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E2F2 transcription factor promotes a cholestatic MASH phenotype by regulating hepatobiliary metabolism through miR-34a-5p

Maider Apodaka-Biguri[1] | André L. Simão[2] | Francisco González-Romero[1] | Daniela Mestre[1] | Pedro M. Rodrigues[3,4,5] | Igor Aurrekoetxea[1,6] | Beatriz Gómez-Santos[1,6] | Xabier Buqué[1] | Ane Nieva-Zuluaga[1] | Mikel Ruiz de Gauna[1] | Idoia Fernandez-Puertas[1] | Paul Gomez-Jauregui[1] | Natalia Sainz-Ramirez[1] | Kendall Alfaro-Jiménez[1] | Ane Ortiz-Palma[1] | Estibaliz Castillero[1] | Ainhoa Iglesias-Ara[7] | Jone Mitxelena[5,7] | Ainhoa Eriz[7] | Ana M. Aransay[3,8] | Juan-José Lozano[3,9] | Jose J.G. Marin[3,10] | Laura Izquierdo-Sanchez[2,3,4] | Maria J. Perugorria[3,4] | Luis Bujanda[3,4] | Jesus M. Banales[3,4,5,11] | César Martín[12,13] | Lorena Mosteiro[5] | Gaizka Errazti[5] | Wing-Kin Syn[1,14] | Luis Castaño[15,16] | Ana M. Zubiaga[7] | Rui E. Castro[2] | Patricia Aspichueta[1,3,6]

[1]Department of Physiology, Faculty of Medicine and Nursing, University of the Basque Country UPV/EHU, Leioa, Spain

[2]Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal

[3]National Institute for the Study of Liver and Gastrointestinal Diseases (CIBERehd, Carlos III Health Institute), Madrid, Spain

[4]Department of Liver and Gastrointestinal Diseases, Biogipuzkoa Health Research Institute, Donostia University Hospital, University of the Basque Country UPV/EHU, San Sebastian, Spain

[5]IKERBASQUE, Basque Foundation for Science, Bilbao, Spain

[6]Biobizkaia Health Research Institute, Cruces University Hospital, UPV/EHU, Spain

[7]Department of Genetics, Physical Anthropology and Animal Physiology, Faculty of Science and Technology, University of the Basque Country UPV/EHU, Leioa, Spain

[8]Genome Analysis Platform, Center for Cooperative Research in Biosciences (CIC bioGUNE), Derio, Spain

[9]Bioinformatic Platform, Clinic Hospital, Barcelona, Spain

[10]Experimental Hepatology and Drug Targeting (HEVEPHARM), University of Salamanca, IBSAL, Salamanca, Spain

[11]Department of Biochemistry and Genetics, School of Sciences, University of Navarra, Pamplona, Spain

[12]Department of Biochemistry and Molecular Biology, University of the Basque Country (UPV/EHU), Leioa, Spain

[13]Department of Molecular Biophysics, Biofisika Institute [University of the Basque Country and Consejo Superior de Investigaciones Científicas (UPV/EHU, CSIC)], Leioa, Spain

[14]Division of Gastroenterology and Hepatology, Saint Louis University, St Louis, Missouri, USA

[15]Faculty of Medicine and Nursing, University of the Basque Country (UPV/EHU), Leioa, Spain

[16]Biobizkaia Health Research Institute, Cruces University Hospital, UPV/EHU, CIBERDEM/CIBERER, Endo-ERN, Barakaldo, Spain

Abbreviations: BDL, biliary duct ligation; BMI, body mass index; CD, chow diet; ChD-HFD, choline-deficient high-fat diet; ChIP, chromatin immunoprecipitation; DCA, deoxycholic acid; DEN, diethilnitrosamine; DG, diglycerides; FAO, fatty acid oxidation; HFD, high-fat diet; MASH, metabolic dysfunction–associated steatohepatitis; MASL, metabolic dysfunction–associated steatotic liver; MASLD, metabolic dysfunction–associated steatotic liver disease; NCTP, sodium taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine methyltransferase; SIRT1, Sirtuin 1; T2DM, type 2 diabetes mellitus; TG, triglycerides; THR, thyroid hormone receptor; UDCA, ursodeoxycholic acid; WT, wild-type.

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Correspondence
Rui E. Castro, Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal.
Email: ruieduardocastro@ff.ulisboa.pt

Patricia Aspichueta, University of the Basque Country UPV/EHU, Barrio Sarriena s/n, 48940 Leioa, Spain.
Email: patricia.aspichueta@ehu.eus

Abstract

Background and Aims: Metabolic dysfunction–associated steatotic liver disease (MASLD) affects a heterogeneous group of patients. Among them, those with a cholestatic profile show worse outcomes. Here, we investigated whether E2F2 is involved in MASLD-associated cholestasis and, if so, the role of miRNAs.

Approach and Results: *E2f2*-knockout ($E2f2^{-/-}$) and wild-type (WT) mice were fed a choline-deficient high-fat diet (ChD-HFD) or an HFD after injection of diethylnitrosamine (DEN-HFD) to induce metabolic dysfunction–associated steatohepatitis (MASH). E2F2 was overexpressed in the liver by AAV8. Cholestasis was induced by bile duct ligation or by a 3,5-diethoxycarbonyl-1,4-dihydrocollidine-enriched diet. microRNA sequencing was performed. Two biopsy-proven MASLD patient cohorts were used. E2F2 deficiency resulted in increased synthesis and excretion of cholesterol, phosphatidylcholine, and bile acids, reducing their storage in the liver while increasing their presence in feces. This was consistent with increased expression of genes involved in biliary lipid metabolism, reduced inflammation and fibrosis, and the generation of a distinct miRNA profile, thereby preventing MASH. Liver-specific induction of E2F2 *in vivo* hampered the transcriptional program involved in biliary lipid metabolism and upregulated miR-34a-5p, which was downregulated in $E2f2^{-/-}$ mice. The protective effects observed in $E2f2^{-/-}$ mice were lost when a miR-34a-5p mimic was used. Hepatic miR-34a-5p levels were elevated in patients with advanced fibrosis, inflammation, steatosis score, cholelithiasis, and increased serum bile acids and biliary lipids. *E2f2* deficiency conferred protection against cholestatic liver injury.

Conclusions: E2F2 deficiency protects against MASH and cholestasis, preventing cholesterol accumulation, fibrosis, and inflammation through modulation of miR-34a-5p. This could provide therapeutic benefits for patients with cholestatic MASH.

Keywords: E2F, metabolism, miR-34a-5p, bile, bile acids, cholestasis, cholesterol, phospholipids, MASH, MASLD

INTRODUCTION

Metabolic dysfunction–associated steatotic liver disease (MASLD) has become a global pandemic, affecting many different countries and regions.[1,2] It is the most common form of chronic liver disease worldwide, with a prevalence of around 35%, and has been consistently increasing with the growing global epidemic of obesity and type 2 diabetes mellitus (T2DM).[1,3] During MASLD, patients with isolated steatosis (metabolic dysfunction–associated steatotic liver, MASL) may progress to metabolic dysfunction–associated steatohepatitis (MASH), which is characterized by steatosis, hepatocellular ballooning and inflammation, often accompanied by fibrosis, which can further progress to cirrhosis, and/or hepatocellular

carcinoma (HCC).[4] The high heterogeneity of patients with MASLD makes it challenging to find appropriate "one fits all" treatments, for which an accurate stratification of patients is even more critical. In this regard, it was recently reported that MASLD patients with a cholestatic profile are more prone to liver disease progression, liver decompensation events, and overall mortality.[5] Alterations in bile acid metabolism with increased levels of hepatic, circulating, fecal, and/or urinary bile acids have been found in MASH patients.[6,7] The production and secretion of bile acids, the major organic components of bile required for lipid digestion and absorption, involve cholesterol metabolism. As such, alterations in the synthesis or secretion of these lipids affect bile acid formation and hepatic efflux or return via the enterohepatic circulation.[8] Consistent with this, disturbances in cholesterol metabolism influence MASLD progression.[9,10] Specifically, alterations in the expression of hepatic CYP7A1/CYP27A1, as well as of ABCG5/8, involved in the conversion of cholesterol to bile acids and in the secretion of cholesterol to bile, respectively, have been described in patients with MASH.[11–13]

Bile acids are not only responsible for lipid digestion and absorption, but are also important signaling molecules that activate receptors and transcription factors involved in energy metabolism.[14] However, the accumulation of bile acids and biliary lipids in hepatocytes, resulting from impaired hepatobiliary secretion and cholestasis, also leads to cytotoxicity and inflammation, thereby contributing to MASLD progression.[5,15]

E2F transcription factors regulate cell cycle as well as hepatic fatty acid metabolism.[16] In fact, E2F overexpression induces the accumulation of neutral lipids in lipid droplets, while the absence of E2F1 or E2F2 ameliorates the development of MASLD-related HCC, attributed, at least in part, to increased fatty acid oxidation (FAO).[16] Although transcriptome analyses have shown that key altered biological pathways conferring resistance to MASLD-related HCC include those related to lipid metabolism,[16] it is still not known whether E2F2 is involved in biliary metabolism in cholestatic-MASH. Crosstalk between members of the E2F family and miRNAs has been reported, with E2Fs acting as both regulators and targets of some miRNAs.[17] In turn, several miRNAs have been identified as regulators of hepatic and/or biliary metabolism during MASLD progression.[18–20] Here, we investigated whether the E2F2 transcription factor promotes a cholestatic-MASH phenotype and, if so, whether specific miRNAs are also involved. Overall, our findings suggest that the E2F2–miR-34a-5p axis constitutes an appealing therapeutic target for patients with MASH and a cholestatic profile.

