14 expression independent of MT.

### Transcription of the Zip14 Gene

Hepatocytes from C57BL/ 6 mice were incubated with 100μM SNAP for up to 16 h and the steady-state mRNA level for Zip14 was measured for three independent experiments by qPCR. Similar results were obtained when the AML12 hepatocyte cell line was used in a separate independent experiment (data not shown). An initial 2-fold increase of Zip14 mRNA occurred 2 h after treatment, and was sustained for 8h (Fig. 3-2A). To assess the transcriptional activity of Zip14, the same samples used for mRNA quantification were used to measure the short-lived hnRNA. An almost identical biphasic response was found measuring the Zip14

hnRNA as with the mRNA, strongly suggesting a transcriptional control mechanism for NO regulation of Zip14. Further evidence of Zip14 transcriptional activity was revealed by treating AML12 hepatocytes for 12 h with 100μM SNAP, followed by RNA Pol II ChIP analysis of the Zip14 promoter (Fig. 3-2B). Collectively, these results indicate that RNA Pol II is recruited to the Zip14 promoter and carries out active transcription following NO exposure.

**Transcription Factor c-Fos Associates with the Zip14 Promoter in Response to NO**

One candidate transcription factor that might be involved in the NO responsiveness of the Zip14 gene was AP-1 (c-Fos/ c-Jun heterodimer) (Kronke 2003). By using the MatInspector bioinformatics program, we were able to map a putative AP-1 binding site to -486/ -477 of the Zip14 promoter (Quandt et al., 1995). Analysis of this potential c-Fos/c-Jun binding site was carried out by ChIP followed by qPCR to amplify a region of the Zip14 promoter containing the -486 to -477 (5'-CGTGAGTCAAG-3') proposed AP-1 binding site. AML12 hepatocytes were treated with 100 μM SNAP for 12 h. Immunopreciptiation of protein/ DNA complexes was performed with an anti-c-Fos antibody or non-specific rabbit IgG (negative control) (Fig. 3-3). The data shows that c-Fos is significantly enriched at the Zip14 promoter beginning after 2 h of SNAP treatment, with the greatest amount of enrichment occurring after 4 h. Analysis of promoter DNA from the negative control IgG by qPCR always resulted in a quantity of at least 10-fold less than with the c-Fos antibody.

**NO Increases ZIP14 Expression and Function at the Plasma Membrane of Hepatocytes**

WT hepatocytes were treated with SNAP for 12 h and expression of ZIP14 was analyzed by western blotting. Two immuno-reactive bands of approximately 50 kDa are increased after SNAP treatment (Fig. 3-4A, upper panel). Densitometric analysis of these immuno-reactive bands showed an approximately two-fold change in Zip14 abundance after SNAP treatment (2.75 for SNAP treated versus 1 for control). Immunofluorescence revealed a greater

concentration of ZIP14 at the plasma membrane in SNAP treated cells (Fig. 3-4B) than in untreated hepatocytes (Fig. 3-4C). These results were attained with non-permeabilized hepatocytes, however similar results were achieved when permeabilized hepatocytes were used. Identical gain was used for both images.

Hepatocytes were isolated from WT and iNOS$^{-/-}$ mice, treated with IL-1β, followed by incubation with or without the ZIP14 antibody. IL-1β stimulation of WT hepatocytes caused an increase in intracellular labile zinc, as measured by an increase in fluorescence from FluoZin-3AM that was not seen in iNOS$^{-/-}$ hepatocytes (Fig. 3-5A vs 3-5B). The peptide used to generate the antibody is from an extracellular epitope and should block ZIP14-mediated zinc uptake. Note that no increase in fluorescence was observed in response to IL-1β when hepatocytes were pre-incubated with the ZIP14 antibody prior to measurement of zinc uptake (Fig. 3-5C). Furthermore, when WT hepatocytes were pre-incubated with an antibody not directed against ZIP14, uptake of zinc was not blocked (Fig. 3-5D). These functional studies show that the increase in intracellular zinc was dependent on up-regulation of ZIP14 by an IL-1β-induced mechanism.

## Discussion

The results presented in the current study describe a nitric oxide-induced mechanism for increasing liver zinc uptake during hepatic inflammation. We have previously shown that ZIP14 may be the major zinc transporter responsible for the hypozincemia associated with inflammation and the acute-phase response (Liuzzi et al., 2005). Our prior research utilized two different model systems to examine Zip14 expression. In the turpentine model of inflammation, IL-6 was necessary for the *in-vivo* induction of Zip14 expression and hypozincemia, whereas the LPS model did not show an absolute requirement for IL-6. These differences in Zip14 regulation may be related to the cytokines produced by each stimulus. Turpentine administration, for the

most part, leads to an IL-6 specific response, while LPS cytokine induction is more complex, involving TNFα, IL-1α, IL-1β, leptin, and IL-6 (Faggioni et al., 1998). Therefore, since increased NO production is a downstream response to IL-1β, we hypothesized that stimulation of hepatic NO by IL-1β may be another mechanism for up-regulation of Zip14 expression.

Nitric oxide regulates a broad spectrum of physiological responses (Ignarro et al., 1999). These NO-activated responses are mediated by signaling cascades that elicit changes in blood pressure, neurotransmission, smooth muscle contraction, and mineral metabolism (Hemish et al., 2003). NO may also regulate genes in order to modulate immune responses and inflammation, and promote or inhibit tumor progression and metastasis (reviewed in Fukumura et al., 2006). The exact mechanisms underlying these NO-induced effects are not entirely known. However, certain signal transducers and transcription factors have been identified that are necessary for regulating these genes in response to NO, including HIF-1α, NF-κB, PI3K, PKC, p53, and the Ras-Raf-MEK-ERK pathway which leads to activation of AP-1 (Hemish et al 2003; reviewed in Fukumura et al., 2006).

Genetic and pharmacological techniques have revealed both protective and toxic roles of NO on liver cell injury, depending on the NO concentration (Klaassen et al., 1999). However, the level of NO donors used here are not likely to produce nitrosative stress or cell injury. Previous studies have indicated that primary hepatocytes are resistant to nitrosative stress with greater than 90% viability (L.A. Lichten and R.J. Cousins, unpublished observations). It has been shown that at high concentrations of NO donors, nitrosative stress results in the release of intracellular zinc (Kroncke et al., 2003). Therefore, the present experiments may present a more physiologic view of the NO-Zn interaction at the level of zinc transport.

The transcription complex, AP-1, often appears in screens of genes that are activated by NO (Fukumura et al., 2006; Kroncke 2003; Salazar-Montes et al., 2006; Schlieper et al., 2007). The AP-1 family of transcription factors consists of three main groups: the Fos proteins (c-Fos, FosB, Fra1, and Fra2), the Jun proteins (c-Jun, JunB, and JunD), and the activating transcription factors (ATF2, ATF3, and B-ATF) (Chimenov and Kerppola 2001). These various family members can form homo- or heterodimers that make up the active AP-1 complex. The DNA-binding and transactivation potential of the AP-1 complex is not only regulated by the dimer composition, but by transcription of the genes, and post-translational protein modifications (Sheerin et al., 2001; Troen et al., 2004). Of importance is the observation that c-Fos transcription and AP-1 activation occur quickly in response to NO (Hemish et al., 2003). The mouse Zip14 gene promoter harbors two putative AP-1 binding sites 5´-TGAGTCA-3´ (Halazonetis et al., 1988), the first of which lies at position -481 relative to the transcription start site. Phylogenetic footprinting analysis of this promoter region shows conservation between mice and humans (Quandt et al., 1995). We therefore investigated whether AP-1 was involved in regulation of Zip14 gene expression by ChIP analysis. In these experiments, we focused on c-Fos because of the documented quality of data produced with the c-Fos antibody used here. Significant enrichment of c-Fos was observed at the Zip14 promoter post-SNAP treatment, suggesting that AP-1 is involved in activation of the Zip14 gene after NO exposure.

Up-regulation of zinc transporters may have positive or negative physiologic consequences, depending on the stimuli involved and/or cellular location of the transporter. Interestingly, exposure of dendritic cells to LPS affects expression of many of the zinc transport proteins, resulting in a net increase in Zn transport out of cells (reviewed in Muakami and Hirano 2008). Overexpression of Zip6 (Slc39a6), whose abundance is reduced by LPS, suppresses

dendritic cell maturation most likely by increasing intracellular zinc levels (Kitamura et al., 2006). Examination of the closest evolutionary neighbour to the Zip14 gene, *Zip8* (Slc39a8), reveals both protective and cytotoxic effects of this transporter. An increase in the expression of Zip8 in primary human lung epithelia by TNFα causes an increase in intracellular zinc levels, which leads to protection against TNFα-induced cytotoxicity (Besecker et al., 2008). However, expression of Zip8 is associated with sensitivity to cadmium (Cd) toxicity specifically in vascular endothelial cells of the testis (Dalton et al., 2005). Similarly, ZIP14 was shown to have a high affinity for cadmium and this ability to transport Cd displaces manganese Mn and $Zn^{2+}$ when expressed in mouse fetal fibroblasts, leading to unwanted cell death due to the toxic nature of Cd (Girijashanker et al., 2008). While ZIP14 was shown to tranport Cd, on a physiological basis we have observed that, expression of Zip14 does not seem to be deleterious to cells, and even overexpression in HEK, AML12, or SF9 insect cells is not cytotoxic (Liuzzi et al., 2004; Liuzzi et al., 2006). Although no significant cellular stress was observed here (data not shown), up-regulation of Zip14 by NO, may serve a protective purpose by increasing intracellular zinc and metallothionein. Numerous reports have documented the beneficial effects of both zinc and/or MT on liver function and hepatocyte survival after exposure to deleterious agents such as ethanol (Klaassen et al., 1999; Tomita et al., 2004; Zhou et al., 2005), Cd, carbon-tetrachloride ($CCl_4$) (Davis and Cousins 2000), radiation and oxidative damage, and contributing to control of cellular proliferation and apoptosis (Klaassen et al., 1999; Lazo et al., 1998). The role of MT in apoptosis has been extensively studied, with the majority of studies showing that MT plays a protective role with respect to apoptosis (Shimoda et al., 2003). In this regard, ZIP14 may be characterized as a positive acute phase protein, possibly protecting the liver during inflammation.

In reference to metal metabolism, the current report is not the first demonstration of NO regulating the transcription of a metal transporter gene. The transport protein involved in intestinal uptake and cellular iron release, divalent metal transporter-1 (DMT-1), is down-regulated in response to NO (Paradkar and Roth 2006). The mechanism for down-regulation of DMT-1 is not at the post-transcriptional level as is usual for regulation of the gene by iron (Gunshin et al., 2001). Rather, NO increases binding of the transcription factor NFκB to the DMT-1 promoter leading to transcriptional repression of the gene in neuronal cells. Of possible relevance is transcriptional up-regulation of DMT-1 in respiratory epithelial cells by modulators of the inflammatory response such as LPS, IFNγ, and TNFα (Wang 2005).

Hypoferremia is a primary marker of the anemia of inflammation, and is thought to occur via increased tissue iron uptake, specifically liver uptake, and decreased intestinal and macrophage iron export (reviewed in Moshage 1997). We recently reported that ZIP14 mediates non-Tf-bound iron (NTBI) uptake into hepatocytes, which would be consistent with inflammation and NO increasing the expression of this transporter (Liuzzi et al., 2006). Furthermore, the transport of zinc from plasma to hepatocytes during inflammation is related to ZIP14 expression and is fully (turpentine-induced inflammation), or partially (LPS –induced inflammation) controlled by IL-6 (Liuzzi et al., 2005). In the present report, we demonstrate that NO could functionally activate ZIP14, thereby increasing zinc uptake. In agreement with that hypothesis, IL-1β increased fluorescently labelled intracellular zinc, but required both iNOS expression and functional ZIP14 to do so. Of particular importance is the predicted topology of the ZIP14 protein. By using bioinformatics and experimental data, we predicted that the histidine-rich region contained within the large peptide loop connecting transmembrane domains 3 and 4 was extracellular (Liuzzi et al., 2004). The ability of the ZIP14 antibody to block zinc

transport results supports our predicted topology. However, when immunofluorescence studies are conducted on permeabilized, rather than non-permeabilized cells, a greater fluorescent intensity from Alexa Fluor 594 labelled ZIP14 is observed (data not shown). Therefore, it is not possible to rule out the possibility that the histidine-rich loop may become cytoplsmic during a transition state. Collectively, our results show that IL-1β can stimulate NO production, elevate Zip14 expression via signalling pathways leading to AP-1 activation which in turn leads to hepatic zinc accumulation. Overall, regulation of the zinc transporter Zip14, by NO adds a new dimension to our understanding of hepatic zinc homeostasis in health and disease.

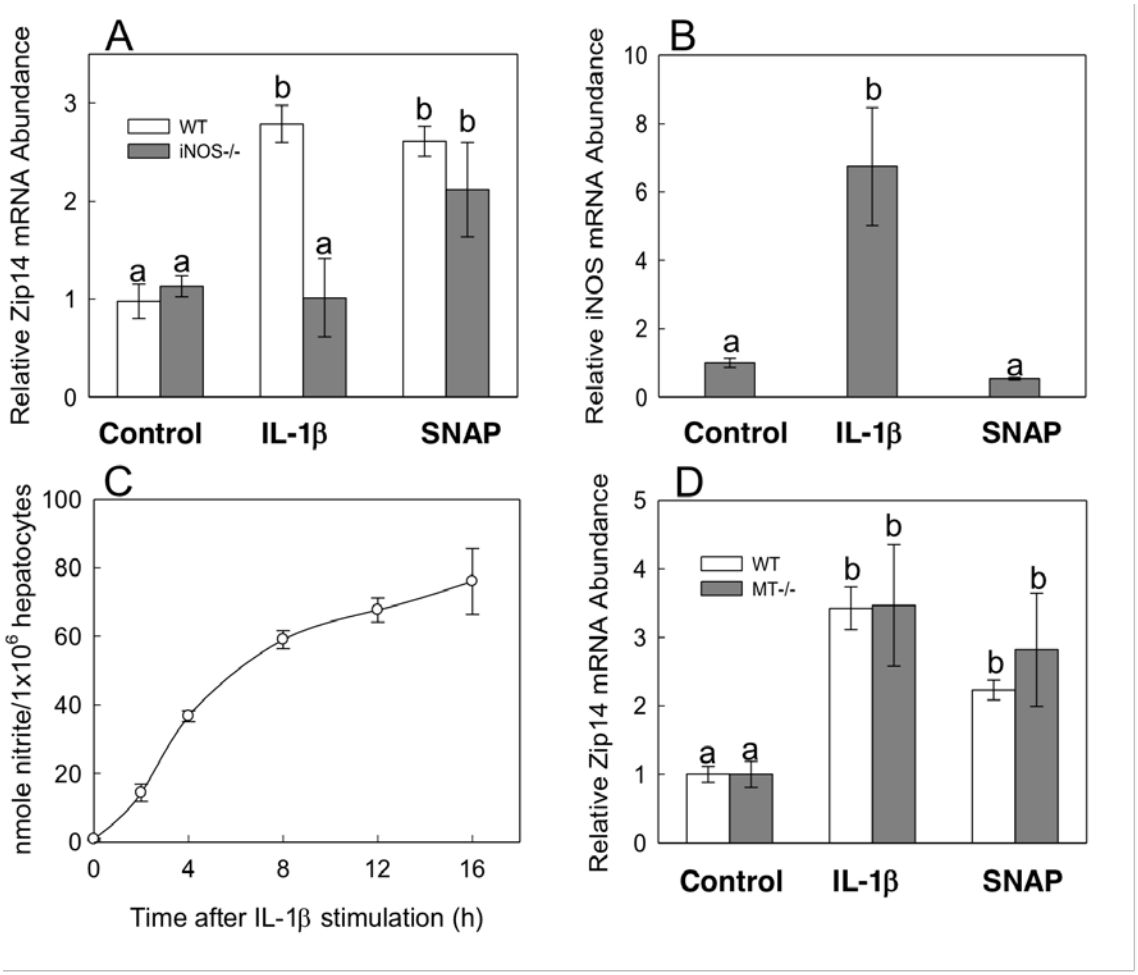


Figure 3-1. Influence of IL-1β and NO on Zip14 expression. A) Hepatocytes from iNOS$^{-/-}$ and C57BL/6 (WT) mice were treated with the NO donor, or IL-1β (100 U/ml), SNAP (100 μM), for 8 h and Zip14 mRNA was measured by qPCR (B). Hepatocytes from C57BL/6 mice were exposed to either IL-1β (100 U/ml) or SNAP (100 μM) for up to 16 h. Relative iNOS mRNA abundance was measured. Similar results were achieved with hepatocytes from 129S3/SvImJ mice. C) WT hepatocytes were exposed to IL-1β (100 U/ml) for up to 16 h. The nitrite concentration of the medium in response to IL-1β was measured by using the Griess reaction, and normalized to the total cell number per well. D) The contribution of MT to regulation of Zip14 by NO was investigated by incubating MT$^{-/-}$ and corresponding control strain hepatocytes with or IL-1β (100 U/ml) or SNAP (100 μM) for 8 h. Values are means ± SD of three independent experiments. Values with different letters are significantly different ($P < 0.001$).

