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E2F1 Is a Novel Fibrogenic Gene That Regulates Cholestatic Liver Fibrosis Through the Egr-1/SHP/EID1 Network

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E2F transcription factor 1 (E2F1) is an important regulator of metabolic diseases; however, its role in liver function remains elusive. This study unraveled a regulatory cascade involving E2F1, early growth response-1 (Egr-1), nuclear receptor small heterodimer partner (SHP, NR0B2), and EIA-like inhibitor of differentiation 1 (EID1) in cholestatic liver fibrosis. Liver E2F1 messenger RNA (mRNA) and protein expression was strongly up-regulated in human nonalcoholic steatohepatitis (NASH) and alcohol cirrhosis; the latter was inversely correlated with diminished SHP expression. E2F1 was also highly induced by 3,5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) feeding and bile-duct ligation (BDL) in mice. *E2F1*$^{-/-}$ mice exhibited reduced biliary fibrosis by DDC as determined by Masson Trichrome and Picro Sirius red staining, and decreased serum bile acid (BA), BA pool size, and fecal BA excretion. In addition, cholestatic liver fibrosis induced by BDL, as determined by immunohistochemistry analysis of a1 collagen expression, was increased in *SHP*$^{-/-}$ mice but attenuated in hepatocyte *SHP*-overexpressed transgenic (STG) mice. Egr-1 exhibited marked induction in livers of *SHP*$^{-/-}$ mice compared to the wild-type mice in both sham and BDL groups, and reduction in STG livers. Egr-1 promoter was activated by E2F1, and the activation was abrogated by expression of SHP and its co-repressor EID1 in hepatoma cells Huh7, Hepa1, and stellate cells LX2. Chromatin immunoprecipitation assays further confirmed the association of E2F1, SHP, and EID1 proteins with the Egr-1 promoter, and their direct protein interactions were determined by glutathione S-transferase pull-down assays. Interestingly, E2F1 activated Egr-1 expression in a biphasic fashion as described in both human and mouse hepatocytes. *Conclusion:* E2F1 is a fibrogenic gene and could serve as a potential new diagnostic marker for nonalcoholic and alcoholic liver fibrosis/cirrhosis. (HEPATOLOGY 2014;60:919-930)

Cholestatic liver injury has been shown to be an important stimulus for the development of fibrosis.[1] Cholestasis results in intrahepatic accumulation of cytotoxic bile acids which ultimately leads to fibrosis and cirrhosis, the latter is the advanced stage of fibrosis.[2] Hepatic fibrosis is a scarring process of the liver that includes components of both increased and altered deposition of extracellular matrix (ECM) and reduced breakdown of ECM components.[3,4] The progression of fibrosis induced by bile duct ligation (BDL), alpha smooth muscle actin (α-SMA), and type I collagen are highly expressed by hepatic stellate cells (HSCs), which are classically considered as indicators and markers of cell activation to myofibroblast-like cell. In addition, fibrogenic cells can also derive from portal fibroblasts,[5] circulating fibrocytes,[6] bone marrow,[7] and epithelial-mesenchymal cell transition.[8]

Abbreviations: α*-SMA, alpha smooth muscle actin; BA, bile acid; BDL, bile-duct ligation; CA, cholic acid; CDCA, chendeoxycholic acid; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; Egr-1, early growth response gene 1; EID1, EIA-like inhibitor of differentiation 1; FXR, farnesoid X receptor; HSC, hepatic stellate cell; LPS, lipopolysaccharide; NASH, nonalcoholic steatohepatitis; RGZ, rosiglitazone; SHP, small heterodimer partner.*

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The E2F family of transcription factors play crucial roles in controlling the expression of genes involved in cell proliferation, differentiation, and apoptosis. E2F1, the most characteristic member of the E2F family, was originally classified as activator of transcription, which binds to DNA and regulates the expression of genes involved in cell cycle progression.[9,10] In addition to its well-known function in cell death and proliferation, an emerging role of E2F1 in regulation of metabolism has been recently identified.[11,12] Increased basal metabolism and resistance to fatigue were reported in *E2f1*$^{-/-}$ mice.[13] Despite these advances, the function of E2F1 in bile acid metabolism and liver fibrosis has never been studied.

Bile acid homeostasis is controlled by coordinated feedback and feedforward regulation of genes involved in bile acid uptake, efflux, and biosynthesis.[2] Nuclear receptor small heterodimer partner (*SHP*, NR0B2) is a crucial component in the negative feedback regulation of bile acid synthesis.[14] *SHP*$^{-/-}$ mice appeared less sensitive to bile acid-induced liver damage[15]; however, they were more prone to BDL-induced cholestasis.[16] The differential responses of *SHP*$^{-/-}$ mice to bile acid feeding and BDL suggest a complex mechanism controlling cholestatic liver injury, which may involve the regulation of unidentified transcription factors. However, the underlying molecular basis by which SHP regulates cholestatic liver fibrosis remains largely unknown.

Zinc finger transcription factor early growth response-1 (Egr-1) is mainly expressed in hepatocytes and to a less extent in other nonparenchymal cells in the liver. The expression of Egr-1 is increased in patients with cholestatic liver disease[17] and is induced by BDL in mice.[18] Induction of Egr-1 by bile acids contributes to the up-regulation of chemokines and hepatic neutrophil accumulation,[18] and Egr-1-deficiency reduces liver inflammation and injury by BDL. A potential significance of Egr-1 in human fibrotic/cirrhotic livers was established[19]; however, its transcriptional regulation remains elusive.

In the present study we identified E2F1 as a novel fibrogenic gene, which interacts with SHP and its co-repressor EID1 to control Egr-1 expression and cholestatic liver fibrosis. Because E2F1 was induced in human cirrhotic livers to a much higher extent as compared to α-SMA and α1-collagen, we propose that E2F1 could be considered a new biomarker for liver fibrosis and cirrhosis.

Materials and Methods

Human Liver Specimens. Human liver specimens were obtained through the Liver Tissue Procurement and Distribution System (Minneapolis, MN) and have been described.[19,20] In brief, the human biospecimens were obtained from liver explants taken during time of surgery from patients with nonalcoholic steatohepatitis (NASH) cirrhosis, alcohol cirrhosis, and donor livers histologically diagnosed as normal livers. Institutional Review Board approval was obtained from the University of Utah Human Subjects Committee.

Animal Treatment. *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6 (wild-type, WT), *SHP*$^{-/-}$ (SKO), SHP nontransgenic control (NC), hepatocyte specific SHP transgenic (STG), farnesoid X receptor (*FXR*$^{-/-}$) mice were described previously.[14,21,22] The treatment of mice with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) supplemented diet, BDL, and FXR synthetic ligand GW4064 has been described previously.[19,20,22] Protocols for animal use were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center and University of Utah.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR). Total RNA isolation and complementary DNA (cDNA) synthesis were conducted with command standard methods. The specific primers for quantitative polymerase chain reaction (qPCR) are shown in Supporting Table S1.