METHODS

Human samples

In this study, 2 cohorts of obese patients [body mass index (BMI) >30 kg/m^2] with biopsy-proven MASLD were used: the Biogipuzkoa cohort (Donostia University Hospital, San Sebastian, Spain) with 158 patients (Table 1); and the Biobizkaia cohort (Cruces University Hospital, Bilbao, Spain) with 45 patients (Supplemental Table S1, http://links.lww.com/HEP/J965). In both cases, tissue samples were obtained during bariatric surgery. Liver miR-34a-5p expression, NAS score, ballooning, and inflammation were determined in each sample. In the Biobizkaia cohort, liver E2F2 protein levels were also analyzed. Serum lipidomic analysis was performed on 141 patients from the Biogipuzkoa cohort and on 39 patients from the Biobizkaia cohort. Participants in the study were diagnosed with MASLD according to the Kleiner criteria.[21] All participants gave written consent after being fully informed about the study. The study adhered to the ethical guidelines of the Declaration of Helsinki and local and national legislation. The study procedures were approved by the Human Ethics Committee of Euskadi, of the Donostia University Hospital and of the University of the Basque Country UPV/EHU.

Animal models

Male *E2f2*-knockout (*E2f2*$^{-/-}$) and corresponding wild-type (WT) mice (of mixed genetic background; C57BL/6J and 129/Sv) were bred at the UPV/EHU animal facility. Animal procedures were approved by the Animal Welfare Ethics Committee of the University of the Basque Country UPV/EHU, in line with the European Union guidelines for animal experimentation.

To induce MASH, 14-day-old *E2f2*$^{-/-}$ and WT mice were injected with diethylnitrosamine (DEN; 25 mg/kg) intraperitoneally. After weaning, mice were fed a high-fat diet (HFD) (Bio-Serv 60% HFD; F3282) for 24 weeks. Alternatively, 1-month-old *E2f2*$^{-/-}$ mice and their WT littermates were fed a choline-deficient high-fat diet (ChD-HFD) (Research Diets, 45% fat without added choline; D05010402) for 24 weeks.[22]

Two different models were developed to induce cholestasis. Three-month-old *E2f2*$^{-/-}$ mice and their WT littermates were subjected to bile duct ligation (BDL) as previously described,[23] and sacrificed 7 days later, preventing marked fibrosis or inflammation given the context of mild-MASH. Sham-operated animals were used as controls. In a second model, 3-month-old *E2f2*$^{-/-}$ and WT mice were administered a 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-enriched diet for 2 weeks, as previously described.[24,25]

TABLE 1 Baseline characteristics of the Biogipuzkoa cohort

Characteristics	Overall (n = 158)	High miR-34a-5p (n = 66)	Low miR-34a-5p (n = 92)
Age	47.63 ± 13.09 (21–82)	52.87 ± 12.68 (27–82)	43.85 ± 12.09 (21–73)
Weight (kg)	124.83 ± 24.17 (65–220)	122.39 ± 27.16 (65–220)	126.44 ± 22.00 (75–187)
BMI (kg/m^2)	45.08 ± 7.35 (25–64)	44.47 ± 8.58 (25–64)	45.47 ± 6.34 (26–60)
ALP (IU/L)	75.09 ± 27.37 (9–186)	81.49 ± 26.60 (27–186)	69.56 ± 27.00 (9–156)
ALT (IU/L)	31.18 ± 20.05 (9–154)	37.21 ± 22.89 (12–154)	26.83 ± 16.53 (9–133)
AST (IU/L)	24.02 ± 11.92 (9–107)	28.01 ± 14.51 (12–107)	21.13 ± 8.63 (9–65)
Glucose (mg/dL)	111.78 ± 26.03 (70–221)	116.92 ± 29.87 (70–216)	108.18 ± 22.44 (82–221)
Cholesterol (mg/dL)	202.62 ± 43.63 (76–351)	204.39 ± 50.56 (98–351)	201.18 ± 37.34 (76–278)
Triglycerides (mg/dL)	147.31 ± 87.10 (48–580)	158.89 ± 94.40 (68–580)	137.85 ± 80.09 (48–429)
Histological characterization, n (%)			
Steatosis			
Grade 0 (< 5%)	25 (15.8)	8 (12.1)	17 (18.5)
Grade 1 (5%–33%)	56 (35.4)	15 (22.7)	41 (44.6)
Grade 2 (34%–66%)	37 (23.4)	24 (36.4)	13 (14.1)
Grade 3 (> 66%)	40 (25.3)	19 (28.8)	21 (22.8)
Lobular inflammation			
Grade 0 (none)	3 (1.9)	0 (0)	3 (3.3)
Grade 1 (< 2 foci)	65 (41.1)	22 (33.3)	43 (46.7)
Grade 2 (2–4 foci)	60 (38.0)	29 (43.9)	31 (33.7)
Grade 3 (> 4 foci)	30 (19.0)	15 (22.7)	15 (16.3)
Ballooning			
Grade 0 (none)	29 (18.4)	10 (15.2)	19 (20.7)
Grade 1 (poor–mild)	73 (46.2)	25 (37.9)	48 (52.2)
Grade 2 (moderate–high)	56 (35.4)	31 (47.0)	25 (27.2)
NAS			
0–4 (simple steatosis)	82 (51.9)	27 (40.9)	55 (59.8)
5–8 (MASH)	76 (48.1)	29 (59.1)	37 (40.2)
Fibrosis			
0 (none)	18 (11.4)	7 (10.6)	11 (12.0)
1 (mild–moderate zone 3 perisinusoidal)	63 (39.9)	19 (28.8)	44 (47.8)
2 (zone 3 perisinusoidal+periportal)	66 (41.8)	29 (43.9)	37 (40.2)
3 (bridging fibrosis)	8 (5.1)	8 (12.1)	0 (4.2)
4 (cirrhosis)	3 (1.9)	3 (4.5)	0 (3.2)

Note: Liver samples from patients with obesity from Biogipuzkoa (Donostia University Hospital) (n = 158) were obtained by liver biopsy. Data are shown as mean (range) ± SD or n (%).
Abbreviation: BMI, body mass index.

Liver-specific *E2f2* overexpression was achieved in 2-month-old mice fed a chow diet (CD) after administration of a recombinant adeno-associated virus serotype 8 with the E2F2 sequence upstream of the albumin promoter (*AAV8-Alb-mE2F2*). *AAV8-Alb-GFP* was used as a control. Mice were sacrificed 1 month later.

Statistical analysis

Data are presented as mean ± SD. Student *t* test was used to examine differences between groups. A *p*-value < 0.05 was considered significant. For correlation studies in human samples, the D'Agostino and Pearson normality test was used to check the normality of the data. The Spearman correlation was then used to assess matched gene expression levels. Further details are provided in the figure legends.

Statistical analyses were conducted using GraphPad Prism (version 8.0, GraphPad Inc.). Graphical visualizations were created with GraphPad Prism version 8.

Additional information is provided in the Supplementary Material and Methods, http://links.lww.com/HEP/J965.

