De-silencing Grb10 contributes to acute ER stress-induced steatosis in mouse liver

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Abstract

The growth factor receptor bound protein GRB10 is an imprinted gene product and a key negative regulator of the insulin, IGF1 and mTORC1 signaling pathways. GRB10 is highly expressed in mouse fetal liver but almost completely silenced in adult mice, suggesting a potential detrimental role of this protein in adult liver function. Here we show that the Grb10 gene could be reactivated in adult mouse liver by acute endoplasmic reticulum stress (ER stress) such as tunicamycin or a short-term high-fat diet (HFD) challenge, concurrently with increased unfolded protein response (UPR) and hepatosteatosis. Lipogenic gene expression and acute ER stress-induced hepatosteatosis were significantly suppressed in the liver of the liver-specific GRB10 knockout mice, uncovering a key role of Grb10 reactivation in acute ER stress-induced hepatic lipid dysregulation. Mechanically, acute ER stress induces Grb10 reactivation via an ATF4-mediated increase in Grb10 gene transcription. Our study demonstrates for the first time that the silenced Grb10 gene can be reactivated by acute ER stress and its reactivation plays an important role in the early development of hepatic steatosis.

Introduction

GRB10 is an adaptor protein that negatively regulates insulin and IGF1 signaling by binding directly to the kinase domains of these receptors (Liu & Roth 1995, Lim et al. 2004). As an imprinted gene, Grb10 is predominantly expressed from the maternally inherited allele in periphery tissues such as fat, muscle and liver tissues (Lim et al. 2004). Recently, we have identified GRB10 as a negative-feedback regulator of the mTORC1 signaling pathway promotes lipolysis and thermogenesis in adipose tissue (Liu et al. 2014). GRB10 has also been found to negatively regulate muscle mass and impair glucose metabolism in muscle (Holt et al. 2012, Mokbel et al. 2014). However, the roles of GRB10 in the liver are largely unknown. GRB10 is highly expressed at the fetal stage in mouse liver, but its hepatic expression is almost completely suppressed in adult mice (Wang et al. 2007, Zhang et al. 2012). There is some evidence suggesting that GRB10 expression could be stimulated by stress stimulation. For example, ATP-depletion increased GRB10 expression in renal proximal tubule cells (Singh et al. 2013). In addition, the expression of GRB10 in mouse adipose tissue was induced by cold stress (Liu et al. 2014). However, it is current unknown whether GRB10 expression could be induced by other stress factors and whether GRB10 plays a role in stress-induced metabolic dysfunction in the liver.
Hepatic steatosis is characterized by an excessive accumulation of lipids that ultimately leads to disruption of tissue architecture and organ dysfunction. It typically occurs in response to over-nutrition and is a prominent pathological feature of obesity and type 2 diabetes. Increased lipid loading in the liver causes ER stress, leading to UPR and impaired lipid homeostasis (Ron & Walter 2007, Rutkowski et al. 2008, Walter & Ron 2011). While long-term HFD feeding and genetic obesity have been well-documented to induce chronic ER stress associated with hepatic steatosis and insulin resistance (Kammoun et al. 2009, Yoshiuchi et al. 2009, Ye et al. 2010), there is also evidence showing that a three to four days of HFD (acute HFD) feeding promotes hepatic steatosis and insulin resistance (Kraegen et al. 1991, Samuel et al. 2004, Lee et al. 2011, Ji et al. 2012), coinciding with UPR activation (Birkenfeld et al. 2011).

In humans, acute dietary fat intake increases hepatic lipid storage accompanied by changes of metabolic gene expression which are similar to changes in patients with type 2 diabetes or non-alcoholic fatty liver disease (Hernandez et al. 2017). This study implies that early changes in the liver metabolism may have long-lasting effects on hepatic lipid dysregulation.

In this study, we show that short-time HFD feeding-induced acute ER stress transcriptionally reactivates the silenced Grb10 gene in adult mouse liver, leading to enhanced impairment of lipid accumulation. We also show that acute ER stress-induced Grb10 reactivation is mediated by the ATF4 signaling pathway. Liver-specific knockout of Grb10 protects mice from tunicamycin- and short-term HFD-induced hepatic steatosis, uncovering an important role of Grb10 gene reactivation in acute ER stress-induced hepatic lipid accumulation.

Materials and methods

Animals

All protocols for animal use were approved by the Second Xiangya Hospital Animal Care and Use Committee of the Central South University.

