Hepatic ZIP14-mediated zinc transport is required for adaptation to endoplasmic reticulum 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 that zinc is essential to maintain normal ER function. However, a role for zinc during hepatic ER stress is largely unknown despite important roles in metabolic disorders, including obesity and nonalcoholic liver disease. We have explored a role for the metal transporter ZIP14 during pharmacologically and high-fat diet–induced ER stress using Zip14−/− (KO) mice, which exhibit impaired hepatic zinc uptake. Here, we report that ZIP14-mediated hepatic zinc uptake is critical for adaptation to ER stress, preventing sustained apoptosis and steatosis. Impaired hepatic zinc uptake in Zip14 KO mice during ER stress coincides with greater expression of proapoptotic proteins. 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 WT mice. During ER stress, the UPR–activated transcription factors ATF4 and ATF6 transcriptionally up-regulate Zip14 expression. We propose 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 showed greater hepatic PTP1B activity during ER stress. These results show the importance of zinc trafficking and functional ZIP14 transporter activity for adaptation to ER stress associated with chronic metabolic disorders.

The endoplasmic reticulum (ER) is a cellular organelle where appropriate folding, assembly, and modification of proteins occur (1). Normal ER function can be compromised by pharmacological stimuli, such as tunicamycin (TM) or thapsigargin (2, 3), and by physiological stimuli, such as a high-fat diet (HFD), viral infection, oxidative stress, or chronic alcohol consumption (4–8). Perturbed ER function is collectively defined as ER stress. When ER stress occurs, mammalian cells activate the unfolded protein response (UPR), which comprises three discrete signaling pathways: the activating transcription factor 6 (ATF6) branch, the inositol-requiring enzyme 1 (IRE1) branch, and the dsRNA-activated protein kinase R-like ER kinase (PERK) branch (9). These pathways act to relieve the protein burden of the ER by enhancing protein-folding capacity through the expression of ER chaperones, including 78-kDa glucose-regulated protein (GRP78/BiP) and 94-kDa glucose-regulated protein (GRP94) (11). Cells survive if ER function is recovered through the activation of these adaptation pathways. However, if components of the UPR are compromised or ER stress is too severe, the UPR leads cells to apoptotic cell death via PERK–mediated phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (12). Phosphorylated eIF2α (p-eIF2α) selectively enhances translation of activating transcription factor 4 (ATF4) mRNA, which leads to up-regulation of C/EBP-homologous protein (CHOP), a transcription factor that induces expression of apoptosis-associated components. Thus, enhanced activation of the p-eIF2α/ATF4/CHOP pathway is a hallmark of maladaptation against ER stress. Sustained apoptosis from ER stress influences numerous pathological conditions, including diabetes and obesity (8, 13). In the liver, apoptosis disrupts homeostasis of lipid regulation, causing hepatic steatosis (14).

Zinc is an essential mineral required for normal cellular functions playing catalytic, structural, and regulatory roles (15). 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 Transporter; SLC30) and ZIP (Zrt-, Irt-like protein; SLC39) (16–18). Physiological stimuli have been shown to regulate the expression and function of some of these transporters. Ablation of some of these transporters results in zinc dyshomeostasis and metabolic defects. Ablation of Zip14 (Slc39a14) in mice leads to a phenotype that includes endotoxemia, hyperinsulinemia, increased body fat, and impaired hepatic insulin receptor trafficking (19–21). Zinc and zinc transporters have been implicated in ER stress and the UPR. Zinc deficiency may induce or exacerbate ER stress and apoptosis. In yeast and some mammalian cells, the UPR was activated by zinc restriction (22, 23). A rat model of alcoholic liver disease created by zinc deficiency was shown to trigger ER stress–induced apoptosis (24). We reported that consumption of a zinc-deficient diet by mice exacerbated ER stress–induced apoptosis and hepatic steatosis during TM-induced ER stress (25). Those recent studies showed that zinc can modulate the proapoptotic p-eIF2α/ATF4/CHOP pathway during ER stress. A number of zinc transporters have also 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 (23). In other vertebrate cells, knockdown (KD) of ZnT3, ZnT5, ZnT7, and

**Significance**

Unresolved endoplasmic reticulum (ER) stress corresponds with various chronic diseases, such as hepatic steatosis and diabetes. Although cellular zinc deficiency has been implicated in causing ER stress, the effect of disturbed zinc homeostasis on hepatic ER stress and a role for zinc during stress are unclear. This study reveals that ER stress increases hepatic zinc accumulation via enhanced expression of metal transporter ZIP14. Unfolded protein response–activated transcription factors ATF4 and ATF6α regulate Zip14 expression in hepatocytes. During ER stress, ZIP14–mediated zinc transport is critical for preventing prolonged apoptotic cell death and steatosis, thus leading to hepatic cellular adaptation to ER stress. These results highlight the importance of normal zinc transport for adaptation to ER stress and to reduce disease risk.

