THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 6, pp. 4405–4415, February 8, 2013

C/EBP Homologous Protein (CHOP) Contributes to Suppression of Metabolic Genes during Endoplasmic Reticulum Stress in the Liver*

Received for publication, October 30, 2012, and in revised form, December 13, 2012 Published, JBC Papers in Press, December 31, 2012, DOI 10.1074/jbc.M112.432344

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Background: ER stress regulates metabolic gene expression in liver.
Results: The ER stress-regulated pro-apoptotic transcription factor CHOP binds to the promoters of metabolic genes and is necessary for their suppression.
Conclusion: CHOP contributes to the metabolic alterations that accompany ER stress *in vivo*.
Significance: These findings suggest that CHOP has a non-apoptotic role in regulating metabolic physiology.

The unfolded protein response (UPR) senses stress in the endoplasmic reticulum (ER) and initiates signal transduction cascades that culminate in changes to gene regulation. Long recognized as a means for improving ER protein folding through up-regulation of ER chaperones, the UPR is increasingly recognized to play a role in the regulation of metabolic pathways. ER stress is clearly connected to altered metabolism in tissues such as the liver, but the mechanisms underlying this connection are only beginning to be elucidated. Here, working exclusively *in vivo*, we tested the hypothesis that the UPR-regulated CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) participates in the transcriptional regulation of metabolism during hepatic ER stress. We found that metabolic dysregulation was associated with induction of eIF2α signaling and CHOP up-regulation during challenge with tunicamycin or Velcade. CHOP was necessary for suppression of genes encoding the transcriptional master regulators of lipid metabolism: *Cebpa*, *Ppara*, and *Srebf1*. This action of CHOP required a contemporaneous CHOP-independent stress signal. CHOP bound directly to C/EBP-binding regions in the promoters of target genes, whereas binding of C/EBPα and C/EBPβ to the same regions was diminished during ER stress. Our results thus highlight a role for CHOP in the transcriptional regulation of metabolism.

The unfolded protein response (UPR)[2] initiates a series of signaling cascades that culminate in extensive transcriptional regulation in response to endoplasmic reticulum (ER) stress. Paradigmatically, the genes regulated by the UPR improve ER protein folding and processing and include ER chaperones, co-chaperones, oxidases, and thiol isomerases; ER-associated degradation factors; amino acid metabolism factors; defenses against oxidative stress; and genes involved in other processes directly connected to ER function (1). However, a role is emerging for the UPR in the regulation of parallel physiological pathways that are at least in principle unconnected to ER protein folding. Most notable among these is metabolic function in tissues such as the pancreas, adipose, and liver (2).

Although UPR activation alleviates ER stress by non-transcriptional mechanisms such as transient inhibition of protein synthesis (3, 4) and of ER protein translocation (5), each arm of the UPR also activates a distinct transcriptional pathway. Oligomerization and autophosphorylation of IRE1α (inositol-requiring enzyme-1α) result in splicing of *Xbp1* mRNA and consequent production of a frame-shifted active transcription factor (6–8). Activated PKR-like ER kinase (PERK) phosphorylates the translation initiation factor eIF2α, which leads to preferential synthesis of ATF4 (activating transcription factor 4) (3). Finally, ATF6α and its paralog ATF6β are cleaved by regulated intramembrane proteolysis in the Golgi, yielding a cytosolic fragment that localizes to the nucleus to activate transcription (9, 10). Each of these proteins activates gene transcription, and their target gene sets, which in many cases overlap, have for the most part intuitive connections to ER protein folding and processing functions (11–13).

The phenotypes of UPR-compromised animals to dietary and pharmacological challenges have more recently revealed a profound influence of ER stress on metabolism in the liver. This influence has been attributed to both direct and indirect actions of UPR effectors on metabolic processes. XBP1 was found to directly regulate the expression of lipogenic genes (14) and also to stimulate lipoprotein secretion through up-regulation of protein-disulfide isomerase (15). In contrast, activated IRE1α was shown to suppress lipogenesis and lipoprotein biogenesis (16), at least in part through direct degradation of key mRNAs by the regulated IRE1-dependent decay pathway (17, 18).

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 DK084058 from NIDDK (to D. T. R.). This work was also supported by the Carver Medical Research Trust Initiative.
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[2] The abbreviations used are: UPR, unfolded protein response; ER, endoplasmic reticulum; PERK, PKR-like ER kinase; C/EBP, CCAAT/enhancer-binding protein; TM, tunicamycin; CHOP, C/EBP homologous protein; IHC, immunohistochemistry; ADRP, adipocyte differentiation-related protein; TRAPα, translocon-associated protein α; qPCR, quantitative real-time PCR; TSS, transcriptional start site.

Atf4$^{-/-}$ mice show altered hepatic lipid metabolism (19), and this might be partly attributable to direct regulation of expression of the lipogenic gene *Lipin2* by ATF4 (20). The PERK/eIF2α pathway can also regulate metabolic gene expression indirectly through translational control over the metabolic master regulators CCAAT/enhancer-binding protein α (C/EBPα) and C/EBPβ (21). ATF6α is not known to regulate any metabolic genes directly but is capable of sequestering the transcriptional coregulator CRTC2 (CREB-regulated transcription factor 2), thereby inhibiting gluconeogenesis (22). These studies point to complex and multifaceted mechanisms for regulation of metabolism by the UPR, which remain incompletely understood.

Mice impaired in UPR signaling or with an intact UPR but compromised ER protein folding developed profound hepatic steatosis upon challenge with the ER stress-inducing agent tunicamycin (TM) (23, 24). This steatosis was accompanied by prolonged suppression of the expression of a host of metabolic genes. The commonality of this phenotype among the various UPR and ER folding mutants suggested that it was a consequence of ER stress *per se*, and indeed, a similar but more rapidly resolving suppression of these same metabolic genes was observed even in wild-type animals challenged with ER stress (23, 24).

Up-regulation of the UPR target gene *Chop* was also a common feature of ER stress induced in each steatotic mutant animal (23). C/EBP homologous protein (CHOP) was originally identified as a repressive member of the C/EBP family of transcription factors (25), although it is now understood to be capable of either transcriptional repression or activation, depending upon context (26). CHOP influences ER function and cell viability through its actions on target genes that promote protein synthesis and oxidative protein folding (27, 28). However, CHOP has been implicated in transcriptional control of processes as diverse as myelination, cell adhesion, and iron metabolism (29–31). Our earlier work suggested that CHOP might play a role in suppression of genes involved in lipid metabolism (23); here, we investigated the contribution of CHOP to the UPR-mediated regulation of metabolic gene expression.

