

FXR controls CHOP expression in steatohepatitis

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The farnesoid X receptor (FXR) and C/EBP homologous protein (CHOP) have critical functions in hepatic lipid metabolism. Here, we aimed to explore a potential relationship between FXR and CHOP. We fed wild-type (WT) and FXR KO mice a MCD diet (model of steatohepatitis) and found that Chop mRNA expression is upregulated in WT but not FXR KO mice. The absence of FXR aggravates hepatic inflammation after MCD feeding. In HepG2 cells, we found that Chop expression is regulated in a FXR/Retinoid X receptor (RXR)-dependent manner. We identified a FXR/RXR-binding site in the human CHOP promoter, demonstrating a highly conserved regulatory pathway. Our study shows that FXR/RXR regulates Chop expression in a mouse model of steatohepatitis, providing novel insights into pathogenesis of this disorder.

Keywords: inflammation; nonalcoholic fatty liver disease (NAFLD); nonalcoholic steatohepatitis (NASH); nuclear receptor

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and is the most common liver disease in the Western countries. More than 40% of the general population and 75% of obese individuals suffer from NAFLD [1,2]. Adipocytes have the capacity to store excess free fatty acid (FA) as triglycerides (TG) [3], while nonadipose tissue cells such as hepatocytes have very limited capacity to store excess lipids. If the cellular FA buffering capacity is reached (or exceeded), the increased amount of free FAs (FFA) can become cytotoxic in a series of events called lipotoxicity [4,5]. Therefore, the balance between FA synthesis, TG formation, and secretion is the pivotal key for proper lipid homeostasis. The nuclear bile acid (BA) receptor farnesoid X receptor (FXR) has a central role in the

control of hepatic BA, lipid and glucose metabolism [6] as well as inflammation [7,8]. As such, FXR negatively regulates *de novo* lipogenesis and subsequently TG formation by suppressing the lipogenic master regulator Srebp1c [9]. Interestingly, C/EBP homologous protein (CHOP) also represses (via the transcription factor C/ebp α) metabolic genes involved in hepatic lipid metabolism such as *de novo* lipogenesis and FAs oxidation [10]. Mice with targeted disruption of the *Chop* gene are more susceptible to (fatty) liver injury reflected by increased steatosis, inflammation and fibrosis development after high-fat diet feeding [11], suggesting a potential role of CHOP in NAFLD development and progression towards more severe stages of disease such as nonalcoholic steatohepatitis (NASH). However, whether there is a relationship between FXR

Abbreviations

ACOX, acyl-CoA oxidase; ACTD, actinomycin D; ALT, alaninaminotransferase; AP, alkaline phosphatase; AST, aspartataminotransferase; BA, bile acid; BAD, Bcl2-associated agonist of cell death; C/EBP, CCAAT/enhancer-binding protein alpha; CDCA, chenodeoxycholic acid; CHOP, C/EBP homologous protein; CHX, cycloheximide; cisRA, cis Retinoid acid; ERDJ4, endoplasmic reticulum DNA J domain-containing protein 4; FA, fatty acid; FASN, fatty acid synthase; FXR, farnesoid X receptor; GRP78, glucose-regulated protein, 78 kDa; MCD, methionine choline deficient; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SREBP1c, sterol regulatory element-binding protein 1c; TG, triglyceride; TNF, tumor necrosis factor alpha; WT, wild-type.

and CHOP in counteracting development from simple steatosis to steatohepatitis needs to be determined. Therefore, we aimed to explore a potential regulatory link between FXR and CHOP.

Material and methods

Animal experiments and diet

Age-matched C57BL/6 WT and FXR KO mice were kindly provided by Dr. FJ Gonzalez and housed with a 12 : 12 h light/dark cycle with water and a methionine choline-deficient (MCD) diet for 5 weeks. Experimental protocols were approved by the local Animal Care and Use Committee according to criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by US National Academy of Sciences (National Institutes of Health publication 86e23, revised 1985). Control and MCD diet was obtained from SAFE diets (Scientific Animal Food & Engineering, Strasbourg, France).