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Zip13 induced or potentiated ER stress (26–28). The role for ZIP14 in ER stress has not been defined.

In the present study, we explored a role of ZIP14 during pharmacologically, HFD-induced ER stress using a Zip14<sup>−/−</sup> (KO) mouse model, which exhibits impaired hepatic zinc uptake. We report that during UPR activation, ATF4 and ATF6 transcriptionally regulate Zip14 expression and that ZIP14-mediated hepatic zinc accumulation is critical for adaptation to ER stress by preventing sustained apoptosis and steatosis. We propose that ZIP14-mediated zinc transport suppresses protein-tyrosine phosphatase 1B (PTP1B) activity, which may contribute to overcoming ER stress.

**Results**

**TM Administration Alters Hepatic Zinc Homeostasis and Zinc Transporter Expression, Particularly ZIP14.** First, to define the effect of ER stress on zinc homeostasis, TM, a potent ER stress inducer, was injected i.p. into mice to induce systemic ER stress. TM is a compound that blocks N-glycosylation of newly synthesized protein. As measured by atomic absorption spectrophotometry, TM-injected mice showed significantly higher hepatic zinc concentrations, along with hypozincemia, compared with vehicle-injected mice (Fig. 1A and C). At 12 h after administration, liver zinc concentrations of the TM group were ∼15% higher than liver zinc concentrations of the control group. Administration of 65Zn by gavage confirmed markedly increased zinc uptake by the liver after administration of TM (∼1.6-fold) (Fig. 1B). No difference in zinc uptake was observed 12 h after administration of TM in tissues that are known to be highly affected by ER stress: the pancreas, kidney, and white adipose tissue (Fig. S1 B–D; WT). Therefore, the liver was our focus of further experiments. Because zinc homeostasis is maintained by zinc transporter activity, we examined expression of 24 known zinc transporter genes in the liver. The primer/probe sequences of genes are provided in Table S1. Among ZIP family transporters, Zip14 expression was most highly up-regulated by TM (∼8.2-fold) (Fig. 1D). Among ZnT family transporters, ZnT3 mRNA expression was markedly increased after administration of TM (∼15-fold) (Fig. 1E). However, ZnT3 has a low hepatic abundance (16), and was not further evaluated. ZIP14 mRNA and protein peaked at 12 h after administration of TM (Fig. 1F and G), which coincided with maximum zinc accumulation (Fig. 1A). The increased hepatic zinc concentration and ZIP14 expression were also observed in mice injected with another ER stress inducer, thapsigargin, an inhibitor of ER Ca<sup>2+</sup>-ATPase (Fig. S2 A and B). Because liver tissue is composed of multiple cell types, we used human hepatoma HepG2 cells to support the in vivo studies. To measure total cellular zinc level in HepG2 hepatocytes, cell lysates were incubated with the zinc fluorophore FluoZin3-AM. Intensity of fluorescence represents the total cellular labile zinc concentration. Consistent with data from the liver of TM-treated mice, TM treatment significantly increased fluorescence (∼2.1-fold after 12 h) (Fig. 1H), which coincided with increased ZIP14 expression (Fig. 1I).

**Zip14 KO Mice Display Impaired Hepatic Zinc Uptake and Higher Apoptosis During TM-Induced ER Stress.** Next, we examined a potential role of ZIP14 during ER stress because increased hepatic