EXPERIMENTAL PROCEDURES

Materials—TM was from EMD Biosciences (San Diego, CA). Velcade was purchased from the University of Iowa Pharmacy. Antibodies used for immunoblotting and/or immunohistochemistry (IHC) were as follows: CHOP (for immunoblotting and IHC; sc-7351, Santa Cruz Biotechnology, Santa Cruz, CA), β-tubulin (TUB2.1, Sigma-Aldrich), BiP (binding protein; 610978, BD Biosciences), phospho-eIF2α (44-728G, Invitrogen), adipocyte differentiation-related protein (ADRP; NB110-40877, Novus Biologicals, Littleton, CO), and eIF2α (9722, Cell Signaling Technology, Danvers, MA). Anti-translocon-associated protein α (TRAPα) antibody was a kind gift of R. S. Hegde (Medical Research Council, Cambridge, United Kingdom). ChIP antibodies were as follows: CHOP (2895, Cell Signaling Technology), C/EBPβ (sc-150, Santa Cruz Biotechnology), and C/EBPα (sc-61, Santa Cruz Biotechnology). These anti-C/EBPα and anti-C/EBPβ antibodies were also used for immunoblotting. Secondary antibodies were from Thermo Scientific.

Animal Experiments—All animal procedures were approved by the University of Iowa Institutional Animal Care and Use Committee. Mice (C57BL/6J, *Chop*$^{-/-}$) were housed in a pathogen-free facility at the University of Iowa on a 12-h light/dark cycle. TM, Velcade, or vehicle dissolved in 150 mM dextrose or phosphate-buffered saline was injected intraperitoneally. Liver tissues were harvested at the indicated time points after injections and either frozen immediately and fixed in formalin (for IHC) or minced and fixed in formaldehyde (for ChIP).

Adenovirus Experiments—Mouse *Chop* cDNA was cloned into pAd5mcsIRESeGFP using standard methods to create *Ad-Chop* and was prepared by the University of Iowa Gene Transfer Vector Core. Control virus expressing GFP only (*Ad-Gfp*) was also obtained from the Vector Core. The viruses were amplified using standard procedures (32). Viruses were tested for the expression of CHOP and GFP at protein and mRNA levels in A549 cells using immunoblotting and quantitative real-time PCR (qPCR) analysis. 2×10^{11} viral particles were administered through tail vein injections for hepatic expression. Experiments were performed 24 h after adenoviral delivery to guard against potential confounding hepatotoxic effects of CHOP expression.

Molecular Analysis—RNA and protein analyses were performed as described (23). Immunoblots were imaged using the ChemiDoc-It imaging system (UVP, LLC, Upland, CA) with on-chip integration and auto-exposure settings, and images were processed using Adobe Photoshop. Black hairlines are solely to aid in visual assessment. Primer sequences and methods utilized for real-time PCR analysis have been published previously (13, 23, 33).

Chromatin Immunoprecipitation—400 mg of liver tissue from animals injected with TM or vehicle was minced and fixed immediately in 1% formaldehyde for 30 min, and cross-links were quenched with 125 mM glycine for 10 min. Following fixing, tissue was rinsed twice with ice-cold PBS, Dounce-homogenized in PBS, and filtered through a 70-μm cell strainer to remove connective tissue. Tissue was resuspended in ChIP cell lysis buffer (10 mM Tris-Cl (pH 8.0), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and one mini EDTA-free protease inhibitor tablet (Roche Applied Science)) and incubated at 4 °C for 10 min. The nuclei were pelleted and resuspended in 2 ml of ChIP nuclear lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris-Cl (pH 8.1), and protease inhibitor). This lysate was sonicated in ice water using a Virsonic 600 probe sonicator (VirTis Co., Inc.) with 12 cycles of 30 s on and 60 s off at 24–27 watts. After DNA shearing, the lysates were centrifuged at high speed to pellet the cell debris, and the supernatant was stored at −80 °C before further processing. Thawed lysates were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, and 16.7 mM Tris (pH 8.1)). After preclearing the lysates with protein G-salmon sperm DNA-agarose beads (Millipore), 1% of the lysate was set aside to quantitate input, and immunoprecipitation was then carried out overnight using 5 μg of antibody (CHOP, C/EBPα, C/EBPβ, or control normal mouse IgG (12-371, Millipore)). The samples were then incubated with protein G-salmon sperm DNA-agarose beads for at least 1 h at 4 °C. Beads were washed with low salt buffer

TABLE 1
ChIP qPCR primers

Primer	Sequences
Ppara	
PPARA-L3019	TCTGTGAAGTGGGGGTAGGT
PPARA-R2914	TCCTAGGACTGGCACTCACC
PPARA-L2946	TGTTTCCTGGATGGTGAGTG
PPARA-R2830	TGCTGATGGAAAATAGGAAGC
PPARA-L2853	CCGCTTCCTATTTTCCATCA
PPARA-R2751	GGATAGTGGCCTCTGATCTCC
PPARA-L1154	TGATCAGCAAAGGTGGGTTT
PPARA-R1062	ACTTTCAGCGCACATCTCCT
PPARA-L978	TGGCATAGCACACATTTCGT
PPARA-R893	TCTTCAGTCACGGAATGCAC
PPARA-L589	TCTCCCCATTTCTCATCCTG
PPARA-R480	GAGCTGGTCTAGATCGCACA
PPARA-L278	CTAAATGGGCATCGAGGAGA
PPARA-R175	CTCAGGGCGAGACACACC
Srebf1c	
Sr-C-Lt1227	GTTGGGGTCAGGGCTCTT
Sr-C-Rt1033	TGTGTCTGACATTCCCCTGA
SR-C-LT929	AGTCCCTGGTCTGCCAACTA
SR-C-RT751	ATGCCAAATCTGTCCCTGTC
SR-C-LT629	CTGGAGGGAAGACACTGACC
SR-C-RT513	TCCCTCATGAAACTGCCTTC
SR-C-LT377	AAATCTTGCTGCTGCCATTC
SR-C-RT258	CCCGGAAGCTCTGTGTTC
SR-C-LTTS43	GGGCCTGACAGGTGAAATC
SR-C-RTTS157	TCCTCCCCAAAGTACCTTCA
Cebpa	
CEA-L4003	GGAGAAGGCATGCTCATGTT
CEA-R3902	TTTATGGCATTTTGGTGTCG
CEA-L786	TGGAGACGCAATGAAAAAGA
CEA-R640	CCACGGGCTCTTCAGAGTAG
Gadd34	
GADD34Up548	GTTGGCGCAGATTGAGTCAG
GADD34Dn697	GGTTCATGTCGCCCTCAG
Fga	
FGA-9869L	CCAACTGCTGACTGCACAAT
FGA-0012R	AAGCAGCCAAAGCTCACAGT
Eif2S2	
EIF2s2–8526L	CCACGAGAAGCACGTAAGGT
EIF2s2–8667R	CTTCTCACAGCACCGCACTA
Irrel	
IRREl FOR	CTGGACTTCCCTCATGAACC
IRREl REV	CTGTCACCAGGACACCAGAA

(0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl (pH 8.1), 150 mM NaCl), high salt buffer (low salt with 500 mM NaCl), and lithium chloride buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-Cl (pH 8.1)) and twice with 10 mM Tris (pH 8) and 1 mM EDTA. Bead complexes were eluted with 1% SDS and 0.1 M $NaHCO_3$ for 30 min at room temperature. Cross-links were reversed in 200 mM NaCl at 65 °C overnight. Samples were treated with RNase A (Thermo Scientific) and proteinase K (New England Biolabs). DNA was purified using phenol/chloroform extraction and ethanol precipitation. qPCR analysis was used to estimate the relative recovery of different promoter regions. The ChIP primers used for real-time reactions were validated for specificity by melting curve analysis and for efficiency by serial dilution analysis of template genomic DNA. Primer sequences used for ChIP analysis are given in Table 1.

