Endoplasmic reticulum (ER) stress activates the adaptive unfolded protein response (UPR) and represents a critical mechanism that underlies metabolic dysfunctions. Fibroblast growth factor 21 (FGF21), a hormone that is predominantly secreted by the liver, exerts a broad range of effects upon the metabolism of carbohydrates and lipids. Although increased circulating levels of FGF21 have been documented in animal models and human subjects with obesity and nonalcoholic fatty liver disease, the functional interconnections between metabolic ER stress and FGF21 remain incompletely understood. Here, we report that increased ER stress along with the simultaneous transcriptional expression of Fgf21 in mice alleviated tunicamycin-induced liver steatosis, in parallel with reduced eIF2α-ATF4-CHOP signaling. Taken together, these results suggest that FGF21 is an integral physiological component of the cellular UPR program, which exerts beneficial feedback effects upon lipid metabolism through counteracting ER stress.

In eukaryotes, the endoplasmic reticulum (ER) is the major site of protein folding and maturation as well as lipid biosynthesis. Accumulation of unfolded or misfolded proteins or perturbation of lipid metabolism at the ER causes ER stress, activating the adaptive cellular response termed the unfolded protein response (UPR) (1–3). In mammals, three canonical signaling branches of the UPR act coordinately to relieve ER stress. These include the ER-resident transmembrane protein inositol-requiring enzyme 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (1, 2). IRE1 is evolutionarily the most conserved sensor of ER stress, possessing both protein Ser/Thr kinase and endoribonuclease (RNase) activities (2, 4, 5). Under ER stress conditions, IRE1 is activated through trans-autophosphorylation and dimerization/oligomerization (6, 7), catalyzing the removal of a 26-nucleotide intron within the mRNA that encodes the transcription factor X-box-binding protein 1 (XBP1) (8). This non-conventional splicing event generates an active spliced form of XBP1 (XBPs1) to initiate a critical UPR program (8). Upon sensing ER stress, PERK mediates the second UPR branch through phosphorylating the ubiquitously protein translation

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4 The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; FGF21, fibroblast growth factor 21; NALFD, nonalcoholic fatty liver disease; PERK, PKR-like endoplasmic reticulum kinase; CHOP, C/EBP homologous protein; LFD, low fat diet; HFD, high fat diet; LKO, liver-specific IRE1α knockout; Tm, tunicamycin; PPAR, peroxisome proliferator-activated receptor; ANOVA, analysis of variance; ERSE, ER stress-response element; Luc, luciferase; EGFP, enhanced GFP.
initiation factor eIF2α, thereby inhibiting cellular mRNA translation (1, 2). In addition, PERK phosphorylation of eIF2α leads to simultaneous induction of the transcription factor ATF4 (9), which in turn drives the expression of its target gene, transcription factor C/EBP homologous protein (CHOP) (10, 11). Together with ATF6, these UPR programs function to maintain homeostasis of the ER and play a pivotal part in managing ER stress to allow for cell survival.

 Emerging lines of evidence have also implicated the UPR pathways in metabolic homeostasis (3, 12). ER stress represents an important mechanism that underlies metabolic disorders (13–15), including the development of nonalcoholic fatty liver disease (NAFLD), a hallmark of metabolic syndrome and a major health burden in both developed and developing countries (16, 17). A number of clinical studies have shown increased ER stress markers in both liver and adipose tissues from human subjects with obesity and NAFLD (18–21). Studies in animal models with genetic disruption of the IRE1α, eIF2α, or ATF6α pathway (22, 23) indicated that the three UPR branches may act in concert to prevent the development of hepatic steatosis, which is linked to perpetuated expression of CHOP arising from unresolved ER stress. Despite this recent progress, the physiological mechanism by which each individual UPR branch can affect lipid metabolism during hepatic steatosis has yet to be completely delineated.

Fibroblast growth factor 21 (FGF21), an atypical member of the FGF family, functions as a hormone that has a wide range of endocrine, autocrine, and pharmacological actions on carbohydrate and lipid metabolism (24). It has been shown in mouse models that FGF21 is secreted from the liver in response to extended periods of fasting, and its expression is controlled by the FGF family, functions as a hormone that has a wide range of physiological mechanism by which each individual UPR branch can affect lipid metabolism during hepatic steatosis has yet to be completely delineated.