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**Fig. 1.** TM-mediated ER stress increases hepatic zinc uptake and ZIP14. Hepatic Zn concentration (A) and 65Zn uptake (B) in mice (n = 3–4) are shown after administration of TM (2 mg/kg) or vehicle. (C) Serum Zn concentration in mice (n = 3–4) 18 h after administration of TM (2 mg/kg) or vehicle. Relative expression of members of the ZIP family (D) and ZnT family transporter (E) genes in the liver of mice (n = 3–4) are shown after administration of TM (2 mg/kg) or vehicle for 12 h. Time-dependent expression of Zip14 mRNA (n = 3–4) (F) and immunoblot analysis of ZIP14 and markers of ER stress (G) in liver lysates (n = 3–4, pooled samples) are shown after administration of TM or vehicle for the indicated times. (H) Total cellular Zn concentrations in HepG2 cells were determined by measurement of fluorescence after incubation with FluoZin3-AM (5 μM) following treatment with TM (1 μg/mL) or vehicle (n = 5). RFU, relative fluorescent unit. (I) 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, **P < 0.01, ***P < 0.001. Cont, control.
ZIP14 expression and zinc accumulation after TM coincided with a marked reduction of CHOP expression (Fig. 1G). To test the hypothesis that ZIP14-mediated zinc uptake is critical to suppress apoptosis, we used conventional Zip14−/− (KO) mice (Fig. 2A). During steady-state conditions, the liver of Zip14 KO mice did not show any indices of UPR activation (Fig. S3). Following TM, Zip14 KO mice exhibited less significant change in hepatic zinc uptake, whereas WT mice displayed significant zinc accumulation (Fig. 2B). Measurement of radioactivity after 65Zn administration by gavage also showed impaired zinc uptake in Zip14 KO mice during ER stress (Fig. 2C). Expression of UPR pathway components, including proapoptotic pathway proteins (p-eIF2α, ATF4, and CHOP) and adaptation pathway proteins (BiP and GRP94), was examined. WT mice showed a marked reduction of proapoptotic protein expression 24 h after administration of TM, whereas Zip14 KO mice displayed sustained expression (Fig. 2D). TUNEL assays of liver sections showed a significantly greater number of TUNEL-positive cells in Zip14 KO mice (~2.5-fold), indicating that the KO mice had a greater level of apoptosis after TM administration (Fig. 2E). Serum alanine aminotransferase activity, a common marker for liver damage, was also greater in Zip14 KO mice after administration of TM (~2.5-fold) (Fig. 2F). Furthermore, Zip14 KO mice expressed significantly less GRP94 than WT mice (~0.6-fold) (Fig. 2D), which may indicate an impaired protein folding function.

To test if there is a direct effect of zinc in the HepG2 hepatocytes, we knocked down Zip14 with siRNA and then supplemented zinc acetate along with pyrithione, a zinc ionophore. Pyrithione was added to improve zinc access under Zip14 KD conditions. The efficiency of Zip14 KD using siRNA transfection was ~90% (Fig. S4A). TM-treated Zip14 KD cells showed an ~24% lower cellular zinc level compared with TM-treated control cells, as measured by Fluorozin3-AM (Fig. 3A, first four bars). To establish an optimal level of zinc supplementation, we treated cells with zinc acetate, ranging from 2.5 to 20 μM. A maximal level of cellular zinc was obtained with TM-treated Zip14 KD cells when 5 μM zinc acetate was added along with pyrithione (Fig. 3A). Thus, 5 μM zinc acetate was used subsequently to test the effects of zinc supplementation. Higher expression of ATF4 (~2.6-fold) and CHOP (~1.8-fold) and lower expression of GRP94 (~0.4-fold) were observed in Zip14 KD cells after TM treatment (Fig. 3B, lanes 3 and 4). In response to TM, zinc-supplemented Zip14 KD cells expressed markedly reduced ATF4 (~34%) and CHOP (~50%) proteins compared with non-zinc-supplemented Zip14 KD cells (Fig. 3B, lanes 4 and 6). In addition, expression of GRP94 was increased (~1.6-fold) after zinc supplementation of the cells. TM treatment increased p-eIF2α expression, which was reduced after zinc supplementation in Zip14 KD cells (Fig. 3B). The lower cell viability shown in TM-treated Zip14 KD cells (Fig. 3C) was eliminated by zinc supplementation (Fig. 3D).

**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. Greater levels of lipid droplet accumulation in Zip14 KO mice were observed with H&E staining of the liver sections after administration of TM (~1.9-fold) (Fig. 4A). Quantification of triglyceride (TG) accumulation in the liver supported this observation (Fig. 4B). Because hepatic lipid homeostasis is mainly maintained by four mechanisms, namely, de novo fatty acid (FA) synthesis, FA oxidation, FA uptake into the liver, and lipoprotein secretion, we measured expression of genes involved in these pathways in the Zip14 KO mice to elucidate which mechanism(s) led to severe steatosis. With TM administration, genes involved in de novo FA synthesis, such as Srebpl, Acc, Fasn, and Scd1, were significantly suppressed in WT mice by ~60%, ~85%, and ~74%, respectively (Fig. 4C). In contrast, expression of Srebpl, Acc, Fasn, and Scd1 in TM-treated Zip14 KO mice was ~2.5-fold, ~2.2-fold, ~3.2-fold, and ~fourfold greater, respectively, than in TM-treated WT mice, indicating that FA synthesis in Zip14 KO mice was enhanced during the stress. In the same setting, there were no significant differences in expression of genes involved in FA oxidation, FA uptake, and lipoprotein secretion (Fig. 4D).