Liver-specific Grb10 knockout mice (Grb10<sup>KO</sup>) were generated by breeding male Alb-Cre transgenic mice (the Jackson Laboratory) and female Grb10 floxed mice on C57BL/6 background (Zhang et al. 2012, Liu et al. 2014). 8–10-week-old adult mice were used. For tunicamycin injection experiments, equal male or female Grb10<sup>KO</sup> (Grb10<sup>KO</sup>/Alb-Cre) mice and the floxed control littermates (Grb10<sup>floxed</sup>) were injected intraperitoneally with tunicamycin (1 mg/kg body mass) to induce acute ER stress and hepatic lipid accumulation. As a control, mice were injected with vehicle (1% DMSO in 150 mM dextrose). For glucose tolerance tests (GTT) and insulin tolerance tests (ITT), 8-week-old male mice were used directly, or were fed a normal diet (ND) or a HFD (60% fat, 20% carbohydrate, 20% protein; D12492; Research Diets, New Brunswick, NJ) for the indicated time period. GTT were performed by injection of glucose (2 g/kg body weight) into overnight fasted mice. ITT were performed by injection of insulin (0.75 U/kg body weight) into 4 h-fasted mice. Blood was withdrawn from the tail vein and blood glucose were measured at 0, 15, 30, 60, 90, and 120 min after glucose or insulin injection.

Materials

Tunicamycin, thapsigargin and collagenase Type IV (100 U/mL) for primary hepatocyte isolation were purchased from Sigma-Aldrich. Metformin was purchased from Selleck Chemicals (Houston, USA). Actinonycin D was from Solarbio (Beijing, China). Synthetic oligonucleotides were purchased from Sangon Biotech (Shanghai, China) and Ribobio (Guangzhou, China). Lipofectamin 3000 was purchased from Life Technologies. Primary antibodies were from Cell Signaling Technology except antibodies against Actin and GAPDH purchased from Millipore Corporation, GRP78, GRP94 from Abcam, pIRE1alpha from Epitomics (Burlingame, USA) and Novus Biological (Littleton, USA), PPARalpha from Thermo Fisher Scientific, MTTP from Novus Biological, and Grb10 polyclonal antibody was homemade (Zhang et al. 2012). Secondary antibodies conjugated to Horse radish peroxidase were from Thermo Fisher Scientific.

Primary hepatocyte isolation, treatment and transient transfection

Primary hepatocyte isolation

Mouse primary hepatocyte isolation was performed by a modified two-step collagenase procedure as previously described (Glick et al. 2012). Briefly, primary hepatocytes were isolated from 8 to 10-week-old mice by perfusing the liver with hank’s balanced salt solution (HBSS), followed with type IV collagenase digestion medium. The liver was then excised and dissociated in cold DMEM isolation medium. Primary hepatocytes were then passed through a 100μm-cell strainer, collected and washed for three times. The viability of hepatocytes was more than 80% as assessed by trypan blue staining. The primary hepatocytes

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were seeded onto a 6-well plate (0.8 x 10⁶ cell/well) in MEM alpha medium (Thermo Fisher Scientific) supplemented with 0.1μm dexamethasone, 10% fetal bovine serum (FBS), 100IU/mL penicillin and 100mg/mL streptomycin. After cell spreading, the culture medium was deprived of FBS for 4h prior to initiating all treatments.

Pharmacological treatment
Tunicamycin (0.1μg/mL) or thapsigargin (1μM) was used to induce acute ER stress in cultured hepatocytes in the presence or absence of the transcription inhibitor actinomycin D (ACD, 5μg/mL), which was added to culture medium one hour before tunicamycin treatment.

Transient transfection
Small interfering RNAs (siRNAs) were designed and purchased from Sangon Biotech company. pCMV-ATF4-myc and pCMV-myc plasmids were gifts from Dr Feifan Guo of the Institute for Nutritional Sciences of Chinese Academy of Sciences. Each pair of siRNA (100pmol/well) and plasmids were transfected into hepatocytes using the Lipofectamine 3000 Reagent according to protocols of the manufacturer. Thirty hours after siRNAs transfection, cells were treated for another 12h with or without 0.1μg/mL tunicamycin and then collected. Forty-eight hours after plasmids transfection, cells were collected. The mRNAs and proteins were then isolated and followed by expression measurement. The siRNA sequences are siAtf4: 5'-GCACUUCAAACCUCUAUGGdtdTdT-3', siXbp1: 5'-CTGCTAGTCTGGAGGAACTdT dT-3', siAtf6: 5'-GTGAAAGGCCATTTGCTTTdTdT-3'.

