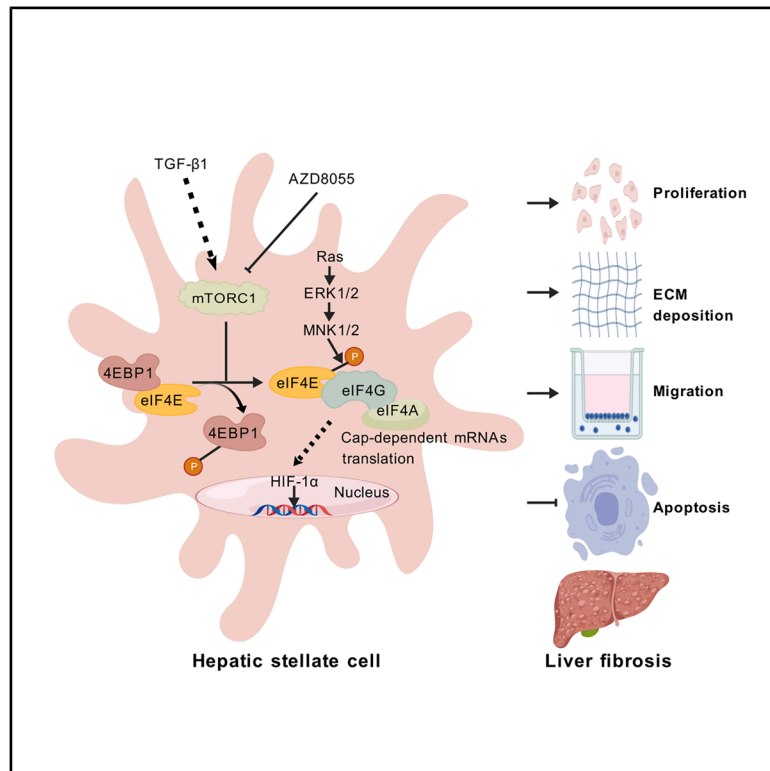


4EBP1 phosphorylation mediates TGF- β 1-induced hepatic fibrosis and upregulates HIF-1 α expression

Graphical abstract



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In brief

Fibrosis; Biochemistry; Cell biology

Highlights

- p-4EBP1 is important for CCl₄ and BDL-induced liver fibrosis
- 4EBP1 ameliorated TGF- β 1-induced fibrosis related genes in hepatic stellate cells
- 4EBP1 suppressed CCl₄ and BDL-induced liver fibrosis
- p-4EBP1 improved HIF-1 α expression



Article

4EBP1 phosphorylation mediates TGF- β 1-induced hepatic fibrosis and upregulates HIF-1 α expression

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SUMMARY

Eukaryotic translation initiation factor 4E binding protein 1 phosphorylation (p-4EBP1) has been involved in the production of excess extracellular matrix (ECM) and the fibrosis of various organs. We first confirmed that the p-4EBP1 and HIF-1 α expressions were significantly elevated in transforming growth factor β 1 (TGF- β 1)-activated LX-2 cells and fibrotic liver samples. *In vivo* experiments supported the antifibrotic effect of targeting p-4EBP1 in the liver. Genetic or drug-targeted p-4EBP1 activity *in vitro* decreased TGF- β 1-induced activation, proliferation, migration, epithelial-mesenchymal transition (EMT), and the HIF-1 α protein expression in LX-2 cells and promoted their apoptosis. Moreover, the short hairpin RNA-mediated knockdown of 4EBP1 was the opposite. In conclusion, our findings suggest that p-4EBP1 is a critical signaling node in TGF- β 1-induced hepatic fibrosis and identified what we believe to be a previously unrecognized p-4EBP1/HIF-1 α signaling for hepatic stellate cell (HSC) fibrosis transformation.

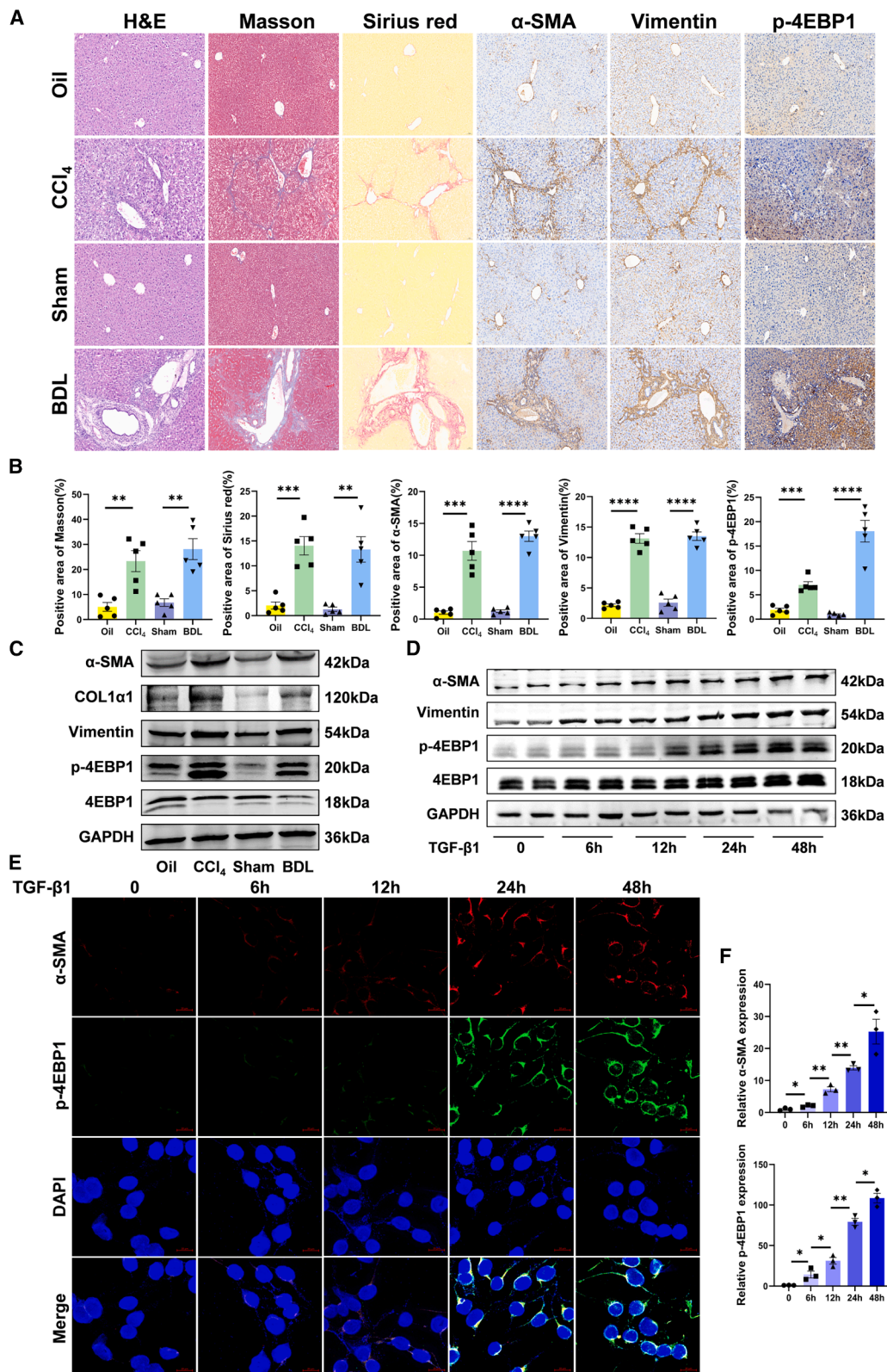
INTRODUCTION

Hepatic fibrosis can progress to cirrhosis, which has a high mortality and morbidity due to the risk of progression to hepatocellular carcinoma¹ and chronic liver failure.² Although some molecular mechanisms of hepatic fibrosis have been revealed, strategies for liver fibrosis treatment or reversion remain limited.^{3,4} Hepatic stellate cells (HSCs) are called fibrotic effector cells, activated to obtain a phenotype of stromal production, proliferation, migration, and contraction, and play a crucial role in liver fibrosis progression.⁵ When receiving extracellular signals, including transforming growth factor β 1 (TGF- β 1), phosphoinositide-3-kinase (PI3K) dimerizes and increases protein kinase B phosphorylation, the downstream signaling molecule of PI3K. Subsequent activation of downstream mechanistic target of rapamycin (mTOR) signals ultimately upregulate the expression of proliferation-related genes and activation-related genes,⁶ which involves influencing fibroblast proliferation responses and TGF- β 1-induced collagen production and differentiation in myofibroblasts.⁷ As a downstream factor of TGF- β pathway, mTOR plays an essential role in regulating various basic cellular processes through two enzyme complexes, mTOR complexes 1 and 2 (mTORC1/mTORC2).⁸

Eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) is a significant translation inhibitor that inhibits cap-

dependent translation by binding to eukaryotic translation initiation factor 4E (eIF4E) and preventing the recruitment of eukaryotic translation initiation factor 4G (eIF4G) and the subsequent formation of translation initiation complexes.⁹ Stepwise phosphorylation by mTORC1 to at least four 4EBP1 key checkpoints (threonine residues 37, 46, and 70 and serine residue 65) led to the separation of 4EBP1 from eIF4E and facilitated eIF4G to bind eIF4E to enable cap-dependent translation.¹⁰ 4EBP1 overexpression in phosphorylation checkpoint defects caused a reduced G1/S phase transition rate,¹¹ suggesting that 4EBP1 plays a key role in cell cycle progression. Furthermore, 4EBP1 inhibits the translation of cell metastasis and invasion mRNAs.¹² The loss of 4EBP1 function induces epithelial-mesenchymal transition (EMT) and increases the metastatic capacity of cancer cells.¹³ The application of ectopic expression of the constitutively active form of 4EBP1 mutants caused the negative regulation of eIF4E, significantly reducing TGF- β 1-induced α -smooth muscle actin (α -SMA) levels and upregulating E-cadherin levels in RLE-6TN cells.¹⁴ Another study has reported that 4EBP1 is involved in regulating LX-2 cell activation.¹⁵ Neuropeptide Y stimulation upregulates the expression of phosphorylated mTOR/4EBP1, which in turn promotes LX-2 cell proliferation and migration and subsequent translation initiation.¹⁶ Moreover, 4EBP1 has a proapoptotic effect in normal and transformed fibroblasts.¹⁷





(legend on next page)

A series of *in vivo* experiments revealed the involvement of 4EBP1 phosphorylation (p-4EBP1) in multiorgan fibrosis. In high glucose-induced peritoneal fibrosis, the p-4EBP1 protein was positively correlated with the expressions of α -SMA and collagen type I.¹⁸ Rapamycin alleviated myocardial fibrosis in mice with dilated cardiomyopathy through mTOR/4EBP1 pathway downregulation.¹⁹ When activated by an upstream signal, mTORC1 promotes the activation of renal interstitial fibroblasts and renal fibrosis by phosphorylating 4EBP1.²⁰ mTORC1/4EBP1 signaling plays a crucial role in collagen synthesis in TGF- β 1-driven human tenon fibroblasts.²¹ The expression of p-4EBP1 increases in mice with systemic sclerosis, which in turn promotes skin fibrosis.²² mTORC1 phosphorylates 4EBP1 and increases the collagen type I expression in lung mesenchymal cells, which is involved in pulmonary fibrosis formation.²³ Woodcock et al.²⁴ demonstrated the significance of the mTORC1/4EBP1 axis in the synthesis of idiopathic pulmonary fibrosis-derived fibroblast collagen. However, the role of 4EBP1 in hepatic fibrosis has not been completely investigated.

