UPR Pathways Combine to Prevent Hepatic Steatosis Caused by ER Stress-Mediated Suppression of Transcriptional Master Regulators

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SUMMARY

The unfolded protein response (UPR) is linked to metabolic dysfunction, yet it is not known how endoplasmic reticulum (ER) disruption might influence metabolic pathways. Using a multilayered genetic approach, we find that mice with genetic ablations of either ER stress-sensing pathways (ATF6α, elf2α, IRE1α) or of ER quality control (p58IPK) share a common dysregulated response to ER stress that includes the development of hepatic microvesicular steatosis. Rescue of ER protein processing capacity by the combined action of UPR pathways during stress prevents the suppression of a subset of metabolic transcription factors that regulate lipid homeostasis. This suppression occurs in part by unresolved ER stress perpetuating expression of the transcriptional repressor CHOP. As a consequence, metabolic gene expression networks are directly responsive to ER homeostasis. These results reveal an unanticipated direct link between ER homeostasis and the transcriptional regulation of metabolism, and suggest mechanisms by which ER stress might underlie fatty liver disease.

INTRODUCTION

Cellular protein folding homeostasis is protected when the depletion of chaperone reserve leads to the activation of proximal signaling molecules, which ultimately results in alterations in gene expression to alleviate stress. In the endoplasmic reticulum (ER), three principal pathways are activated in response to ER stress and comprise the unfolded protein response (UPR): pancreatic ER eukaryotic translation initiation factor (eIF)-2α kinase (PERK), inositol-requiring enzyme (IRE)-1, and activating transcription factor (ATF)-6. PERK and IRE1 are ER-resident transmembrane kinases that lead to translational inhibition through elf2α phosphorylation, and production of X-box binding protein (XBP)-1 transcription factor by an unconventional splicing mechanism, respectively. ATF6 is a transmembrane transcription factor liberated by stress-regulated intramembrane proteolysis. Each pathway culminates in transcriptional regulation of gene expression and contributes to the overall maintenance of homeostasis in the ER during stress (Ron and Walter, 2007; Schröder, 2008; Wek and Cavener, 2007; Wu and Kaufman, 2006). Gene expression profiling has demonstrated that numerous cellular processes beyond protein folding in the ER are regulated by UPR activation (Harding et al., 2003; Shen et al., 2005; Travers et al., 2000). However, how these processes are activated and temporally regulated by ongoing stress, and how such regulation alters cellular functions only tangentially related to secretory pathway function to improve the chances for adaptation, is not understood.

ER stress has been associated with metabolic dysfunction caused by dietary demand (Lee et al., 2008; Oyadomari et al., 2008; Özcan et al., 2004, 2006). How the various pathways of the UPR protect cells from stress in vivo, and how these pathways intersect with other cellular functions, such as metabolism, in different circumstances is not clear. Thus there is a need to examine the functions of the individual UPR signaling pathways in parallel and in vivo. The liver presents an ideal model system for studying these connections, because it is a key organ for both protein secretion (principally in the form of lipoproteins and other serum factors) and metabolism (Postic et al., 2004). The coupling of these processes involves families of transcriptional activators and coregulators. Among these are members of the CCAAT/enhancer binding protein (C/EBP), peroxisome proliferator-activated receptor (PPAR), PPARγ coactivator (PGC)-1, and sterol response element binding protein (SREBP) families, which reprogram liver metabolic mRNA expression as...
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Environmental cues dictate (Ferre and Foufelle, 2007; Lee et al., 2003; Lekstrom-Himes and Xanthopoulos, 1998; Lin et al., 2005). Liver function is also sensitive to environmental or genetic perturbation, and a number of these perturbations, including both alcoholic and nonalcoholic steatopathies, viral hepatitis, hyperhomocysteinemia, acute exposure to hepatotoxins, and high carbohydrate or high fat diets, have been suggested to lead to hepatic dyslipidemia (Jaeschke et al., 2002; Lee et al., 2008; Nguyen et al., 2005). Liver function is also sensitive to environmental or genetic perturbation, and a number of these perturbations, including both alcoholic and nonalcoholic steatopathies, viral hepatitis, hyperhomocysteinemia, acute exposure to hepatotoxins, and high carbohydrate or high fat diets, have been suggested to lead to hepatic dyslipidemia. Liver function is also sensitive to environmental or genetic perturbation, and a number of these perturbations, including both alcoholic and nonalcoholic steatopathies, viral hepatitis, hyperhomocysteinemia, acute exposure to hepatotoxins, and high carbohydrate or high fat diets, have been suggested to lead to hepatic dyslipidemia.

ATF6α protects ER function by augmenting the upregulation of ER protein processing factors, such as chaperones and ER-associated protein degradation (ERAD) machinery during stress (Adachi et al., 2008; Wu et al., 2007; Yamamoto et al., 2007). ATF6α deletion sensitizes cells and animals to persistent ER stress. In vivo, this failure to recover from ER stress results in fatty liver, uncovering a potential connection between ER stress and lipid metabolism (Wu et al., 2007). In this study, we have used a genetic approach to identify the proximal mechanistic connections between activation of the UPR by ER stress and metabolic control. This approach has revealed that the three arms of the UPR cooperate to maintain ER function, and that failure to do so leads to unresolved ER stress and suppression of several key regulators of metabolic gene expression independent of any specific UPR pathway. These results suggest that an intact UPR guards liver function by improving ER protein processing to maintain lipid homeostasis.