**HFD-Mediated ER Stress Is Exacerbated in Zip14 KO Mice.** Feeding an HFD has been used to trigger ER stress in rodents and provides a model with high physiological relevance (8). Thus, we analyzed indices of ER stress after feeding mice with an HFD (60 kcal% fat) or chow diet (12 kcal% fat) for 16 wk. Body weights of HFD-fed mice increased 10.2 ± 2.6 g, whereas chow-fed mice gained 2.5 ± 0.2 g. Both genotypes gained weight at the same rate during HFD feeding. WT mice showed enhanced ZIP14 expression after the HFD (Fig. 5A and B). Hepatic zinc concentrations in HFD-fed WT mice were ~17% higher compared with chow-fed WT mice, indicating that an HFD increases zinc uptake (Fig. 5C). However, zinc levels in HFD-fed Zip14 KO mice were unchanged, indicating that ZIP14 facilitates hepatic zinc accumulation. Although Zip9 mRNA expression was also increased by the HFD (Fig. 5A), its hepatic abundance is known to be low (16); thus, it is unlikely that ZIP9 contributes markedly to hepatic zinc uptake. This conclusion
is also supported by showing that hepatic zinc does not increase due to HFD consumption in Zip14 KO mice (Fig. 5C). Both genotypes showed elevated expression of UPR components, including p-eIF2α, ATF4, CHOP, BiP, and GPR94, indicating UPR activation in response to the HFD (Fig. 5D). However, HFD-fed Zip14 KO mice expressed greater levels of proapoptotic signatures, including p-eIF2α, ATF4, and CHOP, compared with HFD-fed WT mice, indicating that ablation of Zip14 worsens ER stress-associated
apoaptosis in this setting. Expression of ATF4 and CHOP was ~3.7-fold and ~3.2-fold greater, respectively, in Zip14 KO mice. Of particular note is the similarity of these results to the results attained with TM-induced ER stress. Specifically, the HFD-fed Zip14 KO mice expressed a lower level of GRP94 than HFD-fed WT mice (~0.6-fold). Hepatic TG accumulation after the HFD tended to be higher in Zip14 KO mice (Fig. 5E). Moreover, this observation coincided with significantly higher mRNA expression of Srebplc (~1.7-fold), Fasn (~1.8-fold), and Scd1 (~1.9-fold) (Fig. 5F), suggesting a greater level of FA synthesis led to more hepatic TG accumulation in the Zip14 KO mice.

**Increased PTP1B Activity Is Observed in Zip14 KO Mice During ER Stress.** As a possible mechanism underlying the increased ER stress in Zip14 KO mice, we focused on PTP1B, an enzyme that has been implicated in ER stress. PTP1B expression was increased in ER stress induced by TM and the HFD, and deletion of the protein in vivo and in vitro significantly reduced ER stress due to impaired zinc uptake, and thus disrupt adaptation to ER stress. Consistent with previous reports (30, 31), expression of proapoptotic proteins was significantly decreased in TM-KD condition. This finding supported by markedly higher cell viability in Ptp1b KD cells compared with TM-treated control cells (Fig. 6A). ATF4 and CHOP expression was reduced by ~42% and ~30%, respectively, in the Ptp1b KD condition. This finding was supported by markedly higher cell viability in Ptp1b KD cells after TM challenge (Fig. 6B). At steady state, Zip14 KO mice expressed less PTP1B protein than WT mice (~0.6-fold), but PTP1B activity was comparable (Fig. 6C and D). Following TM administration, hepatic PTP1B expression was increased in both genotypes (Fig. 6C, TM). However, PTP1B activity was significantly greater in Zip14 KO mouse liver (~1.7-fold) (Fig. 6D). The same pattern was observed in HFD-fed mice. PTP1B activity was significantly greater in the liver of Zip14 KO mice compared with WT mice (~1.5-fold), although the amount of protein expression was not different (Fig. 6E and F). In HepG2 hepatocytes, Zip14 siRNA KD did not alter PTP1B protein levels (Fig. 6G). In contrast, PTP1B activity was increased in the hepatocytes following Zip14 siRNA KD and TM treatment, but it was significantly reduced with zinc supplementation (Fig. 6H). These data demonstrate a direct effect of zinc on PTP1B activity in this setting. Collectively, these data suggest that normal hepatocytes suppress PTP1B activity during ER stress by regulating cellular zinc availability via ZIP14 induction. Impaired zinc uptake in the KO mice or by siRNA KD disturbs these events. A model underlying ZIP14-mediated adaptation and defense against ER stress is presented in Fig. 6I.