Immunohistochemistry—The liver tissue samples were fixed in 10% formalin, paraffin-embedded, sectioned, and stained with H&E by the University of Iowa Comparative Pathology Laboratory. For IHC, sections were deparaffinized, rehydrated, unmasked, and blocked before primary antibody incubation. Slides were stained after ADRP IHC using the 3,3′-diaminobenzidine substrate kit (Vector Laboratories Inc., Burlingame, CA). For CHOP IHC, Alexa Fluor 488 (Invitrogen) secondary antibody was used. Tissue sections were mounted, and pictures were taken on Nikon Microphot-FX epifluorescence microscope with a Nikon Digital Sight DS-Fi1 camera.

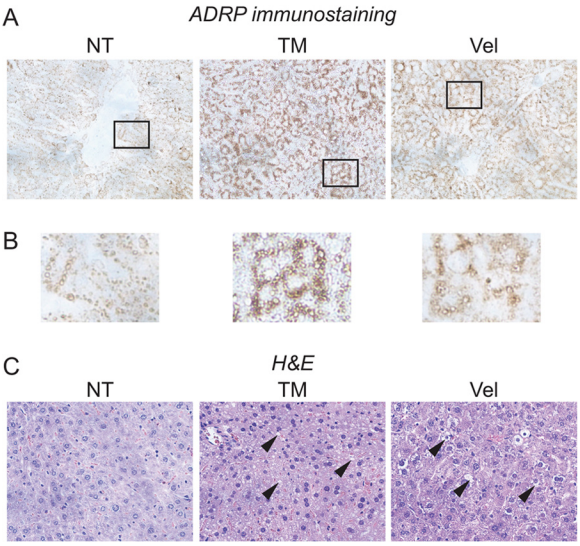

FIGURE 1. **TM and Velcade promote hepatic lipid accumulation.** *A*, wild-type C57BL/6J mice were injected intraperitoneally with TM or Velcade (*Vel*) at 1 mg/kg of body weight or with vehicle (non-treated (*NT*)). 24 h after challenge, livers were resected, and formalin-fixed paraffin-embedded sections were analyzed by IHC using an antibody against the lipid droplet marker protein ADRP. Samples were imaged using a 20× objective. Representative images from multiple animals are shown. *B*, zoomed-in images from *A* are shown. *C*, samples prepared similarly to those in *A* but stained with H&E are shown. *Arrowheads* denote non-eosinophilic cytoplasmic vacuoles.

RESULTS

Activation of PERK/eIF2α Signaling Is Associated with Hepatic Lipid Dysregulation—In prior work, we demonstrated that exposure of animals to the inhibitor of *N*-linked glycosylation and well known ER stress-inducing agent TM was accompanied by down-regulation of genes encoding transcriptional master regulators of lipid metabolism in the liver. Our results suggested that CHOP might be involved in this regulation (23). Thus, we wished to understand the contribution of CHOP to the process.

We first reasoned that if CHOP contributes to metabolic gene regulation, then other stimuli that induce CHOP expression should show similar alterations to gene regulation. We thus compared the response of mice to challenge with either TM or Velcade (bortezomib/PS-341), which is a proteasome inhibitor used to treat multiple myeloma and mantle cell lymphoma (34) and which we previously showed leads to up-regulation of the lipid droplet marker protein ADRP (adipophilin) by immunoblotting (23). Consistent with previous results, TM led to hepatic lipid accumulation as assessed by immunohistochemical staining for ADRP, which can be seen surrounding cytoplasmic lipid vesicles (Fig. 1, *A* and *B*). These vesicles also failed to stain with eosin (Fig. 1*C*). We also observed similar results with Velcade by these criteria (Fig. 1, *A*–*C*). These markers of steatosis correlate with other assays for lipid accumulation, including direct measurement of triglycerides and elec-

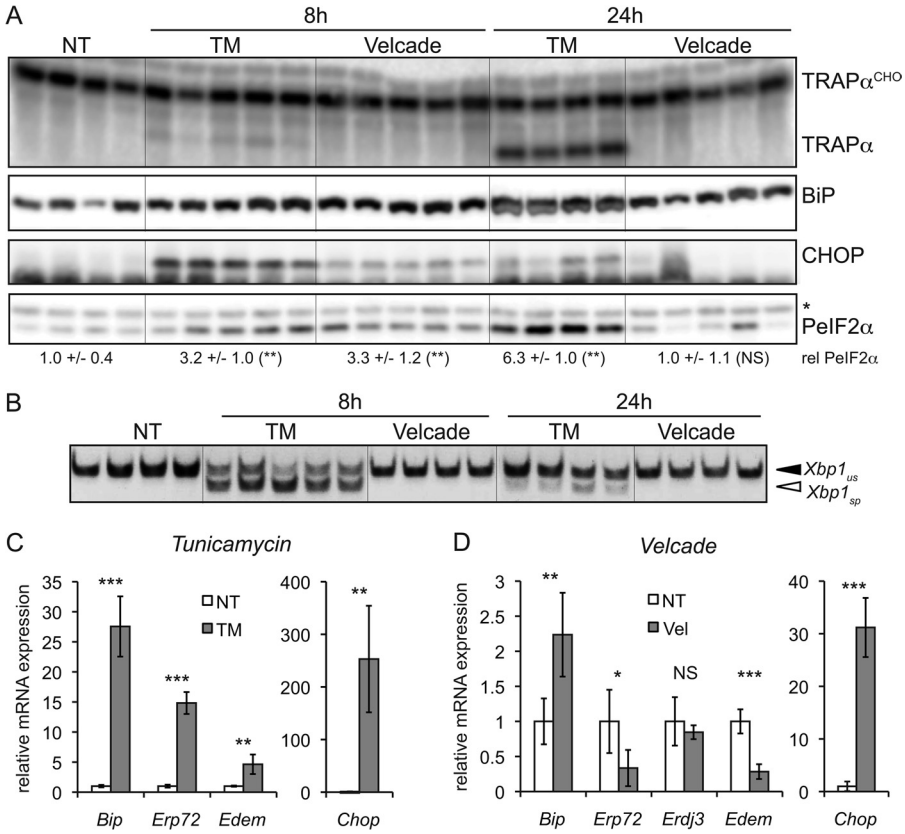

FIGURE 2. **eIF2α signaling is common to TM and Velcade challenge.** *A*, wild-type animals were challenged with TM or Velcade as described in the legend to Fig. 1, and livers were taken after 8 or 24 h of challenge. Samples were analyzed by immunoblotting for the indicated proteins. Glycosylated (*TRAPα^{CHO}*) and unglycosylated forms of the ER-resident glycoprotein TRAPα are indicated. The *asterisk* represents a nonspecific band that indicates equivalent loading. The relative (*rel*) amount of phosphorylated eIF2α (*PeIF2α*) was quantitated by densitometry and is given below the blot. *NT*, non-treated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *NS*, $p > 0.05$ by two-tailed Student's *t* test. Error term represents means ± S.D. *B*, RNA was isolated from liver samples of animals treated as described for *A*, and the presence of spliced (*sp*) and unspliced (*us*) *Xbp1* mRNAs was detected by RT-PCR. The image is black-to-white inverted for visual clarity. *C* and *D*, RNA prepared as described for *B* was analyzed by qRT-PCR for expression of the indicated genes in animals treated for 8 h with TM or Velcade (*Vel*). Expression was normalized to *Gapdh* and *Hprt* and is expressed relative to the level in vehicle-treated animals (n = three to five animals per group).