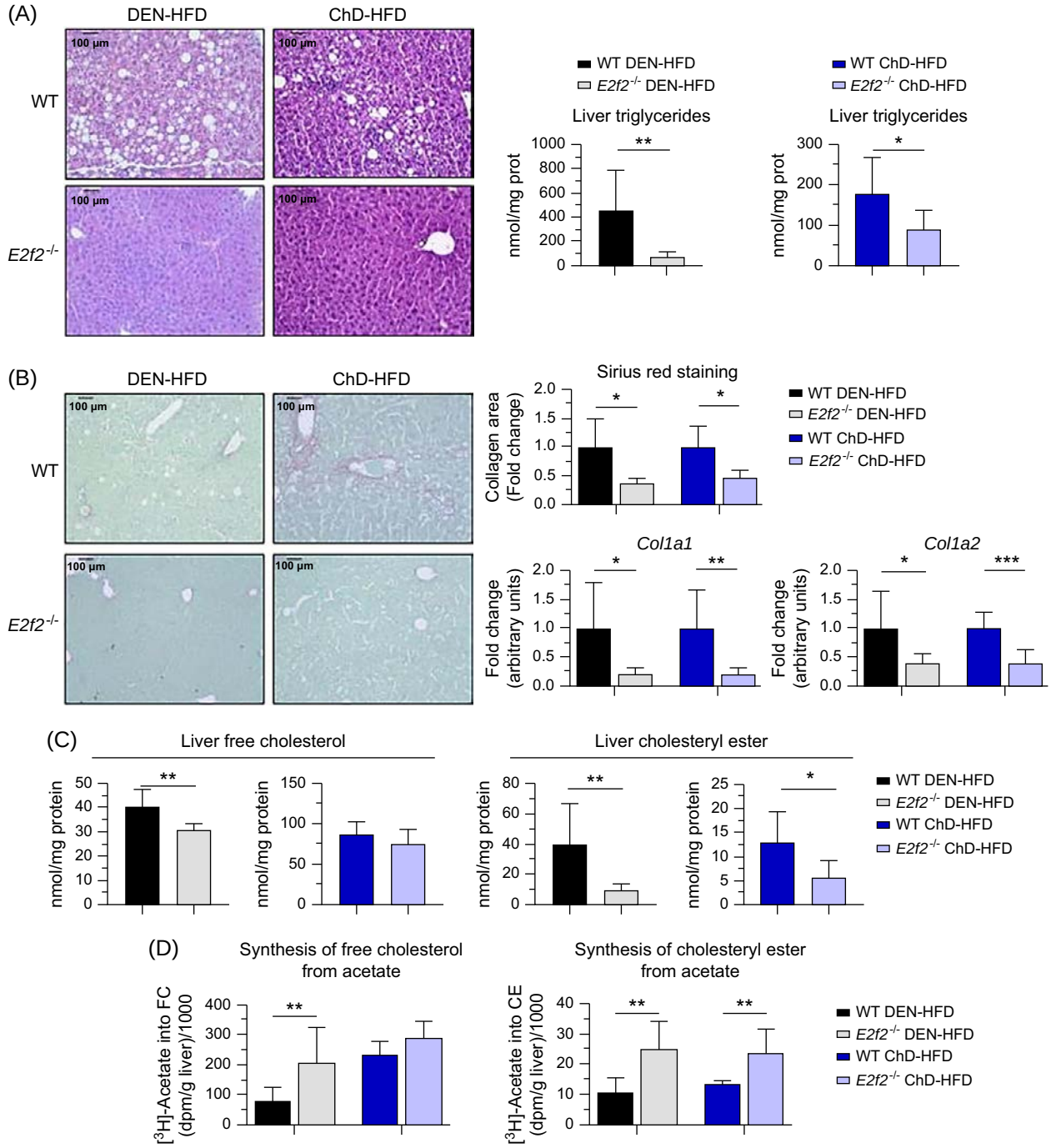

FIGURE 1 *E2f2*-deficient mice are protected against MASH and accumulate less cholesterol in the liver. Male *E2f2*-knockout ($E2f2^{-/-}$) and wild-type (WT) mice were fed a choline-deficient high-fat diet (ChD-HFD) or an HFD after injection of diethylnitrosamine (DEN-HFD) for 6 months. (A) Representative liver sections stained with hematoxylin/eosin and liver triglyceride content of wild-type (WT) and $E2f2^{-/-}$ mice (n = 7–8). (B) Representative liver sections stained with Sirius red and liver collagen area, *Col1a1* and *Col1a2* mRNA levels of WT and $E2f2^{-/-}$ mice (n = 8). (C) Liver free cholesterol and cholesteryl ester content (n = 7–9). (D) Metabolic fluxes of synthesis of free cholesterol and cholesteryl ester through incorporation of [^{3}H]-acetate (n = 4–11). Values represent mean ± SD. Significant differences are shown as $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Abbreviation: MASH, metabolic dysfunction–associated steatohepatitis.

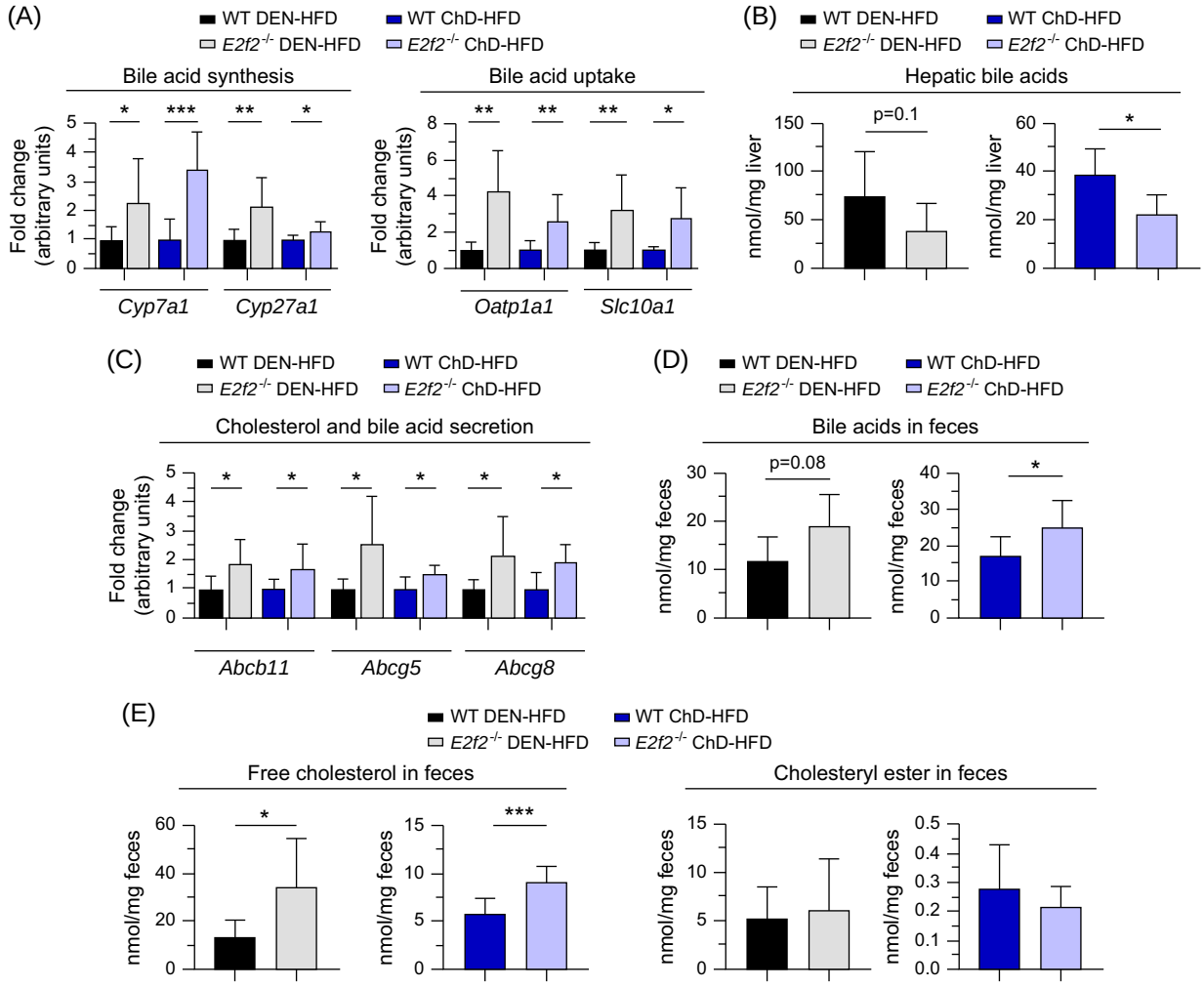

FIGURE 2 E2F2 deficiency promotes the synthesis and release of biliary lipids. Male *E2f2*-knockout ($E2f2^{-/-}$) and wild-type (WT) mice were fed a choline-deficient high-fat diet (ChD-HFD) or an HFD after injection of diethylnitrosamine (DEN-HFD) for 6 months. (A) Hepatic *Cyp7a1*, *Cyp27a1*, *Oatp1a1*, and *Slc10a1* mRNA levels of WT and $E2f2^{-/-}$ mice (n = 7–11). (B) Liver bile acid content of WT and $E2f2^{-/-}$ mice (n = 5–6). (C) Hepatic *Abcb11*, *Abcg5*, and *Abcg8* mRNA levels of WT and $E2f2^{-/-}$ mice (n = 7–11). (D) Bile acid content in feces of WT and $E2f2^{-/-}$ mice (n = 5–7). (E) Free cholesterol and cholesteryl ester content in feces of WT and $E2f2^{-/-}$ mice (n = 6–8). Values represent mean ± SD. Significant differences are shown as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

RESULTS

Reprogramming in biliary lipid and bile acid metabolism underlies protection against MASH in *E2f2*-deficient mice

E2f2 mRNA and protein levels were increased in 2 different preclinical models of MASH (mice exposed to DEN-HFD or ChD-HFD for 6 months), compared with their respective controls (Supplemental Figure S1A, http://links.lww.com/HEP/J965). In the absence of *E2f2*, mice were protected against triglyceride (TG) accumulation (Figure 1A), fibrosis (Figure 1B), and liver injury (Supplemental Figure S1B, http://links.lww.com/HEP/J965). Expression of *E2f1* and *E2f3*, the other recognized cell cycle activators of the E2F transcription family, remained unchanged in $E2f2^{-/-}$ mice compared with the corresponding WT mice (Supplemental Figure S1C, http://links.lww.com/HEP/J965).

Protection from MASH in $E2f2^{-/-}$ mice was further associated with decreased storage of hepatic free and esterified cholesterol (Figure 1C), which did not result from decreased fluxes regulating the *de novo* synthesis, which were found to be increased (Figure 1D).

Hepatic cholesterol is the precursor of bile acids and is secreted from the body as free cholesterol or in the form of bile acids into bile (Supplemental Figure S1D). Our results showed that expression levels of *Cyp7a1* and *Cyp27a1*, which are involved in the conversion of cholesterol into bile acids, were increased in the 2 preclinical models of MASH in the absence of *E2f2* (Figure 2A). The uptake of bile acids from circulation by the sodium taurocholate co-transporting polypeptide (NTCP) and to a lesser extent by several organic anion transporting polypeptides (OATPs) modulates hepatic bile acid availability in mice.[26] Here, our results showed that *E2f2* deficiency induced upregulation of the