Histological Analysis of Liver Sections. Sections were stained with hematoxylin and eosin (H&E) for evaluation of morphology, Masson Trichrome, Picro Sirius red, and type I collagen immunostaining for fibrosis[19] as described in the Supporting Material.

Analysis of Serum Bile Acid (BA), BA Pool Size, and Fecal BA Extraction. The detailed methods have been described[15] and are provided in the Supporting Material.

Cell Culture and Promoter Activity Assays. The transfection of mouse Egr-1 promoter luciferase reporter (Egr-1 Luc) and expression plasmids[19] in

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human hepatoma cell line Huh7 (Health Science Research Resources Bank JCRB0403), mouse hepatoma cell line Hepa1 (ATCC CRL-1830), and human stellate cell line LX2 cells (a kind gift from Dr. Scott Friedman) are described in the Supporting Material.

Chromatin Immunoprecipitation (ChIP) Assays. ChIP assays were performed in Hepa1 cells following procedures described elsewhere[19,23] and described in the Supporting Material. Three sets of primers (Table S1) were designated P1 and P2 specific for the E2F1 binding sites on mouse Egr-1 promoter, whereas P3 was located 2 kb upstream from TSS thus served as a negative control.

Glutathione S-Transferase (GST) Pull-Down and Western Blotting. Detailed methods can be found in our previous publication[24] and are described in the Supporting Material.

Primary Hepatocytes Isolation and Adenovirus Infection. Primary hepatocytes were isolated as described.[25] Adenovirus infection is described in the Supporting Material.

Statistics Analysis. All the experiments were done in triplicate and repeated at least three times. The data are presented as the mean values ± standard error of the mean (SEM). Statistical analysis was carried out using Student *t* test for unpaired data to compare the values between the two groups; $P < 0.05$ was considered statistically significant.

Results

E2F1 Is Induced in Human Cirrhotic Liver. Cirrhosis is the advanced stage of fibrosis. To assess the potential clinical relevance of E2F1 alteration in liver diseases, we examined E2F1 expression in human cirrhotic livers. qPCR revealed a strong induction of E2F1, Egr-1, α-SMA, and α1-collagen messenger RNA (mRNA) and reduction of SHP mRNA in eight NASH cirrhotic livers versus five normal livers (Fig. 1A). The extent of E2F1 induction was much higher than the commonly used fibrotic markers α-SMA and α1-collagen (Fig. 1B). E2F1 mRNA was also significantly elevated in 17 alcohol cirrhosis livers as compared to an additional 13 normal liver specimens (Fig. 1C). The protein levels of E2F1 showed consistent impressive elevation in NASH and alcohol cirrhosis versus controls, similar to its mRNA (Fig. 1D). The basal levels of E2F1 protein in normal livers were detected upon a longer exposure time (not shown). The results suggest that E2F1 up-regulation is a common phenomenon in human fibrotic/cirrhotic livers.

Liver Fibrosis Induced by DDC Is Diminished in E2F1$^{-/-}$ Mice. To further evaluate the function of E2F1 in liver fibrosis, we used a DDC induced biliary type liver fibrosis model. Cholestatic phenotype in this model was related to the induction of a reactive phenotype of bile duct epithelial cells with development of bile duct injury, leading to portal-portal fibrosis and large duct disease.[26]

The gross architecture of the *E2F1*$^{-/-}$ liver was normal, with no overt defects in hepatocyte morphology. When challenged with the DDC diet for 6 weeks, *E2F1*$^{+/+}$ mice showed collagen deposition and periductal portal-portal fibrosis as revealed by both Masson Trichrome staining and Picro Sirius Red staining (Fig. 2A; Supporting Fig. S1). The liver fibrosis induced by DDC was further confirmed by induction of fibrogenic genes α-SMA and α1-collagen mRNA (Fig. 2B). In contrast, a diminished liver fibrosis was observed in *E2F1*$^{-/-}$ mice (Fig. 2A; Fig. S1). Notably, liver E2F1 mRNA was markedly increased by DDC or BDL in *E2F1*$^{+/+}$ mice (Fig. 2B), indicating a biological relevance of E2F1 alteration in cholestatic liver diseases. DDC also induced Egr-1 protein in *E2F1*$^{+/+}$ mice (Fig. 2B), consistent with the induction of E2F1 by DDC.

We next determined whether BA homeostasis was altered by E2F1 deficiency. Compared to *E2F1*$^{+/+}$ mice, serum total BA level, BA pool size, and fecal BA excretion were decreased in *E2F1*$^{-/-}$ mice (Fig. 2C). The inflammatory genes tumor necrosis factor alpha (TNFα) and intercellular adhesion molecule 1 (ICAM1) showed slight down-regulation in *E2F1*$^{-/-}$ mice but were similarly induced by DDC in *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ mice (Fig. 2D). The induction of chemokine (C-C motif) ligand 2 (CCL2) by DDC, on the other hand, was markedly diminished in *E2F1*$^{-/-}$ mice compared with *E2F1*$^{+/+}$ mice. Overall, these results demonstrated the *in vivo* physiological relevance of E2F1 in controlling BA metabolism and cholestatic liver fibrosis.

BDL-Induced Liver Fibrosis Is Attenuated by Overexpressing SHP. Early studies showed that the *SHP*$^{-/-}$ mice were more sensitive to BDL-induced cholestasis.[16] However, the underlying mechanism by which SHP regulates cholestasis-induced liver fibrosis remains unknown. To further assess the E2F1/SHP/Egr1 regulatory network in cholestatic liver fibrosis, we conducted BDL in SKO and the newly developed hepatocyte specific SHP transgenic (STG) mice. Despite no obvious necrotic appearance on the liver surface, histologic examination of the livers showed more severe disseminated liver cell necrosis and bile infarcts in SKO than in WT mice in BDL (Fig. 3A, left). In contrast, the