RESULTS AND DISCUSSION

Unresolved ER Stress Perturbs Hepatic Lipid Homeostasis

Animals nullizygous for the Atf6α gene display no overt phenotype under unchallenged conditions, being viable, fertile, and grossly normal. However, when injected intraperitoneally with an ordinarily sublethal dose of the ER stress-inducing agent tunicamycin (TM), Atf6α-null animals succumb and show evidence of persistent ER stress in the liver and kidneys (Wu et al., 2007). Consistent with our previous findings, TM-challenged Atf6α-null animals displayed prolonged upregulation of the apoptotic protein, C/EBP-homologous protein (CHOP), and its target GADD34 at a time when the expression of these genes is attenuated in wild-type animals (Figure 1A). While adaptation to or recovery from ER stress is accompanied by rapid degradation of Chop mRNA and protein (Rutkowski et al., 2006), persistent CHOP expression of tubulin (loading control) BIP, CHOP, GADD34, and the phosphorylated form of eIF2α. Efficacy of the TM was reflected in inhibition of TRAPs' glycosylation, for which the glycosylated (closed arrow) and unglycosylated (open arrow) species are indicated. (B) Wild-type and Atf6α−/− mice were injected with 1 mg/kg TM and livers were visualized in situ at the indicated times postinjection. (C) Wild-type and Atf6α−/− mice were injected with TM. Cryosections (6 μm) of liver isolated 48 hr postinjection were stained with H&E and visualized at 400× magnification. (D) Wild-type and Atf6α−/− mice were injected with vehicle (NT, not treated) or TM (1 mg/kg). Livers were surgically removed 48 hr postinjection, fixed in 2.5% glutaraldehyde, then prepared for transmission electron microscopic analysis. ER, endoplasmic reticulum; LD, lipid droplets; N, nuclei; M, mitochondria. Scale bar, 500 nm. (E) Protein lysates from liver, isolated 8 or 48 hr postinjection, were probed by immunoblot for TRAP processing to maintain lipid homeostasis. Connections between activation of the UPR by ER stress and metabolic control. This approach has revealed that the three arms of the UPR cooperate to maintain ER function, and that failure to do so leads to unresolved ER stress and suppression of several key regulators of metabolic gene expression independent of any specific UPR pathway. These results suggest that an intact UPR guards liver function by improving ER protein processing to maintain lipid homeostasis.
expression correlates with unresolved stress. The most likely cause of this persistent stress is a failure of Atf6α-null animals to fully upregulate ER protein folding and processing machinery (Wu et al., 2007; Yamamoto et al., 2007), which renders them less able to recover from acute challenge.

Also consistent with our previous results (Wu et al., 2007), wild-type or heterozygous animals challenged with TM showed initial liver perturbation at the molecular and morphological levels, but recovered thereafter, while Atf6α−/− animals failed to recover. By 48 hr after injection, livers of Atf6α−/− animals were much lighter in color than wild-type counterparts, and produced a distinct fat cap upon homogenization and sedimentation (Figure 1B and data not shown). Hematoxylin and eosin (H&E) failed to stain large swaths of Atf6α−/− liver tissue after TM injection, Oil Red O staining confirmed the presence of fat deposits, and substantially more intracellular triglyceride was found in Atf6α-null animals than in the wild-type (Figure 1C, and data not shown; see Figure S1 available online). Ultrastructural analysis revealed a uniform accumulation of cytosolic lipid droplets in knockout livers, indicative of microvesicular steatosis (Figure 1D). In addition, while TM induced breakdown of the lamellar structure of the ER into smaller vesicles in wild-type animals, Atf6α-null animals appeared to show an even greater loss of structural integrity of the ER (Figure 1D). Confirming the accumulation of lipid, the cytosolic lipid droplet protein marker adiponutrient-related protein (ADRP) (Martin and Parton, 2006) was upregulated by ER stress in all genotypes, but more so in Atf6α-null animals (Figure 1E). This accumulation of lipid occurred concomitant with the persistent presence of XBPI protein derived from spliced mRNA, which reflects ongoing stress (Figure 1E and Figure S2).

Thus, lipid accumulation in the livers of Atf6α-null animals is an active process that accompanies persistent UPR signaling. Similar fat accumulation and ADRP upregulation were seen in the kidneys of TM-challenged Atf6α-null animals (Figure S3). ER stress and fat deposition were not observed in other tissues, type upon challenge, but did not otherwise result in lethality (in the kidneys of TM-challenged Atf6α-null animals). Confirming the accumulation of lipid, the cytosolic lipid droplet protein marker adiponutrient-related protein (ADRP) (Martin and Parton, 2006) was upregulated by ER stress in all genotypes, but more so in Atf6α-null animals (Figure 1E). This accumulation of lipid occurred concomitant with the persistent presence of XBPI protein derived from spliced mRNA, which reflects ongoing stress (Figure 1E and Figure S2). Thus, lipid accumulation in the livers of Atf6α-null animals is an active process that accompanies persistent UPR signaling. Similar fat accumulation and ADRP upregulation were seen in the kidneys of TM-challenged Atf6α-null animals (Figure S3). ER stress and fat deposition were not observed in other tissues, type upon challenge, but did not otherwise result in lethality (in the kidneys of TM-challenged Atf6α-null animals). Confirming the accumulation of lipid, the cytosolic lipid droplet protein marker adiponutrient-related protein (ADRP) (Martin and Parton, 2006) was upregulated by ER stress in all genotypes, but more so in Atf6α-null animals (Figure 1E). This accumulation of lipid occurred concomitant with the persistent presence of XBPI protein derived from spliced mRNA, which reflects ongoing stress (Figure 1E and Figure S2).