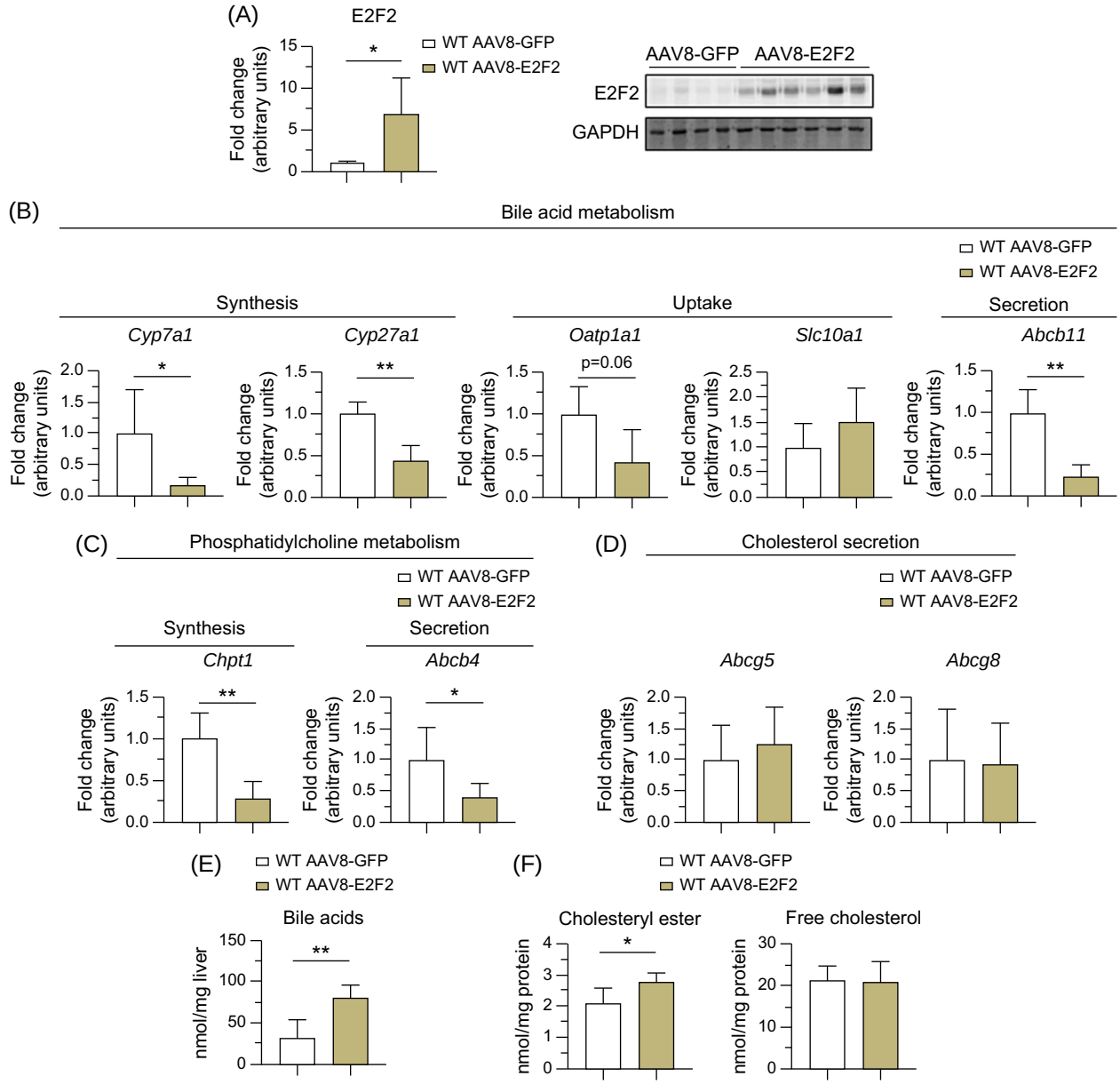

FIGURE 3 Liver-specific E2F2 overexpression leads to the accumulation of bile acids and cholesteryl ester by regulating the transcriptome program. (A) Hepatic E2F2 protein levels in chow diet–fed 3-month-old mice overexpressing E2F2 in livers and in the corresponding controls (n = 4–6). (B) Hepatic *Cyp7a1*, *Cyp27a1*, *Abcb11*, *Oatp1a1*, and *Slc10a1* mRNA levels in chow diet–fed 3-month-old mice overexpressing E2F2 in livers and in the corresponding controls (n = 4–6). (C) Hepatic *Chpt1* and *Abcb4* mRNA levels in chow diet–fed 3-month-old mice overexpressing E2F2 in livers and in the corresponding controls (n = 4–6). (D) Hepatic *Abcg5* and *Abcg8* mRNA levels in chow diet–fed 3-month-old mice overexpressing E2F2 in livers and in the corresponding controls (n = 4–6). (E) Bile acid content in the liver of WT and mice overexpressing E2F2 (n = 5–6). (F) Liver free cholesterol and cholesteryl ester content (n = 4–5). Values represent mean ± SD. Significant differences are shown as $^{*}p < 0.05$ and $^{**}p < 0.01$.

expression of NTCP gene (*Slc10a1*) and of *Oatp1a1* in both models of MASH (Figure 2A). Noteworthy, despite increased expression of proteins involved in bile acid synthesis and uptake, *E2f2*$^{-/-}$ mice presented with decreased levels of hepatic bile acids (Figure 2B). In fact, in both preclinical models of MASH, expression levels of *Abcb11* (BSEP gene) and of *Abcg5* and *Abcg8*, which are involved in the secretion of bile acids and cholesterol into bile, respectively, were increased in *E2f2*$^{-/-}$ mice compared with the WT mice (Figure 2C). This phenotype was associated with increased concentrations of bile acids and free cholesterol in feces, while no changes were observed in cholesteryl ester (Figures 2D, E). Given that synthesis of phosphatidylcholine (PC) has been shown to promote bile acid secretion in mice,[27] expression levels of genes involved in PC synthesis and secretion in bile were also analyzed. Synthesis of PC is driven through 2 processes: the phosphorylation of choline, also known as the Kennedy pathway; and the methylation of phosphatidyethanolamine (PE),

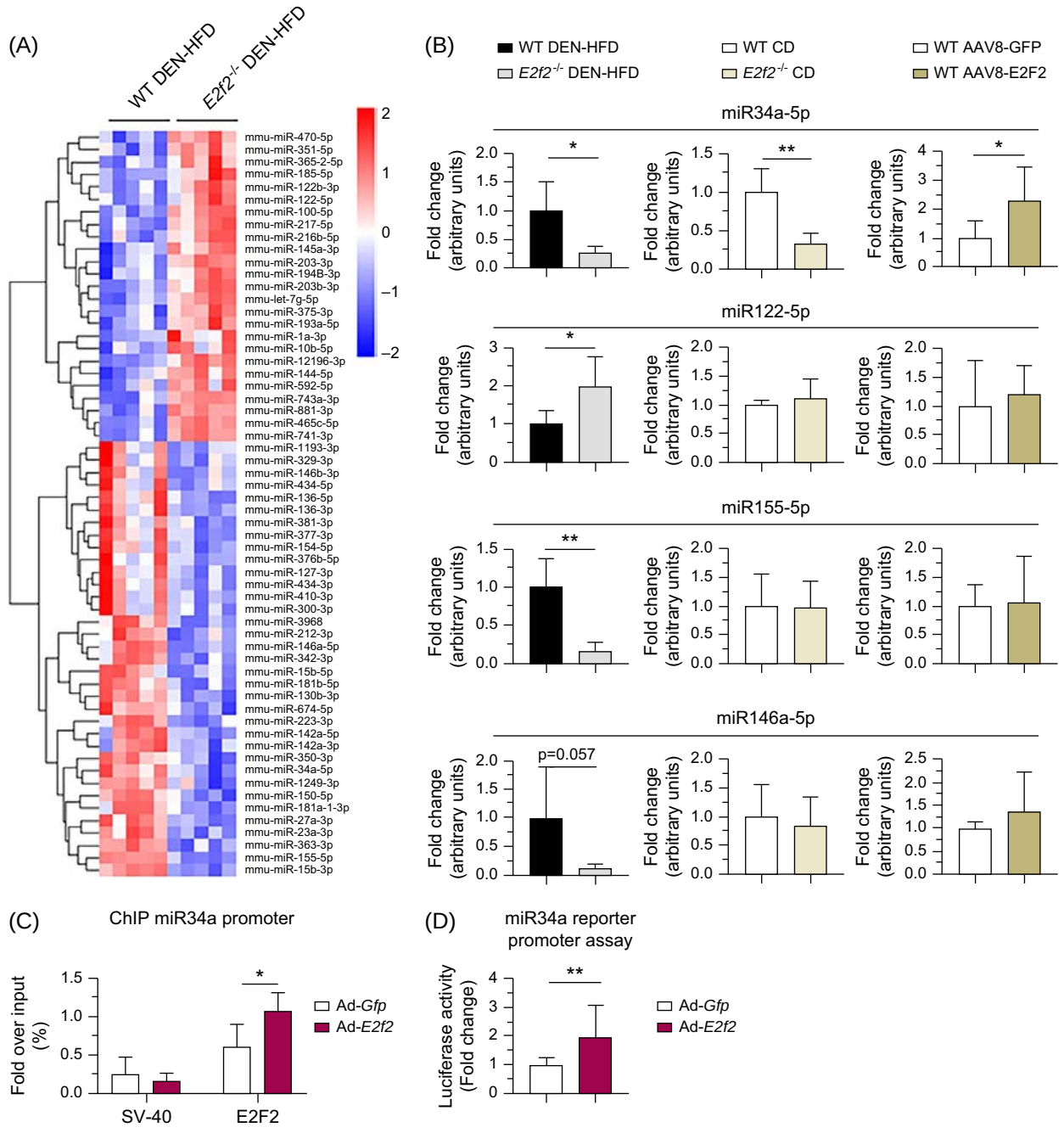

FIGURE 4 miR-34a-5p is a target of E2F2. (A) Heatmap depicting differentially expressed miRNAs in the liver of *E2f2*$^{-/-}$ and wild-type (WT) mice after 9 months of diethylnitrosamine-high-fat diet (DEN-HFD) (n = 5). (B) Hepatic miR-34a-5p, miR-122-5p, miR-155-5p, and miR-146a-5p levels in mice after 6 months of DEN-HFD, in chow diet (CD)–fed 3-month-old mice and in CD-fed 3-month-old mice overexpressing E2F2 in livers (n = 4–6) and in the corresponding controls. (C) ChIP-qPCR analysis of the miR-34a promoter region in primary hepatocytes infected with either Ad-*E2f2* or Ad-*Gfp*. Chromatin was immunoprecipitated using anti-E2F2 or anti-SV40T antibodies (the latter serving as a negative control), followed by qPCR with primers specific to the miR-34a promoter. (D) Luciferase reporter assay of miR-34a promoter activity in AML12 cells infected with either Ad-*E2f2* or Ad-*Gfp*. Experiments were performed in triplicate and produced consistent results. Values represent mean ± SD. Significant differences are shown as *$p < 0.05$ and **$p < 0.01$.