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Diagnosis of NAFLD was according to the guidelines proposed by the Asia-Pacific Working Party (45). Tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C. All samples had been examined by a pathologist, who was blinded to the study. Hepatic steatosis was classified as grade 0 (1–5%), grade 1 (6–33%), grade 2 (34–66%), and grade 3 (67–100%) (46). Tissue samples used for protein and total RNA preparation were from five patients with severe NAFLD (i.e. hepatic steatosis scored as grade 2 or 3) and five controls (i.e. hepatic steatosis grade 0), and none of which was pathologically diagnosed as having nonalcoholic steatohepatitis or cirrhosis. The study procedures were approved by the local ethics committee, following the principles of the Declaration of Helsinki. Written voluntary consent was obtained from all subjects before their participation.

Cell Culture and Treatment—Human hepatoma cell line HepG2 was grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) (Invitrogen). For ER stress experiments, cells were incubated in DMEM containing DMSO, 1 μM thapsigargin (Sigma), or 10 μg/ml tunicamycin (Sigma).

Recombinant Adenoviruses—Recombinant adenoviruses for the overexpression of EGFP (control), the wild-type, or the mutant forms of IRE1α were generated as described previously (47). Adenoviruses for XBP1s overexpression and knockdown were the generous gifts from Dr. Ling Qi (Cornell University). The knockdown control adenovirus Ad-sh-LacZ was generated as described with the BLOCK-iT adenoviral RNAi expression system (Invitrogen) in HEK293A cells according to the manufacturer’s instructions (48). For infection of mouse primary hepatocytes, viruses were used at a multiplicity of infection of 40, which was measured according to the manufacturer’s instructions.

Primary Hepatocytes and Adenoviral Infection—Primary hepatocytes were isolated from male mice at 8–12 weeks of age according to the procedure previously described in detail (48).
Briefly, collagenase perfusion was performed through the portal vein of mice after anesthetizing with 50 ml of perfusion buffer. Livers were aseptically removed and cut in a sterile 10-cm cell culture dish with 20 ml of ice-cold perfusion buffer without collagenase. After aspirating with a large-bore pipette, hepatocytes were filtered through a 70-μm cell strainer (BD Falcon) into a 50-ml centrifuge tube before centrifugation at 50 × 1,100 g for 2 min at 4 °C. Cells were washed with cold hepatocyte wash medium (Invitrogen) three times and resuspended in 15 ml of cold HepaToZYME-SFM medium (Invitrogen) supplemented with 2 mM L-glutamine, 20 units/ml penicillin, and 20 μg/ml streptomycin. After trypan blue staining for determination of viability, cells were plated at 6 × 10^5 cells/well in 6-well culture dishes or at 3 × 10^5 cells/well in 12-well dishes pre-coated with collagen. Cells were cultured for at least 8 h before further use. Hepatocytes were infected with adenoviruses for 48 h in the overexpression experiments or for 72 h in the knockdown experiments. Cells were subsequently treated with the desired reagents prior to protein extraction for immunoblotting analysis or total RNA isolation for quantitative real time RT-PCR analysis.

Chemicals, Antibodies, and Immunoblotting—ERK inhibitor U0126 was purchased from Cayman. p-IRE1α antibody was purchased from Novus Biologicals. Antibodies against IRE1α, p-eIF2α, eIF2α, ERK, p-ERK, and lamin A/C were all from Cell Signaling. XBPI1 antibody was from BioLegend; α-tubulin antibody was from Sigma; ATF4 antibody was from Santa Cruz Biotechnology; ATF6 antibody was from Imgenex, and FGF21 antibody was from Abcam. For immunoblotting, total proteins were extracted from cells or liver tissues by RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.4), and nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane filters (Millipore). After incubation with the desired antibodies, the blots were developed with SuperSignal West Pico Chemiluminescent substrate (Pierce) or Immobilon Western Chemiluminescent HRP substrate (Millipore).