Analysis of allelic expression
Brain and liver tissues were collected from neonatal pups. RNAs were extracted from those tissues and used as positive controls. RNAs were also extracted from primary adult hepatocytes with or without tunicamycin treatment. We analyzed the expression of various GRB10 isoforms in the same samples using a previous identified RT-qPCR primers (Arnaud et al. 2003). For maternal-specific expressed transcripts, we used primers separately for mouse GRB10 isoform alpha (mGRB10alpha) and mouse GRB10 isoform delta (mGRB10delta). For parental-specific expressed transcripts mouse GRB10 isoform beta (mGRB10beta), we used primers for both mGRB10beta1 and mGRB10beta2. Results of RT-qPCR were showed in DNA gel with Actin as internal control.

Total RNA isolation and real-time quantitative PCR (RT-qPCR)
Total RNAs were purified from frozen liver tissue using TRIzol (Thermo Fisher Scientific) according to the manufacturer’s instructions. cDNA synthesis was performed using one μg mRNA and the PrimeScript RT reagent kit with gDNA Eraser (Takara Bio). RT-qPCR was done in triplicate using the FastStart Universal SYBR Green Master kit (Roche Mannheim) in a final reaction volume of 10μL. Measurements were performed on an ABI Prism 7900 sequence detector system (Applied Biosystems). The relative mRNA levels were calculated using actin as the reference gene. A control liver served as calibrator sample for the calculation of fold induction in gene expression. Primer sequences are available upon request.

Western blot analysis
Tissues were rapidly removed from sacrificed mice and homogenized in lysis buffer (2% SDS supplemented with proteinase inhibitors and phosphatase inhibitors). Primary hepatocytes were lysed in sample loading buffer. Equal amounts of proteins were loaded and resolved by SDS-PAGE, transferred to PVDF membrane (Pall Corporation, Port Washington, USA), blocked with 5% BSA for one hour, and incubated with primary antibody dilution overnight at 4°C. Next day, the membranes were washed with PBST (0.05% tween in PBS), and incubated with secondary antibodies diluted in PBST with 5% BSA for 1h at room temperature. Protein bands were visualized with the BIO-RAD ChemiDoc XRS+ imaging system and quantified with ImageJ.

Oil red O and BODIPY (boron-dipyrromethene) staining
Mouse livers were excised freshly and coated with OCT (optimal cutting temperature compound) (Tissue-Tek) at ~80°C. Frozen tissue blocks were sectioned (10μm) and stained with oil red O solution followed by hematoxylin for nucleic staining. To stain primary hepatocytes, cells were washed, fixed with 4% PFA, and then stained with oil red O solution. Tissue BODIPY staining was performed using the BODIPY 493/503 staining kit (Thermo Fisher Scientific). All slides were then washed, mounted with prolong gold anti-fade reagent (Thermo Fisher Scientific) and finally imaged using the OLYMPUS IX71 microscope.
Transmission electron microscopy

Liver tissues (3 mm pieces) were fixed in 2% glutaraldehyde 2% paraformaldehyde in 0.1 M cacodylate buffer followed by post-fixation in the same fixative buffer for overnight. Ultrathin tissue sections were counterstained with 2% uranyl acetate and lead citrate, and imaged using a transmission electron microscope (Hitachi).

Statistical analysis

Statistical significance was determined using GraphPad Prism 5.0 with Student's t-test. More than three animals of each genotype were used for in vivo experiments and three replicates for in vitro experiments and real-time qPCR. Data are expressed as mean ± s.e.m. P values <0.05 were considered statistically significant.

