C/EBP Homologous Protein-induced Macrophage Apoptosis Protects Mice from Steatohepatitis

Harmeet Malhi1,5, Erin M. Kropp1,4, Vinna F. Clavo1, Christina R. Kobrossi1, JaeSeok Han1,3, Amy S. Mauer3, Jing Yong3, Randal J. Kaufman1,2,3

1 Department of Biological Chemistry, 2 Department of Internal Medicine, University of Michigan Medical Center, 1500 West Medical Center Drive, Ann Arbor, MI 48109
3 Center for Neuroscience, Aging, and Stem Cell Research, Sanford Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037
4 Present Address: Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226
5 Present Address: Division of Gastroenterology and Hepatology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905

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To whom correspondence should be addressed: Randal J. Kaufman, Ph.D., Director, Degenerative Disease Research, Center for Neuroscience, Aging, and Stem Cell Research, Sanford Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, T: 858-795-5149, F: 858-795-5273, mail: rkaufman@sanfordburnham.org

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**Background:** We hypothesized that C/EBP homologous protein mediates hepatocyte apoptosis in nonalcoholic steatohepatitis.

**Results:** Paradoxically, Chop deletion protects from steatohepatitis, by inducing apoptosis in activated macrophages.

**Conclusion:** CHOP-dependent macrophage apoptosis in NASH highlights the cell type-specific complexity of the ER stress response.

**Significance:** Therapeutic manipulation of mediators of ER stress response may have opposite effects in different cell populations; therefore, such studies should be interpreted cautiously.

**SUMMARY**

Nonalcoholic fatty liver disease (NAFLD) is a heterogeneous disorder characterized by liver steatosis; inflammation and fibrosis are features of the progressive form nonalcoholic steatohepatitis (NASH). The endoplasmic reticulum (ER) stress response is postulated to play a role in the pathogenesis of NAFLD and NASH. In particular, C/EBP homologous protein (CHOP) is undetectable under normal conditions, but is induced by cellular stress, including ER stress. Chop wildtype (Chop+/-) and knockout (Chop-/-) mice were used in these studies to elucidate the role of CHOP in the pathogenesis of fatty liver disease. Paradoxically, Chop-/- mice developed greater liver injury, inflammation and fibrosis than Chop+/- mice, with greater macrophage activation. Primary, bone marrow-derived and peritoneal macrophages from Chop+/- and Chop-/- were challenged with palmitic acid, an abundant saturated FFA in plasma and liver lipids. Where, palmitic acid treatment activated Chop+/- and Chop-/- macrophages, Chop-/- macrophages were resistant to its lipotoxicity. Chop-/- mice were sensitized to liver injury in a second model of dietary steatohepatitis using the methionine-choline deficient diet. Analysis of bone marrow chimeras between Chop-/- and Chop+/- mice demonstrated that Chop in macrophages protects from liver injury and inflammation when fed the methionine-choline deficient diet. **Conclusion:** Chop deletion has a proinflammatory effect in fatty liver injury apparently due to decreased cell
death of activated macrophages, resulting in their net accumulation in the liver. Thus, macrophage CHOP plays a key role in protecting the liver from steatohepatitis likely by limiting macrophage survival during lipotoxicity.

Nonalcoholic fatty liver disease (NAFLD) is a group of heterogeneous disorders characterized by obesity and insulin resistance (1). Nonalcoholic steatohepatitis (NASH), a progressive form, is present in 10% to 30% of NAFLD patients. It is characterized by liver injury, which can result in cirrhosis. Histologically NASH is distinguished from NAFLD by lobular inflammation and fibrosis (2). Due to increasing prevalence and associated morbidity and mortality, understanding the mediators of these disorders is important. Furthermore, identification of factors involved in the pathogenesis of NASH is key to the development of preventive strategies. Data from animal and human studies point toward insulin resistance, hepatocyte apoptosis and inflammation as keystones of disease pathogenesis (3-5). Animal models have implicated changes in lipid metabolism, adipocytokines, FFA and innate immunity in the onset of hepatic insulin resistance with subsequent steatosis (6,7). These factors also modulate hepatocyte apoptosis, which is a salient feature, correlating with histologic severity and fibrosis in NASH (8). Inflammation on initial liver biopsy in patients with NASH was an independent predictor of progression to advanced fibrosis (5). In spite of all of these potential and emerging mediators, the exact cellular and subcellular molecular mediators of these processes are not fully defined.

The endoplasmic reticulum (ER) has a well-defined stress response and an emerging role in the regulation of lipid and carbohydrate metabolism (9). Classically, upon the accumulation of misfolded proteins in the ER lumen, the unfolded protein response (UPR) is activated under conditions of ER stress. The UPR is mediated by three ER transmembrane proteins, inositol requiring enzyme (IRE) 1α, PKR-like ER kinase (PERK) and activating transcription factor (ATF) 6α. The UPR mediators activate signals that reduce translation rate thus limiting new proteins entering the ER and increase the folding capacity of the ER. The sum total of these events is aimed at restoring ER homeostasis. However, under conditions of a sustained and unrelenting ER stress apoptosis ensues. The exact pathways that mediate ER stress-induced apoptosis are not well defined. C/EBP homologous protein (CHOP), a transcription factor, activated downstream of PERK, is a potent mediator of ER stress-induced apoptosis (10). However the role of CHOP in mediating either hepatocyte apoptosis or hepatic inflammation in NASH is unknown.

CHOP is a bZIP transcription factor, undetectable in normal cells, induced by cellular stress including ER stress (11,12). CHOP deficient cells are resistant to cell death induced by ER stress-inducing agents (10). Mice lacking CHOP, Chop−/−, develop normally, and under normal chow conditions female mice develop increased adiposity over several months (13). Chop−/− mice are protected from diabetes in several mouse models of type 2 diabetes mellitus by decreasing beta cell apoptosis and improving beta cell proliferation (14). In isolated cells CHOP is implicated in palmitic acid (PA)-induced expression of the proapoptotic protein PUMA, and potentially in mediating hepatocyte apoptosis under high concentrations of PA (15,16). However, CHOP has both proapoptotic and proinflammatory properties, depending on the stimulus, disease and tissue context, and its precise role in fatty liver disease remains unclear (17). Herein, we examined the role of CHOP in animal models of NASH. Our principal findings are that CHOP protects mice from the development of steatohepatitis. CHOP deletion sensitizes to the development of liver injury and inflammation, the latter due to sustained survival of activated hepatic macrophages in CHOP deficient mice, thus potentiating steatohepatitis. These findings demonstrate a novel role for CHOP as a disease-preventing factor in NASH and highlight the complexity of the UPR in specific hepatic cell types.

EXPERIMENTAL PROCEDURES
Animal husbandry-Chop−/− mice have been described (10). They were a gift from Dr. David Ron. Experimental protocols involving the use of animals were approved by the University Committee on Use and Care of Animals at the University of Michigan, the Institutional Animal
Use and Care Committee at Mayo Clinic, and at the Sanford Burnham Medical Research Institute. Mice were housed with 12 hour light and dark cycles and ad libitum access to water and diets. 45% Kcal high fat diet (HFD) (Catalog D07081501) or a 10% Kcal fat control diet (CD) (Catalog D12450B) were from Research Diets, and fed for 16 weeks. The methionine and choline deficient (MCD, Catalog 960439) diet and its control methionine and choline sufficient (MCS, Catalog 960441) diet were from MP Biochemicals and fed for 3 weeks.

Animal Procedures- Body mass and fasting blood glucose levels were measured after a six hour fast. Glucose and insulin tolerance tests were performed after a 6 hour fast and pyruvate tolerance tests were performed after a 16 hour fast as previously described (14). Chimeric mice were generated as described (18). Allowing 6 weeks for reconstitution, mice were fed either MCS or MCD diet for three weeks. Blood and liver tissues were collected from euthanized mice.