**Zip14 Is Transcriptionally Regulated by ATF4 and ATF6α During TM Treatment.** Because we observed an induction of Zip14 during TM- and HFD-induced ER stress, our next focus was to identify transcription factor(s) that regulate Zip14. For efficient gene manipulation, we performed in vitro experiments using HepG2 hepatocytes. During TM treatment, Zip14 mRNA was increased in a dose-dependent manner, and treatment with actinomycin D, a transcription inhibitor, suppressed induction, indicating that Zip14 induction by TM is regulated at the transcriptional level (Fig. 7A). Transcriptional regulation was supported by the time-dependent increases in Zip14 mRNA and heterogeneous nuclear RNA. Both exhibited similar expression patterns following TM treatment (Fig. 7B). Global gene expression screening data provided clues regarding the potential Zip14-regulating transcription factors ATF4 and ATF6α (34, 35). ATF4 and ATF6α have been shown to have a strong binding affinity to the
cAMP-response element (CRE) sequence [TGACGT(C/A)(G/A)] (Fig. 7C). Matinspector software analysis revealed that the Zip14 promoter has a potential binding site for both ATF4 and ATF6α at −94 to −89, which matches with a core motif of CRE, and was conserved in mice and humans (Fig. 7D). To test the expression of Zip14 under ATF4-KD or ATF6α-KD conditions, HepG2 hepatocytes were transfected with either Atf4 or Atf6α siRNA (Fig. S4 B and C). KD of Atf4 resulted in significantly reduced Zip14 induction at both 6 h and 24 h after TM treatment (Fig. 7E), whereas KD of Atf6α reduced induction only at 24 h after TM treatment (Fig. 7G). To ensure actual binding of transcription factors to the potential binding site of the Zip14 promoter, we conducted ChIP-PCR. Detection of DNA enrichment revealed high ATF4 DNA binding 6 h after TM treatment. Binding was reduced thereafter (Fig. 7F). Western blotting showed that ATF4 expression was induced until 12 h after TM treatment, and was then decreased at 24 h (Fig. 7I). This finding suggests a time-dependent regulation by ATF4 and ATF6α in which ATF4 first binds to the Zip14 promoter due to increased expression and higher binding affinity and ATF6α then binds to the promoter after ATF4 expression is decreased.

Discussion

In this report, we demonstrate that TM- and HFD-induced ER stress triggers ZIP14-mediated zinc accumulation in mouse liver. Both routes to ER stress yield remarkably comparable results (Figs. 2 and 5). This event is critical for adaptation to ER stress by preventing prolonged apoptosis and steatosis, possibly via inhibition of PTP1B activity. During TM-induced ER stress, Zip14 up-regulation in HepG2 hepatocytes is modulated at the transcriptional level by the UPR-activated transcription factors ATF4 and ATF6α. These findings are summarized in Fig. 8.

During ER stress, cellular metabolism is largely altered by activation of UPR pathways in an effort to restore ER homeostasis. This activation includes enhancing expression of proteins that help the ER folding function, such as ER chaperones, and reducing translation of mRNA to reduce cellular protein burden (11). Several lines of evidence indicate that ER stress also alters the metabolism of some metals. For example, mice injected with
TM have been shown to exhibit hypoferremia and iron sequestration in the spleen and liver (36). Regarding zinc metabolism and ER stress adaptation, it has been shown recently in mice that TM administration alters hepatic gene expression of multiple zinc transporters (23). That observation is supported by our present study, in which ZIP14-mediated hepatic zinc uptake was observed, along with hypozincemia, after TM and thapsigargin treatments (Fig. 1A–C and Fig. S2A–D), demonstrating that acute ER stress induction alters zinc metabolism. We focused on the liver because no difference in zinc uptake was observed in the pancreas, kidney or white adipose tissue, which are known to be highly affected by ER stress (Fig. S1B–D).

We also examined ER stress with an HFD model, which is more physiologically relevant. It was previously demonstrated that 16 wk of HFD feeding to mice induces ER stress in the liver (8). The HFD also produced ZIP14-mediated hepatic zinc accumulation in WT mice, whereas Zip14 KO mice did not accumulate zinc during that time period (Fig. 5C). The impaired hepatic zinc uptake of Zip14 KO mice possibly produced a greater signature of ER stress-induced apoptosis and steatosis, which is consistent with the TM-induced ER stress model (Fig. 5D–F). Phenotypic changes in Zip14 KO mice, such as hepatic zinc accumulation and steatosis, are remarkably similar in those two models. However, the magnitude of change is relatively marginal in the HFD model compared with TM model. This difference may be caused by the chronic vs. acute nature of these two models of ER stress. The HFD is a well-known model of the metabolic syndrome, causing insulin resistance or glucose insensitivity (37). Our recent report has shown that HFD-fed (6 wk) Zip14 KO mice do not develop a disturbed metabolic phenotype, such as hepatic insulin resistance or glucose insensitivity (20), indicating that the impaired stress adaptation of Zip14 KO mice is probably not influenced by glucose stress, which is a known cause of ER stress (38).