Results

Acute ER stress induces Grb10 gene reactivation in adult mouse liver

Consistent with previous reports (Dong et al. 1997, Frantz et al. 1997, Zhang et al. 2012), the protein levels of GRB10, which were detected as multiple bands resulted from alternative mRNA splicing and/or different protein modification, were extremely low in primary hepatocytes isolated from adult mice (Fig. 1A). Treating mouse primary hepatocytes with ER stress inducers such as tunicamycin (Fig. 1A) or thapsigargin (Fig. 1B) greatly promoted GRB10 and several ER stress markers such as phosphorylation of IRE1alpah, CHOP and stress chaperones GRP78 and GRP94 expression. Likewise, GRB10 expression, which was almost silenced in adult mouse liver, was markedly and selectively induced by intraperitoneal injection of tunicamycin (Fig. 1C). To confirm the role of ER stress in the induction of GRB10 expression, we treated mouse primary hepatocytes with ER stress inhibitor tauroursodeoxycholic acid (TUDCA) before tunicamycin treatment. We found that tunicamycin-induced GRB10 expression was significantly inhibited in TUDCA-treated cells (Fig. 1D). In line with this finding, metformin, an anti-diabetic drug that inhibits ER stress (Kim et al. 2010a, 2015, Jung & Choi 2016), greatly repressed the tunicamycin-induced GRB10 expression (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article). These results demonstrate that ER stress reactivates Grb10 gene expression in adult mouse liver.

Time course studies revealed that the highest hepatic Grb10 mRNA levels could be detected at approximately 8 h after tunicamycin injection, which correlated with the highest expression of the ER stress marker Grp78 (Fig. 1E). This correlation was further supported by the finding that tunicamycin treatment stimulated Grb10 and ER stress marker genes transcription in primary hepatocytes (Fig. 1F). Actinomycin D, an inhibitor of transcription, completely suppressed the promoting effects of tunicamycin on the expression of Grb10, Grp78 and Atf4 (Fig. 1F). Taken together, these results indicate that ER stress induces GRB10 expression at the transcription level.

Generation of liver-specific Grb10-knockout mice

To determine the potential role of acute ER stress-induced hepatic GRB10 expression in vivo, we generated liver-specific Grb10-knockout mice (Grb10 KO) by crossing the Grb10 floxed mice with Alb-Cre mice. Since hepatic GRB10 expression was dramatically decreased after birth and almost completely silenced after postnatal day 14 (Fig. 2A), mice at postnatal day 9 were used to confirm liver-specific knockout of GRB10. The expression of GRB10 protein was detected in the liver and several other tissues of the floxed control mice at postnatal day 9, but it was greatly suppressed in the liver of age-matched Grb10 KO mice (Fig. 2B). The higher levels of GRB10 expression in 9-day-old Grb10 KO mice is consistent with the finding of Postic et al. who showed that DNA excision mediated by the Alb-cre transgene in liver is only about 40% at birth but increases to 60% by 1 week and to 75% at 3 weeks of age (Postic & Magnuson 2000).

These findings suggest that insufficient deletion of the Grb10 gene in early development is most likely due to a low expression of the albumin-mediated cre gene. The Grb10 KO mice were viable, fertile and grew at a normal rate (Fig. 2C), and both male and female Grb10 KO mice displayed similar food intake compared to control mice (Data not shown). No significant difference in glucose tolerance and insulin sensitivity was detected between 8-week-old Grb10 KO mice and control mice (Fig. 2D and E). Notably, tunicamycin-induced Grb10 re-expression occurred in adult control mice but not in Grb10 KO mice confirmed the successful generation of the liver-specific Grb10-knockout mice (Fig. 2F).
Hepatic deletion of the Grb10 gene alleviates tunicamycin- and acute HFD feeding-induced lipid accumulation

Treating mice with tunicamycin for 16 h dramatically induced hepatic steatosis, as demonstrated by the pale liver appearance (Fig. 3A). The tunicamycin-induced hepatosteatosis was greatly reduced in the liver of Grb10LKO mice compared with control mice, as demonstrated by oil red O staining and BODIPY staining experiments (Fig. 3A and C). In line with these findings, electronic microscopic studies revealed a significantly lower content of tunicamycin-induced accumulation of lipid droplets in the liver of Grb10LKO mice compared to control mice (Fig. 3D). Consistently, less lipid droplets were detected in tunicamycin-treated primary hepatocytes isolated from Grb10LKO mice compared to those from control mice (Fig. 3E), indicating that Grb10 disruption alleviates tunicamycin-induced hepatic steatosis is a cell autonomous event.

Short-term (3–4 days) HFD feeding has been shown to cause acute lipid loading stress in the liver, leading to increased eukaryotic translation initiation factor 2 alpha (eIF2alpha) signaling, rapid hepatic lipid accumulation and insulin resistance (Kraegen et al. 1991, Samuel et al. 2004, Birkenfeld et al. 2011, Lee et al. 2011, Ji et al. 2012). In order to verify whether Grb10 could be reactivated by acute HFD challenge, we fed mice with HFD for 1–7 days.