tron microscopic confirmation of lipid droplets (23). Thus, both TM and Velcade induce metabolic dysregulation.

We next characterized the effects of both agents on UPR activation. Both TM and Velcade elicited up-regulation of the UPR target proteins BiP and CHOP in the liver after 8 h, and both stimulated phosphorylation of the translation initiation factor eIF2α, although Velcade induced CHOP less robustly than did TM (Fig. 2*A*). However, by 24 h, CHOP up-regulation was diminished in TM-treated animals and was no longer up-regulated by Velcade (Fig. 2*A*), consistent with recovery from stress and attenuation of eIF2α signaling (33). Therefore, CHOP is likely to exert any potential influence on metabolism during the early phases of the responses to TM and Velcade.

Unlike TM, Velcade treatment did not stimulate splicing of *Xbp1* mRNA (Fig. 2*B*), consistent with a previous report that proteasome inhibitors disrupt IRE1α/XBP1 signaling (35). Similarly, although TM led to up-regulation of a full spectrum of UPR-dependent mRNAs (Fig. 2*C*) (data not shown), Velcade treatment did not up-regulate expression of the IRE1α- and ATF6α-dependent genes *Edem*, *Erp72*, and *Erdj3* (Fig. 2*D*) (13, 36). *Bip* mRNA was modestly up-regulated by Velcade challenge (Fig. 2*D*), consistent with this gene being responsive to PERK/eIF2α activation (37). In contrast and consistent with protein expression data, both TM and Velcade induced up-regulation of *Chop* (Fig. 2, *C* and *D*). Thus, to whatever extent TM and Velcade elicit lipid dysregulation through a common mechanism, this mechanism likely involves eIF2α signaling, including, potentially, CHOP.

Suppression of Ppara, Srebf1, and Cebpa Requires CHOP—To directly test a role for CHOP in regulation of metabolic genes, we challenged either wild-type or *Chop*$^{-/-}$ animals with TM or Velcade for 8 h and examined the expression of key transcriptional master regulators of lipid metabolism known to be down-regulated by ER stress (23). By virtue of their control over entire metabolic pathways, these genes are likely to represent the most proximal transcriptional connection between the UPR and metabolic regulation. They include *Cebpa*, which has diverse roles in regulating numerous aspects of liver metabolism; *Ppara*, a regulator of fatty acid oxidation; *Srebf1*, a regulator of lipogenesis; and *Srebf2*, a regulator of cholesterologenesis (38).

Consistent with our previous work, TM led to a suppression of all of these genes (except *Srebf2*) in wild-type animals (Fig. 3*A*). Similar results were seen in animals challenged with Velcade, except that *Srebf2* expression was also diminished (Fig. 3*B*). We found that TM treatment suppressed *Cebpa* and *Ppara* to a significantly lesser extent in *Chop*$^{-/-}$ animals than in wild-type animals (Fig. 3*A*). In animals challenged with Velcade,

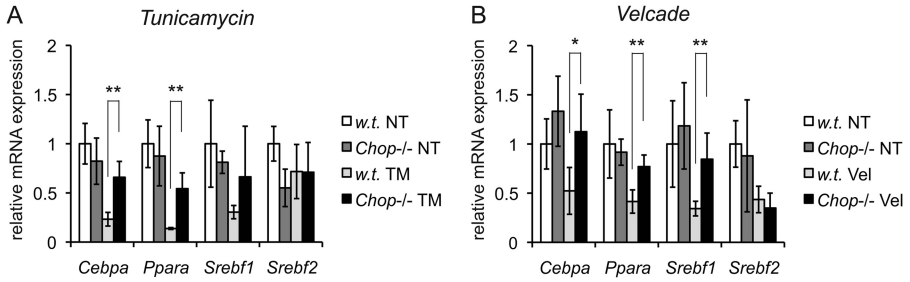

FIGURE 3. **Expression of metabolic master regulators is suppressed in a CHOP-dependent manner.** *A* and *B*, wild-type or *Chop*$^{-/-}$ mice were challenged with TM or Velcade for 8 h. Expression of *Cebpa*, *Ppara*, *Srebf1*, and *Srebf2* in the liver was assessed by qRT-PCR as in the legend to Fig. 2 (n = three to five animals per group). *, $p < 0.05$; **, $p < 0.01$. *NT*, non-treated.

Cebpa, *Ppara*, and *Srebf1* suppression depended on CHOP (Fig. 3*B*). Thus, each of these master regulators (except *Srebf2*) showed some degree of CHOP dependence in its suppression.

We next tested whether CHOP expression alone is sufficient for metabolic gene suppression. Wild-type animals were infected with recombinant adenovirus expressing either GFP (*Ad-Gfp*) or CHOP (*Ad-Chop*) (Fig. 4*A*). We found no significant down-regulation of any of the metabolic genes assessed in animals expressing CHOP (Fig. 4*B*) even though the efficiency of adenoviral transduction was near 100% (*e.g.* Fig. 4*C*). Thus, CHOP alone is insufficient to suppress these genes. We then tested whether CHOP expression could suppress them in the presence of a contemporaneous ER stress signal. We did this by infecting *Chop*$^{-/-}$ animals with either *Ad-Gfp* or *Ad-Chop* and then challenging the animals with TM (Fig. 4*D*). We used *Chop*$^{-/-}$ animals for this experiment so that there would be no confounding influence of endogenous CHOP. In this case, animals expressing CHOP, but not animals infected with control virus, showed significant down-regulation of *Ppara* and *Srebf1*, with *Cebpa* falling near the significance threshold, lending credence to the idea that these genes are downstream of CHOP (Fig. 4*E*). Importantly, there was no evidence that CHOP expression exacerbated the ER stress burden in these animals based on either eIF2α phosphorylation or *Xbp1* splicing, suggesting that CHOP does not act on these genes indirectly simply by augmenting ER stress (Fig. 4*F*). Taken together, these data suggest that at least *Ppara*, *Srebf1*, and *Cebpa* are downstream of CHOP. We thus sought to test whether CHOP acts upon them directly.