The early stages of chronic liver disease (CLD) may rapidly cause tissue and cellular hypoxic responses and increased expression of hypoxia inducible factor 1 subunit alpha (HIF-1 α),²⁵ which regulates the expression of genes essential for collagen synthesis and plays a key regulatory role in TGF- β 1/mTOR-activated HSCs.²⁶ Interestingly, p-4EBP1 can promote HIF-1 α activation,²⁷ suggesting that 4EBP1 can regulate the excessive aggregation of the extracellular matrix (ECM) induced by HIF-1 α activation. Therefore, we attempted to establish a link between 4EBP1/HIF-1 α signaling and TGF- β 1-induced hepatic fibrosis.

RESULTS

P-4EBP1 expression was closely associated with the tissue repair mechanism of hepatic fibrosis

Liver injury was observed in mice with liver fibrosis using H&E staining, and the collagen area was indicated using Masson's trichrome and Sirius red staining (Figure 1A). Immunohistochemical analyses demonstrated that CCl₄- and BDL-induced mice exhibited increased p-4EBP1 expression (Figure 1A). The quantitative results for α -SMA, vimentin, and p-4EBP1 are shown in Figure 1B. Moreover, we noted elevated Acta2, collagen type I alpha 1 (Col1a1), vimentin mRNAs and α -SMA, COL1 α 1, vimentin, and p-4EBP1 protein levels in CCl₄- and BDL-induced liver fibrosis mice (Figures 1C, S1A, and S1B). Interestingly, RT-qPCR verified the downward trend of 4EBP1 in the livers of mice that received CCl₄ treatment or BDL surgery (Figure S1A), which could occur during liver fibrosis or result in liver fibrosis

progression. Furthermore, for the *in vitro* study, the LX-2 cell line was selected. We treated the LX-2 cells with TGF- β 1 10 ng/mL according to previous studies.²⁸ Furthermore, we noted increased ACTA2 and vimentin and decreased 4EBP1 mRNAs in TGF- β 1-induced LX-2 cells (Figure S1C). Notably, the α -SMA, vimentin, and p-4EBP1 expression levels were enhanced in a time-dependent manner in TGF- β 1-stimulated LX-2 cells (Figures 1D and S1D). Immunofluorescence double staining of α -SMA and p-4EBP1 in TGF- β 1-stimulated LX-2 cells for a time gradient also provided similar results (Figures 1E and 1F). Tables 1 and 2 provides specific information regarding all the antibodies and the primer sequences for all genes.

4EBP1-4A attenuated hepatic fibrosis in animal models

Specific information regarding AAV transduction, CCl₄, and BDL administration in the mouse models is described in Figures 2A and 3A. The expression efficiency of 4ebp1-4A mRNA levels and the coexpressed marker of FLAG-tagged protein levels with saline, AAV2/8-scramble, or AAV2/8-4ebp1-4A-3xflag administration were determined using RT-qPCR and western blotting (Figures 2B and 2C). Immunofluorescence analysis of the liver tissue revealed that the AAV2/8 vector could be continuously expressed in the mouse models (Figure S1E). H&E staining images indicated that the AAV2/8-4ebp1-4A-3xflag fibrotic mice had a better appearance and a more regular liver lobule structure (Figures 2D and 3B). Sirius red and Masson's trichrome staining of mouse liver tissue slices demonstrated that when stimulated with CCl₄ or accepted BDL surgery, AAV2/8-4ebp1-4A-3xflag mice exhibited fewer hepatic fibrotic features, including less collagen deposition than AAV2/8-scramble mice (Figures 2D, 2E, 3B, and 3C). Consistently, immunohistochemical staining verified the downward trend of α -SMA and vimentin in AAV2/8-4ebp1-4A-3xflag mice at the protein level (Figures 2D, 2E, 3B, and 3C). AAV2/8-4ebp1-4A-3xflag mice demonstrated significantly lower serum levels of ALT and AST, which are liver function markers (Figures 2F and 3D). Furthermore, mice that underwent BDL surgery had higher TBIL and DBIL levels, whereas AAV2/8-4ebp1-4A-3xflag overexpression reversed the upregulation (Figure 3D). In the liver fibrosis model mice, AAV2/8-4ebp1-4A-3xflag overexpression attenuated the upregulation of TGF- β 1 and IL-6, which are liver inflammation markers (Figures 2G and 3E). AAV2/8-4ebp1-4A-3xflag group mice were accompanied by reduced α -SMA, COL1 α 1, vimentin, fibronectin, and HIF-1 α protein levels, whereas the upward trend of 4EBP1 and E-cadherin in AAV2/8-4ebp1-4A-3xflag mice was verified using western blotting (Figures 2H, 3F, S1F, and S1G). Immunofluorescence results revealed α -SMA and HIF-1 α

Figure 1. p-4EBP1 was upregulated in hepatic fibrosis

(A) Images of H&E, Masson's trichrome, Sirius red, and immunohistochemistry of α -SMA, vimentin, and p-4EBP1 staining of representative liver sections ($\times 200$ magnification).

(B) Representative Masson and Sirius red staining of liver tissues and positive content (%) of α -SMA, vimentin, and p-4EBP1 staining ($n = 5$).

(C) The protein expression of α -SMA, COL1 α 1, vimentin, p-4EBP1, and 4EBP1 in liver tissues was analyzed using western blotting.

(D) The protein expression of α -SMA, vimentin, p-4EBP1, and 4EBP1 in TGF- β 1-activated LX-2 cells for 0, 6, 12, 24, and 48 h.

(E and F) Immunofluorescence double staining of α -SMA and p-4EBP1 in TGF- β 1-stimulated LX-2 cells for 0, 6, 12, 24, and 48 h ($\times 400$ magnification). Values are the mean $\pm$ SD (unpaired two-sample Student's *t* test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. α -SMA, alpha-smooth muscle actin; BDL, bile duct ligation; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; TGF- β 1, transforming growth factor β 1.

Table 1. Antibodies and reagents

Catalog	Antibody	Species	MW (KD)	Company	Application and radio
CY6583	4 EBP1	Rabbit	18	Abways	WB:1:1000
AY8172	p-4EBP1(T46)	Rabbit	20	Abways	WB:1:1000 IHC:1:50
AF3830	p-4EBP1 (T37/T46)	Rabbit	20	Affinity	IF:1:100
BM3902	α -SMA	Rabbit	42	Boster	WB:1:1000 IHC:1:50
BM0002	α -SMA	Mouse	42	Boster	IF:1:1000
14695-1-AP	Collagen I	Rabbit	120	Proteintech	WB:1:1000
CY5134	Vimentin	Rabbit	54	Abways	WB:1:1000 IHC:1:100
CY5404	Cyclin D1	Rabbit	34	Abways	WB:1:1000
M00013-4	HIF-1 α	Rabbit	120	Boster	WB:1:500 IF:1:50
AB0030	FLAG Tag	Rabbit	–	Abways	WB:1:5000
81115-1-RR	β -actin	Rabbit	42	Proteintech	WB:1:5000
PB9561	E-cadherin	Rabbit	97	Boster	WB:1:500
15613-1-AP	Fibronectin	Rabbit	263	Proteintech	WB:1:2000
AF7022	Cleaved-Caspase 3	Rabbit	17	Affinity	IF:1:100
A23920	DyLight 800	Goat	–	Abbkine	WB:1:7000
A-11008	Alexa Fluor™ 488	Goat	–	Invitrogen	IF:1:200
A-11005	Alexa Fluor™ 594	Goat	–	Invitrogen	IF:1:200
A00227-1	GAPDH	Rabbit	36	Boster	WB:1:20000
Catalog	Regent	–	–	Company	–
E-CK-A211	Annexin V-FITC/PI Apoptosis Kit	–	–	Elabscience	–
CA1510	DNA Content Quantitation Assay(Cell Cycle)	–	–	Solarbio	–
GC16380	AZD8055	–	–	GLPBIO	–
PY2270	Recombinant Human TGF- β 1	–	–	Abways	–
SB-CCK8	Cell Counting Kit-8	–	–	Share-bio	–

upregulation, along with α -SMA and HIF-1 α colocalization in fibrotic liver tissues of mice, whereas 4ebp1-4A expression reversed the upregulation (Figures 2I, 2J, 3G, and 3H). According to the aforementioned data, we showed that 4EBP1 represents a common fibrotic signaling center in liver fibrosis.