Figure 1

GRB10 expression is transcriptionally induced by ER stress in adult mouse liver. The expression of GRB10 in primary mouse hepatocytes treated with DMSO or 0.1μg/mL tunicamycin (A) or 1μM thapsigargin (B) for 24 h. Actin was used as a loading control. (C) The protein levels of GRB10 protein in several tissues of adult mice !h after vehicle (−) or 1mg/kg body mass tunicamycin (+) injection. GAPDH was used as a loading control. (D) The protein levels of GRB10 and GRP78 in primary mouse hepatocytes treated with DMSO (−) or with 0.1μg/mL tunicamycin (+) in the presence or absence of indicated concentration of TUDCA for 24 h. Actin was used as a loading control. (E) Real-time qPCR analysis of Grb10 and Grp78 mRNA in livers of adult mice injected intraperitoneally with vehicle (Veh) or tunicamycin (1mg/kg body mass) for the indicated times. Results are shown as mean ± s.e.m. (3-5 mice per group). *P<0.05; **P<0.01; ***P<0.001 compared with mice injected with vehicle. (F) Real-time qPCR analysis of Grb10, Grp78 and Atf4 mRNAs in primary hepatocytes treated with DMSO or 0.1μg/mL tunicamycin in the presence or absence of 5μg/mL actinomycin D (ACD). Results are shown as mean ± s.e.m. (n=3). **P<0.01; ***P<0.001 compared with primary hepatocytes treated with DMSO. (G) Real-time qPCR analysis of Grb10, Grb7 and Grb14 mRNAs in primary hepatocytes treated with DMSO or 0.1μg/mL tunicamycin. mRNA levels were normalized to actin. Results are shown as mean ± s.e.m. (n=3). ***P<0.001 compared with primary hepatocytes treated with DMSO. TM, tunicamycin; TG, thapsigargin.
GRB10 deficiency in the liver decreases the expression of genes involved in fatty acid synthesis

Hepatic steatosis involves excessive deposition of lipids droplets by impairing any stages of hepatic lipid metabolism including degradation (β-oxidation), synthesis and/or import (uptake) and export of triacylglycerol (Cohen et al. 2011). To determine the mechanisms responsible for the decreased hepatosteatosis in the tunicamycin-treated Grb10KO mice, we examined the expression levels of Cd36, which is known to play an important role in fatty acid uptake (Koonen et al. 2007). No significant difference in the expression levels of Cd36 and other lipid uptake-related genes such as fatty acid-binding protein 1 (FABP1) and fatty acid transport protein 1 (FATP1) was observed between tunicamycin-injected Grb10KO mice and their control littermates (Fig. 5A). Liver-specific knockout of GRB10 also had no significant effect on the protein levels of genes involved in β-oxidation (Reddy & Rao 2006) such as peroxisome proliferator activated receptor mRNA levels of Grb10 and Atf4 were significantly induced by 3 days of HFD feeding (Fig. 4A). Acute HFD challenge significantly increased the protein levels of GRB10, GRP78, and phosphorylation of eIF2alpha (Fig. 4B). Liver-specific deletion of GRB10 significantly inhibited acute HFD-induced GRB10 re-expression (Fig. 4C) and lipid accumulation in the liver (Fig. 4D). These findings demonstrate that acute HFD feeding also induces ER stress and consequent reactivation of the Grb10 gene in the liver, which contributes to the increased hepatic lipid accumulation.

Figure 2

Generation of liver-specific Grb10 knockout mice. (A) Expression pattern of GRB10 protein in livers of wild-type mice of different ages. E15.5, embryonic day 15.5; P, postnatal day; 8 w, 8 weeks old. Actin was used as a loading control. (B) The protein levels of GRB10 in liver, pancreas, brain and subcutaneous inguinal white adipose (swAT) tissues of mice at postnatal day 9. Actin was used as a loading control. (C) Body weight of control and Grb10KO mice of different ages with normal diet feeding. Results are shown as mean ± s.e.m. (8–10 mice per group). n.s., non-significant compared with control mice. (D) Glucose tolerance test was performed on 8-week-old control and Grb10KO mice fed with normal diet. Results are shown as mean ± s.e.m. (10 mice per group). (E) Insulin tolerance test was performed on 8-week-old control and Grb10KO mice fed with normal diet. Results are shown as mean ± s.e.m. (8 mice per group). n.s., non-significant compared with control mice. (F) The mRNA and protein levels of GRB10 in livers of 8-week-old control and Grb10KO mice 16h after vehicle (Veh) or tunicamycin (1 mg/kg body mass) injected. mRNA levels were normalized to actin. Results are shown as mean ± s.e.m. (n=4). *p<0.05 compared with control mice injected with vehicle. Actin was used as a loading control. Ctr, control floxed mice; KO, Grb10KO mice; TM, tunicamycin. A full color version of this figure is available at https://doi.org/10.1530/JME-18-0018.