Promoter/Enhancer Regions of Ppara, Srebf1, and Cebpa Are Bound by CHOP—We first used ChIP to test whether CHOP binds directly to this promoter/enhancer region of *Ppara*, for which the strongest case for CHOP dependence can be made. Wild-type or *Chop*$^{-/-}$ animals were treated with TM for 8 h, and the efficacy of treatment was verified by immunoblotting for the glycoprotein TRAPα (data not shown). Prior to ChIP, chromatin was sheared to a 100–500-bp range, and recovery after ChIP was assessed by qPCR, monitoring qPCR products of ~100 bp in length. We detected modest enrichment by CHOP ChIP of two regions within the 1-kb proximal promoter region (Fig. 5*A*); we also found stronger enrichment of a site ~2.8 kb upstream of the *Ppara* transcriptional start site (TSS), which was encompassed by two separate qPCR products (Fig. 5*A*). We immediately noticed that within this region, there was a site that was similar to the previously defined consensus sequence for C/EBPα-CHOP heterodimers (Fig. 5*B*) (39). The enrichment of this region by ChIP depended upon ER stress and peaked at 8 h (Fig. 5*C*).

We next carried out a similar analysis for CHOP binding to the *Srebf1* and *Cebpa* promoters/enhancers. Sequence analysis of the *Srebf1c* promoter/enhancer revealed a site that, like the region in the *Ppara* promoter, was very similar to the C/EBPα-CHOP consensus sequence (Fig. 6*A*). The region containing this sequence, but not others more distal to the TSS, was significantly enriched in wild-type but not *Chop*$^{-/-}$ livers (Fig. 6*B*) (data not shown). We also found a region of the *Cebpa* promoter that was significantly enriched during ER stress (Fig. 6*C*).

The changes in gene regulation elicited by ER stress are poorly reconstituted in cultured hepatocytes *in vitro* (data not shown), limiting our ability to manipulate these sequences in *cis*. Thus, we cannot yet conclude that CHOP binding to the enriched regions is required for CHOP-dependent suppression. We can conclude, however, that CHOP binding is not sufficient. CHOP can be recovered from the *Srebf1* and *Ppara* promoters to similar extents in wild-type animals overexpressing *Ad-Chop* in the absence of ER stress and in wild-type animals overexpressing *Ad-Gfp* but in the presence of stress (Fig. 6, *D* and *E*) (data not shown). However, mere overexpression of CHOP was insufficient to suppress *Ppara* or *Srebf1* expression (Fig. 4*B*). Taken together, our data demonstrate direct CHOP binding to the promoter/enhancer regions of at least three metabolic genes whose expression is influenced by the absence of CHOP yet require an independent stress signal for repression. These data point to a direct but contributory role for CHOP in their regulation.

Strongest CHOP Binding Occurs at C/EBP Sites—The binding of C/EBPα to sites within the mouse genome has been exhaustively mapped by ChIP-seq (40). The region of apparent CHOP binding to the *Ppara* promoter, in addition to containing a putative C/EBPα-CHOP consensus sequence, overlaps with a region of documented C/EBPα binding from that study, as does the CHOP-binding region in the *Srebf1* promoter (Fig. 7*A*) (data not shown). Bioinformatic analysis suggests that this region in the *Ppara* promoter also contains several potential binding sites for C/EBPβ (data not shown). Given the demonstrated ability of CHOP to heterodimerize with both C/EBPα and C/EBPβ (25), we examined the binding characteristics of both proteins at CHOP-binding sites.

As we have shown previously (23) and consistent with mRNA expression data, ER stress suppressed expression of C/EBPα

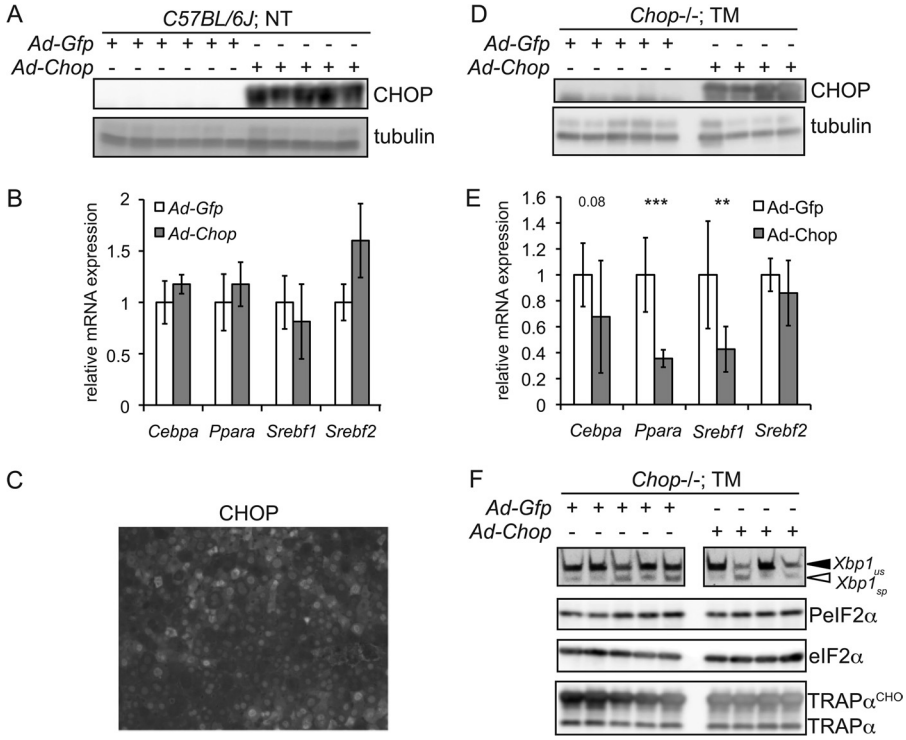

FIGURE 4. **CHOP suppresses metabolic genes in the presence of a concomitant ER stress signal.** *A*, wild-type mice were infected with recombinant adenovirus expressing either GFP (*Ad-Gfp*) or CHOP (*Ad-Chop*) by tail vein injection. Livers were analyzed by immunoblotting for expression of CHOP or tubulin as a loading control. *NT*, non-treated. *B*, expression of the indicated mRNAs from animals in *A* was assessed by qRT-PCR. *C*, formalin-fixed paraffin-embedded liver sections from *Chop*$^{-/-}$ animals injected with *Ad-Chop* were assessed for expression of CHOP by fluorescent IHC. A representative image is shown. Note that the majority of cells show CHOP-positive nuclei. *D*, *Chop*$^{-/-}$ animals were infected with *Ad-Gfp* or *Ad-Chop*. Animals were then challenged with 1 mg/kg TM for 8 h. Expression of CHOP and tubulin was assessed by immunoblotting of liver lysates. *E*, expression of the indicated mRNAs from two separate experiments as described for *D* was assessed by qRT-PCR (*Ad-Gfp*, $n = 8$; *Ad-Chop*, $n = 9$). **, $p < 0.01$; ***, $p < 0.001$. *F*, livers from animals treated as described for *D* were assessed for *Xbp1* splicing by RT-PCR or for eIF2α phosphorylation (*PeIF2α*) and TRAPα glycosylation (*TRAPα*^{CHO}) by immunoblotting. Spliced (*sp*) and unspliced (*us*) *Xbp1* mRNAs are shown.