Quantitative Real Time RT-PCR Analysis—Total RNAs were isolated from cells or liver tissues by TRIzol reagent (Invitrogen), and cDNA was generated by Moloney murine leukemia virus-reverse transcriptase with random hexamer primers (Invitrogen). Real time quantitative PCR was conducted with an ABI Prism 7500 sequence detection system, using the SYBR Green PCR Master Mix (Applied Biosystems). β-Actin was used as an internal control for normalization. The oligonucleotide primers used for each target gene are listed as follows: mouse ActB, sense 5’-AGTGTGAGTCTGGACATCCTGTA-3’ and antisense 5’-GCCAGAGGATATCTCCCTTT-3’; mouse Xbp-1s, sense 5’-CTGAGTCGGATCAGTGAGCAG-3’ and ant-
sense 5'-GTCCTATGGAAGATGTTTCG-3'; mouse Xbp1, sense 5'-TGGCGGCTCTCTAGATCCGG-3' and antisense 5'-CAGGGGTGAAGTCTTGCG-3'; mouse Chop, sense 5'-CTGGAGAGCTTTGATGAGGA-3' and antisense 5'-CGGATACACCTGATGAGGAT-3'; mouse Fgf21, sense 5'-CCGCTGAGAGGTTTCTCAGAG-3' and antisense 5'-GTGTGGGACTTTCGGCAG-3'; human Actb, sense 5'-CTTCCTAAGTGTAAGGCTC-3' and antisense 5'-CTCATCAAGTGTCGCAAG-3'; human Fgf21, sense 5'-GACGGACCCCTAAAGTTCTG-3' and antisense 5'-GCGCACTGCTGTCTCAG-3'; human Chop, sense 5'-CAAGAGTTCCAGTGTCTCTGAGATGAGA-3' and antisense 5'-CCCGAGATGTCGCTCT-3'; human Fgf21, sense 5'-ACACCGAGCGGCTTTATACG-3' and antisense 5'-CCATTCTCTTGGAGGCCAAC-3'; mouse Atf4 and human Atf4, sense 5'-CTTCTGGATGCTCGATGATG-3' and antisense 5'-GCTTGGCCGGAAAGAGCTCC-3'; mouse Fgfr1, sense 5'-CTGGAAGGGAGGTTCTCAGATG-3' and antisense 5'-GTGCGATGTCGCTCT-3'; mouse Fgfr2, sense 5'-CACCACCGAACAAAGCAGTTG-3' and antisense 5'-TGTCAGACCTGAGAGATGAA-3'; mouse Fgfr3, sense 5'-AGATGCTGAAAGATGAGGCT-3' and antisense 5'-ATGATGCTGAAAGATGAGGCT-3'; mouse Fgfr4, sense 5'-CCAGAGCGCTTGGATGAGGAT-3' and antisense 5'-AGGCTGCAAAATCCTTGTC-3'; and mouse Klb, sense 5'-CAGAAGAAGAGAGGTGAGG-3' and antisense 5'-CAGCACCCTGCTTAAGGTA-3'.

Luciferase Reporter Assays—The luciferase (Luc) reporter plasmids for the mouse Fgf21 promoter spanning the region from −1983 to +5 (WT) and its deletion mutant version (ΔCCACG) were constructed in pGL3 (Promega) utilizing a PCR-based cloning strategy. For luciferase activity assays, 293T cells were co-transfected with the Fgf21 promoter-Luc and β-galactosidase plasmids before treatment with thapsigargin (1 μM) or tunicamycin (10 μg/ml) for 6 h or co-transfected with the Luc reporter plasmid, pCMV-XBP1s and β-galactosidase plasmids. Luciferase activity was measured using Dual-Luciferase™ reporter assay system (Promega) following the manufacturer's instructions, and β-galactosidase activity was used for normalization.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed with the agarose ChIP kit (Pierce) according to the manufacturer's instructions. Cells or liver tissues were subjected to cross-linking with 1% formaldehyde. Glycine solution was then added, and nuclear extracts were prepared. Chromatin-XBP1s complexes were immunoprecipitated with normal rabbit IgG or anti-XBP1s antibodies by incubation at 4 °C overnight on a rocking platform, followed by incubation with the beads from the ChIP kit (Pierce) or protein G-Sepharose beads (GE Healthcare) at 4 °C for 1 h with gentle rocking. After washing five times with the washing buffer, the complexes were eluted with the elution buffer from the beads and were subjected to PCR analysis using the oligonucleotide primers that correspond to the −280 to −24 region of the mouse Fgf21 promoter as follows: sense 5'-CTCCAGACGCAAGGACGACA-3' and antisense 5'-TGAAAGGCAGAACACACAGC-3'. Regular PCR was conducted with TaKaRa Taq kits (Takara), and real time quantitative PCR was performed with the SYBR Green PCR system (Applied Biosystems).
Statistical Analysis—Data are presented as the mean ± S.E. Statistical analysis was performed using unpaired two-tailed t test and one-way or two-way analysis of variance (ANOVA) followed by Bonferroni’s post test with GraphPad Prism 4.0. p < 0.05 was considered statistically significant.