Cell culture experiments

HepG2 cells (LGC standard, Wesel, Germany) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 1% streptomycin/penicillin, sodium pyruvate, glutamine, and nonessential amino acids (PAA) at 37 °C in a humidified 5% CO₂ atmosphere. The medium was changed every 48 h. HepG2 cells were treated with low (5 mM) and high levels (25 mM) of glucose with or without chenodeoxycholic acid (CDCA) (Sigma-Aldrich, Vienna, Austria) as well as with or without 9-cis retinoic acid at the indicated concentrations for 12 h in the absence of serum.

Routine serum biochemistry

Blood was collected at harvesting and centrifuged for 20 min at 300 g. Serum was stored at -80 °C until analysis. Assays for alanine aminotransferase, alkaline phosphatase, cholesterol and triglycerides were routinely measured, whereas serum bile acid (BA) concentration was determined by using Bile Acid Kit (Ecoline S+ from DiaSys Diagnostic Systems GmbH, Holzheim, Germany) on a cobas analyser (Roche Diagnostics, Mannheim, Germany). Lipoprotein subfractions were determined by quantitative agarose gel electrophoresis (Helena Biosciences, Gateshead, UK). Glucose levels were assessed with an ACCU-Check Active analyzer (Roche, Mannheim Germany).

Hepatic triglyceride analysis

Total lipids were extracted from frozen liver tissue (100 mg) homogenates with chloroform/methanol/glacial acetic acid (66 : 33 : 1, v/v), and phase separation was

achieved by the addition of water. Dried lipids were redissolved in 1% (v/v) Triton X-100, and TG content was measured using the reagent for quantitative TG measurement (DiaSys, Holzheim, Germany).

Histology

The H&E staining as well as F4/80 immunohistochemistry (IHC) was assessed as described previously [12].

Messenger RNA analysis and Polymerase Chain Reaction (PCR)

RNA isolation, complementary DNA synthesis, and real-time PCR were performed as described previously [13]. All data were normalized to 36b4 or 18sRNA. Oligonucleotide sequences are available upon request.

Chromatin immunoprecipitation

Chromatin immunoprecipitation kit was achieved from Epigentek Group Inc. (D-69120 Heidelberg, Germany) and used according to the manufacturer's instructions.

For immunoprecipitation Antibodies for farnesoid X receptor (FXR) (sc-1204; Santa Cruz Biotechnology, INC.) and retinoic X receptor alpha (RXR α) (sc-553; Santa Cruz biotechnology; D-69115 Heidelberg, Germany) were used.

Statistical analysis

Animal studies were evaluated using SPSS V.18.0. Statistical analysis was performed using Kruskal-Wallis followed by Mann-Whitney test. Data are reported as means of four to six animals per group \pm SD. A *P* value \leq 0.05 was considered significant.

Results

MCD feeding induces steatosis independent of FXR

C57BL/6 WT and FXR KO mice were subjected to MCD feeding for 5 weeks as a model of steatohepatitis. A drop in body weight was seen in mice fed MCD diet, independent of the genotype (Fig. S1). Serum levels of liver transaminases (ALT and AST) as well as alkaline phosphatase (AP) and bile acids (BAs) were increased by MCD feeding, independent of the genotype (Fig. 1A). Total cholesterol as well as free fatty acid (FFA) levels were reduced by MCD diet with a more pronounced reduction in WT mice (Fig. 1A). Serum glucose levels were decreased to the same extent in both WT and FXR KO mice upon dietary challenge (Fig. 1A).