(Fig. 7*B*). However, no such suppression was seen for C/EBPβ (Fig. 7*B*), although ER stress might lead to an increase in the short inhibitory LIP (liver-enriched transcriptional inhibitory protein) isoform of C/EBPβ, which has previously been shown to be up-regulated by ER stress in cultured cells (41). In the absence of ER stress, C/EBPα bound to the CHOP-binding regions of the *Ppara* and *Gadd34* promoters (Fig. 7*C*), but not to the CHOP-binding region of the *Srebf1* promoter (data not shown). In this experiment, the *Fga* and *Eif2s2* promoters, identified as C/EBPα-binding regions (40), served as positive controls (Fig. 7*C*). At most of these loci, C/EBPα binding was substantially diminished upon TM treatment, consistent with the decrease in C/EBPα expression (Fig. 7*C*). Likewise, C/EBPβ binding was observed at the *Ppara* and *Srebf1* CHOP-binding regions and was also diminished by ER stress (Fig. 7*D*), although this diminishment could not be accounted for by any change in C/EBPβ expression (Fig. 7*B*). These data indicate that there is substantial overlap between CHOP- and C/EBPα/β-binding regions in the promoters of metabolic genes and suggest that either cooperative or antagonistic interactions among these proteins might be ultimately responsible for shaping metabolic gene expression during ER stress.

DISCUSSION

The aim of this work was to test whether CHOP has a role in the transcriptional regulation of metabolism. Our results allow us to conclude that CHOP contributes to this regulation and probably does so by direct action on the promoters of metabolic genes. They contribute to the expanding body of data indicating that CHOP is not strictly an apoptotic regulator but carries out non-apoptotic physiological functions as well. Our data suggest that CHOP augments metabolic gene suppression but does not suppress these genes on its own during ER stress.

The ability of CHOP to avidly form heterodimers with other C/EBP family members has been well documented (*e.g.* Refs. 25, 39, and 42–44). It was originally proposed that CHOP inhibited transcription by simple DNA-independent titration of these family members, and this appears to be the case in at least some instances (25, 31). However, CHOP can also bind to DNA when in complex with other proteins, including both C/EBP family members and ATF family members (45, 46). Although heterodimers containing CHOP can activate transcription (46, 47), such heterodimers most commonly appear to attenuate induction of transcription compared with the activity of the binding partner when CHOP is absent (39, 44, 46).

We favor a model in which CHOP forms inhibitory heterodimers with C/EBPα and/or C/EBPβ. At a minimum, our data demonstrate that there is considerable rearrangement of CHOP and C/EBP binding at the promoters studied. In addition, two factors led us to this model: the CHOP-binding regions of the *Ppara* and *Srebf1* promoters contain a sequence

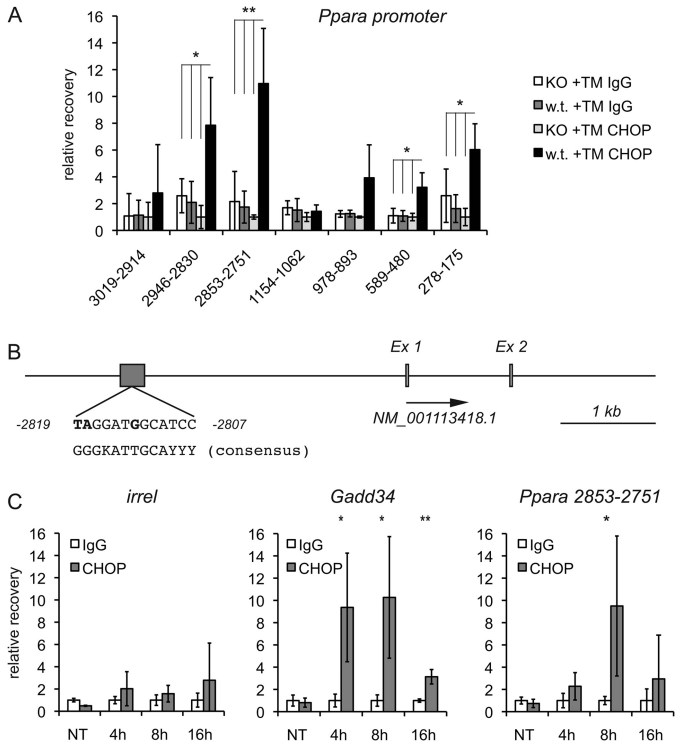

FIGURE 5. **CHOP binds directly to the *Ppara* promoter/enhancer.** *A*, wild-type or *Chop*$^{-/-}$ mice were treated with 1 mg/kg TM for 8 h. Livers were fixed in formaldehyde, homogenized, and sonicated to shear chromatin, and immune complexes were purified using anti-CHOP monoclonal antibody or an equal mass of normal mouse IgG. Recovered DNA was amplified by qPCR, and recovery is expressed relative to that in *Chop*$^{-/-}$ animals using anti-CHOP antibody after quantitating recovery relative to immunoprecipitation input. The *numbers* indicate bases upstream of the *Ppara* TSS. *t* tests were performed comparing recovery in wild-type TM-treated animals using anti-CHOP antibody against all other conditions. The most conservative *p* value among these comparisons is shown and is indicated only when all three comparisons were significant. *Error bars* represent S.D. from four animals per group. *, $p < 0.05$; **, $p < 0.01$. *KO*, knock-out. *B*, a scaled schematic of the *Ppara* promoter/enhancer region shows the TSS (*arrow*) and the region of CHOP binding, along with a comparison of the *Ppara* sequence and the C/EBPα-CHOP consensus sequence, with non-matching bases in *boldface*. *Ex*, exon. *C*, wild-type animals were treated with vehicle (non-treated (*NT*)) or 1 mg/kg TM for the indicated times, followed by ChIP using anti-CHOP or control antibody. The strongest CHOP-binding region of the *Ppara* promoter, the known CHOP-binding region of the *Gadd34* promoter (28), or an irrelevant promoter sequence (*irrel*) was amplified by qPCR. Recovery is expressed relative to non-immune IgG at each time point ($n = 3$).

that is very similar to that previously identified for C/EBP-CHOP heterodimers (39), and CHOP binds to regions from which C/EBPα and/or C/EBPβ can be recovered when CHOP is absent. Thus, we favor this model even though binding of both C/EBPα and C/EBPβ diminishes at these sites during ER stress. Indeed, perhaps a diminishment of C/EBPα and C/EBPβ concentrations at the relevant promoter regions increases the likelihood that CHOP will bind to them because C/EBPα and C/EBPβ are generally expressed at a vast stoichiometric excess compared with CHOP (48). However, it is clear that binding of CHOP to these sites is insufficient to inhibit metabolic gene expression because overexpressed CHOP shows such binding but does not lead to gene suppression in the absence of ER stress (Figs. 4*B* and 6*E*). This observation suggests that even if C/EBP-CHOP complexes form, an ER stress signal is necessary to potentiate its action. Perhaps ER stress modifies either CHOP or C/EBP by phosphorylation or other means (49, 50). Alternatively, ER stress might alter local chromatin structure and so influence CHOP activity indirectly. It has been previously demonstrated that ER stress *per se* can influence the activity of C/EBP-CHOP dimers (39). The relatively inefficient recovery of CHOP from ChIP reactions has so far made it impossible to identify by sequential ChIP the factor(s) with which CHOP interacts at metabolic promoters, which will be an essential element in addressing this issue.