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Expression of ER chaperones and cochaperones (Grp94, Bip, BiP, Grp78, Grp58, Derl3, Edem1, Derl1), as well as genes involved in ERAD [Grp94, Derl3, Edem1], was attenuated in Atf6α-null livers (Figure 2B). In addition, early upregulation of the gene encoding the proapoptotic transcription factor CHOP depended partially upon ATF6 activation. In addition, early upregulation of the gene encoding the proapoptotic transcription factor CHOP depended partially upon ATF6 activation. In addition, early upregulation of the gene encoding the proapoptotic transcription factor CHOP depended partially upon ATF6 activation. In addition, early upregulation of the gene encoding the proapoptotic transcription factor CHOP depended partially upon ATF6 activation. In addition, early upregulation of the gene encoding the proapoptotic transcription factor CHOP depended partially upon ATF6 activation. In addition, early upregulation of the gene encoding the proapoptotic transcription factor CHOP depended partially upon ATF6 activation.

Notably, there was no obvious connection between Atf6α deletion and expression of genes involved in hepatic lipid metabolism, consistent with the phenotypic separation between genotypes developing progressively and manifesting more so at later time points rather than only 8 hr after challenge. However, we observed that a number of key mediators of lipid homeostasis were significantly downregulated by TM independent of geno- type (Figure 2C and Table S1). These included genes encoding the transcription factors and cofactors, SREBP1, C/EBPα, PPARα, FOXO1, and PGC1α, and components of lipid metabolism, including microsomal triglyceride transfer protein (MTP), fatty acid desaturases and synthetases (FADS-1 and -2, and fatty acid synthase [FASN]), and 3-hydroxy-3-methylglutaryl ER Stress Suppresses Expression of Genes Involved in Maintaining Energy and Lipid Homeostasis

Because ATF6α is a transcription factor, we reasoned that the connection between Atf6α deletion and disruption of lipid homeostasis might be most readily identified by global transcriptional analysis. Microarray profiling 8 hr after TM challenge revealed that, in mouse embryonic fibroblasts (MEFs) (Adachi et al., 2008; Wu et al., 2007), Atf6α deletion had little effect on basal gene expression (Figure 2A and Supplemental Data). However, when animals were challenged with TM for 8 hr, a subset of ER stress-regulated genes was altered in their expression by Atf6α deletion (Figure 2A). The majority of these genes that were upregulated by stress in wild-type liver, and either to a lesser or greater extent in Atf6α-null animals. As in MEFs, the expression of ER chaperones and cochaperones (Erp72, Erp57, p58IPK, Grp94), as well as genes involved in ERAD (Derl1, Edem1), was attenuated in Atf6α-null livers (Figure 2B). In addition, early upregulation of the gene encoding the proapoptotic transcription factor CHOP depended partially upon ATF6α.

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Figure 2. Persistent ER Stress In Vivo Suppresses Expression of a Subset of Metabolic Genes

(A) Cluster analysis of gene expression and transcriptional profiling analysis of liver RNAs were performed as described in the Experimental Procedures. Graphic representation of the average expression levels of 4262 differentially expressed genes is shown. Each vertical bar represents a single gene. Green coloration indicates lower expression, and red indicates higher expression.

(B and C) The expression levels of two subsets of mRNAs from microarray analysis are shown, normalized against vehicle-injected wild-type samples. Note that the differences in expression levels of these genes in the absence of ER stress were generally not significant. Data represent means ± SD from three animals in each group. For (B), all genes shown were significantly (p < 0.05) less induced by TM in Atf6α-null animals than wild-type animals. For (C), all genes shown were significantly (p < 0.05) downregulated by stress in both genotypes. The ATF4-dependent tryptophanyl tRNA synthetase (Wars) is here Atf6α dependent, likely because of the failure of Atf6α-null mice to stimulate eIF2α phosphorylation in response to TM challenge.

(D) Real-time RT-PCR analysis was used to assess the expression of selected mRNAs in wild-type or Atf6α-null livers 8 or 48 hr after TM injection. Data represent means ± SD from two (vehicle) or three (TM) animals. Expression values were normalized against β-actin levels and are shown relative to the expression level in wild-type unchallenged animals. Red lettering indicates genes significantly (p < 0.05) different in expression comparing TM-challenged Atf6α-null animals to unchallenged animals.
coenzyme A (CoA) reductase. This regulation was selective only for a subset of metabolic genes in that the expression of many others was unaltered after 8 hr of ER stress (Table S1).