RESULTS

Hepatic FGF21 Expression Is Associated with ER Stress and Hepatic Steatosis—To investigate whether hepatic FGF21 expression was linked to ER stress during the development of hepatic steatosis, we employed a diet-induced mouse model of
FGF21 Acts as a UPR Effector

Expression of FGF21 Is Induced upon Experimental ER Stress—To determine whether FGF21 expression is directly linked to ER stress, we first treated HepG2 cells for different time intervals with tunicamycin and thapsigargin, the two chemical ER stressors. Both tunicamycin and thapsigargin caused activation of the UPR, including increases in IRE1α phosphorylation and XBP1s protein abundance (Fig. 3A), greater accumulation of nuclear ATF6 and ATF4 proteins (Fig. 3A), and elevations in the splicing of XBP1 mRNA (Fig. 3B) and the mRNA abundance of ATP4 and CHOP (Fig. 3C). Tunicamycin or thapsigargin also resulted in increased levels of FGF21 protein (Fig. 3A), along with elevated mRNA abundance of FGF21 but not FGFI (Fig. 3C). Consistently, higher secreted FGF21 protein levels were detected from the culture medium of HepG2 cells treated with tunicamycin or thapsigargin (Fig. 3D).

Next, we treated mice with tunicamycin for 24 h, which caused marked increases in liver TG content (Fig. 4A) and serum levels of FGF21 (Fig. 4B). Tunicamycin activated the three UPR branches in the liver (Fig. 4, C and D), while prominently increasing the level of hepatic FGF21 protein (Fig. 4C) as well as the Fgf21 mRNA abundance (Fig. 4E). Thus, hepatic FGF21 expression could be induced under experimental ER stress conditions both in vitro and in vivo. Notably, tunicamycin treatment resulted in considerable decreases in liver mRNA levels of FGF1, -3, and -4 (Fig. 4F), the potential FGF21 receptors (49, 50), without influencing the expression of β-klotho, the co-factor required for FGF21 activity (51). These results indicate that ER stress might attenuate FGF21 signaling through down-regulating its receptor expression. In addition, we also observed elevations in the mRNA abundance of Fgf21 in the kidney, white adipose tissue, and muscle of tunicamycin-treated mice (Fig. 5, A–C). This implies that FGF21 is also likely to exert cytoprotective functions in other tissues such as the kidney, an organ that can be very sensitive to ER stress.

IRE1α-XBP1 Pathway Regulates the Transcriptional Expression of FGF21—Given that FGF21 was reported as an ATF4-regulated gene (42, 43), we tested whether FGF21 is a common downstream target of multiple UPR pathways using liver-specific IRE1α knock-out (LKO) mice that we created (44). Interestingly, when compared with flox/flox control mice, tunicamycin-treated LKO mice had lower serum FGF21 levels (Fig. 6A). In primary hepatocytes, hepatic IRE1α deficiency not only

FIGURE 4. ER stress causes liver steatosis with up-regulated expression of hepatic FGF21 in mice. Male C57BL/6 mice at 12 weeks of age were treated for 24 h through intraperitoneal injection with PBS (vehicle) or Tm (1 mg/kg body weight). A, liver triglycerides were determined. B, serum levels of FGF21 were measured. Data are shown as the mean ± S.E. (n = 5/group). *, p < 0.05; **, p < 0.01 by t test. C, immunoblot analyses of the UPR markers, including p-IRE1α and p-eIF2α, and FGF21 protein abundance in whole liver lysates. Representative results are shown for three individual mice per group. Ratios of p-IRE1α/IRE1α and p-eIF2α/αIF2α as well as FGF21 protein levels were quantified. D, immunoblot analyses of nuclear XBP1s, ATF4, and ATF6 protein levels. Representative results are shown for three individual mice per group, and quantization was done by normalization to lamin A/C. E, Xbp1 mRNA splicing and the mRNA abundance of ATP4, Chop, Fgf21, and Fgf1 were analyzed by quantitative RT-PCR. F, analysis by quantitative RT-PCR of the mRNA abundance of the FGF21 receptors Klb, Fgfr1, Fgfr2, Fgfr3, and Fgfr4. Data in C–F were normalized to the values of the vehicle control group and are presented as mean ± S.E. (n = 5/group). *, p < 0.05; **, p < 0.01 by t test.