Histologic analyses- Steatohepatitis was evaluated in H&E stained formalin fixed paraffin embedded 5μM liver sections, according to a validated system for NAFLD (2). Hepatocyte apoptosis was identified through TUNEL staining of 5 μM formalin fixed tissue sections by the Cancer Center Research Laboratory Tissue Core using the Apoptag peroxidase in situ apoptosis detection kit (Millipore) according to the manufacturer’s instructions. Picosirius red staining for collagen was as described (19). Immunohistochemistry was performed using F4/80 antibody (eBioscience) at 1:50 dilution, as described (18). Quantification of F4/80 positive surface area in each tissue section was performed with KS400 Image Analysis Software (Carl Zeiss, Inc.), and expressed as a percent of total surface area of each liver section.

Detection of apoptotic macrophages-The Click-iT TUNEL Alexa Fluor 488 imaging assay (Life Technologies) was used to detect apoptotic cells in 5μM cryosections according to the manufacturer’s instructions. Next, F4/80 was detected as described, using 1:500 diluted Alexa fluor 594 donkey anti rat secondary antibody (Life Technologies). Sections were then mounted in Prolong antifade with DAPI and images acquired with an inverted Zeiss laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss Inc.). For quantification dual F4/80 and TUNEL positive cells were counted in 10 random 400x fields by a blinded observer.

Serum Measurements-Total serum cholesterol at weeks 4 and 8 was determined with the Cholesterol E Assay (Wako). The terminal cholesterol and triglyceride (TG) levels were analyzed by the Metabolomics and Obesity Center, University of Michigan. Serum FFA at weeks 4, 8, and 16 were measured using HR series NEFA-HR assay (Wako). ALT levels were determined using a Siemens Advia 2400 auto analyzer at the University of Michigan, Department of Chemical Pathology.

Quantitative Polymerase Chain Reaction (qPCR)-Total RNA was isolated from the livers. mRNA expression was normalized to 18S or β-actin, performed as previously described (20,21), using primers summarized in Table 1 (22,23).

Protein Isolation and Western Blotting- Liver protein extracts in RIPA buffer were resolved by SDS-PAGE as described (18). The following primary antibodies were used: p-eIF2α (Invitrogen), eIF2α (Cell Signaling Technology), CHOP, ATF4, C/EBPα, C/EBPβ, PPARγ, Fas, and Actin, (Santa Cruz Biotech), and alpha-tubulin (Sigma). Densitometry was performed using Image J (NIH). For each experiment the protein of interest was normalized to its loading control and then expressed relative to vehicle control treated conditions.

Macrophage isolation-Bone marrow harvested from hind legs of euthanized mice by flushing with Hank’s balanced salt solution was plated in sterile petri dishes in bone marrow differentiation medium, consisting of RPMI, 10% fetal bovine serum and 20% L929 cell-conditioned medium(18). Medium was changed on days 3 and 5, and differentiated macrophages used for experiments on day 7. Cells were treated with PA as previously described, and apoptosis assessed by DAPI stained nuclear morphology (24).

Lipidomics- Lipidomics analysis for liver total lipid fatty acid content and serum FFA was performed by the Metabolomics and Obesity Center at the University of Michigan. TG and FFA were separated by thin-layer
chromatography. Fatty acid composition was done by tranmethylation to their methyl esters and gas chromatography. Liver TG content was measured using the Infinity TG reagent (Sigma) as described (25).

**RESULTS**

*CHOP deletion sensitizes mice to the development of dietary obesity-HFD-fed mice gained weight; however, Chop<sup>+/+</sup> mice gained significantly more weight, 110±3% at week 16, compared to HFD-fed Chop<sup>−/−</sup> mice, 84±8% (p<0.005, Fig. 1A). Weight gain was associated with features of insulin resistance in both genotypes. HFD-fed Chop<sup>−/−</sup> and Chop<sup>+/+</sup> mice developed significant fasting hyperglycemia (Fig. 1B). Glucose tolerance testing (GTT) performed at weeks 5, 10 (data not shown) and 14 (Fig. 1C), demonstrated significantly greater increase in blood glucose and delayed glucose disposal in HFD-fed compared to CD-fed mice, i.e., the area under the glucose disposal curve upon GTT (Fig. 1D). Similarly, hepatic gluconeogenesis, tested by pyruvate tolerance testing at week 15 (Fig. 1E), demonstrated sustained elevation of blood sugars in the HFD-fed groups compared to the CD-fed groups. Insulin tolerance testing at week 16 (Fig. 1F) demonstrated impaired insulin sensitivity among the HFD-fed mice. Given the role of FFA in lipotoxicity, circulating levels were measured at weeks 4, 8 and 16, and were not elevated in the HFD-fed Chop<sup>−/−</sup> mice, in spite of greatest weight gain (Table 2). Total cholesterol levels were significantly elevated at week 4 in both Chop<sup>−/−</sup> and Chop<sup>+/+</sup> HFD-fed mice, and remained elevated at weeks 8 and 16, compared with the CD-fed mice (data not shown and Table 2). Overall, both HFD-fed Chop<sup>−/−</sup> and Chop<sup>+/+</sup> mice were obese with several features of insulin resistance compared with CD-fed mice; however, HFD-fed Chop<sup>−/−</sup> mice demonstrated a greater increase in body mass but not greater insulin resistance.

Chop<sup>−/−</sup> mice develop steatohepatitis-HFD-fed Chop<sup>−/−</sup> mice demonstrated significant hepatomegaly. Due to significant differences in body weight gain liver mass was normalized to body weight and was significantly elevated in the Chop<sup>−/−</sup> HFD-fed mice (p<0.05, Fig. 2A). Both HFD-fed groups demonstrated liver steatosis, although the histologic steatosis score (Fig. 2B) was significantly greater in the HFD-fed Chop<sup>−/−</sup> mice (p<0.001). Alanine transaminase (ALT) levels (Fig. 2C, p<0.05) and lobular inflammation were significantly greater in HFD-fed Chop<sup>−/−</sup> mice compared to Chop<sup>+/+</sup> mice (Fig. 2D, p<0.05), indicative of significant liver injury and inflammation, respectively. Representative liver sections are shown in Fig. 2E. The increase in liver mass was due to excess lipid deposition in hepatocytes. Liver total lipid content was three fold greater in the HFD-fed Chop<sup>−/−</sup> mice as compared to Chop<sup>+/+</sup> mice (Fig. 3A). The increase in lipid in the HFD-fed Chop<sup>−/−</sup> liver was associated with increased expression of PPARγ protein and mRNA as compared with the Chop<sup>+/+</sup> mice (Fig. 3B and C). The expression of C/EBPα, C/EBPβ, PPARα and PPARδ was unchanged (Fig. 3C-E). Thus, CHOP deletion sensitized to the development of steatosis and steatohepatitis, as Chop<sup>−/−</sup> mice developed hepatomegaly, liver injury and histologic steatohepatitis upon HFD-feeding.