recognized as the PE methyltransferase (PEMT) pathway (Supplemental Figure S2A, http://links.lww.com/HEP/J965).[27] *E2f2* deficiency led to increased expression of genes involved in PC synthesis (*Chpt1* and *Pemt*) (Supplemental Figure S2B, http://links.lww.com/HEP/J965) and secretion (*Abcb4,* the MDR2 gene) (Supplemental Figure S2C, http://links.lww.com/HEP/J965), while liver PC and PE levels remained unaltered (Supplemental Figure S2D, http://links.lww.com/HEP/J965). Fecal phospholipid levels were found to be

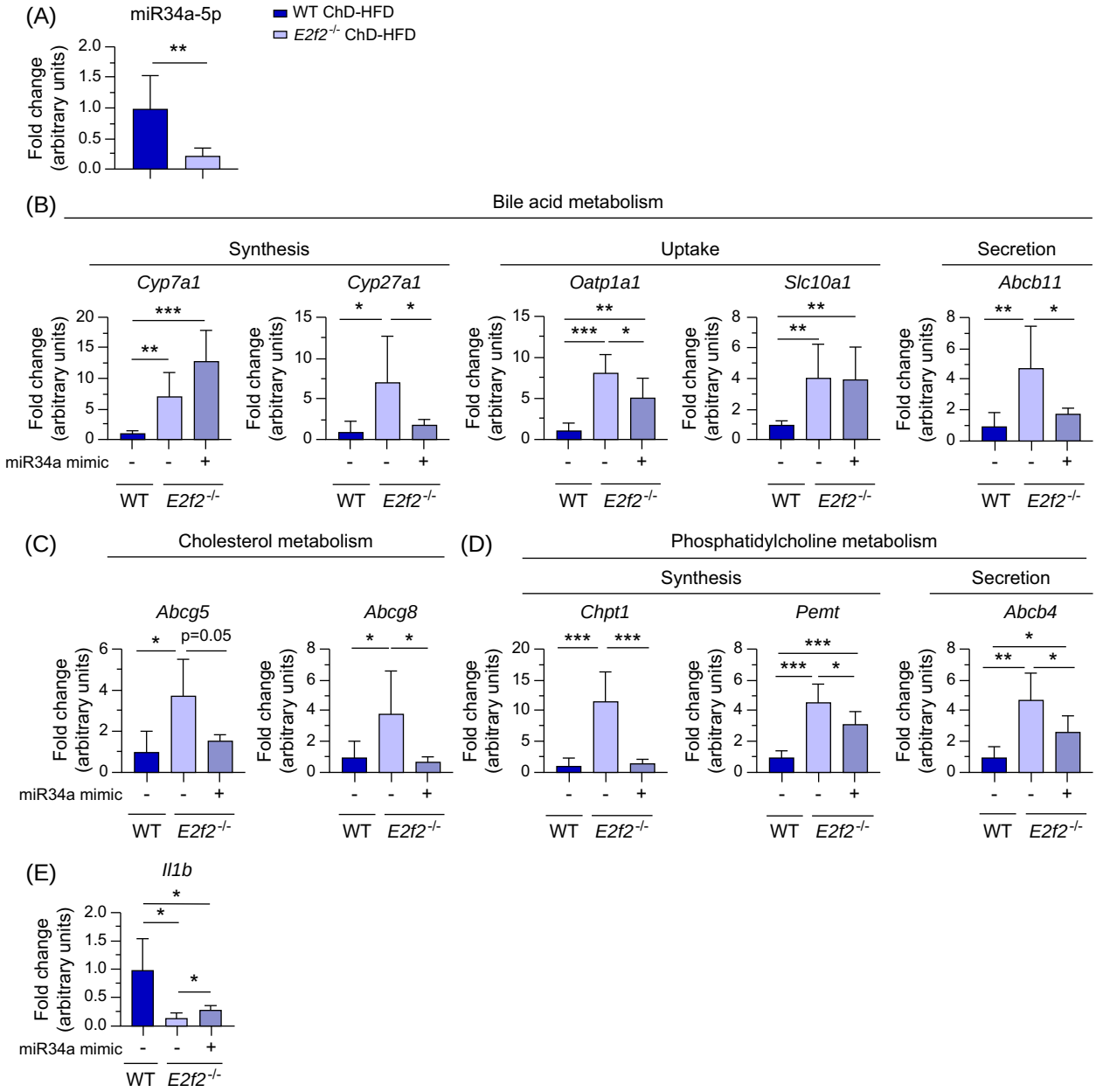

FIGURE 5 miR-34a-5p mimic prevents the E2F2 deficiency-mediated protection against MASH. Male *E2f2*-knockout ($E2f2^{-/-}$) and wild-type (WT) mice were fed a choline-deficient high-fat diet (ChD-HFD) for 6 months. (A) Hepatic miR-34a-5p levels in WT and $E2f2^{-/-}$ mice (n = 10–11). (B) Hepatic *Cyp7a1*, *Cyp27a1*, *Oatp1a1*, *Slc10a1*, and *Abcb11;* (C) *Abcg5* and *Abcg8*; (D) *Chpt1*, *Pemt*, and *Abcb4*; and (E) *Il1b* mRNA levels in liver sections from $E2f2^{-/-}$ mice treated with the miR-34a-5p mimic (n = 5–6) or a scrambled negative control (n = 5–6). Liver sections from WT mice treated with a scrambled mimic were used as a control (n = 5–6). Values represent mean ± SD. Significant differences are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Abbreviation: MASH, metabolic dysfunction–associated steatohepatitis.

increased in $E2f2^{-/-}$ mice compared with the WT mice (Supplemental Figure S2E, http://links.lww.com/HEP/J965).

Considering that E2F2 is increased in MASLD patients[16] and in preclinical models of MASH (Supplemental Figure S1A, http://links.lww.com/HEP/J965), we next evaluated whether liver-specific overexpression of E2F2 could modulate the expression of enzymes and transporters involved in biliary lipid metabolism (Figure 3). Results showed that liver-specific E2F2 overexpression (Figure 3A), mainly induced in hepatocytes due to the presence of the albumin promoter, decreased the expression of enzymes involved in bile acid synthesis and secretion (Figure 3B) and phosphatidylcholine synthesis and secretion in bile (Figure 3C), as compared with the corresponding WTs. However, it did not induce changes in the expression of *Abcg5/8* (Figure 3D). This transcriptome profile induced by liver-specific E2F2 overexpression led to the accumulation of bile acids (Figure 3E) and cholesteryl esters

(Figure 3F), thereby preventing lipotoxic free cholesterol storage, which remained unchanged (Figure 3F). These results illustrate that remodeling of biliary lipid metabolism in *E2f2*$^{-/-}$ mice is liver-specific and suggest that hepatic overexpression of E2F2 in human MASH is associated with cholestatic-MASH.

E2F2 coordinates biliary lipid synthesis and efflux in hepatocytes by modulating miR-34a-5p

E2F transcription factors can act as regulators of miRNAs.[17] For instance, E2F2 was previously shown to directly bind to the promoter of the mir-17-92 cluster,[28] while miR-17 and miR-20a can jointly target ~30 UTRs of E2F2 and E2F3.[29] As such, we next performed miRNA sequencing in livers from 9-month-old DEN-HFD-fed *E2f2*$^{-/-}$ and WT mice. The microRNA sequencing data have been uploaded to the NCBI Gene Expression Omnibus (GEO) database and can be accessed using the accession number GSE276277. *E2f2*$^{-/-}$ mice exhibited distinct liver miRNA expression compared with WT mice (Figure 4A), suggesting that the protective effects of E2F2 deficiency against MASH may depend on miRNA targeting. In fact, among the modulated microRNAs, several have been previously associated with MASLD/MASH and/or fibrosis, including miR-34a-5p, miR-122-5p, miR-155-5p, or miR-146a-5p.[17–20,30,31] Validation of the expression of these 4 miRNAs after 6 months of DEN-HFD confirmed the effect in *E2f2*$^{-/-}$ mice when compared with their corresponding WTs (Figure 4B). To better evaluate a putative direct transcriptional effect of E2F2 on these miRNAs, their expression was also measured in the liver of 3-month-old *E2f2*$^{-/-}$ and WT mice fed a chow diet (CD). In addition, to query for a direct effect of E2F2 in the liver, miRNA expression was also measured in CD-fed 3-month-old mice in which E2F2 was specifically overexpressed in the livers (Figure 4B). Results showed that liver miR-34a-5p expression was decreased in the absence of *E2f2* and, conversely, increased in mice with specific E2F2 liver overexpression (Figure 4B). This was not observed for miR-122-5p, miR-155-5p, or miR-146a-5p, suggesting that, among these miRNAs, only miR-34a-5p is a putative direct target of hepatic E2F2. Noteworthy, we and others have shown that liver miR-34a-5p upregulation is a key pathogenic event during experimental and human MASLD progression.[20,32,33] Expression of miR-34a-5p remained unchanged in the absence of *E2f1* in CD-fed and DEN-HFD–fed mice (Supplemental Figure S3A, http://links.lww.com/HEP/J965), suggesting that only E2F2 modulates miR-34a-5p. At the same time, it should be noted that several other modulated miRNAs (Figure 4A) may be direct or indirect targets of E2F2.

miR-34a was recently shown to enhance cholestasis-associated ductular reaction and liver fibrosis.[34] In fact, we have previously shown that ursodeoxycholic acid (UDCA), the main treatment for primary biliary cholangitis, ameliorates hepatic steatosis-mediated inhibition of Sirtuin 1 (SIRT1) via a miR-34a-dependent pathway.[32] In turn, deoxycholic acid (DCA) activates a hepatic JNK1/miR-34a/SIRT1 proapoptotic circuit both in vitro and in vivo.[35] As such, given the relevance of miR-34a in controlling bile acid metabolism, we next performed in silico analysis, querying the crosstalk between genes upregulated in the hepatic transcriptome of *E2f2*$^{-/-}$ mice, resistant to MASLD-associated HCC, and miR-34a-5p predicted target genes. Results showed that *Cyp27a1*, *Abcb11*, *Slc10a1*, *Oatp*, and *Chpt1* were common target genes, according to the miRWalk database (Supplemental Figure S4A, http://links.lww.com/HEP/J965). Among these, *Oatp* and *Slc10a1* were also identified in TargetScan (Supplemental Figure S4B, http://links.lww.com/HEP/J965).