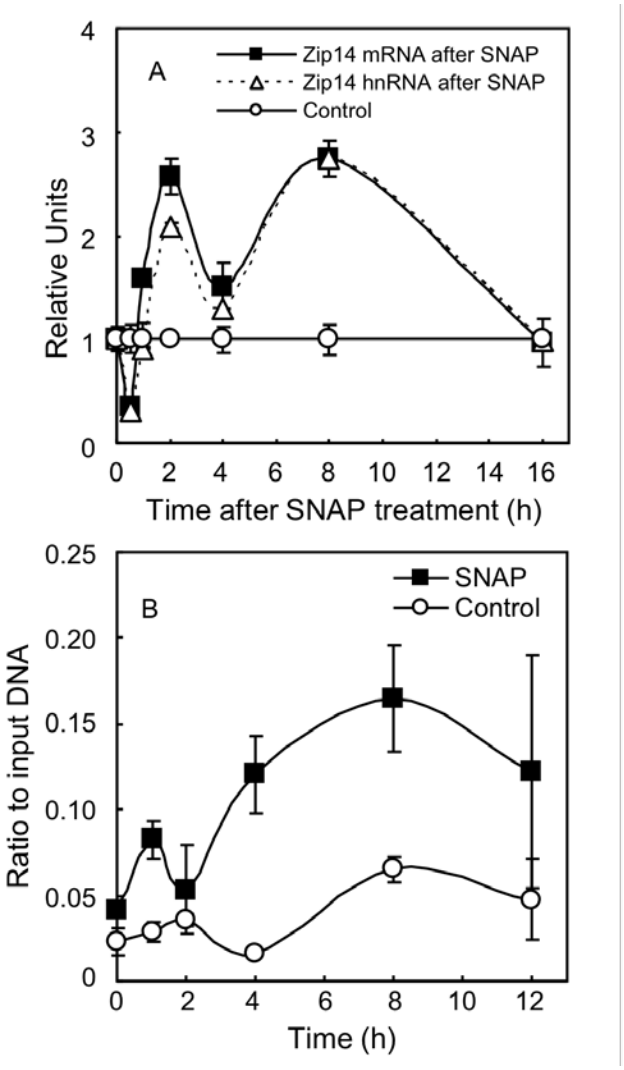


Figure 3-2. Effect of NO on Zip14 steady-state mRNA levels and transcriptional activity. Primary hepatocytes from WT (C57BL/6) mice were incubated with SNAP (100μM) for up to 16 h. At the times indicated, total RNA was isolated and analyzed by qPCR. (A) Transcriptional activity of the Zip14 gene was assessed by measurement of the hnRNA, utilizing primers corresponding to the junction of exon 4 and intron 4. From the same samples, steady-state mRNA levels for Zip14 were determined as in Fig 1. The Zip14 hnRNA and mRNA abundance is plotted as arbitrary units normalized to 18s rRNA, relative to control values, and based on an RNA standard curve. Each data point represents the mean ± SD of three independent experiments. (B) Murine AML12 hepatocytes were used to provide further evidence for transcriptional activity of the Zip14 gene. The cells were treated with SNAP (100μM) for 0-12 h, and ChIP analysis was performed using RNA Pol II antibody. Relative binding of Pol II to the Zip14 promoter was analyzed by qPCR. Data were plotted as the ratio immunoprecipitated DNA to a 1:20 dilution of input DNA. Background immunoprecipitation values were obtained by using a non-specific rabbit IgG, and never achieved a ratio higher than 0.01 to input DNA. Each data point represents the mean ± SEM for three replicate experiments.

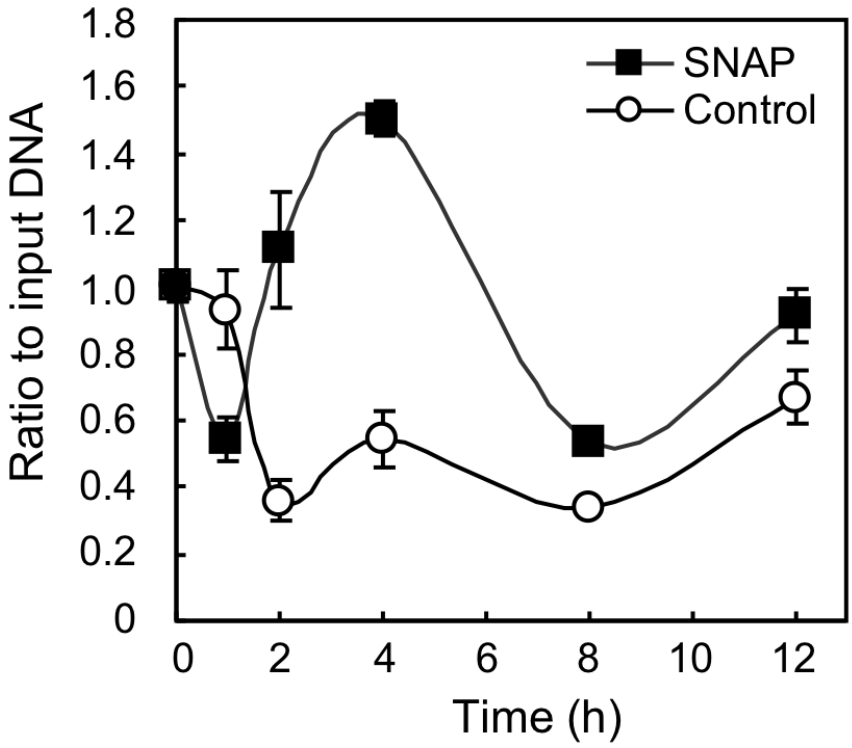


Figure 3-3. ChIP analysis shows c-Fos binds to the Zip14 promoter in response to nitric oxide. Murine AML12 hepatocytes were incubated with SNAP (100 μM) for 12 h. ChIP analysis was performed using an anti-c-Fos antibody, followed by qPCR. A non-specific rabbit IgG was used as a negative control. Data were plotted as the ratio of immunoprecipitated DNA to a 1:20 dilution of input DNA. Background immunoprecipitation levels were always below a ratio of 0.01 (to input DNA). Each data point represents the mean ± SEM for three independent experiments.

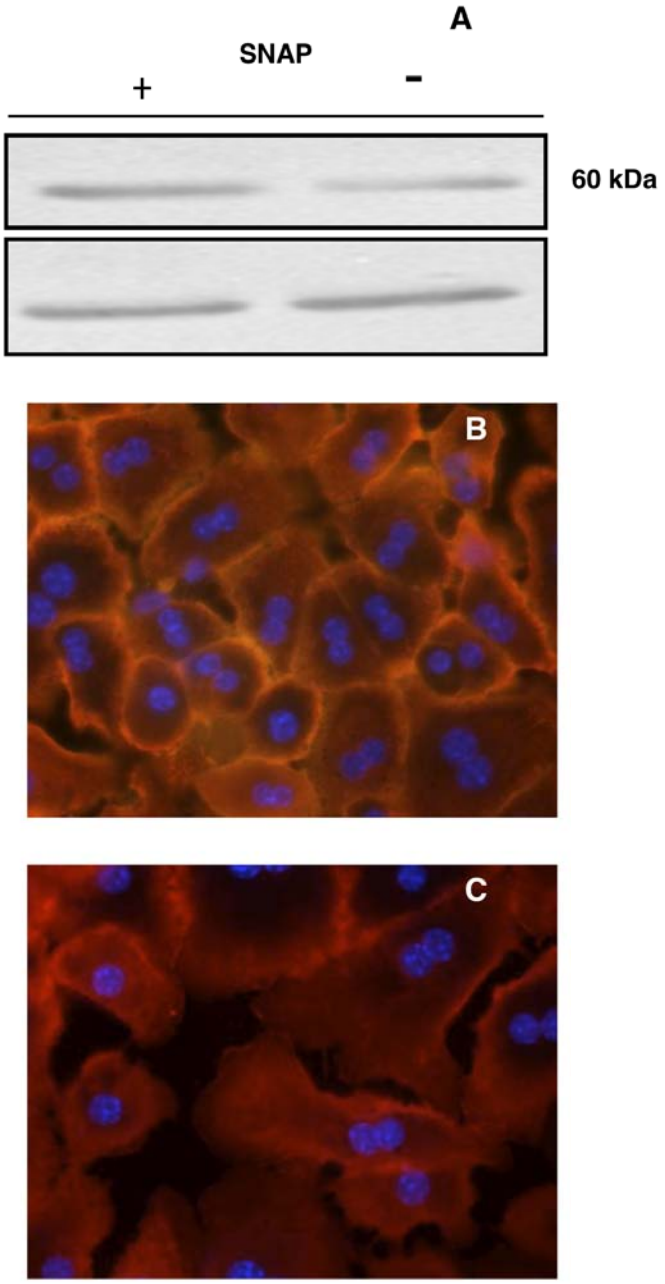


Figure 3-4. Nitric oxide up-regulates ZIP14 protein expression in liver parenchymal cells. Hepatocytes from WT (C57BL/6) mice were incubated with SNAP for 8 h. A) Total cell lysates were separated by SDS-PAGE, and ZIP14 was detected by Western blot analysis using an affinity purified ZIP14 antibody. Upper blot shows the increased ZIP14 expression produced by SNAP, while the lower blot is the tubulin loading control. B) Non-permeabilized primary hepatocytes were stained with 4,6 - diamidino-2-phenylindole (DAPI) for visualization of the nucleus, and an affinity purified ZIP14 antibody was used for immunolocalization of ZIP14. C) Untreated cells were used as a control. Representative images from SNAP-treated and untreated cells using identical gain settings are shown.

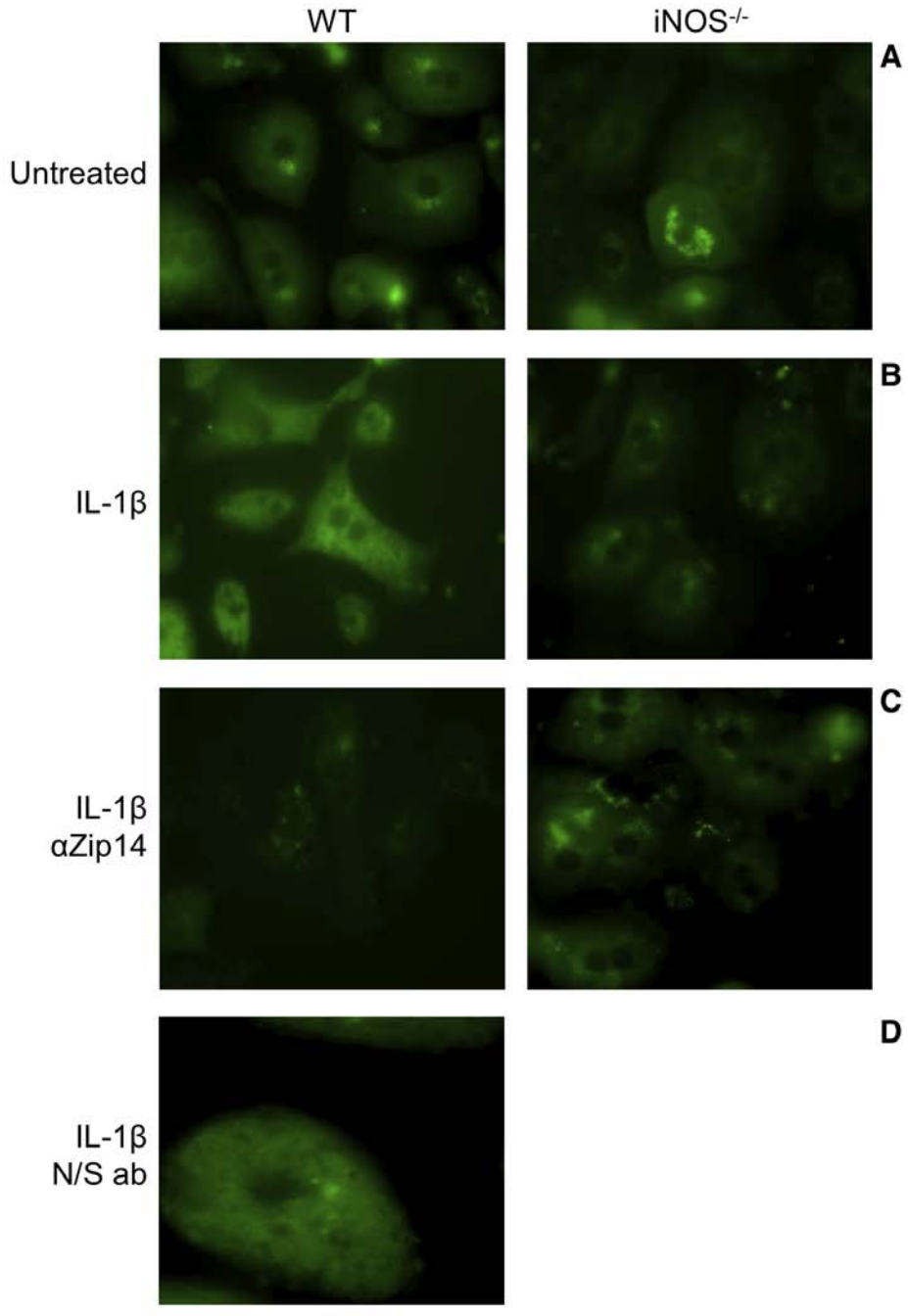


Figure 3-5. Fluorescent detection of NO-mediated zinc uptake in hepatocytes from WT and iNOS-/- mice using FluoZin3-AM. Hepatocytes were treated without A) or with B) Il-1β for 8h, followed by incubation for with 40 μM zinc for 5 min, and then immediately visualized by fluorescence microscopy. C) Hepatocytes were incubated with IL-1β for 8 h and then an anti-ZIP14 antibody was added prior to addition of zinc to the medium. D) Hepatocytes were treated as above, however a non-specific antibody (N/S ab) was used instead of an anti-ZIP14 antibody.

# CHAPTER 4
# NITRIC OXIDE INCREASES THE TRANSCRIPTION OF METALLOTHIONEIN AND ZINC TRANSPORTER-1 GENES THROUGH ACTIVATION OF THE TRANSCRIPTION FACTOR MTF-1

## Introduction

The metal-thiolate clusters of MT possess the unique ability to function as a redox unit, potentially allowing this protein to be involved in a redox sensitive signaling pathway (Maret 2009). Indeed, nitric oxide (NO) has been shown to S-nitrosylate MT and release zinc from cultured pulmonary artery endothelial cells as confirmed by fluorescence fusion-protein modified MT that undergoes conformational changes in the presence of NO (Pearce et al., 2000). Furthermore, a study involving MT-KO and wild-type (WT) mice illustrated a specific increase in intracellular labile zinc from WT, but not MT-KO lung fibroblasts after exposure to the NO donor S-nitrocysteine (St. Croix et al., 2002). The interaction of NO with MT is specific. In vitro studies have demonstrated a preferential release of the 3 zinc atoms in the N-terminal β-domain of MT, leaving the 4 zinc atoms in the c-terminal α-domain intact (Zangger et al., 2001). These alterations in cellular zinc homeostasis suggest the protective effects of MT against nitric oxide toxicity occur through intracellular zinc signaling (Schwarz et al., 1995).

The link between NO and MT may be the activation of the metal response element binding protein-1 (MTF-1). MTF-1 responds to changes in intracellular zinc by translocating from the cytosol to the nucleus where it binds to metal response elements (MREs) of metal responsive genes (Heuchel et al., 1994). MTF-1 has been shown to translocate to the nucleus under a variety of stress conditions as well, including heavy metal and oxidative stress (Saydam et al., 2001). MTF-1 is involved in the cellular response to oxidative stress (Dalton et al., 1996), hypoxia (Murphy et al., 1999), heavy metal stress (Heuchel et al., 1994), and amino acid deprivation (Adilakshmi et al., 2002). Cellular oxidative stress produced through the use of

H2O2 and tert-butylhydroquinone, has been shown to increase MTF-1 binding to the MREs of the MT promoter (Dalton et al., 1996). MTF-1 contains six zinc fingers of the C2H2 type. These zinc fingers provide the sequence-specific interaction with DNA at the MRE consensus sequence 5'-TGCRCNC-3'. In addition to MT, the zinc transporter-1 (ZnT-1) is also known to contain MREs, and be responsive to intracellular zinc status (Langmade et al., 2000). Through activation of MTF-1 the labile zinc released from MT by NO may provide the means for turning on genes that are involved in zinc homeostasis and ultimately cellular protection.

## Results

### SNAP Causes Intracellular Labile Zinc Release

We use the NO-donor SNAP in primary murine hepatocytes to investigate the effect of NO on the intracellular release of zinc, as measured by FluoZin3-AM fluorescence (Fig. 4-1). As would be expected, very little labile zinc is present in hepatocytes (Fig. 4-1A). However, after exposure to SNAP, a large increase in FluoZin3-AM fluorescence was observed in CK hepatocytes, suggesting release of labile zinc. As was shown in lung fibroblasts, the labile zinc release is dependent upon the presence of MT (Fig. 4-1C). These results suggest that MT is necessary for the intracellular release of labile zinc caused by NO.

### NO Increases Expression of MT and ZnT1 Genes

As a first step in examining the effect of NO on MT and ZnT1 gene expression, primary hepatocytes from WT mice were exposed to the NO donor SNAP (100 μM) or 100 u/ml IL-1β (Fig. 4-2). Exposure to exogenous or endogenous NO led to approximately a four-fold induction of both genes. Next, these same parameters were used for primary hepatocytes from $iNOS^{-/-}$ mice (Fig. 4-2). Once again utilizing the exogenous nitric oxide donor SNAP led to a similar increase in both MT and ZnT1 gene expression. However, because $iNOS^{-/-}$ hepatocytes can no longer produce NO in response to IL-1β stimulation, no increase in MT or ZnT1 gene expression

was observed. These results indicate that IL-1β increases MT and ZnT1 gene expression via production of NO.