*Chop<sup>−/−</sup> mice develop hepatocyte apoptosis and fibrosis-Hepatocyte apoptosis is a pathogenic event in progressive forms of NASH.(8) Given the presence of histologic steatohepatitis in HFD-fed Chop<sup>−/−</sup> mice, hepatocyte apoptosis was assessed. Indeed, hepatocyte apoptosis was increased in HFD-fed Chop<sup>−/−</sup> and Chop<sup>+/+</sup> mice compared with CD-fed Chop<sup>−/−</sup> mice (Fig. 4A and B, p<0.05). Increased death receptor expression correlates with apoptosis in NASH, therefore liver mRNA expression for the death receptors Fas and death receptor 5 (DR-5) was assessed by qPCR. The HFD-fed Chop<sup>−/−</sup> had significantly greater increase in expression of both death receptors (p<0.05, Fig. 4C), though no increase was observed in their ligands, FasL and TRAIL. Furthermore, whole liver Fas protein levels were increased in HFD-fed Chop<sup>−/−</sup> mice (Fig. 4D). Whole liver DR5 expression could not be assessed due to lack of specific antibodies. We next dissected the contribution of isolated hepatocytes and macrophages to increased Fas and DR5 expression using an *in vitro* model of PA lipotoxicity (24). We found a slight increase in Fas protein in PA-
treated Chop⁻/⁻ macrophages (Fig. 4E), without a corresponding increase in hepatocytes. Fas and DR5 mRNA were also induced upon PA treatment in Chop⁺/+ and Chop⁻/⁻ macrophages (Fig. 4F). Fas and DR5 mRNA were increased only in Chop⁺/+ hepatocytes (Fig. 4F). These findings suggest that macrophages contribute to the significantly greater Fas and DR5 mRNA expression, and Fas protein expression, observed in vivo, due to greater macrophage accumulation under these conditions (Fig. 5B and C). In this experiment CHOP deletion did not protect hepatocytes from apoptosis, in fact, apoptosis was greatest in HFD-fed Chop⁻/⁻, consistent with histologic and biochemical steatohepatitis. In keeping with chronic inflammation and ongoing apoptosis, in HFD-fed Chop⁻/⁻ mice pericellular fibrosis was increased compared with the HFD-fed Chop⁺/+ mice (Fig. 4H). The expression of key genes that regulate hepatic fibrosis, transforming growth factor β1, alpha-2 smooth muscle actin, collagen 1a1, and tissue inhibitor of metalloproteinases 1 were all increased in HFD-fed Chop⁻/⁻ mice (Fig. 4G). Taken together, these data identify paradoxically greater death receptor expression and hepatic fibrosis in Chop⁻/⁻ mice on a HFD.

**CHOP deletion promotes inflammation and macrophage activation**-Inflammation distinguishes NASH from benign steatosis. Therefore, in the HFD-fed Chop⁻/⁻ and Chop⁺/+ mice inflammatory gene expression was analyzed. Hepatic expression of inflammatory mediators, interleukin 1β, macrophage inflammatory protein-1α (MIP-1α), macrophage chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α, was significantly greater in the HFD-fed Chop⁻/⁻ mice (Fig. 5A). Both MIP-1α and MCP-1 are produced by activated macrophages and are key chemokines in the ensuing inflammatory response (26-28). Injury activated macrophages in the liver may be derived from Kupffer cells, liver resident macrophages, or recruited from bone marrow-derived monocytes (29,30). Activated macrophages form a feed-forward loop in perpetuating liver inflammation and injury, therefore we assessed macrophage activation in this model of NASH by F4/80 immunohistochemistry, a murine macrophage specific marker. An increase in F4/80 positive macrophages was evident in HFD-fed Chop⁻/⁻ mice (Fig. 5B). Quantification of F4/80 positivity (Fig. 5C) was consistent with a significant increase in F4/80 positive cells under HFD in Chop⁻/⁻ mice. Altogether these data demonstrate greater numbers of activated macrophages in HFD-fed Chop⁻/⁻ mice.

**Palmitate activates macrophages and induces the UPR-Based** on the activation of macrophages seen histologically in HFD-fed Chop⁻/⁻ mice, a mouse macrophage cell line was challenged with PA. PA was utilized in these studies as it is physiologically abundant, a known mediator of apoptosis in models of lipotoxicity, and enriched in the sera and livers of HFD-fed mice in these studies (Fig. 6). RAW 264.7 cells were activated upon treatment with PA in a concentration- (200µM, not shown) and time-dependent manner (Fig. 7A). PA-induced activation was associated with the UPR including increased CHOP expression (Fig. 7B). mRNA abundance of several UPR markers, BiP, spliced XBP1, Erp72, and CHOP was significantly increased. CHOP protein expression was also enhanced as shown by immunoblot analysis of PA-treated RAW 264.7 cells (Fig. 7C). PA-induced phosphorylation of eIF2α, and increased protein expression of ATF4 and CHOP. Thus, PA activated macrophages; this activation was associated with the UPR. PA-induced macrophage activation and its association with the UPR were further confirmed in primary bone marrow (BM)-derived macrophages. CHOP was detected only in macrophages from Chop⁺/+ mice, and induced by PA (Fig. 7D). Consistent with the data from RAW 264.7 cells, signaling events emanating from activation of IRE1α and PERK and suggestive of activation of ATF6α were detected in primary macrophages as well (Fig. 7E). UPR markers were comparable between Chop⁺/+ and Chop⁻/⁻ macrophages. Both Chop⁺/+ and Chop⁻/⁻ macrophages were comparably activated upon PA treatment (Fig. 7F). As CHOP is a mediator of apoptosis under conditions of sustained ER stress and its deletion prevents ER stress-induced apoptosis, we assessed apoptosis in primary macrophages from Chop⁺/+ and Chop⁻/⁻ mice upon treatment with PA. In Chop⁺/+ macrophages, PA treatment caused apoptosis in a
time- and concentration-dependent manner (Fig. 8A). Apoptosis in Chop<sup>−/−</sup> macrophages was significantly reduced (p<0.05). Apoptotic BM-derived macrophages are shown in Fig. 8B. Thus, CHOP mediates apoptosis in macrophages under conditions of PA-induced activation and UPR; this is abrogated in Chop<sup>−/−</sup> macrophages. Next, we assessed macrophage apoptosis in vivo by colocalization using immunofluorescence for F4/80 and the TUNEL assay. Macrophage apoptosis was a rare event (0.02 cells/400x field Chop<sup>+/+</sup> CD); however observed most frequently in HFD-fed Chop<sup>+/−</sup> (0.2 cells/400x field) and not observed in HFD-fed Chop<sup>−/−</sup> livers, consistent with a role for CHOP in mediating macrophage apoptosis in vivo (Fig 8C).