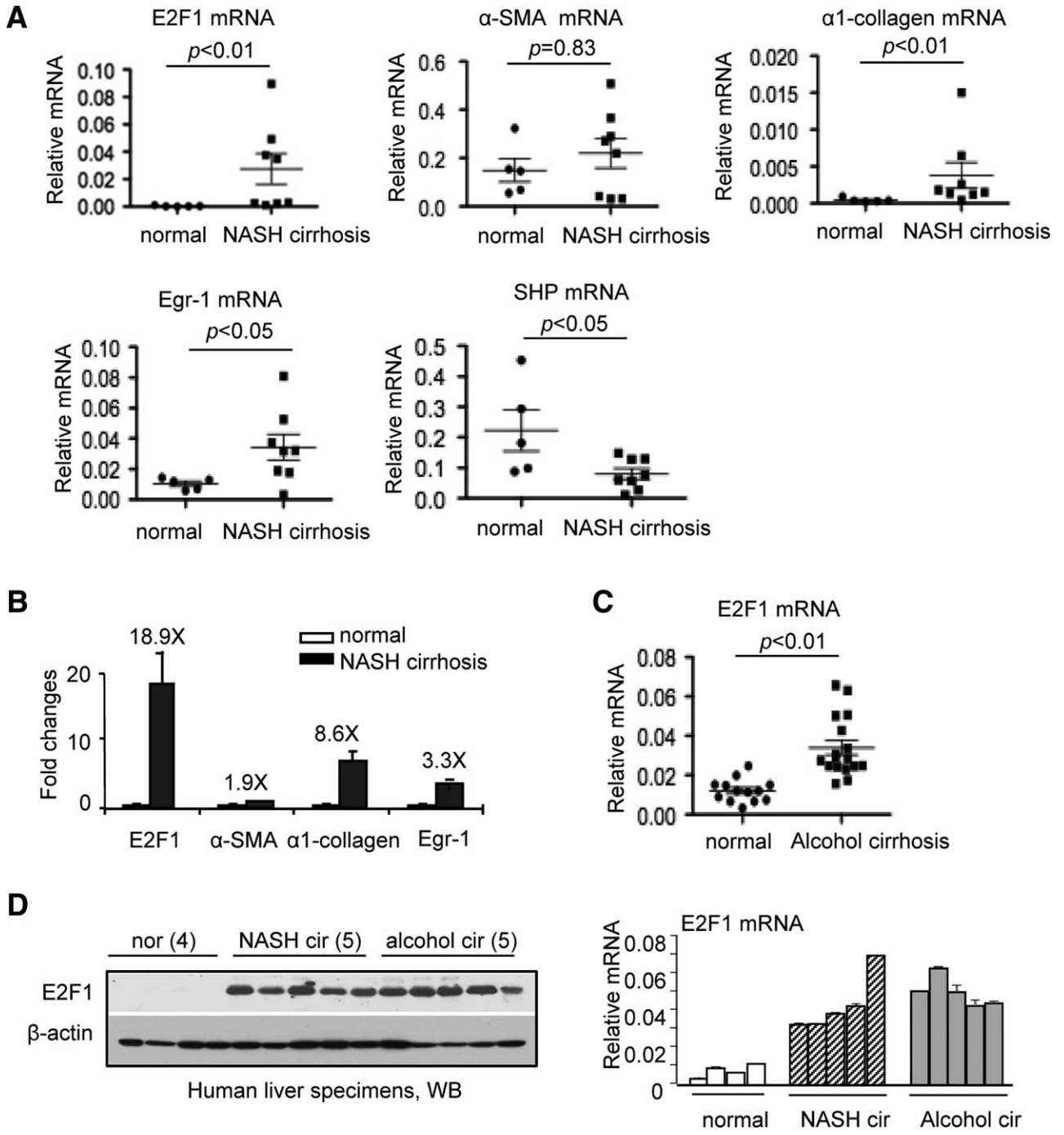

Fig. 1. Gene expression of E2F1, α-SMA, α1-collagen, Egr-1, and SHP in human cirrhotic livers. (A,B) qPCR of E2F1, α-SMA, α1-collagen, Egr-1, and SHP mRNA in five normal (nor) and eight NASH cirrhotic (cir) human livers. The average of gene induction relative to normal livers is shown in B. (C) qPCR of E2F1 mRNA in 13 normal (nor) and 17 alcohol cirrhotic (cir) human livers. (D) Western blots (WB) of E2F1 protein and qPCR of E2F1 mRNA in four normal, five NASH cirrhotic, and five alcohol cirrhotic human liver specimens.

liver damage appeared to be less severe in STG BDL compared to the NC BDL (Fig. 3A, right).

Type I collagen is the most abundant collagen of mature scar tissue. Hepatic fibrosis induced by BDL is unique in that the primary pathological lesions occur surrounding the bile duct epithelium. As expected, BDL-induced hepatic fibrotic lesions were evident in all four types of mice. In sham animals, the deposition of hepatic type I collagen was similar between WT and NC livers. However, SKO livers displayed stronger staining for type I collagen, which was to a less extent in STG livers (Fig. 3B; Fig. S2). The data suggest that

