REGULATION AND FUNCTION OF ZINC AND ZINC TRANSPORTERS DURING ER STRESS

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To my family

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometry
ACC	Acetyl-CoA Carboxylase
ACOX1	Acyl-CoA Oxidase
Act D	Actinomycin D
AE	Acrodermatitis Enteropathica
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
APOB	Apolipoprotein B
APOE	Apolipoprotein E
ATF4	Activating Transcription Factor 4
ATF6α	Activating Transcription Factor 6α
BW	Body Weight
C/EBP	CCAAT-enhancer-binding Proteins
cAMP	Cyclic Adenosine Monophosphate
CD36	Cluster of Differentiation 36
ChIP	Chromatin Immunoprecipitation
CHOP	C/EBP-homologous Protein
CHREBP	Carbohydrate-responsive Element-binding Protein
CPT1α	Carnitine Palmitoyltransferase 1α
CRE	cAMP-response Element
CREBH	cAmp-response Element-binding Protein H
d	Days
DNA	Deoxyribonucleic Acid
eIF2α	Eukaryotic Initiation Factor 2α

ER	Endoplasmic Reticulum
ERAD	ER-associated Degradation
FA	Fatty Acid
FABP	Fatty Acid-binding protein
GRP78/BiP	78 kDa Glucose-regulated Protein
GRP94	94 kDa Glucose-regulated Protein
HFD	High Fat Diet
H&E	Haematoxylin and Eosin
hnRNA	Heterogeneous Nuclear RNA
hr	Hours
IRE1	Inositol-requiring Enzyme 1
KO	Knockout
LPS	Lipopolysaccharide
MMP	Matrix Metalloproteinase
MRE	Metal Response Element
mRNA	Messenger RNA
MT	Metallothionein
MTF	MRE-binding Transcription Factor
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NHI	Non-heme Iron
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDI	Protein Disulfide Isomerase
PERK	Double-stranded RNA-activated Protein Kinase-like ER Kinase
PMSF	Phenylmethylsulfonyl Fluoride

PPARα	Peroxisome Proliferator-activated Receptor
PTP1B	Protein Tyrosine Phosphatase 1B
RNA	Ribonucleic Acid
SCD1	Stearoyl-CoA Desaturase-1
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
siRNA	Small Interfering Ribonucleic Acid
SLC	Solute Carrier
TBP	TATA-binding Protein
TdT	Terminal Deoxynucleotidyltransferase
TG	Thapsigargin
TM	Tunicamycin
TPEN	N,N,N,N-tetrakis-(2-pyridyl-methyl)ethylenediamine
TUNEL	TdT-mediated dUTP Nick End Labeling
UPR	Unfolded Protein Response
WAT	White Adipose Tissue
wk	Weeks
WT	Wild Type
XBP1	X-box Binding Protein 1
ZIP	Zrt-, Irt-like Protein
ZnA	Zinc-adequate
ZnD	Zinc-deficient
ZnS	Zinc-supplementation
ZnT	Zinc Tranporter

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REGULATION AND FUNCTION OF ZINC AND ZINC TRANSPORTERS DURING ER STRESS

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Extensive endoplasmic reticulum (ER) stress damages the liver causing apoptosis and steatosis, despite the activation of the unfolded protein response (UPR). Restriction of zinc from cells can induce ER stress, indicating zinc is essential to maintain normal ER function. Zinc transporter ZIP14 (SLC39A14) is abundantly expressed in liver. ZIP14 transports extracellular and organellar zinc into the cytosol of cells. We found ZIP14 expression was highly increased in mouse liver after administration with tunicamycin (TM), a potent ER stress inducer. However, the precise roles of zinc and/or ZIP14 in the UPR are unclear. This project has explored a role for ZIP14 during induced ER stress using *Zip14*$^{-/-}$ (KO) mice, which exhibit impaired hepatic zinc uptake. Major finding of the project is that ZIP14-mediated hepatic zinc uptake is critical for adaptation to ER stress by preventing sustained apoptosis and steatosis. Impaired hepatic zinc uptake in *Zip14* KO mice during ER stress coincides with greater expression of pro-apoptotic proteins in the UPR pathway. In addition, ER stress-induced *Zip14* KO mice show greater levels of hepatic steatosis due to higher expression of genes involved in *de novo* fatty acid synthesis, which are suppressed in ER stress-

induced wild type (WT) mice. During ER stress, the UPR-activated transcription factors, activating transcription factor 4 (ATF4) and activating transcription factor 6α (ATF6α), transcriptionally up-regulate *Zip14* expression. Mechanistically, ZIP14 mediates zinc transport into hepatocytes to inhibit protein-tyrosine phosphatase 1B (PTP1B) activity, which acts to suppress apoptosis and steatosis associated with hepatic ER stress. *Zip14* KO mice show greater hepatic PTP1B activity during ER stress. Furthermore, WT mice were fed different levels of zinc to examine the importance of dietary zinc intake on the adaptation to TM-induced ER stress. Mice fed zinc deficient diet exhibit increased hepatic apoptosis and steatosis during TM-challenge, which coincides with greater PTP1B activity. These results show the importance of zinc trafficking and functional ZIP14 transporter activity for adaptation to ER stress.

CHAPTER 1
INTRODUCTION

ER Stress and the UPR Signaling Pathway

ER Stress and the UPR

The endoplasmic reticulum (ER) is a cellular organelle where appropriate folding, assembly, modification, and trafficking of proteins happens (1). In addition, the ER is a site for calcium storage and lipid biosynthesis. Since the ER is a specialized organelle that synthesizes and secretes proteins and lipids, it must have a fine quality control system to prevent aggregation and accumulation of unfolded or misfolded proteins. Only properly folded proteins are transferred to Golgi, whereas improperly folded proteins are retained in the ER lumen and then degraded through the ER-associated degradation (ERAD) process (2).

The normal ER function can be compromised by pharmacological stimuli such as the exogenous chemicals, tunicamycin (TM) and thapsigargin (TG) (3, 4), and by physiological stimuli such as consumption of a high fat diet (HFD), viral infection, oxidative stress or chronic alcohol consumption (5-9). The perturbed ER function is collectively termed as ER stress. Prolonged ER stress is associated with many diseases and developmental abnormalities (10). In response to ER stress, mammalian cells activate a special pathway known as the unfolded protein response (UPR) to prevent the prolonged stress. The UPR comprises three discrete signaling pathways: activating transcription factor 6 (ATF6) branch, inositol-requiring enzyme 1 (IRE1) branch, and double-stranded RNA-activated protein kinase-like ER kinase (PERK) branch (11). ATF6, IRE1 and PERK are sensors that localize on ER membrane where they monitor ER homoeostasis. During an unstressed state, ER luminal domains of ATF6, IRE1 and

PERK are bound to a ER chaperone, 78 kDa glucose-regulated protein (GRP78/BiP), which acts to prevent activation of these sensors. Upon ER stress, ATF6, IRE1 and PERK are dissociated from GRP78, which cause activation of IRE1 and PERK by transautophosphorylation, and the activation of ATF6 by proteolytic cleavage. These three branches, so called UPR adaptation pathways, operate in concert during ER stress, but utilize unique signal transduction.

These adaptation pathways act to relieve the protein burden of the ER by employing two adaptive mechanisms to return normal ER function (12). First, ATF6 and IRE1 branches enhance the folding capacity of the ER via induction of ER-resident molecular chaperones and folding enzymes including GRP78 and 94 kDa glucose-regulated protein (GRP94) (13-15). GRP78 and GRP94 are constitutively expressed under steady-state in many organs. During ER stress, their expression is highly up-regulated to restore ER homeostasis by playing various roles in protein folding, assembly and degradation (13). Second, the IRE1 and PERK branches reduce the biosynthetic load of the ER by attenuating protein synthesis at both the transcriptional and translational level. At the transcriptional level, IRE1 increases gene expression involved in ERAD thereby facilitating the clearance of unfolded proteins (16). At the translational level, PERK-mediated phosphorylation of eukaryotic initiation factor 2α (eIF2α) induces global attenuation of mRNA translation (17). If ER stress is mitigated and ER homeostasis is reestablished in a timely fashion, cells can survive without further insult.

UPR-Mediated Apoptotic Cell Death

Under chronic or irreversible ER stress conditions, the adaptive pathway of the UPR fails to mitigate ER stress and ER function remains perturbed. UPR signaling will

then instead trigger apoptotic cell death (12). Short-term induction of apoptosis during severe ER stress may be beneficial to the organism as it can prevent the potential for aberrant signaling from damaged cells (14). However, sustained apoptosis from ER stress results in numerous pathological conditions such as hepatic steatosis, neurodegenerative diseases, diabetes, atherosclerosis, renal failure, and obesity (9, 18). Therefore, prevention of ER stress-induced apoptosis has been considered as a therapeutic target for ER stress-associated diseases (19, 20).

Several mediators have been implicated in ER stress-induced apoptosis. One of the major mechanisms of UPR-mediated apoptotic cell death includes sequential steps, beginning with PERK-mediated phosphorylation of eIF2α (21). Although p-eIF2α assists cellular survival during early and reversible ER stress by attenuating global mRNA translation, the protein acts differently during chronic ER stress to induce apoptotic cell death. p-eIF2α selectively enhances the translation of activating transcription factor 4 (ATF4), which then upregulates C/EBP-homologous protein (CHOP), which is a transcription factor that induces expression of apoptosis-associated components. Thus, enhanced and sustained activation of the pro-apoptotic p-eIF2α/ATF4/CHOP pathway is a hallmark of unresolved ER stress.

The transcription factor CHOP is the most extensively studied pro-apoptotic component in the UPR signaling pathway. CHOP has been linked to mechanisms of such as protein phosphatase 1 regulatory subunit 15A (PPP1R15A), Bcl-2-like protein 11 (BCL2L11), Tribbles homolog 3 (TRIB3), and Bcl-2-binding component 3 (BBC3) (22). Studies using *Chop*$^{-/-}$ mice have shown that CHOP is required for ER stress-induced apoptotic cell death under multiple pathological conditions (23-25). *Apoe*$^{-/-}$ mice

are a commonly used animal model of atherosclerosis. *Chop*$^{-/-}$ and *Apoe*$^{-/-}$ double knockout mice display reduced levels of apoptosis and plaque growth compared to normal *Apoe*$^{-/-}$ mice (25). In addition, deletion of *Chop* exhibited a protective effect from ER stress-induced apoptosis in the renal tubular epithelium in mice administered with TM (26). These findings suggest that suppression of the pro-apoptotic p-eIF2α/ATF4/CHOP pathway, especially by lowering CHOP expression, could be a promising therapeutic target to ameliorate chronic ER stress and its associated diseases.

ER Stress in Liver Disease

The liver is a central organ, which plays multiple roles in normal systemic metabolism including carbohydrate metabolism, glycogen storage, biosynthesis of amino acids, plasma protein synthesis and secretion, regulation of lipoprotein secretion, cholesterol biosynthesis, and xenobiotic metabolism (27). Therefore, it is not surprising that hepatocytes are enriched in ER to facilitate those functions. ER stress has been implicated in various liver diseases such as hepatic steatosis, chronic viral hepatitis B and C, alcohol-induced liver injury, hyperhomocysteinemia, and ischemia-reperfusion injury (19). Various factors may contribute to hepatic ER stress, including oxidative stress, hepatic viral infections, metabolic disorders, chronic alcohol consumption, drug abuse, and chemical toxicity (18).

Multiple studies modeling hepatic steatosis have shown that excessive accumulation of triglycerides is highly associated with ER stress (9, 28, 29). TM treatment suppresses gene expression of transcription factors and components involved in lipid synthesis such as *Srebp1c* and *Fasn* (29). Furthermore, ablation of UPR pathway components results in development of hepatic steatosis in liver of mice

administered TM, indicating ER stress and UPR signaling are related to lipid homeostasis. ER stress-induced hepatic steatosis is associated with prolonged expression of CHOP, suggesting apoptotic cell death also influences the disturbed hepatic lipid homeostasis (29, 30). In *ob/ob* mice, GRP78 overexpression reduced ER stress markers, which coincided with reduced levels of hepatic triglyceride and cholesterol contents, and improved insulin sensitivity (28). These data suggest that ER stress is a major contributor to hepatic steatosis.

Mechanistically, major UPR components such as X-box binding protein 1 (XBP1), ATF6, GRP78 and GRP94 may regulate hepatic lipid metabolism. Liver-specific *Xbp1* knockout (KO) mice exhibited significantly decreased level of hepatic lipid production, showing hypocholesterolemia and hypotriglyceridemia (31). During TM challenge, *Atf6* KO mice accumulated a markedly greater level of triglycerides with higher mortality, whereas WT mice could recover from the insults (32). A hepatoprotective role of ER stress-responsive chaperones, GRP78 and GRP94, also has been reported. Liver-specific *Grp78* KO mice displayed fatty liver and exacerbated liver injury in response to alcohol feeding, high fat diet, and toxins, which are well-known mediators of hepatic ER stress (33, 34). Similar to *Grp78*, liver-specific deletion of *Grp94* in mice led to focal steatosis and liver injury (35). Furthermore, the KO mice showed hyperproliferation of liver progenitor cells, and subsequent accelerated development of liver tumors. Collectively, these studies suggest that functional UPR activation is essential to overcome ER stress and to prevent further hepatic damage.

Zinc and Zinc Transporters

Zinc and Its Physiologic Functions

Zinc was first established as an essential mineral in plants in 1869, and in experimental animals in 1934 (36). Essentiality of zinc in humans was recognized in 1961 when the observation was made in the middle eastern dwarfs who were severely zinc-deficient (37). Total body zinc is normally between 1.5 – 2.5 g in adult human, and is ubiquitously distributed in tissues. In particular, skeletal muscle, bone, skin, and liver contain a great proportion of it. About 95% of zinc is located in the intracellular space, especially in the cytosolic vesicles. Free zinc exists at very low concentrations inside of the cell. Abundant sources of dietary zinc include oysters, organ meats, and flesh of mammals, crustaceans, and fish (38). Although severe zinc deficiency is rare, nearly half of the world population is reported to be at risk of a marginal zinc deficiency (39). Typical symptoms of severe zinc deficiency are growth retardation, hypogonadism, delayed sexual maturation, dermatitis, diarrhea and intestinal inflammation (40). In experimental settings, zinc-deficient animals and humans have shown decreased cell proliferation, tissue damage, stress intolerance, impaired development, and immune deficiency (41-43).

It is now well established that zinc is required for normal cellular functions, as it has catalytic, structural, and regulatory roles (44). Zinc does not exhibit redox chemistry, unlike iron and copper, which allows the metal to be involved in various physiological events without risk of oxidative damage. Zinc serves a catalytic role in more than 50 zinc metalloenzymes, including tissue-nonspecific alkaline phosphatase (45). The structural function for zinc is illustrated in zinc finger proteins, which have zinc-binding motifs (46). These cysteine- and histidine-containing motifs require zinc to produce a

tetrahedral complex. Removal of zinc from zinc finger proteins causes loss of function due to misfolded structures. In addition, zinc can regulate gene expression and signal transduction. The regulatory role of zinc was first identified when it was shown to display a regulatory mechanism for metallothionein (MT) gene expression (47). The metal response element (MRE)-binding transcription factor (MTF) is a zinc-dependent transcription factor that is influenced by zinc status. Zinc has been shown to modulate activity of a number of kinases and phosphatases through which the metal controls various signaling pathways (48).

Mammalian Zinc Transporters

To maintain zinc homeostasis, mammalian cells use 24 known zinc transporters that tightly control the trafficking of zinc in and out of cells and subcellular organelles. These transporters are within two families: ZnT (Zinc Tranporter; SLC30) and ZIP (Zrt-, Irt-like protein; SLC39) (49-51). The ZnT family transports zinc from the cytoplasm to the extracellular space or organelles, thereby reducing intracellular zinc levels. On the other hand, the ZIP family transports zinc into cells from the extracellular space or organelles to the cytoplasm in order to increase intracellular zinc levels.

The expression of mammalian zinc transporters is regulated both transcriptionally and posttranscriptionally. Some of the transporter genes, such as *ZnT1* and *Zip10,* are regulated by MTF-1, a zinc-responsive transcription factor, by which cellular zinc availability transcriptionally controls expression (52, 53). During high zinc status, MTF-1 induces the expression of *ZnT1*, whereas it suppresses the expression of *Zip10*, which acts as an important mechanism for cellular zinc homeostasis. On the other hand, expression of *Zip4,* which is a major intestinal zinc transporter that import zinc at apical membrane, is controlled at both the transcriptional and posttranscriptional level (54, 55).

It has been shown that *Zip4* mRNA is stabilized during zinc deficiency, which leads to enhanced level of ZIP4-mediated zinc uptake. On the contrary, *Zip4* mRNA is destabilized and degraded during high zinc conditions in order to prevent excessive zinc accumulation. Zinc deficiency also induces the transcription factor KLF4 which in turn regulates *Zip4* (56). A number of physiological stimuli including cytokines and hormones have also been shown to regulate the expression of these transporters. For example, treatment of lipopolysaccharide (LPS) altered expression of many zinc transporters in dendritic cells, thereby reducing intracellular zinc (57). Similarly in mouse liver, administration of LPS or turpentine altered gene expression of multiple zinc transporters including *Zip14*, thereby contributing to hypozincemia during the acute-phase response (58).

Ablation of some of these zinc transporters results in zinc dyshomeostasis and numerous metabolic defects. One well-known example is the mutation of *Zip4* in humans which causes acrodermatitis enteropathica (AE) (59). AE is characterized by dermatitis, alopecia, and diarrhea caused by systemic zinc deficiency due to lack of a functional ZIP4. Targeted deletion of *Zip1*, *Zip2* and *Zip3* produces a phenotype that is more sensitive to dietary zinc deficiency during pregnancy, as shown in mouse models (60, 61). Mutation of *Zip13* was found in the human Ehlers-Danlos syndrome, of which symptoms include impaired joints and aberrant scar formation (62). Studies using *Zip13* KO mice have demonstrated that mutation of *Zip13* results in zinc deficiency in the ER due to zinc trapping in vesicular stores (63).

Function and Regulation of ZIP14

ZIP14, a member of the LZT (LIV-1 subfamily of ZIP zinc transporters) subfamily of zinc transporters, is a metal transporter encoded by *Slc39a14* gene (64). ZIP14

contains multiple transmembrane domains, localizing at the plasma membrane and in endosomes where the transporter transports zinc into the cytoplasm (65, 66). In human tissues, the expression of ZIP14 is shown to be most abundant in liver, and is also significant in intestine, pancreas, and heart (64). ZIP14 was initially well-characterized as a zinc transporter, but further studies have shown that it also transports non-transferrin-bound iron, manganese and cadmium under certain circumstances (65, 67, 68), although ZIP14 transport affinities may differ among these metals.

ZIP14 is responsive to inflammation. Administration of LPS or turpentine increased *Zip14* gene expression in mouse liver (58). Interleukin-6 (IL-6), a pro-inflammatory cytokine, is involved in the ZIP14 upregulation since the event was not observed in mice lacking IL-6. Of note is that the IL-6-dependent upregulation of hepatic ZIP14 expression is an important event for inflammation-induced hypozincemia, which is potentially associated with host defense by reducing available serum zinc (69). Additionally, interleukin-1β (IL-1β) and nitric oxide, mediators of inflammation, are also involved in ZIP14 expression in murine hepatocytes (70). ZIP14 expression was also elevated in response to LPS treatment in cultured sheep pulmonary cells (71). Development of the *Zip14* KO mouse model further revealed the physiological role of ZIP14. Phenotypically, *Zip14* KO mice exhibited low grade chronic inflammation (metabolic endotoxemia) due to impaired gut barrier function (72). The genotype also displays enlarged pancreatic islets with hyperinsulinemia and greater body fat (73), which is a general feature of type 2 diabetes and obesity. In adipocytes, ablation of *Zip14* resulted in increased cytokine production, serum leptin level, hypertrophied adipocytes, and dampened insulin signaling (74). Of importance is that ZIP14 is

essential for hepatic zinc uptake as *Zip14* KO mice showed impaired hepatic zinc uptake (73, 75), indicating the transporter serves as a major hepatic zinc channel. ZIP14-mediated zinc uptake was shown to be critical during liver regeneration and hepatocyte proliferation (75).

ER Stress, Zinc and Zinc Transporters

The ER is an important organelle for cellular zinc metabolism as it is an intracellular zinc storage site (76). Zinc is an essential cofactor for numerous proteins including metalloenzymes and transcription factors (77). Since these proteins acquire zinc at an early period in the secretory pathway, it is not surprising that normal zinc homeostasis is required for maintaining ER function. At the same time, requirement for zinc in normal ER function highlights the importance of functional zinc transporters in delivering zinc to the organelle.

Disturbed zinc homeostasis and zinc transporter activities have been implicated in ER stress and UPR activation. In *Saccharomyces cerevisiae*, zinc depletion using limited zinc medium induced UPR activation, indicating zinc is required for ER function (78). Similarly, treatment with N,N,N,N-tetrakis-(2-pyridyl-methyl)ethylenediamine (TPEN), a potent zinc chelator, induced UPR activation in some mammalian cell lines such as HeLa and HepG2 cells (78, 79). A rat model of alcoholic liver disease created by zinc deficiency was shown to trigger ER stress-induced apoptosis, indicating the importance of zinc for adaptation to ER stress (80).

Since zinc homeostasis is maintained by zinc transporters, these proteins have been associated with ER stress and UPR. Administration of TM altered expression of numerous zinc transporter genes in mouse liver during induction of ER stress (79). These included *ZnT1, ZnT3, ZnT5, ZnT7, ZnT10* among the ZnT family, and *Zip1*, *Zip3*,

Zip4, *Zip5*, *Zip6*, *Zip13* and *Zip14* among the ZIP family. Indeed, ablation of zinc transporters was shown to influence ER stress and UPR activation. Knockdown of *ZnT5* and *ZnT7* in HeLa cells resulted in exacerbated ER stress (81). ZnT5 and ZnT7 are localized in the early secretory compartments of the ER. Therefore, this indicates the importance of zinc transport into the ER to maintain ER function. Similar to ZnT5 and ZnT7, ZIP7 is an intracellular zinc transporter that localizes to the ER (50). In mice lacking *Zip7*, intestinal epithelium showed induction of ER stress in proliferative progenitor cells, resulting in massive level of apoptosis (82). Mutation in *Zip13* resulted in cellular zinc deficiency due to zinc trapping in vesicles, and the event was associated with a significant induction of ER stress (63). In the human neuroblastoma cell line, SH-SY5Y, ZnT3 displayed a protective role during ER stress (83). In response to TM challenge, cells with knockdown of *ZnT3* showed less cell viability due to greater ER stress compared to control cells. Of note is that *ZnT3* was most highly up-regulated transporter among ZnT family following TM administration in mouse liver (79). Among ZIP family, *Zip14* was most highly up-regulated transporter, suggesting a potential role for ZIP14 during ER stress considering the protein's high abundance in liver. However, no studies have been conducted to show a role for ZIP14 during ER stress.

PTP1B, ER Stress and Zinc

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed protein phosphatase that is involved in multiple signaling pathways (84). PTP1B is localized to the ER. At the ER, the catalytic site of PTP1B is exposed to the cytoplasm, although the protein can be cleaved and released from the ER in response to certain stimuli (85). PTP1B can dephosphorylate multiple receptor tyrosine kinases including the insulin receptor, epidermal growth factor receptor, and insulin-like growth factor 1 receptor.

These allow PTP1B to be implicated in many human diseases such as cancer and metabolic syndromes (86, 87).

PTP1B has been implicated in ER stress and UPR signaling. It was first characterized using embryonic fibroblasts isolated from *Ptp1b* KO mice, where lack of PTP1B resulted in decreased levels of ER stress-induced apoptosis and UPR activation in response to pharmacological ER stress inducers (88). The study indicated that PTP1B was involved in the IRE-1 branch of UPR signaling via an unclear mechanism. In liver tissue, PTP1B may play a significant role during ER stress since PTP1B expression was markedly increased in livers of mice under chronic ER stress induced by high fat diet feeding and genetic obesity (89, 90), and in hepatic cells treated with pro-inflammatory cytokines and free fatty acids to induce ER stress (91, 92). Using liver-specific *Ptp1b* KO mouse, it was demonstrated *in vivo* that PTP1B is a critical mediator of hepatic ER stress and UPR signaling as the liver-specific *Ptp1b* KO mice exhibited significantly reduced level of ER stress compared to wild-type mice (93). In another study, the liver-specific *Ptp1b* KO mouse showed decreased expression of phosphorylated eIF2α, ATF4, and CHOP, which are pro-apoptotic pathway components of UPR as well as reduced activation of IRE1 and its down-stream effectors (91). Collectively, these data indicate that ablation of PTP1B can influence multiple branches of UPR pathway, potentially ameliorating ER stress at least in the liver tissue. This raises a possibility that PTP1B inhibitors may be considered as a therapeutic target in the treatment or prevention of ER stress-related diseases (94).