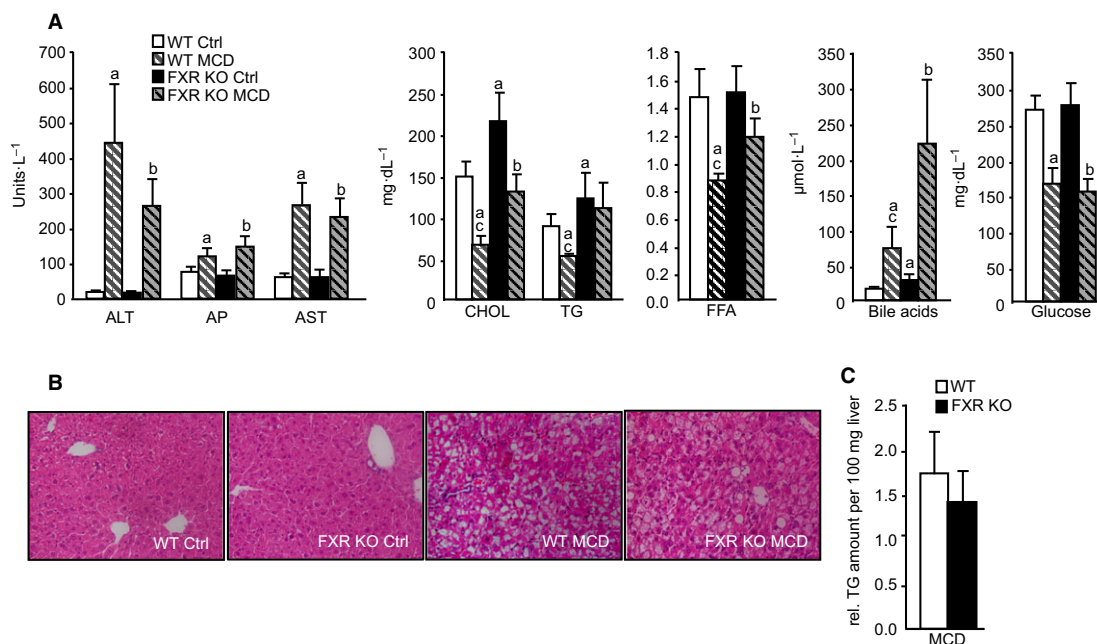


Fig. 1. Increased liver injury due to MCD feeding. (A) WT and FXR KO mice fed a MCD diet for 5 weeks showed increased serum levels of AST, AP, ALT, and bile acids. Total cholesterol and glucose levels were decreased in the presence of MCD independent of the genotype. Serum TG levels were decreased in MCD fed WT mice and not affected in challenged FXR KO mice compared with the corresponding control groups. a indicates a significant difference from untreated WT mice (Ctrl); b indicates a significant difference from untreated FXR KO mice; c indicates a significant difference from treated FXR KO mice; $P < 0.05$. (B) H&E stainings reflect increased steatosis after MCD feeding in both, WT and FXR KO mice. (C) Quantitation of hepatic TGs revealed no difference between MCD-fed WT and FXR KO mice. ($N = 4$ mice per group).

The H&E staining demonstrated MCD-induced steatosis reflected by increased amounts of hepatic lipid droplets in both WT as well as FXR KO mice following MCD feeding (Fig. 1B). In line, biochemical quantification of hepatic TGs revealed no significant differences between WT and FXR KO MCD-fed mice (Fig. 1C). This observation is underlined by similar reductions of peroxisome proliferator-activated protein alpha (Ppar α) and acyl CoA oxidase (Acox) mRNA expression as indicators of impaired β oxidation in WT and FXR KO MCD-fed mice (Fig. S2A). Similarly, *de novo* lipogenesis was repressed under MCD feeding to similar degrees in WT and FXR KO mice as demonstrated by reduced mRNA expression levels of sterol response element-binding protein 1 c (Srebp1c) and its downstream target fatty acid synthase (FasN) (Fig. S2B). Decreased *de novo* lipogenesis in the MCD-fed animals may also explain the relatively minor degree of evident steatosis development in these mice.

Absence of FXR aggravates inflammation due to MCD feeding

Since in addition to steatosis, inflammation is a key hallmark of steatohepatitis, we next investigated

inflammatory parameters such as tumor necrosis factor alpha (Tnf α), F4/80, monocyte chemoattractant protein 1 (Mcp1) and interleukin 1b (IL1b). Notably, mRNA expression levels of all markers were increased in FXR KO mice after MCD feeding to a higher degree than in WT mice. Mcp1 and IL1b levels were already significantly increased in WT mice under MCD feeding (Fig. 2A). In line, F4/80 IHC showed increased numbers of F4/80 positive cells in MCD fed WT and (to a higher extent in) FXR KO mice (Fig. 2B).