Sensitization to liver injury is independent of obesity and mediated by macrophages Due to greater weight gain and obesity in HFD-fed Chop<sup>−/−</sup> mice, the sensitization to liver injury could be attributed to greater obesity and hepatic steatosis. Therefore, Chop<sup>−/−</sup> were challenged with an alternative diet. The methionine and choline deficient diet (MCDD), and its CD which is supplemented in methionine and choline diet (MCSD) were fed to Chop<sup>+/−</sup> and Chop<sup>−/−</sup> mice. MCDD-fed mice developed similar steatosis (histologic steatosis score: 1.25±0.25 Chop<sup>+/−</sup> and 1.5±0.23 Chop<sup>−/−</sup>) and TG content (Fig. 9A) and had similar body weights and liver masses (Fig. 9B and C). Similar to the HFD data, Chop<sup>−/−</sup> mice had an increase in hepatocyte apoptosis (Fig. 9D and E). MCDD-fed Chop<sup>+/−</sup> and Chop<sup>−/−</sup> mice developed steatohepatitis (Fig. 10A). Chop<sup>−/−</sup> mice had a significant increase in inflammatory foci (Fig. 10B), liver injury as reflected by the greater increase in serum ALT (p<0.05, Fig. 10C) and macrophage accumulation (Fig. 10D and E). To further dissect the contribution of CHOP in macrophages to liver injury, reciprocal bone marrow transplantation was performed to generate chimeric mice. Where Chop mRNA was not detected in Chop<sup>−/−</sup> mice transplanted with Chop<sup>−/−</sup> bone marrow, it was detected in the liver in whole body Chop<sup>−/−</sup> mice transplanted with Chop<sup>+/−</sup> bone marrow (Fig. 11A), indicative of bone marrow engraftment and presence of bone marrow-derived cells in the liver. ALT elevation, liver inflammation and macrophage accumulation were significantly reduced in the chimeric mice, compared to whole body Chop<sup>−/−</sup> mice fed the MCDD (Figs 10B,C, and D and 11B, p<0.05 compared to MCDD-fed Chop<sup>−/−</sup> mice). We had fewer chimeric Chop<sup>−/−</sup> mice transplanted with Chop<sup>−/−</sup> bone marrow, and Chop<sup>+/−</sup> transplanted with Chop<sup>+/−</sup> bone marrow. However, they were a phenocopy of the whole body genotypes. The ALT in Chop<sup>−/−</sup> to Chop<sup>−/−</sup> was 480 IU/mL, the NASH activity score was 7.5, and the TG content was 0.66 µg TG/µg liver protein. The ALT in Chop<sup>+/−</sup> to Chop<sup>+/−</sup> was 203.5 IU/mL, the NASH activity score was 2.15, and the TG content was 0.4±0.13 µg TG/µg liver protein. TG content of reciprocally transplanted MCDD-fed chimeric mice was comparable to the Chop<sup>−/−</sup> to Chop<sup>−/−</sup> and Chop<sup>+/−</sup> to Chop<sup>−/−</sup> transplanted mice. It was 0.4±0.05 µg TG/µg liver protein in Chop<sup>+/−</sup> transplanted with Chop<sup>−/−</sup> bone marrow, and 0.5±0.1 µg TG/µg liver protein in Chop<sup>+/−</sup> transplanted with Chop<sup>−/−</sup> bone marrow. The NASH activity score was 3.6±0.3 for Chop<sup>−/−</sup> mice reconstituted with Chop<sup>−/−</sup> marrow, and 3.4±0.5 for Chop<sup>+/−</sup> reconstituted with Chop<sup>−/−</sup> marrow. Thus, data from MCDD-feeding demonstrate that CHOP deletion sensitizes to the development of liver injury independent of obesity and hepatic steatosis. Unexpectedly, chimeric Chop<sup>+/−</sup> mice reconstituted with Chop<sup>−/−</sup> bone marrow also demonstrated a protection from liver injury, inflammation and macrophage accumulation. However, the abrogation of liver injury, inflammation and macrophage accumulation in chimeric Chop<sup>−/−</sup> mice transplanted with Chop<sup>−/−</sup> bone marrow supports a role for CHOP-mediated macrophage apoptosis as a modulator of the injury-inflammation response, as any and all CHOP expression in this group is donor-derived, and we see a corresponding decrease in injury, inflammation and macrophage accumulation.

**DISCUSSION**

The principle findings of this study demonstrate a novel role for C/EBP homologous protein in nonalcoholic steatohepatitis. The loss of CHOP led to i) development of steatohepatitis, ii) activation of the innate immune system in steatohepatitis, and iii) decreased macrophage
apoPsis under conditions of FFA-induced ER
stress.

CHOP deletion led to the development of significant steatohepatitis in mice. Chop\(^{-}\)/\(^{-}\) male mice became more obese compared with their littermate controls, consistent with published studies.\(^{13,31}\) Obese Chop\(^{-}\)/\(^{-}\) mice developed hepatomegaly. Histologically hepatocyte steatosis and biochemically total liver TG were greatly elevated in the Chop\(^{-}\)/\(^{-}\) mice. Increased hepatic expression of PPAR\(\gamma\) protein and mRNA were found in Chop\(^{-}\)/\(^{-}\) mice. We propose increased PPAR\(\gamma\) expression as the mechanism for the enhanced steatosis observed in Chop\(^{-}\)/\(^{-}\) mice as recently shown in adipose tissue\(^{31}\). CHOP binds to C/EBP\(\beta\) and prevents its DNA binding, thus functioning as an inhibitory regulator\(^{12}\). In the absence of CHOP, the transcriptional activity of C/EBP\(\beta\) is increased. C/EBP\(\beta\) upregulates PPAR\(\gamma\), and is the likely mechanism for the increased PPAR\(\gamma\) expression observed in our experiments\(^{32}\).

Hepatomegaly in Chop\(^{-}\)/\(^{-}\) mice was accompanied by an increase in liver injury and inflammation, consistent with the development of steatohepatitis. Liver injury assessed by serum ALT, a marker of hepatocellular damage, was significantly elevated in the HFD-fed Chop\(^{-}\)/\(^{-}\) mice. Histologically, this group of mice had features of lobular inflammation and pericellular fibrosis, both characteristic features of NASH. In addition, inflammatory and fibrogenic gene expression were significantly increased in the HFD-fed Chop\(^{-}\)/\(^{-}\) mice. These unique findings in the liver were independent of insulin resistance, serum lipids and circulating FFA, which were identical among the HFD-fed Chop\(^{-}\)/\(^{-}\) and Chop\(^{+/+}\) mice. The sum of these findings suggests that CHOP is a negative regulator of steatosis and inflammation in the liver.

Previous studies have demonstrated a disease-promoting role for CHOP in mouse models of type II diabetes mellitus\(^{14,33}\) and in isolated hepatocytes have shown a role for CHOP in PA-induced expression of the proapoptotic protein PUMA, and delaying PA-induced cell death; however cell death was not studied \textit{in vivo} in the liver\(^{15,16}\). In contrast to our expectations, we found no decrease in hepatocyte apoptosis \textit{in vivo} in HFD-fed Chop\(^{-}\)/\(^{-}\) mice. This occurred in spite of increased lipid storage in hepatocytes and adipose tissue, both of which may protect from lipotoxicity due to effective storage of excess fat. This also suggests that the UPR in steatotic hepatocytes is not of a magnitude sufficient to activate apoptosis. As under such conditions, a decrease in apoptosis would be expected in the HFD-fed Chop\(^{-}\)/\(^{-}\) mice.

Inflammation is a key feature of NASH. In fact, progressive fibrosis correlates with the degree of inflammation in patients with NASH, and those without inflammation do not develop progressive disease\(^{5}\). Macrophages mediate liver injury. In models designed to dissect the contribution of each of these populations to injury-associated macrophages, evidence supports substantial recruitment of circulating BM-derived monocytes in liver injury states\(^{30,34,35}\). Activated macrophages, manifest both, as clusters of cells and increased numbers of macrophages are seen in livers from NASH patients and in HFD-fed rats\(^{29}\). In experimental steatohepatitis with the MCD diet, depletion of the macrophage population ameliorates liver injury and inflammation\(^{36}\). In our study macrophages were significantly increased in the HFD-fed and the MCD-fed Chop\(^{-}\)/\(^{-}\) mice. This suggests a role for CHOP in the recruitment, activation, or persistence of activated macrophages in liver disease. It is possible that other liver and immune cell types contribute to injury and inflammation in these models, though our data support a role for macrophages.

Previous studies in atherosclerosis disease models have demonstrated that CHOP deletion decreases macrophage apoptosis both in cultured macrophages and \textit{in vivo} in atherosclerotic plaques\(^{37,38}\). Furthermore, in RAW 264.7 macrophages nitric oxide (NO)-induced apoptosis is CHOP-dependent\(^{39}\). In our experiments PA treatment activated RAW 264.7 macrophages and induced the UPR. We utilized PA as it is physiologically abundant, and present in highest concentrations compared with other fatty acids in HFD-fed livers in this experiment. In addition to RAW 264.7 macrophages, the UPR was induced in BM-derived macrophages upon treatment with PA, and they were activated as well. Chop\(^{-}\)/\(^{-}\) and Chop\(^{+/+}\) BM-derived macrophages were equally activated.
upon PA treatment. This activation was not due to lipopolysaccharide contamination of bovine serum albumin (BSA), as control cells were treated with BSA. Treatments with cholesterol and nitric oxide activate the UPR in macrophages, similar to our results with PA. Additionally, the deletion of CHOP protected macrophages from PA-induced apoptosis, suggesting that activation-induced macrophage cell death appears to be due to a sustained ER stress, and that activated macrophages likely persist in Chop–/– livers, thus amplifying inflammation. Macrophage apoptosis was rarely detected, in vivo, yet greatest in HFD-fed Chop+/+ livers and undetected in HFD-fed Chop–/– livers, consistent with CHOP-dependent macrophage cell death.