In this context, of note is that zinc is a well-known inhibitor of many phosphatases including PTP1B (95). Indeed, it has been demonstrated that zinc binds to PTP1B to

inhibit its activity (96), and this was supported by increased PTP1B activity in a setting of impaired zinc uptake caused by *Zip14* knockdown in hepatocytes (75). However, whether zinc acts to inhibit PTP1B activity in a model of ER stress has not yet been studied.

Study Aims

In conclusion, observations discussed above led to the hypothesis that zinc and functional zinc transporter activity are required for adaptation to ER stress. To investigate this hypothesis, four specific aims were set in this dissertation project:

1. Determination of changes in zinc metabolism and zinc transporter expression during ER stress.
2. Delineation of a specific role of ZIP14 during ER stress.
3. Identification of the transcription factor(s) that regulate *Zip14* expression during ER stress.
4. Determination of the impact of zinc deficiency on ER stress *in vivo*.

CHAPTER 2
MATERIALS AND METHODS

Mice and Diets

Development and characterization of the murine *Zip14* KO mice were previously described (73). A colony of Zip14$^{+/-}$ heterozygotes on the C57BL/6/129S5 background was used to produce KO and *Zip14*$^{+/+}$ (WT) mice. Young adult (8 - 16 wk of age) male WT and KO mice were used throughout these studies. The same response to treatments was seen in female mice, but only males were used for the experiments reported here. Mice had free access to a chow diet (Harlan Teklad 7912) and tap water, and were maintained with a 12 hr light-dark cycle. To model high fat diet-induced ER stress, WT and *Zip14* KO mice were fed either the chow diet (17 kcal% fat) containing 63 mg zinc/kg or a high fat diet (HFD; 60 kcal% fat, Research Diets, New Brunswick, NJ; D12492) containing 39 mg zinc/kg for 16 wk. For the dietary zinc manipulation study, mice were given a 5 day acclimation period, then were fed egg white-based purified diets (AIN-76A) that contained <1 mg Zn/kg diet (zinc deficient diet; ZnD), 30 mg Zn/kg diet (zinc adequate diet; ZnA), or 180 mg Zn/kg diet (zinc supplementation diet; ZnS) for 2 wk with free access to deionized drinking water. Other compositions among the three purified diets were identical except zinc contents (97). All purified diets were purchased from Research Diets (New Brunswick, NJ). For controlled zinc intake experiments, each mouse was maintained individually in a shoebox cage with a wire mesh floor to prevent zinc recycling through an intake of excreted feces or urine. Mice were anesthetized by isofluorane inhalation prior to injections and euthanasia by cardiac puncture. All research protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Treatments

ER stress was induced by intraperitoneal administration of TM (Sigma, St. Louis, MO) or TG (Sigma) dissolved in 1% DMSO/150 mM glucose at 2 mg/kg bw or 1 mg/kg bw, respectively. All mice were sacrificed between 9 AM and 10 AM. Collected tissues were snap frozen in liquid nitrogen and stored at -80°C.

Biochemical Analyses

Tissue and serum zinc concentrations were measured using flame atomic absorption spectrophotometry (AAS) as described previously (73). Hepatic non-heme iron (NHI) concentrations were analyzed colorimetrically (98). Serum alanine aminotransferase (ALT) activity was measured using a colorimetric end point assay (75). Liver triglycerides were measured using a colorimetric assay (BioVision Research, Mountain View, CA) according to the manufacturer's instructions. In some experiments, to assess zinc absorption and tissue distribution, ^{65}Zn (2 µCi, Perkin Elmer, Waltham, MA) was given to mice by oral gavage 3 hr prior to sacrifice. Accumulated ^{65}Zn in tissue and plasma was measured by gamma scintillation spectrometry.

Histological Analysis

Liver tissues were fixed in 10% formalin in phosphate-buffered saline (PBS), embedded in paraffin, and sectioned to 4 µm in thickness. For histological analysis, the sections were stained with hematoxylin and eosin. Apoptotic cells in the liver were detected by terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay by using a In Situ Apoptosis Detection Kit (Abcam, Cambridge, MA) according to the manufacturer's instructions. Briefly, liver sections were rehydrated with xylene and ethanol, then incubated with proteinase K for 20 min at room temperature. This was followed by incubation with TdT labeling reaction mix for 90 min

in a humidified chamber, where TdT binds to exposed 3'-OH ends of DNA fragments generated during apoptosis and catalyzes the attachment of biotin-labeled deoxynucleotides. The biotinylated nucleotides were detected via a streptavidin-horseradish peroxidase conjugate, which produces a brown substrate with an addition of diaminobenzidine.

PTP1B Assay

PTP1B activity was measured as described previously (75) with slight modifications. Briefly, total lysates were obtained by homogenization of tissues using a HEPES buffer supplemented with protease inhibitor cocktail (Thermo Fisher Scientific) and Bullet Blender (Next Advance). After homogenization and centrifugation, protein lysates were incubated with PTP1B substrate (ELEF-pY-MDYE-NH2) (AnaSpec, Fremont, CA) dissolved in 20mM HEPES buffer for 30 min at 30°C. Sodium orthovanadate, a nonspecific phosphatase inhibitor (Sigma), and 3-(3,5-dibromo-4-hydroxybenzoyl)-2-ethyl-N-[4-[(2-thiazolylamino)sulfonyl]phenyl]-6-benzofuransulfonamiade, a PTP1B-specific inhibitor (Calbiochem, San Diego, CA), were used for positive controls of phosphatase activity inhibition. Released inorganic phosphate levels were measured using a colorimetric phosphate assay (Biovision, Mountain View, CA). Assays were normalized to total protein concentration using the bicinchoninic acid assay (BioRad, Richmond, CA).

Cell Culture and siRNA Knockdown

The human hepatocellular carcinoma cell line HepG2 (ATCC, Manassas, VA) was maintained in Dulbecco's Modification of Eagle's Medium (Corning, NY) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma). Cells were maintained at 37°C in 5% CO_2. HiPerFect transfection reagent (Qiagen,

Valencia, CA) was used to transfect siRNA for human *Zip14* (Thermo Fisher 4392420), *Atf4* (Qiagen SI03018345), *Atf6* (Ambion 115887), *Ptp1b* (Cell Signaling 13348) or negative control siRNA (Dharmacon, Pittsburgh, PA) into cells at a final concentration of 5 nM according to the manufacturer's instructions. The efficiency of knockdown was detected using qPCR and immunoblotting. The transfected cells were treated with TM (1 µg/ml, unless specifically indicated) or vehicle (DMSO) to induce ER stress. In zinc supplementation experiments, zinc acetate (Sigma; 2.5 - 20 µM) and pyrithione (2-Mercaptopyridine N-oxide sodium salt) (Sigma; 50 µM) were added to the culture medium for 30 min.

Western Blotting

Tissue samples or cells were homogenized in RIPA lysis buffer

Santa Cruz, Dallas, TX) supplemented with protease and phosphatase inhibitors (Thermo Fisher, San Jose, CA) using Bullet Blender (Next Advance, Averill, NY) or a sonicator (Thermo Fisher). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. A polyclonal rabbit antibody against ZIP14 was raised in-house as described previously (58). Purchased antibodies were GRP78, CHOP, phosphorylated eIF2α (Ser^{52}) (Santa Cruz Biotechnology, Santa Cruz, CA), ATF4, GRP94, PTP1B (Cell Signaling, Boston, MA), ATF6 (Novus Bio, Littleton, CO) and Tubulin (Abcam, Cambridge, MA). Immunoreactivity was visualized using enhanced chemiluminescence reagents (Thermo Fisher).

Quantitative Real-Time PCR (qPCR)

Total RNA from tissue samples or cells was isolated using TRIzol reagent (Ambion, Austin, TX) and then homogenized using the Bullet Blender (Next Advance). Isolated RNA was treated with Turbo DNA-free reagent (Ambion) to prevent DNA

contamination. To determine mRNA expression, qPCR was performed using EXPRESS One-Step Superscript Mix (Invitrogen, Carlsbad, CA). Amplification values were normalized to a value of TATA-binding protein (TBP) mRNA. The primer/probe sequences of genes involved in ER stress and lipid homeostasis are provided in Table 2-1. The primer/probe sequences for zinc transporters are provided in Table 2-2 and 2-3. In experiments detecting the transcriptional activity of *Zip14*, primers spanning exon 5 and intron 5 junction of *Zip14* were designed to measure unspliced heterogeneous nuclear RNA (hnRNA). The hnRNA was quantified by qPCR using SYBR Green (Applied Biosystems). The primer sequences used are provided in Table 2-4. The reaction conditions for PCR were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec, and a final cycle at 60°C for 1 min. Melting curves were obtained after PCR to ensure only a single product was amplified.

MTT Assay

The MTT assay in HepG2 cells was performed using the MTT Cell Proliferation Assay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's manual.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as described previously (99) with slight modifications. Briefly, TM or vehicle-treated HepG2 cells were cross-linked with 1.1% (v/v) formaldehyde for 10 min, followed by addition of 0.125 M glycine to stop cross-linking. Cells were lysed with nuclei swelling buffer (5 mM PIPES, pH 8.0, 85 mM KCL, 0.5% NP-40), centrifuged at 6000 xg for 5 min at 4°C, and the supernatant was discarded. The pellet (nuclei) was re-suspended in RIPA buffer followed by sonication using a BioRuptor® (Diagenode, Liege, Belgium) for 15 min (15 cycles of 30 sec on and 30 sec off on high power) to produce 200 to 500-bp DNA fragments. DNA fragment size

after sonication was ensured by electrophoresis using a 1.6% agarose gel. DNA was immunoprecipitated with ChIP-grade ATF6α antibody (Novus Bio) or ATF4 antibody (Cell Signaling). Thereafter, cross-links between protein/DNA complex were removed by incubating with 5M NaCl. DNA was analyzed by qPCR with primers designed to detect the *Zip14* promoter binding site spanning potential ATF6α or ATF4 binding sites or another downstream region that did not include the putative binding sites. The *Zip14* promoter primers used are provided in the Table 2-4.

Table 2-1. Primers and probes for ER stress- and lipid metabolism-associated genes

Gene		Sequence (5’ to 3’)
Grp78	Forward	TTCTGCCATGGTTCTCACTAAA
	Reverse	TTGTCGCTGGGCATCATT
	Probe	FAM-AGACTGCTGAGGCGTATTTGGGAA-BHQ1
Chop	Forward	CAGCGACAGAGCCAGAATAA
	Reverse	CAGGTGTGGTGGTGTATGAA
	Probe	FAM-TGAGGAGAGAGTGTTCCAGAAGGAAGT-BHQ1
Chrebp	Forward	CTGGGGACCTAAACAGGAGC
	Reverse	GAAGCCACCCTATAGCTCCC
Acc	Forward	TGACAGACTGATCGCAGAGAAAG
	Reverse	TGGAGAGCCCCACACACA
Scd1	Forward	CCGGAGACCCCTTAGATCGA
	Reverse	TAGCCTGTAAAAGATTTCTGCAAACC
Cd36	Forward	TGGAGCTGTTATTGGTGCAG
	Reverse	TGGGTTTTGCACATCAAAGA
Fabp	Forward	GCTGCGGCTGCTGTATGA
	Reverse	CACCGGCCTTCTCCATGA
Pparα	Forward	CTGCAGAGCAACCATCCAGAT
	Reverse	GCCGAAGGTCCACCATTTT
Cpt1α	Forward	TGGCATCATCACTGGTGTGTT
	Reverse	GTCTAGGGTCCGATTGATCTTTG
Acox1	Forward	GCCCAACTGTGACTTCCATC
	Reverse	GCCAGGACTATCGCATGATT
Apoe	Forward	GCTGGGTGCAGACGCTTT
	Reverse	TGCCGTCAGTTCTTGTGTGACT
Apob	Forward	CGTGGGCTCCAGCATTCTA
	Reverse	TCACCAGTCATTTCTGCCTTTG
Tbp	Forward	TCTGCGGTCGCGTCATT
	Reverse	GGGTTATCTTCACACACCATGAAA
	Probe	FAM-TCTCCGCAGTGCCCAGCATCA-BHQ1

Table 2-2. Primers and probes for ZIP transporter genes

Gene		Sequence (5' to 3')
Zip1	Forward	TCCTCAAGGTCATTCTGCTCCTA
	Reverse	CCCTTTCTCTTGAAGCACCTTAGA
	Probe	FAM-CTGCTCACTGGCCTTCTCTTTGTCCAA-BHQ1
Zip2	Forward	CTGCTTGCTCTTCTGGTTCTCA
	Reverse	GACCTGTAGCTGCATCCATCTG
	Probe	FAM-ACTGGGCTGTGGCCTTACTCCCATCTAC-BHQ1
Zip3	Forward	CTGGGCTACGCCGTTCTG
	Reverse	GGGACGTGCTCTGTGTCCTT
	Probe	FAM-CTTTCTCAAGTGGTGAGCCCTGAATCCC-BHQ1
Zip4	Forward	CTCTGCAGCTGGCACCAA
	Reverse	CACCAAGTCTGAACGAGAGCTTT
	Probe	FAM-CAATCTCCGACAGTCCAAACAGACCCAT-BHQ1
Zip5	Forward	GGGCAGCCTCATGTTTACCA
	Reverse	CCACATCAGCCGTCAGGAA
	Probe	FAM-CCCTATTGGAGGAGCAGCTAGTGCCC-BHQ1
Zip6	Forward	GCCACAGCCAGCGCTACT
	Reverse	ATCACCATCCAGGCCAATGT
	Probe	FAM-CGGCGTCCTTCAGCTCCTCTCGA-BHQ1
Zip7	Forward	AGGCATCAAACACCACCTGG
	Reverse	TGCGGAGATCAGCACTGTG
	Probe	FAM-CTGTCACCCTCTGGGCCTACGCACT-BHQ1
Zip8	Forward	CTAACGGACACATCCACTTCGA
	Reverse	CCCTTCAGACAGGTACATGAGCTT
	Probe	FAM-ACTGTCAGCGTTGTATCCCTCCAGGATG-BHQ1
Zip9	Forward	AAATTCCCGTTTGCTTGGAA
	Reverse	CAGTTTCGAAAGGCGCTTAGG
	Probe	FAM-ACCACGCGTTTAAACA-BHQ1
Zip10	Forward	CGGCAGTCGGTCAGTATGC
	Reverse	AACATGCCGGCAGTGATTG
	Probe	FAM-AACAACATCACACTCTGGAT-BHQ1
Zip11	Forward	CACTGAGTGGAAGGCATCTTTCT
	Reverse	TGAGGTGTTGAAGTTGAGTCTAGTGA
	Probe	FAM-TCGAGGCTAACCCCTACTTGTCCCACC-BHQ1
Zip12	Forward	GGTTGTAAATTTGTCCTGCATGAA
	Reverse	TTGGGCTTGGGTTGTGTTG
	Probe	FAM-CCTCCCATTCACCC-BHQ1
Zip13	Forward	AGGAATGTGAACTGGAAGAATGC
	Reverse	GGTGTCAGCCAAGGGAAATAGT
	Probe	FAM-AAGCCATAATCCCC-BHQ1
Zip14	Forward	GTAAACCTTGAGCTGCACATTAGC
	Reverse	TGCAGCCGCTTCATGGT
	Probe	FAM-TGGCCTCACCATCCTGGTATCCGT-BHQ1

Table 2-3. Primers and probes for ZnT transporter genes

Gene		Sequence (5’ to 3’)
ZnT1	Forward	CACGACTTACCCATTGCTCAAG
	Reverse	CTTTCACCAAGTGTTTGATATCGATT
	Probe	AGTCTGCTCTCATTCTTCTACAAACTGTCCCTAAGC
ZnT2	Forward	CCGACCAGCCACCAAGAC
	Reverse	TGGAAAGCACGGACAACAAG
	Probe	FAM-CGGCTCGATGCCAGCCGAA-BHQ1
ZnT3	Forward	GGTGGTTGGTGGGTATTTAGCA
	Reverse	CAAGTGGGCGGCATCAGT
	Probe	FAM-ACAGCTTGGCCATCAT-BHQ1
ZnT4	Forward	GCTGAAGCAGAGGAAGGTGAA
	Reverse	TCTCCGATCATGAAAAGCAAGTAG
	Probe	FAM-CAGGCTGACCATCGCTGCCGT-BHQ1
ZnT5	Forward	CTGCTCGGCTTTGGTCATG
	Reverse	CGGCCATACCCATAGGAGGA
	Probe	FAM-TTTGCTGCCCTGATGAGCCGC-BHQ1
ZnT6	Forward	TCCCAGGACTCAGCAGTATCTTC
	Reverse	GCCCCAGCAAGATCAATCAG
	Probe	FAM-TGCCCCGCATGAATCCGTTTG-BHQ1
ZnT7	Forward	CCTCTCTTTCGCTTTTGTGGAA
	Reverse	GTGGAAGGAGTCGGAGATCAAG
	Probe	FAM-ACTCTACGGCATCTGGAGCAACTGCCT-BHQ1
ZnT8	Forward	TGGGTGGTATCGAGCAGAGAT
	Reverse	ACACCAGTCACCACCCAGATG
	Probe	FAM-TCGGTGCCCTGCTGTCTGTCCTT-BHQ1
ZnT9	Forward	GCACTGGGCATCAGCAAAT
	Reverse	GAAAAGCCGTACGGGTGAGA
	Probe	FAM-TGTTCAAACACCAGATCC-BHQ1
ZnT10	Forward	GCACTGGGCATCAGCAAAT
	Reverse	GAAAAGCCGTACGGGTGAGA
	Probe	FAM-CTCTGAACTGGAGTGAGC-BHQ1

Table 2-4. *Zip14* primers used for hnRNA detection and ChIP-PCR

Gene		Sequence (5’ to 3’)
Zip14 (hnRNA)	Forward	TCCAAGTCTGCAGTGGTGTT
	Reverse	ACAATTGGGCCTCACCCAT
Zip14 (ChIP)	Forward	TTCCGGAGGCAGGAGGA
	Reverse	CAGCTTAGCCGGTGCGT

CHAPTER 3
DETERMINATION OF CHANGES IN ZINC METABOLISM AND ZINC TRANSPORTER EXPRESSION DURING ER STRESS

Introductory Remarks

Zinc is an essential mineral required for normal cellular functions (100). To maintain zinc homeostasis, mammalian cells use 24 known zinc transporters that tightly control the trafficking of zinc in and out of cells and subcellular organelles. These transporters are within two families: ZnT and ZIP (51). A number of physiological stimuli have been shown to regulate the expression and the function of these transporters. Ablation of some of these transporters results in zinc dyshomeostasis and numerous metabolic defects. Zinc and zinc transporters have been implicated in ER stress and UPR activation. Zinc deficiency may induce or exacerbate ER stress and apoptosis. In yeast and some mammalian cells, the UPR was activated by zinc restriction (78, 79). A number of zinc transporters also have been associated with ER stress and the UPR. Administration of TM altered expression of numerous zinc transporter genes in mouse liver including *ZnT3*, *ZnT5*, *ZnT7*, *Zip13* and *Zip14* (79). Overall, these previous studies suggest that ER stress alters zinc homeostasis, and that disturbed zinc homeostasis may cause ER stress. However, those studies were mostly conducted in *in vitro* experimental setting. Furthermore, Homma et al. examined expression of zinc transporters at the transcript level in mouse liver, but did not determine actual zinc concentrations in liver tissue and did not examine protein expression of zinc transporters during ER stress (79). Thus, it remains uncertain whether ER stress changes zinc metabolism. As use of TM is an extreme experimental model to induce ER stress, examination of zinc metabolism in response to physiologically relevant ER stress model such as HFD feeding is required.

Therefore, the purpose of the research reported in this chapter is to determine changes in zinc metabolism and zinc transporter expression during ER stress. This focus will include the changes in zinc homeostasis following pharmacologically- and HFD-induced ER stress in tissues such as liver, pancreas, white adipose tissue (WAT), and kidney, all of which are known to be affected by ER stress. In addition, the responses of zinc transporters, especially ZIP14, to ER stress are described.

Results

TM Administration Alters Hepatic Zinc Homeostasis

First, to test the effect of ER stress on zinc homeostasis, TM, a potent ER stress inducer, was intraperitoneally injected into mice (2 mg/kg) to induce systemic ER stress. TM is an enzyme that blocks N-glycosylation of newly synthesized proteins. Thus treatment with TM triggers accumulation of misfolded or unfolded proteins in the ER lumen, which induces ER stress and activates the UPR. When measured by AAS, TM-injected mice showed significantly higher levels of hepatic zinc concentration along with hypozincemia compared to vehicle-injected mice (Figure 3-1A and 3-1B). Especially 12 h after administration, liver zinc concentration of TM group was ~15% higher than the control group. ^{65}Zn administration by oral gavage confirmed markedly increased zinc uptake after TM in liver (~1.6-fold) (Figure 3-2A), but no difference in zinc uptake was observed after TM in other tissues such as pancreas (Figure 3-1C, 3-2C), WAT (Figure 3-1D, Figure 3-2D), spleen (Figure 3-1E), and kidney (Figure 3-2E). Thus, following experiments focused mainly on liver tissue.

TM Administration Alters Zinc Transporter Expressions Including ZIP14

As zinc homeostasis is maintained by zinc transporters, hepatic gene expression of zinc transporters were examined. Among ZIP family transporters, *Zip14* gene

expression was most highly upregulated by TM (~8.2-fold) (Figure 3-3A), and its time-dependent gene and protein expressions showed its expression peaked at 12 h after TM, which coincided with extra zinc uptake (Figure 3-3C and D). Significant changes in *Zip2*, *Zip6*, *Zip7*, and *Zip8* mRNA expression were also observed (Figure 3-3A). In addition, a number of ZnT family transporters including *ZnT1*, *ZnT3*, *ZnT5*, *ZnT7*, *ZnT8*, and *ZnT10* showed significantly altered gene expressions by TM (Figure 3-3B). The increased hepatic zinc concentration and ZIP14 expression were also observed in mice injected with another ER stress inducer, TG (1 mg/kg), an inhibitor of the ER Ca^{2+}-ATPase (Figure 3-4A and B). As liver tissue is composed of multiple cell types, human hepatoma HepG2 cells were used to support an *in vivo* study. To measure total cellular zinc level in HepG2 cells, TM-treated cells were intensively sonicated to disrupt all cellular membranes, then the lysates were incubated with the zinc fluorophore, FluoZin3-AM. Thus, intensity of fluorescence represents the cellular labile zinc concentration. In agreement with *in vivo* data, TM treatment significantly increased fluorescence (~2.1-fold after 12 h) (Figure 3-5A), which coincided with increased ZIP14 expression (Figure 3-5B). Collectively, these results suggest that pharmacologically induced ER stress increases zinc uptake in liver through zinc transporter regulation.

HFD-Mediated ER Stress Increases Hepatic Zinc Uptake and ZIP14 Expression

Use of TM is an extreme experimental model to induce ER stress. HFD feeding has been used to trigger ER stress in rodents and has more physiological relevance (9). Therefore, indices of ER stress that were measured in the TM model were also analyzed after feeding WT and *Zip14* KO mice with a HFD (60 kcal% fat) or chow diet (12 kcal% fat) for 16 wk. After 16 wk, body weight of HFD-fed mice increased 10.21 ± 2.60 g, whereas chow-fed mice gained 2.78 ± 0.15 g, assuring validity of HFD feeding.

Hepatic zinc concentrations in HFD-fed mice were ~17% greater compared to chow-fed mice, indicating that HFD increases zinc uptake (Figure 3-6A). qPCR and western blotting showed an enhanced hepatic ZIP14 expression by HFD (Figure 3-6B and D), indicating increased ZIP14 expression might contribute to the elevated hepatic zinc accumulation. No difference was observed in gene expression of ZnT family transporters after HFD (Figure 3-6C).