FXR is not involved in the development of ER stress and/or apoptosis under MCD feeding

Since inflammation is an important trigger for endoplasmic reticulum (ER) stress and/or apoptosis we analyzed mRNA expression of key markers of ER stress (glucose-regulated protein 78—Grp78, ER DNA J domain-containing protein 4—ErDj4, Chop) and apoptosis (Bcl2-associated agonist of cell death—Bad, Chop). Interestingly, expression levels of Grp78, ErDj4, and Bad did not differ between MCD fed animals and control mice, whereas Chop was higher expressed in WT MCD-fed mice in comparison to FXR KO MCD mice and WT Ctrl mice (Fig. 3).

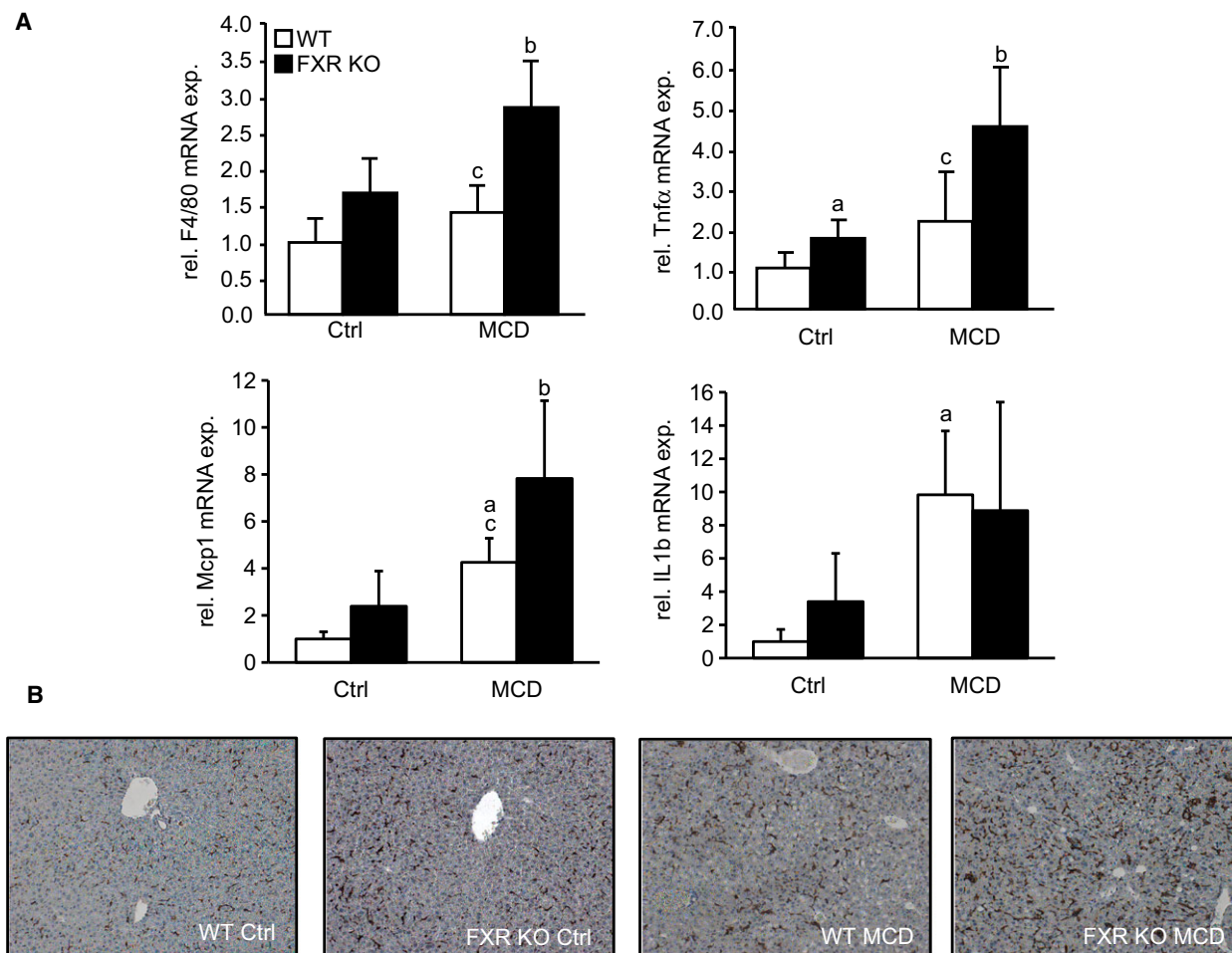


Fig. 2. Absence of FXR results in aggravation of hepatic inflammation. (A) Real-time PCR was used to assess mRNA levels of inflammatory markers F4/80, Tnf α , Mcp1, and IL1b. Expression levels of inflammatory markers were increased by MCD feeding in WT and FXR KO mice (but to a bigger extent in FXR KO mice). a indicates a significant difference from untreated WT mice (Ctrl); b indicates a significant difference from untreated FXR KO mice; c indicates a significant difference from treated FXR KO mice; $P < 0.05$. (B) F4/80 immunohistochemistry showed increased number of F4/80-positive cells in FXR KO mice compared WT mice under MCD feeding.

Bile acids and low glucose coordinately regulate Chop mRNA expression in HepG2 cells via FXR and RXR

To mimic the *in vivo* situation in mice under MCD diet, exhibiting low levels of glucose and high levels of BAs, HepG2 cells were treated with low and (high) dose of glucose and chenodeoxycholic acid (CDCA). This *in vitro* model system was used to understand whether and how BA-activated FXR regulate CHOP expression. Interestingly, CHOP mRNA levels were increased by CDCA incubation in normal glucose concentrations (5 mM), an effect which was lost at high glucose concentrations (25 mM) (Fig. 4A).