Deletion of Atf6α does not immediately sensitize cells to stress, but instead renders them less able to recover from and adapt to ongoing stress (Wu et al., 2007). Indeed, the initial response of the liver to TM injection in Atf6α-null mice was very similar to that of wild-type mice in terms of activation of ER stress pathways, and it was only at later time points that knockout animals showed persistent ER stress when wild-type animals had recovered (Figure 1A). Thus, we speculated that Atf6α deletion might sensitize animals to ER stress, not by loss of direct regulation of metabolic gene expression by ATF6α, but instead by indirectly leading to ongoing stress in the liver because of failure to properly improve the protein folding environment in the ER. In support of this hypothesis, quantitative RT-PCR demonstrated that expression of the genes Mttp, Srebp1, Pgc1α, and Pparγ was suppressed by ER stress in both wild-type and Atf6α-null animals 8 hr after TM injection, but to a much greater extent in Atf6α-null animals 48 hr after injection (Figure 2D). Expression of C/ebpα mRNA and protein followed a similar trend, being rapidly suppressed by TM in both genotypes, but remaining suppressed in Atf6α-null animals over the course of the experiment (Figure 2E and data not shown). C/EBPα protein is synthesized in both long (p42) and short (p30) forms by virtue of translational regulation (Calkhoven et al., 2000) and, consistent with the decrease in C/ebpα mRNA, both forms were suppressed in Atf6α-null animals. We also observed upregulation of c-Jun at both the mRNA and protein levels (Figure 2E and data not shown). This is notable, because elevated hepatic expression of c-Jun accompanies C/ebpα deletion (Floodby et al., 1996).

We considered four possible mechanisms to account for fatty liver in TM-challenged Atf6α-null mice: (1) enhanced de novo synthesis of fatty acids in the liver; (2) increased mobilization and uptake of fatty acids from adipose tissue; (3) diminished fatty acid oxidation by the liver; and (4) decreased lipoprotein secretion. Because the genotype-specific changes in gene expression upon ER stress became evident at later time points after TM challenge, we used quantitative RT-PCR to probe the expression of several categories of genes involved in lipid and carbohydrate homeostasis 48 hr after challenge, when wild-type animals have largely recovered. The transcriptional regulators C/ebpα (gluconeogenesis and lipogenesis), Pparγ (fatty acid oxidation and gluconeogenesis), Pgc1α (fatty acid oxidation and gluconeogenesis), Srebp1α (lipogenesis), and ChREBP (a coregulator of lipogenesis with SREBP1) all remained significantly downregulated by persistent ER stress in Atf6α-null livers (Figure 2F). In contrast, other regulators including other C/ebp family members, Pparγ, Creb, and Lxrα, were not significantly affected by ER stress in these animals. Consistent with these results, we sampled the expression of key targets of these transcriptional regulators, finding that genes involved in fatty acid oxidation, gluconeogenesis, and lipogenesis were all suppressed in Atf6α-null animals (Figure 2G). In addition, genes involved in lipoprotein synthesis and transport were similarly suppressed, suggestive of a defect in activity of the transcription factor SREBP2. Immunoblot confirmed that both uncleaved and cleaved forms of SREBP1 and SREBP2 were downregulated in Atf6α-null animals, suggesting that the primary regulation of their activity after TM injection is transcriptional (data not shown). Since key proteins involved in lipogenesis (stearoyl-CoA desaturase-1, FASN, diacylglycerol acyltransferase-2) are downregulated in Atf6α-null animals, and a key protein that regulates the formation of new lipid droplets (FIT2 [Kadereit et al., 2008]) is not affected, de novo lipogenesis in the liver is an unlikely source of cytosolic lipid droplet formation. Likewise, Fabp1 suppression implies limited mobilization of fatty acids from adipose tissue. Taken together, these data suggest that lipid accumulates in the livers of Atf6α-null animals because of a defect in fatty acid oxidation, possibly augmented by impaired lipoprotein secretion. In support of this idea, microvesicular steatosis can accompany genetic defects in both fatty acid oxidation (Rao and Reddy, 2001) and lipoprotein secretion (Raabe et al., 1999).

ER Stress Sensing Pathways Cooperate to Maintain Homeostasis and Restore Metabolic Gene Expression

We next turned our attention to the mechanism by which ER stress leads to the suppression of these transcription factors. The rapid downregulation of genes encoding metabolic transcription factors by ER stress independent of genotype does not support the idea that ATF6α directly regulates these genes. Rather, there more likely exists a difference between wild-type and Atf6α-null mice that is exacerbated as the latter fail to recover from TM challenge, which is regulated indirectly by ATF6α. One noticeable difference between the responses of wild-type and Atf6α-null livers to TM is that Xbp1 mRNA splicing and production of XBPl protein from the spliced product persisted in the latter (Figure 1E and Figure S2). Thus, we considered the possibility that suppression of metabolic gene expression might depend upon signaling through the IRE1/XBP1 axis. Ire1α- and Xbp1-null animals die during embryogenesis because of liver defects (Reimold et al., 2000; Zhang et al., 2005). To circumvent this problem, we created animals with a floxed Ire1α allele and mated them with mice expressing the CRE recombinase under the control of the albumin promoter, thus generating a liver-specific deletion of Ire1α. Confirming efficient Ire1α deletion, these animals showed very little splicing of Xbp1 mRNA in the liver in response to TM injection (Figure S7).

Rather than being dependent upon IRE1α, the suppression of metabolic gene expression was exacerbated in liver-specific Ire1α-null animals in a manner very similar to Atf6α-null animals. Both forms of C/EBPα protein were downregulated in Ire1α-null livers 30 hr after TM injection to a much greater extent than in wild-type animals, while expression of C-JUN was upregulated (Figure 3A). Liver-specific Ire1α-null animals developed fatty liver

(E) Protein lysates from livers of wild-type or Atf6α−/− mice injected with vehicle or TM (1 mg/kg) were probed by immunoblot for either C/EBPα (which exists in a 42 kDa long form and 30 kDa short form) or C-JUN.