Discussion

When protein folding capacity of ER is disrupted, cellular metabolism is largely altered by activation of UPR pathways to restore the ER homeostasis. This includes enhancing expression of ER chaperones and reducing translation of mRNA to reduce the cellular protein burden (11). Several lines of evidence point to the metabolism of some metals being altered during ER stress. Mice injected with TM exhibited hypoferremia and iron sequestration in spleen and liver (101). The change in iron metabolism during ER stress was regulated by induction of hepcidin, a master regulator of iron metabolism. Regarding zinc metabolism, it has been shown that TM administration into mice alters hepatic gene expression of multiple zinc transporters (79). This observation is supported by the present study in which ZIP14-mediated hepatic extra zinc uptake was observed along with hypozincemia after TM and TG treatment (Figure 3-1A, B, Figure 3-2A, and Figure 3-4A, B), demonstrating ER stress also alters zinc metabolism. As pharmacological induction could be an extreme model, a HFD-induced ER stress model was also examined, which would be more physiologically relevant experimental model. It was previously demonstrated that 16 wk of HFD feeding to mice could induce ER stress in liver (9). In response to HFD,

additional hepatic zinc uptake, possibly via ZIP14 activity, was similar to that seen in pharmacological models of ER stress (Figure 3-6).

More zinc may be required to assist protein folding process under this stress condition as zinc is a structural component of many proteins or as a regulatory factor (15). This is particularly true in the liver, a key organ for protein synthesis and maturation. As ER function is dependent on zinc availability to provide zinc for zinc-metalloproteins (102), increased zinc may play a role in the restoration of ER homeostasis. This notion is supported by previous reports where zinc deficiency-induced ER stress was illustrated, indicating zinc is essential for normal ER function.

The research question addressed was whether the increased hepatic zinc uptake after ER stress would be beneficial or harmful in terms of stress adaptation. Zinc may be beneficial in facilitating adaption to ER stress by mechanisms discussed above. This notion is supported by the western blotting data where a significant reduction of CHOP expression, a common marker of ER stress-induced apoptosis, was seen alongside increased zinc accumulation and ZIP14 expression. This observation suggests that ZIP14-mediated zinc accumulation may be required to suppress apoptosis. On the other hand, in the sense that metals may exhibit toxicity when their cellular concentration is too high, it is also possible that elevated zinc level may aggravate ER stress. For example, excessive accumulation of some metals such as manganese (103-105), cadmium (106-108), and fluoride (109) can induce ER stress and UPR activation. These research questions are investigated in chapter 4.

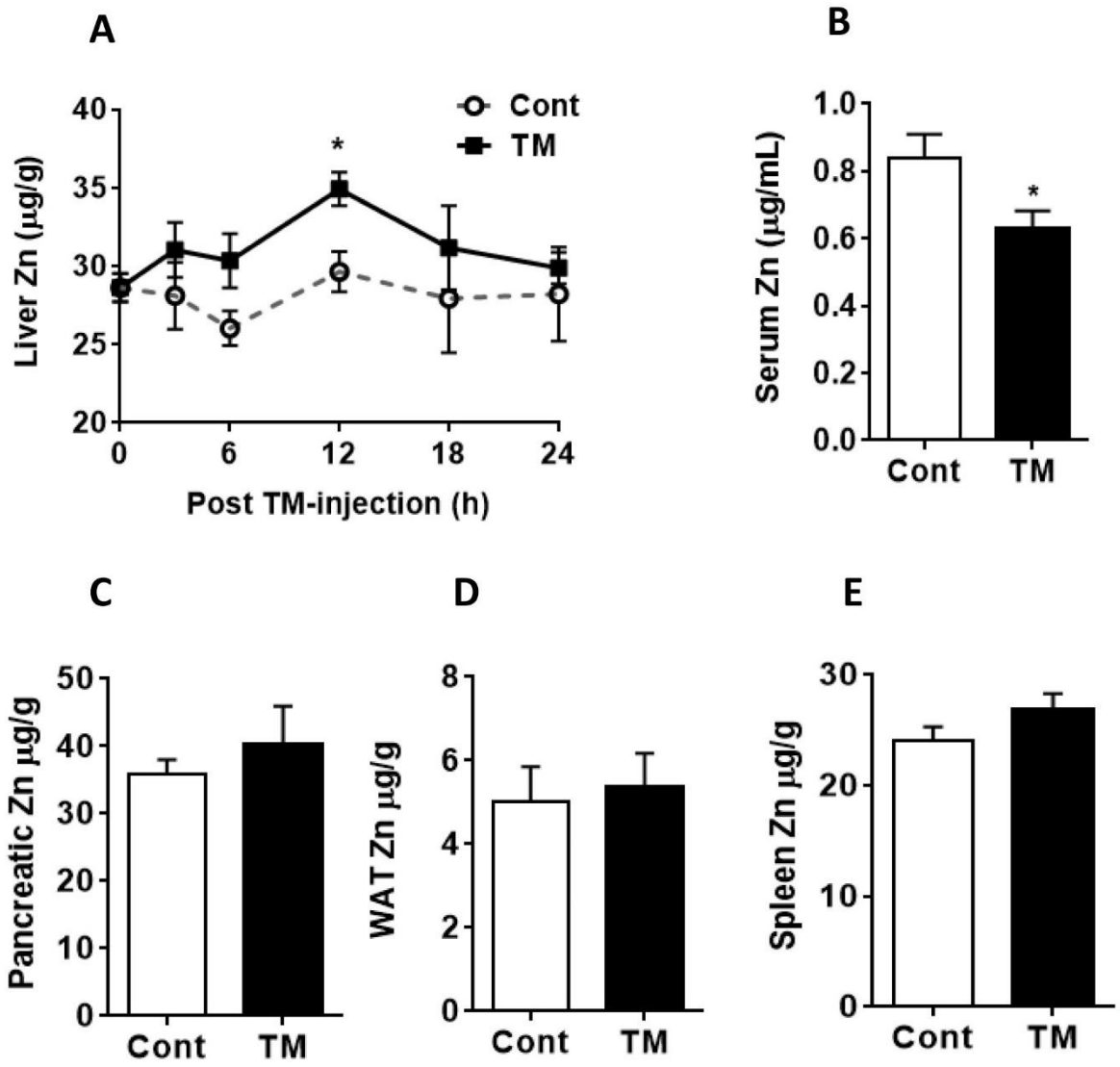

Figure 3-1. TM-mediated ER stress increases hepatic zinc uptake measured by AAS. Mice were administered with TM (2 mg/kg) or vehicle for up to 24 h. Serum was collected via cardiac puncture and liver, pancreas, WAT and spleen were collected for measurement of zinc concentrations. Zinc concentration of liver (A), serum (B), pancreas (C), WAT (D) and spleen (E) measured by AAS. All data are represented as mean ± SD. n = 3-4 mice. *$p < 0.05$.

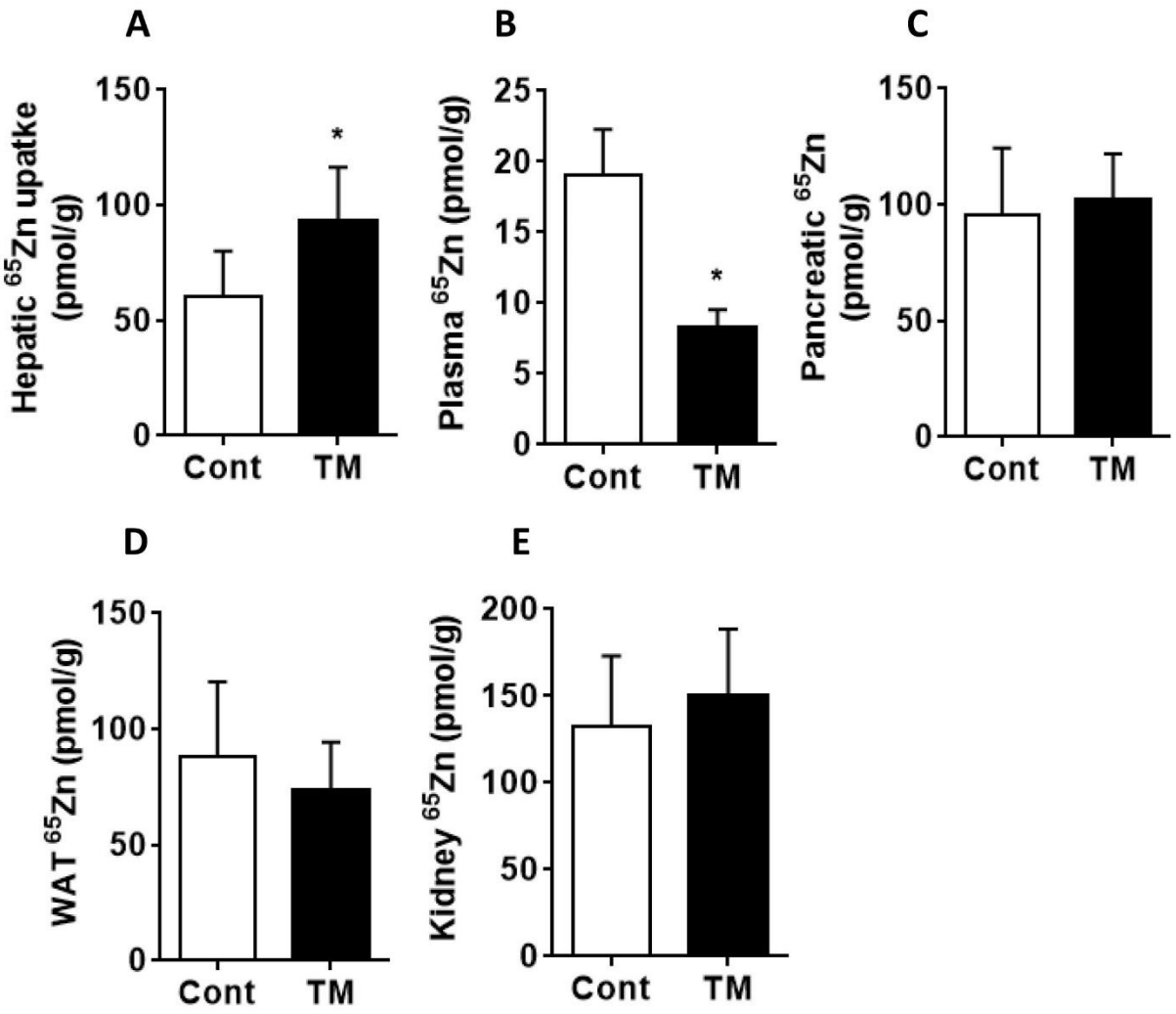

Figure 3-2. TM-mediated ER stress increases hepatic zinc uptake measured by ^{65}Zn uptake. Mice received 2 µCi of ^{65}Zn by oral gavage, which was followed by TM injection. Mice were sacrificed 12 h after TM administration. Radioactivity of liver (A), plasma (B), pancreas (C), WAT (D) and kidney (E) was measured and divided by tissue weight. All data are represented as mean ± SD. n = 3-4 mice. *$p < 0.05$.

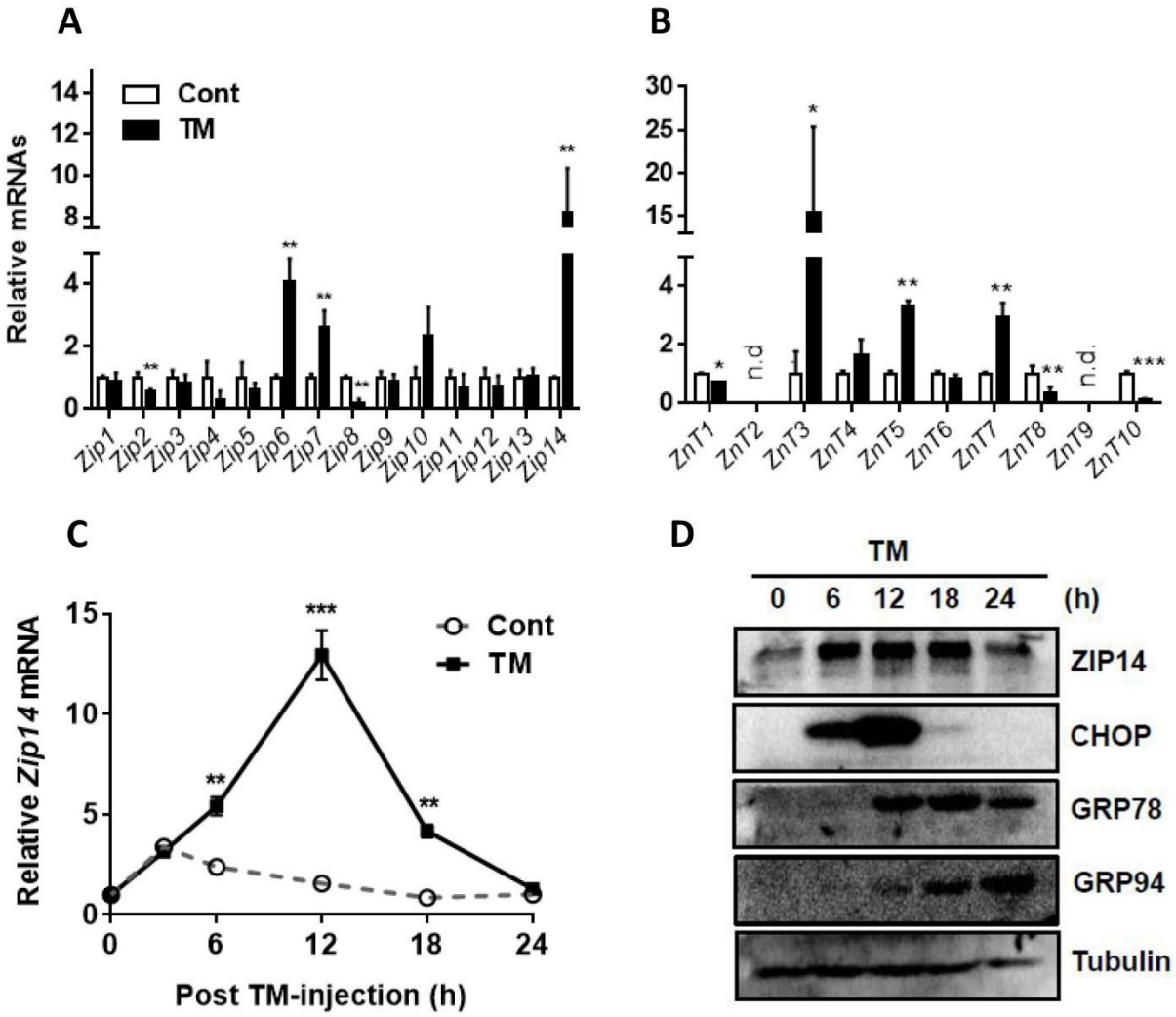

Figure 3-3. TM-mediated ER stress changes hepatic expression of multiple zinc transporters including ZIP14 in mouse liver. (A and B) Relative expression of members of the ZIP family (A) and ZnT family transporter (B) genes in livers of mice after administration of TM (2 mg/kg) or vehicle. (C and D) Time-dependent expression of *Zip14* mRNA (C) and immunoblot analysis of ZIP14 and markers of ER stress (D) in liver lysates after TM administration for the indicated times. All data are represented as mean ± SD. n = 3-4 mice. *p < 0.05, **p < 0.01, ***p < 0.001.

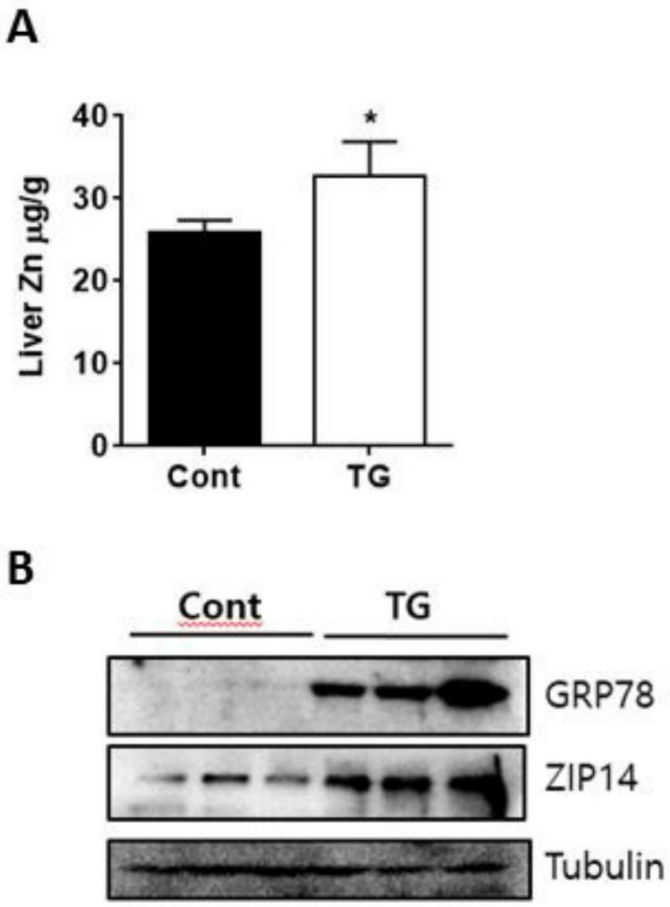

Figure 3-4. TG-mediated ER stress increases zinc concentrations and ZIP14 expression in mouse liver. Mice were administered with TG (1 mg/kg) or vehicle for 6 h. (A) Hepatic zinc concentration measured by AAS. (B) Immunoblot analysis of GRP78 and ZIP14 in liver lysates. All data are represented as mean ± SD. n = 3-4 mice. *$p < 0.05$.

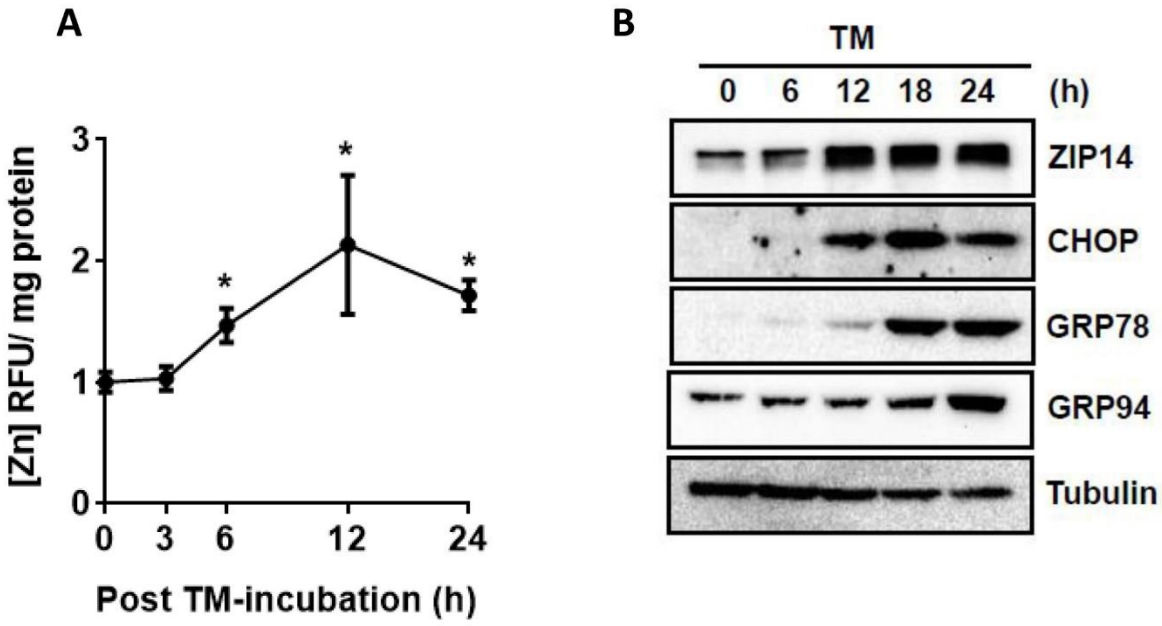

Figure 3-5. TM-mediated ER stress increases total cellular zinc concentrations and ZIP14 expression in HepG2 hepatocytes. (A) Total cellular zinc concentrations were determined by measurement of fluorescence after incubation with FluoZin3-AM (5 µM) following treatment with TM (1 µg/ml) or vehicle. (B) Immunoblot analysis of ZIP14 and markers of ER stress in lysates of HepG2 cells after TM (1 µg/ml) or vehicle treatment. All data are represented as mean ± SD. *$p < 0.05$.

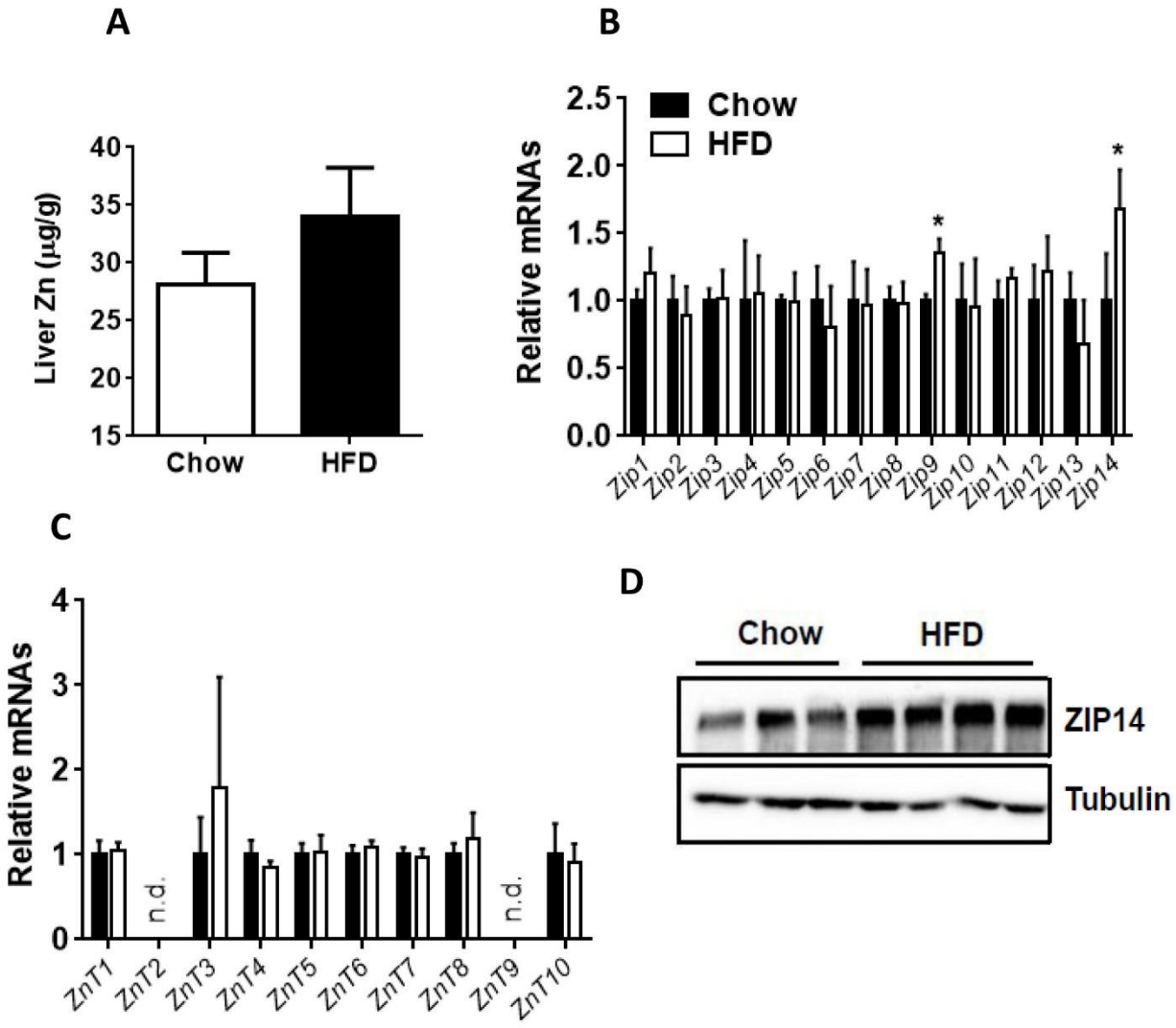

Figure 3-6. HFD-mediated ER stress increases zinc concentration and ZIP14 expression in mouse liver. Mice were fed the HFD or a chow diet for 16 wk. (A) Hepatic zinc concentration of mice. (B and C) Relative gene expression of members of the ZIP family (B) or ZnT family (C) transporter in mouse liver. (D) Immunoblot analysis of ZIP14 from liver lysates of mice. All data are represented as mean ± SD. n = 4 mice, *$p < 0.05$.