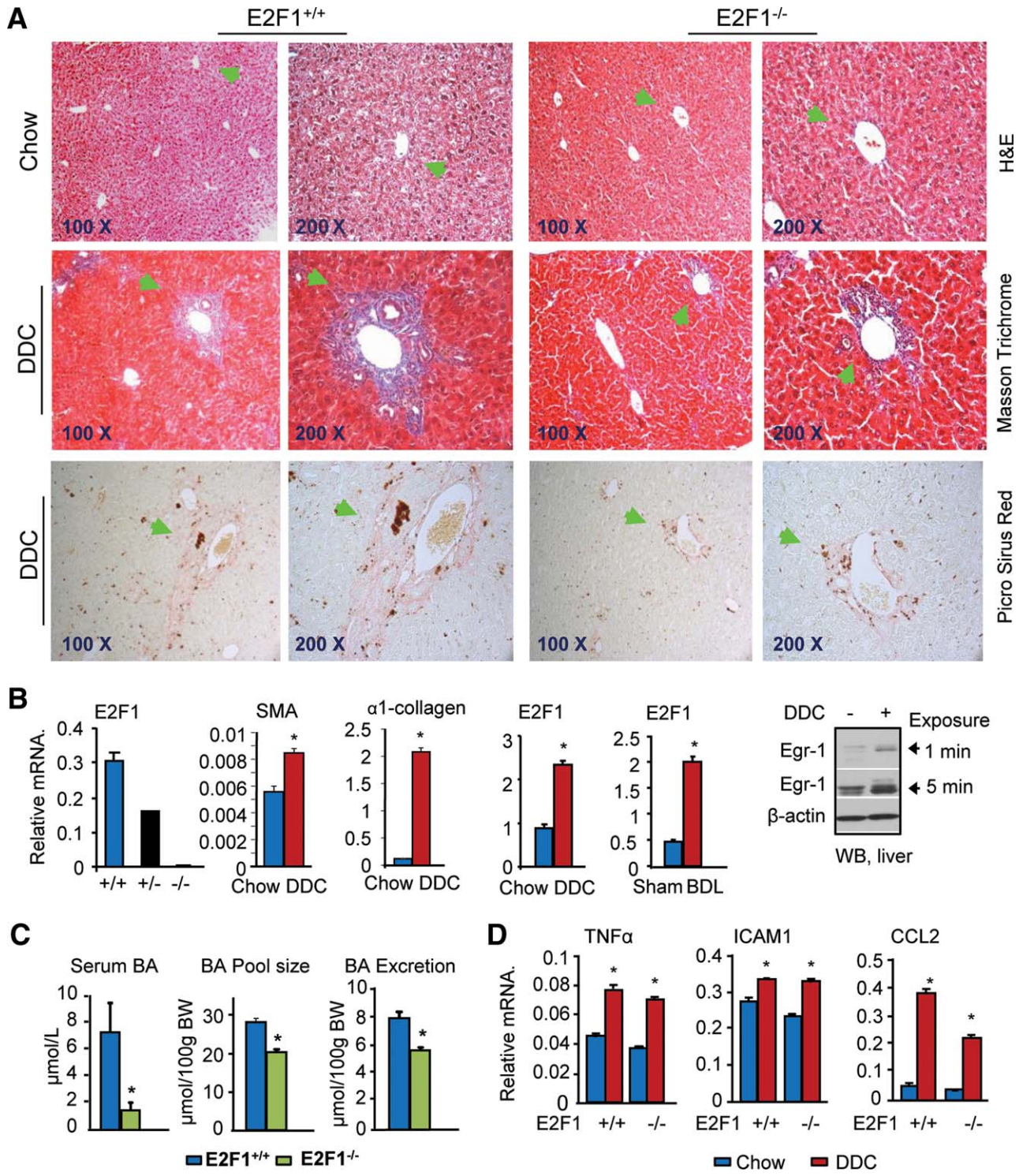

Fig. 2. *E2F1*$^{-/-}$ mice exhibited diminished liver fibrosis by DDC diet and altered bile acid homeostasis. (A) H&E, Masson Trichrome and Picro Sirius Red staining of collagen in liver sections from *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ mice fed with a DDC diet. (B) Left: qPCR of E2F1 mRNA in *E2F1*$^{+/+}$, *E2F1*$^{+/-}$, *E2F1*$^{-/-}$ livers. Middle: qPCR of SMA, α1-collagen, and E2F1 in wild-type mice subjected to DDC or BDL. Right: western blot of Egr-1 protein in wild-type mice fed a DDC diet. *$P < 0.01$ versus corresponding chow or sham control. (C) Analysis of serum BA concentration (left), BA pool size (middle), and fecal BA extraction (right) in *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ mice. *$P < 0.01$ versus *E2F1*$^{+/+}$. (D) qPCR of inflammatory markers in *E2F1*$^{+/+}$ and *E2F1*$^{-/-}$ mice fed with control or DDC diet. *$P < 0.01$ versus chow.

loss of SHP results in severe liver fibrosis response to BDL; in contrast, elevated levels of SHP in liver ameliorate hepatic biliary fibrosis caused by BDL.

In parallel, elevated levels of serum alanine aminotransferase (ALT) were observed in SKO mice after BDL (Fig. S3A, left), which corresponded to the

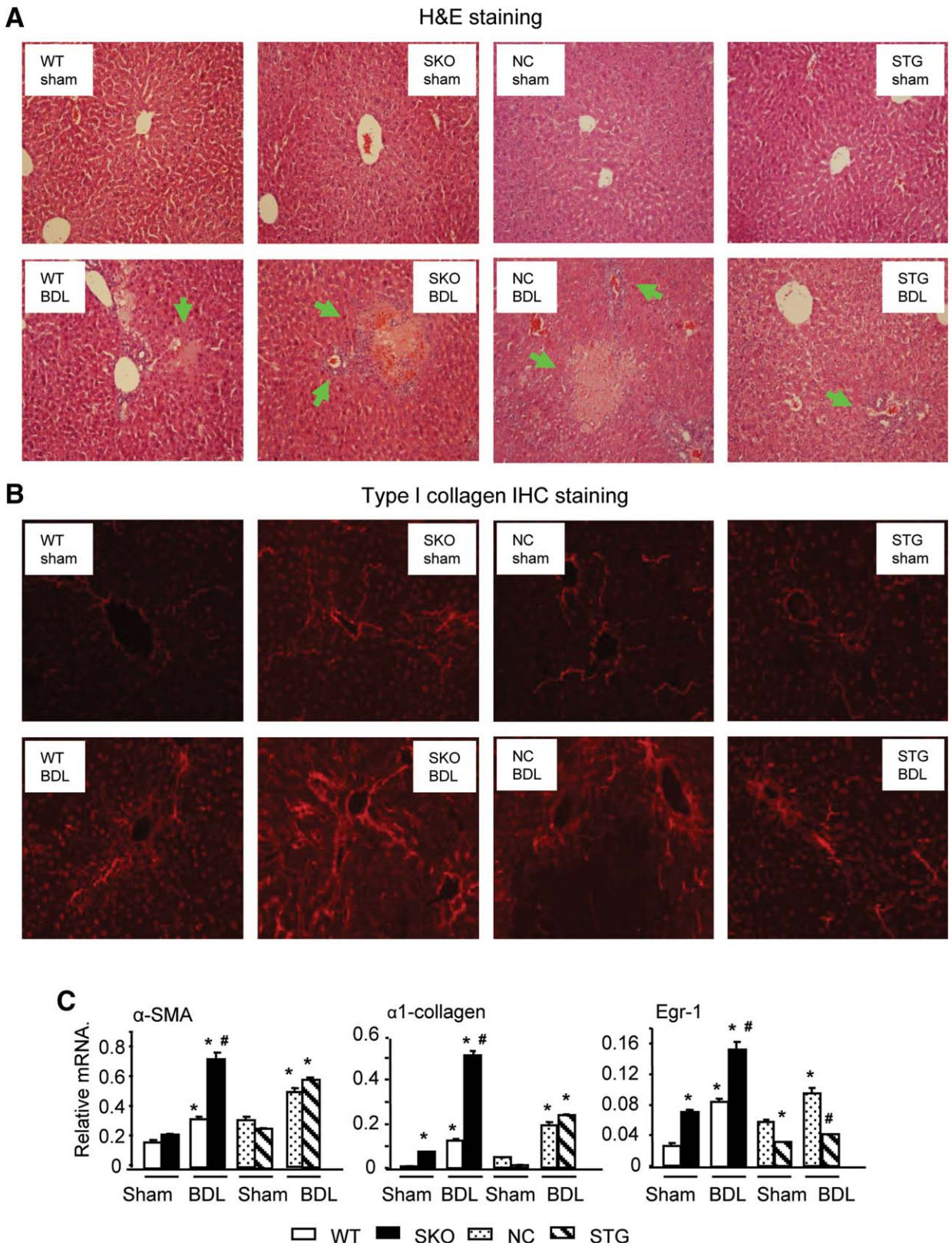

Fig. 3. Histological analysis of collagen accumulation and qPCR analysis of gene expression in livers from mice subjected to bile duct ligation (BDL). (A) H&E staining of liver sections. Arrows indicate regions of coagulative hepatocyte necrosis. (B) Type I collagen immunohistochemistry (IHC) staining of liver sections. The quantified results are presented in Fig. S1. (C) qPCR of α-SMA, α1-collagen, and Egr-1 gene expression from sham and BDL operated mice. Liver RNA was isolated from sham or BDL operated WT and SKO, NC and STG mice (n = 5-10). Data are expressed as means ± SEM. $*P < 0.01$ versus corresponding sham; $\#P < 0.01$ versus corresponding BDL.

increased severity of fibrosis in these mice. In contrast, serum ALT contents were moderately reduced in STG mice versus NC controls after BDL (Fig. S3A, right). The levels of total serum BA, triglycerides, cholesterol, and free cholesterol were increased in SKO mice after BDL, although no difference was seen between the NC and STG mice (Fig. S3B). Consistently, the sera of SKO mice appeared to be more yellowish than the WT mice, which was indicative of increased lipid accumulation (Fig. S3C).