4EBP1-4A reduced ECM deposition induced by activated HSCs

To investigate the exact mechanism by which 4EBP1 influences hepatic fibrosis, we performed RNA sequencing (RNA-seq) analyses (NovoMagic, Beijing, China) in TGF- β 1-activated LX-2 cells

Table 2. Primer sequences for RT-qPCR

Gene (ID)	Species	Forward (5'-3')	Reverse (5'-3')
<i>GAPDH</i>	Mouse	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
<i>Acta2</i>	Mouse	GTCCAGACATCAGGGAGTAA	TCGGATACCTCAGCGTCAGGA
<i>Col1a1</i>	Mouse	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
<i>Vimentin</i>	Mouse	CGGAAAGTGAATCCTTGACAGG	AGCAGTGAGGTCAGGCTTGAA
<i>4ebp1-4A</i>	Mouse	ACTACAGCACCGCTCCGG	GCTTTGGCCACAGGTGCGTT
<i>4ebp1</i>	Mouse	ACTACAGCACCGCTCCGG	GCTTTGGCCACAGGTGCGTT
<i>GAPDH</i>	Human	GGAGCGAGATCCCTCCAAAT	GGCTGTTGTCTACTTCTCATGG
<i>ACTA2</i>	Human	CGTGCTGGACTCTGGAGATGGT	CCGATGAAGGATGGCTGGAACA
<i>Vimentin</i>	Human	AGGCAAAGCAGGAGTCCACTGA	ATCTGGCGTTCCAGGGACTCAT
<i>4EBP1-4A</i>	Human	CAAGGGATCTGCCCCACATT	AACTGTGACTCTTCCCGCC
<i>4EBP1</i>	Human	CTATGACCGGAAATTCCTGATGG	CCCGCTTATCTTCTGGGCTA

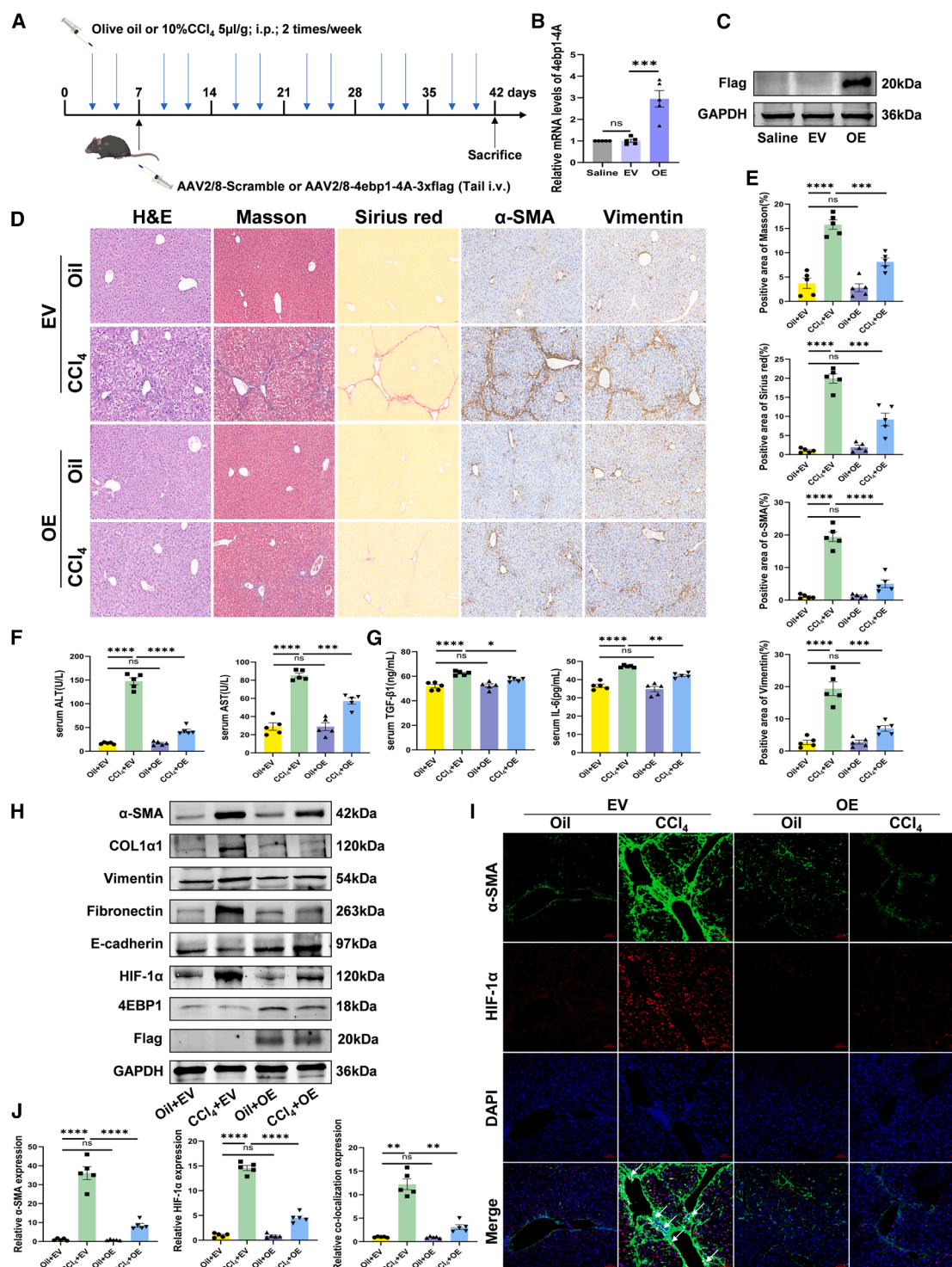


Figure 2. 4EBP1-4A attenuated CCl₄-induced hepatic fibrosis in mice

(A) Illustration of adeno-associated virus, AAV transduction and CCl₄ administration in mice models. (B and C) RT-qPCR analysis of 4ebp1-4A levels and (C) western blotting analysis of FLAG-tagged protein levels with saline, AAV2/8-scramble, or AAV2/8-4ebp1-4A-3xflag administration. (D) Images of H&E, Masson's trichrome, Sirius red, and IHC of α-SMA and vimentin staining of representative liver sections (×200 magnification). (E) Representative Masson and Sirius red staining of liver tissues and positive content (%) of α-SMA and vimentin staining (n = 5).

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transduced with LV-empty or LV-4EBP1-4A-3xflag. In total, 2,490 significantly upregulated and 2,026 significantly downregulated genes were identified in this analysis (Newcore, Shanghai, China) (Figure 4A). A significant downregulation was observed on fibrosis-related genes compared with LV-empty, including the HSC activation marker gene platelet-derived growth factor subunit B, ECM deposition-related gene collagen type VI alpha 6, collagen type VIII alpha 1, collagen type IX alpha 2, and fibulin 2 (Figure 4A). In addition, the ECM breakdown-related gene matrix metalloproteinase 1 and antifibrosis-related gene decorin demonstrated a significant upregulation (Figure 4A). Moreover, GO and Reactome functional annotation analyses revealed that the differentially expressed genes in LV-empty and LV-4EBP1-4A-3xflag were significantly associated with ECM formation and mesenchymal cell differentiation, which is a crucial stage in the hepatic fibrosis process (Figures 4B–4D). We performed KEGG enrichment analyses of upregulated and downregulated genes (Figure 4E). Differential genes were enriched for ECM-receptor interactions and the PI3K/Akt signaling pathway, which coincided with the Reactome functional annotation analysis results (Figure 4E). Furthermore, GSEA enrichment analysis revealed that 4EBP1-4A overexpression caused apoptosis-related genes upregulation (Figure 4F). Results in the expression of cleaved caspase-3 and α -SMA by immunofluorescent staining revealed that the colocalization of α -SMA and cleaved caspase-3 increased in the liver sections of fibrotic mice injected with AAV2/8-4ebp1-4A-3xflag (Figures 4G and 4H). These findings implicated that 4EBP1 was inseparable from the ECM deposition, fibrosis process, and apoptotic process in HSCs of mice fibrotic liver tissues.

4EBP1-4A inhibited the fibrosis phenotype of HSCs

The cell processing protocol is shown in Figure 5A. The expression efficiency of 4EBP1-4A mRNA levels and the coexpressed marker of FLAG-tagged protein levels with control, LV-empty, or LV-4EBP1-4A-3xflag administration were determined using RT-qPCR and western blotting (Figures 5B and 5C). The cell transduction observed under a fluorescence microscope is depicted in Figure S2A. We observed that the LV-4EBP1-4A-3xflag expression significantly reduced the proliferation of LX-2 cells stimulated with TGF- β 1 by the CCK-8 analysis (Figure 5D). Following the observation of 4EBP1-mediated growth inhibition, we investigated whether 4EBP1 impacts the cell cycle. The LV-4EBP1-4A-3xflag expression significantly increased the percentage of cells in the G1 phase in TGF- β 1-stimulated LX-2 cells (Figures 5E and 5F). In addition, the expression levels of the regulatory subunit cyclin D1, which is a cell-cycle arrest marker (G1/S checkpoint effectors), were investigated using western

blotting analysis. Similarly, treatment of LX-2 cells with LV-4EBP1-4A-3xflag significantly reduced the cyclin D1 expression (Figures 5G and 5H). Following LV-4EBP1-4A-3xflag transduction into LX-2 cells, the α -SMA, COL1 α 1, vimentin, fibronectin, and HIF-1 α protein expressions were markedly downregulated, whereas the 4EBP1 and E-cadherin levels increased (Figures 5I and S2B). The fluorescence intensity of α -SMA and HIF-1 α protein expression in LX-2 cells significantly increased under TGF- β 1 stimulation, whereas LV-4EBP1-4A-3xflag transduction reversed the effect of TGF- β 1 (Figures 5J and S2C). Transwell experiments revealed that LV-4EBP1-4A-3xflag transduction reduced TGF- β 1-stimulated LX-2 cell migration (Figures 5K and 5L). The supported results of protein expressions in the cell migration-related biomarkers, vimentin, and E-cadherin, were shown in the previous section (Figures 5I and S2B). Compared to the model group, the apoptotic rate in the LV-4EBP1-4A-3xflag group significantly increased (Figures 5M and 5N).

4EBP1 knockdown promoted the fibrosis phenotype of HSCs

As p-4EBP1 levels were induced in TGF- β 1-activated LX-2 cells, we proved the potential influence of 4EBP1 knockdown (simulated full phosphorylated 4EBP1) on LX-2 cell activation. The cell processing protocol is shown in Figure 6A. The efficiency of 4EBP1 silencing was measured and confirmed using RT-qPCR and western blotting (Figures S2D–S2F). The control and silencing sequences are listed in Table 3. For further experiments, we used the 4EBP1-shRNA3 sequence. We used AZD8055, a structurally unique, potent, and highly selective ATP-competitive mTOR kinase inhibitor. AZD8055 dose-dependently inhibited LX-2 cell proliferation, and the half-maximal inhibitory concentration of AZD8055 was 0.53 μ M (Figure 6B). We decided to use 0.5 μ M for further experiments. We noted that 4EBP1 silencing significantly promoted proliferation and induced the altered AZD8055-inhibited proliferation of TGF- β 1-stimulated LX-2 cells (Figure 6C). The results of AZD8055- and downregulated 4EBP1-induced alterations in cell cycle progression and cyclin D1 expression were consistent with the aforementioned results (Figures 6D–6G). Western blotting analyses indicated that AZD8055 treatment significantly suppressed the α -SMA, COL1 α 1, vimentin, fibronectin, and HIF-1 α protein expression levels, whereas the opposite happened in E-cadherin (Figures 6H and S2G). Notably, silencing 4EBP1 reversed the effect of AZD8055 (Figures 6H and S2G). The results of the fluorescence intensity in α -SMA and HIF-1 α protein expression also supported this point (Figures 6I and S2H). The number of migration was significantly decreased following

(F and G) Serum (F) ALT, AST and (G) TGF- β 1, IL-6 in each group.