CHAPTER 4
DELINEATION OF SPECIFIC ROLE OF ZIP14 DURING ER STRESS

Introductory Remarks

To maintain zinc homeostasis, mammalian cells use 24 known zinc transporters that tightly control the trafficking of zinc in and out of cells and subcellular organelles. A number of physiological stimuli have been shown to regulate the expression and function of some of these transporters. Ablation of some of them results in various types of dyshomeostasis. For example, ZIP14 (SLC39A14), a zinc transporter abundantly expressed in liver, pancreas, and intestine, is upregulated in response to inflammatory stimuli in mouse liver, which led to hepatic zinc accumulation and hypozincemia, a component of the acute-phase response (58, 73, 75).

Some zinc transporters have been shown to be associated with ER stress and the UPR pathway. Administration of TM altered gene expression of multiple zinc transporters in mouse liver including *ZnT3*, *ZnT5*, *ZnT7*, *Zip6*, *Zip7*, *Zip13* and *Zip14* (79). These observations are supported by the data in chapter 3, where expression of many zinc transporters including ZIP14 were altered (Figure 3-3). It has been shown that these ER stress-responsive zinc transporters influence ER stress adaptation. Indeed, TM-induced ER stress was exacerbated in *ZnT5*$^{-/-}$*ZnT7*$^{-/-}$ cells by limiting zinc transport into the secretory pathway (81). Knockdown of *Zip13* in HeLa cells activated the UPR by trapping zinc in vesicles (63). These data imply that ER stress controls zinc metabolism via regulation of zinc transporters, and thus, dysfunctional zinc transporter activity may cause ER stress.

Chronic ER stress is involved in the development of various liver diseases such as hepatic steatosis, chronic viral hepatitis B and C, alcohol-induced liver injury,

hyperhomocysteinemia, and ischemia-reperfusion injury (19). In particular, the development of hepatic steatosis has been shown to be highly associated with ER stress (9). Treatment with TM suppresses expression of genes involved in lipid synthesis such as *Srebp1c* and *Fasn*. Ablation of UPR pathway components results in development of hepatic steatosis in the TM-administered mouse liver, indicating ER stress and UPR signaling are related to lipid homeostasis (29). In particular, ER stress-associated hepatic steatosis is associated with prolonged expression of CHOP, suggesting apoptotic cell death also influences the disturbed hepatic lipid homeostasis (29, 30).

PTP1B is a ubiquitously expressed protein phosphatase that is involved in multiple signaling pathways such as the insulin signaling pathway (84). Dysregulation of PTP1B has been implicated in ER stress. PTP1B expression was increased in ER stress induced by TM and HFD, and deletion of the protein *in vivo* and *in vitro* significantly reduced ER stress-associated apoptosis and steatosis. (88, 91, 93). Using liver-specific *Ptp1b* KO mice, it was demonstrated *in vivo* that PTP1B is a critical mediator of hepatic ER stress and UPR signaling as these mice exhibited significantly reduced levels of ER stress compared to wild-type mice (93). In further study, the liver-specific *Ptp1b* KO mice showed decreased expression of phosphorylated eIF2α, ATF4, and CHOP, which are pro-apoptotic components of UPR. This indicates that ablation of PTP1B can ameliorate ER stress at least in the liver. As zinc is a known inhibitor of PTP1B activity via binding to a specific site (75, 96), there is a possibility that zinc-mediated suppression of PTP1B may positively influence ER stress adaptation.

Of interest is that ZIP14-mediated extra hepatic zinc uptake observed in chapter 3 (Figure 3-1A and 3-3D) may be critical to suppress ER stress-induced apoptosis, as the enhanced hepatic ZIP14 expression after TM administration coincides with extra zinc uptake. The liver is a site where increased zinc accumulation was observed after TM administration. Therefore, the purpose of the research reported in this chapter is to delineate a role for ZIP14 during TM- and HFD-induced ER stress. The effect of ZIP14-mediated zinc accumulation on PTP1B activity is also examined. To examine the specific role of ZIP14, the conventional *Zip14* KO mouse model is primarily used. To support observations found in the mice, HepG2 hepatocytes were transfected with *Zip14* siRNA to knockdown *Zip14*.

Results

Zip14 KO Mice Do Not Display Hepatic ER Stress under Steady-State Conditions

This chapter aims to examine the potential role of ZIP14 during ER stress. ZIP14 was focused on among zinc transporters because ZIP14 is a major zinc importer of the liver, and the enhanced hepatic ZIP14 expression seen after TM administration coincided with extra zinc uptake (Figure 3-1A and 3-3D), which may suggest that ZIP14 is responsible for the event. Given that the hepatic extra zinc uptake was followed by a marked suppression of CHOP, a pro-apoptotic UPR protein (Figure 3-3D), a hypothesis was produced that ZIP14-mediated extra hepatic zinc uptake is critical to suppress ER stress-induced apoptosis. To test the hypothesis, conventional *Zip14* KO mice were used. Consistent with chapter 3, hepatic ZIP14 expression was markedly elevated following TM administration (Figure 4-1A). Under steady-state conditions, livers of *Zip14* KO mice did not show any indices of UPR activation such as enhanced expression of GRP78, GRP94, or CHOP compared to WT mice (Figure 4-1B-D).

Zip14 KO Mice Display Impaired Zinc Uptake during TM-Induced ER Stress

Zip14 KO mice have been shown to display an impaired hepatic zinc uptake (73). As expected, following TM administration, *Zip14* KO exhibited significantly less hepatic zinc uptake compared to WT mice (Figure 4-2A). After 12 h of TM administration, zinc concentrations of *Zip14* KO mice were ~78% compared to that of WT. Radioactivity measurements after ^{65}Zn administration also showed impaired hepatic zinc uptake in *Zip14* KO mice during ER stress (Figure 4-2B). Consistent with Chapter 3, no difference in ^{65}Zn level was observed in other tissues including pancreas, kidney, and WAT (Figure 4-2D-F), further supporting the rationale to focus on the liver. Since ZIP14 can contribute to uptake of manganese and non-transferrin-bound iron under certain circumstances, the hepatic concentration of these metals after TM administration was measured. The level of the non-heme iron (NHI) and manganese were comparable between WT and *Zip14* KO mice during TM challenge, indicating compared with iron and manganese, zinc is the only metal of which hepatic concentration is different in this setting (Figure 4-3A and B).

Zip14 KO Mice Display Higher Apoptosis during TM-Induced ER Stress

Next, expression of UPR pathway components including pro-apoptotic pathway proteins (p-eIF2α, ATF4 and CHOP) and adaptation pathway proteins (GRP78 and GRP94) were examined to determine how the KO mice responded to ER stress. Immunoblot analysis of WT mice showed a marked suppression of pro-apoptotic pathway proteins after 24 h TM (Figure 4-4A). Compared to WT mice, *Zip14* KO mice displayed greater expressions of p-eIF2α, ATF4, and CHOP. Densitometric analysis showed that ATF4 and CHOP expression were respectively ~2.6-fold, and ~2-fold higher in *Zip14* KO mice. TUNEL assay in mouse liver sections showed a significantly

greater number of TUNEL-positive cells in *Zip14* KO compared to WT (~2.3-fold), indicating that *Zip14* KO experienced higher levels of apoptosis during ER stress (Figure 4-4B). Serum ALT levels were also higher in *Zip14* KO after TM (~2.5-fold), indicating greater liver damage (Figure 4-4C), which perhaps was caused by increased apoptosis. GRP78 and GRP94 are ER chaperones induced by UPR during ER stress to assist in protein folding. *Zip14* KO mice expressed significantly less GRP94 compared to WT mice (~ 0.6-fold) (Figure 4-4A), which may indicate impaired ER protein folding. In HepG2 cells, higher expression of ATF4 (~4.5-fold) and CHOP (~ 2-fold), and less expression of GRP94 (~ 0.5-fold) were also observed after TM treatment when *Zip14* was knocked down (Figure 4-6C, first 4 lanes). The quality of *Zip14* knockdown using siRNA transfection was ~90% (Figure 4-6A).

To directly test the effect of zinc, it was examined if dietary zinc supplementation could ameliorate the extensive ER stress-induced apoptosis shown in *Zip14* KO mice. WT and *Zip14* KO mice were fed either a zinc adequate diet (ZnA; 30 mg Zn/kg diet) or a zinc supplemented diet (ZnS; 180 mg Zn/kg diet) for 2 wk, after which mice were injected with TM (2 mg/kg) for 24 h. However, ZnS-fed *Zip14* KO mice did not show any reduction in pro-apoptotic protein expression (Figure 4-5). Although the result was different from what was initially hypothesized, this evidence was not enough to conclude that zinc does not influence the ER stress-induced apoptosis. This may be because additional zinc could not be taken up into the liver without ZIP14, a major liver zinc transporter. To overcome this, an *in vitro* experiment was conducted where we knocked down *Zip14*, and then supplemented zinc acetate along with pyrithione, a zinc ionophore. Pyrithione was added to improve cellular zinc access under these *in vitro*

conditions. TM-treated *Zip14* knockdown cells showed ~24% less cellular zinc level compared to its control, when it was measured by FluoZin3-AM (Figure 4-6B). To determine an optimal zinc supplementation condition, we tested various doses of zinc acetate ranging from 2.5 µM to 20 µM. When more than 5 µM of zinc acetate were added along with pyrithione, TM-treated *Zip14* knockdown cells exhibited a similar level of cellular zinc, based on FluoZin-3 fluorescence, to that of TM-treated control cells (Figure 4-6B). Thus 5 µM of zinc acetate was used afterwards to model zinc supplementation. In response to TM, zinc supplemented *Zip14* knockdown cells expressed markedly reduced expression of ATF4 (~41%) and CHOP (~37%) proteins compared to non-zinc supplemented *Zip14* knockdown cells (Figure 4-6C). After supplementation, ATF4 and CHOP expression in *Zip14* knockdown cells were not significantly different from control cells. In addition, expression of GRP94 was increased (~1.9-fold) after zinc supplementation in *Zip14* knockdown cells. The decrease in cell viability shown in TM-treated *Zip14* knockdown cells was ameliorated by zinc supplementation (Figure 4-6D and E). Collectively, these data indicate that supplementation of zinc prevents the severe ER stress shown in the ZIP14-ablated condition, demonstrating a direct effect of zinc on preventing ER stress-induced apoptosis.

Zip14 KO Mice Show a Greater Level of Hepatic Steatosis during TM-Induced ER Stress

Prolonged ER stress in the liver has been linked to the occurrence of hepatic steatosis, and may result from damage after the UPR fails to restore ER protein folding homeostasis. Therefore, lipid homeostasis in *Zip14* KO mice was examined during TM challenge. Greater levels of lipid droplet accumulation in *Zip14* KO mice were observed

in H&E staining of liver sections after TM administration (~1.9-fold) (Figure 4-7A). Quantification of triglycerides in the liver also showed that the *Zip14* KO mice accumulated significantly higher levels of triglyceride compared to WT (~1.8-fold) (Figure 4-7B). As hepatic lipid homeostasis is mostly maintained by four mechanisms including de novo fatty acid (FA) synthesis, FA oxidation, FA uptake into the liver, and lipoprotein secretion from the liver, expression of genes of key enzymes in these mechanisms were measured to elucidate where lipid metabolism is dysregulated in *Zip14* KO mice. After TM administration, genes involved in de novo FA synthesis such as *Srebp1c*, *Acc*, *Fasn*, and *Scd1* were significantly suppressed in WT mice (Figure 4-7C). Expression levels of *Srebp1c, Fasn,* and *Scd1* were reduced ~60%, ~85%, and ~74%, respectively. In the same setting, *Zip14* KO mice showed reduced gene expressions of *Srebp1c*, *Acc*, *Fasn*, and *Scd1*, which were ~2.5-fold, ~2.2-fold, ~3.2-fold, and ~4-fold higher than WT, respectively, indicating FA synthesis in *Zip14* KO mice is higher during ER stress. There was no significant difference in gene expression related to other pathways of lipid metabolism including FA oxidation, FA uptake, and lipoprotein secretion (Figure 4-7D). Collectively, these data suggest that *Zip14* KO mice have higher levels of ER stress-mediated hepatic steatosis due to greater FA synthesis.

Zip14 KO Mice Display Impaired Hepatic Zinc Accumulation, which Coincides with Higher Apoptosis and Hepatic Steatosis during HFD-Induced ER Stress

To investigate the effects of ER stress in *Zip14* KO mice in a more physiologically relevant setting, indices of ER stress that were measured in the TM model were analyzed after feeding WT and *Zip14* KO mice with a HFD (60 kcal% fat) or chow diet (12 kcal% fat) for 16 wk. As shown in Chapter 3, hepatic zinc concentrations in HFD-fed WT mice were ~17% higher compared to chow-fed WT mice, indicating that HFD

increases zinc levels in the liver. However, hepatic zinc levels in HFD-fed *Zip14* KO mice were unchanged compared to chow-fed *Zip14* KO mice (Figure 4-8A), possibly due to impaired zinc uptake.

In accordance with a previous report (9), both genotypes showed UPR activation in response to HFD feeding, which was validated by elevated protein levels of UPR components including p-eIF2α, ATF4, CHOP, GRP78 and GPR94 (Figure 4-8B). However, HFD-fed *Zip14* KO mice expressed greater levels of pro-apoptotic p-eIF2α (~2.1-fold), ATF4 (~4-fold) and CHOP (~3-fold) compared to HFD-fed WT mice, indicating that ablation of *Zip14* worsens ER stress-associated apoptosis in this setting. Similar to the TM-administration model, HFD-fed *Zip14* KO mice expressed a lower level of GRP94 than HFD-fed WT mice (~0.5-fold). Regarding steatosis, hepatic TG accumulation after HFD feeding was ~42% higher in *Zip14* KO mice, although it was not statistically significant (Figure 4-8C). This observation coincided with significantly higher mRNA expressions of *Srebp1c* (~1.7-fold), *Fasn* (~1.8-fold), and *Scd1* (~1.9-fold) (Figure 4-8D), suggesting a greater level of FA synthesis during HFD in *Zip14* KO mice. These results from the HFD model support the hypothesis that ZIP14-mediated zinc uptake is critical for suppressing ER stress-induced apoptosis and hepatic steatosis.

Increased PTP1B Activity is Observed in *Zip14* KO Mice during ER Stress

The next research aim was to elucidate the possible mechanism underlying the phenotypes shown in ER-stress-induced *Zip14* KO mice. PTP1B, a protein phosphatase that regulates several pathways such as the insulin signaling pathway, has been implicated in ER stress. PTP1B expression was increased in ER stress induced by TM and HFD. Deletion of the protein *in vivo* and *in vitro* significantly reduced ER stress-associated apoptosis and steatosis. (88, 91, 93). As zinc is a known non-competitive

inhibitor of PTP1B activity via binding to a specific site (75, 96), a hypothesis was produced that *Zip14* KO mice would exhibit increased PTP1B activity during ER stress due to impaired zinc uptake, thereby resulting in greater apoptosis and steatosis. In agreement with previous reports, knockdown of *Ptp1b* in HepG2 hepatocytes resulted in significantly reduced expression of p-eIF2α, ATF4 and CHOP during TM challenge compared to control cells, suggesting suppression of PTP1B reduces ER stress-induced apoptosis (Figure 4-9A). This was supported by measurement of cell viability using the MTT assay. During TM treatment *Ptp1b* knockdown cells displayed higher cell viability than control cells (Figure 4-9B).

Although *Zip14* KO mice expressed less PTP1B expression than WT (~0.6-fold), their PTP1B activity was not different (Figure 4-10A and C). Following TM administration, hepatic PTP1B expression was increased in both WT and *Zip14* KO mice, and the expression levels were not statistically different between the two genotypes. (Figure 4-10A). However, measurement of PTP1B activity revealed a significantly higher level of PTP1B activity in *Zip14* KO mice compared to WT (~1.7-fold) (Figure 4-10B). The same pattern was observed in the HFD model. PTP1B activity was significantly greater in HFD-fed *Zip14* KO mice compared to HFD-fed WT (~1.5-fold), although the protein levels were similar between HFD-fed WT mice and HFD-fed KO mice (Figure 4-10C and D).

In vitro studies were conducted to test a direct effect of zinc on PTP1B activity. HepG2 hepatocytes were transfected with *Zip14* siRNA or control siRNA, then an established zinc supplementation protocol (Figure 4-6B) was used to treat cells either with or without concurrent TM treatment. Similar to *in vivo* results, cells lacking *Zip14*

showed significantly higher PTP1B activity in response to TM treatment than control cells (~1.5-fold) (Figure 4-6F). However, the increased PTP1B activity was significantly reduced with zinc supplementation. No additional effect of zinc supplementation was observed in TM-treated control cells. Collectively, these data suggest that normal cells suppressed PTP1B activity during ER stress by facilitating extra zinc via ZIP14 induction, whereas the *Zip14* KO mice's impaired zinc uptake altered this process. This could be a potential mechanism underlying zinc-mediated adaptation against ER stress.

Discussion

The major finding reported in this chapter was that ZIP14-mediated hepatic zinc accumulation provides a beneficial effect in suppressing suppress apoptosis and steatosis induced by ER stress. In response to ER stress, *Zip14* KO mice expressed greater levels of CHOP as well as its upstream modulators, p-eIF2α and ATF4, (Figure 4-4A). This was confirmed with greater numbers of TUNEL-positive cells in *Zip14* KO mice (Figure 4-4B). Additionally, in *Zip14* knockdown HepG2 cells, supplementation of zinc could reverse the higher expression of pro-apoptotic proteins and corresponding cell death (Figure 4-6D and E), demonstrating a direct effect of zinc on ER stress-induced apoptosis. As prolonged apoptosis causes many pathogenic disorders, suppression of p-eIF2α/ATF4/CHOP pathway is important for adapting to ER stress. In particular, suppression of CHOP, a downstream effector of the pro-apoptotic pathway, could be a therapeutic target that leads to ER stress adaptation. This was illustrated in an experiment using *Chop* KO mice where the genotype exhibited an apoptosis-resistant phenotype in response to ER stress. These data collectively demonstrate that ZIP14-mediated hepatic zinc uptake influences the p-eIF2α/ATF4/CHOP pathway by which apoptosis is suppressed.

A potential direct target of zinc that influences the p-eIF2α/ATF4/CHOP pathway was examined. In this chapter, the data suggests that zinc can modulate the p-eIF2α/ATF4/CHOP pathway, possibly through suppression of PTP1B. This enzyme is an ER-resident protein tyrosine phosphatase which regulates various pathways including insulin and leptin signaling (110). Dysregulation of PTP1B activity was demonstrated to contribute to the pathogenesis of various diseases such as cancer and diabetes. Furthermore, there has been a significant effort to develop PTP1B inhibitors as drug targets (111). Additionally, PTP1B has been implicated in ER stress (112). Since ER stress increases PTP1B expression, its deletion was effective at improving ER stress adaptation (91). *In vitro*, *Ptp1b* knockdown in fibroblasts made them more resistant to ER stress induced apoptosis (88). *In vivo*, liver-specific *Ptp1b* KO mice showed decreased expression of the p-eIF2α/ATF4/CHOP pathway along with reduced indexes of metabolic syndrome during TM- and HFD-induced ER stress (91, 93). Although it is still unclear how inhibition or deletion of PTP1B can directly inhibit ER stress-induced apoptosis, these reports strongly suggest that suppression of the activity of PTP1B can be a therapeutic target to overcome ER stress. Zinc is a known inhibitor of PTP1B by physically binding to it (96). It has been reported that overexpression of ZIP14 in AML12 hepatocytes could suppress PTP1B activity (75). Based on these reports, a hypothesis was produced that ER stress-induced mice would increase hepatic ZIP14 expression in order to suppress PTP1B activity by facilitating additional zinc uptake. In response to TM administration and HFD-feeding, PTP1B protein expression was increased (Figure 4-10A and C). However, only *Zip14* KO mice showed significantly elevated PTP1B activity whereas the activity of WT mice remained

unchanged (Figure 4-10B and D). The direct effect of zinc on PTP1B activity in *Zip14* knockdown HepG2 cells was demonstrated using zinc supplementation (Figure 4-10F). However, further investigation will be required since these data are still observational. Although knockdown of *Ptp1b* reduced apoptosis in hepatocytes (Figure 4-9), it would be interesting if zinc supplementation could suppress the ER stress response if PTP1B was overexpressed. Apoptosis could also be potentiated with *Zip14* ablation due to lack of GRP94 expression during ER stress. GRP94, an ER chaperone, binds to misfolded proteins and assists in their appropriate folding (113). Deletion of GRP94 has been shown to potentiate ER stress (114). *Zip14* KO mice did not express GRP94 at the levels shown in WT mice (Figure 4-4A and 4-8B). Supplementation of zinc in *Zip14* knockdown cells increased GRP94 expression after TM treatment (Figure 4-6C), indicating impaired zinc uptake obstructed the induction of GRP94. The proteomic profile analysis revealed that zinc supplemented pigs (2425 mg zinc/kg) had increased pancreatic GRP94 expression (115). Although additional research is needed, it is possible that zinc modulates GRP94 expression, or that GRP94 may require zinc as a cofactor like other ER folding proteins such as Calreticulin and Calnexin (116, 117).

Disrupted hepatic lipid homeostasis is another feature of unresolved ER stress. This has been observed in cells with compromised UPR function. Genetic ablation of the UPR components including ATF6α, IRE1α, and eIF2α resulted in the development of hepatic steatosis (118). Loss of ATF4 increased free cholesterol in rodent livers (119), and liver-specific deletion of XBP1 produced hypocholesterolemia and hypotriglyceridemia (31). Similarly, livers of *Zip14* KO mice showed potentiated triglyceride accumulation after TM administration (Figure 4-7A and 4-7B). Hepatic

steatosis in *Zip14* KO mice resulted from higher expression of genes involved in *de novo* FA synthesis including *Srebp1c*, *Acc*, *Fasn,* and *Scd1* (Figure 4-7C), as there was no significant difference in gene expression involved in FA oxidation, FA uptake, and lipoprotein secretion between KO and WT mice (Figure 4-7D). This is different from other ER stress-associated hepatic steatosis models such as the *Atf6α* KO mouse in which dysregulation of FA oxidation and lipoprotein secretion caused increased lipid accumulation (29). Similar to apoptosis, zinc-mediated PTP1B suppression may explain the cause of hepatic steatosis in *Zip14* KO mice since liver-specific *Ptp1b* KO mice showed improved lipid metabolism during HFD feeding compared to WT mice (93). *Ptp1b* KO mice exhibited markedly less expression of *Srebp1c*, *Fasn*, and *Acc*. All of these were increased in TM-injected, and HFD-fed *Zip14* KO mice in report from the experiments in this dissertation. Thus, ZIP14-mediated zinc uptake is required to suppress FA synthesis during ER stress possibly through inhibition of PTP1B activity.