FIGURE 5. ER stress induces the expression of FGF21 in kidney, white adipose tissue, and muscle. Mice were treated with PBS (vehicle) or Tm for 24 h. Xbp1 mRNA splicing and the mRNA abundance of Fgf21 and Fgfi1 in kidney (A), fat tissue (B), and muscle (C) were determined by quantitative RT-PCR. Data were normalized to the values of the vehicle control group and are presented as mean ± S.E. (n = 5/group). *, p < 0.05; **, p < 0.01 by t test.

obesity. When fed a high fat diet (HFD) for 16 weeks, mice displayed significant increases in body weight and liver content of triglycerides (TG) relative to control mice fed a low fat diet (LFD) (Fig. 1A). HFD-fed mice also had significantly elevated serum levels of FGF21 (Fig. 1B). Immunoblotting analyses showed that FGF21 protein levels were increased in livers of HFD-fed obese mice, which were accompanied by increased phosphorylation of IRE1α and eIF2α (Fig. 1C), along with increased nuclear accumulation of XBP1s, ATF4, and ATF6 proteins (Fig. 1D). Consistently, the splicing of Xbp1 mRNA and the mRNA abundance of Atf4 and Chop were significantly increased in livers of HFD-fed mice, in parallel with up-regulated mRNA expression of Fgf21 but not Fgfi1 (Fig. 1E). Similarly, activation of all the three UPR branches was also detected in livers of human NAFLD patients when compared with those of control subjects without NAFLD (Fig. 2, A–C), and this was accompanied by increased levels of FGF21 protein (Fig. 2A) and FGF21 mRNA (Fig. 2C). These results demonstrated the occurrence of metabolic ER stress during hepatic steatosis, which was associated with the up-regulation of hepatic FGF21 expression in both animals and humans.
Fgf1 (Fig. 7) expression of viral overexpression of XBP1s (Fig. 7) resulted in significant reductions in ER stress-induced expression in hepatocytes from male tunicamycin (1 mg/kg body weight) for 8 h. Serum levels of FGF21 were measured and are shown as the mean ± S.E. (n = 3/group). B and C, primary hepatocytes from male flox/flox or LKO mice were treated for 6 h with dimethyl sulfoxide (DMSO), thapsigargin (Tg, 1 μM), or Tm (10 μg/ml). B, immunoblot analysis of IRE1α protein expression and quantitative RT-PCR analysis of Xbp1 mRNA splicing. C, analyses of the mRNA abundance of Atf4, Fgf21, and Fgf1. Data were normalized to values of DMSO-treated flox/flox hepatocytes and are shown as the mean ± S.E. (n = 3 independent experiments). **, p < 0.01 by two-way ANOVA. D and E, primary hepatocytes from male C57BL/6 mice were infected for 2 days with adenoviruses expressing EGFP or the wild-type (WT) or kinase-dead K599A mutant human IRE1α protein. D, immunoblot analysis of IRE1α and quantitative RT-PCR analysis of Xbp1 mRNA splicing. E, analyses of the mRNA abundance of the indicated genes. Data were normalized to values from Ad-EGFP control cells and are shown as the mean ± S.E. (n = 3 independent experiments). *, p < 0.05; **, p < 0.01 by one-way ANOVA.