To further dissect the dependence of liver injury on obesity and steatosis, and confirm our findings in a second model of liver injury, we utilized the MCD diet. This diet induces steatohepatitis without other features of NASH including insulin resistance. Consistent with our HFD data, MCD diet-fed Chop–/– mice developed significantly greater steatohepatitis, both histologically and by ALT elevation, than wildtype controls. This is consistent with a published study in which Chop–/– mice developed greater liver injury, inflammation and steatosis upon MCD diet challenge (40). Thus, the sensitizing effects of CHOP to steatohepatitis were independent of obesity, being present both in the HFD-fed mice and the MCD diet-fed mice. Furthermore, the reduction in liver injury and inflammation in MCD diet-fed Chop–/– mice transplanted with Chop+/+ bone marrow, supports a role for CHOP-induced macrophage apoptosis in vivo. Unexpectedly, liver injury and inflammation were also mitigated in reciprocally transplanted mice. This may be due to technical limitations of using bone marrow transplantation, such that chimeric mice (Chop+/+ mice reconstituted with Chop–/– bone marrow) have sufficient residual Chop+/+ function in resident macrophages. We utilized a knockout strain; therefore our transplants were performed with syngeneic donors. Furthermore, we used same sex donors, to mitigate gender-based differences in inflammation, therefore we are unable to assess the relative proportions of donor-derived and recipient derived macrophages in Chop+/+ mice reconstituted with Chop–/– bone marrow. We hope to address this with future studies with tissue-specific deleted mice.

To summarize, in this study we have demonstrated a dual role for CHOP in preventing steatohepatitis. In our model, CHOP protects hepatocytes from excess storage of ectopic TG. In the absence of CHOP, hepatocytes develop exuberant steatosis likely driven by the uninhibited lipogenic action of PPARγ. In addition, in liver macrophages, CHOP mediates ER stress-induced cell death (Fig. 12). Macrophages deficient in CHOP are protected from cell death. We propose CHOP as a disease susceptibility gene in the pathogenesis of NASH, especially relevant as it has pervasive in vivo effects. Our future studies are focused on creating tissue specific deletion mutants to delineate the relative contribution of each of these novel roles of CHOP in preventing the onset and progression of nonalcoholic steatohepatitis. Since CHOP deletion has been shown to protect different cell types from ER stress-induced cell death (14,33,38,41), there is interest in testing the potential for CHOP antagonists to ameliorate human disease. In this context, there should be caution associated with the damaging effects of CHOP deletion in the liver.
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FOOTNOTES

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The abbreviations used are: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ER, endoplasmic reticulum; UPR, unfolded protein response; IRE1\textsubscript{\alpha}, inositol requiring enzyme 1 alpha; PERK, PKR-like ER kinase; ATF6\textsubscript{\alpha}, activating transcription factor alpha; CHOP, C/EBP homologous protein; bZIP, basic leucine zipper domain; GADD153, growth arrest and DNA damage 153; HFD, high fat diet; CD, control diet; MCD, methionine and choline deficient diet; MCS, methionine and choline sufficient diet; TG, triglyceride; PPAR\textsubscript{\gamma}, peroxisome proliferator-activated receptor gamma; C/EBP\textsubscript{\alpha}, ccaat-enhancer-binding protein alpha; C/EBP\textsubscript{\beta}, ccaat-enhancer-binding protein beta; ALT, alanine transaminase; DR-5, death receptor 5; qPCR, quantitative real-time PCR; TGF-\beta1, transforming growth factor \beta1, \alpha2-SMA, alpha-2 smooth muscle actin; Col-1A1, collagen 1a1; TIMP1, and tissue inhibitor of metalloproteinases; IL-1\beta, interleukin 1 beta; MIP-1\alpha, macrophage inflammatory protein-1 alpha; MCP-1 macrophage chemoattractant protein-1; TNF-\alpha, tumor necrosis factor-alpha; BiP, binding immunoglobulin protein; sXBP-1, spliced X-box binding protein-1; eIF2\alpha, eukaryotic translation initiation factor 2 alpha.

FIGURE LEGENDS

**Figure 1.** Metabolic characteristics of high fat diet (HFD) fed and CD-fed littermate CHOP wildtype (Chop\textsuperscript{+/+}) and knockout (Chop\textsuperscript{-/-}) mice (n=5-6 per group). (A) Percent increase in body mass is shown for all four groups. Chop\textsuperscript{-/-} HFD-fed mice demonstrated the greatest increase in body mass, this was significant at week 4, and remained significant for the duration of the study (*p<0.05). (B) Fasting blood glucose was measured at weeks 11 and 16. HFD-fed mice developed progressive hyperglycemia (p<0.05, compared with CD-fed mice). No differences were observed between Chop\textsuperscript{+/+} and Chop\textsuperscript{-/-} mice (p=ns). (C) Glucose disposal curves following i.p. injection of glucose at week 14. HFD-fed mice developed progressive glucose intolerance. No differences were seen between Chop\textsuperscript{+/+} and Chop\textsuperscript{-/-} mice. (D) Area under the glucose tolerance test curve done at weeks 5, 10 and 14 demonstrated progressive glucose intolerance in the HFD-fed mice (p<0.05, compared with CD-fed mice). No differences were observed between Chop\textsuperscript{+/+} and Chop\textsuperscript{-/-} mice (p=ns). (E) Pyruvate tolerance testing done at week 15 showed enhanced hepatic glucose production among HFD-fed Chop\textsuperscript{+/+} and Chop\textsuperscript{-/-} mice (p<0.05, compared with CD-fed mice), without a genotypic difference between the two groups (p=ns). (F) Intraperitoneal insulin tolerance test at week 16 demonstrated progressive insulin resistance in the HFD-fed mice (p<0.05, compared with CD-fed mice), without genotypic differences between Chop\textsuperscript{+/+} and Chop\textsuperscript{-/-} mice (p=ns).

**Figure 2.** Liver injury develops in high fat diet (HFD) fed CHOP knockout (Chop\textsuperscript{-/-}) mice (n=5-6 per group). (A) Relative liver weight of all four experimental groups is shown. Chop\textsuperscript{-/-} mice developed significant hepatomegaly upon HFD-feeding for 16 weeks (*p<0.05). (B) Histologic steatosis score for all four groups is shown. HFD-fed Chop\textsuperscript{-/-} mice had greatest hepatic steatosis, significantly greater than the Chop\textsuperscript{+/+} group (*p<0.001). (C) Alanine transaminase levels from all four groups are shown. HFD-fed
Chop\textsuperscript{−/−} mice had greatest elevations in serum alanine transaminase (\(*p<0.05\), compared to HFD-fed Chop\textsuperscript{+/+}). (D) Histologic inflammation score for the four groups is shown. Significantly greater inflammation was observed in the HFD-fed Chop\textsuperscript{−/−} mice (\(p<0.05\), compared to HFD-fed Chop\textsuperscript{+/+}). (E) Representative H&E stained liver sections from all four groups are shown. The bottom right panel from HFD-fed Chop\textsuperscript{−/−} mice demonstrates characteristic features of steatohepatitis. The arrows point to inflammatory foci surrounding steatotic hepatocytes.