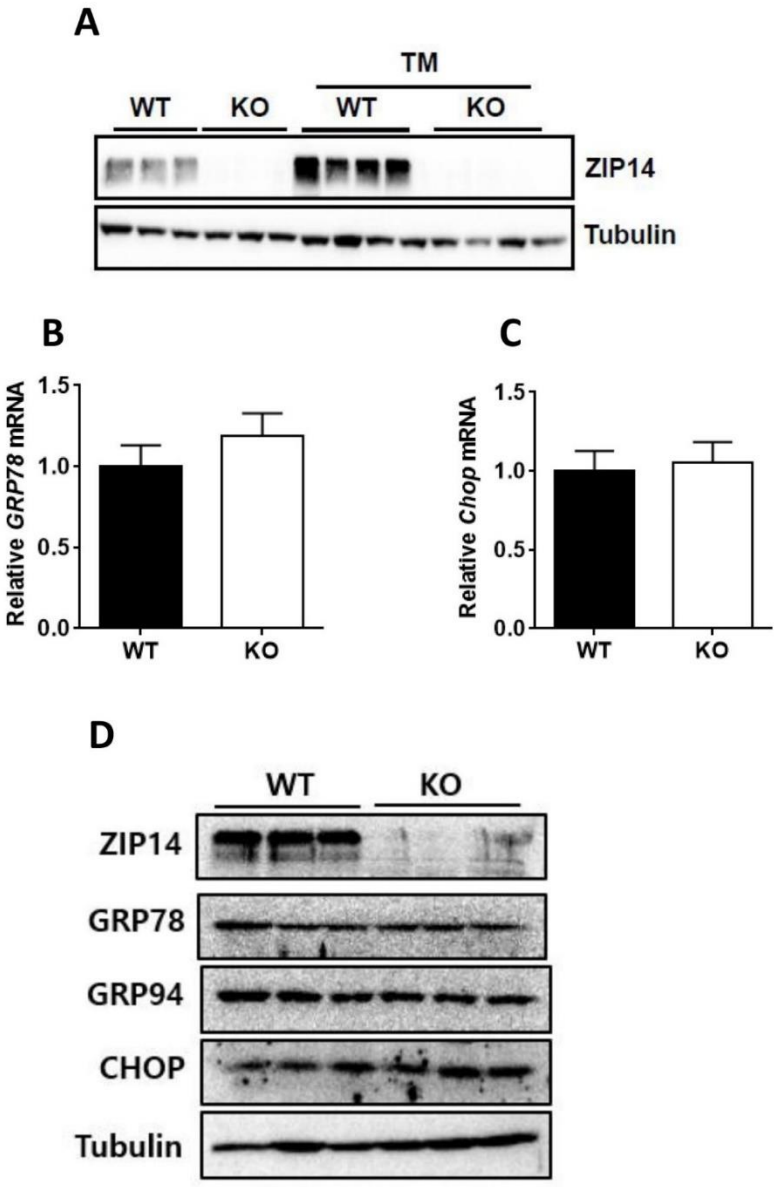

Figure 4-1. *Zip14* KO mice do not show hepatic ER stress under steady-state conditions. (A) Immunoblot analysis of ZIP14 from liver lysates of WT and *Zip14* KO mice 12 h after TM (2mg/kg) or vehicle administration. (B and C) Relative gene expression of *Grp78* (B) and *Chop* (C) in liver of WT and *Zip14* KO mice under steady-state. (D) Immunoblot analysis of ZIP14 and markers of ER stress in liver lysates of WT and *Zip14* KO mice under steady-state. All data are represented as mean ± SD. n = 3-4 mice.

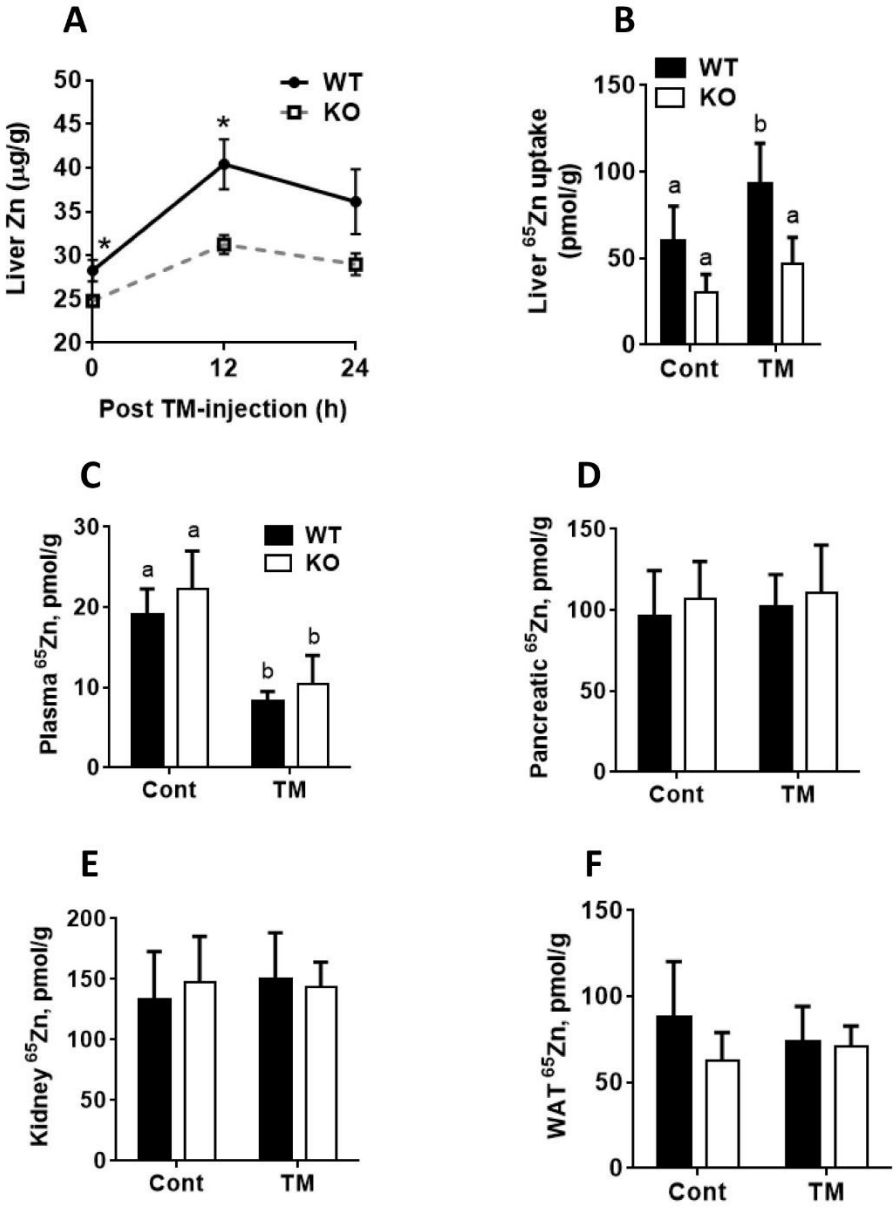

Figure 4-2. *Zip14* KO mice display impaired hepatic zinc uptake during TM-induced ER stress. (A) Hepatic zinc concentrations measured by AAS in mice after administration of TM (2mg/kg) or vehicle for up to 24 h. (B-F) Mice received 2 μCi of ^{65}Zn by gavage, which was followed by TM (2mg/kg) injection. Mice were sacrificed 12 h after TM administration. ^{65}Zn uptake in mouse liver (B), plasma (C), pancreas (D), kidney (E) and WAT (F). All data are represented as mean ± SD. n = 3-4 mice. *$p < 0.05$. Labeled means without a common letter differ significantly ($p < 0.05$).

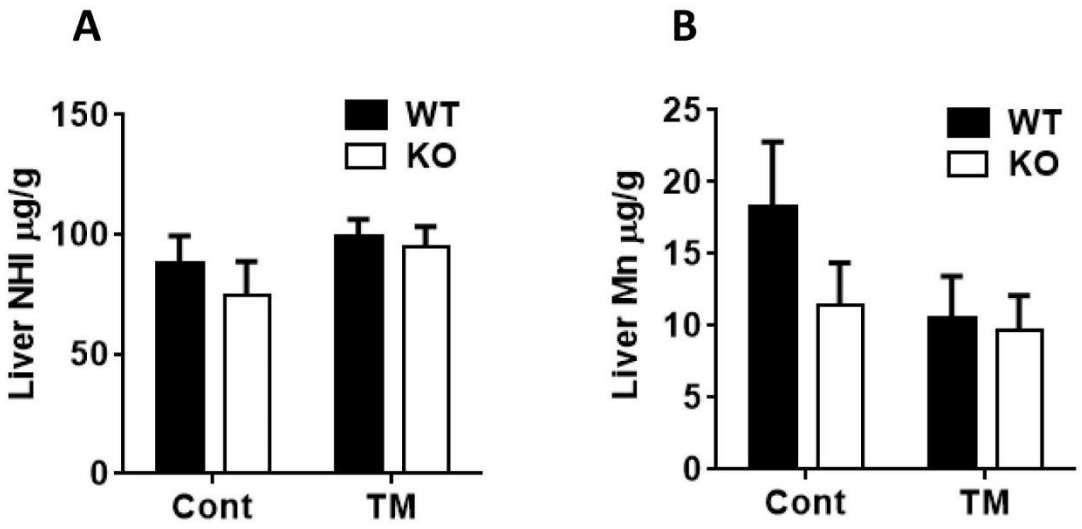

Figure 4-3. Hepatic concentration of NHI and manganese are comparable between WT and *Zip14* KO mice during TM challenge. WT and *Zip14* KO mice were administered with TM (2mg/kg) or vehicle for 12 h, and were sacrificed. (A) Hepatic NHI concentrations were analyzed colorimetrically. (B) Hepatic manganese concentrations were measured by AAS. All data are represented as mean ± SD. n = 3-4 mice.

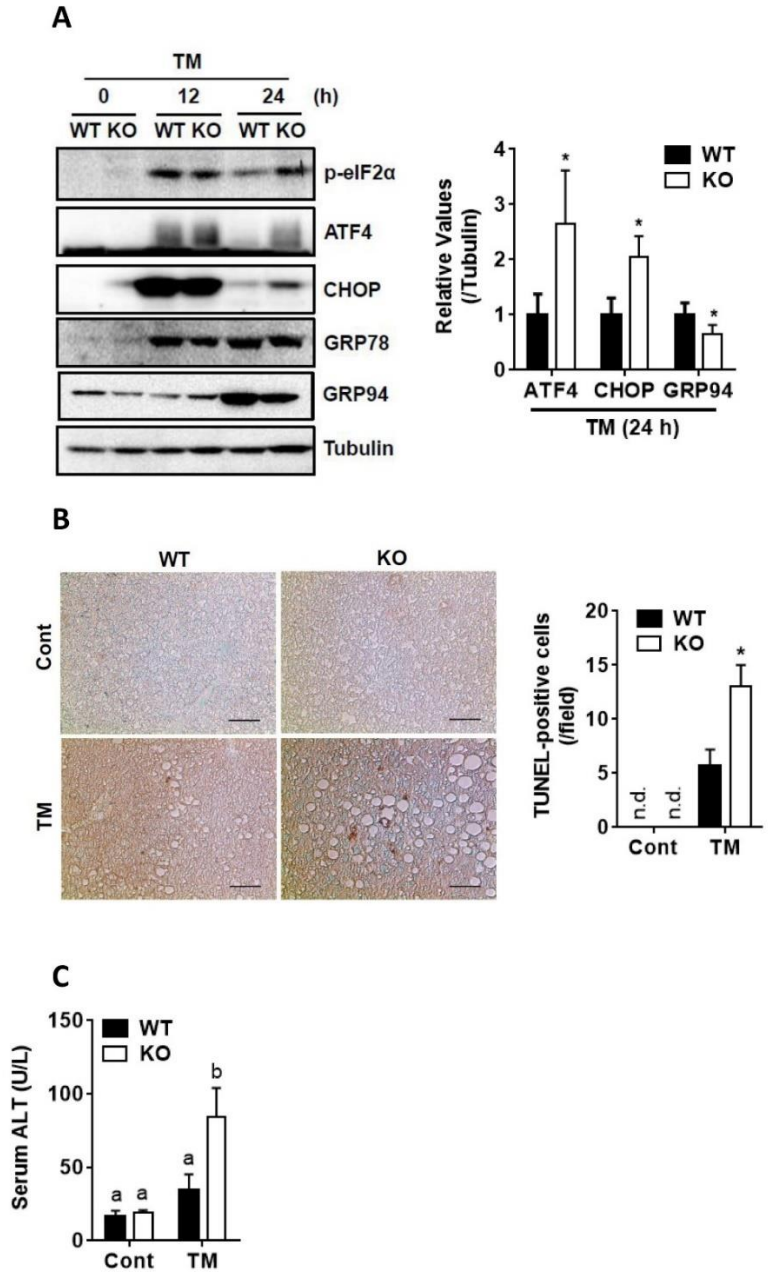

Figure 4-4. *Zip14* KO mice display increased apoptosis during TM-induced ER stress. (A) Immunoblot analysis of ER stress markers from liver lysates of WT and *Zip14* KO mice (n= 3-4, pooled samples) after administration of TM (2mg/kg). Individual blots (24 h after TM, n = 4) were measured using digital densitometry. (B) Representative images of TUNEL assays of liver sections of WT and *Zip14* KO mice 24 h after administration of TM (2mg/kg) or vehicle. TUNEL-positive cells in field were quantified. Images are at 40X magnification; bars = 25 µm. (F) Serum ALT activity of WT and *Zip14* KO mice 24 h after administration of TM (2mg/kg) or vehicle (n = 3-4). All data are represented as mean ± SD. *p < 0.05. Labeled means without a common letter differ significantly (p < 0.05).

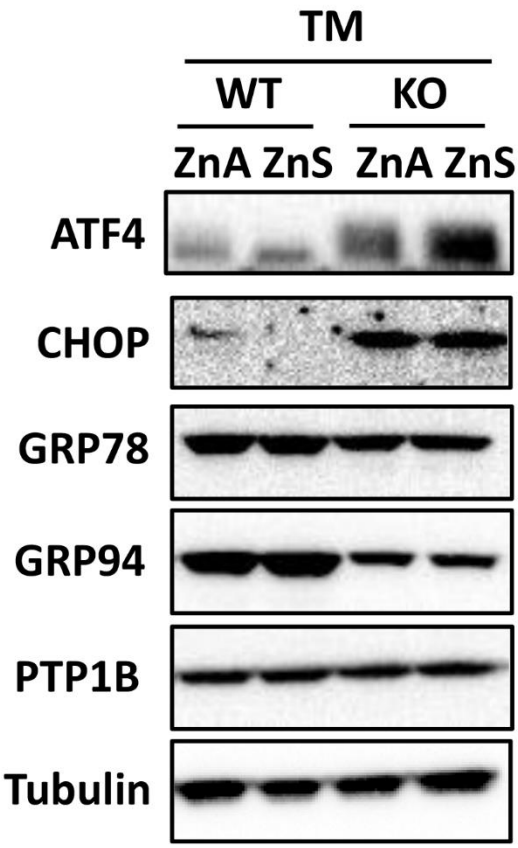

Figure 4-5. Dietary zinc supplementation does not reduce the hepatic expression of pro-apoptotic proteins in *Zip14* KO mice. WT and *Zip14* KO mice were fed either with zinc adequate diet (ZnA; 30 mg Zn/kg diet) or zinc supplementation diet (ZnS; 180 mg Zn/kg diet) for 2 wk, after which mice were injected with TM (2 mg/kg) for 24 h. Immunoblot analysis of ER stress markers from liver lysates of WT and *Zip14* KO mice (n= 4, pooled samples) after administration of TM (2mg/kg).

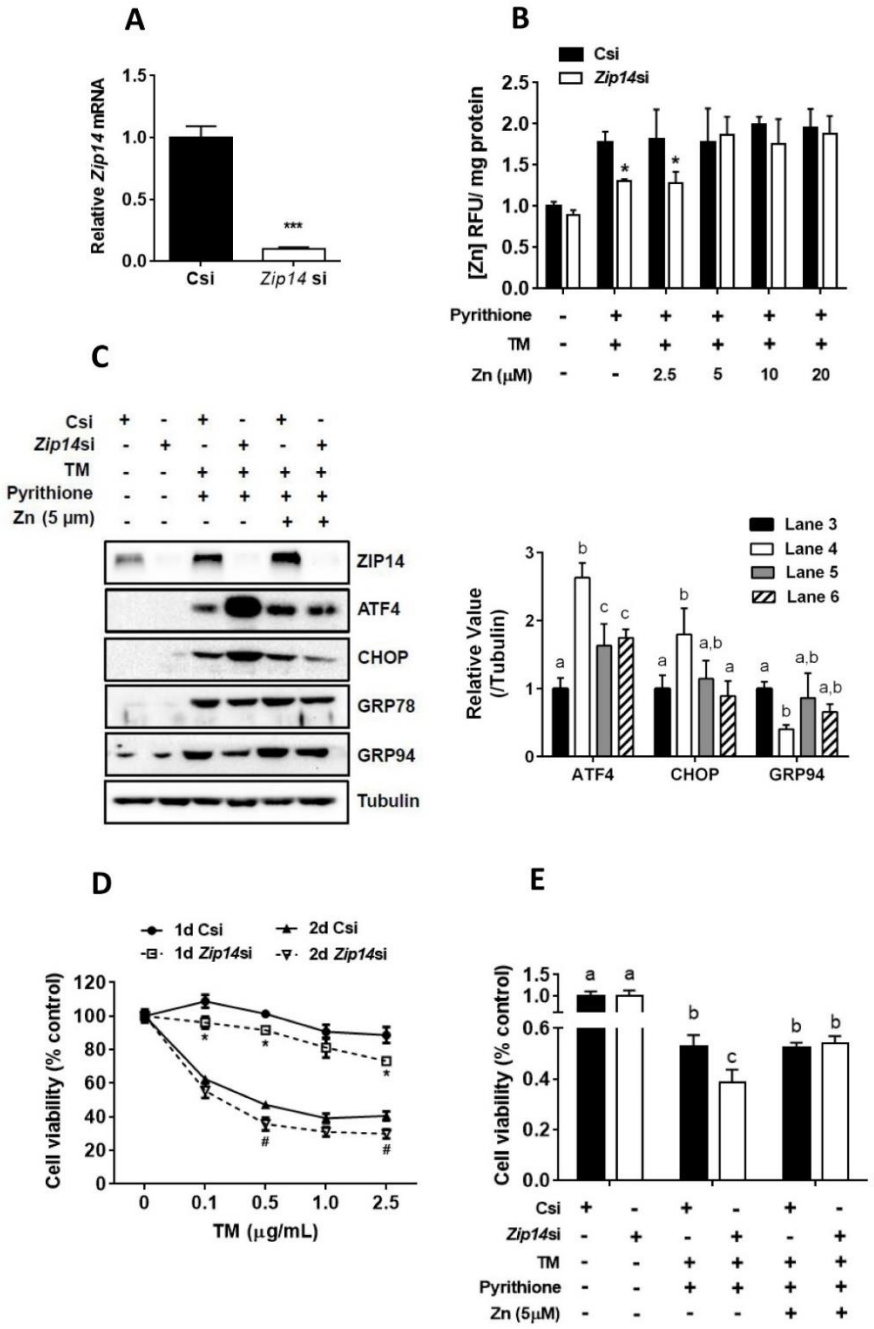

Figure 4-6. Zinc supplementation rescues ER stress-mediated apoptosis in *Zip14*-knockdown hepatocytes. (A) Relative expression of *Zip14* mRNA in HepG2 hepatocytes transfected with *Zip14* siRNA or control siRNA. (B-E) HepG2 cells were incubated for 30 min with zinc acetate and pyrithione (50 μM), which was followed by incubation with TM (1 μg/ml) or vehicle. (B) Total cellular zinc concentrations were determined by measurement of fluorescence after incubation with FluoZin3-AM (5 μM). (D and E) Cell viability was measured using the MTT assay. All data are represented as mean ± SD. *, $^{\#}p < 0.05$. Labeled means without a common letter differ significantly ($p < 0.05$).

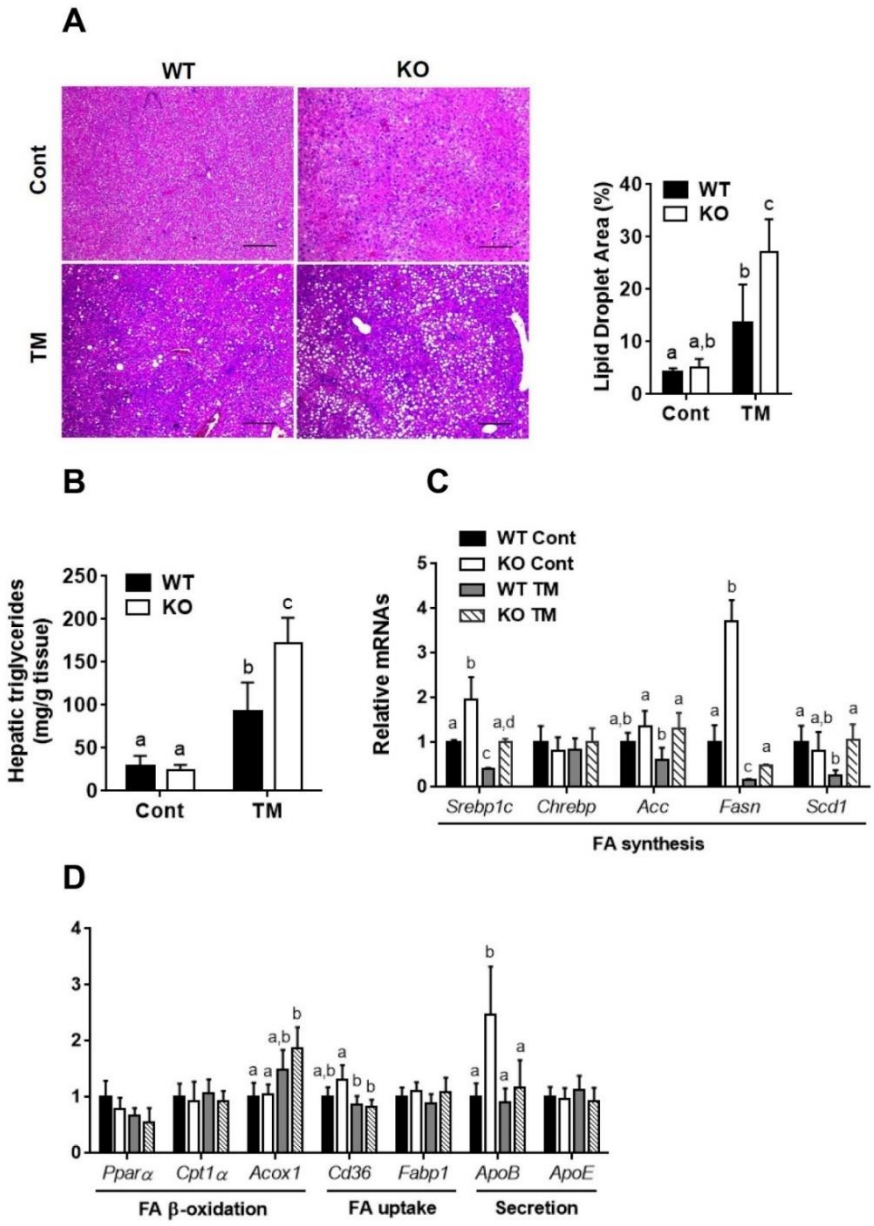

Figure 4-7. *Zip14* KO mice exhibit a greater level of hepatic triglyceride accumulation after TM-induced ER stress. (A) Representative images of H&E-stained liver sections of WT and *Zip14* KO mice 24 h after administration of TM (2mg/kg) or vehicle. The lipid droplet area in the field was measured. Images are at 10X magnification; bars = 100 μm. (B) Liver triglyceride levels of WT and *Zip14* KO mice were measured 24 h after administration of TM (2mg/kg) or vehicle (n = 3-4). (C and D) Relative expression of genes that regulate FA synthesis (C), FA β-oxidation, FA uptake, and lipoprotein secretion (D) were measured in livers of WT and *Zip14* KO mice 12 h after administration of TM (2mg/kg) or vehicle (n= 3-4). All data are represented as mean ± SD. Labeled means without a common letter differ significantly ($p < 0.05$).