**NO Induces Transcription of MT and ZnT1 Genes**

To analyze what role NO plays in increasing expression of MT and ZnT1 genes, primary hepatocytes were treated with 100 μM SNAP for up to 24 h (Figs. 4-3, 4-4). Steady-state mRNA levels were measured for both MT and ZnT1 over the entire course of treatment. A maximal increase (approximately four fold) in MT mRNA abundance was observed 2 h post SNAP treatment, but was greater than control levels for at least an additional 8 h. A similar increase of ZnT1 mRNA levels was observed. Additionally, zinc treatment was used as a positive control for these two metal-inducible genes. A predictable increase in steady-state mRNA levels was achieved for both genes, with a seven-fold increase in MT mRNA at 4 h, and a nine-fold increase in ZnT1 at 2 h. Elevations in both transcripts were maintained for up to 12 h post zinc treatment.

To determine whether NO is increasing the transcription of MT and ZnT1 genes or stabilizing the transcripts, the abundance of hnRNA was measured over the same time period as the steady-state mRNA levels. First, zinc treatment was again used as a positive control for transcriptional regulation of these genes. A clear increase in hnRNA abundance was observed during the same time frame as the steady state mRNA levels indicating a transcriptional mode of regulation for MT and ZnT1. Furthermore, SNAP also increased hnRNA levels of both MT and ZnT1 in a manner that mirrored the mRNA levels. These results imply that NO upregulates MT and ZnT1 expression by increasing transcription of the genes, similar to the classic response to zinc.

**MTF-1 Mediates the NO-Induced Increases in MT and ZnT1 Expression**

Nuclear translocation of MTF-1 is necessary for metal-induced transcription of target genes (Saydam et al., 2001). In a study by Stitt et al., SNAP induced nuclear localization of an

EGFP-MTF-1 chimeric protein in cells containing MT but not in MT null cells. We examined the effect of SNAP on endogenous MTF-1 localization in WT hepatocytes (Fig. 4-5). The results suggest an increase in nuclear abundance of MTF-1 after SNAP treatment, as well as zinc, indicating that MTF-1 may be responsible for SNAP induced changes in MT and ZnT-1 gene expression in primary hepatocytes.

MTF-1 regulates expression of MT and ZnT1 in response to zinc (Heuchel et al., 1994). Therefore, zinc was once again used as a positive control for MTF-1 mediated expression of MT and ZnT1 (Fig. 4-6). Knocking down expression of MTF-1, by using MTF-1 siRNA, inhibited zinc activation of MT and ZnT1 genes. Therefore, this would provide a good basis to determine whether or not NO induces expression of these genes through MTF-1. Once again SNAP or IL-1β could increase MT and ZnT1 gene expression, meaning NO could activate these genes from either an endogenous or exogenous source (i.e., SNAP). However, in the absence of MTF-1, NO could not induce the expression of either MT or ZnT1. Taken together, these results suggest that MTF-1 mediates the NO-induced upregulation of MT and ZnT1.

**NO Downregulates Zip10 Expression through MTF-1**

Unlike activation of ZnT-1 by MTF-1, Zip10 expression is suppressed by induction of MTF-1 (Wimmer et al., 2002). This was the first Zip gene identified as an MTF-1 target, and moreover Zip10 was the first gene repressed by metal induction (Cd) of MTF-1. After establishing a link between NO and MTF-1 activation of MT and ZnT1, we hypothesized that NO would decrease Zip10 expression via MTF-1. In agreement with our hypothesis, SNAP decreased Zip10 mRNA abundance by nearly 10 fold, and this suppression was maintained for 10 h (Fig. 4-7).

Although NO suppresses Zip10 expression similar to zinc, we needed to determine whether MTF-1 was the mediator of this downregulation. A recent study in zebrafish

demonstrated that MTF-1 was responsible for zinc-induced repression of Zip10, through binding to a downstream-intronic MRE (Zheng et al., 2008). We therefore used MTF-1 siRNA to determine if MTF-1 mediated the SNAP induced repression of Zip10. qPCR analysis demonstrated that Zip10 repression after SNAP exposure, could be alleviated by MTF-1 siRNA (Fig. 4-8). Together these results suggest that MTF-1 also mediates NO-induced repression of Zip10.

## Discussion

The metallothionein genes were the first identified and the best characterized target genes of MTF-1. Heavy metal exposure (Heuchel et al., 1994), reactive oxygen species (Dalton et al., 1996) and hypoxia (Murphy et al., 1999) have all been shown induce MT gene transcription through activation of MTF-1. While the mechanisms of activation are only partially understood, recent evidence (Daniels et al., 2002; Jiang et al., 2003; Zhang et al., 2003) supports the hypothesis that metals and oxidants activate MTF-1 by causing a redistribution of zinc in the cell (Andrews 2000; Giedroc et al., 2001). MTF-1 is regulated by micromolar concentrations of zinc (Bittel et al., 1998), and may function as a sensor of cytoplasmic zinc via a subset of zinc fingers with lower binding affinities for the metal than those for most zinc-binding transcription factors (Bittel et al., 2000; Giedroc et al., 2001).

Previous evidence has indicated that NO increases labile zinc in pulmonary endothelial cells and induces nuclear translocation of MTF-1 (St. Croix et al., 2002; Spahl et al., 2003). The absence of these NO-induced effects on zinc homeostasis and MTF-1 localization in cells derived from MT-null mice revealed that MT was central to both processes. Furthermore, cadmium, copper or hydrogen peroxide induced transcription only in the presence of zinc-saturated MT. Additionally, the apo-protein, thionein, inhibited the activation of MTF-1, presumably by sequestering zinc.

The three most common DNA binding motifs are helix-loop-helix, leucine zipper, and zinc finger. The zinc finger structure is accounts for 80% of the transcription factor DNA binding motifs (Andreini et al., 2006). NO has demonstrated both positive and negative effects on transcription depending on NO concentration and cell type. NO disrupts the zinc fingers of LAC9 (Kroncke et al., 1994), Sp1 and EGR-1 (Berendji et al., 1997) causing reversible inactivation of gene transcription. While the zinc clusters located in the β-domain of MT are definitive targets for NO (Zangger et al., 2001), the zinc fingers of MTF-1 do not appear to be targets for NO, because disruption of the zinc thiolate bonds would actually be expected to weaken the DNA binding affinity of MTF-1 (Giedroc et al., 2001). In support of this theory, I have shown NO could only induce the expression of MT and ZnT1 in the presence of MTF-1, indicating functional transactivation of gene expression by MTF-1.

The nuclear localization of MTF-1 appears to be essential, but not necessarily sufficient, for the transcriptional activation of zinc responsive genes (Saydam et al., 2001). Some studies indicate phosphorylation via multiple kinases including protein kinase C (PKC), c-Jun N-terminal kinase (JNK) and tyrosine kinase, may be involved in the metal-dependent transactivation of MTF-1 (LaRochelle et al., 2001; Saydam et al., 2001). In addition, multiple MAP kinase signaling pathways, including JNK, have been implicated in the cellular responses to NO related species (Hall et al., 2000; Lander et al., 1996) via S-nitrosylation of critical cysteine residues (Park et al., 2000). While this evidence suggests that NO could directly affect the phosphorylation of MTF-1, phosphorylation alone was not sufficient to induce translocation of MTF-1 in MT-null cells (Stitt et al., 2005).

Endogenous NO and NO donors have both been shown to increase MT mRNA expression in bovine aortic endothelial cells (Spahl et al., 2003). In support of these findings, the data

presented here in primary murine hepatocytes confirm that MT mRNA is induced in response to NO. In these studies, NO was also shown to differentially effect the expression of two zinc transporters, ZnT1 and Zip10. The changes in gene expression observed for these three genes was also linked to activation of MTF-1. Endogenous NO has been shown to induce glutathione (GSH) synthesis in vascular endothelial cells, in part through increased expression of gamma-glutamylcysteine synthase (γGCS) (Moellering et al., 1999). While MTF-1 is necessary for basal transcription and expression of γGCS (Gunes et al., 1998) the mechanism involved in the NO-dependent signaling for γGCS remain unclear. However, in murine hepatocytes we show a direct link between NO-induced Zn release from MT, nuclear translocation of MTF-1, and subsequent changes in MT, ZnT1, and Zip10 expression observed only in the presense of MTF-1.

These results are intriguing in the overall context of zinc homeostasis during inflammation. The concentration of cellular zinc is maintained by zinc binding proteins (e.g., MT) and zinc transporters. For the first time, we show a differential response of two zinc transporters to NO. Furthermore, ZnT1 and ZIP10 have opposing cellular transport functions. These new studies, along with many demonstrations of increased MT abundance after NO exposure, suggest that liver parenchymal cells maintain zinc homeostasis by decreasing uptake, increasing efflux, and increasing binding of zinc to proteins. However, recent studies of another ZIP family member, Zip14, demonstrate gene activation activation and subsequent zinc uptake during inflammation-induced NO production (Lichten et al., 2009). This discrepancy in transporter activation/ inactivation during inflammation may be of a temporal nature (i.e., Zip10 mRNA responds to NO before Zip14 mRNA) or more likely is a result of the importance of tissue-specific expression of these two genes. While Zip14 is abundantly expressed in the liver (Liuzzi et al.,

2006), expression of Zip10 is lower in the liver, and approximately 30-fold higher in the brain (Lichten et al. unpublished observations). Therefore, further experiments utilizing murine KO models of Zip14 or Zip10 would be useful to determine what roles these transporters play in zinc homeostasis during inflammation.

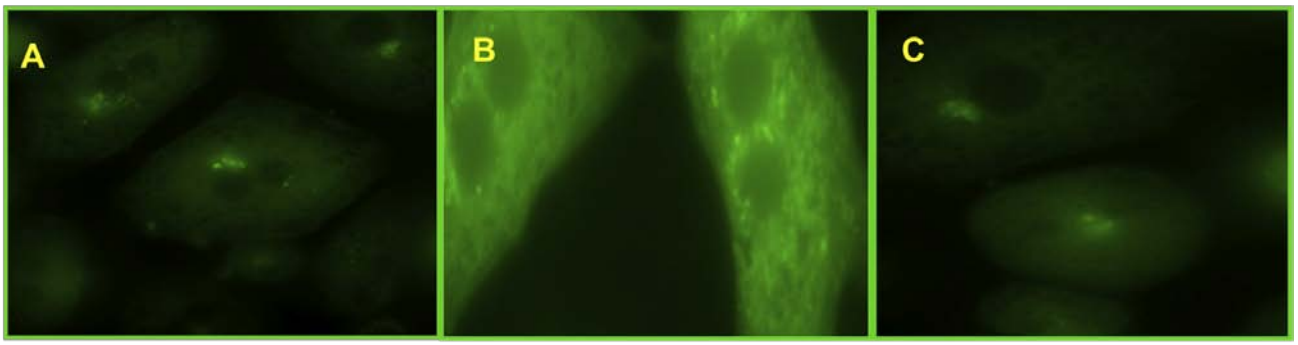


Figure 4-1. FluoZin 3-AM labled intracellular Zn2+ from primary hepatocyte cultures. A) Untreated WT hepatocytes. B) WT hepatocytes incubated with SNAP (100 μM). C) MT-/- hepatocytes incubated with SNAP (100 μM).

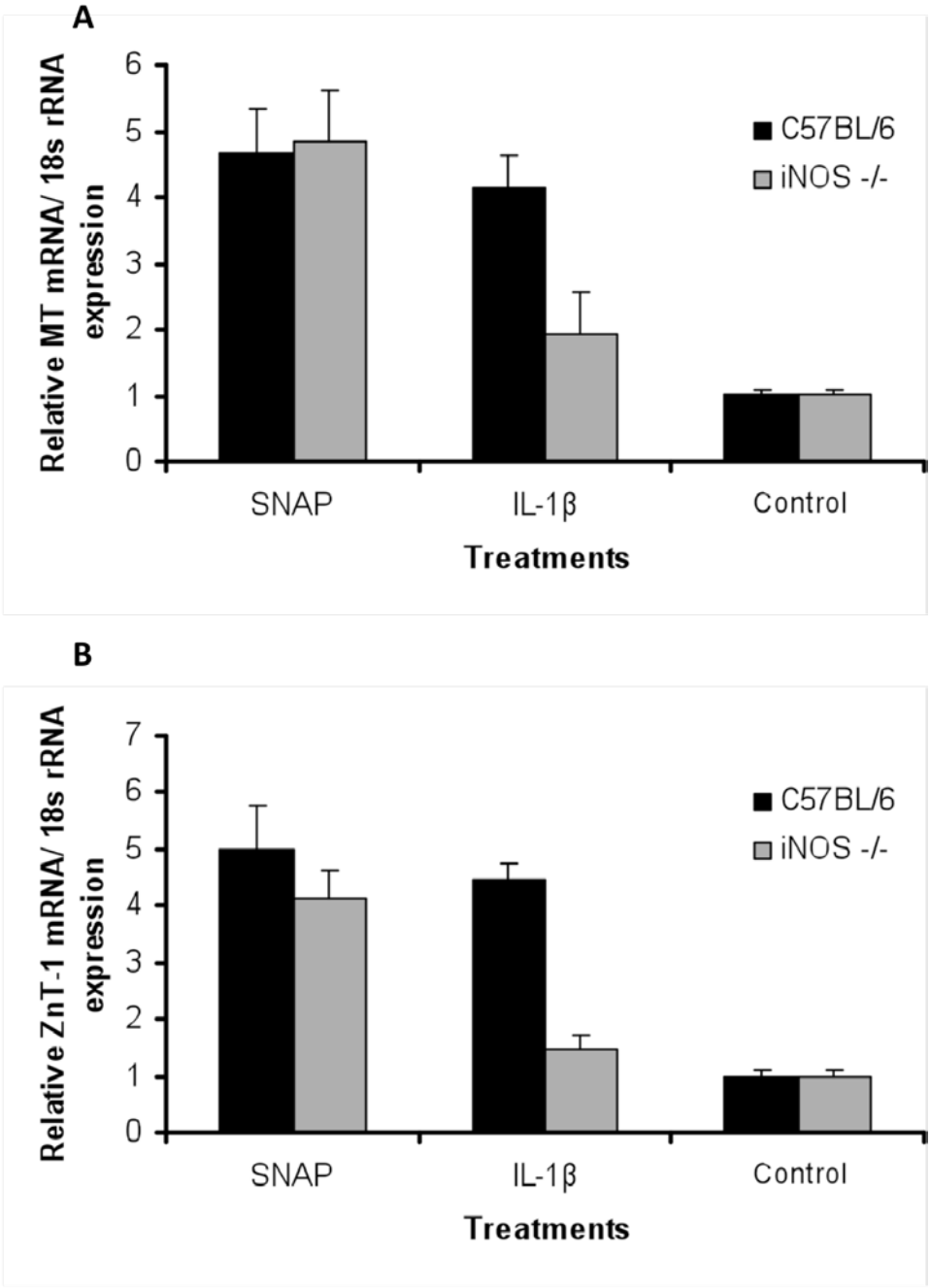


Figure 4-2. Endogenous or exogenous NO modulates MT and ZnT1 gene expression. A) qPCR measurement of MT mRNA expression after 6h treatment with SNAP (0.1 mM) or IL-1β (100 u/ ml). B) qPCR measurement of ZnT1 mRNA levels under the same conditions as in A. Values represent the mean ± S.D. of 2 independent experiments.

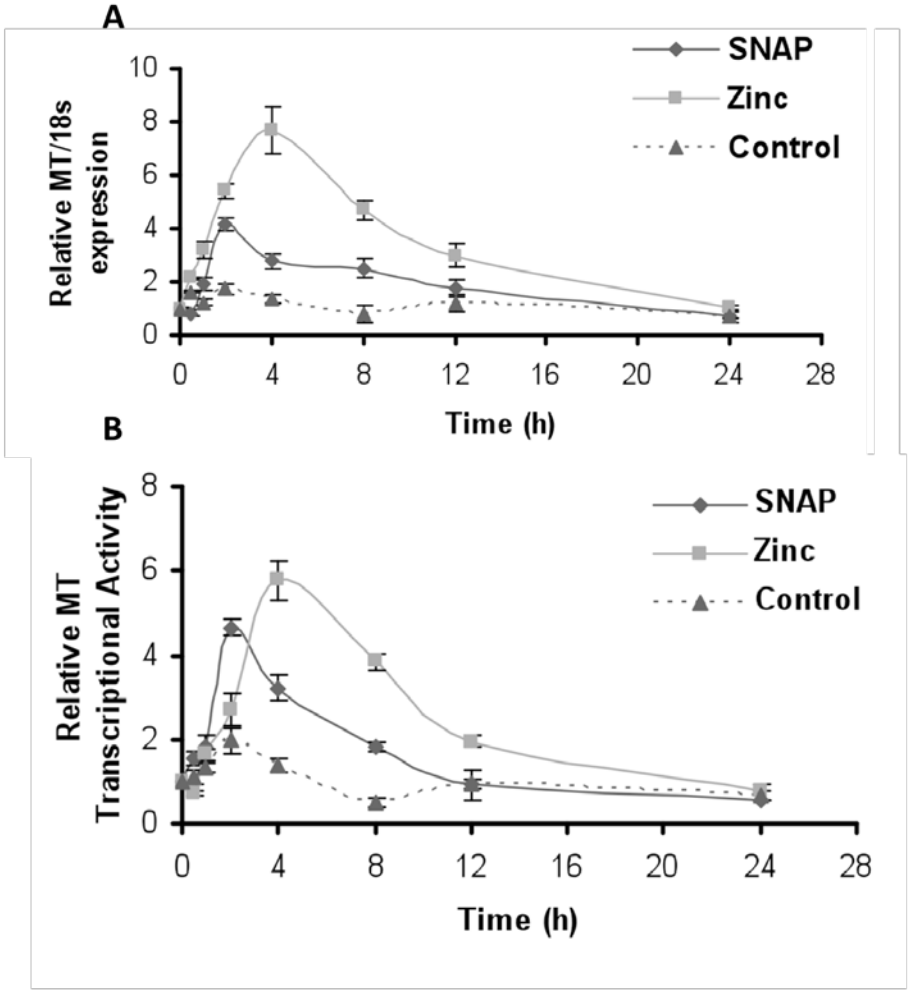


Figure 4-3. SNAP increases MT gene transcription and steady-state mRNA levels. A) qPCR measurement of MT mRNA expression after treatment with 0.1 mM SNAP or 40 μM $zinc^{2+}$. B) qPCR measurement of MT hnRNA levels under the same conditions as in A. Values represent the mean ± S.D. of 3 independent experiments.