Figure 3

Liver-specific GRB10 knockout alleviates tunicamycin-induced hepatic lipid accumulation. (A) Liver appearance of adult control and Grb10KO mice 16h after vehicle or tunicamycin (1 mg/kg body mass) injection. (B and C) Imaging of lipid droplets in liver tissue sections of adult control and Grb10KO mice 16h after vehicle or tunicamycin (1 mg/kg body mass) injection. (B) Oil red O staining (ORO), bar, 25 μm. (C) BODIPY staining, bar, 25 μm. (D) Electron microscopy of liver from adult control and Grb10KO mice 16h after tunicamycin (1 mg/kg body mass) injection. Lipid droplets are indicated by asterisk. Bar, 250 nm. (E) Oil red O staining of lipid droplets in primary mouse hepatocytes isolated from control and Grb10KO mice with DMSO or 0.1 μg/mL tunicamycin treatment for 24h. Insets show higher magnification of the cells. Bar, 50 μm. Ctr, control floxed mice; KO, Grb10KO mice; N, nucleus; TM, tunicamycin.
alpha (PPARalpha) and carnitine palmitoyltransferase 1A (CPT1A) (Fig. 5B), as well as genes involved in the assembly of very low-density lipoprotein (VLDL) (Noiva 1999) such as microsomal triglyceride transfer protein (MTTP) and protein disulfide isomerase (PDI) in the livers of tunicamycin-treated Grb10<sup>−/−</sup> mice and control mice (Fig. 5C). The protein levels of fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and ATP citrate lyase (ACL) were significantly decreased in the liver of Grb10<sup>−/−</sup> mice compared to controls in response to tunicamycin treatment (Fig. 5D), despite similar mRNA levels of these proteins in the livers of Grb10<sup>−/−</sup> mice and controls (Fig. 5E). These findings suggest that GRB10 deficiency decreases the expression of lipid synthesis-related genes, which could contribute to reduced lipid content in the liver.

**Discussion**

In the current study, we show that acute ER stress or short-time HFD challenge activates the silenced Grb10 gene in adult mouse liver. Liver-specific knockout of Grb10 alleviates acute HFD challenge or tunicamycin-induced hepatosteatosis, uncovering an important role of GRB10 of mGRB10alpha and mGRB10delta isoforms were detected in mouse peripheral tissues (Arnaud et al. 2003, Lim et al. 2004). In order to investigate the parental origin of GRB10 re-expression by ER stress, we investigated the isoform-specific expression of GRB10 in tunicamycin-treated hepatocytes. We found that the expression of mGRB10beta was restricted to the brain of neonatal mice while the expression of mGRB10alpha and mGRB10-delta isoforms was detected in the liver (Fig. 6A). Tunicamycin greatly induced the mRNA levels of mGRB10alpha and mGRB10delta but not mGRB10beta in primary hepatocytes isolated from adult mice (Fig. 6A), indicating that tunicamycin treatment promotes Grb10 gene reactivation from the maternal allele.

ER stress activates three major UPR signaling pathways including the inositol-requiring enzyme 1 alpha (IRE1alpha), the protein kinase RNA (PKR)-like ER kinase (PERK) and the activating transcription factor 6 (ATF6)-mediated pathways (Schroder & Kaufman 2005, Ron & Walter 2007). IRE1alpha is a protein kinase possessing endonuclease (RNase) domain in the ER membrane (Tirasophon et al. 2000). When activated, IRE1alpha splices Xbp1 mRNA to produce an active transcriptional factor XBP1s. PERK phosphorylates eIF2alpha, which selectively promotes Atf4 transcription. The increased expression of XBP1s and ATF4 as well as ATF6 cleavage activates respective downstream target genes such as chaperone genes glucose-regulated protein (Gpp78, Gpp94) or C/EBP homologous protein (Chop) to restore ER homeostasis (Walter & Ron 2011). To determine which pathway contributes to the acute ER stress-induced GRB10 expression, we suppressed the expression of Atf4, Atf6 and Xbp1 by small interfering RNA (siRNA) in primary hepatocytes. Suppressing the expression of Atf4 (Fig. 6B) but not Atf6 nor Xbp1 (Fig. 6C and D) in primary hepatocytes significantly blocked tunicamycin-induced expression of Grb10 and Vldlr, a downstream target of ATF4 (Jo et al. 2013). However, overexpression of ATF4 had no significant effect on the mRNA or protein levels of GRB10 (Fig. 6E and F), suggesting that ATF4 is necessary but not sufficient to mediate acute ER stress-induced GRB10 re-expression.
in regulating hepatic lipid metabolism. We also find that the effect of acute ER stress on Grb10 re-expression is largely mediated by ATF4-mediated transcription. Taken together, these findings reveal for the first time that the silenced Grb10 gene could be reactivated by acute ER stress and that Grb10 reactivation plays an important role in promoting hepatic lipid accumulation at the onset of fatty liver disease (Fig. 6G).