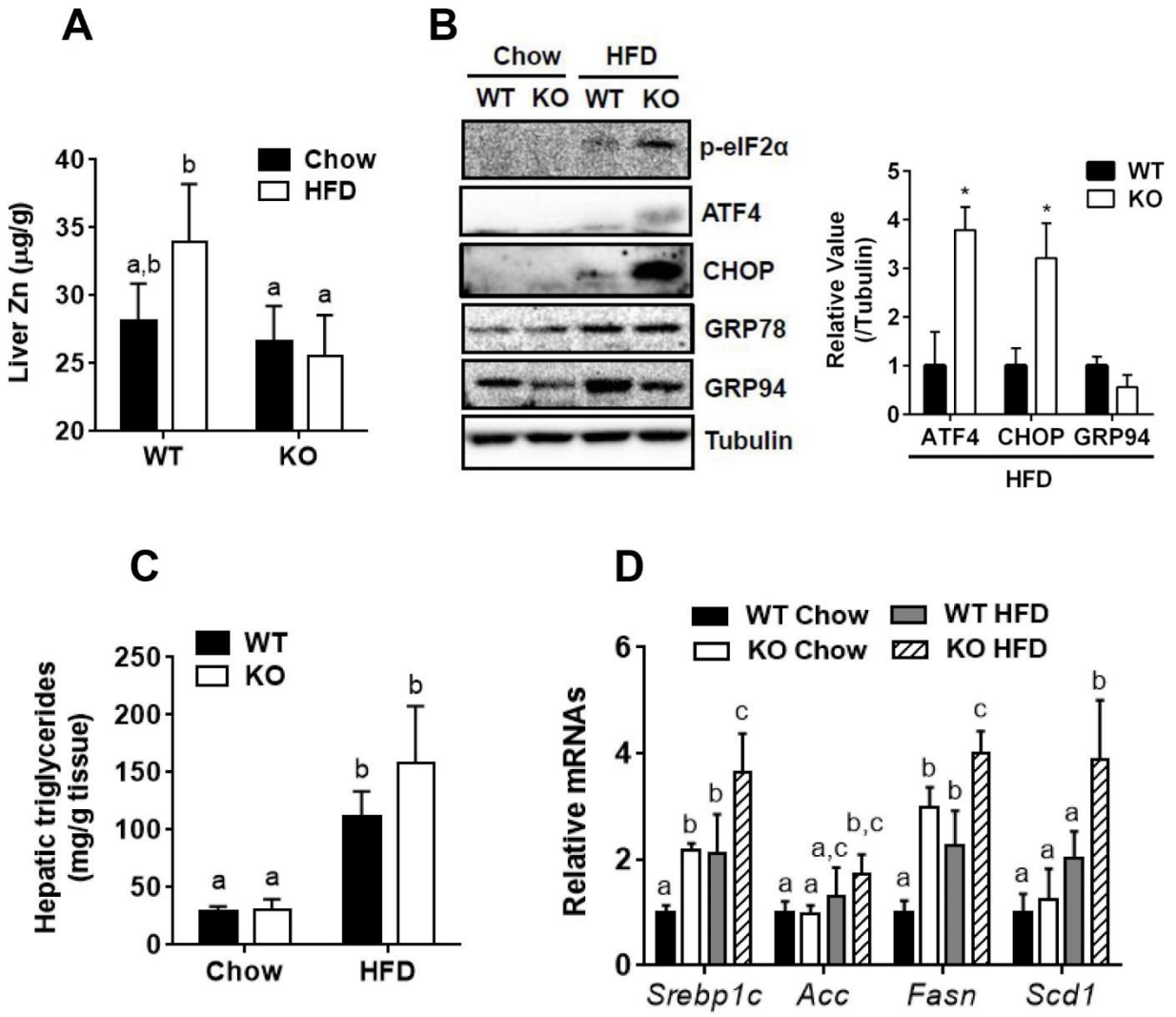

Figure 4-8. HFD-fed *Zip14* KO mice show greater hepatic ER stress-induced apoptosis and triglyceride accumulation. Mice were fed the HFD or a chow diet for 16 wk.(A) Hepatic zinc concentration of WT and *Zip14* KO mice (n = 4). (B) Immunoblot analysis of ER stress markers from liver lysates of WT and KO mice (n = 4, pooled samples used). Individual blots (HFD, n = 4) were measured using digital densitometry. (C) Liver triglyceride levels were quantified in WT and *Zip14* KO mice (n = 4). (D) Relative expression of genes that regulate FA synthesis were measured in livers of WT and *Zip14* KO mice (n = 4). All data are represented as mean ± SD. *p < 0.05. Labeled means without a common letter differ significantly (p < 0.05).

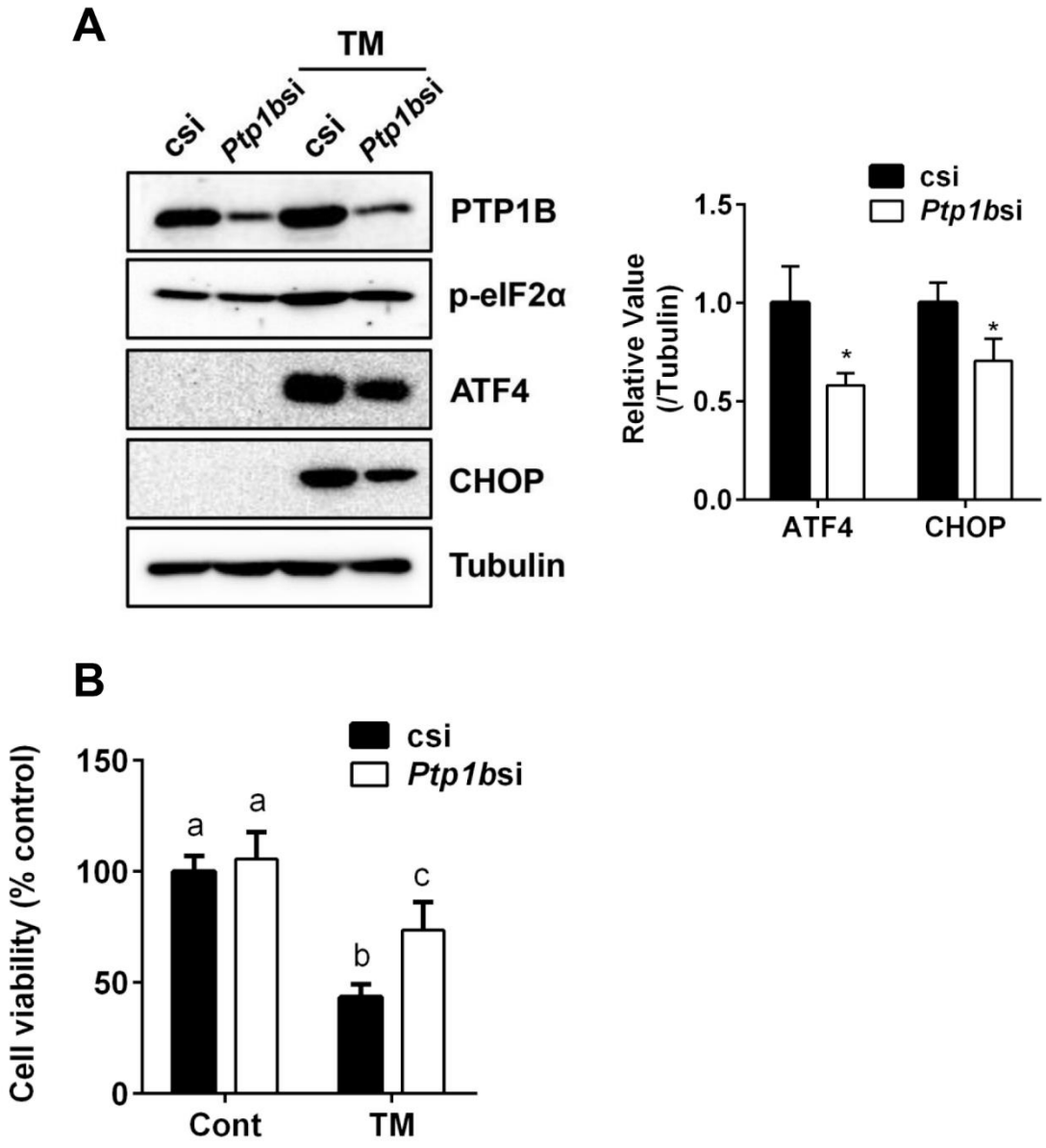

Figure 4-9. ER stress-induced apoptosis is reduced in *Ptp1b*-knockdown hepatocytes. HepG2 hepatocytes were transfected with *Ptp1b* siRNA or control siRNA, which was followed by incubation with TM (1 μg/ml) or vehicle for 24 h. (A) Immunoblot analysis of PTP1B and ER stress markers from cell lysates. (n = 3, pooled samples used). Individual blots (n = 3) were measured using digital densitometry. (B) Cell viability was measured using the MTT assay. All data are represented as mean ± SD. *$p < 0.05$. Labeled means without a common letter differ significantly ($p < 0.05$).

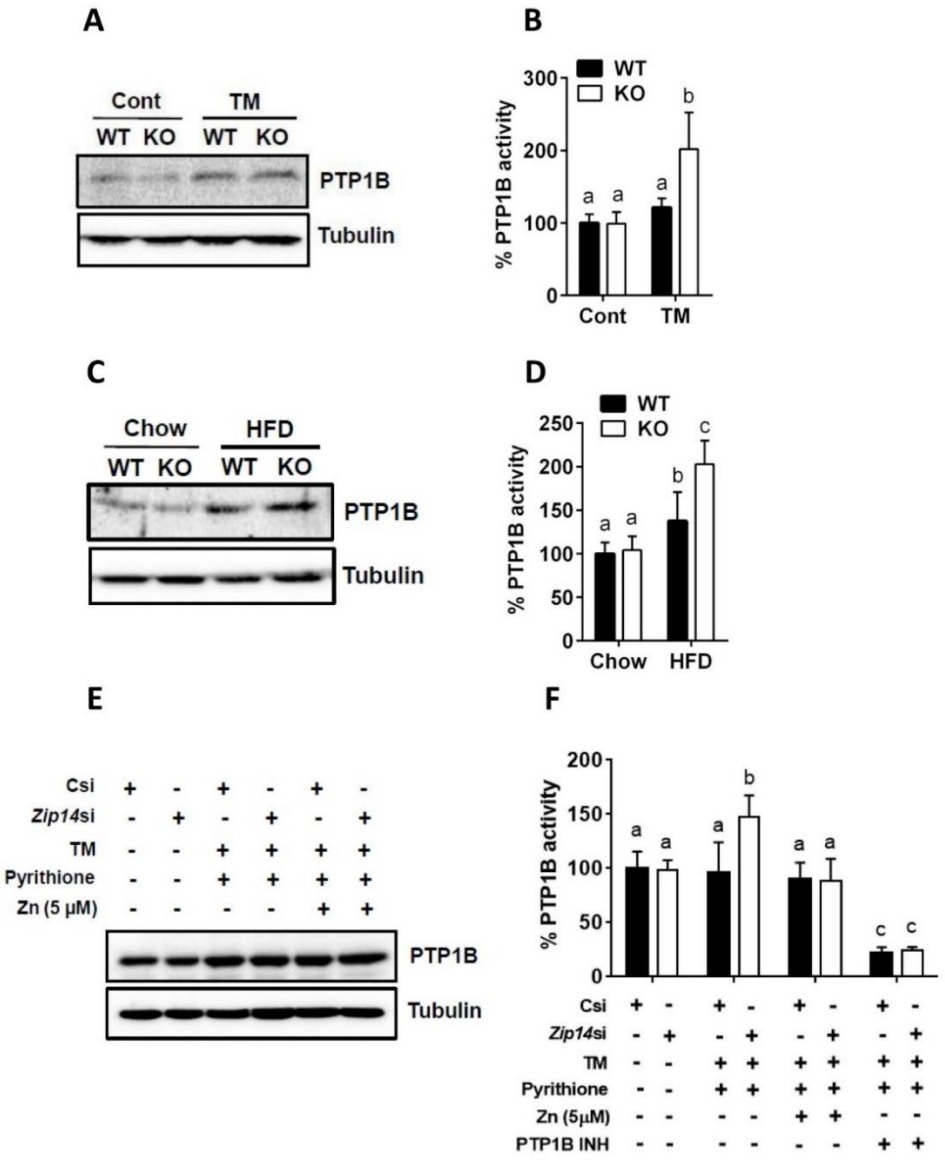

Figure 4-10. ZIP14 is required to suppress hepatic PTP1B activity after TM administration and HFD. (A and B) Immunoblot analysis of PTP1B protein (A) and measurement of PTP1B activity (B) in livers of WT and *Zip14* KO mice 12 h after administration of TM (2mg/kg) or vehicle (n = 3-4, pooled samples used for panel A). (C and D) Immunoblot analysis of PTP1B protein (C) and measurement of PTP1B activity (D) in livers of WT and *Zip14* KO mice fed with HFD or Chow for 16 wk (n = 4, pooled samples used for panel C). (E and F) Immunoblot analysis of PTP1B protein (E) and measurement of PTP1B activity (F) in HepG2 hepatocytes transfected with *Zip14* siRNA or control siRNA. Cells were pre-treated with zinc acetate (5 μM) and pyrithione (50 μM) for 30 min before TM (1 μg/ml) treatment for 12 h All data are represented as mean ± SD. Labeled means without a common letter differ significantly ($p < 0.05$).

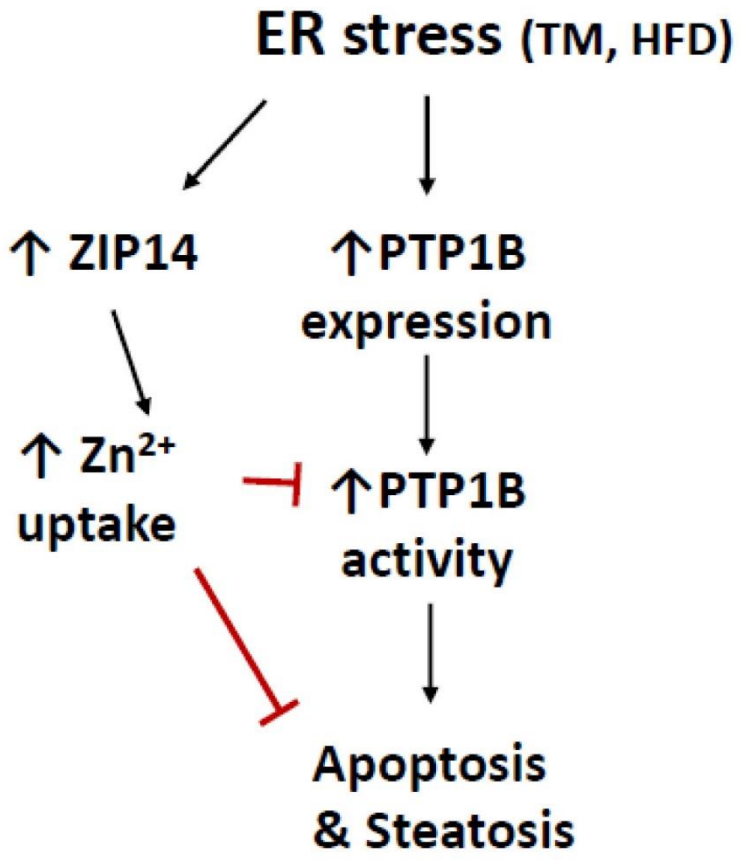

Figure 4-11. Proposed model for ZIP14-mediated zinc transport and inhibition of PTP1B activity.

CHAPTER 5
IDENTIFICATION OF THE TRANSCRIPTION FACTOR(S) THAT REGULATE ZIP14 EXPRESSION DURING ER STRESS

Introductory Remarks

UPR activation induces a variety of genes involved in protein folding, degradation, and trafficking to restore ER homeostasis via activation of transcription factors such as ATF6α, ATF4, and XBP-1 (12). For example, genes such as *Grp78*, *Grp94*, and *Protein disulfide isomerase* (*Pdi*) are transcriptionally up-regulated by ATF6α and XBP-1 during the UPR activation (120). In the pro-apoptotic pathway of the UPR, ATF4 transcriptionally up-regulates *Chop* mRNA expression (121).

In chapter 3, it was demonstrated that *Zip14* gene expression is significantly elevated by TM administration in mouse liver and HepG2 hepatocytes (Figure 3-3 and 3-5). Previous reports that performed global transcriptional profiling suggest that *Zip14* is one of the genes targeted by transcription factors involved in UPR signaling. RNA sequencing data from liver-specific *Atf4* KO mice showed significantly reduced *Zip14* mRNA induction by TM administration in these KO mice compared to WT mice (119) . Similarly, RNA sequencing analysis from isolated *Atf6α* KO fibroblasts showed no induction of *Zip14* mRNA in response to TM treatment (122). These data suggest that the transcription factors ATF4 and/or ATF6α may upregulate *Zip14* mRNA during TM treatment.

The purpose of the research reported in this chapter is to identify the transcription factor(s) that regulate *Zip14* gene expression during TM-induced ER stress. For efficient gene manipulation, HepG2 hepatocytes were used for these experiments. This chapter will focus on identification of transcriptional regulation of the *Zip14* gene by TM treatment and analysis of the *Zip14* promoter region in order to find

potential binding site(s) for ATF4 and ATF6α. In addition, transcription factor binding affinity to the *Zip14* promoter region was examined using ChIP-PCR.

Results

Zip14 mRNA Expression is Regulated at Transcriptional Level during TM Treatment

During TM treatment of the HepG2 cells, mRNA expression of *Zip14* was increased in dose-dependent manner, and treatment with actinomycin D (Act D), a transcription inhibitor, suppressed its induction (Figure 5-1A). This indicated that *Zip14* induction is likely regulated at the transcriptional level. This was supported by the time-dependent increases in both *Zip14* mRNA and *Zip14* hnRNA, a pre-mRNA present before splicing. Their similar expression patterns following TM treatment (Figure 5-2B), demonstrated transcriptional regulation of *Zip14* mRNA by TM.

Zip14 is Transcriptionally Regulated by ATF4 and ATF6 during TM Treatment

ATF4 and ATF6α have been shown to have a strong binding affinity to the cAMP-response Element (CRE) sequence (TGACGT(C/A)(G/A)) (Figure 5-2A). Matinspector software analysis revealed that the *Zip14* promoter has a strong potential binding site for ATF4 and ATF6α at -94 to -89, which matches with the core motif of the CRE (Figure 5-2B). This sequence was conserved in mouse and human *Zip14*. HepG2 cells were transfected with either *Atf4* or *Atf6α* siRNA to determine which transcription factor was responsible for *Zip14* upregulation (Figure 5-3A and B). Knockdown of *Atf4* resulted in significantly reduced *Zip14* induction both 6 h and 24 h after TM treatment (Figure 5-3C), whereas knockdown of *Atf6α* reduced induction only 24 h after TM treatment (Figure 5-3E). This suggests that *Zip14* is regulated by both ATF4 and ATF6α, but there may be a time-dependent regulation. To ensure actual binding of transcription factors to

the potential binding site of the *Zip14* promoter, ChIP-PCR was conducted. Detection of DNA enrichments revealed that ATF4 highly bound 6 h after TM treatment, then the binding was reduced thereafter (Figure 5-3D). However, enhanced binding of ATF6α was only detected 24 h after TM (Figure 5-3F). Western blotting showed that ATF4 expression was induced until 12 h after TM treatment, and then decreased by 24 h (Figure 5-4A). This may account for the time-dependent regulation of *Zip14* by ATF4 and ATF6α in which ATF4 binds first due to higher binding affinity, but ATF6α is able to bind after ATF4 expression is reduced (Figure 5-4B). These data demonstrated that *Zip14* is transcriptionally regulated by ATF4 and ATF6α during TM-induced ER stress.

Discussion

UPR activation induces a variety of genes involved in restoring ER homeostasis via the activation of transcription factors such as ATF6α, ATF4, and XBP-1 (12). The data in the current chapter indicates that one aim of UPR pathway is to modulate zinc metabolism based on the finding that *Zip14* is a transcriptional target of ATF4 and ATF6α as described in Figure 6-4B. ATF4 and ATF6α exhibit significant binding affinity to the CRE sequence (5'-TGACGTGA-3') (123-125). The *Zip14* promoter has a CRE-like sequence (5'-TGACGcGc) (Figure 5-2A), and it was demonstrated that both ATF4 and ATF6α bound to that CRE-like sequence after TM treatment in a time-dependent manner, leading to production of *Zip14* transcripts. These data confirm the observation from previous global transcriptional profiling which showed significantly reduced *Zip14* gene expression after TM treatment in liver-specific *Atf4* KO mice (119), and in *Atf6α* KO fibroblasts (122). The ATF4- and ATF6α-mediated *Zip14* gene modulation may indicate that the UPR aims to increase cellular zinc availability through this transcriptional mechanism. This is supported by a previous report where XBP-1-mediated h*ZnT5*

upregulation was demonstrated during ER stress via direct binding to its promoter (81). As ZnT5 is a zinc importer that provides zinc into the early secretory pathway, the upregulation of ZnT5 through the UPR pathway implies an increased zinc requirement in the ER during ER stress. Impaired hepatic GRP94 expression during ER stress shown in *Zip14* KO mice (Figure 4-4A and 4-8B) supports this notion, suggesting functional zinc transporter activity is required for complete induction of UPR targets. Although further investigation is required, GRP94 may be an enzyme regulated by zinc or the zinc-responsive transcription factor, MTF-1.

Similar to the regulation of zinc transporters such as *Zip14* and *ZnT5*, it has been reported that ER stress transcriptionally up-regulates hepcidin, which is a master regulator hormone for iron metabolism (101). CREBH has been shown to bind to the *Hepcidin* promoter region, causing iron accumulation in mouse liver and spleen. Indeed, *Crebh* KO mice did not induce *Hepcidin* mRNA expression following TM administration. Of note is that the *Zip14* promoter lacks the sequence required for the CREBH binding, therefore CREBH-mediated *Zip14* regulation was not examined in this project.

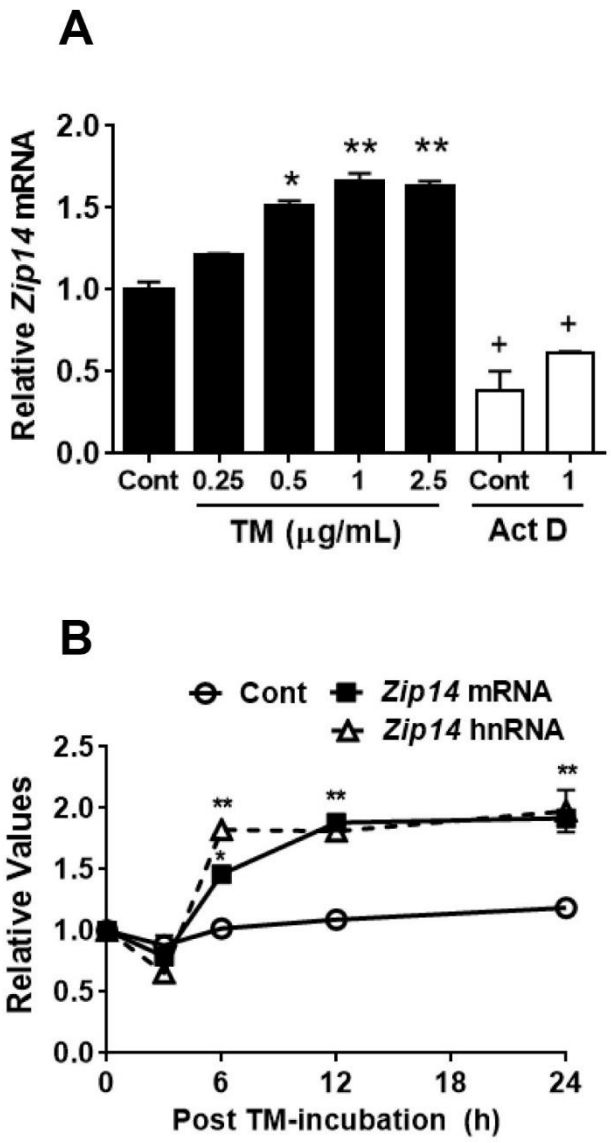

Figure 5-1. Up-regulation of *Zip14* mRNA by TM treatment is regulated at the transcriptional level in hepatocytes. (A) Relative expression of *Zip14* mRNA in HepG2 hepatocytes treated with TM (1 μg/ml) and/or actinomycin D (2 μg/ml) for 12 h. (B) Relative expression of *Zip14* mRNA and hnRNA in HepG2 hepatocytes treated with TM (1 μg/ml) or vehicle.