(H) The protein expression of α -SMA, COL1 α 1, vimentin, fibronectin, E-cadherin, 4EBP1, FLAG, and HIF-1 α in liver tissues analyzed using western blotting.

(I) Immunofluorescence double staining of α -SMA and HIF-1 α in the liver sections of mice ($\times$ 100 magnification). The arrow heads represent colocalization of staining.

(J) Relative quantification of α -SMA, HIF-1 α , and colocalization of α -SMA and HIF-1 α . EV, AAV2/8-scramble; OE, AAV2/8-4ebp1-4A-3xflag. Values are the mean $\pm$ SD (unpaired two-sample Student's *t* test). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001; ns, not significant. α -SMA, alpha-smooth muscle actin; AAV, adeno-associated virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; HIF-1 α , hypoxia inducible factor 1 subunit alpha; IL-6, interleukin 6; RT-qPCR, quantitative reverse transcription polymerase chain reaction; TGF- β 1, transforming growth factor beta 1.

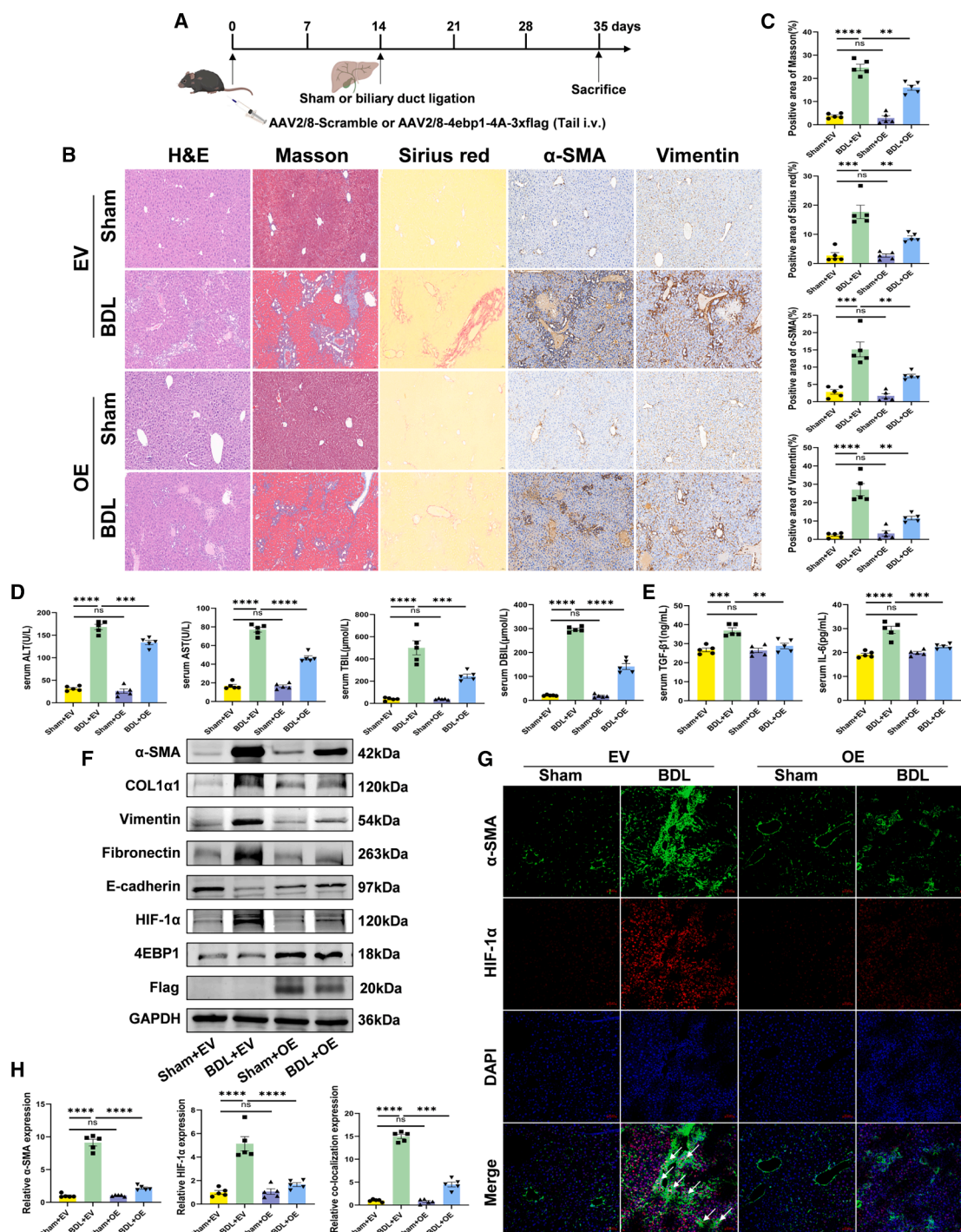


Figure 3. 4EBP1-4A attenuated BDL-induced hepatic fibrosis in mice

(A) Illustration of AAV transduction and BDL administration in mouse models.

(B) Images of H&E, Masson's trichrome, Sirius red, and IHC of α-SMA and vimentin staining of representative liver sections (×200 magnification).

(C) Representative Masson and Sirius red staining of liver tissues and positive content (%) of α-SMA.

and vimentin staining (n = 5).

(D and E) Serum (D) ALT, AST, TBIL, DBIL, and (E) TGF-β1, IL-6 in each group.

(F) The protein expression of α-SMA, COL1α1, vimentin, fibronectin, E-cadherin, 4EBP1, FLAG, and HIF-1α in liver tissues was analyzed using western blotting.

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treatment with AZD8055 in LX-2 cells, and silencing 4EBP1 reversed the downregulation (Figures 6J and 6K). Furthermore, AZD8055 significantly increased the proportion of apoptotic cells, and silencing 4EBP1 reversed the effect of AZD8055 (Figures 6L and 6M).

DISCUSSION

Stable activation of various profibrotic signaling pathways is a recognized marker for transformed HSCs in hepatic fibrotic tissues. 4EBP1, a member of the translation inhibitor protein family, plays a crucial role in mTORC1 signaling, controlling cell proliferation and translation.²⁹ The livers of fibrotic mice in different models demonstrated decreased 4EBP1 mRNA expression, consistent with previous studies in human cirrhosis,³⁰ suggesting that the function of 4EBP1 is closely associated with the tissue repair mechanism of liver diseases. We found a decrease in 4EBP1 mRNA levels in TGF- β 1-induced LX-2 cells, which is consistent with the results of *in vivo* experiments. In response to the activation of the TGF- β 1/mTORC1 signaling pathway, 4EBP1 undergoes phosphorylation and subsequent inactivation, a process that is regulated at the protein level. Both the downregulation of 4EBP1 at the mRNA level and its phosphorylation at the protein level are indicative of the progression of fibrosis, and the biological functions induced by these changes are consistent. The nonphosphorylated mutant of 4EBP1 can compositionally bind eIF4E and inhibit cap-dependent translation.²⁴ Previous findings have suggested that 4EBP1-4A effectively blocks eIF4E-mediated translation initiation downstream of cap-dependent mTORC1.³¹ H&E, Masson tricolor, and Sirius red staining revealed that hepatocyte structural disorder and collagen deposition were alleviated in mice with continuous AAV2/8-4ebp1-4A-3xflag expression. The 4EBP1-4A expression *in vitro* and *in vivo* reversed the increased COL1 α 1, α -SMA, vimentin, and fibronectin expressions in mice with liver fibrosis, indicating that the function of 4EBP1 is associated with the changes in ECM and fibroblast differentiation during liver fibrosis.

The liver injury-induced HSC activation, proliferation, and transformation are significant factors in liver fibrosis.³² LX-2 cells have the molecular characteristics and functions of primary activated HSCs, which are immortalized human HSCs and highly transfectable.³³ Data obtained by knocking down 4EBP1 or overexpressing 4EBP1-4A using lentiviral transduction, combined with data acquired by 4EBP1 shRNA rescuing the ATP-competitive mTOR inhibitor AZD8055 from the inhibitory effect of TGF- β 1-induced fibrosis, strongly support our key observations regarding the critical role of 4EBP1 in fibrogenesis. Regarding identifying the mechanisms by which 4EBP1 influences the fibrotic response of TGF- β 1, first, AZD8055 attenuates the TGF- β 1-induced fibrotic response; second, the effect of

AZD8055 on the TGF- β 1-induced fibrotic response can be salvaged by targeting 4EBP1 silencing; Third, inducing the expression of 4EBP1 dominant negative phosphorylation mutants blocks the TGF- β 1-induced fibrotic response. EMT is the process by which epithelial cells undergo cytoskeletal changes and lose epithelial cell-cell contact, all of which favor the acquisition of migration and invasion. These changes are accompanied by changes in the expression of related proteins, including those involved in cytoskeletal proteins (e.g., loss of cytokeratin and gain of vimentin and α -SMA) and proteins in cell contact (e.g., loss of E-cadherin and gain of N-cadherin).³⁴ We noted that TGF- β 1-activated p-4EBP1 expression induced EMT, which can be blocked by p-4EBP1-targeting genetic or pharmacological interventions. Based on these results, we hypothesized that 4EBP1 can play a negative regulatory role in liver fibrosis by inhibiting the fibrotic phenotype of HSCs.

Theoretically, mutations in the phosphorylation sites of 4EBP1 (4EBP1-4A) render it unable to be phosphorylated by mTORC1, thereby allowing it to bind to eIF4E and exert an inhibitory effect on cap-dependent mRNA translation. This process is independent of p-4EBP1. Overexpression of 4EBP1-4A compensates for the series of effects caused by 4EBP1 functional inactivation (4EBP1 phosphorylation). Therefore, we hypothesize that 4EBP1-4A overexpression produces anti-fibrotic biological effects independently of the activation of the mTORC1/4EBP1 pathway (i.e., the high-level expression of p-4EBP1). On the other hand, low expression of 4EBP1 mimics 4EBP1 phosphorylation; consequently, a large amount of eIF4E that is not bound to 4EBP1 initiates cap-dependent mRNA translation, thereby inducing pro-fibrotic biological effects. We found that in TGF- β 1-stimulated LX-2 cells, compared with the control group, knockdown of 4EBP1 further promoted phenotypic changes associated with HSC activation, exerting a synergistic effect with the action of TGF- β 1 (Figure 6). This further supports the biological function of unphosphorylated 4EBP1 in exerting anti-fibrotic effects.