To elucidate whether E2F2 transcriptionally regulates miR-34a-5p in mouse liver cells, we performed chromatin immunoprecipitation (ChIP) and luciferase reporter assays. Results showed that overexpression of *E2f2,* driven by adenovirus, increased the abundance of E2F2 in the miR-34a promoter (Figure 4C) and resulted in a ~2-fold increased luciferase activity from a reporter vector containing the most well-characterized promoter region of miR-34a (Figure 4D). The same increase in luciferase activity was observed when using HepG2 cells co-transfected with a human E2F2 plasmid (Supplemental Figure S4C, http://links.lww.com/HEP/J965).

To investigate whether the effects of E2F2 in biliary lipid synthesis and efflux were dependent on miR-34a-5p, liver slices from *E2f2*$^{-/-}$ mice fed the ChD-HFD for 6 months, where miR-34a-5p was downregulated (Figure 5A), were treated with a miR-34a-5p mimic or a scrambled (negative) control. Compared with control *E2f2*$^{-/-}$ liver slices, miR-34a-5p-overexpressing liver slices displayed decreased expression of *Cyp27a1*, *Oatp1a1*, and *Abcb11* (Figure 5B); *Abcg8* (Figure 5C); as well as *Chpt1*, *Pemt*, and *Abcb4* (Figure 5D). In parallel, decreased expression of *Il1b* in ChD-HFD–fed *E2f2*$^{-/-}$ mice liver slices was partially restored upon treatment with the miR-34a-5p mimic (Figure 5E).

Because the miR-34a-5p mimic failed to modulate expression of *Cyp7a1* or *Slc10a1* and presented a limited effect on *Abcg5* and *Il1b* expression, we queried whether E2F2 could be directly modulating these genes. ChIP results showed that E2F2 was able to bind to the *Cyp7a1*, *Slc10a1*, *Abcg5*, and *Il1b* promoters, according to the percentage of enrichment, when compared with the corresponding controls (Supplemental Figure S5A, http://links.lww.com/HEP/J965).

Additional genes are expected to be modulated by E2F2 in a miR-34a-5p–independent manner. For

instance, we have previously described that, during MASLD, E2F2 controls FAO by modulating *Cpt2* expression.[16] In fact, expression of *Cpt2*, *Acsl1*, and *Acsm1*, involved in FAO, are upregulated in *E2f2*$^{-/-}$ mice livers (Supplemental Figure S5B, http://links.lww.com/HEP/J965). Still, despite these genes also being predicted targets of miR-34a-5p, according to miRWalk and TargetScan, none were further modulated in miR-34a-5p mimic-treated liver slices (Supplemental Figure S5B, http://links.lww.com/HEP/J965).

We next inhibited miR-34a-5p, using a specific mirVana miRNA inhibitor (anti–miR-34a-5p) in liver slices from WT and *E2f2*$^{-/-}$ mice fed a ChD-HFD for 6 months (Supplemental Figure S6A, http://links.lww.com/HEP/J965). Expression of select genes involved in bile acid synthesis (*Cyp7a1* and *Cyp27a1*), cholesterol (*Abcg8),* bile acid *(Abcb11*), and PC secretion (*Abcb4*) was upregulated in WT mice upon inhibition of miR-34a-5p but not in *E2f2*$^{-/-}$ mice, where the expression levels were already increased (Supplemental Figure S6B, http://links.lww.com/HEP/J965). Curiously, inhibition of miR-34a-5p also increased the expression of *E2f2* and of its targets, members of the MCM family (*Mcm2*, *Mcm3*, and *Mcm4*), in WT mice (Supplemental Figure S7A, http://links.lww.com/HEP/J965). This effect was confirmed in human hepatocytes (THLE2 cell line) exposed to palmitic acid, where inhibition of miR-34a-5p upregulated *E2F2* and *MCMs* expression (Supplemental Figure S7B, http://links.lww.com/HEP/J965). Because we previously reported that increased E2F2 expression induces lipid accumulation,[16] we quantified the amount of lipids in mouse liver slices and human hepatocytes. Results showed that miR-34a-5p inhibition resulted in the accumulation of triglycerides and diglycerides in ChD-HFD–fed WT mice, compared with controls, an effect that was not observed in *E2f2*$^{-/-}$ mice, where lipid levels were already low (Supplemental Figure S7C, http://links.lww.com/HEP/J965). Similarly, inhibition of miR-34a-5p in human hepatocytes exposed to palmitic acid also led to lipid accumulation (Supplemental Figure S7D, http://links.lww.com/HEP/J965). Silencing of miR-34a failed to modulate sterol levels in liver slices or in human hepatocytes; however, cholesteryl ester was already decreased in ChD-HFD–fed *E2f2*$^{-/-}$ mice when compared with WT mice (Supplemental Figure S7C, http://links.lww.com/HEP/J965), in agreement with whole-liver results (Figure 1). Overall, these results suggest that the benefits of targeting E2F2 in the context of MASLD integrate both miR-34a-5p–dependent and miR-34a-5p–independent effects.

E2F2 and miR-34a-5p expression is increased in the liver from patients with advanced MASLD and dyslipidemia

We and others have shown that miR-34a is a key regulator of MASLD development and progression.[32,36] Our results herein show that liver miR-34a-5p expression increases with disease severity in MASLD patients, namely liver steatosis, ballooning inflammation, and fibrosis (Figure 6A and Table 1). Serum lipidomic analysis further showed that patients with higher hepatic miR-34a-5p expression also presented with elevated levels of circulating TG and diglycerides (DG) subspecies (Figure 6B), a hallmark of MASLD-associated dyslipidemia. Furthermore, patients with cholelithiasis also displayed increased hepatic miR-34a-5p expression and increased levels of serum bile acids (Figure 6C). Consistent with these findings, patients with higher hepatic miR-34a-5p expression also presented with increased levels of several bile acid species in serum (Figure 6D).

As mentioned before, E2F2 has been described to be increased in the liver of MASLD patients.[16] In fact, our results show that E2F2 protein levels are increased in patients with MASH (Supplemental Figure S8A, http://links.lww.com/HEP/J965) and that both E2F2 protein levels (Supplemental Figure S8B, http://links.lww.com/HEP/J965) and miR-34a-5p levels (Supplemental Figure S8C, http://links.lww.com/HEP/J965) are increased in patients with higher degrees of steatosis, ballooning, and inflammation. In addition, serum AST (Supplemental Figure S8D, http://links.lww.com/HEP/J965), as well as serum total-cholesterol and LDL-cholesterol, were found to be increased in patients with levels of E2F2 and miR-34a-5p above average, compared with those with E2F2 or miR-34a-5p levels below average (Supplemental Figure S8E, http://links.lww.com/HEP/J965). Serum lipidomic analysis further showed that patients with higher levels of E2F2 and miR-34a-5p presented with dyslipidemia with increased levels of TG, DG, and PC subspecies, compared with patients with lower levels of E2F2 or miR-34a-5p (Supplemental Figure S8F, http://links.lww.com/HEP/J965).

E2F2 deficiency protects against cholestasis

Patients with MASLD and a cholestatic liver enzyme pattern are more likely to progress to liver decompensation events and mortality.[5] Indeed, higher levels of hepatic and serum bile acids have been reported in patients with advanced MASH.[6] Therefore, we queried whether E2F2 could also mediate liver injury induced via high concentrations of hepatic and circulating bile acids, namely cholestasis. For this, cholestasis was induced *in vivo* in *E2f2*$^{-/-}$ and WT mice by bile duct ligation (BDL) or by feeding a DDC-enriched diet. In both models, cholestasis increased E2F2 protein levels (Supplemental Figures S9A, B, http://links.lww.com/HEP/J965). In agreement with our previous results, miR-34a-5p expression was downregulated in *E2f2*$^{-/-}$ BDL mice and in *E2f2*$^{-/-}$ DDC mice (Figure 7A). This is