We next determined the relative mRNA abundance of fibrogenic genes in mouse livers by qPCR. Both α-SMA and α1-collagen mRNA levels were significantly increased in WT BDL livers, as well as in sham-operated SKO livers, and were further induced to a much greater extent in SKO BDL livers (Fig. 3C, left and middle), which was consistent with the results of type I collagen staining. Surprisingly, the expression of α-SMA and α1-collagen was slightly decreased in sham STG livers compared to NC controls but was increased to a similar extent in BDL models from STG and NC mice. Because SHP was specifically overexpressed in the hepatocytes of STG mice, one possible explanation could be the minimal effect of SHP on the regulation of α-SMA and α1-collagen expression in stellate cells. Of particular interest, Egr-1 mRNA was induced in WT BDL liver (Fig. 3C, right), which was consistent with induction of E2F1 by BDL (Fig.2B). Importantly, Egr-1 exhibited a marked induction in SKO livers compared to the WT controls in both sham and BDL groups, and a reduction in STG livers before and after BDL as compared to the NC controls. The data strongly suggest that the transcription of Egr-1 is negatively regulated by SHP in cholestatic liver fibrosis.

E2F1 Activates Egr-1 Promoter Which Is Repressed by SHP and EID1. The potential significance of Egr-1 in human fibrotic/cirrhotic livers has been reported; however, its transcriptional regulation still remains elusive.[19] We next focused our effort to determine whether Egr1 is a direct target of E2F1. We searched transcription factor binding motifs and identified multiple potential sites for E2F1 within the Egr-1 gene 5′ flanking region (Fig. 4B). Nuclear receptor SHP has been shown to repress Egr1 transcription.[19] To determine the role of E2F1 in crosstalk with SHP in regulating Egr-1 promoter activity, Huh7 cells were cotransfected with the Egr-1 promoter luciferase reporter and expression plasmids for E2F1, SHP, and EID1. Ectopic expression of E2F1 markedly activated Egr-1 promoter in a dose-dependent manner (Fig. 4A, left). Coexpression of SHP with E2F1 did not inhibit E2F1 activation in Huh7 cells, but enhanced the inhibitory effect of EID1 (Fig. 4A, middle) in human stellate LX2 cells (Fig. 4A, right).

The ChIP assay was commonly used to determine protein association with target gene promoter. Due to the lack of specific antibodies for SHP and EID1, this experiment was done using an overexpression cell system. Hepa1 cells were transfected with Flag-E2F1, GFP-SHP, and HA-EID1 and chromatin was analyzed for the recruitment of each protein to the Egr-1 promoter. Two sets of PCR primers specific for the Egr-1 promoter containing E2F1 site enriched region (P1 and P2) were designed and a third primer set (P3) covering a region that does not contain E2F1 binding motif was used as a negative control (Fig. 4B, upper). The recruitment of E2F1 was markedly enriched to the Egr-1 promoter region that was amplified by P1 but not by P2 and P3 primers (Fig. 4B, lower). The binding of E2F1 was decreased by EID1 alone or together with SHP (P1, IP: Flag, 32× cycles). In contrast, SHP or EID1 binding to the Egr-1 promoter was not affected by the presence of E2F1 (P1, IP: GFP and IP: HA, 35× cycles). Therefore, EID1 and SHP inhibit E2F1 activity through reducing its binding to the Egr-1 promoter.

We next propose that SHP and EID1 proteins interact directly with E2F1 protein to repress its transactivation of the Egr-1 promoter. Because SHP is known to interact with EID1,[27] we employed GST pull-down assays to determine the protein-protein interactions between E2F1 and SHP or E2F1 and EID1. E2F1 was observed to directly interact with SHP interaction domain (Int, 2) and repression domain (Rep, 3) (Fig. 4C). Interestingly, the strongest interaction of E2F1 with SHP was observed when both the Int and Rep domains of SHP were present (2+3 versus 2 or 3). The interaction between E2F1 and SHP appeared to be weaker in the presence of N-terminal of SHP (FL, full length) as compared with N-terminal deleted SHP (FL versus 2+3). E2F1 also showed a strong interaction with both mouse and human EID1 protein (Fig. 4D). Deletion of a putative heptad repeat domain LXCXE in the C-terminal region of mEID1 did not affect E2F1-EID1 interaction. Taken together, these results identified SHP and EID1 as new E2F1 interaction proteins.

Induction of Egr-1 by E2F1 Is Inhibited by SHP and EID1. In agreement with its activation of Egr-1 promoter, E2F1 overexpression induced Egr-1 mRNA and protein in both Hepa1 and Huh7 cells (Fig. 5A,B). It was noted that the highest level of E2F1 protein correlated with a low level of Egr-1 protein