Our results point to a repressive mechanism for CHOP distinct from those previously identified. The direct binding of CHOP to metabolic promoters argues against a mechanism whereby CHOP simply acts as a dominant-negative titrating agent against C/EBP family members. CHOP has also been shown to attenuate transcriptional up-regulation by forming heterodimers with ATF4. In this case, ATF4 alone induced transcription of the *Asns* gene, whereas CHOP-ATF4 heterodimers led to a less robust induction (46), and as in our work, CHOP binding could be seen at other genes, including *Snat2*, *Vegf*, and *Cat-1*, for which CHOP binding alone was insufficient to alter expression (46). However, in the case of CHOP-ATF4 dimers, depletion of CHOP allowed for a stress-dependent up-regulation of *Asns*, whereas in our case, CHOP deletion instead prevented a decline in expression (Fig. 3), suggesting that here CHOP is attenuating basal transcription rather than attenuating stimulated transcription. In addition, CHOP-ATF4 dimers bound to amino acid response elements (46, 51), which we have not identified in the ChIP-enriched regions of the genes studied here. Thus, it appears that there are multiple mechanisms by which CHOP can attenuate or repress transcription, and these are probably all highly dependent on the complement of C/EBP and other bZIP proteins expressed under a given condition.

The most parsimonious interpretation of our data is that CHOP suppresses the metabolic genes studied here by direct binding. However, an important caveat is that because this metabolic regulation is not recapitulated in *in vitro* studies, we cannot yet formally exclude the possibility that its presence at these promoters is coincidental, *i.e.* that CHOP has some intrinsic affinity for C/EBP sites that is captured here but that CHOP functions through some other mechanism to inhibit metabolic gene expression. Testing the necessity of CHOP binding will require either reconstituting CHOP-dependent repression in cultured cells (*e.g.* primary hepatocytes) or creating reporter constructs that can be expressed and monitored *in vivo* through adenoviral expression or hydrodynamic DNA delivery.

What is the relevance of CHOP action on these genes to normal and pathological metabolic physiology? At a minimum, our findings predict that *Chop*$^{-/-}$ animals should show altered hepatic lipid metabolism either basally or during chronic stresses that induce the UPR such as obesity, viral hepatitis, and both alcoholic and non-alcoholic steatohepatitis (52). A recent report shows that *Chop*$^{-/-}$ animals show enhanced basal hepatic steatosis compared with wild-type controls, although it is not clear whether this phenotype is autonomous to the liver (53). CHOP deletion has a confounding effect on pancreatic β cell survival and function (54, 55). Thus, liver-specific inducible deletion will be required to rigorously explore the contribution of CHOP to lipid metabolism in the liver.

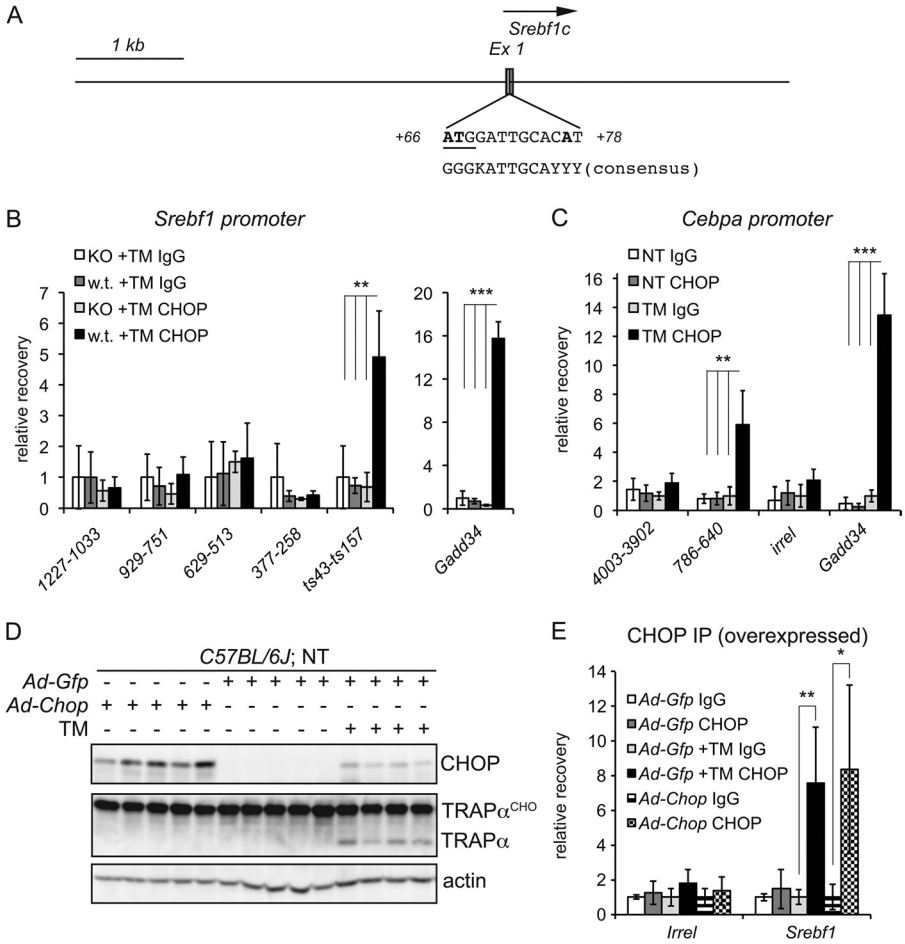

FIGURE 6. **CHOP binds to the *Srebf1c* and *Cebpa* promoter/enhancer regions.** *A*, a scaled schematic of the *Srebf1c* promoter/enhancer region shows the TSS (*arrow*) along with the putative CHOP-binding region, which overlaps exon 1 (*Ex 1*) and contains the indicated C/EBPα-CHOP-like binding site. Non-matching bases are in *boldface*. The site overlaps the SREBF1c start codon, which is *underlined*. *B*, binding of CHOP to the *Srebf1c* 1-kb proximal promoter was analyzed by ChIP as described in the legend to Fig. 5*A*. *ts43-ts157* denotes a region 43–157 bp downstream of the TSS and encompassing the putative C/EBPα-CHOP site. Binding is given relative to *Chop*$^{-/-}$ animals using a non-immune antibody. For comparison, CHOP binding to the *Gadd34* promoter from the same experiment is shown ($n = 4$). **, $p < 0.01$; ***, $p < 0.001$. *KO*, knock-out. *C*, CHOP binding to a region ~700 bp upstream of the *Cebpa* TSS is shown, along with a more distal region 4 kb upstream and an irrelevant genomic sequence (*irrel*) as negative controls and the *Gadd34* promoter as a positive control ($n = 4$). *NT*, non-treated. *D*, wild-type animals were injected with *Ad-Gfp* or *Ad-Chop* as described in the legend to Fig. 4, and one group of *Ad-Gfp*-injected animals was treated with TM for 8 h to induce endogenous CHOP expression. Expression of CHOP and efficacy of TM treatment as determined by immunoblotting are shown. *TRAP*α^{CHO}, glycosylated TRAPα. *E*, binding of CHOP to an irrelevant genomic region or to the *ts43-ts157* region of the *Srebf1* promoter was tested by ChIP in four animals per group from the experiment shown in *D*. *, $p < 0.05$.