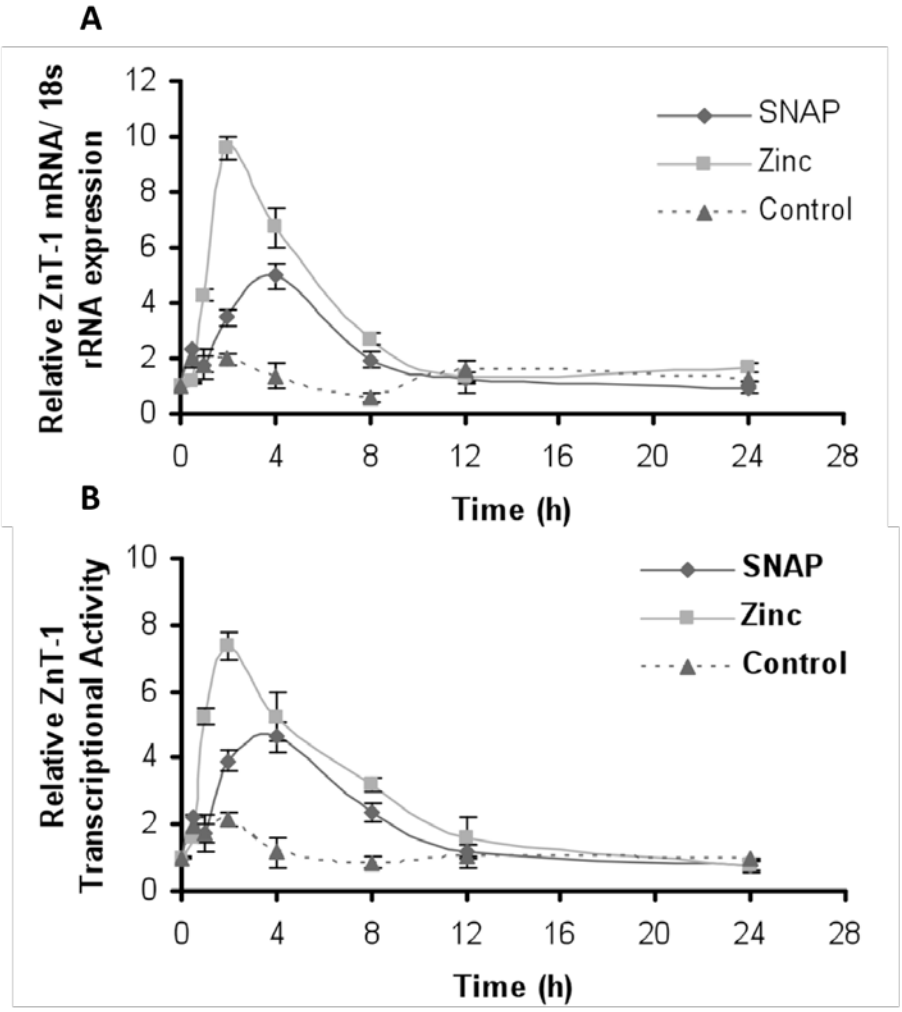


Figure 4-4. SNAP increases ZnT1 gene transcription and steady-state mRNA levels. A) qPCR measurement of ZnT1 mRNA expression after treatment with 0.1 mM SNAP or 40 μM $zinc^{2+}$. B) qPCR measurement of ZnT1 hnRNA levels under the same conditions as in A. Values represent the mean ± S.D. of 3 independent experiments.

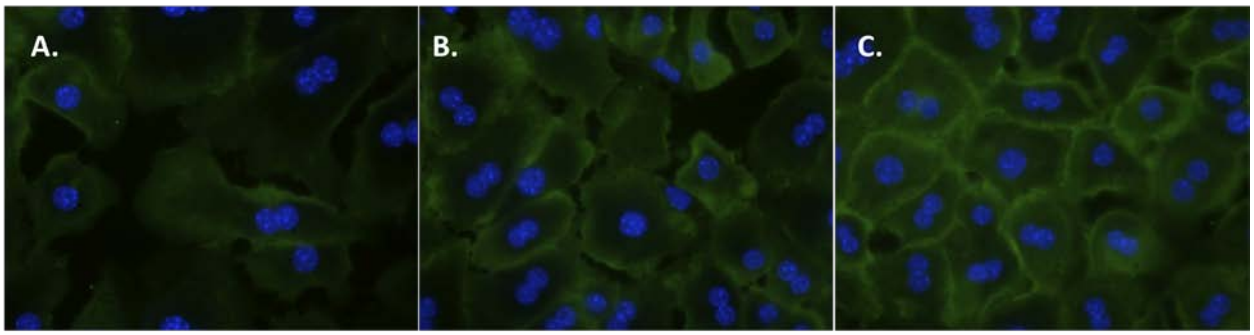


Figure 4-5. NO induces MTF-1 nuclear translocation in primary hepatocytes. Hepatocytes from WT mice were permeabilized with 0.1% triton X-100 and incubated with an anti-MTF-1 antibody followed by secondary incubation with Alexafluor 594 for visualization. A) Untreated hepatocytes. B) Hepatocytes exposed to SNAP for 3 h. C) Hepatocytes exposed to zinc for 3 h.

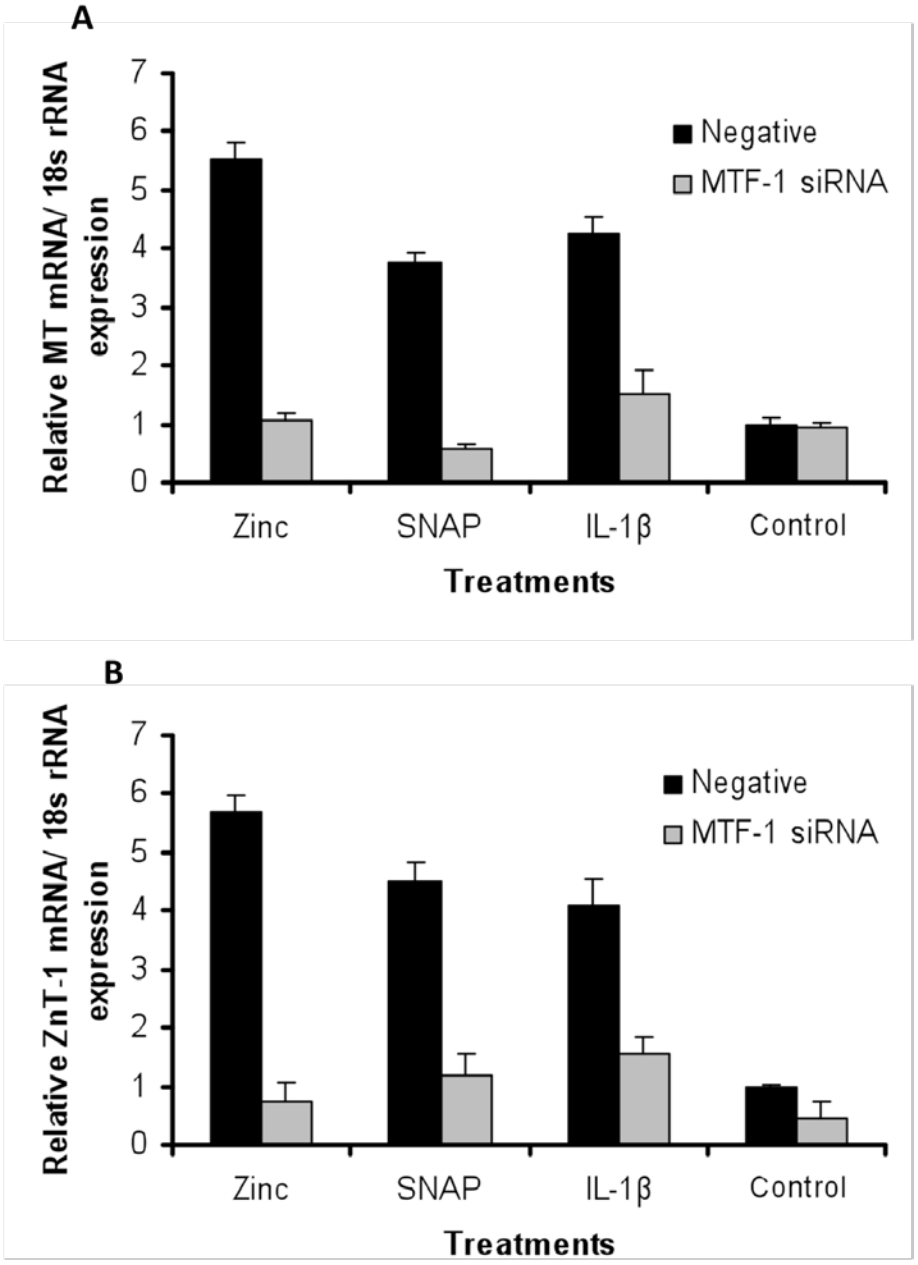


Figure 4-6. MTF-1 mediates increases in MT and ZnT-1 gene expression. A) qPCR measurement of MT mRNA expression 48h after transfection with MTF-1 siRNA (15 nM), followed by 6h treatment with Zinc2+ (40 μM), SNAP (0.1 mM), or IL-1β (100 u/ mL). B) qPCR measurement of ZnT-1 mRNA levels under the same conditions as in A. Values represent the mean ± S.D. of 3 independent experiments.

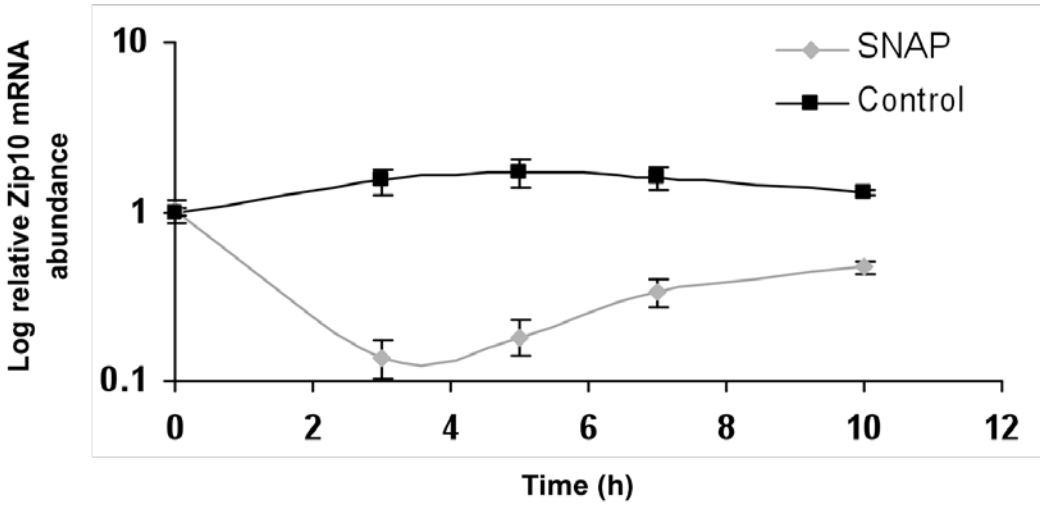


Figure 4-7. SNAP increases Zip10 steady-state mRNA levels. qPCR measurement of Zip10 mRNA expression after treatment with 0.1 mM SNAP. Values represent the mean ± S.D. of 3 independent experiments.

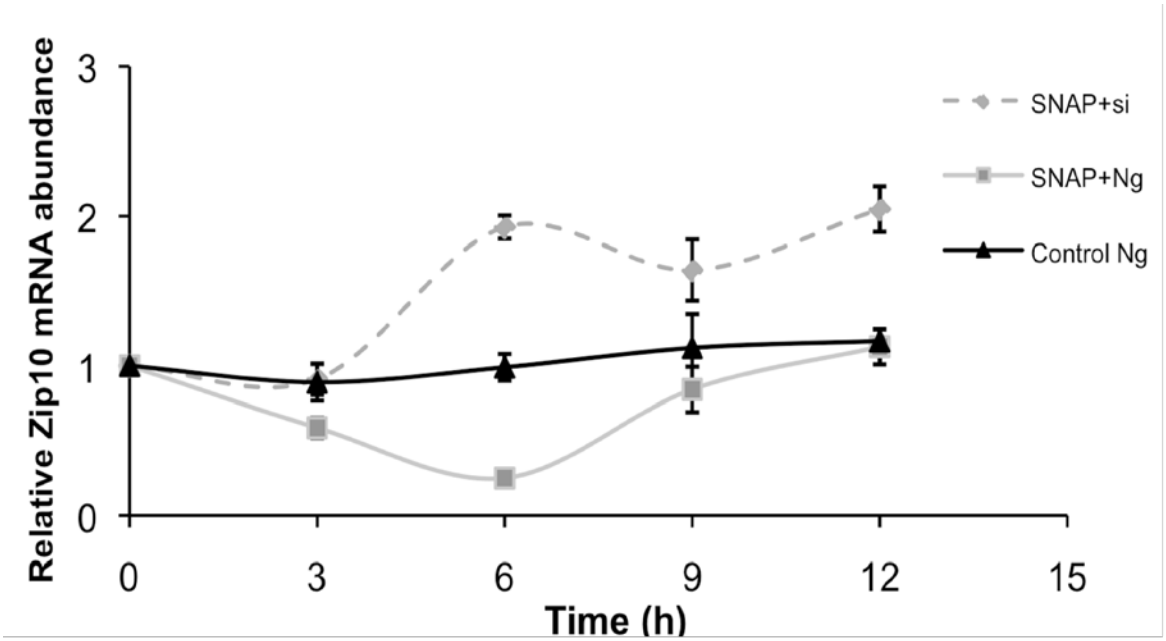


Figure 4-8. MTF-1 mediates SNAP induced repression of Zip10 expression. qPCR measurement of Zip10 mRNA expression 48h after transfection with MTF-1 siRNA (15 nM), negative control siRNA (Ng), followed by up to 12 h treatment with SNAP (0.1 mM). Values represent the mean ± S.D. of 3 independent experiments.

# CHAPTER 5
# REGULATION OF THE MURINE ZINC TRANSPORTER ZIP10 (SLC39A10) BY DIETARY ZINC RESTRICTION.

## Introduction

Zinc is an essential dietary component for all species ranging from plants to humans. More than ten percent of the human genome codes for zinc containing proteins (Andreini et al., 2006). The functions of zinc in biology are numerous, but can be separated into three main categories: catalytic, regulatory, and structural roles. Not surprisingly, deficiency of this single mineral nutrient is associated with diverse pathology, including impaired immunity, retarded growth, neurological disorders, and delayed wound healing (King and Cousins 2005). However, the mechanisms leading to these clinical manifestations of zinc deficiency remain elusive.

Zip10 belongs to the ZIP class of zinc transporters, which oppose the ZnT transporters by increasing cellular zinc concentrations through plasma membrane zinc uptake or vesicular efflux (Liuzzi and Cousins 2004). Unlike MTs or ZnT1, activation of MTF-1 by Cd, followed by binding to the Zip10 promoter, inhibited gene expression (Wimmer et al., 2005). *In silico* promoter examination and EMSA analysis revealed one functional MRE located at +17 relative to the Zip10 transcription start site (Wimmer et al., 2005). This was the first demonstration of potential metal-dependent transcriptional gene repression by MTF-1. However the mechanism of repression is unclear.

Although 24 zinc transporters exist, only two (i.e., ZnT1 and Zip4) have been tested and shown to have clear responsiveness to dietary zinc deficiency. Mechanistically, during zinc restriction down-regulation of ZnT1 occurs through decreased MTF-1 activation and ZnT1 promoter binding (Langmade et al., 2000). The functional outcome of less ZnT1 expression is decreased cellular zinc efflux. Concomitant to decreased ZnT1 expression is an increase in the primary intestinal apical zinc transporter, Zip4, which results in increased dietary zinc uptake.

Up-regulation of intestinal Zip4 by dietary zinc deficiency and down-regulation by supplemental zinc is independent of MTF-1 activity (Dufner-Beattie et al., 2003; Weaver et al., 2007; Liuzzi et al. 2009).

Here I demonstrate the dynamic responsiveness of Zip10 to zinc deficiency and excess. These studies also show for the first time that dietary zinc deficiency modulates nuclear MTF-1 translocation in murine brain and liver tissues, and the absence of nuclear MTF-1 results in an increase in Zip10 expression. Furthermore, ChIP analysis revealed in vivo association of MTF-1 with the Zip10 promoter in mouse hepatocytes after zinc supplementation. Finally, MTF-1 siRNA and Luciferase reporter constructs show repression of Zip10 expression occurs via MTF-1 activity and DNA binding to the downstream MRE of Zip10. Therefore, this study identifies an important zinc transporter potentially involved in zinc accumulation by the brain and liver, as well as revealing a novel role for MTF-1 in regulation of zinc transport.