**Figure 3.** PPAR\textsubscript{γ} gene expression in HFD-fed mouse livers. (A) Liver lipid content in CD-fed Chop\textsuperscript{+/+}, Chop\textsuperscript{−/−}, and high fat diet (HFD) Chop\textsuperscript{+/+} and Chop\textsuperscript{−/−} is shown (B) Western blot analysis for PPAR\textsubscript{γ} in CD-fed Chop\textsuperscript{+/+} (lanes 1-3), Chop\textsuperscript{−/−} (lanes 4-6), and HFD-fed Chop\textsuperscript{+/+} (lanes 7-10), and Chop\textsuperscript{−/−} (lanes 11-15) is shown. Lane 16 is epididymal fat included as control. Alpha-tubulin is the loading control. (C) Relative mRNA expression for PPAR\textsubscript{α}, PPAR\textsubscript{δ}, and PPAR\textsubscript{γ} from all four experimental groups is shown. PPAR\textsubscript{γ} expression was significantly increased in HFD-fed Chop\textsuperscript{−/−} mice compared with HFD-fed Chop\textsuperscript{+/+} mice (\(*p<0.05\)). (D) Relative mRNA expression for C/EBP\textalpha, C/EBP\beta, and C/EBP\gamma from all four groups shows that levels were comparable. (E) Western blot analysis for C/EBP\alpha and C/EBP\beta in CD-fed Chop\textsuperscript{+/+} (lanes 1-3), Chop\textsuperscript{−/−} (lanes 4-6), and HFD-fed Chop\textsuperscript{+/+} (lanes 8-10), and Chop\textsuperscript{−/−} (lanes 11-15) is shown. Lane 16 is tunicamycin injected Chop\textsuperscript{+/+} liver. Lane 7 is unloaded by error. Alpha-tubulin is the loading control. Non-specific bands were seen for both C/EBP\alpha and C/EBP\beta, therefore the arrows point to the correct bands based on predicted molecular weight. C/EBP\beta isoforms are indicated, the full length liver-enriched transcriptional activator protein (LAP) protein designated LAP\*, and LAP (21 amino acids are truncated at the N-terminus), and liver-enriched transcriptional inhibitory protein (LIP).

**Figure 4.** Hepatocyte apoptosis and fibrosis occur in mice with steatohepatitis (n=5-6 per group). (A) Representative photomicrographs of liver sections stained with Apoptag to detect apoptotic nuclei are shown from all four groups. The arrows point to apoptotic hepatocyte nuclei. The far right panel is from Chop\textsuperscript{−/−} mice. (B) Apoptag stained liver sections were quantified for each of the CD-fed and high fat diet (HFD)-fed Chop\textsuperscript{+/+} and Chop\textsuperscript{−/−} livers. Increased hepatocyte apoptosis was seen in the high fat diet (HFD)-fed Chop\textsuperscript{−/−} mice, when compared with the HFD-fed Chop\textsuperscript{+/+} mice (\(***p<0.05\)). Increased apoptosis was also seen in HFD-fed Chop\textsuperscript{+/+} mice compared to CD-fed Chop\textsuperscript{+/+} mice (\(*p<0.05\)). (C) Death receptor expression by quantitative PCR in all four groups is shown. HFD-fed Chop\textsuperscript{−/−} mice had significant increase in mRNA expression of Fas and Death Receptor 5 (DR-5) (\(*p<0.05\)). (D) Whole liver Fas expression by western blotting is shown. Lane numbers, dietary groups and genotypes are indicated. The arrow points to the band specific for Fas based on predicted molecular weight. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (E) Western blot for Fas in isolated hepatocytes (left panel) from Chop\textsuperscript{+/+} (WT) and Chop\textsuperscript{−/−} is shown. The arrow points to the predicted Fas band. GAPDH was used as loading control. (F) Western blot for Fas in isolated bone marrow derived macrophages (right panel) from Chop\textsuperscript{+/+} (WT) and Chop\textsuperscript{−/−} is shown. The arrow points to the predicted Fas band. Actin was used as loading control. (F) Relative mRNA expression for DR5 and Fas in isolated primary hepatocytes and bone marrow derived macrophages treated with 800\mu M PA for 8 hours or vehicle (VC) is shown. Both Fas and DR5 were induced in primary wildtype hepatocytes (\(*p<0.05\), compared to WT-VC) upon PA treatment. In macrophages, mRNA for DR5 and Fas were induced in wildtype (\(p<0.05\), compared to WT-VC), and induced in Chop\textsuperscript{−/−} macrophages (\(**p<0.05\), compared to Chop\textsuperscript{-/-}-VC). (G) Expression of genes that regulate hepatic fibrosis by qPCR in all four groups is shown. HFD-fed Chop\textsuperscript{−/−} mice had significant increase in mRNA expression of transforming growth factor-B1 (TGF-B1), alpha2 smooth muscle actin (\(\alpha2\-SMA\)), collagen 1-alpha 1(Col1-A1), and tissue inhibitor of metalloproteinases 1(TIMP1) (\(*p<0.05\)). (E) Representative photomicrographs of Sirius red stained liver sections from all four groups are shown. The arrow points to characteristic pericellular fibrosis seen in the HFD-fed Chop\textsuperscript{−/−} mice.
Figure 5. Inflammation and Kupffer cell activation in HFD-fed CHOP knockout (Chop<sup>−/−</sup>) mice. (A) Relative gene expression of several inflammatory markers from livers of CD and high fat diet (HFD) fed mice is shown. A significant increase in expression of interleukin 1β (IL-1β), MIP-1α, MCP-1, and TNF-α was seen (*p<0.05, compared to HFD-fed Chop<sup>+</sup>+/+). (B) Representative photomicrographs of F4/80 stained liver sections from CD and HFD-fed Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> mice are shown. The arrow in the bottom right picture points to a cluster of F4/80 positive cells in HFD-fed Chop<sup>−/−</sup> liver. (C) Quantitative morphometry for F4/80 positive area expressed as a percent of total area of each liver section is shown. In HFD-fed Chop<sup>−/−</sup> there was a significant increase in F4/80 positive surface area (p<0.05 compared to HFD-fed Chop<sup>+</sup>+/+).

Figure 6. Serum and liver fatty acid composition. (A) Serum FFA content from CD-fed and HFD-fed wildtype (Chop<sup>+</sup>+/+) and Chop<sup>−/−</sup> mice is shown. (B) Liver total lipid fatty acid composition from CD-fed and HFD-fed wildtype (Chop<sup>+</sup>+/+) and CHOP knockout (Chop<sup>−/−</sup>) mice is shown.

Figure 7. Macrophage activation and UPR induction by PA. (A) Relative mRNA expression of inflammatory markers from RAW 264.7 macrophages treated with PA is shown. A significant increase in expression of TNF-α, IL-6, MIP-1α and MCP-1 was seen (*p<0.05). (B) Relative mRNA expression of UPR markers from RAW 264.7 macrophages treated with PA is shown. A significant increase in expression of BiP, sXBP1, and CHOP was seen (*p<0.05). Erp72, was also induced, though this did not achieve statistical significance. (C) Representative Western blot analysis of PA-treated RAW 264.7 macrophages showing phosphorylation of elf2α, induction of ATF4 and CHOP protein is shown (left panel). Alpha-tubulin is the loading control. Densitometric quantification of bands relative to vehicle treated conditions is shown under each western blot image. The panel on the right shows quantification of these bands from 4 experiments (*p<0.05, compared to vehicle). (D) Relative CHOP mRNA expression of PA-treated wildtype primary bone marrow-derived macrophages (BMDM) shows a concentration- and time-dependent induction of CHOP mRNA. CHOP mRNA was not detected in BMDM from Chop<sup>−/−</sup> mice. (E) Relative gene expression of several UPR markers from PA-treated BMDM is shown. A significant increase in expression of sXBP1, WFS-1, PDI, ATF4, Gadd34, Erp57 and Erp72 was seen (*p<0.05) in BMDM from Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> mice. (F) Relative gene expression of several inflammatory markers in PA treated BMDM from Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> mice showing a significant increase in expression of IL-6, IL-1β, TNF-α, MIP-1α and MCP-1 (*p<0.05).