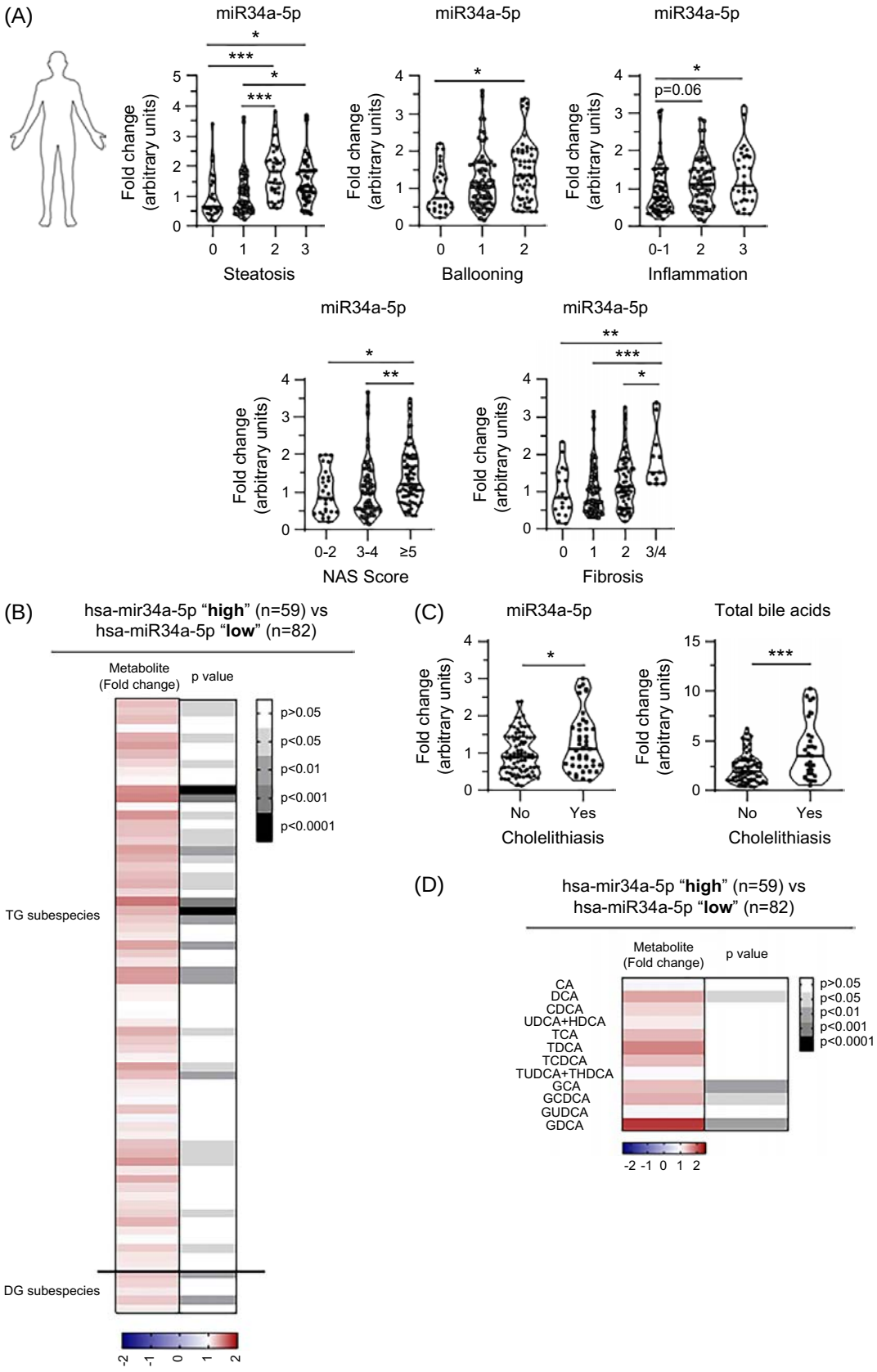

FIGURE 6 Liver miR-34a-5p is increased in patients with advanced MASLD. (A) miR-34a-5p levels in the livers of patients with different steatosis, ballooning inflammation, NAS score, and fibrosis grades. (B) Heatmap of the hepatic triglyceride (TG) and diglyceride (DG) subtypes of patients with MASLD and with miR-34-5p hepatic expression above the mean ("High") (n = 59) compared with those patients with miR-34-5p expression below the mean ("Low") (n = 82). (C) miR-34a-5p and bile acid levels in patients with or without cholelithiasis. (D) Heatmap of the

hepatic bile acids of patients with MASLD and with miR-34-5p hepatic expression above the mean ("High") (n = 59) compared with those patients with miR-34-5p expression below the mean ("Low") (n = 82). Significant differences are shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. The Spearman correlation coefficient is shown. Abbreviation: MASLD, metabolic dysfunction–associated steatotic liver disease; NAS, NAFLD activity score.

associated with lower ALT and AST levels (Figure 7B); decreased liver fibrosis by Sirius red staining (Figure 7C); and decreased expression of genes involved in fibrogenesis (Supplemental Figure S9C, http://links.lww.com/HEP/J965) and liver inflammation (Figure 7D). These results suggest that *E2f2*$^{-/-}$ mice are protected against high levels of hepatic and serum bile acids when compared with WT mice, even though the gene expression of the bile acid uptake transporter *Slc10a1* was increased (Supplemental Figure S9D, http://links.lww.com/HEP/J965). The analysis of the biliary lipids in liver and serum demonstrated that in both preclinical models of cholestasis, *E2f2* deficiency in mice resulted in decreased levels of free and esterified cholesterol in the liver (Supplemental Figure S9E, http://links.lww.com/HEP/J965) and serum (Supplemental Figure S9F, http://links.lww.com/HEP/J965), compared with the corresponding WTs. Similar results were obtained with the phospholipids (Supplemental Figure S9E, F, http://links.lww.com/HEP/J965).

To confirm that miR-34a-5p was involved in the protection that *E2f2*$^{-/-}$ mice exhibited when exposed to high levels of bile acids, miR-34a-5p was overexpressed in primary hepatocytes isolated from WT and *E2f2*$^{-/-}$ mice and challenged with bile acids. Results showed that hepatocytes from *E2f2*$^{-/-}$ mice were protected from inflammation, as shown by the decreased expression of *Il1b* and *Il10*, a phenomenon that was abrogated when miR-34a-5p was overexpressed (Figure 7E).

DISCUSSION

The global prevalence of metabolic disorders, including obesity, diabetes, and MASLD, is steadily increasing.[1,3] Although the exact mechanisms responsible for disease progression in certain individuals remain unclear, it is well established that an imbalance in lipid homeostasis within hepatocytes can lead to the accumulation of harmful lipids, a characteristic feature of MASH.[37] Intracellular accumulation of toxic bile acids can further destabilize cell membranes by solubilizing fatty acids, cholesterol, and phospholipids. In addition, circulating bile acid composition has been shown to be abnormal in MASH patients,[38] while the considerable heterogeneity of the pathology makes it challenging to find a specific treatment.[39] Still, a thyroid hormone receptor (THR)-beta selective agonist (Resmetirom) has recently been approved for the treatment of patients with MASH and advanced fibrosis.[40] Despite this progress, it is still necessary to investigate disease mechanisms involving the simultaneous reprogramming of different metabolic pathways, which should provide the basis for developing more holistic therapeutic strategies. For instance, research on nuclear PPAR receptors has led to the development of several PPAR agonists, such as lanifibranor (PAN-PPAR), which is still in clinical trials,[41] or saroglitazar, a new PPARα and PPARγ agonist[42] , which might be useful for MASLD. In this context, the involvement of other transcription factors in metabolic diseases should be explored.

We have previously shown that the E2F1 and E2F2 transcription factors are able to regulate metabolic pathways involved in the progression of MASLD to HCC.[16] Specifically, deficiency in E2F1 and/or E2F2 resulted in a new transcriptome where the expression of genes involved in mitochondrial generation, fatty acid transport to mitochondria and the electron transport chain, associated with increased FAO, prevented the tumorigenic lipid-rich environment, and the development of MASLD-associated HCC.[16] Here, we analyzed the role of the E2F2 transcription factor in MASH and cholestasis development; and identified miR-34a-5p as a new target of E2F2 that controls pathways involved in cholesterol and biliary metabolism in hepatocytes, hence promoting the progression of MASH and cholestasis. Further, we show that E2F2 is involved in cholestasis and liver inflammation, with both processes being mediated by miR-34a-5p.

Because patients with MASH and a more cholestatic liver enzyme profile experience higher liver decompensation events and mortality,[5] identifying specific therapeutic targets for this group of cholestatic-MASH is of great clinical importance. Moving toward personalized medicine, it is of high relevance to understand the metabolic heterogeneity of MASH. In this regard, demonstrating how modulation of a specific transcription factor affects several pathways involved in different MASH subtypes is of particular clinical interest. Herein, our results show that E2F2 modulates the transcriptome of genes involved in biliary metabolism. In particular, E2F2 ablation in mice promoted the synthesis and secretion of biliary lipids, while its specific overexpression in livers in vivo led to an opposite profile, highlighting a specific effect of E2F2 in modulating biliary lipid metabolism. In the absence of E2F2, the rearranged gene expression profile prevented cholesterol and bile acid storage in the liver,