Farnesoid X receptor is known to form either monomer or heterodimer retinoid X receptor alpha

(RXR α). To determine whether FXR acts as a heterodimer, we incubated HepG2 cells with the highly specific RXR α agonist 9-cis retinoic acid (9-cisRA). As shown in Fig. 4B, RXR α activation under normal glucose levels (5 mM) clearly increased Chop mRNA expression; this effect was even stronger in the presence of CDCA (Fig. 4B). In line, C/ebp α expression was reduced under RXR α -activated conditions (Fig. 4B). Together, these data support the concept that FXR and RXR are required to regulate Chop expression in normal glucose conditions. Next, to determine whether FXR regulates Chop expression at the transcriptional level, we pretreated cells with Actinomycin D (ActD), a polymerase inhibitor, and then incubated in normal glucose condition with CDCA. As shown in Figure 4C, ActD massively reduced Chop

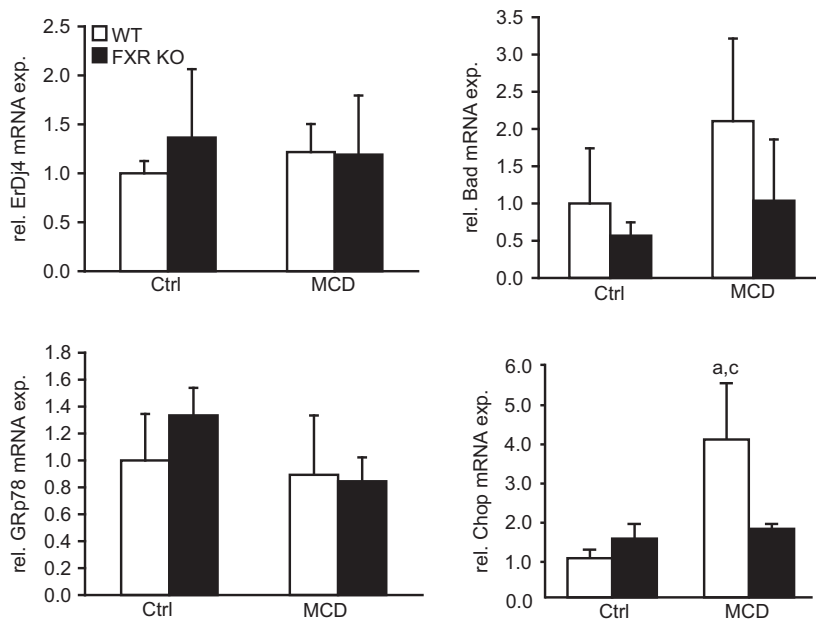


Fig. 3. MCD feeding does not induce ER stress and/or apoptosis in WT and FXR KO mice. Real-time PCR was used to assess mRNA levels of ER stress and apoptotic markers ERdj4, Grp78, Bad, and Chop. Chop mRNA levels were increased in WT mice upon MCD feeding exclusively. Expression levels of ERdj4, Grp78, and Bad did not change due to diet feeding in both, WT and FXR KO mice. a indicates a significant difference from untreated WT mice (Ctrl); b indicates a significant difference from untreated FXR KO mice; c indicates a significant difference from treated FXR KO mice; $P < 0.05$.

induction mediated by CDCA, suggesting that FXR regulates Chop expression mainly at the transcriptional level.