The finding that Grb10 gene is reactivated in adult mouse liver by acute ER stress suggests that GRB10 may function as a stress response protein. Consistently, GRB10 expression has been found to be induced in renal proximal tubule cells under ATP-depletion-induced stress (Singh et al. 2013), in the hypoxia-challenged liver of common sole flounder (Mazurais et al. 2014), and in hypoxic human adipocytes from Simpson-Golabi-Behmel syndrome (SGBS) patients (Leitherer et al. 2014). Our recent study also shows that GB10 expression is upregulated by cold stress in adipose tissue (Liu et al. 2014). A recent study shows that increased phosphorylation of GRB10 at S501/S503 during muscle hypertrophy is associated with activated ER stress response (Hamilton et al. 2014), suggesting that increased expression and/or phosphorylation of GRB10 may be a response for stress-induced metabolic and physiological demand. Thus, GRB10 may function as a stress response protein and play an important role in the regulation of energy homeostasis in vivo.

Cells adapt to persistent chronic stress by altering protein expression patterns that are qualitatively distinct from acute ER stress-induced UPR (Rutkowski et al. 2006, Rutkowski & Kaufman 2007). In fact, acute and chronic ER stress has been reported to have different effects on metabolism (Fu et al. 2012). We found that Grb10 gene reactivation was induced by acute (Fig. 1) but not chronic (data not shown) HFD feeding, highlighting the complexity of hepatic physiological
settings during HFD feeding process. It is tempting to speculate that GRB10 re-expression following acute HFD challenge may represent a rapid hepatic response to environmental change-induced ER stress, which is absent during sustained HFD feeding due to altered pathophysiological setting in the liver. Our results also reveal that although tunicamycin and thapsigargin have been frequently used to study the role of ER stress in metabolic dysfunction, distinct differences exist between acute ER stress induced by these compounds and chronic ER stress induced by other stimuli such as chronic HFD feeding.

Lipid accumulation in a tissue is dependent not only on lipogenesis but also on other mechanisms, such as increased lipid deposition, decreased lipolysis and/or reduced triglyceride output (Cohen et al. 2011). Tunicamycin affects several pathways contributing to the hepatic lipid accumulation, including inhibition of fatty acid oxidation (DeZwaan-McCabe et al. 2017), upregulation of the very-low-density lipoprotein receptor (Jo et al. 2013) and inhibition of efflux of triglyceride (Qiu et al. 2006, Ota et al. 2008). Interestingly, while tunicamycin inhibits the mRNA expression of lipogenic genes such as Fas and SrebF1 in liver (Rutkowski et al. 2008), tunicamycin treatment for either 8h or 24h had no significant effect on lipogenic activity (DeZwaan-McCabe et al. 2017). These findings suggest that other mechanisms, rather than promoting lipogenic gene expression, may play major roles in tunicamycin-induced hepatic steatosis.