completely abolished tunicamycin- or thapsigargin-induced Xbp1 mRNA splicing (Fig. 6B), it also markedly diminished ER stress induction of Fgf21 without affecting the expression of Atf4 or Fgf1 (Fig. 6C). Conversely, adenovirus-mediated overexpression in hepatocytes of the wild-type (WT) but not the kinase-dead K599A mutant IRE1α could significantly increase the splicing of the Xbp1 mRNA (Fig. 6D) and the mRNA abundance of Fgf21 (Fig. 6E). Next, to determine whether IRE1α regulation of Fgf21 expression is indeed mediated by XBP1s, we employed adenovirus-expressed shRNA to knock down the expression of XBP1 in primary hepatocytes (Fig. 7A). This resulted in significant reductions in ER stress-induced expression of Fgf21 but not Atf4 or Fgf1 (Fig. 7B). Conversely, adenoviral overexpression of XBP1s (Fig. 7C) greatly increased the expression of Fgf21 but did not affect the expression of Atf4 or Fgf1 (Fig. 7D). These data demonstrate that the IRE1α-XBP1 branch of the UPR plays a crucial role in regulation of FGF21 expression in response to ER stress.

We then examined whether Fgf21 is a direct transcriptional target of XBP1s. Luciferase reporter assays showed that the mouse Fgf21 promoter could be activated under experimental ER stress conditions (Fig. 8A). Moreover, a putative ER stress-response element (ERSE), CCATT...N(n)...CCACG, was identified, a potential XBP1s-binding core site (52) that is also conserved in the promoter of human and rat Fgf21 genes (Fig. 8B). Importantly, XBP1s co-expression stimulated the mouse Fgf21 promoter activity, and deletion of CCACG from this ERSE abolished the ability of XBP1s to stimulate it (Fig. 8C). Chromatin immunoprecipitation (ChIP) assays showed that XBP1s physically bind to the ERSE-containing region of the Fgf21 promoter (Fig. 8D), and the CCACG core sequence was required for its binding (Fig. 8E). Next, we determined through ChIP assays whether endogenous XBP1s could bind to the Fgf21 promoter in an ER stress-responsive manner in vivo. Indeed, tunicamycin treatment significantly increased XBP1s occupancy of the Fgf21 promoter in the livers of mice (Fig. 8F). These results reveal that the IRE1α-XBP1 pathway drives the transcriptional expression of Fgf21 during ER stress. Therefore, Fgf21 serves as a downstream target gene of the UPR, which is regulated dually by ATF4 and XBP1s.

Fgf21-ERK Signaling Suppresses the eIF2α-ATF4-CHOP Pathway of ER Stress—The observed UPR-responsive expression of Fgf21 suggests that Fgf21 may act as a UPR effector to exert feedback effects on ER stress-associated metabolic changes. To test this idea, we first examined the effect of recombinant mouse Fgf21 protein upon tunicamycin-induced ER stress signaling in mouse primary hepatocytes. Notably, Fgf21 treatment did not cause significant changes in tunicamycin-induced phosphorylation of IRE1α (Fig. 9A) or splicing of Xbp1 mRNA (Fig. 9B), but it significantly decreased the phosphory
lation of eIF2α (Fig. 9A) and the mRNA abundance of Atf4 and Chop (Fig. 9B), leading to a reduced protein level of CHOP (Fig. 9C). To determine the possible mechanism that mediates FGF21’s actions, we examined whether FGF21-induced MAPK phosphorylation (29, 33) is involved. During tunicamycin-induced ER stress in hepatocytes, FGF21 treatment increased ERK phosphorylation (Fig. 10A), which was markedly inhibited by U0126, a chemical ERK inhibitor. Importantly, suppression of ERK activation significantly blunted the attenuating effects of FGF21 upon tunicamycin-induced eIF2α phosphorylation and CHOP expression (Fig. 10A–C). These data indicate that FGF21-directed ERK activation likely mediates its actions in counteracting ER stress, leading to suppression of the eIF2α-ATF4-CHOP pathway.