Our first question regarding the zinc, ZIP14, and ER stress relationship was whether the increased hepatic zinc uptake after ER stress would be a factor in stress adaptation. In the sense that ER function is dependent on zinc availability to provide zinc for incorporation into and folding of zinc metalloproteins (39), increased zinc may play a role in the restoration of ER homeostasis. In support of that notion, it has been shown that a supply of zinc to the early secretory pathway via ZnT5, ZnT6, and

Fig. 7. Zip14 is transcriptionally regulated by ATF4 and ATF6α in a time-dependent manner during TM treatment. (A) Relative expression of Zip14 mRNA in HepG2 cells treated with TM (1 μg/mL) and/or actinomycin D (Act D; 2 μg/mL) for 12 h. (B) Relative expression of Zip14 mRNA and heterogeneous nuclear RNA (hnRNA) in HepG2 cells treated with TM (1 μg/mL) or vehicle. (C) Consensus motif of CRE and binding motifs of ATF4 and ATF6. (D) 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. Relative expression of Zip14 mRNA in TM-treated HepG2 cells (1 μg/mL) after transfection with control siRNA, Atf4 siRNA (E), or Atf6α siRNA (G) is shown. Enrichment of DNA bound to ATF4 antibody (F) or ATF6α antibody (H) was measured by quantitative real-time PCR after ChIP assays in TM-treated HepG2 cells (1 μg/mL). Nonspecific rabbit IgG antibody was used as a negative control. (I) Immunoblot analysis of ATF4 and full-length and cleaved ATF6α in TM-treated HepG2 cells (1 μg/mL). All data are represented as mean ± SD. *P < 0.05, **P < 0.01.
ZIP14 in AML12 hepatocytes suppressed PTP1B activity (33). Based on these reports, we hypothesized that ER stress-induced mice increase hepatic ZIP14 expression to enhance zinc uptake for suppression of PTP1B activity. ZIP14 KO mice showed significantly elevated PTP1B activity, whereas activity in WT mice remained unchanged after TM- and HFD-induced ER stress (Fig. 6D and F), possibly due to impaired hepatic zinc uptake. In vitro, a direct effect of zinc on PTP1B activity inhibition was demonstrated using zinc supplementation, where the greater PTP1B activity in TM-treated ZIP14 KD cells was reversed by zinc supplementation (Fig. 6H). These data are consistent with our previous study, where increased PTP1B activity was observed in mice fed a zinc-deficient diet compared with mice fed a zinc-adequate diet during TM challenge (25).

Disrupted hepatic lipid homeostasis is another hallmark of unresolved ER stress. This defect has been observed in cells with compromised UPR function. Genetic ablations of UPR components, including ATF6α, Ire1α, and eIF2α, result in the development of hepatic steatosis (14, 54, 55). Loss of ATF4 increased free cholesterol in rodent liver (35), and liver-specific deletion of XBP1 produced hypochondroplasia and hypothyrgliceridemia (56). Similar to these observations, the liver of ZIP14 KO mice showed potentiated TG accumulation after TM administration due to enhanced FA synthesis (Fig. 4). Zinc-mediated PTP1B activity suppression may mechanistically explain these data because liver-specific Ptp1b−/− mice showed diminished hepatic FA synthesis and TG storage during HFD feeding (50), which is consistent with the ER stress-induced TG accumulation we showed in ZIP14 KO mice.

UPR activation induces a variety of genes involved in protein folding, degradation, and trafficking to restore ER homeostasis via transcription factors, such as ATF6α, ATF4, and XBP-1 (12). We propose that one arm of UPR pathway activation is to modulate zinc trafficking, based on our finding that ZIP14 is a transcriptional target of ATF4 and ATF6α (Fig. 7E–H). ATF4 and ATF6α have a significant binding affinity for the CRE consensus sequence (57–59). We found that the ZIP14 promoter has a CRE-like sequence, and demonstrated here that both ATF4 and ATF6α bound to the CRE-like sequence after TM treatment in a time-dependent manner, leading to ZIP14 mRNA increases through increased transcription (Fig. 7). Our data agree with the observations from global transcriptional profiling, which showed significantly reduced ZIP14 mRNA levels after TM treatment in liver-specific Atf4−/− mice (35) and in Atf6α−/− fibroblasts (34). ATF4 and ATF6α regulation of ZIP14 suggests that these events lead to increased cellular zinc availability through ZIP14-mediated zinc transport. Of note is that the ZIP14 promoter lacks the sequence required for the liver-specific transcription factor CREBH, which is needed for induction of hepcidin by ER stress (36). Limited evidence indicates that UPR components regulate expression of other zinc transporter genes. XBP-1−mediated hZnT5 up-regulation during ER stress occurs via direct binding to that promoter (26). In addition, ZnT5+/−/ ZnT7+/− cells displayed an exacerbated ER stress response (26). Similarly, KD of Zip7, Zip13, and ZnT3 in cells induced ER stress and higher cell death, indicating the importance of normal zinc homeostasis for ER stress adaptation (27, 28, 60).