(F and G) The expression of metabolic genes subdivided into various categories was determined 48 hr after TM injection by real-time RT-PCR. Red lettering indicates genes significantly (p < 0.05) different in expression comparing TM-challenged Atf6α-null animals to unchallenged animals.

Data represent means ± SD.
FIGURE 3. Each Arm of the Canonical UPR Contributes to Protection from Metabolic Dysregulation

(A) Wild-type mice and mice with a hepatocyte-specific deletion of ire1α were injected with vehicle or TM (2 mg/kg). Protein lysates from liver isolated 30 hr postinjection were probed by immunoblot as indicated. The long (open arrowhead) and short (closed arrowhead) forms of C/EBPα are indicated. Note that the apparent difference in inhibition of TRAPα glycosylation is likely a consequence of this protein itself being a UPR target at least partially regulated at IRE1α (Nagasawa et al., 2007), rather than any difference in the pharmacological efficacy of TM between genotypes. (B) RNA was prepared from wild-type and ire1α−/− liver tissue samples from (A) and analyzed by real-time RT-PCR; n = 3 animals per group. Data represent means ± SD.

(D) Expression of metabolic mRNAs from the animals shown in (C) was quantitated by real-time RT-PCR as in (B); n = 2–4 animals per group. While the difference in upregulation of Adrp mRNA was not statistically significant comparing SA and AA mice after TM challenge, more robust upregulation of ADRP protein was evident (data not shown). Red lettering indicates different expression levels comparing ire1α−/− or AA TM-challenged animals to unchallenged, p < 0.1. Data represent means ± SD.

upon injection (data not shown), and displayed upregulation of Adrp along with suppression of transcription factors involved in lipid metabolism (Figure 3B). As with Atf6α-null animals, Ire1α-deleted livers showed persistent upregulation of the proapoptotic CHOP protein after CHOP upregulation had been largely attenuated in wild-type animals (Figures 3A and 3B). Thus, Atf6α-null and Ire1α-null livers display similar sensitivity to TM and perturbation of metabolic gene expression. While XBP1 has been proposed to regulate basal expression of lipogenic genes (Lee et al., 2008), TM-induced steatosis occurs in the presence of either spliced or unspliced XBP1 (Figures 1E and 3A; Figures S2 and S7B), suggesting that XBP1 does not directly regulate ER stress-induced microvesicular steatosis.

The similar phenotypes in these two genetic models led us to ask whether ablating the third, PERK/eIF2α-dependent arm of the UPR would produce a similar outcome. To test this hypothesis, a constitutively expressed eIF2α transgene flankled by LoxP sites was introduced into mice harboring the nonphosphorylatable S51A mutation in genomic eIF2α. This transgene rescued the otherwise neonatal lethal eIF2α S51A homozygous mutant, and either these homozygous (AA) or heterozygous (SA) animals carrying the transgene were bred to mice expressing CRE under control of the albumin promoter (see Supplemental Experimental Procedures and Figure S8). No phosphorylation of eIF2α was observed in homozygous S51A animals with a deleted transgene upon TM injection (Figure 3C, lanes 13–16), confirming efficient eIF2α transgene depletion. As with ATF6α or Ire1α deletion, we also observed suppression of C/EBPα protein expression, upregulation of C-JUN and ADRP protein, and fatty liver in these animals, but to a lesser extent in mice heterozygous for the genomic S51A locus (Figures 3C, compare lanes 10–12 with lanes 13–16; also, data not shown) Similar results were obtained at the mRNA level (Figure 3D). Thus, while increased eIF2α dephosphorylation protects against steatosis induced by severe dietary stress (Oyadomari et al., 2008), eIF2α phosphorylation is clearly necessary to prevent fat accumulation in response to a direct challenge of ER stress.

Our data demonstrate that ongoing ER stress, irrespective of the UPR pathway ablated, perpetuates suppression of metabolic gene expression. These results suggest that the UPR protects lipid homeostasis, not by selective regulation of gene expression mediated by the ATF6α, Ire1α, or PERK pathways, but instead by the three pathways of the UPR contributing to overall maintenance of ER function. In this view, the suppression of metabolic gene expression should be directly responsive to the ER stress burden in real time, and should correlate with molecular markers of ER dysfunction. This hypothesis is supported by the following observations. First, the suppression of C/EBPα expression and upregulation of C-JUN can be recapitulated in cultured FaO rat hepatoma cells in response to either TM treatment or induction of ER stress by the ER calcium-depleting agent thapsigargin (TG) (Figure 4A). This observation confirms that the effect is not TM specific, but is likely to be a general consequence of ER stress. Second, at least one secretory protein was retained intracellularly in Atf6α-null TM-challenged mice. While the gene encoding serum amyloid protein (SAP) was transcriptionally...
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Figure 4. Disruption of ER Protein Processing Suppresses Metabolic Gene Expression
(A) FaO rat hepatoma cells were treated in duplicate with 500 nM TG or 5 μg/ml TM for 8 or 24 hr, followed by cell lysis and immuno blot for GRP94, BiP, C/EBPα, and C-JUN. In hepatocytes, slower-migrating phospho-C-JUN, induced by ER stress, is also observed.
(B) Wild-type and Atf6α−/− mice were injected with vehicle or TM (1 mg/kg body weight). Protein lysates from liver isolated at the indicated time points after injection were probed by immunoblot for intracellular serum amyloid protein (SAP) (B); Plasma level of SAP was assayed by ELISA (C).
(D) Total cholesterol and triglyceride levels were measured by enzymatic assay from plasma samples of mice injected with vehicle or TM for 24 hr, with a 6 hr fast preceding death. Data represent means ± SD.
(E) Cholesterol and triglyceride represented either as total content, or in the form of LDL and VLDL particles purified by differential precipitation, was measured as in (D). In this case samples were collected 48 hr after TM injection rather than 24 hr. Note that the absolute values are somewhat different comparing (D) and (E), likely due to experimental variation. However, in (D) there is no significant difference in either cholesterol or triglyceride levels between genotypes after TM injection, while cholesterol levels recover to a greater extent in wild-type animals than Atf6α-null animals in (E). The fact that TM has a much greater effect on cholesterol-rich lipoprotein particles than triglyceride-rich particles suggests that TM inhibits liver lipoprotein production but not intestinal production. Data represent means ± SD; *p < 0.05.