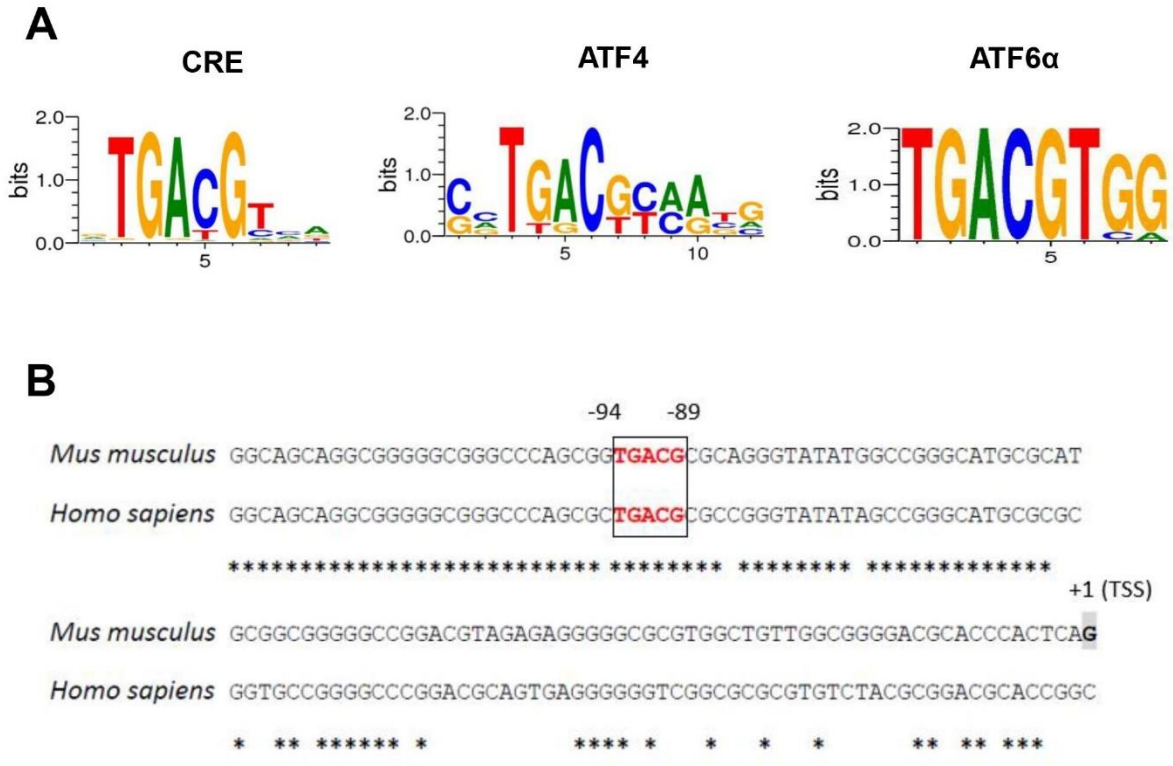

Figure 5-2. The *Zip14* promoter contains a CRE sequence, a potential binding site for ATF4 and ATF6α. (A) Consensus motif of CRE and binding motifs of ATF4 and ATF6. (B) The sequence of mouse and human *Zip14* promoter regions (from -120 to +1). Identical nucleotides are indicated by an *asterisk*. The TGACG sequence (from -94 to -89) is marked by a box.

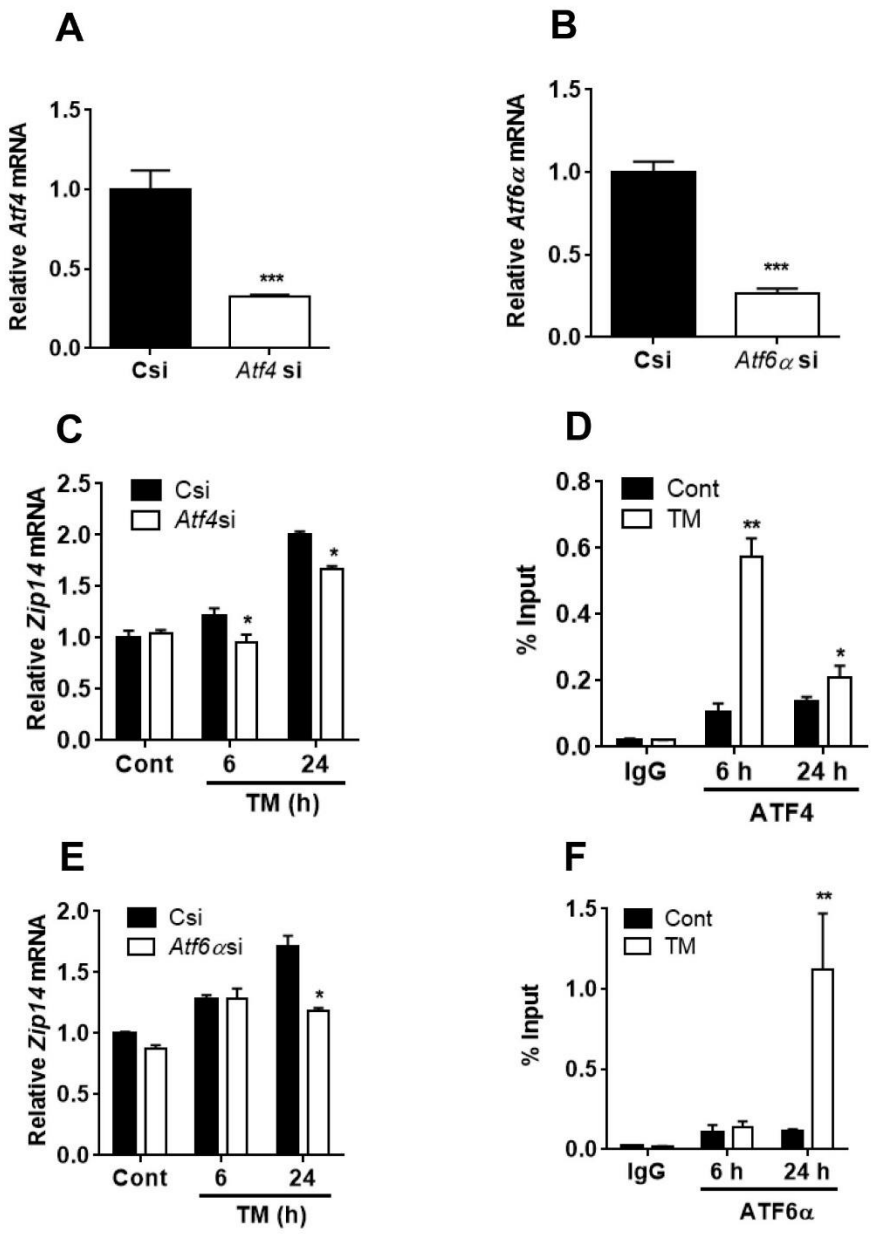

Figure 5-3. *Zip14* is transcriptionally regulated by ATF4 and ATF6α during TM treatment in hepatocytes. HepG2 hepatocytes were transfected with control siRNA, *Atf4* siRNA (A, C, E) or *Atf6α* (B, D, F) siRNA. (A and B) Relative expression of *Atf4* (A) and *Atf6α* mRNA (B) after siRNA transfection. (C and E) Relative expression of *Zip14* mRNA in TM-treated HepG2 cells (1 μg/ml) after transfection with control siRNA, *Atf4* siRNA (C), or *Atf6α* siRNA (E). (D and F) Enrichment of DNA bound to the ATF4 antibody (D) or the ATF6α antibody (F) were measured by qPCR after ChIP assays in TM-treated HepG2 cells (1 μg/ml). Non-specific rabbit IgG antibody was used as a negative control. All data are represented as mean ± SD. *$p < 0.05$, **$p < 0.01$.

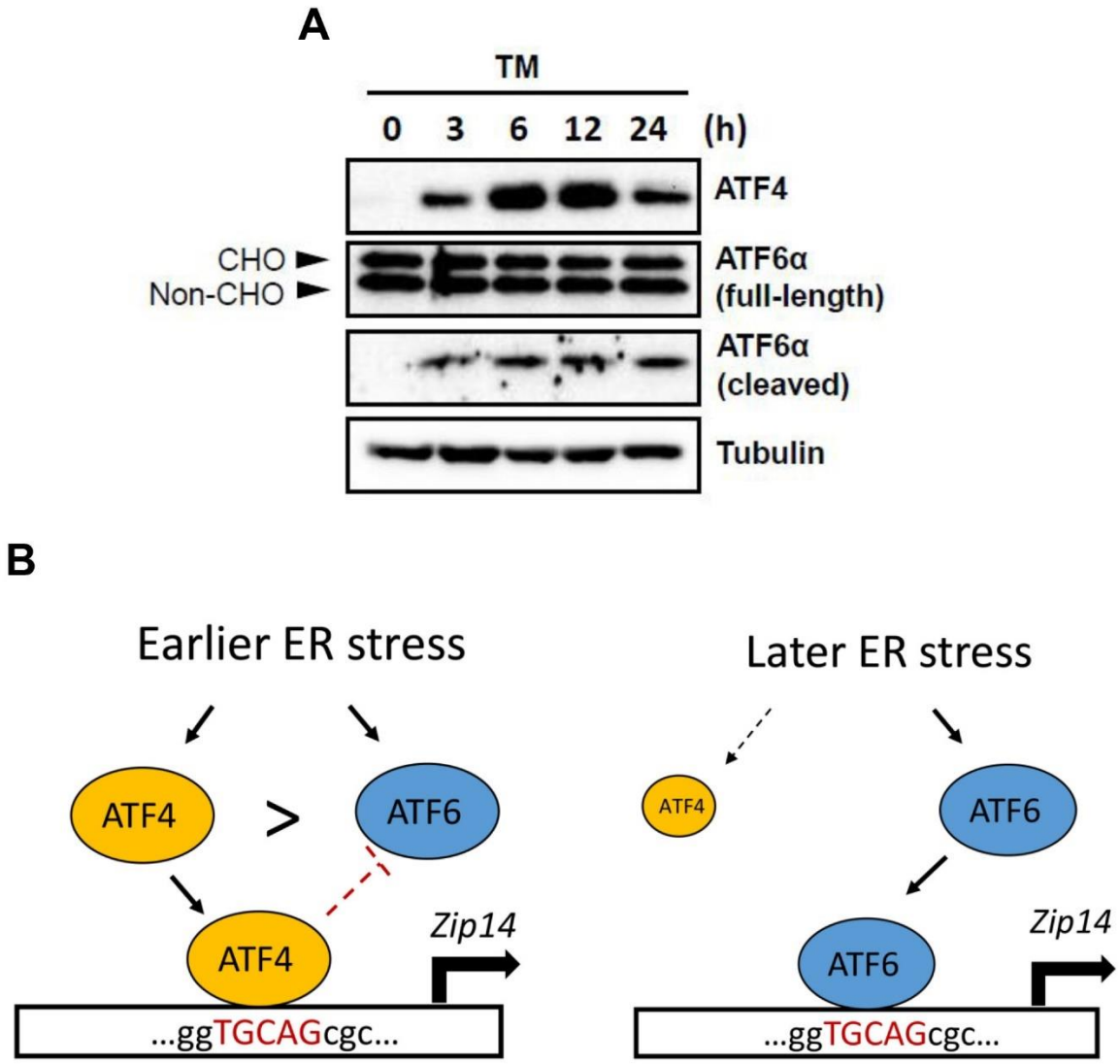

Figure 5-4. Time-dependent regulation of *Zip14* by ATF4 and ATF6α. (A) Immunoblot analysis of ATF4 and full-length and cleaved ATF6α in TM-treated HepG2 cells (1 μg/ml). (B) Proposed model for time-dependent regulation of *Zip14* by ATF4 and ATF6α during earlier and later ER stress. The TGACG sequence, a binding site for ATF4 and ATF6α, is marked with red.

CHAPTER 6
DETERMINATION OF THE IMPACT OF ZINC DEFICIENCY ON ER STRESS IN VIVO

Introductory Remarks

Systemic zinc deficiency has been associated with a number of human disorders such as growth retardation, immune deficiencies, dermatitis, sexual immaturity, and neurodegenerative disorders (100). Furthermore, zinc deficiency has been implicated in ER stress. Zinc restriction mediated by treatment of cells with a cell permeable zinc chelator, TPEN, induces ER stress and results in activation of the UPR in some eukaryotic cell lines and yeast (78, 79). In HeLa cells, zinc deficiency in the cytosol and other organelles induced by knockdown of *Zip13*, which transports vesicular zinc to the cytosol has been shown to cause ER stress (63). It has also been demonstrated that hepatic zinc deficiency mediated by chronic ethanol exposure in rats triggered ER stress-mediated apoptotic cell death (80).

However, it has not been established if consumption of a zinc deficient diet can induce ER stress in an *in vivo* model. Furthermore, the impact of dietary zinc on apoptotic cell death and other insults caused by ER stress is unclear. In order to obtain answers to these questions, the purpose of this chapter is to determine the impact of dietary zinc deficiency on ER stress *in vivo*. To examine this question, mice were fed either a zinc deficient, zinc adequate or zinc supplementation diet for 2 weeks, then TM was administered to model ER stress. This chapter will focus on indices of ER stress including apoptosis and hepatic steatosis in mice fed diets that supply low, adequate or supplemented amounts of zinc. In addition, activity of PTP1B was measured in this setting since previous chapters have presented data indicating that zinc-mediated PTP1B inhibition is important for ER stress adaptation.

Results

No Activation of UPR in Mice Fed ZnD

Mice were fed ZnD (<1 mg Zn/kg diet) or ZnA (30 mg Zn/kg diet) for two weeks. During the dietary zinc manipulation period, there was no statistical difference in weekly food intake (Figure 6-1A) or net body weight change (Figure 6-1B) among groups. After two weeks of the controlled zinc intake period, mice fed ZnD displayed significantly decreased levels of serum, liver, and pancreatic zinc compared to those of ZnA mice, demonstrating ZnD triggered systemic zinc depletion in the mice (Figure 6-2A). As the liver and pancreas are highly affected by ER stress, gene and protein expression of GRP78 and CHOP, which are common markers of UPR activation, were analyzed in these two organs. In general, ER stress leads to induction of GRP78 and CHOP through UPR activation (11). However, no difference in expression of GRP78 or CHOP mRNAs was observed in the tissues of mice fed ZnD (Figure 6-2C, E-G). CHOP protein expression was not detected by immunoblot in either tissue since the protein is only expressed during ER stress. GRP78 protein was not different. These results indicate that diet-mediated zinc deficiency is not a factor that causes ER stress in a mouse model, unlike previously reported *in vitro* experiments that modeled zinc deficiency using a TPEN treatment (78, 79).

Although zinc deficiency mediated by the ZnD diet did not cause differences in markers of ER stress in mice, it is possible that zinc might play a role during induced ER stress. Thus the next research aim was to investigate the response of the liver to pharmacologically induced ER stress when the zinc content of the diet was varied. After two weeks, the levels of serum zinc and liver zinc (Figure 6-3A) reflected the amount of dietary zinc provided to each group. Following the TM injection, extra zinc was

accumulated in the liver during (Figure 6-3A), which was consistent with observations in the Chapter 3. However, the level of extra zinc uptake during the TM challenge followed the dietary zinc level. The ZnS group had a significantly greater level of extra hepatic zinc accumulation by 48 hour after the TM was administered, whereas the ZnD group displayed a delayed accumulation by 12 h after TM administration.

Dietary Zinc is Essential for Suppression of ER Stress-Induced Apoptosis by Regulating the Pro-Apoptotic p-eIF2α/ATF4/CHOP Pathway

To examine whether dietary zinc content influences adaptation to ER stress markers of the UPR were analyzed. In comparison to ZnA group, mice fed ZnD diet expressed greater levels of p-eIF2α, ATF4, and CHOP, especially later in the TM challenge (24 h and 48 h) (Figure 6-3B and C). To the contrary, mice fed ZnS diet showed less expression of those proteins. Expression of GRP78 and GRP94 tended to increase in the ZnD group, suggesting that requirement for ER chaperones might be enhanced in the ZnD group in order to help maintain protein folding. To confirm the western blot data, the extent of apoptosis was evaluated using the TUNEL assay in sectioned liver tissue (Figure 6-4A). Consistent with pro-apoptotic protein expression patterns, greater amounts of TUNEL-positive cells were observed in mice fed ZnD during the TM challenge compared to those fed ZnA and ZnS. There was no difference in TUNEL-positive cells between ZnA and ZnS groups. Taken together, these observations suggest that adequate dietary zinc is required for suppression of ER stress-mediated apoptotic cell death via modulation of the pro-apoptotic p-eIF2α/ATF4/CHOP pathway.

Dietary Zinc is Essential for Suppression of ER Stress-Induced Steatosis

ER stress has been shown to cause hepatic steatosis and tissue damage (29, 32). To test for the accumulation of lipid droplets in mice, liver sections were stained with H&E. Lipid droplets were more abundant in mice fed ZnD diet in response to TM compared to those fed ZnA and ZnS (Figure 6-4B). No difference was observed between ZnA and ZnS groups. This was supported by a direct measurement of liver triglycerides where mice fed ZnD showed significantly greater triglyceride levels compared to other groups during the TM challenge (Figure 6-4C).

Next, serum ALT was measured to analyze liver tissue damage caused by ER stress (Figure 6-5). Serum ALT is a common marker of liver damage, since ALT can leak into the plasma from damaged hepatocytes (30). ZnD mice displayed higher levels of ALT activity in their serum during the TM treatment compared to mice fed ZnA and ZnS groups. No difference was observed between ZnA and ZnS groups.

During ER Stress, PTP1B Activity was Increased in Mice Fed ZnD

As described in detail in the Chapter 4, zinc may contribute to ER stress adaptation by suppressing PTP1B activity. Thus, hepatic PTP1B activity was measured in mice fed diets with the different amounts of zinc. During TM challenge, PTP1B expression was increased, however, the protein expression levels were comparable among the three groups (Figure 6-6A). In contrast, PTP1B activity was significantly elevated in mice fed ZnD, whereas activity in ZnA and ZnS was not different from untreated controls (Figure 6-6B). These results suggest that the higher levels of ER stress-induced apoptosis and steatosis during ZnD diet might result from the elevated PTP1B activity due to low availability of zinc.

Discussion

Zinc is essential for normal ER function, and its deficiency has been suggested to cause ER stress (79, 81). This perception is largely based on *in vitro* experiments that used TPEN, a cell permeable zinc chelator, to restrict available zinc (78, 79). In previous reports, the addition of TPEN to cells activated the UPR in yeast and mammalian cells. These conditions produce an equimolar chelation of TPEN: Zn that leads to cell death (126, 127).

In this chapter, it was demonstrated that after 2 weeks, there was no activation of UPR markers in mice fed a ZnD that yielded signs of dietary zinc deficiency. The discrepancy between previous *in vitro* experiments and current *in vivo* experiments may be derived in part from the systemic homeostatic mechanisms in animals. Human and animals fed a low zinc diet can react to reduced zinc intake to maintain the cellular zinc homeostasis (44). Therefore, consumption of a low zinc diet may not be a critical factor that triggers ER stress *in vivo.* However, it should still be noted that ZnD may potentiate ER stress in a much longer and severe setting of zinc deficiency. In the current study, a 2 week period of dietary zinc manipulation was used, as it provides enough time to induce signatures of systemic zinc deficiency in the mouse model according to previous observations (128, 129). Long-term (e.g. > 1 month) zinc deficient diet feeding was avoided because it triggers a number of complications.

A number of pathological conditions, such as diabetes, neurological disorders, and hepatic steatosis, are related to ER stress (18). Disturbed zinc homeostasis has been implicated in these diseases (80). Thus this chapter aimed to investigate the impact of dietary zinc status on ER stress in mice induced by TM administration. In this study, the liver was focused upon since it is the site where a massive number of

proteins are synthesized, which makes this organ more susceptible to protein misfolding. Additionally, observations from Chapters 3 and 4 showed no changes in zinc homeostasis in other tissues including the pancreas, kidney, WAT, and spleen (Figure 3-1 and 3-2). In agreement with these results, Sun et al. reported that zinc deficiency induces ER-stress mediated apoptosis in livers of rats after chronic ethanol consumption (80). Collectively, our findings suggest that a diet providing an adequate source of zinc is critical for adapting to ER stress and suppressing apoptotic cell death.

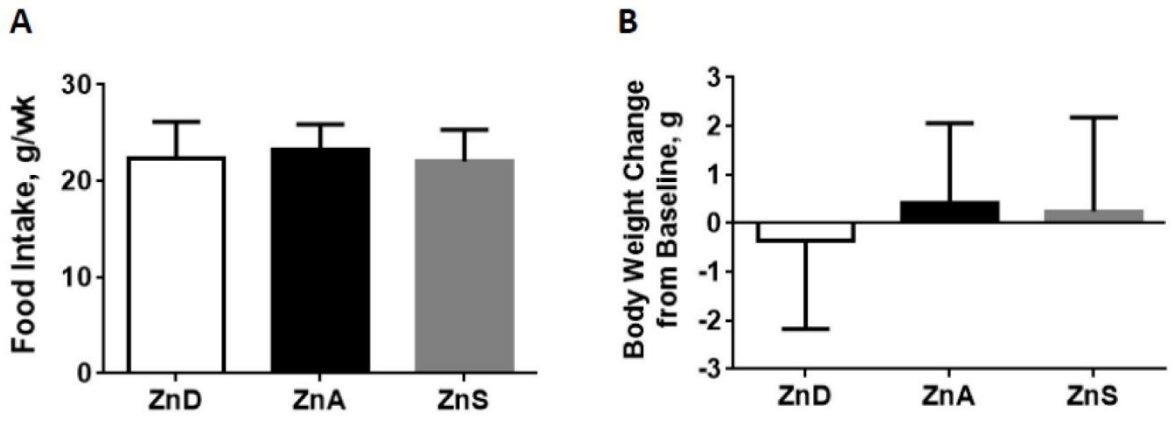

Figure 6-1. No difference in food intake and net body weight change was found among mice fed with zinc-manipulated diets. Mice were fed either ZnD (<1 mg/kg diet), ZnA (30 mg/kg diet), or ZnS (180 mg/kg diet) diets for 2 wk. Weekly food intake (A) and net change in body weight (B) during dietary study. All data are represented as mean ± SD. n = 15 mice.

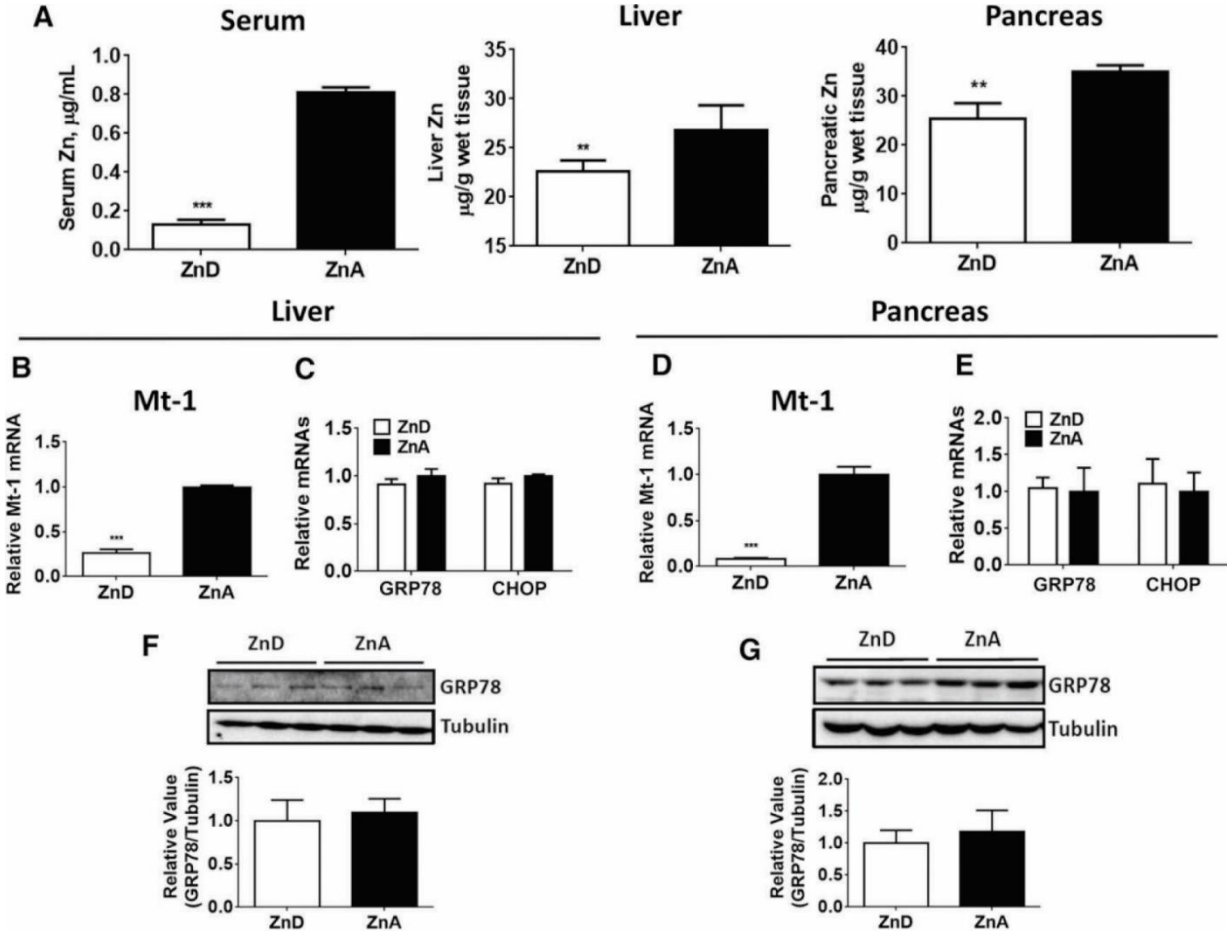

Figure 6-2. No activation of ER stress markers in mice fed a ZnD. Mice were fed either ZnD (<1 mg/kg diet) or ZnA (30 mg/kg diet) diets for 2 wk. (A) Serum, liver, and pancreatic zinc concentration measured by AAS. (B-E) Relative expression of *Mt-1* (B and D) and *Grp78* (C and E) in liver and pancreas. (F and G) Immunoblot analysis of GRP78 in liver (F) and pancreas (G) of mice. In panels F and G, individual blots were analyzed by digital densitometry. All data are represented as mean ± SD, n = 3–4 mice. **p < 0.01, ***p < 0.001.