## Results

### Dietary Zinc Modulates mZip10 Expression in Mice

Liver and brain tissues were collected from mice maintained on a zinc-deficient (ZnD, <1 ppm zinc) or zinc-adequate (ZnA, 30 ppm zinc) diet for 3 weeks. Quantification by qPCR revealed a nearly 3-fold increase in ZIP10 mRNA expression under ZnD conditions in both the liver and brain (Fig. 5-1A). Of particular interest is the approximately 30-fold difference in relative transcript abundance between the liver and brain. This difference indicates there could be an important role for ZIP10 related to zinc transport in the brain. Western analysis of ZIP10 protein in plasma membrane fractions from liver followed the same pattern as mRNA expression (Fig. 5-1B), with an increase in protein abundance under the ZnD condition relative to the loading control. Similarly, immunohistochemical analysis with the anti-ZIP10 antibody revealed a mainly cytoplasmic distribution of ZIP10 in liver cells of ZnA mice (Fig. 5-2A and B). Under

ZnD conditions, ZIP10 is localized to distinct intracellular membranes and the plasma membrane. As shown previously, prior incubation of this antibody with ZIP10 peptide blocked immunoreactivity (Ryu, M.S. et al., 2008). The most prominent areas of ZIP10 localization in the forebrain were the posterior lateral, posterior internal and the posterior medial striatum terminalis (Fig. 5-2C-F). The lateral globus pallidus (LGP), sublentic amygdala (SLEA) and dorsal lateral septal nuclei also showed marked ZIP10 expression in the forebrain (Fig 5-2C and D). Staining was markedly increased in those sections from brains of the ZnD mice. The ventral tegmental area (VTA) was also highly immunopositive for ZIP10 and was increased in the ZnD mice (Fig. 5-2E and F).

**Regulation of mZip10 Expression in AML12 Hepatocytes by Zinc**

To further investigate the regulation of Zip10 by zinc, we focused experiments on the AML12 hepatocyte cell line for the remainder of these studies. Treatment of these hepatocytes with different amounts of zinc revealed a dose-dependant repression of Zip10 transcript abundance (Fig. 5-3A). The decrease in these mRNA levels after zinc treatment (100μM) could be maintained for up to 10 h (Fig. 5-3B). The cells were incubated with either TPEN (5μM) or DTPA (50μM), which lead to a four-fold increase in Zip10 mRNA levels (Fig. 5-3C). Twelve h following zinc or TPEN treatment, membrane proteins were isolated from the hepatocytes for western analysis to examine ZIP10 abundance (Fig. 5-4A). Abundance closely followed that found for Zip10 mRNA. A comparable increase in ZIP10 was observed with DTPA (not shown). Concomitant to an increase in ZIP10 abundance was a distinct pattern of plasma membrane localization in permeabilized hepatocytes under zinc-restricted conditions compared to those not treated with the chelator (i.e. 5μM TPEN) (Fig. 5-4B).

**Zinc Regulated Expression of mZip10 Occurs through Activation of MTF1**

Mechanistically, exposure of cells to zinc causes a rapid translocation of MTF-1 from the cytoplasm to the nucleus, and up-regulation of the zinc-responsive genes *Mt* and ZnT1 occurs via binding of MTF-1 to the corresponding proximal promoter region. However, through EMSA analysis, MTF-1 was shown to bind to a downstream MRE of Zip10 (i.e., +17 relative to the TSS) after Cd exposure (Wimmer et al., 2005). Therefore, I focused on MTF-1 as a possible mediator of Zip10 expression in the liver and brain tissues of ZnD and ZnA mice. As an initial step, I investigated the nuclear and cytosolic abundance of MTF-1 protein. Western blotting with an anti-MTF-1 antibody revealed the presence of nuclear MTF-1 from both liver and brain tissues of ZnA mice, while these changes were not evident in the ZnD mice (Fig. 5-5B). Next, in an attempt to determine if MTF-1 is responsibe for in vivo zinc regulated expression of Zip10, we exposed the hepatocytes to zinc restricted and zinc supplemented conditions (i.e., 5μM TPEN and 100μM zinc, respectively) and analyzed the protein-DNA interactions through chromatin immunoprecipitation (ChIP) (Fig. 5-5A). Binding of MTF-1 to the Zip10 promoter was increased by almost three fold after 1 h of zinc treatment, and decreased to 25% after TPEN treatment.

Having established a correlation between cellular zinc status and nuclear MTF-1 at the Zip10 promoter, I knocked-down the expression of MTF-1 by using siRNA. A clear decrease in MTF-1 expression was evident 48h post siRNA transfection (Fig. 5-6A). Following the successful knockdown of MTF-1, AML12 hepatocytes were treated with zinc (100μM) for 12h, and ZIP10 protein abundance was measured by western blotting. In the absence of a sufficient amount of MTF-1, zinc repression of Zip10 was prevented. Similarly, basal ZIP10 protein expression was increased after treatment with MTF-1 siRNA. I next examined the Zip10 mRNA levels in cells lacking MTF-1. Again the knockdown of MTF-1 was able to reverse the dose-

responsive repression of Zip10 by zinc, and this reversal was maintained for 12h (Fig. 5-6B and 6C).

**MTF-1 Regulates Zip10 Expression through Obstruction of Pol II Elongation**

The functional capacity of the intronic MRE located downstream using Zip10 promoter-Luciferase construct was examined. Upon transfection into AML12 cells, the promoter responded in a positive fashion to addition of 100μM Zn (Fig. A-1). In addition, co-tranfection with MTF-1 siRNA reduced the response of the Zip10 reporter to activation by 100μM Zn. Further, the MRE upon being mutated lost responsiveness to zinc. These results show that the Zip10 promoter as a 5kb fragment which includes the MRE at +17 does not in this reporter system respond to zinc as observed in vivo with mouse liver or AML12 hepatocytes. I therefore investigated the possibility that MTF-1 physically obstructs Pol II movement, preventing active transcription under conditions of adequate cellular zinc. ChIP assays were utilized to immunoprecipitate the initiating and elongating forms of Pol II. Further analysis by qPCR was conducted by amplification of the TSS, exon 2, and exon 10 of Zip10. The ctd of Pol II is phosphorylated at Ser5 during initiation of transcription at the promoter of active genes (reviewed in Saunders et al., 2006). Under conditions of both zinc restriction and zinc supplementation of the cells, an equal amount of Ser5-P Pol II is associated with the TSS of Zip10 (Fig. 5-7A), even though levels of MTF-1 at the promoter are higher under the supplemented conditions (Fig. 5-5A). Active gene transcription involves a switch from the Ser5-P ctd to the Ser2-P ctd of Pol II for elongation. Amplification of either exon 2 or exon 10 of Zip10, under zinc restricted (+ TPEN) conditions show clear elongation activity of Pol II, through increased DNA binding (Fig. 5-7A, B). In contrast, the supplementation with 100μM zinc produced no evidence of Ser2-P or Ser5 Pol II association. Taken together, the results from the Zip10 TSS and downstream exons show that Pol II is recruited to the TSS even under zinc

supplemented conditions, but elongation occurs only under zinc restricted conditions. Furthermore, the *Mt* gene reacts positively to zinc supplementation through activation and binding of MTF-1 to the promoter. Therefore, *Mt* serves as a positive control for transcriptional activity enhanced by MTF-1. In direct contrast to the Zip10, under zinc supplemented conditions, but not zinc restricted conditions the Ser2-P isoform of Pol II was detected downstream of the *Mt* TSS (Fig. 5-7C). These data suggest that MTF-1 creates an obstacle that prevents Pol II movement from the TSS through the downstream remainder of the Zip10 gene.

**Repression of Zip10 Does Not Occur via Histone Modifications**

DNA methylation and histone deacetylation are epigenetic mechanisms that play major roles in eukaryotic gene regulation (reviewed in Dannenberg and Edenberg 2006; Edenberg et al., 2006). To rule out the possibility that zinc suppresses Zip10 expression by DNA methylation and/ or histone deacetylation, we treated the AML12 hepatocytes with 5-aza-2'-deoxycytidine (5-aza-dC) to inhibit DNA methylation and trichostatin A (TSA) to inhibit histone deacetylation. No differences in Zip10 expression were evident after exposure to either inhibitor after zinc treatment (Fig. 5-8). I therefore conclude that Zip10 is not regulated via chromatin modifications but instead is negatively regulated by zinc through recruitment of MTF-1, which obstructs productive transcription elongation.

## Discussion

Zinc homeostasis is maintained through regulation of zinc import and export via Zip and ZnT transporters respectively, and through intracellular zinc-binding proteins (e.g., metallothionein) (Cousins et al., 2006). Deficiency of dietary zinc leads to alterations in zinc transporter abundance and localization. For example, the intestinal zinc transporter, Zip4, increases in abundance and concentrates at the apical surface for luminal zinc uptake (Dufner-Beattie et al., 2003; Liuzzi et al., 2004), whereas the major cellular zinc exporter, ZnT-1,

decreases in abundance (McMahon and Cousins 1998). Dietary zinc deprivation causes a decrease in plasma zinc concentration, but results in only small reductions in the zinc content of most peripheral tissues (King and Cousins 2005). Similarly, the concentration of zinc in the brain overall does not vary under zinc restriction and is therefore tightly regulated (Prohaska 1987; Wallwork et al., 1983; Frederickson et al., 2005). The results presented in this study identify ZIP10 as a murine zinc importer that responds differentially to zinc restriction in both liver and brain and must be placed within the framework of zinc homeostasis in these organs.

The mRNA expression as well as protein abundance of ZIP10 from the liver and brain is increased after 3 weeks of dietary zinc restriction. A particularly interesting finding is the large relative difference in Zip10 transcript abundance between the brain versus the liver, although the magnitude of responsiveness to zinc deficiency remains the same. I observed significant upregulation of ZIP10 expression in the striatum, amygdala, caudate nucleus and substantia nigra as well as other regions which correspond to components of the basal ganglia. These regions play a significant role in movement control. Zinc exerts many neurobiological effects, such as modulating functions of neurotransmitter receptors in the brain. Alterations in zinc homeostasis in the brain may be involved in neurological diseases such as Alzheimer's disease, Parkinson's, and amytrophic lateral sclerosis. Inadequate dietary zinc intake leads to changes in behavior such as reduced activity and responsiveness to stimuli (Golub et al., 1995; Shagal et al., 1980). Zinc restriction during infancy causes impaired learning behavior (Takeda et al., 2000). Therefore, perturbations in ZIP10 function or single nucleotide polymorphisms that cause loss of function or restricted function may affect brain development or the pathogenesis of neurological disorders. Furthermore, the up-regulation of ZIP10 during conditions of zinc deficiency, particularly during development, may have deleterious effects on neurological processes. The

potential for increased zinc influx via ZIP10 controlled import may raise issues about the known relationship between excess zinc and neural cell death (Kim et al., 2000). Since these findings are new, it is possible that ZIP10 up regulation is a homeostatic mechanism to maintain zinc levls in the brain in response to low dietary zinc intake.

Utilizing AML12 murine hepatocytes, I show that Zip10 responds differentially to zinc supplementation and zinc restriction. Zip10 mRNA abundance, protein levels, and plasma membrane localization all increased in response to zinc depletion. These results may indicate an important role for ZIP10 in maintaining zinc homeostasis in liver parenchymal cells during dietary zinc restriction or supplementation, by increasing abundance of the transporter to allow more zinc influx during deficiency, and less zinc uptake when the mineral is in excess. The total zinc concentration of the liver does not decrease during reduced dietary intake of the micronutrient.

Cells respond to zinc through activation and rapid translocation of MTF-1 from the cytoplasm to the nucleus (Smirnova et al., 2000). Typically, metal-responsive genes such as MT and ZnT-1, are up-regulated by binding of MTF-1 to the corresponding proximal promoter region. However, MTF-1 was shown to bind to a downstream MRE of Zip10 (i.e., +17 relative to the TSS) after Cd exposure causing down-regulation of Zip10 expression (Wimmer et al., 2005). Further experiments in zebra fish also show MTF-1 mediated expression of Zip10 (Zheng et al., 2008). I was able to follow the movement of MTF-1 from the cytosol to the nucleus of liver preparations only when zinc status of mice was normal, or after zinc supplementation of hepatocytes in vitro. In addition, in the presence of a normal level of intracellular zinc, MTF-1 translocated to the nucleus and associated with a consensus MRE located downstream of the Zip10 TSS, as revealed by ChIP analysis. Furthermore, suppression

of Zip10 expression by zinc through MTF-1 binding was shown with experiments utilizing siRNA directed against MTF-1. In the absence of this metal-regulatory protein, zinc no longer suppresses Zip10 expression. This finding is of interest in the context of zinc-regulated transporters. For the first time in mice, MTF-1 was shown to be responsible for the down-regulation of a zinc importer. While simultaneously the prototypical metal-responsive transporter, ZnT-1, is up-regulated by zinc and MTF-1. This provides a plausible mechanism for maintaining cellular zinc homeostasis during dietary zinc deficiency or excess, by controlling the transport of zinc into and out of the cell.

Although previous experiments in zebra fish have indicated Zip10 is regulated by MTF-1 through a downstream intronic MRE, the mechanism of suppression was not identified (Zheng et al., 2008). Results of experiments designed to investigate Zip10 gene regulation including actinomycin D inhibition and qPCR measurement of hnRNA, lead me to conclude that Zip10 is regulated at the transcriptional level in response to zinc status (Lichten et al., unpublished observations). However, transcription can be inhibited by various mechanisms, including, but not limited to DNA methylation, transcriptional repressors, and chromatin modifications. The transcriptional activator MTF-1 has been previously shown by others (Zheng et al., 2008; Wimmer et al., 2005) and here, to be responsible for the suppression of Zip10 expression through binding to an MRE downstream of the TSS. In contrast, traditional, promoter-reporter constructs which included the +17 MRE did not exhibit repression of Zip10 (Fig. A-1). Therefore, the positive mode of MTF1-binding to MREs, as shown for Mt and ZnT1, was not expected to yield a negative mode of regulation for Zip10. Furthermore, I have shown that the nuclear abundance of MTF-1 is less when a zinc restricted diet is fed to mice and, through ChIP analysis, that MTF-1 binding is reduced in zinc depletion of AML12 hepatocytes. Hence, I hypothesized that MTF-

1 acts as a repressor of Zip10 when cellular zinc status is adequate, which yields MTF-1 occupancy by zinc and translocation to the nucleus.

As a mechanism to explain the repression of Zip10 during zinc adequate conditions of hepatocytes, I hypothesized that MTF-1 acts as a transcriptional repressor by obstructing movement of Pol II from the Zip10 TSS. Transcription is a complex multistep process involving sequence-specific activators that recruit Pol II and general transcription factors (GTFs) to the TSS for formation of the pre-initiation complex (PIC). Transcription initiation begins with TFIIH (a GTF) phosphorylating Ser5 residues in the carboxy-terminal domain of Rpb1, the large subunit of Pol II (Saunders et al., 2006). I show here that under the zinc restricted, adequate, and supplemental conditions used in these experiments, Pol II is actively recruited to the Zip10 TSS and is poised for transcription initiation as indicated by Ser5 phosphorylation. However, for gene transcription to occur productive elongation must ensue. This entails a shift from Ser5 phosphorylation to Ser2 phosphorylation, and recruitment of various elongation factors (reviewed in Saunders et al., 2006). Only under the conditions of zinc restriction could we detect Ser2 phosphorylated Pol II. We therefore propose that when cellular zinc is adequate, MTF-1 represses Zip10 expression by interfering with the transition from transcription initiation to elongation by impeding the movement of Pol II. Finally, I found no appreciable contribution of epigenetic regulation to the repression of Zip10 expression by zinc, further supporting the interference of Pol II movement by MTF-1 as the mechanism of suppression.

In summary, the experiments described here using intact mice and isolated murine hepatocytes show that MTF-1 is an integral part of ZIP10-related cellular zinc homeostasis in the liver both during zinc restriction and zinc excess. The results show that MTF-1 has physiologic significance and can act as a repressor of Zip10 under normal cellular levels of labile zinc. Upon

reduced cellular zinc, repression is removed as MTF-1 is not translocated to the nucleus allowing enhanced Zip10 transcriptional activation. The apparent differential mode of MTF-1 action, resides in the genomic placement of the MRE downstream of the Zip10 transcription start site. The results also show that ZIP10 is a new target to investigate dietary influences on zinc metabolism by the liver and within specific regions of the brain and therein could help to elucidate the neurobiological effects of zinc that have been elusive thus far.