P-4EBP1 and overexpressed HIF-1 α can promote proliferation and inhibit the apoptosis of HSCs, thereby stimulating hepatic fibrosis development. Consistent with previous findings, HIF-1 α levels in the fibrotic mice liver and TGF- β 1-activated LX-2 were both elevated. Knocking down 4EBP1 in LX-2 cells further upregulated HIF-1 α levels, whereas overexpression of the 4EBP1 dominant negative phosphorylation mutant reduced HIF-1 α protein levels *in vivo* and *in vitro*. We noted that mTORC1 regulates downstream HIF-1 α mainly owing to 4EBP1 phosphorylation and cap-dependent translation increase, and our results are consistent with those of previous studies.^{35,36} Our study revealed a distinct association between 4EBP1 and HIF-1 α , which is significant in the context of hepatic fibrosis and carcinogenic transformation and is associated with several diseases. To the

(G) Immunofluorescence double staining of α -SMA and HIF-1 α in the liver sections of mice ($\times 100$ magnification). The arrowheads represent colocalization of staining.

(H) Relative quantification of α -SMA, HIF-1 α , and colocalization of α -SMA and HIF-1 α . EV, AAV2/8-scramble; OE, AAV2/8-4ebp1-4A-3xflag. Values are the mean $\pm$ SD (unpaired two-sample Student's *t* test). ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001; ns, not significant. α -SMA, alpha-smooth muscle actin; AAV, adeno-associated virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BDL, bile duct ligation; DAPI, 4',6'-diamidino-2-phenylindole; DBIL, direct bilirubin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin-eosin; HIF-1 α , hypoxia inducible factor 1 subunit alpha; IL-6, Interleukin 6; TBIL, total bilirubin; TGF- β 1, transforming growth factor beta 1.

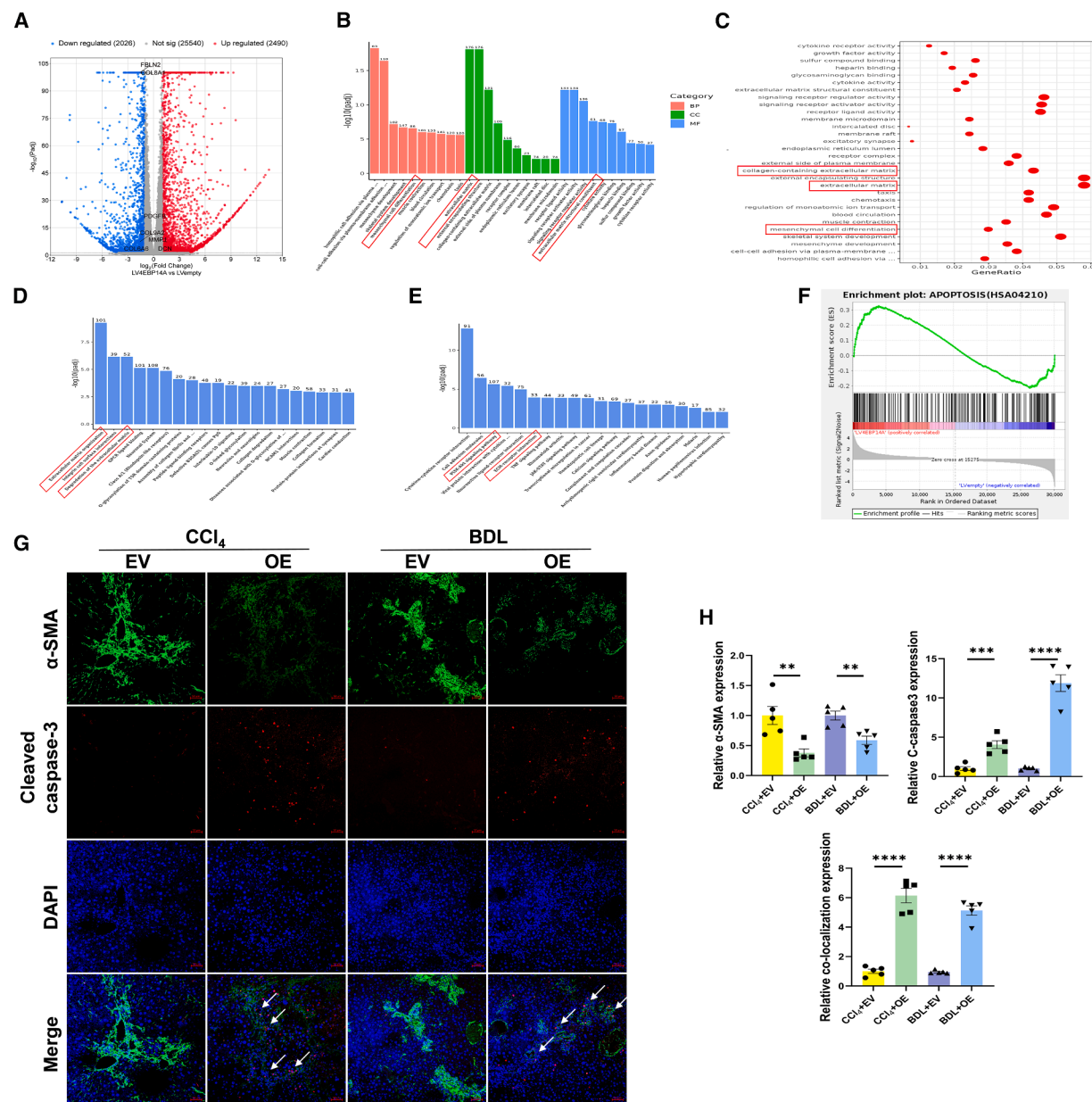


Figure 4. RNA-seq data differential gene analysis of TGF- β 1-activated LX-2 cells transduced with LV-empty or LV-4EBP1-4A-3xflag (five biological replicates per group)

(A) The differential gene volcano map analysis of 2,490 significantly upregulated genes and 2,026 significantly downregulated genes.

(B) Differential genes GO functional annotation analysis, including BP, CC, and MF.

(C) Differential genes GO enrichment dot bubble analysis, with the top 30 being shown.

(D) Reactome enrichment analysis of the differential genes associated with ECM organization, with the top 20 being shown.

(E) Differential gene KEGG enrichment analysis associated with ECM-receptor interactions. The top 20 were shown ($p < 0.05$).

(F) GSEA enrichment analysis associated with apoptosis-related gene expression.

(G) Representative immunofluorescent in the liver sections of fibrotic mice delivered AAV2/8-scramble or AAV2/8-4ebp1-4A-3xflag ($\times 100$ magnification). The arrowheads represent colocalization of staining.

(H) Relative quantification of α -SMA, cleaved caspase-3, and colocalization of α -SMA and cleaved caspase-3. EV, AAV2/8-scramble; OE, AAV2/8-4ebp1-4A-3xflag. Values are the mean $\pm$ SD (unpaired two-sample Student's t test). ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. α -SMA, alpha-smooth muscle actin; AAV, adeno-associated virus; BDL, bile duct ligation; BP, biological process; CC, cellular component; DAPI, 4',6-diamidino-2-phenylindole; ECM, extracellular matrix; LV, lentivirus; MF, molecular function.

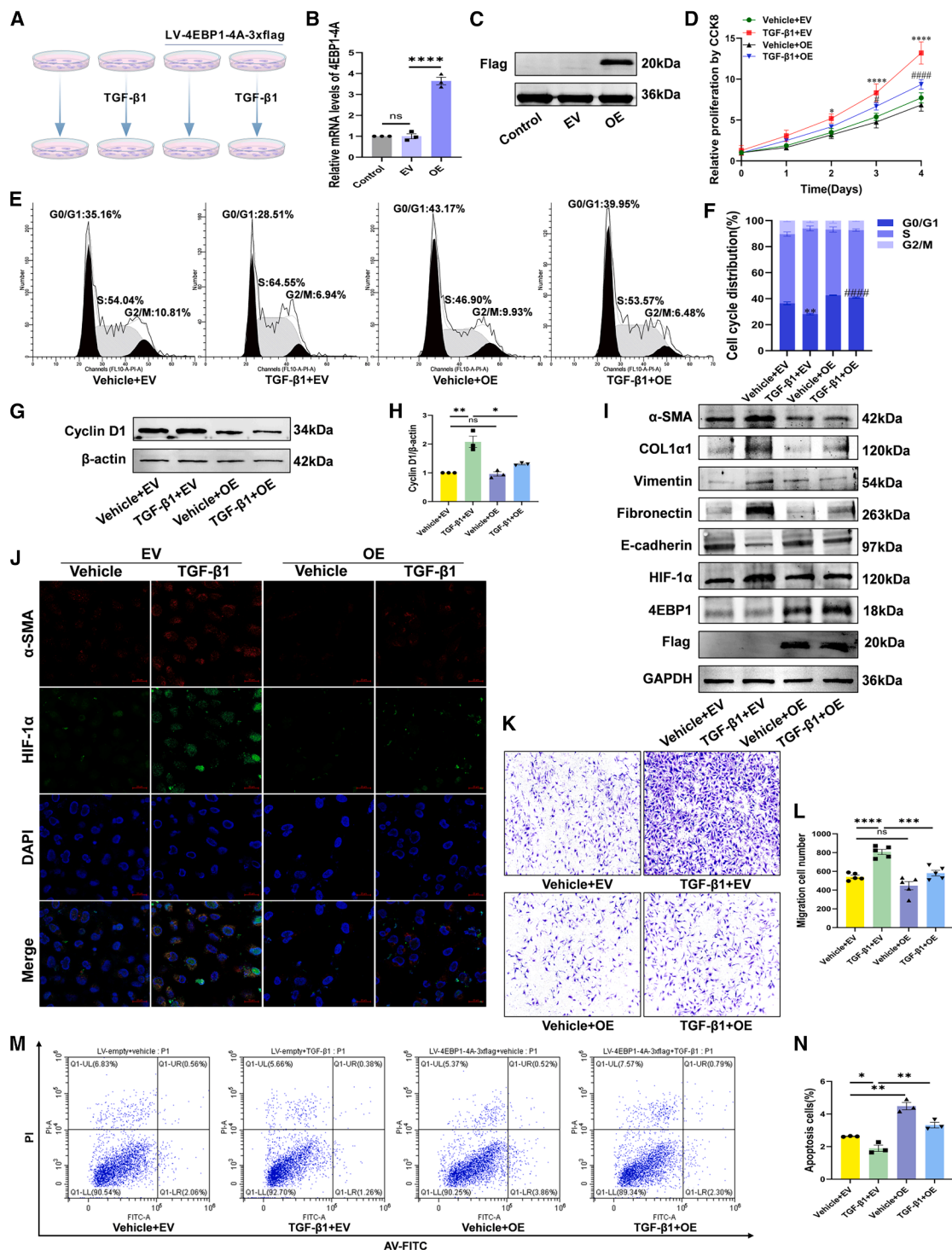


Figure 5. 4EBP1-4A inhibited the fibrosis phenotype of HSCs

(A) The cell processing protocol. (This diagram was made using BioGDP).