Figure 8. Cell death in Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> macrophages. (A) Bone marrow-derived macrophages (BMDM) from Chop<sup>+</sup>+/+ (WT) and Chop<sup>−/−</sup> (KO) were treated with PA. Apoptosis in both was concentration- and time-dependent, though significantly abrogated in BMDM from Chop<sup>−/−</sup> mice (*p<0.05). (B) Representative fluorescent photomicrographs of BMDM nuclei stained with DAPI from PA-treated Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> are shown. The white arrows point to apoptotic nuclei. Cells were treated with 400µM palmitic acid for the indicated times. (C) Immunofluorescence for F4/80 is shown in red and TUNEL assay in green for CD-fed and high fat diet-fed Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> mice. The arrow points to an apoptotic F4/80 positive macrophage in HFD-fed Chop<sup>+</sup>+/+ liver.

Figure 9. Liver steatosis and hepatocyte apoptosis in MCD diet-fed CHOP knockout (Chop<sup>−/−</sup>) mice. (A) Liver TG content of MCS and MCD-fed mouse livers is shown. MCD-fed Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> livers showed a significant increase in liver TG compared with MCS-fed mice of both genotypes. Both MCD-fed Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> mice had comparable TG content. (B) Percent change in body weight of MCS and MCD-fed Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> mice. MCD-fed mice lost body weight, there were no genotypic differences between the Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> groups. (C) Relative liver weight of MCS and MCD-fed Chop<sup>+</sup>+/+ and Chop<sup>−/−</sup> mice. Relative liver weights were comparable across all four groups. (D) Apoptag stained liver sections were quantified for the MCS and MCD diet-fed groups. Hepatocyte apoptosis was significantly
increased in the MCD diet-fed Chop−/− livers (p<0.05). (E) Apoptag stained liver sections from MCS and MCD fed Chop+/+ and Chop−/− mice are shown. The arrows point to apoptotic hepatocytes.

Figure 10. Macrophages mediate liver injury and inflammation in CHOP knockout (Chop−/) mice. Mice were fed the methionine-choline deficient (MCD) diet and its CD, methionine and choline sufficient (MCS) diet. Data from whole body Chop+/+ and Chop−/− mice and from chimeric mice generated by bone marrow transplantation of Chop−/− mice with Chop+/+ bone marrow and reciprocally transplanted Chop+/+ mice with Chop−/− bone marrow are shown. (A) Representative H&E stained liver sections from MCD-fed and MCS-fed Chop+/+ and Chop−/− mice are shown. The arrow points to an inflammatory focus. (B) Histologic scores for inflammation are shown from MCD-fed Chop+/+ and Chop−/− mice (whole body genotype) and chimeric mice from both groups (chimeric genotype) are shown. Inflammatory foci were significantly increased in Chop−/− mice (*p<0.05, compared to MCD-fed Chop+/+). Chimeric mice showed a significant attenuation in inflammation compared to MCD-fed Chop−/− mice (**p<0.05). MCS-fed mice had no liver inflammation, hence are not shown. (C) MCD-fed Chop−/− mice had significantly greater elevation of serum ALT than Chop+/+ mice (*p<0.05); this was attenuated in chimeric mice (**p<0.05). MCS fed Chop+/+, Chop−/− and chimeric mice had normal ALT’s and are not shown. (D) Representative H&E stained liver sections from chimeric mice fed the MCS and MCD diets are shown. (E) Quantitative morphometry for F4/80 positive area expressed as a percent of total area and normalized to MCS fed controls is shown. There was a significant increase in F4/80 positive surface area in MCD-fed Chop−/− (p<0.05 compared to MCD-fed Chop+/+). The increase in F4/80 was significantly reduced in chimeric mice (p<0.05 compared to MCD-fed Chop−/−).

Figure 11. Inflammation and macrophage accumulation in bone marrow chimeric mice. (A). Bone marrow reconstitution was assessed in chimeric mice by qPCR for Chop expression. Chop mRNA was detected in Chop+/+ mice and not detected in Chop−/− mice. mRNA expression was normalized to Chop−/− mouse transplanted with Chop+/+ bone marrow. CHOP was detected in liver samples of chimeric Chop−/− mice transplanted with Chop+/+ bone marrow 9 weeks after transplantation indicating successful bone marrow reconstitution. (B) Relative gene expression of inflammatory markers from livers of MCD diet fed Chop+/+, Chop−/− (whole body genotypes) and reciprocally transplanted chimeric mice is shown. A significant increase in expression of MIP-1α and MCP-1 was seen in MCD-fed Chop−/− mice (*p<0.05, compared to MCD fed-Chop+/+), and this was attenuated in MCD-fed chimeric mice (***p<0.05, compared with MCD fed-Chop−/−). Data are normalized to MCS diet fed control animals. (C) Representative photomicrographs of F4/80 liver sections from MCS and MCD diet-fed Chop+/+ and Chop−/− mice (top row) and reciprocally transplanted chimeric mice (bottom row) are shown.

Figure 12. C/EBP homologous protein prevents steatohepatitis. Our model proposes that in the pathogenesis of nonalcoholic steatohepatitis nutrient stress, manifest as elevated FFA, activates macrophages in the liver. Macrophage activation results in the UPR and endoplasmic reticulum (ER) stress, presumably due to the increased secretory load of activated macrophages. CHOP is activated downstream of the UPR. Activated macrophages undergo CHOP-dependent apoptosis with the biologic consequence of dampened inflammation. However, in the absence of CHOP, activated macrophages die less, and thus persist in the liver with the consequence of enhanced inflammation.
Table 1: Sequences for quantitative PCR primers.

| Gene    | 5’ to 3’ Sequence                  |
|---------|------------------------------------|
| 18S     | F CGCTTCCTTACCTGGTGTGAT            |
|         | R GAGCGACCAAGAGGACCATA             |
| Actin   | F GATCTGGCACCACACCTTCT             |
|         | R GGGGTGTGAAGGCTCCTAA             |
| BiP     | F GTGTGAGCAGGACATCAAGTT            |
|         | R CCCACCTCACAATATCAACTTGA         |
| PDI     | F CAAGATCAAGCCCCACCTGAT            |
|         | R AGTTCGCCCAACCCAGTGAT           |
| Chop    | F CTGCCTTTACCTTGGAGAC             |
|         | R CGTTTCCTGGGGATGAGATA           |
| ATF-4   | F ATGCGCCTATGGATGTGAT             |
|         | R CGAAGTCACACTTCTTCCAGATCC        |
| Gadd34  | F CCCGAGATTCCTCTCTAAAAGC          |
|         | R CCAAGCACAGGAAATGG               |
| sXBP1   | F GAGTCGCCAGCAGGTTG               |
|         | R GTGTCAGAGTCATGGGA              |
| WFS-1   | F GTAGCAAGTGCGGCGGTTTC            |
|         | R TGGATATGGAGGCACTTGGT            |
| Erp72   | F TCCCCATGGCTTAGCAGAAAT            |
|         | R GGGGTAGCCTACATCAAAAT            |
| Erp57   | F CGCCTCCATGTTGTGGA              |
|         | R CAGTCATCCACCTTTCTTA             |
| α2-SMA  | F GTCCCAGACATCAGGAGTAA            |
|         | R TCGGATATCCAGGTCAGGA            |
| Col-1A1 | F GCTCCTCTTAGGGGCCCACC            |
|         | R CCAAGTGCTAGCAATTGGGG           |
| TGF-β1  | F CTCCCCTGGCTTCTATGTC             |
|         | R GCCCTAGTTGGACAGGATCTG          |
| TIMP1   | F AGTGAGTCTCGTTGTATTCT            |
|         | R GTAAGGCTGTAGCTGTGCC           |
| FAS     | F TATCAAGGAGGCCCATTGTTGC         |
|         | R TGGTCCTTTACATGCTGCC          |
| FAS-Ligand | TCCGTGAGTTCACCAACAAA               |
|         | R GGGGTTCCTGGTAAATGG            |
| TRAIL   | F ATGGTGATTTGCCATAGTGATCC        |
|         | R GCAAGCAGGCTCTGTTCAAGA            |
| DR-5    | F CGGCGGATACTACACC                |
|         | R TGTTACTGGAACAAAGACAGGC          |
| Il-6    | F TAGTCCTCTCATCCAATTTCC           |
|         | R TGGTCCTTTAGCCACTTCTTC         |
| Il-1β   | F GCAACTGTTCTGAACACTCAACT      |