In order to understand whether FXR directly regulates Chop expression, we pretreated HepG2 cells with cycloheximide (CHX), (a drug inhibiting protein neosynthesis), and incubated the cells in normal glucose condition with CDCA. CHX massively reduced Chop induction by CDCA, indicating that Chop regulation by FXR did not require *de novo* protein synthesis (Fig. 4C).

Finally, *in silico* analysis revealed a *bona fide* FXR response element in the proximal human Chop promoter. We therefore performed a chromatin immunoprecipitation against FXR and RXR followed by real-time quantitative PCR (Fig. 4D). In control cells, FXR is weakly bound to the site (Ct value of 28,78), whereas RXR is highly present (Ct value of 19,02). After CDCA incubation in normal glucose conditions HepG2 cells incubated with CDCA showed a drastic increase in FXR protein bound to the site (Ct value of 22,47), whereas RXR binding was only modestly lowered (Ct value of 19,47). These data suggest that CDCA incubation under normal glucose conditions (5 mM), results in replacement of FXR homodimer by a FXR/RXR heterodimer on the FXR

response element in the Chop promoter. These findings could indicate a direct mechanistic link between FXR and Chop signaling, possibly interfering with the inflammatory response which was aggravated in FXR KO mice under MCD diet.

Discussion

In this study, we examined the relationship between FXR and CHOP in steatohepatitis. To this purpose, we used a methionine choline-deficient (MCD) dietary model which has been used in previous studies by others to induce steatohepatitis and fibrosis in rodents [14–19]. Although several shortcomings such as weight loss, lack of insulin resistance, and obesity in this model need to be acknowledged [20,21], MCD diet is increasingly used to study the hepatic features of the more severe stages of NAFLD such as inflammation and oxidative stress in mice [22]. Moreover, it has been demonstrated that loss of methionine and choline makes human more susceptible to develop fatty liver [23,24]. Methionine is an intermediate in S-Adenosyl-methionine as well as in glutathione synthesis. Both proteins are important antioxidants [25], therefore methionine deficiency predisposes to oxidative stress, inflammation, and fibrosis [26]. The finding that WT