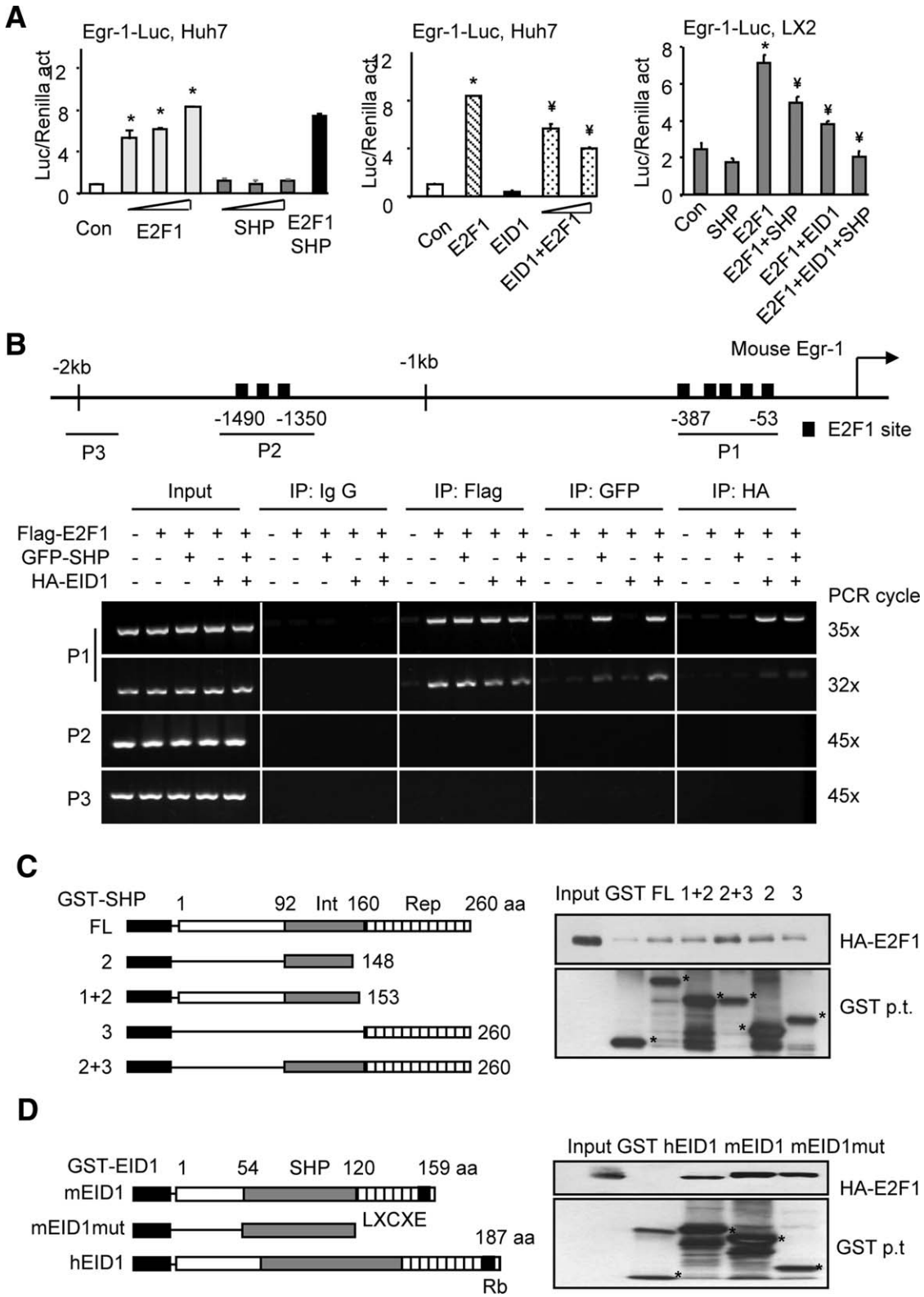

Fig. 4. E2F1 activates Egr-1 promoter that is inhibited by SHP and EID1. (A) Left: Egr-1-Luc reporter assay in Huh7 cells transfected with Egr-1-Luc and plasmids expressing E2F1 and SHP alone or in combination. Middle: Huh7 cells were transfected with Egr-1-Luc and plasmids expressing E2F1 and EID1 alone or in combination. Right: Egr-1-Luc activity regulation by SHP, E2F1, and EID1 in human stellate LX2 cells. $^{*}P < 0.01$ versus con; $^{¥}P < 0.01$ versus E2F1. (B) ChIP assays to determine the association of E2F1, SHP, and EID1 to the Egr-1 promoter in Hepa1 cells. P1 and P2 primers could detect Co-IP of each protein on Egr-1 promoter, whereas P3 was located 2 kb upstream from TSS thus served as a negative control. (C,D) GST pull-down assays to determine *in vitro* interaction of E2F1 with SHP (C) and EID1 (D). HA-E2F1 was *in vitro* translated and used to interact with GST fusion proteins that were expressed from bacterial *Escherichia coli* BL21/DE3/RIL.

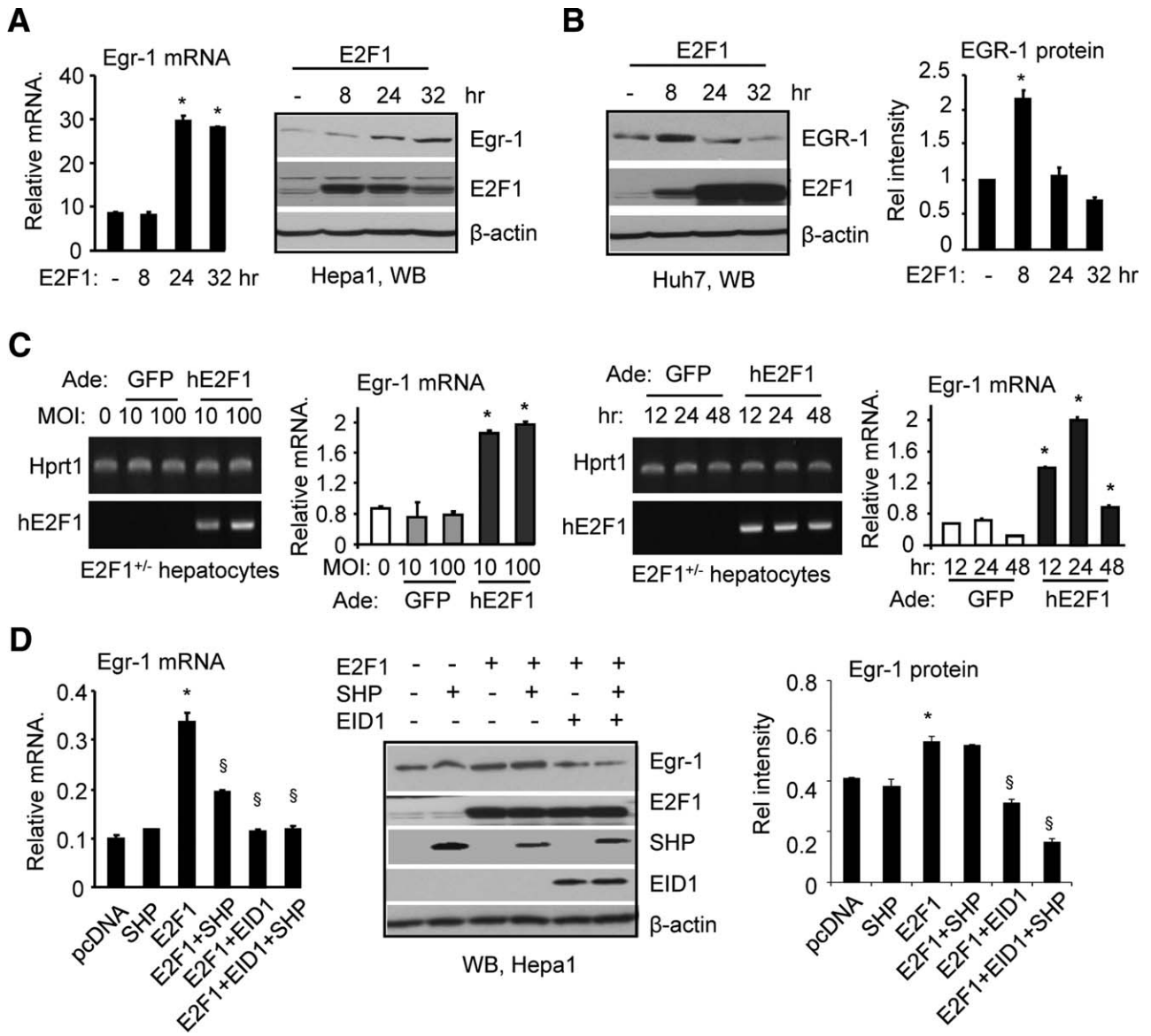

Fig. 5. E2F1 induces Egr-1 expression that is repressed by SHP and EID1. (A) qPCR of Egr-1 mRNA and western blot (WB) of Egr-1 protein in Hepa1 cells overexpressed with E2F1. (B) WB and quantitative analysis of Egr-1 protein in Huh7 cells overexpressed with E2F1. (C) RT-PCR of E2F1 mRNA and qPCR of Egr-1 mRNA in *E2F1*$^{+/-}$ hepatocytes transduced with human E2F1 adenovirus (Ade). *$P < 0.01$ versus GFP. (D) qPCR of Egr-1 mRNA (left) and WB of Egr-1 protein (middle) in Hepa1 cells transfected with E2F1, SHP, and EID1 expression vectors alone or in combination. Quantitative analysis of the band intensity is shown in the right panel. *$P < 0.01$ versus con; §$P < 0.01$ versus E2F1.