In conclusion, we demonstrate that ZIP14-mediated hepatic zinc uptake plays an important role in suppressing ER stress-induced apoptosis and hepatic steatosis (Fig. 8). ZIP14 displayed a protective effect against ER stress by influencing the proapoptotic p-eIF2α/ATF4/CHOP pathway and de novo FA synthesis through zinc-mediated inhibition of PTP1B activity. Deletion of ZIP14 produced a phenotype presenting with hepatic lipid accumulation during ER stress. In addition, we demonstrated that ZIP14 is transcriptionally regulated by ATF4 and ATF6α. The present findings document the importance of functional zinc transporter for adaptation to ER stress.

Fig. 8. Role of ZIP14-mediated zinc transport in ER stress adaptation. Based on the data in this report, we propose a cycle where ER stress sequentially induces expression of ATF4 and ATF6α. The transcription factors increase expression of ZIP14, leading to increased ZIP14 in hepatocytes. Enhanced transporter activity increases intracellular zinc concentration, leading to inhibition of PTP1B activity.
Materials and Methods

Mice and Diets. Development and characterization of the murine Zip14+/− (KO) mice have been described previously (19). A colony of Zip14+/+ heterozygotes on the C57BL/6J background were used to produce KO and Zip14+/− (WT) mice. The colonies were backcrossed on a mixed 129S5 and C57BL/6 background. Male wildtype and KO mice were used for TM experiments. To model HFD-induced ER stress, WT and KO mice were fed either the chow diet (17 kcal% fat) containing 63 mg of zinc per kilogram as ZnO or an HFD (60 kcal% fat, D12492; Research Diets) containing 39 mg of zinc per kilogram as ZnO2 for 16 weeks.

Treatments. ER stress was induced by i.p. administration of TM (Sigma) or thapsigargin (Sigma) dissolved in 1% DMSO/150 mM glucose at 2 mg/kg of body weight (TM) or 1 mg/kg of body weight (thapsigargin), respectively. To assess zinc absorption and tissue distribution, 65Zn (2 μCi; PerkinElmer) was given to mice by oral gavage 3 h before euthanasia. Accumulated 65Zn in tissue and plasma was measured by gamma scintillation spectrometry. Mice were anesthetized by iso-flurane for injections and euthanasia by cardiac puncture. All mice were killed between 9:00 AM and 10:00 AM. Collected tissues were snap-frozen in liquid nitrogen and stored at −80 °C. All animal protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Western Blot Analysis. Tissue samples or cells were homogenized in radio-immunoprecipitation assay lysis buffer (Santa Cruz Biotechnology) supplemented with protease and phosphatase inhibitors (Thermo Fisher) using Basic Lysis Blendor (Next Advance). Proteins were then separated by SDS-PAGE and transferred to a nitrocellulose membrane. Polyclonal rabbit antibody against ZIP14 was raised in-house as described previously (61). Purchased antibodies were BIP, CHOP, and phosphorylated eIF2α (Se27/28) (all from Santa Cruz Biotechnology); ATF4, GRP94, and PTP1B (all from Cell Signaling); ATF6 (Novus Bio); and tubulin (Abcam). Immunoreactivity was visualized by ECL reagents (Thermo Fisher). Immunoblots for ATF4, CHOP, and GRP94 were quantified using densitometry.

Results

Statistical Analyses. Results are expressed as mean ± SD. Statistical significance was analyzed using GraphPad Prism 6 (GraphPad Software). Comparisons between two groups were conducted by the Student’s t test, and comparisons among multiple groups were conducted using ANOVA followed by a Tukey’s test. Statistical significance was set at P < 0.05.

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Supporting Information

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SI Materials and Methods

Biochemical Analyses. Tissue and serum zinc concentrations were measured using flame atomic absorption spectrophotometry as described previously (19). Hepatic nonheme iron concentrations were analyzed colorimetrically (62). Serum alanine aminotransferase activity was measured using a colorimetric end-point assay (33). Liver TGs were measured using a colorimetric assay (BioVision Research) according to the manufacturer’s instructions.