**FGF21 Alleviates ER Stress-induced Hepatic Steatosis**

We then analyzed the metabolic effects of FGF21 in tunicamycin-treated mice. Tunicamycin caused a marked increase in hepatic TG content but a prominent decrease in serum TG levels (Fig. 11A), which likely resulted from suppression of hepatic TG secretion and/or enhanced liver TG biosynthesis/uptake. Interestingly, although causing considerable reductions in both hepatic and serum TG levels in the absence of tunicamycin treatment, FGF21 administration in tunicamycin-treated mice resulted in a significant decrease in hepatic TG overload (Fig. 11A, left panel), but it did not affect the reduction of serum TG levels (Fig. 11A, right panel). This indicates that FGF21 had little effect upon tunicamycin-elicited changes in hepatic TG secretion or uptake. Similar to the observations in hepatocytes, FGF21 showed insignificant effect upon the phosphorylation of IRE1α (Fig. 11B) or splicing of Xbp1 mRNA (Fig. 11C) in the livers of tunicamycin-treated mice, but it substantially blocked the phosphorylation of eIF2α (Fig. 11B) and significantly blunted the elevations in the mRNA abundance of Atf4 and Chop (Fig. 11C). ER stress has been shown to enhance de novo lipid biosynthesis via activation of SREBP1 proteins (22, 23, 53), and we observed that FGF21 administration resulted in appreciable decreases in matured SREBP1 protein levels in livers of tunicamycin-treated mice (Fig. 11D). Given that CHOP is viewed as a transcriptional repressor that is implicated in the disruption of metabolic networks (22), these results suggest that FGF21 may suppress the eIF2α-ATF4-CHOP pathway, thereby counteracting ER stress to ameliorate hepatic steatosis through reducing lipogenesis while promoting lipid oxidation.

**DISCUSSION**

Many studies have established the importance of FGF21 that functions as a hormone to regulate carbohydrate and lipid metabolism (24). Despite its remarkable pharmacological beneficial effects in improving metabolic parameters
such as body weight, insulin sensitivity, and hyperlipidemia, increased circulating levels of FGF21 have been shown to be associated with human obesity, type 2 diabetes, and NAFLD (34–40). It remains largely elusive how changes in FGF21 expression are linked to the pathogenic development of metabolic disorders. In this study, we found in both mice and human subjects that hepatic FGF21 expression is coupled to cellular ER stress response in the face of NAFLD. Furthermore, our results suggest that FGF21 can be dually regulated by the IRE1α-XBP1 and eIF2α-ATF4 pathways (Fig. 11E) and can serve as a direct downstream target of the UPR program; FGF21 can in turn counteract ER stress to alleviate hepatic steatosis.

ER stress is thought to underlie metabolic dysfunctions (13–15), and the three UPR pathways have been implicated in various metabolic processes (3, 12). As a hepatokine, FGF21 expression is responsive to nutritional states (24), and animal model studies have shown that hepatic FGF21 is transcriptionally controlled by the nuclear receptor PPARα.
during prolonged starvation (25, 26). We previously showed that the IRE1α/H9251 pathway is activated in the liver during fasting or starvation (44, 47). Hepatic IRE1α can sense nutrient deprivation and regulate the adaptive shift of fuel utilization through XBP1s-directed regulation of PPARα/H9251. In this scenario, metabolic activation of the IRE1α/H9251-XBP1 pathway promotes the expression of Fgf21 as a PPARα/H9251 target gene, indicating a physiological role for the IRE1α/H9251-XBP1 branch in the starvation response. Our findings herein suggest that XBP1s may also directly up-regulate Fgf21 in a PPARα-independent manner during prolonged fasting. Although XBP1 has been implicated in the regulation of lipogenesis (54), XBP1s-mediated up-regulation of Fgf21 likely represents a feedback mechanism to control liver TG content through lipid oxidation. Under ER stress conditions, the transcriptional expression of Fgf21 is not only up-regulated by XBP1s but also by ATF4 (42, 43), and this dual induction indicates that FGF21 is a bona fide effector of the UPR program. The fact that FGF21 expression is controlled by at least two of the three UPR signaling arms not only supports an important role for FGF21 in the feedback control of ER stress responses, it also reflect the coordinated feature of multiple UPR arms in exerting their metabolic actions.