How the Grb10 gene reactivation promotes acute ER stress-induced hepatic lipid accumulation remains to be further elucidated. We found that Grb10 knockout decreased the expression of hepatic lipid synthesis-related genes, suggesting the protein may contribute to acute ER stress-induced lipid dysregulation by promoting lipid synthesis. However, the role of GRB10 on lipid metabolism seems not to be related to insulin function change. Both we and others have previously found that tunicamycin inhibits the insulin signaling pathway by decreasing the phosphorylation of AKT in the liver (Chen et al. 2011, Han et al. 2016, Feng et al. 2017). However,
we also noticed that knocking out the Grb10 gene in the mouse liver only slightly suppresses the inhibitory effect of tunicamycin on AKT phosphorylation (Supplementary Fig. 2), suggesting that tunicamycin may inhibit insulin signaling through additional mechanisms in conjunction with the induction of Grb10 gene re-expression. Notably, liver-specific knockout of GRB10 significantly decreased the protein but not mRNA levels of enzymes involved in fatty acid synthesis, suggesting that ER stress-induced hepatic Grb10 gene reactivation might play a role in the production or stability of those metabolic enzymes. GRB10 has been reported to interact with E3 ubiquitin ligase NEDD4 (Morrione et al. 1999, Huang & Szebenyi 2010) and mediate or prevent the degradation of specific proteins (Vecchione et al. 2003, Murdaca et al. 2004, Ramos et al. 2006, Monami et al. 2008). A similar mechanism may be used by GRB10 to regulate the protein levels of the enzymes involved in lipid synthesis. More investigation will be needed to test this hypothesis.

We found that overexpression of ATF4 is not sufficient to reactivate the Grb10 gene, suggesting the presence of other acute ER stress-specific components that coordinate with ATF4 to regulate hepatic GRB10 expression. In fact, our unpublished data show that inhibition of the endonuclease activity of IRE1alpha prevents tunicamycin-induced GRB10 protein expression without affecting Grb10 mRNA levels, suggesting that IRE1alpha exerts posttranscriptional regulation on the Grb10 gene expression. Further studies are needed to fully elucidate the mechanisms by which acute ER stress induces Grb10 gene expression in the liver.

The regulation of Grb10 gene expression is more complex in humans, which shows a highly tissue- and isoform-specific imprinting profile (Blagitko et al. 2000, Yoshihashi et al. 2000, Hitchins et al. 2001). The human Grb10 gene is expressed specifically from the paternal allele in the fetal brain and is biallelic in most other tissues, which is different to the imprinting pattern seen in mice (Blagitko et al. 2000, Hitchins et al. 2001). GRB10 regulates insulin and glucagon secretion in human islets (Prokopenko et al. 2014) and the Grb10 gene has been considered as a potential diabetes candidate gene in humans (Kluth et al. 2014). GRB10 expression is increased in acute myeloid leukemia and melanoma metastases (Mirmohammadsadegh et al. 2004, Kazi & Ronnstrand 2013), but is reduced in some other human tumors and suppresses the proliferation of some human tumor cell lines (Yu et al. 2011, Mroue et al. 2015), suggesting that GRB10 expression may be involved in cancer progression. However, the mechanisms regulating GRB10 expression in human tumors, and whether GRB10 loss plays a role in tumorigenesis, are completely unknown.

We found that silencing Grb10 gene expression in the adult mouse liver could minimize hepatic lipid accumulation, thus providing a mechanism to protect mice from excessive ectopic fat deposition in the liver under certain pathophysiological conditions, such as acute fatty liver. Our study also suggests a potential role of GRB10 in other acute ER stress-induced liver dysfunction. In fact, iron or heavy metal loading (Hiramatsu et al. 2007, Lou et al. 2009), toxins such as carbon tetrachloride (Kim et al. 2010b), and even some clinically used drugs such as acetaminophen (Nagy et al. 2007) and bortezomib (Dong et al. 2009) were all shown to induce acute ER stress and activate the UPR. However, further studies will be needed to determine whether Grb10 gene expression could be reactivated in the adult mouse liver by these anticancer drugs.

In summary, our data demonstrates a causal role for acute ER stress in promoting Grb10 gene reactivation in mouse liver. In addition, we show that reactivation of the Grb10 gene contributes to acute HFD challenge-induced hepatic lipid dysregulation. The finding that Grb10 gene is reactivated at the early stage of hepatic steatosis suggests that the development of chronic diseases is a molecularly dynamic process, which provides new insights into the distinct roles of acute and chronic ER stress in regulating hepatic lipid metabolism.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JME-18-0018.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
L P L designed, performed and analyzed most of the experiments and wrote the paper. W X J helped to design and performed the experiments shown in Figs 1 and 4. H L performed the experiments shown in Figs 2 and 6. J C B, P T, Y D, Z P X, H R L and B L L provided technical assistance and contributed to the preparation of the figures. Z G Z contributed to data analysis. B X initiated plan and provided guidance of the project. F L

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conceived the study, helped in experimental design, analyzed the data and wrote the paper with L P L. All authors reviewed the results and approved the final version of the manuscript.

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