(8 hours), and the lowest level of E2F1 with the highest level of Egr-1 protein (32 hours) in Hepa1 cells (Fig. 5A). Similarly, Egr-1 protein was induced by a low level of E2F1 at 8 hours, but gradually decreased as the E2F1 level reached the highest by 32 hours in Huh7 cells (Fig. 5B). Although the maximal expression of E2F1 took longer in Huh7 cells, a biphasic response of Egr-1 to E2F1 was observed clearly in both cell lines. To extend our findings beyond cell lines, we overexpressed human E2F1 in *E2F1*$^{+/-}$ primary hepatocytes using adenovirus. Egr-1 mRNA was significantly induced by E2F1 at both low multiplicity of infection (MOI) 10 and high MOI 100 (Fig. 5C, left). Interestingly, Egr-1 mRNA was induced by E2F1 at 12 hours, reached maximal levels by 24 hours, and then drastically decreased at 48 hours (Fig. 5C, right). It should be noted that although Egr-1 is a known early response gene, its expression pattern regulated by E2F1 cannot be simply regarded as an early response phenomenon. Overall, these results suggest a unique biphasic regulation of Egr-1 by E2F1 in a time-dependent fashion.

We next assessed the regulatory role of SHP and EID1 on the activation of Egr-1 gene by E2F1.

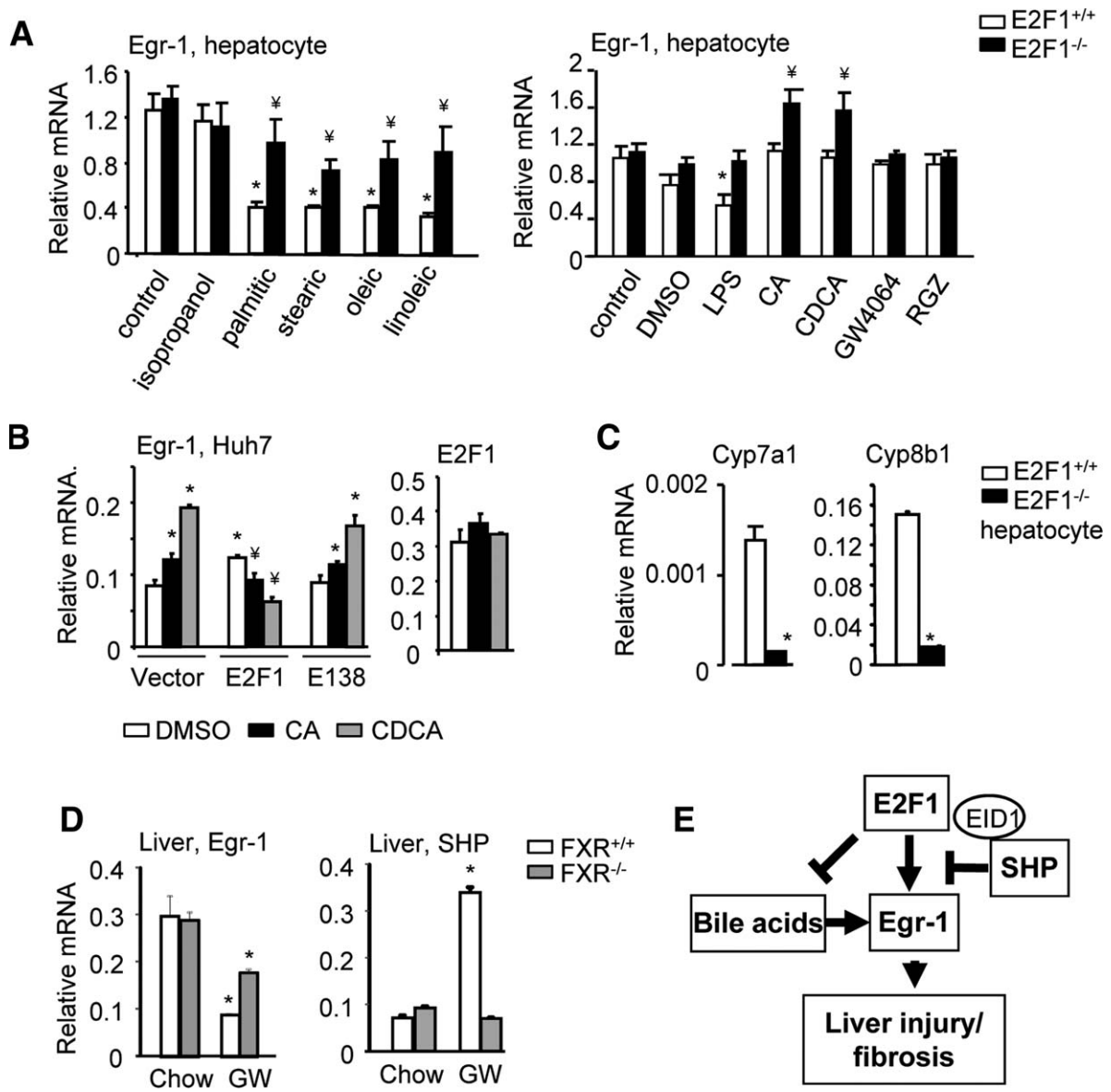

Fig. 6. Egr-1 expression regulation by E2F1, SHP, and FXR. (A) qPCR of Egr-1 mRNA in $E2F1^{+/+}$ and $E2F1^{-/-}$ hepatocytes treated with LPS (10 μg/mL), CA (100 μM), CDCA (100 μM), GW4064 (0.1 μM), RGZ (10 μM), and solvent 0.1% dimethyl sulfoxide (DMSO), or palmitic acid (100 μM), stearic acid (100 μM), oleic acid (100 μM), linoleic acid (100 μM), and solvent 1% isopropanol. $^{*}P < 0.01$ versus solvent; $^{¥}P < 0.01$ versus $E2F1^{+/+}$ hepatocyte. (B) qPCR of E2F1 and Egr-1 mRNA in Huh7 cells transfected with plasmid E2F1 or E138, an E2F1 mutant with no DNA binding activity for 24 hours, then incubated with CA (100 μM) or CDCA (100 μM) for an additional 24 hours. $^{*}P < 0.01$ versus vector DMSO; $^{¥}P < 0.01$ versus vector treatment. (C) qPCR of Cyp7a1 and Cyp8b1 mRNA in $E2F1^{+/+}$ and $E2F1^{-/-}$ hepatocytes. $^{*}P < 0.01$ versus $E2F1^{+/+}$ hepatocytes. (D) qPCR of Egr-1 and SHP mRNA in $FXR^{+/+}$ (WT) and $FXR^{-/-}$ mice fed with FXR specific agonist GW4064. $^{*}P < 0.01$ versus chow. (E) Schematic of the E2F1/Egr-1/SHP/EID1 regulatory network in cholestatic liver fibrosis.