(B and C) (B) RT-qPCR analysis of 4EBP1-4A levels and (C) western blotting analysis of FLAG-tagged protein levels with control, LV-empty, or LV-4EBP1-4A-3xflag administration.

(D) Relative cell proliferation of vehicle or TGF-β1-induced LX-2 cells after transduced with LV-empty or LV-4EBP1-4A-3xflag assessed using the CCK-8 assay (n = 4). *p < 0.05, ****p < 0.0001 vs. vehicle+ LV-empty; #p < 0.05, ###p < 0.0001 vs. TGF-β1+ LV-empty.

(E) Flow cytometry analyses using PI staining were performed to examine the cell cycle of LX-2 cells.

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best of our knowledge, this study is the first to demonstrate the critical role of 4EBP1 and its regulatory effect on HIF-1 α in the pathogenesis of hepatic fibrosis.

mTOR inhibition can ameliorate BDL-induced hepatic fibrosis in rats.³⁷ Our study is consistent with previous finding, which suggests that mTOR inhibitors are an effective treatment for liver fibrosis.³⁸ As mTOR is widely involved in the physiological processes of normal cells, inhibiting mTOR may also result in adverse reactions.³⁹ Targeting 4EBP1 phosphorylation can more precisely and specifically regulate protein synthesis while reducing interference with other nontarget signaling pathways and reducing the risk of side effects. Considering that combination therapy inhibiting several typical signaling pathways that affect translation initiation may lead to cumulative side effects, compounds that mimic the action of 4EBP1 or block the function of eIF4E to inhibit cap-dependent translation are currently being investigated in preclinical or clinical trials.⁴⁰ Both eIF4G and 4EBP1 use alpha helix motifs to bind eIF4E, and a recently designed cell active pin peptide (HCS-4EBP1) mimic can manipulate the eIF4E-eIF4G interaction.⁴¹ A novel eIF4E/eIF4G interaction inhibitor EGPI-1 targets the eIF4E signaling pathway to inhibit lung cancer cell growth and angiogenesis.⁴² P-4EBP1/eIF4E holds great promise as an antifibrotic drug target.

In summary, we identified the critical role of 4EBP1 phosphorylation in regulating key fibrotic proteins and maintaining fibrotic function in HSCs and in the signaling pathway through which TGF- β 1 exerts its powerful fibrotic role (Figure 7). mTORC1 mediates the fibrogenic roles of TGF- β 1 via a 4EBP1-dependent mechanism. HIF-1 α may be a key molecule in profibrotic effect induced by 4EBP1 phosphorylation. The clinical significance and function of 4EBP1 in CLD require further investigation. Elucidating these mechanisms offers potential for identifying novel therapeutic targets for hepatic fibrosis. Selectively targeting increased translation efficiency in fibrotic cells driven by 4EBP1 phosphorylation may be an effective strategy for preventing hepatic fibrosis progression.

Limitations of the study

The genetically inferred circulating concentration of 4EBP1 has been observed to be significantly inversely associated with the risk of portal vein thrombosis, which is frequently encountered in patients with cirrhosis.⁴³ The correlation between the level of circulating 4EBP1 and the progression of liver fibrosis requires further analysis. Although some studies have demonstrated that LX-2 cells are functionally and phenotypically similar to normal human HSCs, we used immortalized human HSCs

(LX-2) rather than primary human HSCs. A recent study has indicated that the data obtained from LX-2 cells are highly consistent with those obtained from primary human or mouse HSCs.⁴⁴ Persistent activation of the TGF- β 1/mTORC1 signaling pathway may induce phosphorylation of overexpressed wild-type 4EBP1, resulting in the functional impairment of exogenous wild-type 4EBP1. Given the accumulation of 4EBP1 phosphorylation in liver fibrosis, we focused on investigating whether 4EBP1-4A, which cannot be phosphorylated, exerts anti-fibrotic biological effects. The differential effects between wild-type 4EBP1 and 4EBP1-4A in liver fibrosis require further evaluation. Our study suggested that the abnormal expression of HIF-1 α is associated with the upregulation of 4EBP1 phosphorylation, thereby affecting liver fibrosis progression. However, the specificity of HIF-1 α in p-4EBP1-mediated hepatic fibrosis and whether 4EBP1 affects the liver fibrosis process through other pathways require further research.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Dianxing Sun (sundx9@163.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization was performed by Y.S. Methodology was established by Y. S., Z.L., and M.S. Investigation was carried out by C.Z., J.C., and Y.M. Visualization was performed by Y.S., D.Z., and M.S. Supervision was performed by D.S. and Y.N.

(F) The distribution of cell cycle in LX-2 cells is shown by a histogram ($n = 3$). $^{**}p < 0.01$ vs. vehicle+ LV-empty; $^{###}p < 0.0001$ vs. TGF- β 1+ LV-empty.

(G) Cyclin D1 expression assessed using western blotting and (H) relative quantification of each band normalized to the loading control.

(I) The protein expression of α -SMA, COL1 α 1, vimentin, fibronectin, E-cadherin, 4EBP1, FLAG, and HIF-1 α in LX-2 cells analyzed using western blotting.

(J) Immunofluorescence double staining of α -SMA and HIF-1 α in vehicle or TGF- β 1-stimulated LX-2 cells transduced with LV-empty or LV-4EBP1-4A-3xflag ($\times 400$ magnification).

(K and L) (K) The representative images and (L) quantification of LX-2 cells migration ($\times 100$ magnification).

(M and N) (M) The apoptosis of vehicle or TGF- β 1-stimulated LX-2 cells transduced with LV-empty or LV-4EBP1-4A-3xflag determined using flow cytometry and (N) percentages of apoptotic cells (early and late apoptotic cells). EV, LV-empty; OE, LV-4EBP1-4A-3xflag. Values are the mean $\pm$ SD (unpaired two-sample Student's t test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$; ns, not significant. α -SMA, alpha-smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF-1 α , hypoxia inducible factor 1 subunit alpha; LV, lentivirus; PI, propidium iodide; RT-qPCR, quantitative reverse transcription polymerase chain reaction; TGF- β 1, transforming growth factor beta 1.

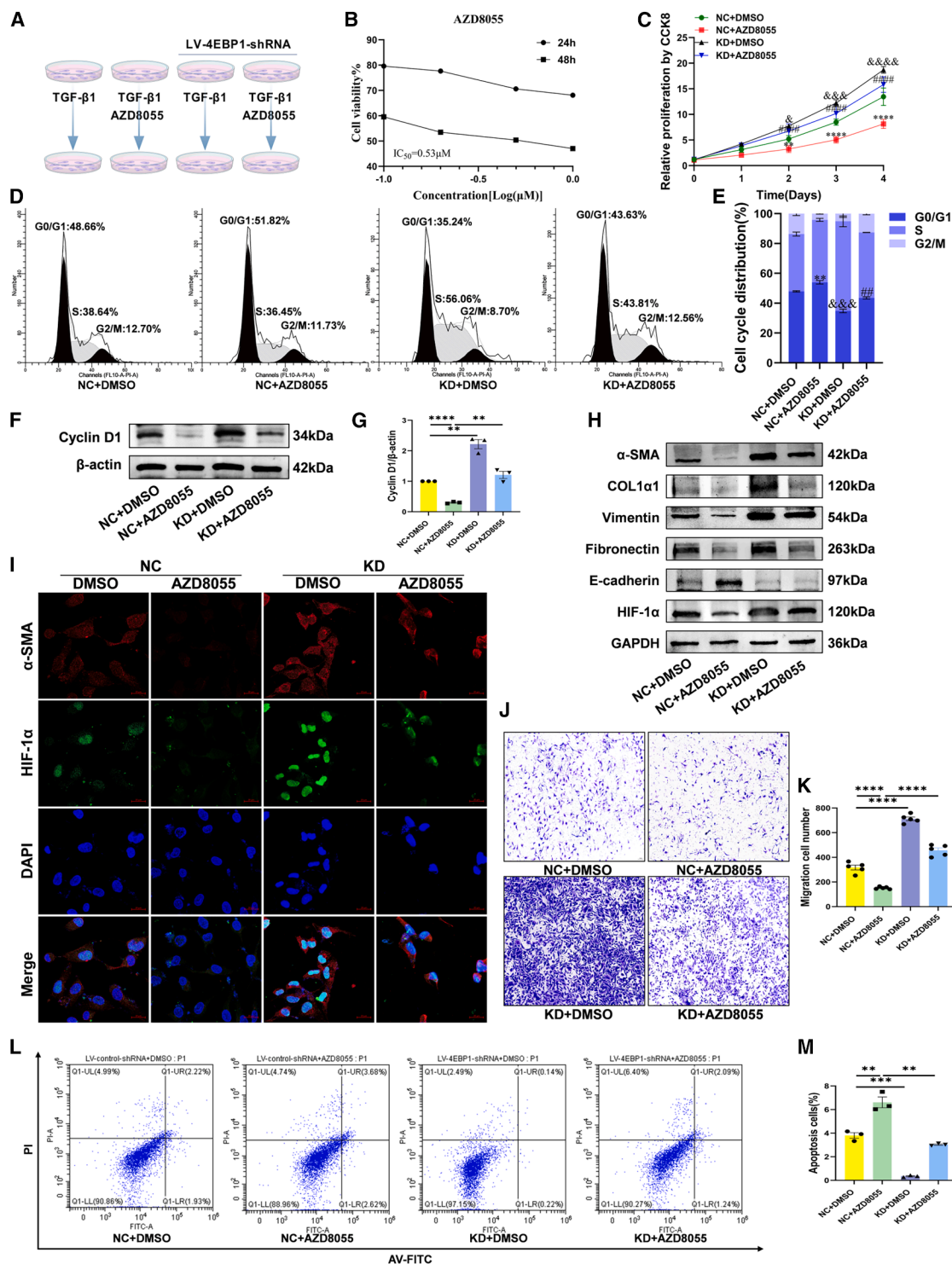


Figure 6. 4EBP1 knockdown promoted the fibrosis phenotype of HSCs

(A) The cell processing protocol. (This diagram was made using BioGDP).