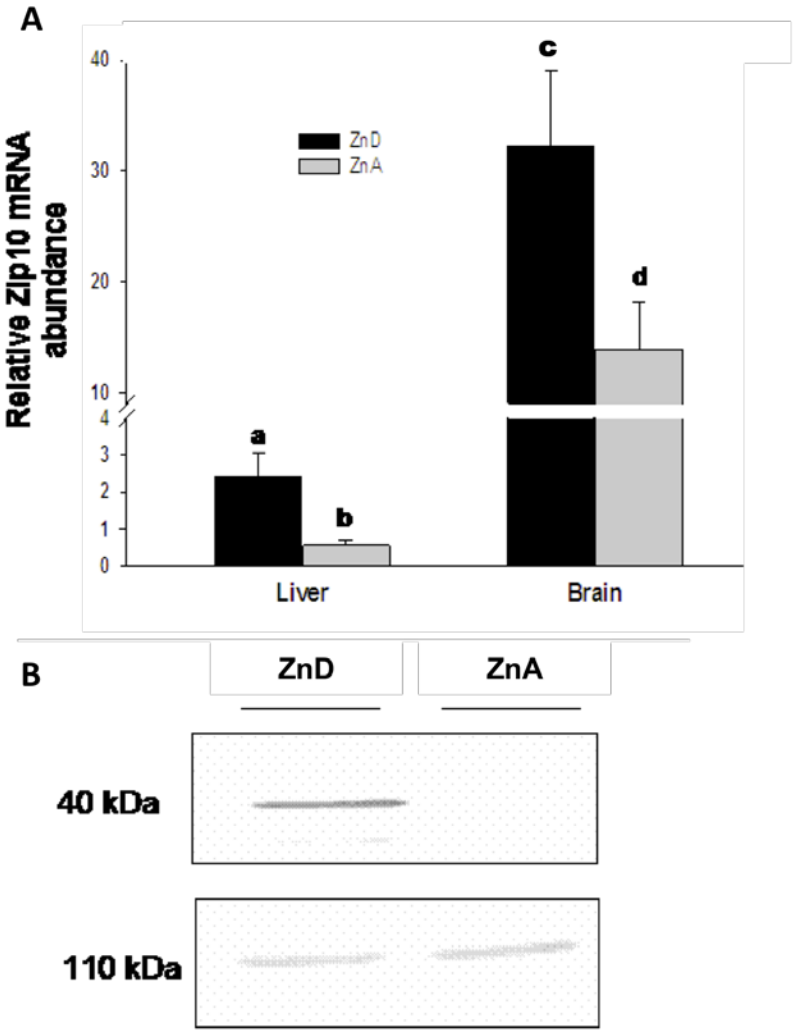


Figure 5-1. Dietary zinc deficiency regulates the expression of ZIP10. A) Total RNA extracts from liver or brain tissues of ZnD or ZnA mice were analyzed by qPCR. Values shown are Zip10 mRNA levels relative to 18s rRNA levels (means ± S.D. n = 4). B) Plasma membrane fractions from the liver of ZnD or ZnA mice were isolated and were separated by SDS-PAGE. Western blotting was performed by using an anti-ZIP10 antibody, then blots were stripped and re-probed using an anti-Na/ K ATPase antibody. A representative blot is shown.

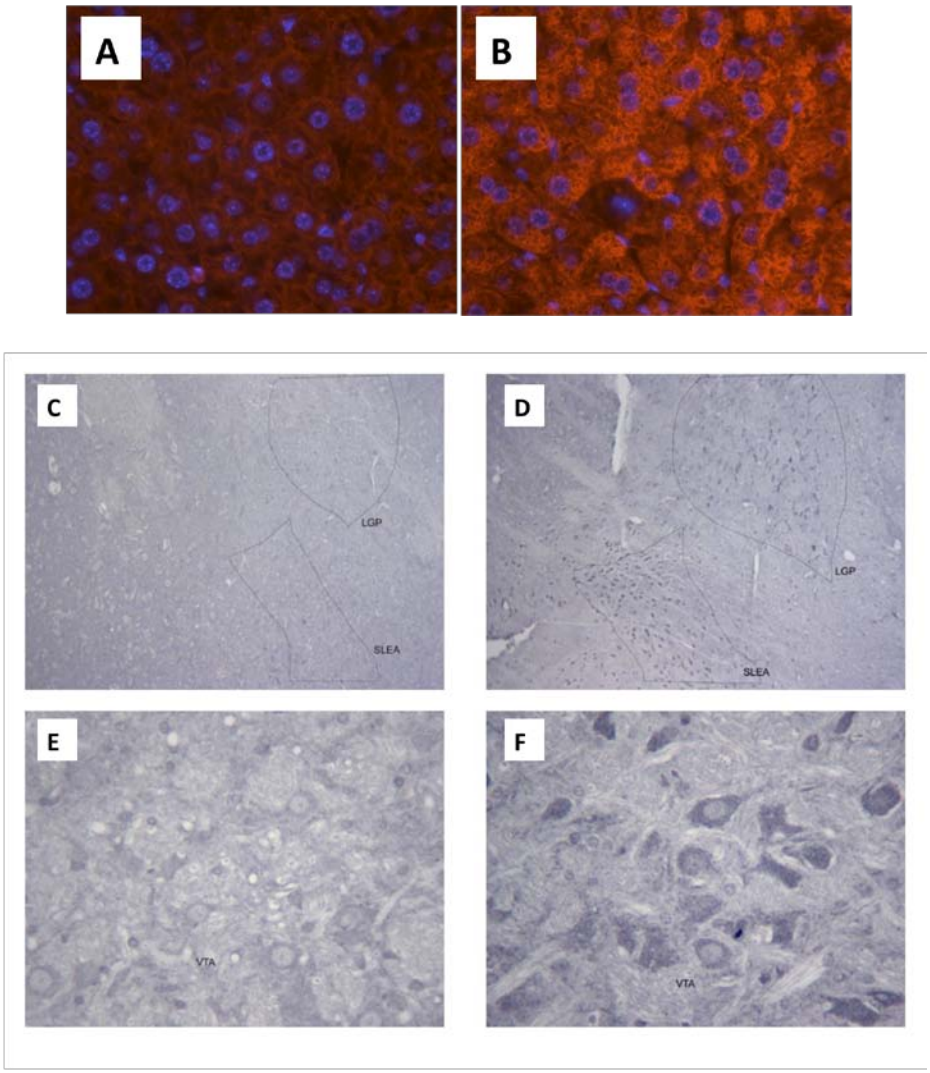


Figure 5-2. Immunohistochemical analysis of ZIP10 expression in the liver and brains of zinc depleted and zinc adequate mice. A, B) Representative liver sections from ZnA and ZnD mice. Immunolocalization of Zip10 was performed by using the anti-Zip10 antibody, and visualization was achieved with an Alexa fluor594 secondary antibody. C, D) Representative regions of the sublentic amygdala (SLEA) and lateral globus pallidus (LGP) and E, F) the mesencephalic ventral tegmental area (VTA) of ZnD and ZnA mice were examined. Horseradish immunoperoxidase was used to localize ZIP10; Sections A, C, and E were derived from ZnA mice and sections B, D, and F were from ZnD mice. Magnification was 60x for A, B, E, F and 40x for C,D.

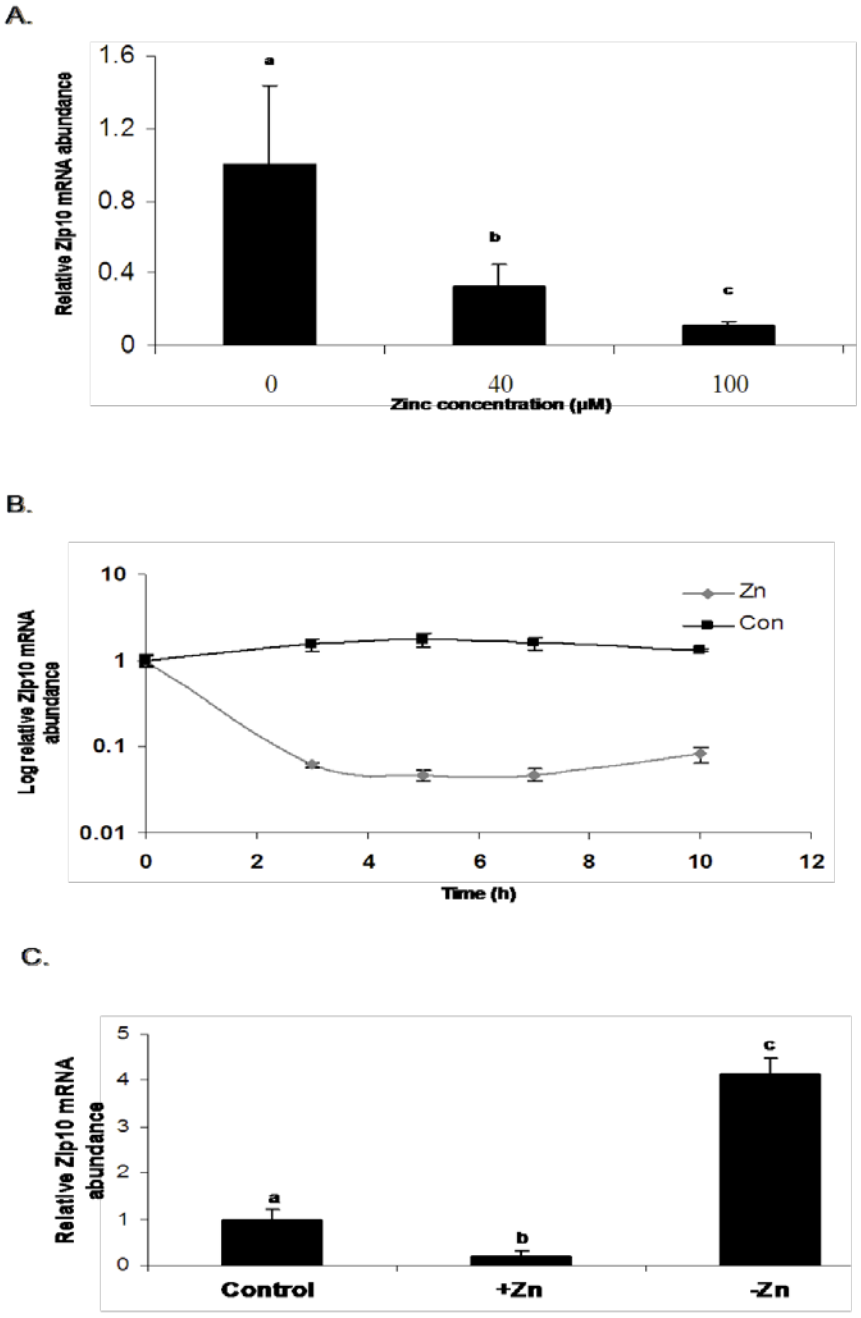


Figure 5-3. Zinc regulates Zip10 expression in AML12 hepatocytes. A) qPCR measurement of Zip10 mRNA expression after 3 h of treatment with 0, 40, or 100 μM zinc. B) qPCR measurement of Zip10 mRNA levels for 0-10h following 100 μM zinc. C) Relative Zip10 mRNA levels 3 h-post 100 μM zinc (+Zn), 50 μM DTPA or 5μM TPEN treatments. Values are relative to 18s rRNA, and represent the mean ± S.D. of three independent experiments.

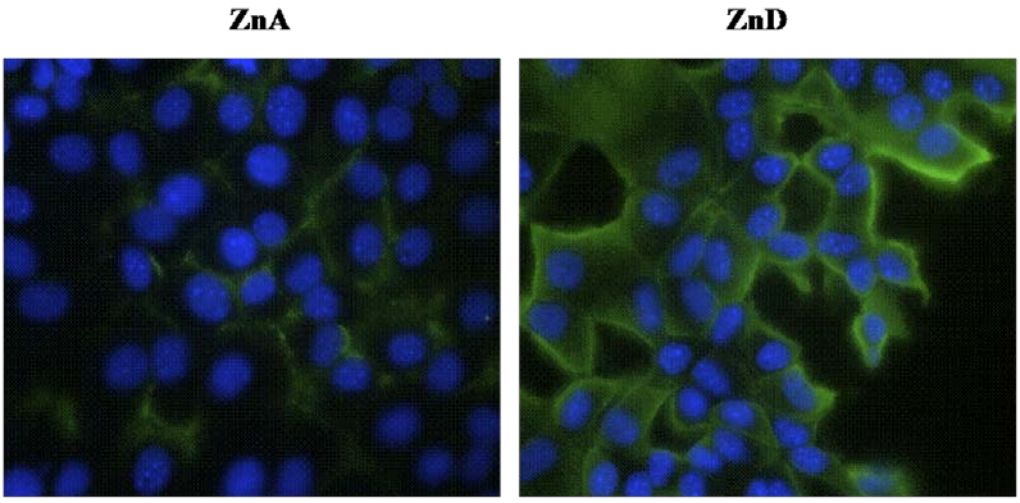


Figure 5-4. Zinc regulates the plasma membrane localization of ZIP10. The cells were incubated for 10h with or without 5μM TPEN. Non-permeabilized hepatocytes were incubated with 4′,6 -diamidino-2-phenylindole (DAPI) for visualization of the nucleus, and the affinity purified ZIP10 antibody was used for immunolocalization of ZIP10 by fluorescence microscopy. Representative images from TPEN-treated and untreated cells using identical gain settings are shown.

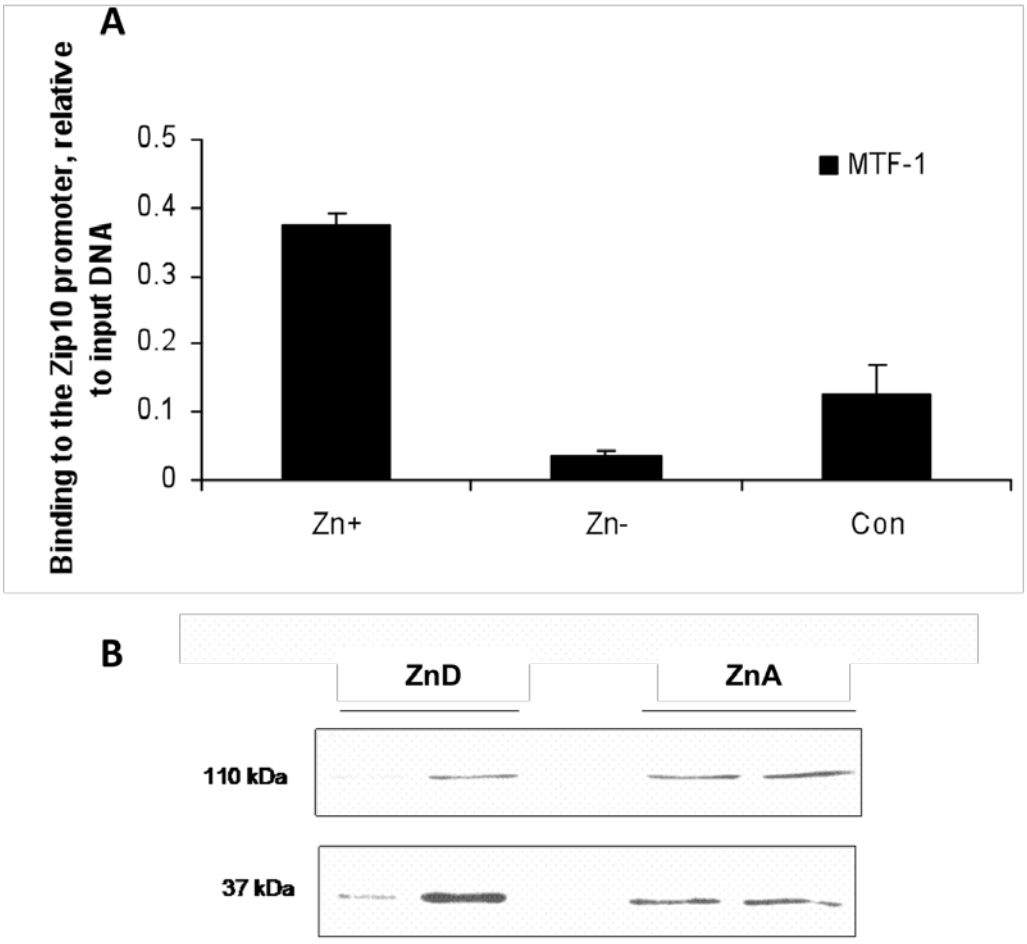


Figure 5-5. MTF-1 associates with the Zip10 promoter during zinc supplementation, but not zinc restriction. A) ChIP analysis reveals in vivo binding of MTF-1 to the Zip10 promoter in response to zinc. Murine AML12 hepatocytes were incubated with zinc (100 μM) or TPEN (5μM) for 3 h. ChIP analysis was performed using an anti-MTF-1 antibody, followed by qPCR. A non specific rabbit IgG was used as a negative control. Data were plotted as the ratio of immunoprecipitated DNA to a 1:20 dilution of input DNA. Background immunoprecipitation levels were always below a ratio of 0.01 (to input DNA). Each data point represents the mean ± S.E.M. for three independent experiments. B) Western blot analysis showing decreased MTF1 in nucleus from liver of the zinc restricted (ZnD) mice described in Figure 1. C. Nuclear fractions were isolated from liver protein extracts and separated by SDS-PAGE. Nuclear abundance of MTF-1 was identified by western blotting, utilizing an anti-MTF-1 antibody. Blots were then stripped and reprobed with anti-TBP antibody.