Individual or coexpression of SHP and EID1 diminished the induction of Egr-1 mRNA by E2F1 in Hepa-1 cells (Fig. 5D, left). Overexpression of SHP also synergistically enhanced EID1 inhibition of Egr-1 protein activation by E2F1 (Fig. 5D, middle and right).

Interestingly, the response of Egr-1 to various stimuli differed markedly in hepatocytes isolated from $E2F1^{+/+}$ and $E2F1^{-/-}$ mice. The reduction of Egr-1 mRNA by lipopolysaccharide (LPS) and fatty acids (palmitic acid, stearic acid, oleic acid, and linoleic acid) occurred only in $E2F1^{+/+}$ but not in $E2F1^{-/-}$ hepatocytes (Fig. 6A). Cholic acid (CA) and chendeoxycholic acid (CDCA), but not FXR agonist GW4064 and peroxisome proliferator-activated receptor gamma (PPARγ) agonist rosiglitazone (RGZ), induced Egr-1 mRNA only in $E2F1^{-/-}$ hepatocytes lacking E2F1. In contrast, the induction of Egr-1 by CA or CDCA was impeded by E2F1 overexpression in Huh7 cells, but not by E138, an E2F1 mutant with no binding

activity (Fig. 6B). It was noted that the expression of E2F1 was not affected by CA and CDCA in Huh7 (Fig. 6B), Hepa1, or hepatocytes (not shown). The results suggest that despite its direct activation of Egr-1 transcription, E2F1 may also indirectly inhibit BA-mediated expression of Egr-1. Interestingly, Cyp7a1 and Cyp8b1 mRNA was largely diminished in *E2F1*$^{-/-}$ hepatocytes (Fig. 6C), suggesting that their down-regulation may contribute to the E2F1 inhibition of BA synthesis (Fig. 2C). In addition, the basal expression of Egr-1 was no different in WT and *FXR*$^{-/-}$ liver and the induction of SHP by FXR agonist GW4064 in WT mice correlated with the reduction of Egr-1 (Fig. 6D). Overall, E2F1, along with SHP and EID1, forms a regulatory network to fine tune the expression of Egr-1 (Fig. 6E).

Discussion

Cholestatic liver disease results when bile acids excretion from liver is interrupted. As a consequence, hepatocyte injury develops and inflammation response occurs. If cholestasis is not corrected, and hepatocyte injury and inflammation persists, biliary fibrosis and eventually cirrhosis ultimately develops. Here, we uncovered a new molecular mechanism of cholestatic liver fibrosis, which involves a cascade of transcriptional regulators including E2F1, Egr1, Shp, and EID1.

The major finding of this study is the identification of a physiological role of E2F1 in liver fibrosis and cirrhosis. We show that hepatic E2F1 expression is highly up-regulated in several mouse liver fibrosis (BDL and DDC) models and human liver cirrhotic (NASH and alcohol) specimens. More important, E2F1-deficiency largely lessened the development of biliary liver fibrosis caused by DDC, providing direct evidence for its crucial importance in the regulation of liver fibrosis. At the molecular level, we identified Egr-1 as a downstream target of E2F1, which offers at least one underlying basis for understanding the fibrogenic function of E2F1. Bile acids could induce Egr-1 by activating MAPK signaling pathway[28] to mediate the inflammatory response.[18] Perhaps the most encouraging observation is the higher induction of E2F1 in human cirrhotic livers than the standard fibrosis markers α-SMA and α1-collagen. These lines of evidence strongly suggest the possibility of developing E2F1 as a potential new diagnostic marker for liver fibrosis/cirrhosis.

It is interesting to note that E2F1 activates Egr-1 expression in a biphasic fashion in human and mouse hepatocytes. The earliest study to apply the term "biphasic" to E2F1 was based on the observation that E2F1 activity was stimulated by low concentrations of androgen but strongly inhibited by high concentrations during LNCaP prostate cancer cells proliferation.[29] Later, it was found that E2F1 expression increased at earlier timepoints then decreased as Myc gene input increased, which was also designated a biphasic or bimodal response.[30] It is possible that the interplay between activation and repression mediated by a common input may contribute to biphasicity. Indeed, despite its direct activation of Egr-1 transcription, E2F1 also indirectly inhibits BA-mediated induction of Egr-1 (Fig. 6E). It is important to emphasize that the biphasic pattern of Egr-1 activation by E2F1 is a specific and unique feature of Egr-1 that is not shared with other new E2F1 targets we are currently investigating. Therefore, the biphasic response constitutes a critical, underappreciated challenge in analyzing phenotypic consequences of E2F1 alteration. We acknowledge that Egr-1 is unlikely the sole factor contributing to E2F1-mediated liver fibrosis. It is thus of importance to identify additional downstream targets of E2F1.

An intriguing question is the role of E2F1 in BA homeostasis. Based on the decreased BA levels in *E2F1*$^{-/-}$ mice and diminished expression of Cyp7a1 and Cyp8b1 in *E2F1*$^{-/-}$ hepatocytes, we predict that BA synthesis may be disrupted by E2F1-deficiency. A previous study reported that during inflammation nuclear factor (NF)-kappaB rapidly recruited E2F1 to fully activate the transcription of NF-kappaB target genes, and that knockdown E2F1 impaired the LPS inducibility of the proinflammatory cytokines.[31] Interestingly, among several inflammatory genes we analyzed, the activation of CCL2 by DDC was impaired by E2F1-deficiency, suggesting its specific regulation by E2F1. It is plausible that both the decreased (toxic) BAs and the impaired inflammation response by loss of E2F1 may contribute to the diminished cholestatic liver fibrosis in *E2F1*$^{-/-}$ mice.

Another important finding from this study is establishing SHP and EID1 as part of the E2F1/Egr-1 regulatory network, which coordinately control the expression of Egr-1. SHP functions as a transcriptional repressor[32] and the differential responses in Huh7, LX2, and Hepa1 cells to SHP inhibition of Egr-1 suggest the requirement and existence of additional corepressors in each cell type. In this regard, EID1 appears to be a potent SHP partner to execute the full repression function of SHP. Overall, our study opens the avenues for further investigation of the E2F1 function in chronic liver diseases.

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