(B) LX-2 cells were treated with 0.1, 0.2, 0.5, or 1 μ M AZD8055 and subjected to the CCK-8 assay at 24 h and 48 h to analyze the cell viability. Absorbance was measured at 450 nm.

(C) Relative cell proliferation of TGF- β 1-induced and DMSO or AZD8055-treated LX-2 cells after transduced with LV-control-shRNA or LV-4EBP1-shRNA assessed using the CCK-8 assay ($n = 4$). ** $p < 0.01$ and **** $p < 0.0001$ vs. LV-control-shRNA+DMSO; ##### $p < 0.0001$ vs. LV-control-shRNA+AZD8055; & $p < 0.05$, && $p < 0.001$, and &&& $p < 0.0001$ vs. LV-control-shRNA+DMSO.

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Table 3. shRNA sequences in LX-2 cells

Gene (ID)	Forward (5'-3')	Reverse (5'-3')
Sh-control	TTCTCCGAACGTGTCACGTAA	TTACGTGACACGTTCGGAGAA
sh4EBP1-1	GACAGTTTGAGATGGACATTTA	TAAATGTCCATCTCAAAGTGT
sh4EBP1-2	GCGCAATAGCCCAGAAGATAA	TTATCTTCTGGGCTATTGCGC
sh4EBP1-3	GCGGTGAAGAGTCACAGTTTGA	TCAAAGTGTACTCTTACCGG

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
 - CCl₄-and bile duct ligation (BDL)-induced hepatic fibrosis models and adeno-associated virus 2/8 (AAV2/8) transduction in mice
 - Cell culture and treatment
- **METHOD DETAILS**
 - Serum detection of biochemical indexes and inflammation indexes
 - Histological analysis and immunohistochemistry (IHC)
 - Immunofluorescence and histochemistry
 - RNA extraction and qRT-PCR
 - Protein extraction and western blotting
 - Cell counting Kit-8 (CCK-8) assay
 - Cell transduction and stable cell lines
 - Flow cytometry
 - Cell migration
 - RNA sequencing and related data analysis
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

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- (E) The distribution of the cell cycle in LX-2 cells is shown by a histogram ($n = 3$). $^{**}p < 0.01$ vs. LV-control-shRNA+DMSO; $^{##}p < 0.01$ vs. LV-control-shRNA+AZD8055; $^{***}p < 0.001$ vs. LV-control-shRNA+DMSO.
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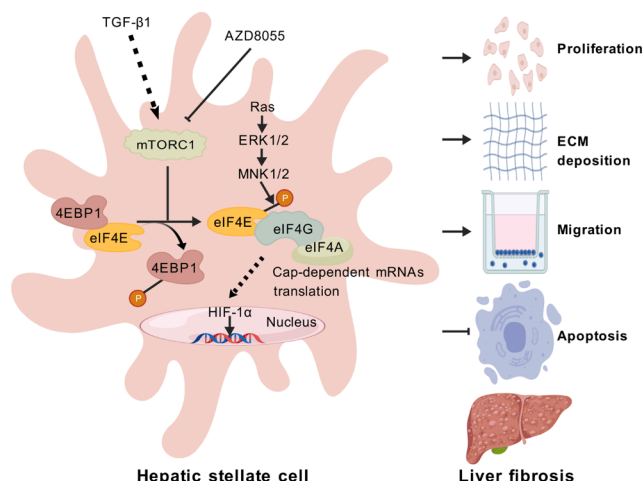


Figure 7. mTORC1/4EBP1 signaling mediates TGF- β 1-induced fibrosis phenotype in HSC

TGF- β 1-stimulated HSCs result in the activation of mTORC1/4EBP1 signaling, followed by cap-dependent mRNA translation, which increases activation, proliferation, ECM deposition, migration, and inhibits apoptosis of HSCs. In addition, TGF- β 1 enhances HIF-1 α activity through mTORC1/4EBP1 signaling. (This diagram was made using BioGDP).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-4EBP1	Abways	Cat#CY6583; RRID: AB_851615
Rabbit monoclonal anti-p-4EBP1(T46)	Abways	Cat#AY8172; RRID: AB_10915563
Rabbit monoclonal anti-p-4EBP1(T37/T46)	Affinity	Cat#AF3830; RRID: AB_10887735
Rabbit monoclonal anti- α -SMA	Boster	Cat#BM3902; RRID: AB_2847835
Mouse monoclonal anti- α -SMA	Boster	Cat#BM0002; RRID: AB_3712128
Rabbit polyclonal anti- Collagen I	Proteintech	Cat#14695-1-AP; RRID: AB_852982
Rabbit monoclonal anti-Vimentin	Abways	Cat#CY5134; RRID: AB_2216132
Rabbit monoclonal anti-Cyclin D1	Abways	Cat#CY5404; RRID: AB_1661031
Rabbit monoclonal anti-HIF-1 α	Boster	Cat#M00013-4; RRID: AB_2927791
Rabbit monoclonal anti-Flag-Tag	Abways	Cat#AB0030; RRID: AB_1960908
Rabbit monoclonal anti- β -actin	Proteintech	Cat#81115-1-RR; RRID: AB_2750915
Rabbit polyclonal anti-E-cadherin	Boster	Cat#PB9561; RRID: AB_397580
Rabbit monoclonal anti-Fibronectin	Proteintech	Cat#15613-1-AP; RRID: AB_2738005
Rabbit monoclonal anti-Cleaved-Caspase 3	Affinity	Cat#AF7022; RRID: AB_2910623
DyLight 800 Goat anti Rabbit IgG	Abbkine	Cat#A23920; RRID: AB_10697505
Alexa Fluor™ 488 Goat anti Rabbit	Invitrogen	Cat#A-11008; RRID: AB_10373266
Alexa Fluor™ 594 Goat anti Mouse	Invitrogen	Cat#A-11005; RRID: AB_10374440
Rabbit polyclonal anti-GAPDH	Boster	Cat#A00227-1; RRID: AB_2651132
Bacterial and virus strains		
AAV2/8-Scramble	HanBio	N/A
AAV2/8-4ebp1-4A-3xflag	HanBio	N/A
LV-empty	HanBio	N/A
LV-4EBP1-4A-3xflag	HanBio	N/A
Chemicals, peptides, and recombinant proteins		
AZD8055	GLP BIO	GC16380
Recombinant Human TGF- β 1	Abways	PY2270
Olive oil	Macklin	N/A
99.9% CCl ₄ solution	Macklin	N/A
DAPI	Solarbio	N/A
Critical commercial assays		
Annexin V-FITC/PI Apoptosis Kit	Elabscience	E-CK-A211
DNA Content Quantitation Assay(Cell Cycle)	Solarbio	CA1510
Cell Counting Kit-8	Share-bio	SB-CCK8
High-Capacity cDNA Reverse Transcription Kit	Takara Bio	RR036A
TBGreen™ Premix Ex Taq™ II	Takara Bio	Tli RNaseH Plus
Experimental models: Cell lines		
Human hepatic stellate cells LX-2	Pricella	CL-0560
Experimental models: Organisms/strains		
C57BL/6J mouse	Beijing HFK Bioscience	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Primer sequences for qRT-PCR, see Table 2	Sangon Biotech	N/A
shRNA sequences in LX-2 cells Sh-control: Forward TTCTCCGAACGTGTCACGTAA; Reverse TTACGTGACACGTCGGAGAA	HanBio	N/A
shRNA sequences in LX-2 cells sh4EBP1-1: Forward GACAGTTTGAGATGGACATTTA; Reverse TAAATGTCCATCTCAAAGTGT	HanBio	N/A
shRNA sequences in LX-2 cells sh4EBP1-2: Forward GCGCAATAGCCCAGAAGATAA; Reverse TTATCTTCTGGGCTATTGCGC	HanBio	N/A
shRNA sequences in LX-2 cells sh4EBP1-3: Forward GCGGTGAAGAGTCACAGTTTGA; Reverse TCAAAGTGTGACTCTTCACCG	HanBio	N/A
Software and algorithms		
Powerpoint 2021	Microsoft	N/A
Excel 2021	Microsoft	N/A
Graphpad Prism9.5	GraphPad Software	N/A
ImageJ	NIH	N/A
Other		
Platereader Envision™	PerkinElmer	Envision™
Leica DMC5400	Leica Microsystems	N/A
Fluorescence microscope	Olympus	N/A
ABI 7500 Real-Time PCR System	Applied Biosystems	N/A
Odyssey Fluorescence Imaging System	LI-COR	N/A
Flow cytometer	Beckman	BD Accuri™ C6
ModFit LT	Verity Software	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**CCl₄-and bile duct ligation (BDL)-induced hepatic fibrosis models and adeno-associated virus 2/8 (AAV2/8) transduction in mice**

Eight-week-old male C57BL/6J wild-type mice (22–24 g) were provided by Beijing HFK Bioscience Co., Ltd, and housed in an environmentally controlled facility under a 12-h light/dark cycle with *ad libitum* access to food and water. The ectopic expression of the dominant negative 4ebp1-4A phosphomutant (with phosphorylated checkpoints Thr36, Thr45, Ser64, and Thr69 substituted with alanine, such that the 4EBP1 protein could not be successfully phosphorylated) was mediated by transduction of liver-specific serotype AAV2/8 in mouse models of CCl₄ attack or BDL surgery. 4ebp1-4A cDNA was PCR-amplified and subcloned into the AAV-CMV-MCS-3flag AAV vector to construct the 4ebp1-4A overexpression (OE) plasmid. The 4ebp1-4A expression cassette was packaged into AAV2/8 to generate AAV2/8-4ebp1-4A vector (AAV2/8-4ebp1-4A-3xflag). Both AAV2/8-4ebp1-4A-3xflag and control AAV2/8-Scramble viruses were obtained from HanBio (Shanghai, China). At the expected time, mice were intravenously administered 2×10^{11} viral genomes of AAV2/8-4ebp1-4A-3xflag or AAV2/8-Scramble control vector via the tail vein in 100 μ L phosphate-buffered saline (PBS, Gibco, Grand Island, NY, USA).