compromised ER protein folding suppresses metabolic gene expression partially through CHOP

The rapid downregulation of C/ebpα mRNA and others during ER stress could be explained by either induced degradation of these mRNAs, or by inhibition of new synthesis. To discriminate between these possibilities, we treated FaO hepatoma cells with TM or TG in the presence or absence of the transcription inhibitor actinomycin D (ActD). In the absence of stress, ActD alone dramatically reduced C/EBPα expression, and it essentially nullified the effect of TM or TG on C/EBPα (Figure 6A). Therefore, C/ebpα mRNA is basally degraded very rapidly in hepatocytes, and its stress-mediated downregulation is most likely due to inhibition of its transcription.

One commonality among the Atf6α, Ire1α, and p58 genetic models presented here is persistent upregulation and nuclear localization of CHOP as a consequence of ongoing stress, while CHOP is rapidly, but only transiently, upregulated in response to TM challenge in wild-type mice (Figures 1A; Figures S10 and S11). CHOP is a member of the C/EBP family of transcription factors, and has been proposed to be a dominant-negative regulator of their function (Ron and Habener, 1992). We considered the possibility that CHOP upregulation during stress is at least partially responsible for the suppression of metabolic gene expression. Upon challenge of Chop-null animals with TM, expression of the transcriptional regulators C/ebpα, Pparα, Pgc1α, and Srebp1 was less suppressed than in wild-type animals (Figure 6B). This was also observed upon analysis of C/EBPα protein (Figure 6C). Chop deletion also partially protected against lipid accumulation, seen in reduced upregulation of ADRP (Figure 6C). Thus, CHOP forms at least a component of the mechanistic

induced by TM treatment to comparable extents in both wild-type and Atf6α-null animals (Figure S9), its secretion was essentially blocked in the latter, and it was instead detected readily in the cellular homogenate (Figures 4B and 4C). In addition, the concentation of plasma cholesterol in the form of ApoB-containing lipoproteins (VLDL and LDL), was diminished by TM in both genotypes 24 hr after challenge, but to a greater extent in Atf6α-null than wild-type animals 48 hr after challenge (Figures 4D and 4E). Together, these data point to suboptimal function of the secretory pathway during challenge in the livers of TM-treated UPR-compromised animals.

If the suppression of metabolic gene expression exacerbated by genetic deficiency of UPR components is in fact caused by disruption of protein folding and processing in the ER rather than loss of specific UPR-mediated signaling cascades, then direct perturbation of ER protein folding independent of the UPR should produce a similar phenotype. To test this prediction, we used TM to challenge animals nullizygous for the ER-resident DnaJ protein and binding protein (BiP) cochaperone p58IPK (Ladiges et al., 2005). p58-null cells and animals are more sensitive to ER stress than wild-type counterparts, most likely due to the role of p58IPK in enhancing BiP-dependent protein folding (Rutkowski et al., 2007). Strikingly, p58-null animals developed fatty liver after TM injection (Figure 5A) and suppressed expression of C/ebpα (Figures 5B and 5C). As with Atf6α-null and Ire1α-null animals, p58-null animals also showed persistent CHOP upregulation after challenge (Figures 5B and 5C). Thus, the suppression of metabolic genes, such as C/ebpα, is a direct consequence of ER stress and not dependent upon any single pathway of UPR signaling.

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linkage between persistent ER stress and the suppression of transcription factors like C/EBPα.

CHOP was persistently upregulated by stress in our genetic models, from early through late time points, despite lack of an apparent stimulation of eIF2α phosphorylation, which is presumably a consequence of persistent upregulation of GADD34 downstream of CHOP (Marciniak et al., 2004; Wu et al., 2007) (Figure 1A). Conversely, in wild-type animals, CHOP upregulation is rapidly attenuated despite persistent stimulation of eIF2α phosphorylation. Surprisingly, we found that ATF4 was also upregulated by ER stress in Atf6α−/− animals at both early and late time points (Figure 6D and data not shown). These data suggest that the upregulation of CHOP, which then influences lipid metabolism, occurs through a dissociation of ATF4 protein levels from the phosphorylation status of eIF2α by a mechanism that is the subject of ongoing investigation.