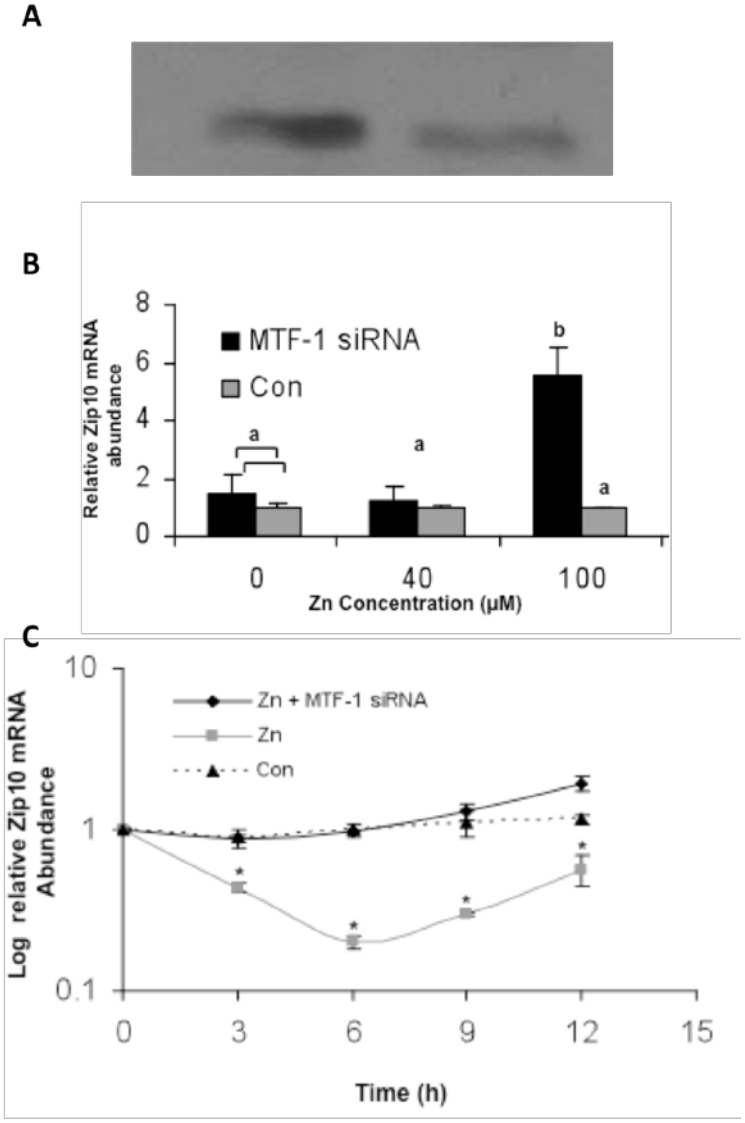


Figure 5-6. MTF-1 knockdown increases Zip10 expression and alleviates zinc-induced gene repression. A) AML12 hepatocytes were transfected with MTF-1 siRNA or negative control siRNA oligonucleotides and allowed to incubate for 48h. Total protein extracts were collected and separated by SDS-PAGE, followed by western blotting using the affinity purified ZIP10 antibody. Equal loading was evaluated using β-Actin. B) Hepatocytes were transfected with siRNA as above, then incubated with 40 and 100 μM of zinc for 3 h. Zip10 mRNA abundance was measured by qPCR, and is relative to 18s rRNA. Values with a different superscript are significant at $P < 0.05$. C) Hepatocytes were transfected as above, and incubated with 100 μM zinc for up to 12h. Zip10 mRNA was measured as in B, and expressed relative to control samples transfected with negative control siRNA. Values represent the mean ± S.D. of three independent experiments.

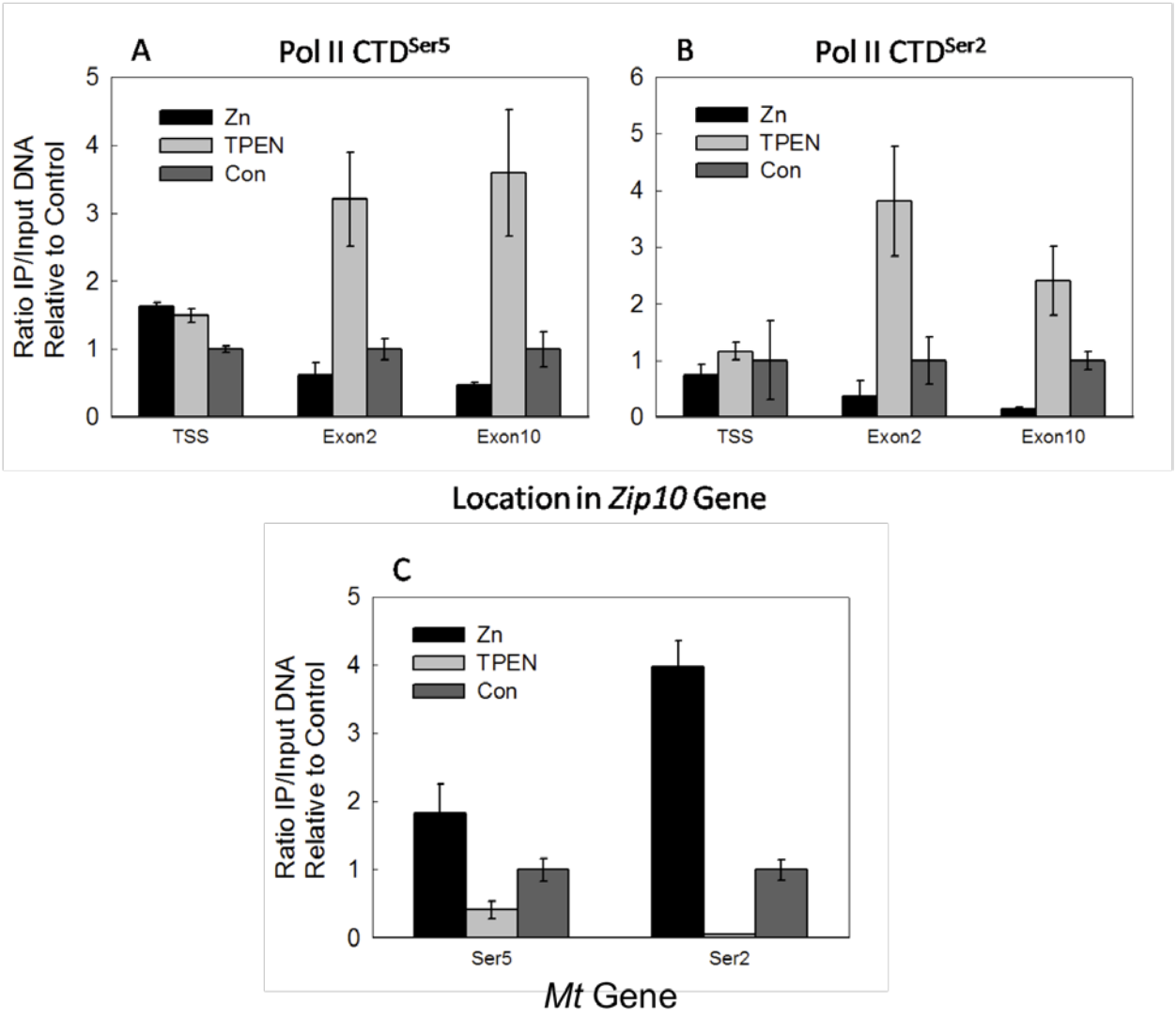


Figure 5-7. Transcriptional elongation by Pol II occurs during zinc deficiency, but not with zinc supplementation. A) ChIP analysis of Pol II throughout the Zip10 gene by using an antibody specific for the Ser5 phosphorylated form of Pol II, 3 h after incubation of the AML12 hepatocytes with 100 μM zinc or 5 μM TPEN. B) ChIP analysis of the elongating form of Pol II, phosphorylated at Ser2. Cells were treated as in A. A non specific rabbit IgG was used as a negative control for both A & B. C) ChIP analysis of phosphorylated Pol II associated with the *Mt* promoter. Data were plotted as the ratio of immunoprecipitated DNA to a 1:20 dilution of input DNA. Background immunoprecipitation levels were always below a ratio of 0.01 (to input DNA). Values represent the mean ± S.D. for two independent experiments.

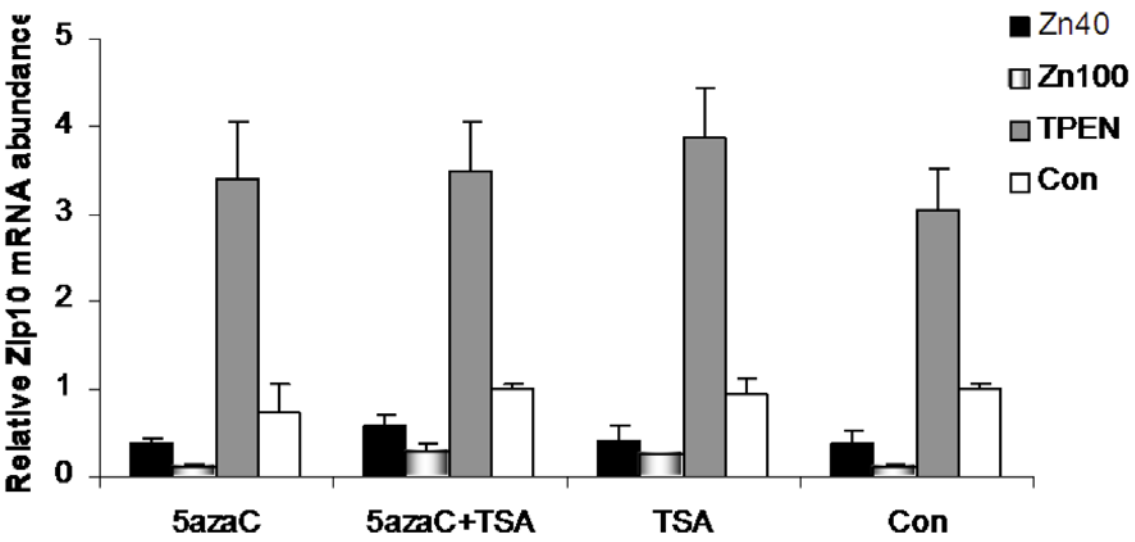


Figure 5-8. MTF-1 mediates Zip10 expression independent of chromatin modifications. Measurement of Zip10 mRNA levels by qPCR after 24 h of 5- azacytidine, (AZA) 12 h trichostatin A (TSA), or both. AML12 hepatocytes were subsequently treated with either 40 μM zinc, 100 μM zinc, 5 μM TPEN or no addition (control) for 3 h. Data were normalized by 18s rRNA, and relative to control levels. Values represented are the mean ± S.D. of three independent experiments.

# CHAPTER 6
# CONCLUSIONS AND FUTURE DIRECTIONS

## Conclusions

Zinc serves structural, regulatory, or catalytic roles in hundreds of proteins (Berg and Shi 1996; Krishna et al., 2003; Ravasi et al., 2003; Vallee and Auld, 1990) and is the second most abundant essential trace metal. When zinc is deficient, through lack of dietary intake or as a consequence of various pathologic conditions, a multitude of 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. Clinical symptoms of zinc deficiency in children and adults include retarted growth, depressed immune function, skin lesions, depressed appetite, skeletal abnormalitites, and impaired reproductive ability. On the other hand, zinc is also toxic when in excess. Therefore, the maintenance of zinc homeostasis is critical and multiple genes have evolved to modulate zinc storage (4 Metallothionein genes in mice), efflux (10 Znt genes), and uptake (14 Zip genes). Furthermore, many mammalian zinc transporters are expressed in a tissue-specific manner and in specific cellular localizations. In addition, they can change cellular localization and stability in response to differing stimuli (e.g., zinc deficiency or excess, hormones, and cytokines) (Cousins et al., 2006; Eide 2006; Kambe et al., 2004). The diverse roles of these transporters in zinc homeostasis are only now being recognized.

The initial results presented here describe a nitric oxide-induced mechanism for increasing liver zinc uptake during hepatic inflammation. We have previously shown that ZIP14 may be the major zinc transporter responsible for the hypozincemia associated with inflammation and the acute-phase response (Liuzzi et al., 2005). Our prior research utilized two different model systems to examine Zip14 expression. In the turpentine model of inflammation, IL-6 was

necessary for the in-vivo induction of Zip14 expression and hypozincemia, whereas the LPS model did not show an absolute requirement for IL-6. These differences in Zip14 regulation may be related to the cytokines produced by each stimulus.

Experiments presented here, utilizing iNOS$^{-/-}$ mice, identified NO as a key regulator of Zip14 expression in the mouse liver. IL-1β could only induce expression and activity of Zip14 in the presence of NO. Moreover, transcription of Zip14 could be induced by the NO-donor, SNAP, supporting the involvement of NO in Zip14 expression. Additionally, ChIP analysis revealed the association of the transcription factor, c-Fos (a component of AP-1), during transcription of the Zip14 gene. Collectively, these results demonstrate that IL-1β can stimulate NO production, elevate Zip14 expression via signalling pathways leading to AP-1 activation which in turn leads to hepatic zinc accumulation. This was a first step in analyzing the influence of NO on zinc transport and the implications for hepatic zinc homeostasis.

Evidence in the literature has indicated that NO causes an intracellular redistribution of zinc in certain cell types by increasing labile zinc, and inducing nuclear translocation of MTF-1 (St. Croix et al., 2002; Spahl et al., 2003). The absence of these NO-induced effects on zinc homeostasis and MTF-1 localization in cells derived from MT-null mice revealed that MT was central to both processes.

The results presented here in primary murine hepatocytes and AML12 cells support zinc release from MT by NO as a necessary step in regulation of MTF-1 responsive genes. By using the zinc specific fluorphore FluoZin3-AM, the release of intracellular zinc after exposure to NO demonstrated a dependence on MT. Furthermore, NO could only regulate expression of MT, ZnT-1, or Zip10 mRNA levels in the presence of MTF-1. While the effects of NO on Zip14 expression are independent of MT, intracellular zinc release, and MTF-1, regulation of *MT*,

*ZnT1*, and Zip10 appear to be dependent upon this metal inducible signaling pathway. Moreover, the novel findings of NO regulation of two additional zinc transporters, *ZnT1* and Zip10, add another layer to the complexity of zinc transport during inflammation.

In studying the effects of NO on Zip10 expression, it became apparent that zinc played a central role in this process. Metal regulation of Zip10 was initially elucidated through development of a liver specific MTF-1 conditional knockout mouse (Wimmer et al., 2005). Unlike the activation of ZnT-1 by MTF-1, Zip10 expression is suppressed by the presence, or Cd induction of MTF-1 (Wimmer et al., 2005). This was the first Zip gene identified as an MTF-1 target, and moreover Zip10 was the first gene repressed by metal induction of MTF-1. Therefore, experiments were designed to determine if zinc alone can regulate Zip10 expression, and the mechanism by which this may occur.

Initially mice were fed either a ZnD or ZnA diet and the mRNA levels for Zip10 were examined in the liver and the brain. Under ZnD conditions, Zip10 mRNA abundance was three-fold greater in both tissues compared to animals fed the ZnA diet. Interestingly, while the magnitude of change in Zip10 expression was similar for both tissues, the overall transcript abundance was approximately 30-fold higher in the brain. These trends were also apparent for ZIP10 protein expression. ZIP10 localized to the plasma membrane of hepatocytes, and specific regions of the brain during zinc restriction. The expression of ZIP10 was significantly upregulated in the striatum, amygdala, caudate nucleus and substantia nigra as well as other regions that correspond to components of the basal ganglia. These regions play a significant role in the control of movement.

While dietary zinc restriction led to an increase in Zip10 expression, supplemental zinc could repress gene expression in vitro. These results showed that Zip10 responds differentially

to zinc availability, but the mechanism was unclear. Initial studies indicated that MTF-1 could bind to a downstream MRE of Zip10 (i.e., +17 relative to the TSS) after Cd exposure causing down-regulation of Zip10 expression (Wimmer, U. et al 2005). Moreover, experiments in zebra fish also showed MTF-1 mediated repression of Zip10 through an intronic MRE (Zheng et al., 2008). Our studies utilizing MTF-1 siRNA support these previous findings, and show that zinc-induced repression of Zip10 is mediated by MTF-1. These results provided only half of the mechanism of Zip10 regulation, because there was still a question of how a transcriptional activator could repress gene expression.

In an effort to further understand zinc-induced repression of Zip10 mediated by MTF-1, ChIP experiments were designed to track the movement of Pol II throughout the gene. By using antibodies directed against Pol II phosphorylated at Ser5 of the CTD (the initiating form), and Pol II phosphorylated at Ser2 of the CTD (elongating form) it was shown that zinc could still induce the recruitment of Pol II to the promoter region of Zip10. However, if the murine hepatocytes were exposed to zinc, the elongating form of Pol II could not be found downstream of the promoter region or MRE. These results suggested that the MTF-1-bound MRE was occluding the forward movement of Pol II. Therefore, a unique mechanism was identified to regulate Zip10 in response to zinc, whereby the primary zinc-induced transcriptional activator, MTF-1, mediated suppression of gene expression by blocking transcription elongation.

## Future Directions

Inflammation and infection are known to be associated with reduced plasma levels of zinc. The decrease in plasma zinc during the acute phase response has been documented in clinical studies. In one study, healthy volunteers administered endotoxin (LPS) had an increase in levels of IL-6 and TNFα, with a concomitant decrease in plasma zinc levels (Gaetke et al., 1997). Children afflicted with acute *falciparum* malaria, had very low levels of baseline plasma zinc

levels and correlated inversely with serum CRP and the degree of parasitemia (Duggan et al., 2005). Patients with burn injuries also present with significantly depressed plasma zinc levels (Boosalis MG et al., 1988), as have children undergoing bone marrow transplantation (Uckan, D. et al., 2003).

The exact mechanisms by which the decrease in plasma zinc occurs are not clear. It has been postulated that zinc is acutely redistributed from the serum to other tissues, particularly the liver, where zinc is required for synthesis of acute phase proteins (Liuzzi et al., 2005). In plasma, zinc is primarily bound to albumin, but the primary functions of zinc are carried out at the intracellular level. Experiments performed in the Cousins' lab demonstrate that ZIP14, a zinc transporter found in the liver, is up-regulated by an increase in IL-6 and NO as a result of the release of IL-1β, contributing to the acute decrease in plasma zinc (Liuzzi et al., 2005; Lichten, et al., 2009). The benefit or detriment of this response, however, is not fully understood. Bacterial pathogens require zinc, which suggests that decreased plasma zinc levels induced by the acute phase response could be protective by limiting zinc availability to bacteria. A temporary decrease of plasma zinc may also limit the cytokine response during inflammation. However, significantly depressed zinc levels have been associated with nonsurvival in patients with septic shock (Wong, et al., 2007).