C/EBP Homologous Protein Protects Mice from Steatohepatitis
|    | R             | F             | R             |
|----|---------------|---------------|---------------|
| CRP| ATCTTTTGGGTCCGTC\(\text{CAACT}\) | ATGGAGAAGCTACTCTGGGTGC | ACACACAGTAAGGTTTCAGTG |
| MIP-1\(\alpha\) | TTCTCTGTACCATGACACTCTTG\(\text{CGAG}\) | GTGGGAGGTTTCGGGACTTGA | ACCGGG\(\text{TTTCCGGGACTTGA}\) |
| MCP-1 | TTAAAAACCTGGATCGGAACCAA | GGAGGAGACCTGCAACAGCAG | CACGGAGACCTGCAACAGCAG |
| TNF-R1 | CCGGAGAAGAGGGATAGCTT | GCATTAGCTTTCAGATTACGGGT | TCGGACAGTCAACTCAACAAAGT |
| TNF-\(\alpha\) | CCCTACA\(\text{CTCATCATCTTC}\) | GCTACGACGTC\(\text{GGGCTACAG}\) | AGAGCCCC\(\text{CATCTGTCTCTC}\) |
| PPAR\(\alpha\) | AGAGCCC\(\text{CATCTGTCTCTC}\) | ACTG\(\text{GTAGCTCTGCAAAAACCA}\) | TCCATCGTCAACAAAGACGGG |
| PPAR\(\delta\) | TCCATCGTCAACAAAGACGGG | ACTTG\(\text{GGCTCAATGATGTCAC}\) | GCTACGACGTC\(\text{GGGCTACAG}\) |
| PPAR\(\gamma\) | TCGG\(\text{AGATGCAGATGCTCTGATT}\) | GAGAG\(\text{GTCCACAGGCTCAAGCAGACGAG}\) | TGGACAAGAAGACAGCAACAGAG |
| C/EBP\(\alpha\) | TGGACAAGAAGACAGCAACAGAG | TC\(\text{ACTGGCTCAACTCCAGCAC}\) | ACCGG\(\text{TTTCCGGGACTTGA}\) |
| C/EBP\(\beta\) | ACCGG\(\text{TTTCCGGGACTTGA}\) | G\(\text{TTCGCTGATGTCAGCTGATGCTC}\) | G\(\text{TTCGCTGATGTCAGCTGATGCTC}\) |
| C/EBP\(\gamma\) | ACGTG\(\text{CCCAATTGAGCAAGCAG}\) | C\(\text{GTGACCACTCCAGCAC}\) | CTGAG\(\text{GAACCTGCTGTAAGC}\) |
Table 2: NEFA at weeks 4, 8, 16 and serum lipids at week 16 in high fat fed mice.

|                      | CD Wildtype | CD Chop<sup>−/−</sup> | HFD Wildtype | HFD Chop<sup>−/−</sup> |
|----------------------|-------------|-----------------------|--------------|------------------------|
| NEFA* (mEq/L)        | 1.172±0.065 | 1.137±0.133           | 1.222±0.125  | 1.134±0.103            |
| NEFA** (mEq/L)       | 0.848±0.087 | 0.688±0.121           | 0.800±0.044  | 0.630±0.020            |
| Triglyceride*** (mg/dL) | 75±7        | 94±8                  | 91±4         | 70±5                   |
| Cholesterol*** (mg/dL) | 101.7±8.7  | 114.8±9.8             | 163.7±9.5    | 188.3±19.2             |
| NEFA*** (mEq/L)      | 0.941±0.139 | 1.193±0.153           | 0.865±0.048  | 1.017±0.118            |

NEFA: nonesterified fatty acid; * Week 4 (fasted), **Week 8 (fasted), ***Week 16 (fed)
Figure 5

A

Relative mRNA Expression

IL-1β  CRP  MIP-1α  MCP-1  TNF-R1  TNF-α

B

Wild Type Control Diet  CHOP −/− Control Diet

Wild Type High Fat Diet  CHOP −/− High Fat Diet

C

F4/80 Positive Area (% Total)

WT  CHOP −/−  WT  CHOP −/−

Control Diet  High Fat Diet
Figure 7

A. Bar graph showing relative mRNA expression of TNFα, IL-6, MIP-1α, and MCP-1.

B. Bar graph showing relative mRNA expression of Bip, sXBP1, Erp72, and CHOP.

C. Western blot analysis showing levels of p-eIF2α, eIF2α, ATF4, and CHOP, with molecular weight (kDa) and time (hrs) indicated.

D. Bar graph showing relative CHOP mRNA expression.

E. Bar graph showing relative mRNA expression of sXBP1, WFS-1, PDI, ATF4, Gadd34, Erp57, and Erp72.

F. Bar graph showing relative mRNA expression of IL-6, IL-1β, TNF-α, MIP-1α, and MCP-1.
Figure 10

A

Wild Type MCS  CHOP -/- MCS  Wild Type MCD  CHOP -/- MCD

B

| Whole Body Genotype | Chimeric Genotype |
|---------------------|-------------------|
| Histologic Inflammation Score |
| WT               | CHOP -/-          |
| CHOP -/- MΦ       | WT MΦ             |
|                   | **                |
| WT               | CHOP -/-          |
| CHOP -/- MΦ       | WT MΦ             |
|                   | **                |

C

| Whole Body Genotype | Chimeric Genotype |
|---------------------|-------------------|
| Alamine Transamnese (units/L) |
| WT               | CHOP -/-          |
| CHOP -/- MΦ       | WT MΦ             |
|                   | **                |
| WT               | CHOP -/-          |
| CHOP -/- MΦ       | WT MΦ             |
|                   | **                |

D

| Whole Body Genotype | Chimeric Genotype |
|---------------------|-------------------|
| Histologic Score |
| WT with CHOP -/- MΦ | CHOP -/- with WT MΦ |
|                     |                    |

E

| Whole Body Genotype | Chimeric Genotype |
|---------------------|-------------------|
| Percent F480 Area (relative to controls) |
| WT               | CHOP -/-          |
| CHOP -/- MΦ       | WT MΦ             |
|                   | **                |
| WT               | CHOP -/-          |
| CHOP -/- MΦ       | WT MΦ             |
|                   | **                |
Figure 11

A

![Graph showing relative CHOP mRNA expression.](image)

B

![Graph showing relative mRNA expression for MCP-1 and MIP-1alpha.](image)

C

![Images showing cellular morphology for different conditions.](image)
Figure 12

Nutrient Stress
Free Fatty Acids

Macrophage

ER stress
CHOP
Apoptosis

Macrophage Cell Death
Dampened Inflammation

Persistence of activated macrophages
Enhanced Inflammation
C/EBP homologous protein-induced macrophage apoptosis protects mice from steatohepatitis
Harmeet Malhi, Erin M. Kropp, Vinna F. Clavo, Christina R. Kobrossi, JaeSeok Han, Amy S. Mauer, Jing Yong and Randal J. Kaufman

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