Figure 5. Disrupted ER Protein Folding Leads to Steatosis Despite an Intact UPR
(A–C) Wild-type and p58IPK−/− mice were injected with vehicle or TM (1 mg/kg) and sacrificed 48 hr postinjection. (A) Livers from TM-challenged mice were visualized in situ. (B) Protein lysates from livers were probed by immunoblot as indicated. (C) RNA was analyzed by real-time RT-PCR. Red lettering represents p < 0.05 comparing p58−/− treated to untreated. Data represent means ± SD.

Hepatic Fat Accumulation Accompanies Diverse ER Stresses
The diversity of physiological causes of hepatic microvesicular steatosis led us to ask whether other less severe forms of ER stress could also lead to fat accumulation and suppression of C/EBPα expression. Remarkably, the proteasome inhibitor bortezomib, which is used clinically to treat multiple myeloma, led to lipid accumulation, as seen by ADRP upregulation (Figure 7A). Published reports suggest possible hepatoxocity of bortezomib (Hernandez-Espinosa et al., 2008; Rosinol et al., 2005). In addition, ADRP and C-JUN up-regulation and suppression of lipogenic genes were also seen as a consequence of overexpression of the misfolding-prone factor VIII clotting protein (Figure S12). Thus, lipid accumulation and suppression of C/EBPα are conserved features of the hepatic response to ER stress in general.

Conclusions
Our data demonstrate that protection of ER homeostasis by the combined action of the three UPR pathways maintains metabolic function in part by suppressing CHOP expression and the attendant downregulation of genes involved in lipid and energy metabolism.
Unresolved ER Stress Causes Steatosis

**Figure 7. Diverse ER Stresses Lead to Lipid Accumulation**

(A) Normal mice were injected intravenously with the proteasome inhibitor Velcade (bortezomib) at a dose of 1 mg/kg and sacrificed 8 hr after injection. Lysates were probed for upregulation of CHOP, ADRP, and C-JUN. (B) A schematic diagram depicting the joint role of the ATF6, PERK, and IRE1 pathways in maintaining lipid homeostasis. Failure of the UPR to adequately protect the ER results in ongoing production of CHOP, which suppresses metabolic genes via C/EBPα and ultimately leads to disruption of fatty acid oxidation, lipoprotein secretion, gluconeogenesis, and other metabolic processes.

Unresolved ER Stress Causes Steatosis

The work is the first to our knowledge to use a comprehensive genetic approach to understand how the pathways of the vertebrate UPR contribute to the homeostasis of an organ in vivo. Our data allow us to propose a model to describe the proximal events that connect ER stress to lipid homeostasis (Figure 7B). Exposure to ER stress activates all three ER-resident sensors, IRE1α, ATF6α, and PERK. Activation of these sensors induces expression of both genes that facilitate adaptation and/or recovery, such as ER chaperones, and also of antiadaptive genes, including Chop. One of the consequences of Chop upregulation is suppression of genes involved in lipid homeostasis, likely mediated through dominant-negative inhibition of C/EBPα family members by CHOP.

We conclude that negative regulation of C/EBPα by CHOP is of key importance in metabolic dysregulation by ER stress, in part because the phenotypes that we observe—fatty liver, hypoglycemia, and depletion of hepatic glycogen—are also observed upon postnatal deletion of C/EBPα, as are many similar changes in gene expression profiles, including expression of metabolic transcriptional regulators (Yang et al., 2005). Indeed, potential binding sites for C/EBPα exist in the promoters of Pparα and Srebp1 (Villacorta et al., 2007). It seems most plausible that ER stress will lead to suppression of C/EBPα, partially through CHOP and consequent suppression of other master regulators of metabolic gene expression, such as Srebp1, Pparα, and Pgc1α. Thus, we speculate that unresolved ER stress recapitulates a knockdown of C/EBPα. Whether C/EBPα suppression alone is sufficient to drive these changes, and whether CHOP alone is sufficient to negatively regulate C/EBPα expression, are the subject of ongoing investigation, and we believe it likely that other as-yet unknown mechanisms contribute as well. Furthermore, which individual metabolic transcription factors actually directly control fatty acid oxidation under these circumstances is not clear. It is possible that steatosis might be driven entirely by downregulation of PGC1α and/or PPARα, both of which ordinarily control fatty acid oxidation. Thus, it will be important to elucidate the hierarchy of genetic regulation downstream of CHOP and C/EBPα.

Our results suggest that an initial perturbation—even a transient one—will initiate changes in gene regulation that impact lipid metabolism. Assuming that the adaptive response is sufficient to overcome the perturbation, the UPR is then largely deactivated, and production of proteins involved in lipid metabolism is restored due to rapid degradation of CHOP (Rutkowski et al., 2006). However, if the stress is too severe (elicited, in this case, by genetic compromise of either UPR pathways or ER quality control), then CHOP-directed suppression of gene expression persists, leading to profound metabolic disruption. Our data support the idea that each of the UPR pathways contributes to adaptation and/or recovery, and that the overlap of UPR targets ensures that the strongest predictor of recovery versus ongoing stress is the expression of adaptive (e.g., ER chaperones) versus antiadaptive (e.g., CHOP) downstream gene products (Rutkowski et al., 2006).