The mice were randomly divided into four groups (n = 5) and received group-specific diets for six weeks: Mice with AAV2/8-Scramble or AAV2/8-4ebp1-4A-3xflag tail vein injection were randomly divided into control and experimental groups. Mice in the experimental groups were injected intraperitoneally with a final concentration of 10 % CCl₄ solution dissolved in olive oil (5 μ L/g, based on the weight of the mice, twice a week for 6 weeks). The control mice were simultaneously injected with a matching volume of olive oil. Olive oil and 99.9 % CCl₄ solution were purchased from Macklin (Shanghai, China). Similarly, mice with AAV2/8-Scramble or AAV2/8-4ebp1-4A-3xflag tail vein injection were randomly divided into the sham groups (sham, n = 5) or the BDL groups (BDL, n = 5) receiving sham operation or bile duct ligation, respectively. CCl₄ and ligation of the common bile duct can lead to liver injury and fibrosis. At the end of six weeks, all animals were fasted overnight and euthanized. Blood and liver samples were collected. Livers were fixed in 10 % neutral-buffered formalin for histological analysis, rapidly frozen in liquid nitrogen, and stored at -80 °C for RNA and protein extraction. We confirm that the study is in accordance with the ARRIVE guidelines. All procedures were conducted following

the guidelines of the Animal Care and Use Committee of Hebei Province and approved by the Animal Experiment Ethics Committee of Hebei Medical University (Ethics approval number: 2024-30).

Cell culture and treatment

Human hepatic stellate cells LX-2 (CL-0560, Pricella, Wuhan, China) were cultured in DMEM (#10566-016, Gibco), supplemented with 10 % FBS (#10091-148, Gibco) and 100 units/ml penicillin and streptomycin (#CC004, Mac-gene) at 37 °C in a humidified atmosphere with 5 % CO₂. LX-2 cells were seeded into 6-well plates (1 × 10⁵ cells/well), and the serum-free medium was replaced for 24 h before stimulation with TGF-β1 (10 ng/mL, PY2270, Abways). Specific concentrations of AZD8055 (GC16380, GLP BIO) were added simultaneously with TGF-β1. After stimulation for 24 h, cells were collected for western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses.

METHOD DETAILS

Serum detection of biochemical indexes and inflammation indexes

Mice peripheral blood serum was obtained by centrifuging at 3000 rpm at 4 °C for 10 min, which was used to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), TGF-β1, and Interleukin 6 (IL-6) levels using appropriate enzyme assay kits on a dedicated automatic analyzer (Chemray 240, Shanghai, China).

Histological analysis and immunohistochemistry (IHC)

Liver tissues were fixed in 10 % neutral formalin and embedded in paraffin blocks. H&E, Masson's trichrome, Sirius Red, and IHC staining were used to stain 5 μm-thick liver sections. For IHC, deparaffinized and hydrated liver tissue slices were incubated with 10 % H₂O₂ for 10 min, followed by 0.01 mol/L sodium citrate buffer for 30 min. The reaction was blocked by incubation with 3 % bovine serum albumin (BSA) for 1 h at room temperature. Sections were incubated with various primary antibodies overnight at 4 °C. Subsequently, PBS was used to wash the sections, which were then incubated with a secondary antibody at room temperature (20–25 °C) for half an hour. Positive immunological interaction signals were observed with the 3,3'-diaminobenzidine solution. All procedures were performed according to the manufacturer's instructions. Images were visualized using a standard light microscope (Leica DMC5400, Leica Microsystems), and the degree of collagen deposition was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). [Table 1](#) provides specific information regarding all the antibodies.

Immunofluorescence and histochemistry

Fixed mouse liver tissues were stained with various primary antibodies and subsequently stained with goat anti-mouse IgG (H+L) (Invitrogen, USA) and goat anti-rabbit IgG (H+L) (Invitrogen, USA) secondary fluorescent antibodies, respectively.

LX-2 cells were grown uniformly on cell-climbing slides. After washing DMEM with PBS, the cells were fixed with 4 % paraformaldehyde for 15 min, followed by permeabilization with 0.1 % Triton-100 for 5 min at room temperature. After culturing with 3 % BSA for 30 min, primary antibodies were used to incubate LX-2 cells overnight at 4 °C in moist conditions, and the next day combined with corresponding secondary antibodies for 1 h at ambient temperature. DAPI (Solarbio, Beijing, China) was used to stain the cell nuclei, and the slides were sealed. Fluorescent images were captured using a fluorescence microscope (Olympus, Tokyo, Japan) from at least three randomly selected fields. Statistical analysis was performed using ImageJ software.

RNA extraction and qRT-PCR

RNA was extracted from cells and mouse liver tissues using the TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Takara Bio, RR036A), according to the manufacturer's instructions. qRT-PCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA) using TBGreenTM Premix Ex TaqTM II (Tli RNaseH Plus; Takara Bio, RR820A), as previously described.⁴⁵ Glyceraldehyde-3-phosphate dehydrogenase expression was used as an internal control. The threshold cycle (2^{-ΔΔCT}) method was used to calculate the fold-change in the corresponding genes. [Table 2](#) shows the primer sequences for all genes.

Protein extraction and western blotting

LX-2 cells and mouse liver tissues were lysed using RIPA lysis solution (Beyotime, Shanghai, China), mixed with PMSF and phosphatase inhibitors, and lysed on ice for 1 h. After centrifugation at 12,000 rpm at 4 °C for 20 min, the proteins in the upper layer were extracted, and the BCA assay was used to detect their concentration. Proteins were separated by 10 % SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany) using the wet transfer method. After being blocked with 5 % skimmed milk or BSA for 1 h at room temperature, membranes were incubated with primary antibodies overnight at 4 °C. On the second day, Tris-buffered saline containing 0.1 % Tween-20 was used to wash the membranes thrice, followed by incubation with a horseradish peroxidase-labeled secondary antibody DyLight 800 goat IgG (1:7,000, A23920, Abbkine Scientific, Wuhan, China) for 1 h at room temperature. Finally, protein bands were detected using the Odyssey Fluorescence Imaging System (LI-COR, USA), and density was analyzed using ImageJ software.

Cell counting Kit-8 (CCK-8) assay

For each treatment group, 9×10^5 cells were suspended in 3 mL Complete Cell Culture Medium. Each group of cells was seeded at a density of 100 μ L of the cell suspension per well in a 96-well plate. The cells were cultured for 0, 24, 48, 72, and 96 h. Ten microliters of CCK-8 reagent (SB-CCK8, Share-bio) was added to each well, and the plate was incubated for 1.5 h. Absorbance was measured at 450 nm using an enzyme-linked immunosorbent assay kit (Infinite F50, Swiss TECAN Company).

Cell transduction and stable cell lines

The 4EBP1-4A (encoding human 4EBP1 with phosphorylated checkpoints Thr37, Thr46, Ser65, and Thr70 substituted with alanine) cDNA was PCR-amplified and subcloned into the LV-CMV-MCS-3flag-EF1-puro lentiviral vector to construct the 4EBP1-4A over-expression plasmid. The 4EBP1-4A expression cassette was packaged into lentiviral particles (LV-4EBP1-4A-3xflag) in 293T cells, with empty vector (LV-empty, no insert) and LV-empty-ZsGreen serving as controls (HanBio, Shanghai, China). LX-2 cells were stably transduced with lentiviral supernatants containing equal amounts of LV-empty, LV-empty-ZsGreen, and LV-4EBP1-4A-3xflag in the complete growth medium. Short hairpin RNAs (shRNAs) targeting 4EBP1 were generated according to the manufacturer's instructions. The cells were transduced with lentiviral supernatants containing equal amounts of sh-control, sh-4EBP1-1, sh-4EBP1-2, and sh-4EBP1-3. LX-2 cells were infected with lentivirus and selected with 1.5 μ g/mL puromycin (Sigma-Aldrich, Milwaukee, WI, USA) for about two weeks to obtain stably transfected cell lines. The control and silencing sequences are listed in Table 3.

Flow cytometry

LX-2 cells in each group were harvested using trypsin-EDTA, blown gently with fresh medium, centrifuged into a pellet, and rinsed with pre-cooled PBS. Then, the cells were fixed with 80 % pre-cooled ethanol. After overnight incubation at 4°C, cells were washed with PBS and incubated with RNase A at 37°C for 30 min. The cells were then incubated with propidium iodide (PI) on ice for half an hour in the dark. Cell cycle distribution was analyzed using a BD Accuri™ C6 flow cytometer (BD Biosciences, USA) with ModFit software.

LX-2 cells were first treated with $1 \times$ Annexin V Binding Buffer (100 μ L) for 10 min, then 2.5 μ L of Annexin V-FITC Reagent and 2.5 μ L of PI Reagent were added. Samples were gently vortexed and incubated at room temperature in the dark for 15 min. $1 \times$ Annexin V Binding Buffer (400 μ L) was added to the samples and mixed thoroughly. Cell apoptosis was analyzed using a BD Accuri™ C6 flow cytometer.

Cell migration

For migration assays, LX-2 cells resuspended in 200 μ L of serum-free DMEM were plated in the upper chambers (Corning Transwell Reservoir, NY, USA) as previously described. The lower chambers used 27 DMEM with 10 % FBS and respective therapeutic drugs as a chemoattractant. After 24 h, non-migrating cells were gently removed from the upper surface using a cotton swab. Cells that migrated through the membrane to the lower surface were fixed with cold methanol for 15 min, washed three times with PBS, and stained with crystal violet (Sigma-Aldrich) for 30 min. The polycarbonate membranes were sealed with neutral balsam. The cells were counted and imaged using a microscope (Leica) with ImageJ software. The experiments were performed in triplicate.

RNA sequencing and related data analysis

TGF- β 1-activated LX-2 cells transduced with LV-empty or LV-4EBP1-4A-3xflag (five biological replicates per group) were pooled and sequenced on an Illumina Novaseq platform, and all operations were performed by professional technicians from Novogene (Beijing, China). DESeq2 (<http://bioconductor.org/packages/stats/bioc/DESeq2/>) was used to analyze the genes that were differentially expressed, and the criterion for the screening of significantly differentially expressed genes was $FDR < 0.05$ & $|\log_2 FC| \geq 1$. GO and Reactome functional annotation analysis, GO enrichment analysis, KEGG pathway enrichment analysis, and GSEA enrichment analysis were performed. Data were analyzed using the online platform of Novomagic Cloud Platform (<https://magic-plus.novogene.com>).

QUANTIFICATION AND STATISTICAL ANALYSIS

All quantitative data are represented as mean $\pm$ standard deviation. GraphPad Prism 9.0 (San Diego, CA, USA) was used for data processing and statistical plotting. A t-test was used to compare the differences between two groups, and one-way ANOVA was used for multi-group analysis. Statistical significance was set at $p < 0.05$. significant.