Cell Culture and siRNA KD. Human hepatocellular carcinoma cell line HepG2 (American Type Culture Collection) was maintained in Dulbecco’s Modification of Eagle’s Medium (Corning) supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma). Cells were maintained at 37 °C in 5% CO2. HiPerFect transfection reagent (Qiagen), or negative control siRNA (Dharmacon) into cells at a final concentration of 5 nM according to each manufacturer’s instructions. The efficiency of KD was detected using quantitative real-time PCR (qPCR) and immunoblotting. Cells were then treated with TM (1 μg/mL, unless specifically indicated otherwise) or vehicle (DMSO) to induce ER stress. In zinc supplementation experiments, zinc acetate (2.5–20 μM; Sigma) and pyrithione (2-mercaptopyridine N-oxide sodium salt, 50 μM; Sigma) were added to the culture medium for 30 min. To measure the total cellular zinc level, cells were sonicated to disrupt all cellular membranes, and the lysates were then incubated with the zinc fluorophore FluoZin3-AM (Invitrogen).

qPCR. Total RNA from tissue samples or cells was isolated using TRIzol reagent (Ambion) and was homogenized using 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). 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 the Table S1. The primer/probe sequences for zinc transporters have been provided previously (33).

In experiments detecting the transcriptional activity of Zip14, primers spanning the 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 S1. The reaction conditions for PCR were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s, and a final cycle at 60 °C for 1 min. Melting curves were obtained after PCR to ensure only a single product was amplified.

Histological Analyses. Collected liver tissues were embedded in paraffin after being fixed in 10% formalin, and were then sectioned to 4 μm in thickness. For histological analysis, the liver sections were stained with H&E. Lipid droplet areas in H&E-stained images were quantified using ImageJ software (National Institute of Mental Health). To detect apoptotic cells in the liver sections, an In Situ Apoptosis Detection Assay Kit (Abcam) was used following the manufacturer’s instructions.

PTP1B Assay. Activity of PTP1B was analyzed as described previously (33), with slight modifications. To briefly describe the process, liver tissues or cells were homogenized in Hepes buffer supplemented with protease inhibitor mixture (Thermo Fisher Scientific). Obtained total lysates were centrifuged at 13,000 × g at 4 °C, and the supernatant was incubated with PTP1B-specific substrate (ELEF-pY-MDYE-NH2; AnaSpec) for 30 min at 30 °C. The inorganic phosphate level released during the incubation was measured using a colorimetric phosphate assay (Biovision). Assay values were normalized by total protein concentration.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in TM-treated HepG2 cells was performed using the MTT Cell Proliferation Assay Kit (Cayman Chemical) according to the manufacturer’s manual.

ChIP Assay. ChIP assays were performed as described previously (63), with slight modifications. Briefly, TM or vehicle-treated HepG2 cells were cross-linked with 1.1% (vol/vol) 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 piperazine-N,N′-bis(2-ethanesulfonic acid) buffer (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40] and centrifuged at 3,000 × g for 5 min at 4 °C, and the supernatant was then discarded. The pellet (nuclei) was resuspended in radioimmunoprecipitation assay buffer, followed by sonication using a BioRuptor (Diagenode) for 15 min (15 cycles of 30 s on and 30 s 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. Target DNA bound with protein was immunoprecipitated with ChIP-grade ATF6α antibody (Novus Bio) or ATF4 antibody (Cell Signaling). Therefore, cross-link reagents and protein were removed. 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 Table S1.
**Fig. S1.** Measurement of $^{65}$Zn uptake in plasma (A), pancreas (B), kidney (C), and white adipose tissue (WAT) (D) of WT and Zip14 KO mice ($n = 3–4$) is shown 12 h after administration of TM (2 mg/kg) or vehicle. Concentration of nonheme iron (E) and manganese (F) in livers of WT and Zip14 KO mice ($n = 3–4$) is shown 12 h after administration of TM (2 mg/kg) or vehicle. All data are represented as mean ± SD. Labeled means without a common letter differ significantly ($P < 0.05$). Cont, control.

**Fig. S2.** (A) Hepatic Zn concentration in mice ($n = 3$) 6 h after administration of thapsigargin (TG; 1 mg/kg) or vehicle. (B) Immunoblot analysis of ZIP14 and BiP in liver lysates ($n = 3$) 6 h after administration of TG (1 mg/kg) or vehicle. All data are represented as mean ± SD. *$P < 0.05$. 

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Fig. S3.  (A) Relative expression of BiP and Chop in livers of WT and Zip14 KO mice (n = 3). (B) Immunoblot analysis of ZIP14 and ER stress markers in liver lysates of WT and Zip14 KO mice (n = 3). All data are represented as mean ± SD.

Fig. S4.  Relative expression of Zip14 (A), Atf4 (B), and Atf6α (C) mRNAs in HepG2 hepatocytes transfected with the indicated siRNAs. Csi, control siRNA.
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