An important question for future work is what components of the ER stress-lipid homeostasis nexus facilitate adaptation, and are thus likely to be observed in the course of adaptation to chronic disruptions of liver function, versus those that are purely consequences of severe stress and represent metabolic dysfunction. Indeed, while steatosis accompanies organismal susceptibility to ER stress in ATF6α-null models, the cause of death of these animals is not yet known, and inhibition of fatty acid oxidation and lipoprotein secretion might even be protective, at least in certain physiological circumstances. The topic of the role of components of the UPR in regulating lipid homeostasis has been the subject of very recent attention. Both XBP1 and eIF2α have been shown to participate in basal and/or diet-induced regulation of lipid metabolism (Lee et al., 2008; Oyadomari et al., 2008). We have observed, in a model cultured cell system, that cells adapted to chronic ER stress paradoxically become more resistant to further challenge than naïve cells (Rutkowski et al., 2006). Thus, the extent to which dietary stresses might allow for gradual adaptation of UPR-deficient cells, while focal stresses might not, could significantly impact the resultant phenotype. Indeed, the observation that C/EBPα expression apparently promotes steatosis in the dietary context (Oyadomari et al., 2008), while its downregulation promotes steatosis in response to ER stress (this work), is reminiscent of the observation that constitutive embryonic deletion of C/EBPα prevents hepatic lipid
accumulation (Floidby et al., 1996; Wang et al., 1995), while deletion in the adult exacerbates it (Yang et al., 2005). Whether our study and the recent XBP1 and eIF2α papers together represent three faces of the same basic signaling pathway or, alternatively, whether they illuminate the mechanisms of different forms of hepatic dysfunction, remains to be investigated.

Our work demonstrates a direct connection between ER dysfunction and the regulation of lipid metabolism through the protective role of the UPR. These findings raise the possibility that ER stress is a contributing factor to the development of hepatic steatosis.

EXPERIMENTAL PROCEDURES

General Notes

Many of the techniques described here, including RNA and protein analysis, utilized previously published reagents and standard experimental techniques (Rutkowski et al., 2006; Wu et al., 2007; and references therein).

Animal Experiments

All protocols for animal use were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan. Animals were fed standard rodent chow and housed in a controlled environment with 12 hr light-dark cycles. Littermate controls were used for all experiments where possible, and age and gender were matched for each experiment, though neither of these variables was observed to have any effect on the phenotype. Plasma from blood samples was collected by cardiac puncture using a BD Microtainer with lithium heparin. Plasma levels of SAP were determined using the murine SAP ELISA kit (ImmunoLogic Consultants Laboratory, Inc.). TM injections were as previously described (Sakaki et al., 2008). Insulin injection was intraperitoneal with 2 U/kg body weight. Plasma and liver cholesterol and triglyceride levels were determined as previously described (Zinszner et al., 1998). Insulin injection was intraperitoneal with 2 U/kg body weight. Plasma and liver cholesterol and triglyceride levels were determined as previously described (Sakaki et al., 2008b). Velcade (bortezomib, from Millenium) was administered intravenously at a dose of 1 mg/kg, and mice were sacrificed 8 hr after treatment.

Array Analysis

Mice were injected intraperitoneally with 2 mg/kg body weight of TM, or vehicle, and livers were isolated 8 hr after injection. Total RNA was isolated using RNeasy (QIAGEN) and analyzed by the University of Michigan Comprehensive Cancer Center Affymetrix and cDNA Microarray Core Facility (Ann Arbor, Michigan), exactly as described previously (Wu et al., 2007).

Protein and RNA Analysis

Briefly, tissue was homogenized using an electronic homogenizer in RIPA buffer containing protease inhibitors or in Trizol RNA reagent (Invitrogen), and centrifuged at 15,000 rpm for 10 min in a microtube at 4°C. RNA was isolated using Trizol RNA reagent or RNeasy (QIAGEN), according to the manufacturers’ protocols. Additional real-time primer sequences are described in the Supplemental Data (Table S2).

Histological Analysis of Tissues

Hepatic tissues were frozen in OCT in liquid nitrogen-cooled 2-methylbutane. The sections were cut to 6 μm. Livers were visualized in situ using a Leica MZ16FA stereomicroscope.

Cell Culture and Analysis

FaO rat hepatoma cells were from ATCC and cultured in DMEM medium with 10% fetal bovine serum. Treatment of cells with TM or TG was as previously described (Rutkowski et al., 2006).

Generation of Mouse Models

Creation of Atf6α-null, p58Ikkα-null, and Chop-null mice has been described elsewhere (Ladiges et al., 2005; Wu et al., 2007; Zinszner et al., 1998).

eIF2αA44V mice were created by introduction of a transgene encoding constitutively expressed wild-type eIF2α flanked by LoxP sites into the eIF2α S51A knockin background (S.H.B. and R.J.K., unpublished data). These mice were then bred to mice expressing CRE recombinase under control of the albumin promoter for liver-specific transgene deletion. Similarly, liver-specific ire1α knockout mice were created by embryonic stem-targeted homologous recombination of exons 16 and 17 flanked by LoxP sites. Mating to albumin-CRE-expressing mice allowed for liver-specific deletion of these exons, resulting in loss of the ire1α kinase domain.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, twelve figures, and three tables and can be found with this article online at http://www.cell.com/developmentalcell/supplemental/S1534-5807(08)00475-9/.

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