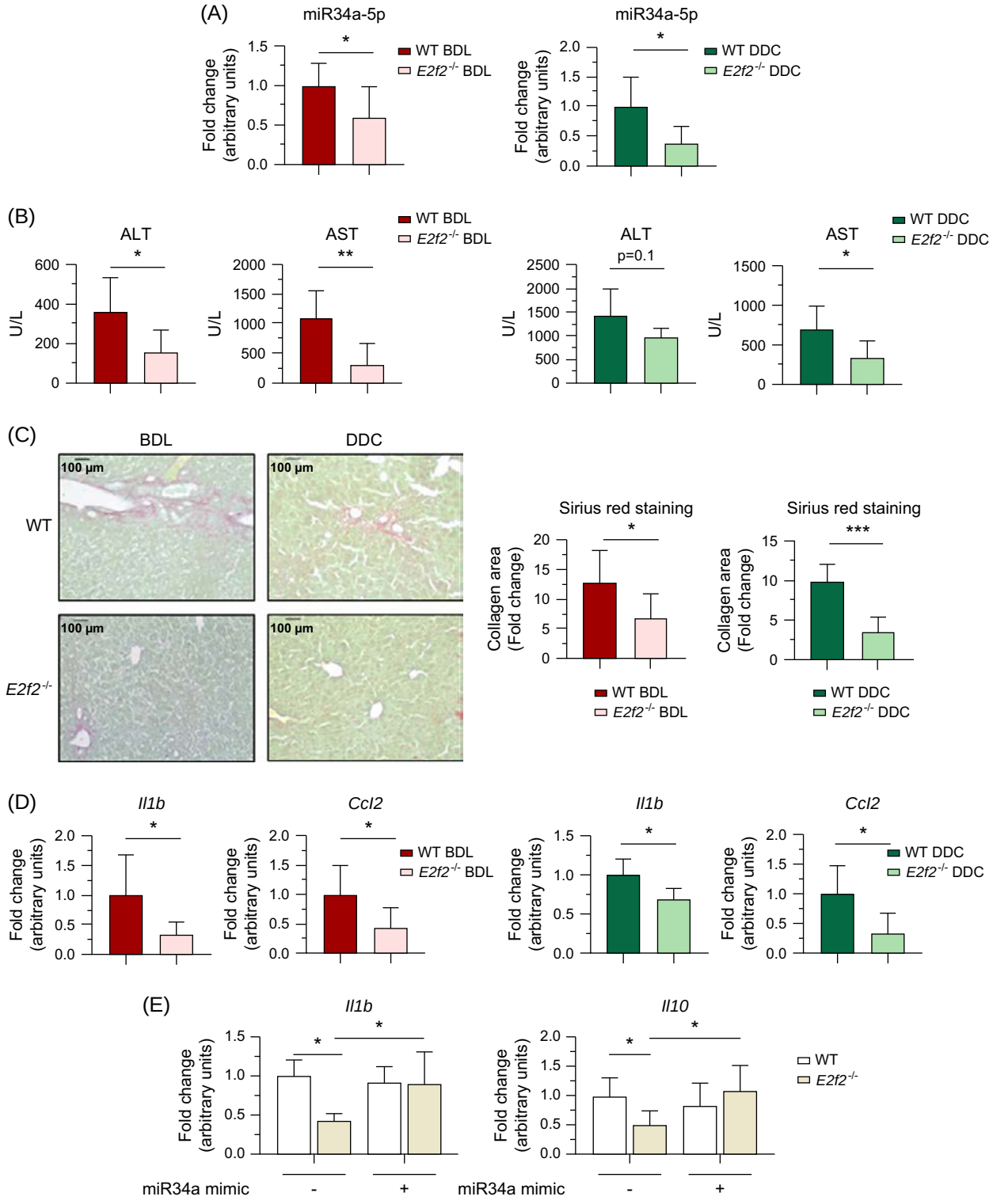

FIGURE 7 The transcription factor E2F2 promotes cholestasis. BDL was performed in male *E2f2*-knockout ($E2f2^{-/-}$) and wild-type (WT) mice, which were sacrificed 7 days later. Male $E2f2^{-/-}$ and WT mice were fed a 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) supplemented diet for 2 weeks. (A) Hepatic miR-34a-5p levels 7 days after BDL and 2 weeks after feeding a 0.1% DDC diet in WT and $E2f2^{-/-}$ mice (n = 5–8). (B) Transaminase levels 7 days after BDL and 2 weeks after feeding a 0.1% DDC diet in WT and $E2f2^{-/-}$ mice (n = 5–8). (C) Representative liver sections stained with Sirius red 7 days after BDL and 2 weeks after feeding a 0.1% DDC diet in WT and $E2f2^{-/-}$ mice (n = 5–8). (D) *Il1b* and *Ccl2* mRNA levels 7 days after BDL and 2 weeks after feeding a 0.1% DDC diet in WT and $E2f2^{-/-}$ mice (n = 5–8). (E) Hepatic *Il1b* and *Il10* levels in primary hepatocytes treated with 50 µM lithocholic acid (LCA) and 50 µM chenodeoxycholic acid (CDCA) with or without miR-34a-5p mimic (n = 4–5). Values represent mean ± SD. Significant differences are shown as $*p < 0.05$ and $**p < 0.01$. Abbreviation: BDL, biliary duct ligation.

protecting against experimental MASH. Furthermore, E2F2 deficiency not only promoted the secretion of biliary lipids but also protected the liver from cholestasis-induced inflammation. Thus, the lack of E2F2 plays a dual protective role, favoring bile acid secretion and preventing liver damage induced by its accumulation.

miRNA expression in DEN-HFD–fed mice was also strikingly distinct in *E2f2*$^{-/-}$ mice, resistant to MASLD-associated HCC, when compared with WT mice. Specifically, miR-34a-5p expression paralleled expression of E2F2 in hepatocytes or total liver, with subsequent experiments demonstrating that E2F2-mediated regulation of biliary metabolism and bile acid-mediated liver damage occurred, at least in part, via miR-34a-5p. Furthermore, miR-34a-5p is suggested to be a target of the E2F2 transcription factor not only during pathogenic conditions (ie, in 2 murine models of MASH) but also under normal physiologic conditions (ie, in healthy mice).

miR-34a-5p has been previously described as a modulator of biliary metabolism in metabolic diseases.[20,32] Despite its relatively low expression in hepatocytes, miR-34a-5p exerts a strong regulatory effect on lipid metabolism.[43] For instance, miR-34a-5p was shown to inhibit VLDL secretion and promote liver steatosis and hypolipidemia by targeting hepatocyte nuclear factor 4α.[44] Similarly, miR-34a-5p has been previously described to inhibit cholesterol synthesis and increase its efflux in hepatocytes.[20] miR-34a also modulates biliary metabolism through the regulation of SIRT1,[32] while its role in regulating *Cyp7a1* appears to be controversial; *Cyp7a1* has been described to be decreased in hepatocytes concomitantly with miR-34a-5p upregulation,[45] which is consistent with our observation that *Cyp7a1* is increased when E2F2 and miR-34a-5p are inhibited. By contrast, other studies have reported that miR-34a-5p downregulation decreases *Cyp7a1* expression.[20] These results suggest that there might be other factors involved in regulating bile acid metabolism during the progression of MASLD. In fact, our findings indicate that E2F2 inhibition has broader ameliorating effects on MASLD compared with inhibiting miR-34a alone. Not only that, but our results also show that miR-34a silencing may negatively feed back on E2F2, which could promote a worse metabolic scenario, by inducing lipid storage, among others. As such, and because we show that E2F2 behaves as a transcriptional activator of miR-34a, it could be more appealing to therapeutically target E2F2 compared with miR-34a. Targeting E2F2 in the context of MASLD would also prevent E2F-driven hepatosteatosis and the activation of cell cycle regulators, which typically promote MASH progression. In any case, miR-34a-5p could be particularly relevant in cholestasis-associated MASLD.

Our results further showed that the lack of E2F2 and miR-34a-5p during experimental MASH increases the synthesis and efflux of cholesterol in hepatocytes. Of note, the results showed that the E2F2–miR-34a-5p axis also regulates PC synthesis and secretion in bile, thus playing a pivotal role in protecting hepatocytes against bile acid-induced cytotoxicity.[46] In patients with obesity, higher hepatic expression of miR-34a-5p was associated with a new serum lipidome where increased levels of glycerolipid subtypes (TG and DG) and bile acids were found. A link between the miR-34a–SIRT1 axis, which is under the control of the FXR/SHP cascade pathway, and gallstones was previously suggested.[47] Here, we observed that miR-34a-5p was increased in patients with cholelithiasis and associated with higher levels of circulating bile acids, as previously described.[47,48] However, no changes in SIRT1 levels were found (data not shown). Changes in bile acid composition are characteristic of both MASLD and cholestatic liver diseases, which share several key pathophysiological mechanisms. Moreover, novel therapeutic approaches for cholestatic and fatty liver diseases currently under investigation are based on shared pathogenetic and therapeutic principles.[15]

We previously described that hepatic levels of E2F2 are increased in obese patients.[16] Here, we found that E2F2 levels positively correlated with steatosis grade and with ballooning; and that patients with increased levels of E2F2 and miR-34a-5p present with more dyslipidemia and higher ALT levels. However, our study was limited by the small number of liver biopsies (both the size of the liver biopsies and the number of patients) available to analyze protein expression levels of E2F2 and miR-34a-5p across the spectrum of MASLD severity.

Overall, the results obtained in preclinical models of MASH and in patients with obesity-related MASLD showed that the transcription factor E2F2 is an important regulator of cholestasis in MASLD. The dysregulation that it induces in biliary metabolism is mediated, at least in part, by miR-34a-5p. Thus, as a conclusion, this study identifies miR-34a-5p as a target of E2F2 and demonstrates the potential value of targeting the E2F2–miR-34a-5p axis, especially among patients who exhibit a cholestatic-MASH phenotype. These results open a new avenue for finding potential treatments for different MASH subtypes.

AUTHOR CONTRIBUTIONS

Conceptualization and design: Maider Apodaka-Biguri, Rui E. Castro, and Patricia Aspichueta; Funding acquisition: Patricia Aspichueta, Rui E. Castro, Xabier Buqué, Jesus M. Banales, and Luis Bujanda; Investigations and data analysis: Maider Apodaka-Biguri, André L. Simão, Francisco González-Romero, Daniela Mestre, Pedro M. Rodrigues, Igor Aurrekoetxea, Beatriz Gómez-Santos, Xabier Buqué, Ane Nieva-Zuluaga, Mikel Ruiz de Gauna, Idoia Fernandez-Puertas, Paul Gomez-Jauregui, Natalia Sainz-Ramirez, Kendall Alfaro-Jiménez, Ane Ortiz-Palma, Estibaliz Castillero,

Ainhoa Iglesias-Ara, Jone Mitxelena, Ainhoa Eriz, Ana M. Aransay, Juan-José Lozano, Jose J.G. Marin, Laura Izquierdo-Sanchez, Maria J. Perugorria, Luis Bujanda, Jesus M. Banales, César Martín, Lorena Mosteiro, Gaizka Errazti, Wing-Kin Syn, Luis Castaño, Ana M. Zubiaga, Rui E. Castro, and Patricia Aspichueta. Supervision: Patricia Aspichueta and Rui E. Castro; Writing first draft: Maider Apodaka-Biguri, Rui E. Castro, and Patricia Aspichueta. Review and editing: all the coauthors.

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CONFLICTS OF INTEREST

Jesus M. Banales consults for and is on the speakers' bureau for AstraZeneca. He consults for and received grants from Albireo and Cymabay. He is on the speakers' bureau and received grants from Incyte. He consults for Ipsen, Jazz, Servier, and OWL. He is on the speakers' bureau for Eisai and Advanz. Wing-Kin Syn consults for Merck and Ipsen. He received grants from Zydus and 89Bio. The remaining authors have no conflicts to report.

ORCID

Maider Apodaka-Biguri https://orcid.org/0000–0002–8459–4752
Patricia Aspichueta https://orcid.org/0000–0002–3553–1755

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