Therefore, the paramount question to be answered becomes: Is ZIP14 necessary for zinc uptake by the liver during inflammation? Initial studies suggest that ZIP14 is critical for zinc uptake by primary hepatocytes during inflammation (Lichten et al., 2009). Further studies tracking $^{65}Zn$ uptake by hepatocytes that have been transfected with Zip14 siRNA during inflammation could also demonstrate the necessity of ZIP14. However, the only way to determine whether or not ZIP14 is required to decrease plasma zinc during systemic infection/

inflammation would be to create conditional knockout of hepatic Zip14. This mouse model could not only determine if Zip14 is a necessary acute phase gene, but if it is, how not having this transporter may affect the mortality of the mouse during infection. Therefore, if ZIP14 is absent during an infection, and plasma zinc does not decrease, is that detrimental to the mouse? Furthermore, are these findings applicable to humans? Are there certain mutations in hZIP14 that can render the protein non-functional? It would be very interesting to see if a polymorphism in Zip14 could affect its transport function. Could a Zip14 mutation predispose certain populations to an increased incidence of illness? Furthermore, zinc supplementation studies in children of developing countries, where dietary zinc deficiency is relatively common, have shown some positive impact on outcomes such as incidence of pneumonia and diarrhea. One large-scale study by Brooks et al., (2005) of the effect of zinc supplementation on prevention of pneumonia and diarrhea showed that patients in the supplementation group had significantly fewer episodes of pneumonia and diarrhea, as well as a significantly lower risk of mortality. Additionally, zinc supplementation of children with severe pneumonia reduced recovery time (Brooks et al., 2004). An interesting question to be answered in the future would be whether or not a Zip14 polymorphism coupled to low dietary zinc intake during infection confers an even greater mortality risk.

Another interesting aspect of the work presented here is the regulation of mZip10 by inflammation and dietary zinc intake. While Zip14 was positively regulated by NO, Zip10 was repressed. At the cellular level, the mechanisms regulating the response of these two genes to NO were different. How this fits in to integrative whole-body zinc homeostasis remains to be determined. It is clearly possible that a hierarchy of zinc transporter activation may exist, whereby certain transporters are activated or inactivated based on the stimuli presented. In this

case inflammation and infection seem to preferentially activate Zip14, which is abundantly expressed in the liver, allowing uptake of zinc from the plasma. Zip10 on the other hand is expressed to a much greater degree in the brain than the liver, and the stimulus for its activation is zinc deficiency.

According to an analysis of data from the Food and Agricultural Organization, the prevalence of zinc deficiency may be as high as 40% worldwide (Brown et al., 2001). Additionally, it has been estimated that Zn deficiency contributes to 800,000 excess deaths annually among children under 5 y (Claufield et al., 2004a) with pneumonia (406,000), diarrhea (176,000), or malaria (207,000) (Claufield et al., 2004b). Remarkably, the documented Zn deficiency was considered relatively mild, with no clinical signs and plasma/serum Zn concentrations that are, at most, moderately depressed (reviewed in Hambidge and Krebs 2008). These clinical studies emphasize the importance of zinc nutrition to overall health. What is unclear however is the role of the zinc transporters in the pathogenesis of various disease states attributed to zinc deficiency.

Mutations of hZip4 lead to acrodermatitis enteropathica (AE), while homozygous deletion of mZip4 is embryonic lethal (Dufner-Beattie et al., 2007). Heterozygous mutation of mZip4 leads to a hypersensitivity to zinc deficiency. Additionally, homozygous deletion of mZnT1 is also embryonic lethal. However, no mutations casuing this phenotype have been identified in humans (Andrews et al., 2004). In contrast mutant mice lacking either mZip1, mZip2, mZip3, or both mZip1 and mZip3 (Double-KO) show no overt phenotype, and are only mildly sensitive to zinc deficiency. Creation of the triple-KO did reveal an increased sensitive to zinc deficiency (Kambe et al., 2008). These studies suggest that while some transporters are critical (i.e., Zip4 and ZnT1) to zinc homeostasis, others may be compensatory or serve redundant purposes.

Therefore, the next step in studying mZip10 should be the creation of mice homozygous and heterozygous for null mutations in Zip10. How these mice deal with alterations in dietary zinc intake will be fascinating to determine.

Due to the abundant expression of Zip10 in the brain, and specifically the basal root ganglia, it would be completely plausible to assume that a Zip10 knock out mouse would be embryonic lethal due to neurodegeneration. However, if the knock out was not lethal could it still cause severe neurological impairments? The globus pallidus contains a relatively high level of zinc in the basal ganglia, and a high level of zinc is associated with Parkinson's disease. Furthermore, alterations in zinc homeostasis in the brain may be involved in other neurological diseases such as Alzheimer's disease, and amytrophic lateral sclerosis (Cuajuncgo and Lees 1997). Inadequate dietary zinc intake leads to changes in behavior such as reduced activity and responsiveness to stimuli (Shagal 1980; Golub et al., 1995). Moreover, zinc restriction during infancy causes impaired learning behavior (Takeda et al., 2000). These findings suggest that alterations in brain-zinc fluxes could be severely detrimental. Additionally, one could also assume that Zip10 heterozygotes would be extremely sensitve to zinc deficiency, similar to Zip4 heterozygotes. By looking at the broader picture of human zinc deficiency, it is easy to recognize how perturbations in Zip10 function or single nucleotide polymorphisms that cause loss of function, or restricted function may affect brain development or the pathogenesis of neurological disorders.

APPENDIX A
ZINC RESPONSIVENESS OF THE ZIP10 PROMOTER

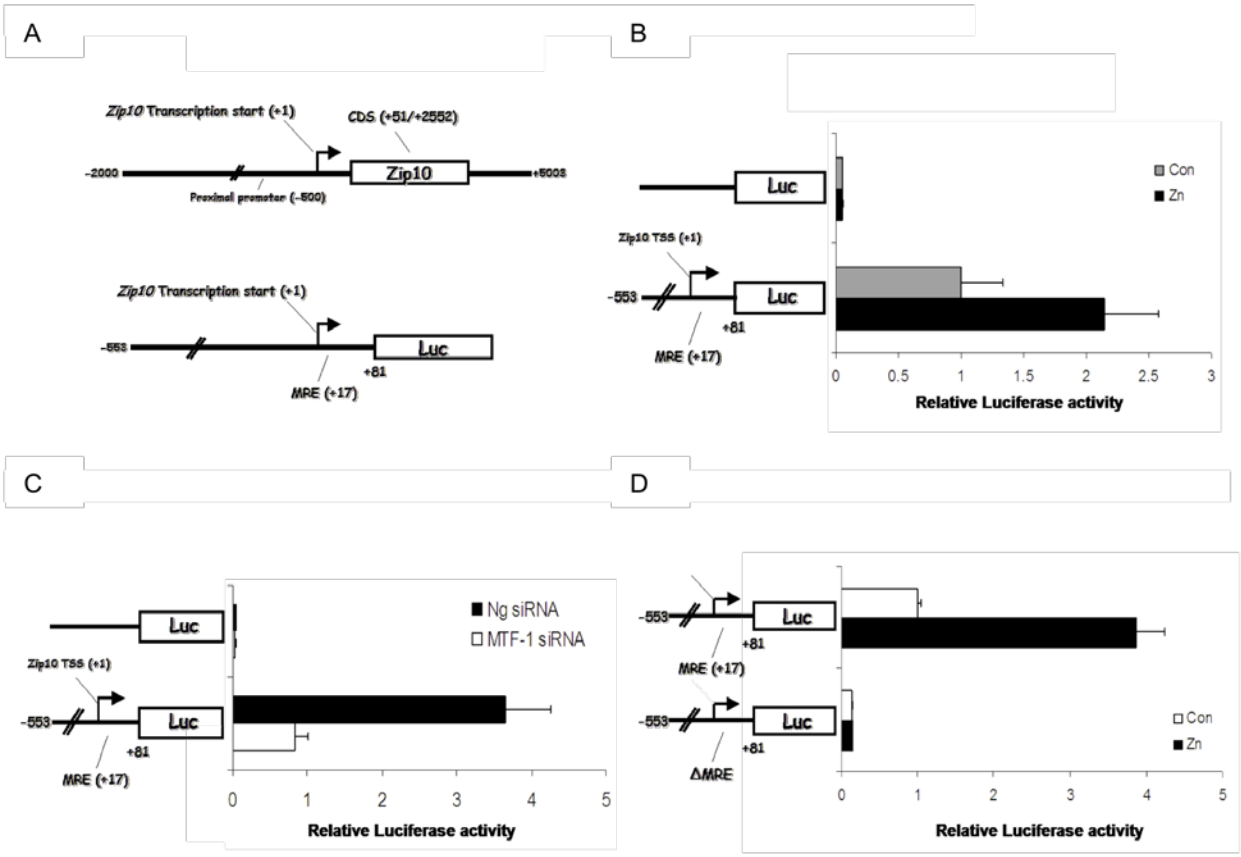


Figure A-1. The Zip10 promoter contains one functional downstream MRE. A. Genomic Zip10 and Zip10 promoter-Luc organization. B. Relative luciferase activity 12 h following 100 μM Zn treatment. C. Co-transfection of MTF-1 siRNA with luciferase constructs. Both the negative siRNA (Ng) and MTF-1 siRNA transfected cells were subsequently treated with 100 μM Zn for 12 h. D. The MRE located at +17 was mutated to contain a StuI restriction site for screening purposes. Both the normal and mutated constructs were then transfected into AML12 hepatocytes and treated with Zn as above.

APPENDIX B
ZIP14 PROMOTER RESPONSIVENESS

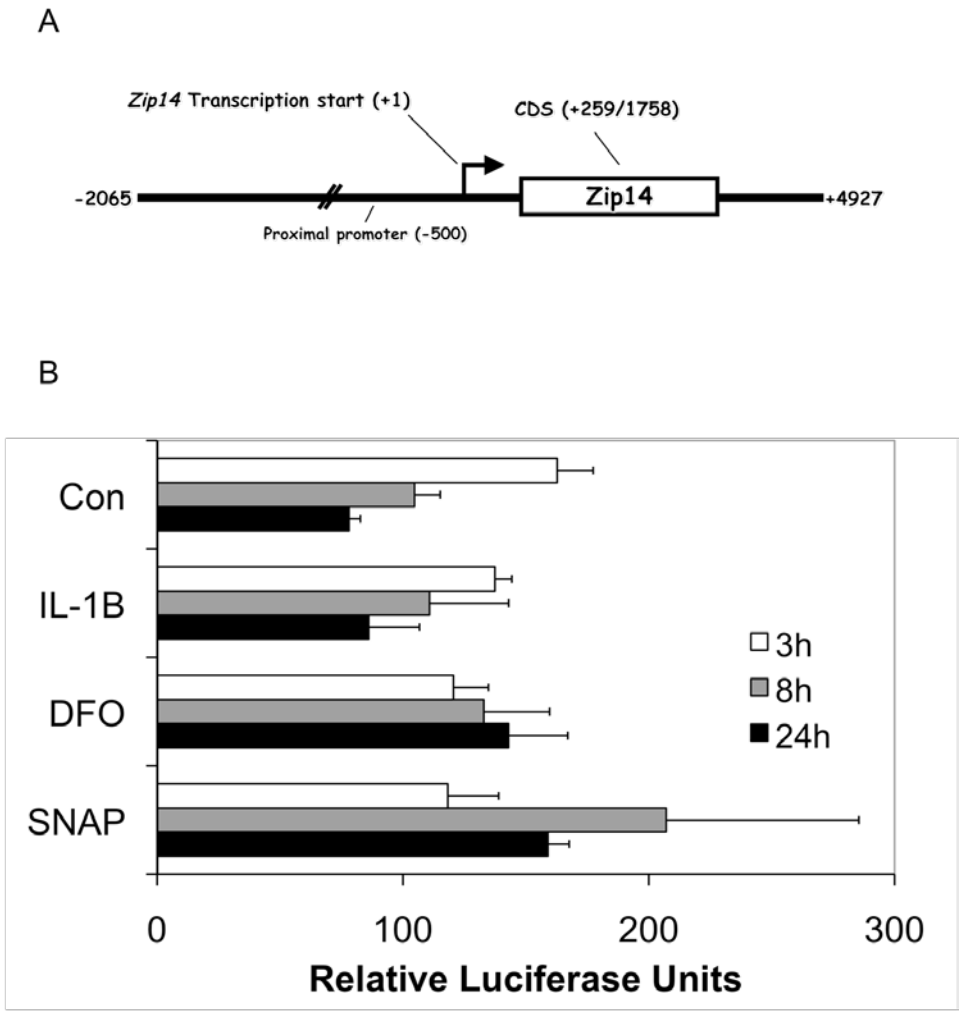


Figure B-1. Zip14 genomic organization and promoter responsiveness. A) Genomic organization of the Zip14 gene, with special attention to the promoter region where -2065 to +100 bp were cloned into the pGL3 vector. B) The Zip14pGL3 plasmid was transfected into AML12 hepatocytes and allowed to incubate for 48 h prior to treatment. The cells were exposed to 50 ng/ ml IL1-β, 50 μM Deferoxamine (DFO), or 100 μM SNAP for the times indicated. Relative luciferase untis were obtained by the normalization of *Firefly* luciferase to a *Renilla* control vector. Data represent the mean ± S.D. of three independet experiments.

APPENDIX C
ZIP14 PROMOTER DELETIONS

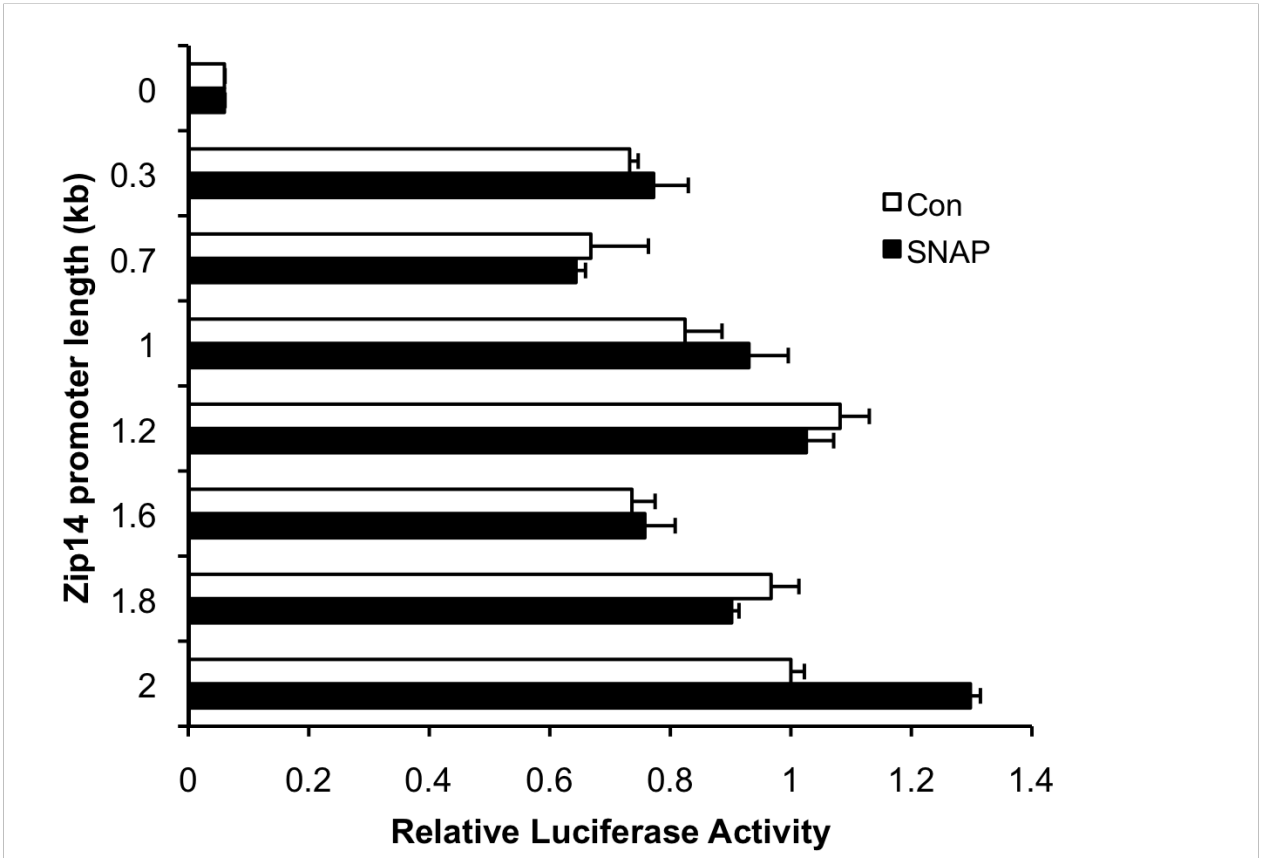


Figure C-1. Response of Zip14 promoter fragments to SNAP. The initial 2.1 Kb Zip14pGL3 promoter construct was digested with ExoIII nuclease to yield promoter fragments of varying sizes. These promoter fragments were then transfected into AML12 cells, and subsequently incubated with SNAP for 24 h. Values given are mean ± S.D. of three independent experiments.

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# BIOGRAPHICAL SKETCH

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