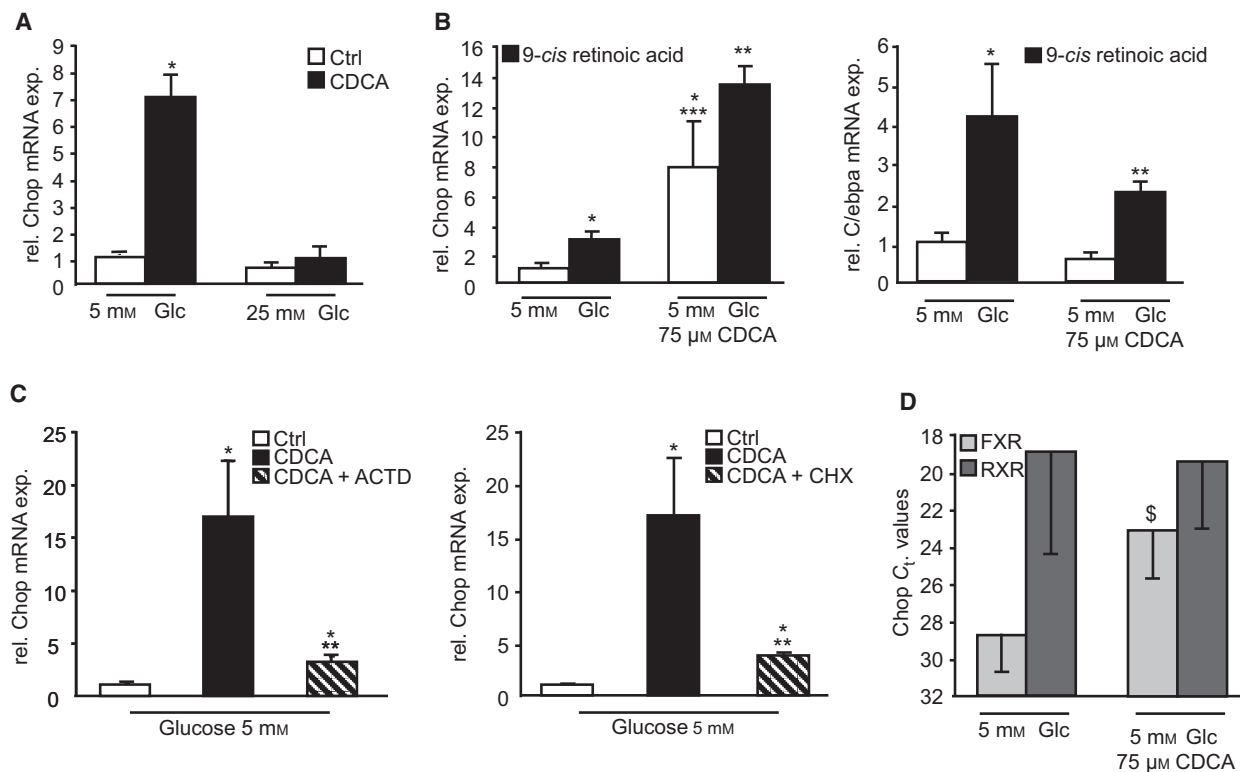


Fig. 4. Bile acid and low glucose regulate Chop mRNA expression transcriptionally in HepG2 cells via FXR and RXR. (A) Chop mRNA expression levels were significantly upregulated in HepG2 cells treated with 5 mM Glc and 75 μ M CDCA compared to cells treated with 5 mM Glc only, while in cells treated with high dose of Glucose with and without CDCA Chop expression was not affected. (B) Chop mRNA levels were upregulated in HepG2 cells treated with 9-cis retinoic acid compared with control groups. * indicates a significant difference from 5 mM Glc-treated control cells (Ctrl); ** indicates a significant difference from cells treated with 5 mM Glc and 9-cis retinoic acid; *** indicates a significant difference from cells treated with 5 mM Glc, CDCA and 9-cis retinoic acid $P < 0.05$. (C) Chop mRNA levels were upregulated in cells treated with 5 mM Glc and CDCA compared with control cells (Ctrl) and decreased in cells treated with CDCA and ActD or CHX. * indicates a significant difference from 5 mM Glc-treated control cells (Ctrl); ** indicates a significant difference from cells treated with 5 mM Glc and 75 μ M CDCA. $P < 0.05$. (D) HepG2 cells were cultured in DMEM without fetal calf serum treated with 5 mM Glc with and without 75 μ M CDCA. Cells were harvested after 6 h of treatment and assessed with chromatin immunoprecipitation with antibodies for FXR and RXR. Precipitated DNA was assessed with real-time PCR and mRNA levels of Chop were increased in samples where FXR and RXR antibodies were used. \$ and # indicates a significant difference from corresponding control group; § indicates a significant difference from cells where FXR antibody was used $P < 0.05$.

mice develop only moderate inflammation and no signs of ER stress (Fig. 2 and 3) may result from a variety of reasons including differences between mouse strains, diet, and housing conditions, factors which can result in changes of gut microbiota, known to have critical function in development of NAFLD/NASH [27]. Of note, an absent unfolded protein response the “classical” ER stress signaling cascade in response to MCD feeding, was also reported by others [28].

Since Chop KO mice fed a MCD diet are prone to development of inflammation, reduced mRNA expression of Chop in FXR KO mice fed a MCD diet uncovered by this study may contribute to the aggravation of inflammation. Loss of Chop results in decreased cell death of activated macrophages,

resulting in their accumulation in the liver [11]. Moreover, anti-inflammatory effects of FXR via NF κ B may be relevant for this observation [7]. In line, inflammatory markers *F4/80*, *Tnf α* , *Mcp1* and *Il1b* were increased in FXR KO MCD-fed mice (Fig. 2A). Furthermore, our *in vitro* studies in HepG2 cells showed a role of FXR in regulating Chop during low glucose and high bile acid conditions (mimicking the *in vivo* conditions under MCD diet), suggesting a beneficial effect of FXR—Chop signaling in hepatocytes possibly by interfering with cytokine secretion [29] and subsequently reduced recruitment and activation, of inflammatory cells (implicating an indirect effect on preventing inflammation). Since FXR is highly expressed in hepatocytes and only moderately