Chop$^{-/-}$ animals appear to accumulate more hepatic lipid than wild-type animals in response to Velcade treatment and perhaps during long-term TM treatment as well (data not shown). This observation suggests that the role of CHOP in inhibiting lipogenesis outweighs its impact on fatty acid oxidation, although increased hepatic lipid accumulation could be due to indirect influences of CHOP that are independent of the gene regulatory events described here. More puzzlingly, it would seem paradoxical that CHOP suppresses both fatty acid oxidation and lipogenesis by CHOP. However, we have previously shown that inhibition of fatty acid oxidation protects the liver from ER stress *in vivo* (56), suggesting that fatty acid catabolism contributes to ER stress. In addition, because lipogenesis occurs at the ER membrane, it also might compromise ER function and thus be targeted for suppression during UPR activation; at a minimum, triglyceride synthesis and production and secretion by the liver of VLDL lead to ER stress (57). It is conceivable that both anabolic and catabolic lipid fluxes tax the ER and that the UPR is primed to suppress both processes. Accordingly, whether CHOP promotes or protects against lipid accumulation probably depends upon the nature of the inducing stimulus and on whether the effects of CHOP are greater on the opposite processes of lipid anabolism (*e.g.* SREBF1) or catabolism (*e.g.* peroxisome proliferator-activated receptor α).

CHOP is conventionally considered to promote apoptosis, and the fact that both cells and animals lacking CHOP are protected against a broad array of pharmacological and physiological insults suggests that, on balance, CHOP compromises cell function, viability, or both (58). However, it is not clear whether CHOP is directly apoptotic or whether cell dysfunction and death arise as a secondary consequence of actions of CHOP that are geared toward protecting ER function. One clear consequence of CHOP deletion is accumulation of reactive oxygen species, which can occur as a result of both CHOP-mediated resumption of protein synthesis via its effect on GADD34 (28) and CHOP-dependent up-regulation of the ERO1α oxidase

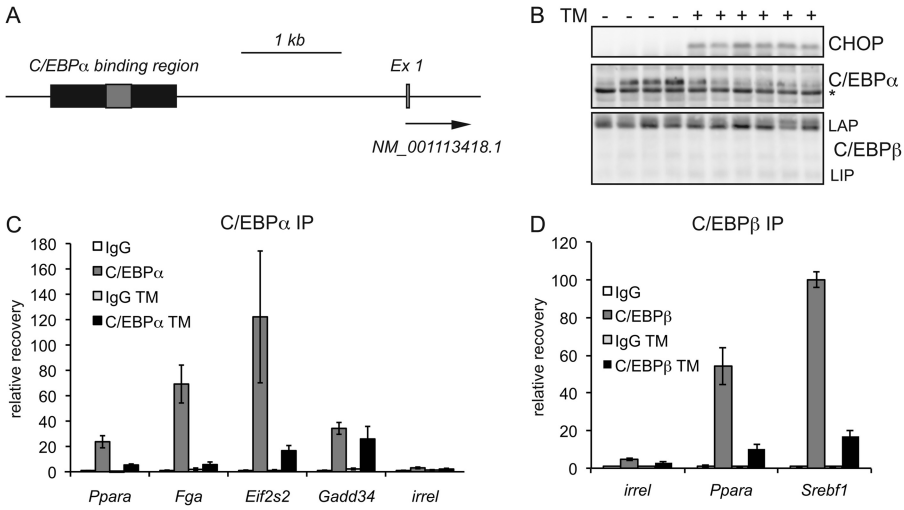

FIGURE 7. **CHOP binds to C/EBPα sites in diverse promoters.** *A*, a schematic of the *Ppara* promoter shows overlap between the ChIP-defined CHOP-binding region (*gray box*) and a C/EBPα-binding region defined by ChIP-seq (*black box*) (40). *Ex 1*, exon 1. *B*, expression of C/EBPα and the LIP and LAP (liver-enriched transcriptional activating protein) forms of C/EBPβ following 8 h of treatment with TM as determined by immunoblotting. The *asterisk* indicates a nonspecific band showing equal loading. For C/EBPα, only the long form of the protein is shown; the 30-kDa short form could not be unambiguously identified. *C*, promoter/enhancer regions from the indicated genes were assessed for C/EBPα binding by ChIP (*IP*). For *Ppara* and *Gadd34*, primers spanning CHOP-binding regions were used for qPCR. For *Fga* and *Eif2s2*, primers covered regions identified by ChIP-seq. Association with an irrelevant genomic sequence (*irrel*) is also shown. ChIP used anti-C/EBPα or control antibody in animals treated for 8 h with vehicle or 1 mg/kg TM ($n = 4$). *D*, binding of C/EBPβ (using an antibody that recognizes both LAP and LIP forms of the protein) to the CHOP-binding sites of the *Ppara* and *Srebf1* promoters or to an irrelevant genomic sequence was assessed by ChIP after 8 h of TM challenge.

(27). Both resumption of protein synthesis and up-regulation of oxidative protein folding might represent appropriate protective measures during minor ER stresses, the consequences of which (reactive oxygen species) can be dealt with after ER stress is alleviated but which are deleterious during either severe or chronic ER stress. Likewise, inhibition of fatty acid oxidation appears to protect ER function by promoting oxidative folding while exacerbating reactive oxygen species production via alterations to glutathione oxidation (56). To the extent that fatty acid oxidation falls under the control of CHOP, *e.g.* through its effects on peroxisome proliferator-activated receptor α, regulation of lipid metabolism might also represent a nominally beneficial role for CHOP that can become maladaptive under certain conditions.

The relationship between ER stress and lipid metabolism is clearly complex, and multiple pathways appear to be at work. Understanding this relationship demands an accounting of the mechanisms that exert transcriptional control over metabolic genes. Our results place CHOP within this framework and so suggest a novel physiological role for this protein.

Acknowledgments—We thank H. Qi, N. Spencer, Y. Zhang, and W. Zhou (Department of Anatomy and Cell Biology, University of Iowa Carver College of Medicine) for advice and technical assistance.

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