Our results from tunicamycin-treated mice showed that administration of recombinant FGF21 could ameliorate hepatic TG overload that was caused by typical ER stress. Interestingly, this was associated with suppression by FGF21 of both the elf2α-ATF4-CHOP pathway of ER stress and ER stress-activated SREBP1 protein maturation. CHOP has been shown to suppress metabolic genes through disrupting the function of C/EBPα, consequently leading to perturbations of fatty acid oxidation, lipoprotein secretion, and other metabolic processes (22). Thus, FGF21-induced suppression of CHOP expression may constitute a possibly major mechanism that mediates its alleviating effect upon liver steatosis during ER stress. Although it remains to be more clearly delineated how FGF21 signaling is mechanistically linked to maintaining ER homeostasis or suppressing the PERK-elf2α-CHOP pathway, our results indicate that FGF21-induced ERK activation contributes to mediating the feedback effect of FGF21 upon ER stress. Moreover, it is worth further dissecting whether FGF21 can exert similar effects upon liver steatosis through mitigating obesity-related...
metabolic ER stress. In addition to modulating lipid metabolism in the liver, the suppressive effect of the FGF21-ERK pathway upon the expression of CHOP implies that FGF21 may also influence ER stress-induced apoptosis, like another FGF family member FGF2 (55). Indeed, we also noticed an alleviating effect of FGF21 upon tunicamycin-induced apoptosis in cultured cells (data not shown), indicating a broader range of functions of FGF21 under ER stress-associated conditions.

In summary, our findings reveal that FGF21 is functionally linked to ER stress in the state of hepatic steatosis. The expression of FGF21 is controlled by dual branches of the UPR, i.e., ATF4 and XBP1s. This suggests that FGF21 can act as an integral component of the cellular UPR program in the management of ER stress. It is worth noting that FGF21 was recently shown to exert its metabolic actions in adipose tissues in an autocrine fashion (56–58) and could even function as a myokine (43). Given that increased ER stress is found to occur in the liver as well as in the adipose tissues and muscles, it remains to be elucidated whether FGF21 can act as a UPR effector to affect the functions of other metabolic tissues besides the liver. In addition, it is also tempting to speculate that FGF21, as a component of the UPR pro-

FIGURE 10. Inhibition of ERK activation blunts the suppressive effect of FGF21 on the eIF2α-ATF4-CHOP pathway. Primary hepatocytes were treated with DMSO (vehicle), recombinant mouse FGF21 (1 μg/ml), Tm (10 μg/ml), or tunicamycin after pretreatment with FGF21 in the absence or presence of ERK inhibitor U0126 as indicated. A, immunoblotting of p-ERK, ERK, p-IRE1α, IRE1α, p-eIF2α, eIF2α, and CHOP. Tubulin was used as the loading control. B, ratios of p-eIF2α/eIF2α and CHOP protein levels were quantified (n = 3 independent experiments). C, quantitative RT-PCR analyses of Xbp1 mRNA splicing and the mRNA abundance of Atf4 and Chop (n = 3 independent experiments). Data were normalized to the values of vehicle control group and are shown as the mean ± S.E. *, p < 0.05; **, p < 0.01 by two-way ANOVA.
FIGURE 11. Administration of FGF21 reduces ER stress-induced hepatic steatosis in mice. Male C57BL/6 mice at 12 weeks of age were injected intraperitoneally with PBS (vehicle) or recombinant mouse FGF21 (1 mg/kg body weight) together with DMSO (vehicle) or Tm (1 mg/kg body weight) (n = 5/group). Mice were sacrificed at 24 h after treatment. A, liver TG contents and serum TG levels. B, immunoblot analysis of p-IRE1α, p-eIF2α, IRE1α, and eIF2α in whole liver lysates. GAPDH was used as the loading control. Representative results are shown for two individual mice per group. Ratios of p-IRE1α/IRE1α and p-eIF2α/eIF2α were quantified and are presented as the mean ± S.E. (n = 5/group). C, quantitative RT-PCR analyses of Xbp1 mRNA splicing and the mRNA abundance of Atf4 and Chop. Data are shown as the mean ± S.E. (n = 5/group), D, immunoblot analysis of matured SREBP1 protein (SREBP1-m) in whole liver lysates. Tubulin was used as the loading control. SREBP1-m levels were quantified and normalized to tubulin (n = 5/group). Data in B–D were normalized to the values of the vehicle control mice. *, p < 0.05; **, p < 0.01 by two-way ANOVA. E, schematic model. Dually controlled by XBP1s and ATF4, FGF21 acts in turn to suppress the eIF2α-ATF4-CHOP pathway and alleviate ER stress-induced hepatic steatosis.
gram, may exert beneficial actions in other ER stress-associated pathologies.

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