expressed in Kupffer cells [30], the situation in hepatocytes (compared to macrophages) might be more relevant to the overall situation seen in the liver. However, further studies have to specify whether there is a direct anti-inflammatory effect of FXR—Chop signaling in macrophages. The observation that the FXR—Chop signaling axis may have anti-inflammatory properties only under low/normal glucose level emphasizes the importance of the strict diabetic management in patients with NAFLD, especially in those receiving FXR agonists. Beneficial FXR effects could be much more pronounced in patients with normal glucose levels.

Treatment with 9-cis retinoic acid as an agonist for retinoic X receptor (RXR) demonstrate that RXR is also involved in the regulation of Chop mRNA expression (Fig. 4B). Furthermore, our *in vitro* studies reveal that the regulation of Chop via FXR and RXR may occur indirectly at a transcriptional level (Fig. 4C) and the two transcription factors need to be available as proteins (Fig. 4D). Overall, our data suggest that under conditions mimicking MCD diet-induced liver injury (low glucose, high bile acids) FXR together with RXR activate Chop mRNA expression and subsequent inhibition of C/ebp α , which may explain suppression of Srebp1c levels in MCD fed WT mice.

Despite observations by Watanabe and coworkers suggesting that BA-activated FXR counteracts hepatic TG accumulation [9] via Srebp1c downregulation, we observed a marked increase in hepatic lipid accumulation in WT MCD fed mice despite a clear reduction in Srebp1c mRNA levels compared with FXR KO MCD-fed mice. Lipid accumulation may result from increased FA flux from adipose tissue to the liver, seen in MCD-fed mice [20] in synergy with reduced VLDL synthesis and secretion [31]. In line with reported findings in the literature, WT MCD-fed mice showed low serum VLDL levels (data not shown) [32]. In addition to the reduced hepatic VLDL secretion due to low levels of phosphatidylcholine [32], it is attractive to speculate whether the increased expression of VLDL receptor found in WT MCD-fed mice (data not shown) may result in hepatic lipoprotein reuptake contributing to hepatic TG accumulation. Furthermore, it has to be mentioned that the inhibitory effect of activated FXR on Srebp1c expression shown by Watanabe *et al.* [9] seems to be an acute response to increased BA levels since after 7 days of cholic acid (CA) feeding mRNA levels of Srebp1c and its downstream targets FasN and Scd1 returned to normal [9]. Therefore, we propose that in our MCD-fed mouse model, Srebp1c expression may be reduced due to the Chop-induced inhibition of C/ebp α .

In conclusion, this study identifies FXR as a regulator of Chop (under MCD conditions), which ameliorates inflammation and subsequently progression of fatty liver disease from the benign stage of steatosis to the more severe steps such as steatohepatitis implicating a potential role of Chop as pharmacological target.

Author contribution

CDF: writing of the manuscript, data collection, statistical analysis and interpretation of data; TC: critical revision of the manuscript for important intellectual content. HS, TS: data collection, critical revision of the manuscript for important intellectual content; MT: study concept and design, interpretation of data, outlining and revising the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. MCD feeding leads to reduction in bodyweight. In both, WT and FXR KO mice, MCD feeding over the period of 5 weeks results in bodyweight reduction. * indicates a significant difference from untreated WT mice, # indicates a significant difference from untreated FXR KO mice; $P < 0.05$.

Fig. S2. Impaired *de novo* lipogenesis and β -oxidation

in WT and FXR KO mice-fed MCD diet. (A) mRNA levels of Ppar α and Acox were downregulated in both, WT and FXR KO mice fed a MCD diet for 5 weeks (B) mRNA levels of Srebp1c and FasN were reduced by MCD feeding in both WT and FXR KO mice upon MCD feeding. a indicates a significant difference from untreated WT mice (Ctrl); b indicates a significant difference from untreated FXR KO mice; c indicates a significant difference from treated FXR KO mice; $P < 0.05$.