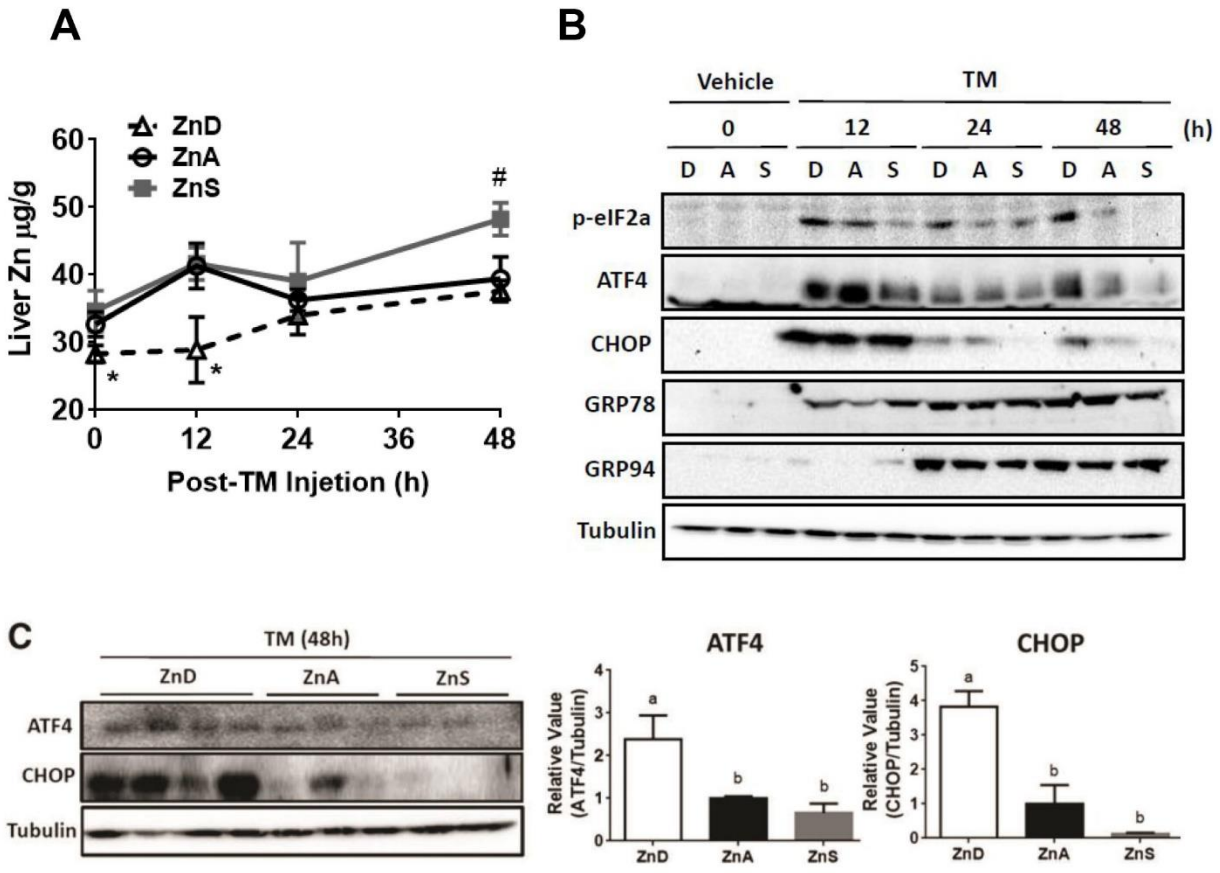

Figure 6-3. Mice fed ZnD display a delayed hepatic zinc accumulation, which coincides with greater expression of pro-apoptotic proteins. Mice were fed either ZnD (<1 mg/kg diet), ZnA (30 mg/kg diet), or ZnS (180 mg/kg diet) diets for 2 wk, which was followed by administration of TM for the indicated time. (A) Hepatic zinc concentrations were measured by AAS. (B) Immunoblot analysis of markers of ER stress in liver lysates of mice. (n= 3-4, pooled samples) (C) Individual samples (48 h after TM, n = 4) were blotted and measured using digital densitometry. All data are represented as mean ± SD. *$p < 0.05$ compared with ZnA or ZnS, #$p < 0.05$ compared with ZnD or ZnA. Labeled means without a common letter differ significantly ($p < 0.05$).

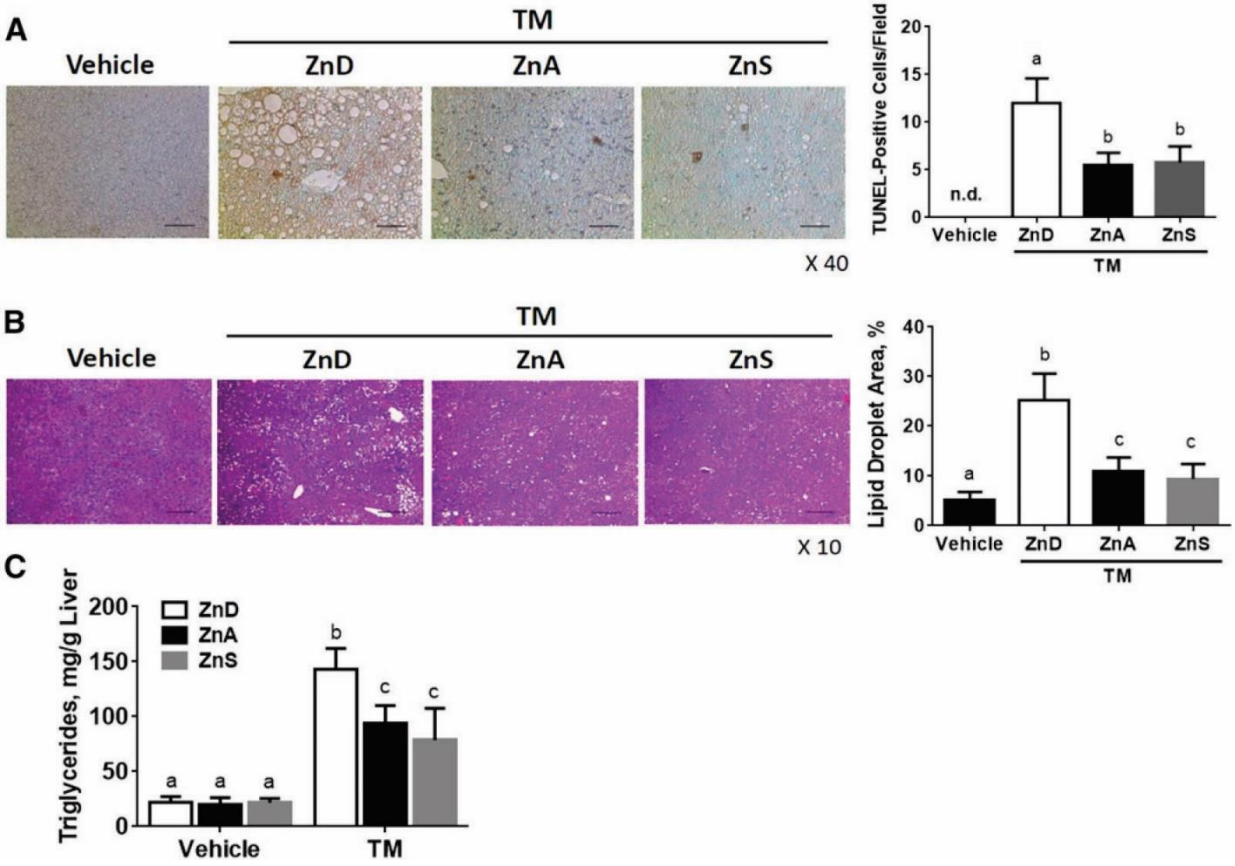

Figure 6-4. Dietary zinc is essential for suppression of ER stress-induced apoptosis and steatosis. Mice were fed either ZnD (<1 mg/kg diet), ZnA (30 mg/kg diet), or ZnS (180 mg/kg diet) diets for 2 wk, which was followed by administration of TM for 48 h. (A) Representative images of the TUNEL assay in liver sections of mice. Images are at 40X magnification; bars = 25 mm. (B) Representative images of H&E staining in liver sections of mice. Images are at 10X magnification; bars = 100 mm. (C) Quantification of triglycerides in livers of mice. All data are represented as mean ± SD. n = 3-4 mice. Labeled means without a common letter differ significantly ($p < 0.05$).

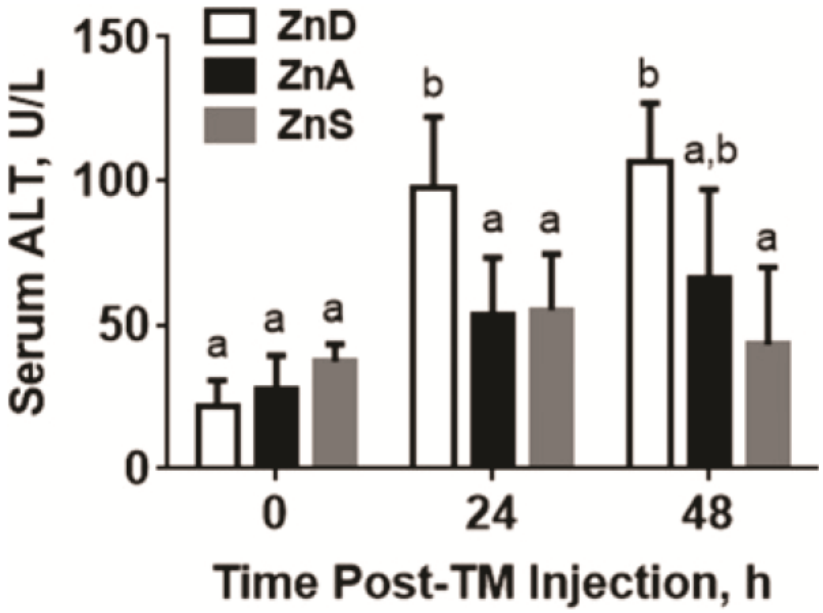

Figure 6-5. Mice fed ZnD display greater liver damage during TM challenge. Mice were fed either ZnD (<1 mg/kg diet), ZnA (30 mg/kg diet), or ZnS (180 mg/kg diet) diets for 2 wk, which was followed by administration of TM for the indicated time. Serum ALT activity of mice was measured. All data are represented as mean ± SD. n = 3-4 mice. Labeled means without a common letter differ significantly ($p < 0.05$).

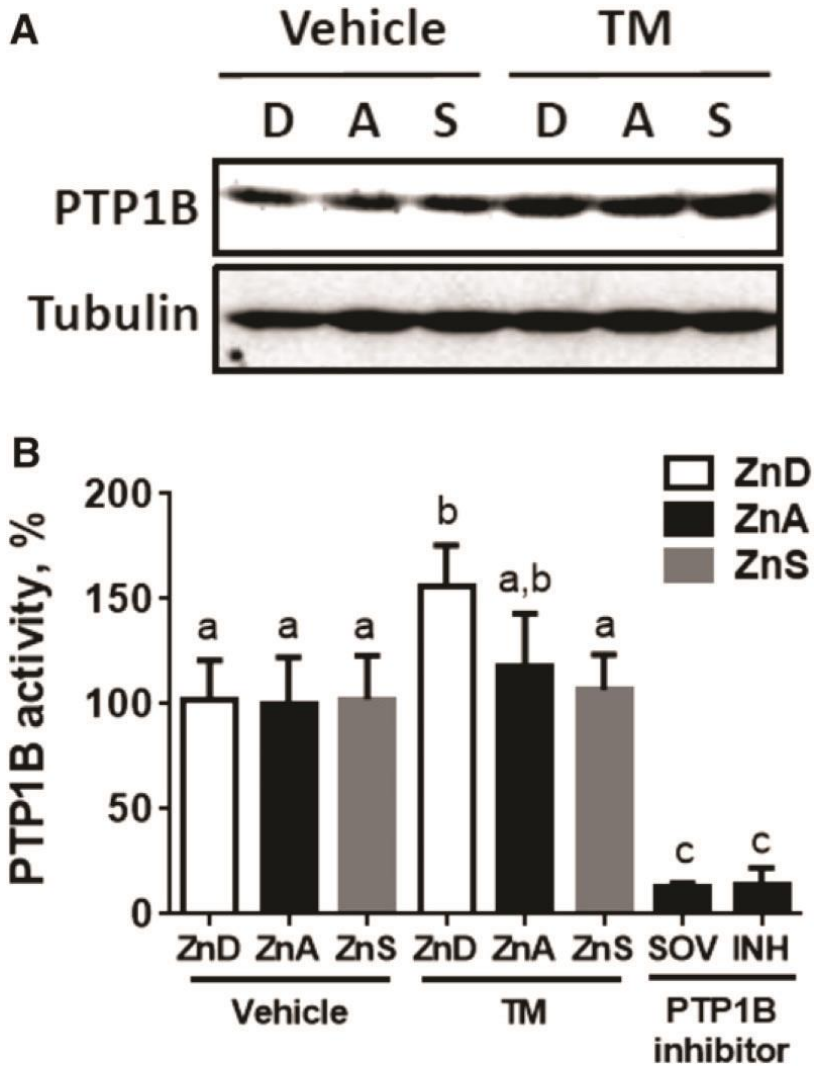

Figure 6-6. Mice fed ZnD display greater hepatic PTP1B activity during TM challenge. Mice were fed either ZnD (<1 mg/kg diet), ZnA (30 mg/kg diet), or ZnS (180 mg/kg diet) diets for 2 wk, which was followed by administration of TM for 48 h (A) Immunoblot analysis of PTP1B in liver lysates of mice (n= 3-4, pooled samples). (B) Measurement of PTP1B activity in livers of mice. PTP1B inhibitors were used as negative controls of the assay. All data are represented as mean ± SD. n = 3-4 mice. Labeled means without a common letter differ significantly ($p < 0.05$).

CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

The general aim of this project was to gain a better understanding of the regulation and function of zinc and zinc transporters during ER stress and in the UPR pathway. To examine this, four specific research aims were set: determination of changes in zinc metabolism and zinc transporter expression during ER stress; delineation of the specific role of ZIP14 during ER stress; identification of the transcription factor(s) that regulate *Zip14* expression during ER stress; and determination of the impact of zinc deficiency on ER stress *in vivo*.

To investigate these aims, the *Zip14* KO mouse model was primarily used for *in vivo* experiments. *Zip14* KO mice are a suitable model for these research aims as these mice display impaired hepatic zinc uptake, which enables us to observe what happens when the zinc requirement is increased during ER stress. In parallel, HepG2 hepatocytes were used to support the animal studies. As described in the schematic model (Figure 7-1), TM-, and HFD-induced ER stress triggers ZIP14-mediated zinc accumulation in mouse liver. During TM-induced ER stress, a cycle is initiated where *Zip14* up-regulation in hepatocytes is modulated at the transcriptional level by UPR-activated transcription factors, ATF4 and ATF6α. ZIP14 mediates zinc transport into hepatocytes to inhibit PTP1B activity, which acts to suppress apoptosis and steatosis associated with hepatic ER stress. In support of this model, impaired hepatic zinc uptake in *Zip14* KO mice during ER stress coincides with greater expression of pro-apoptotic proteins in the UPR pathway including phosphorylated eIF2α, ATF4 and CHOP. In addition, ER stress-induced *Zip14* KO mice show greater levels of hepatic steatosis due to higher expression of genes involved in de novo FA synthesis, which are

suppressed in ER stress-induced wild type mice. During ER stress, the UPR-activated transcription factors, ATF4 and ATF6α, transcriptionally up-regulate *Zip14* expression. And finally, *Zip14* KO mice showed greater hepatic PTP1B activity during TM- and HFD-induced ER stress.

In this project, the function of hepatic ZIP14 was primarily focused upon. Future studies could focus on other zinc transporters of which expression is significantly increased during ER stress. For example, *ZnT3* gene expression was markedly enhanced (~15-fold) in mouse liver following TM administration. Although hepatic abundance of ZnT3 protein is known to be low, its expression after TM could be significant, which raises the possibility of a potential role for hepatic ZnT3 during ER stress. This notion is supported by previous report that showed a protective role of ZnT3 during ER stress in neuroblastoma cells (83). Similar to its function in the brain, ZnT3 may play a protective role in the liver during ER stress. In addition, expression of ZIP2, ZIP6, ZIP7, ZnT1, ZnT5, ZnT7, and ZnT10 were significantly altered in TM-challenged mouse liver, but their function has not been examined using *in vivo* models. In addition, function of ZIP14 in other tissues could be further studied. Although zinc concentration and ZIP14 expression were not changed following TM injection in pancreas, kidney, spleen and adipose tissue at the time of examination (12 h after TM administration), there is still the possibility the zinc homeostasis is altered at different time point during a TM challenge. Of interest is role of ZIP14 in pancreatic tissue as the tissue is known to express significant amounts of ZIP14 and is highly affected by ER stress.

A potential future study could examine a role for zinc in the aggresome, which is a recently discovered cellular structure (130). The aggresome was first identified in the

characterization of a mutation of the cystic fibrosis transmembrane conducting regulator (CFTR), which produces a phenotype of protein misfolding and aggregation (131, 132). Aggresome formation occurs when the capacity of the proteasome is exceeded by the production of misfolded or unfolded proteins. Similar to what the UPR does for ER stress adaptation, aggresomes play a critical role in clearance of ER stress by sequestering aggregated proteins in cells (133). To my knowledge, the effect of zinc or other metals on formation and/or function of aggresome has not been studied. Normal aggresome formation requires a number of proteins. Therefore, it would be interesting to examine if the aggresome formation is influenced in response to cellular zinc status and/or zinc transporter activity, possibly through zinc-regulated or zinc-dependent proteins.

Additionally, the potential role of matrix metalloproteinase (MMP) during ER stress and in the UPR pathway is of interest. MMPs are zinc-dependent metalloenzymes involved in the various physiologic reactions including hydrolytic breakdown of connective tissue (134). Activity of MMPs has been implicated in human pathologies including cancer, arthritis, and heart disease (135). Some MMPs such as MMP-3 plays a role in the ER stress-induced apoptosis. It has been shown that TM treatment increases MMP-3 expression, which participates in neuronal apoptotic signaling (136). Similarly, MMP-9 is also shown to enhance ER stress in motor neurons, triggering neurodegeneration (137). It is well established that zinc is required for functional MMP activity (138, 139), which leads to a hypothesis that cellular zinc status and/or zinc transporter activity influence the MMP-mediated apoptosis during ER stress.

Supporting that notion, it has been shown that hepatic MMP-9 expression is influenced by dietary zinc level and functional ZIP14 activity in sepsis model of mice (140).

As shown in our model (Figure 7-1), ZIP14 is represented as a cell surface protein. Since ZIP14 undergoes endocytosis (66), such re-localization may yield physiologic outcomes that remain to be examined.

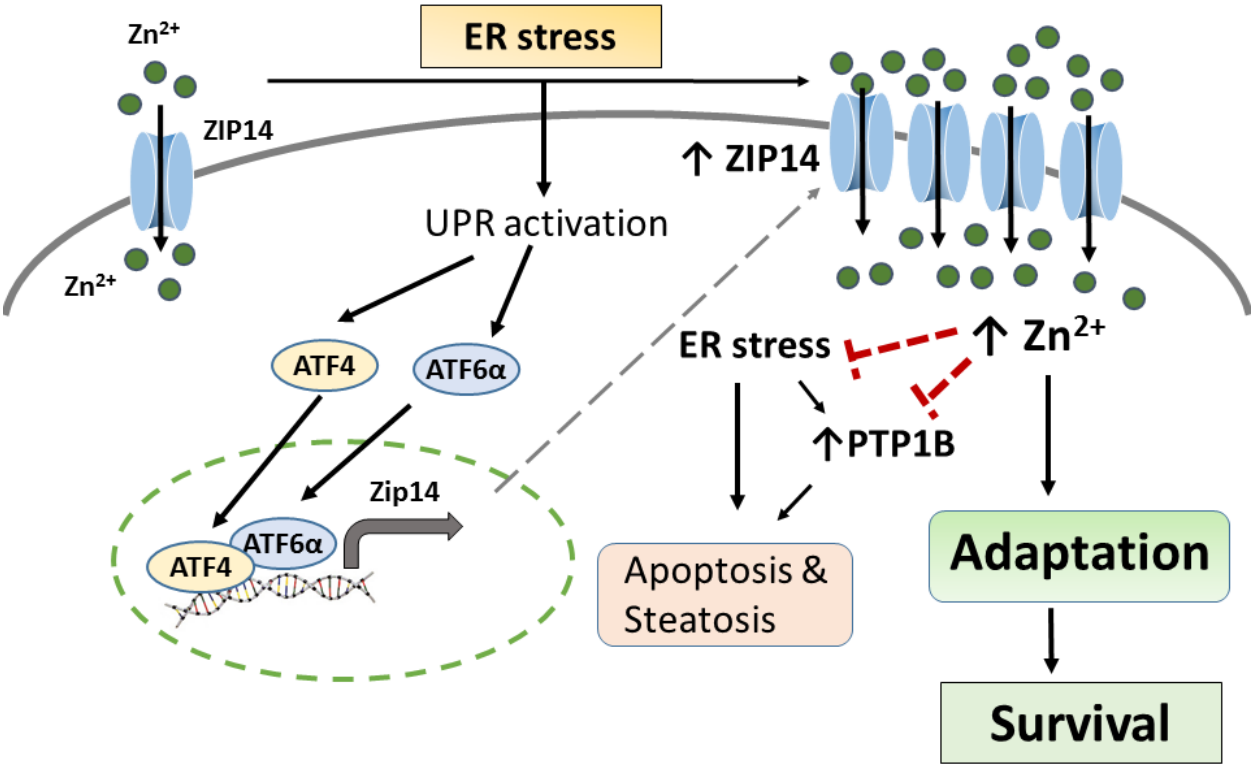

Figure 7-1. Role of ZIP14-mediated zinc transport in ER stress adaptation. Based on the data in this report, a cycle is proposed where ER stress sequentially increases expression of ATF4 and ATF6α. The transcription factors increase transcription of *Zip14* leading to increased ZIP14 in hepatocytes. Enhanced transporter activity increases intracellular zinc concentration leading to inhibition of PTP1B activity.

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BIOGRAPHICAL SKETCH

Min-Hyun Kim was born in Seoul, South Korea. He received his B.S. in food and nutrition from Yonsei University in 2010, and received his M.S. from the same department in 2012. Min-Hyun came to the University of Florida in fall 2013 to begin his Ph.D. studies in nutritional sciences and joined Dr. Robert J. Cousins' laboratory where he focused on the physiologic function of mammalian zinc transporters. He received his Ph.D. from the University of Florida in the summer of 2017.