OMTN, Volume *18*

Supplemental Information

NPAS2 Contributes to Liver Fibrosis by Direct Transcriptional Activation of Hes1 in Hepatic Stellate Cells

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Supplementary Figures:

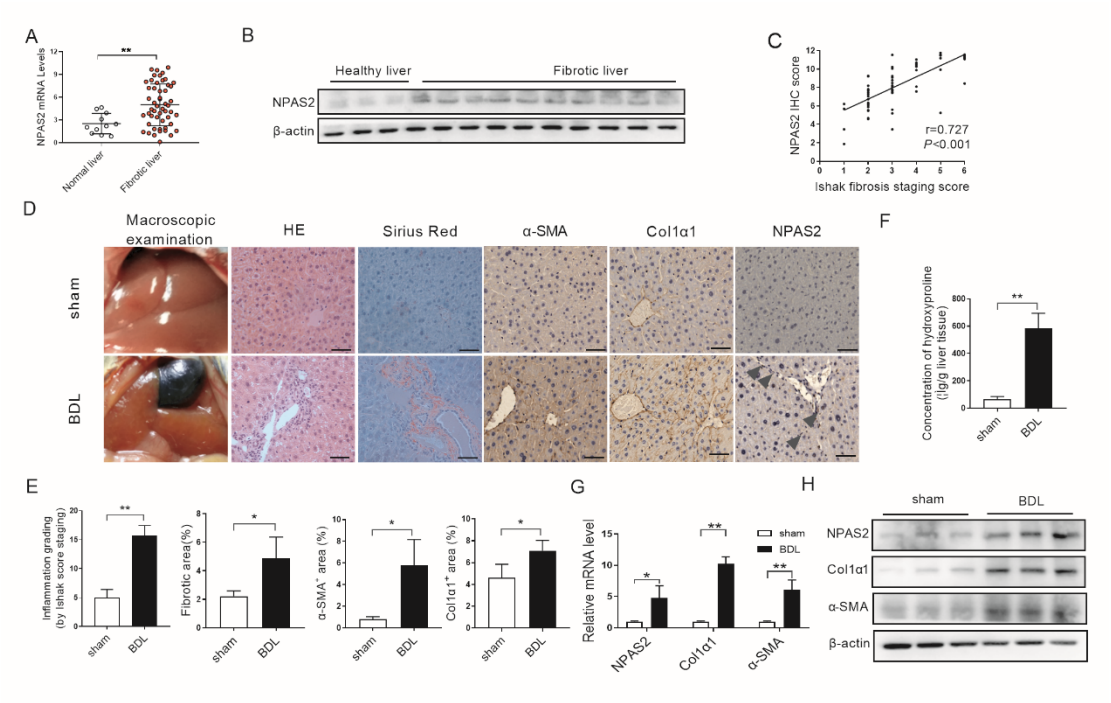

Figure S1. Expression levels of NPAS2 in huamn LF specimens and BDL-induced LF.

(A and B) qRT–PCR and western blotting analyses for NPAS2 expression were performed in human liver tissues (n=63). (C) Pearson's correlation analyses of NPAS2 IHC scores with Ishak scores of picosirius red staining (r=0.727, P<0.01, n=63). (D) Representative results to evaluate liver fibrosis progression. (E) Semiquantification results to statistically analyze fibrosis progression indicated in Figure 1D. (F) Liver hydroxyproline content was analyzed to compare the fibrosis progression after CCl4 or BDL induced injuries. (G and H) Classical fibrosis related genes was analyzed to compare the fibrosis progression stage of different groups at mRNA level and protein level (n=8-10 for each group). Data shown are the mean ± S.E.M. from three independent experiments. $P < 0.05$.

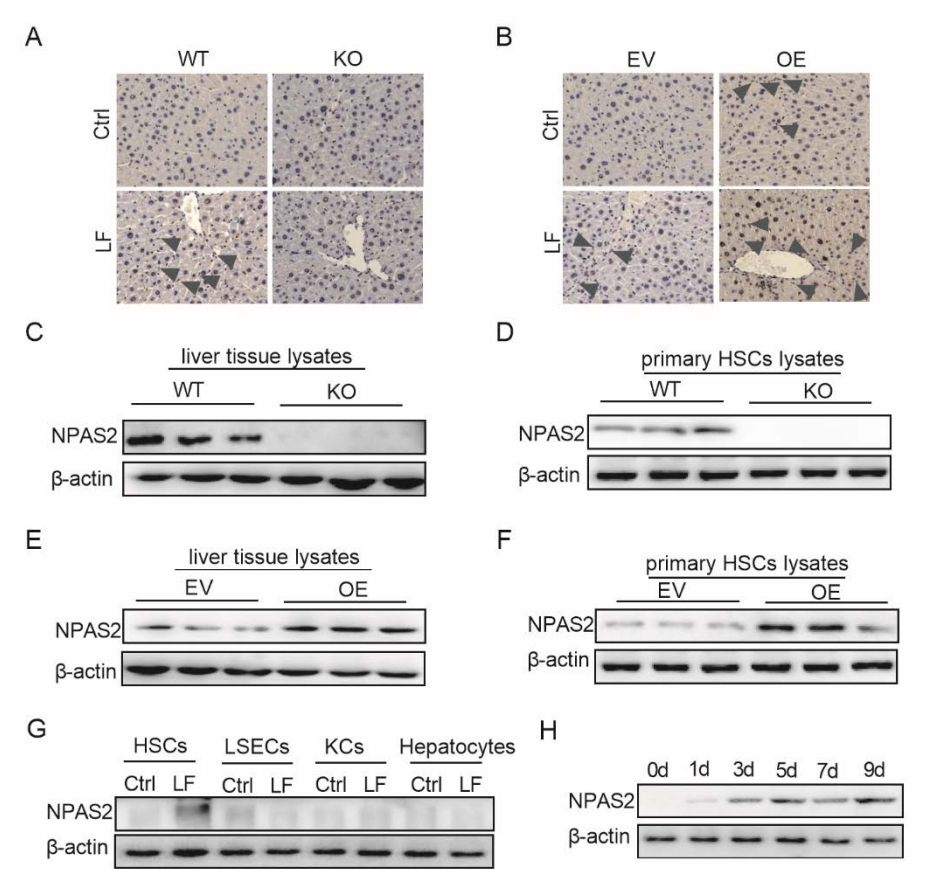

Figure S2. Expression level of NPAS2 in liver lysates and primary cells lysates.

(A) IHC analysis for expression of NPAS2 in WT and KO liver fibrosis mice. (B) IHC analysis for expression of NPAS2 in WT and KO liver fibrosis mice. (C and D) Western blotting analysis for expression of NPAS2 in whole liver lysates and primary HSCs of WT and KO mice. (E and F) Western blotting analysis for expression of NPAS2 in whole liver lysates and primary HSCs of EV and OE mice. (G) Western blotting analysis for expression of NPAS2 in primary hepatocytes, HSCs, LSECs and Kuppfer cells after liver injury. (H) Western blotting analysis for expression of NPAS2 in primary HSCs from WT mice at 0, 1, 3, 5, 7, 9 day.

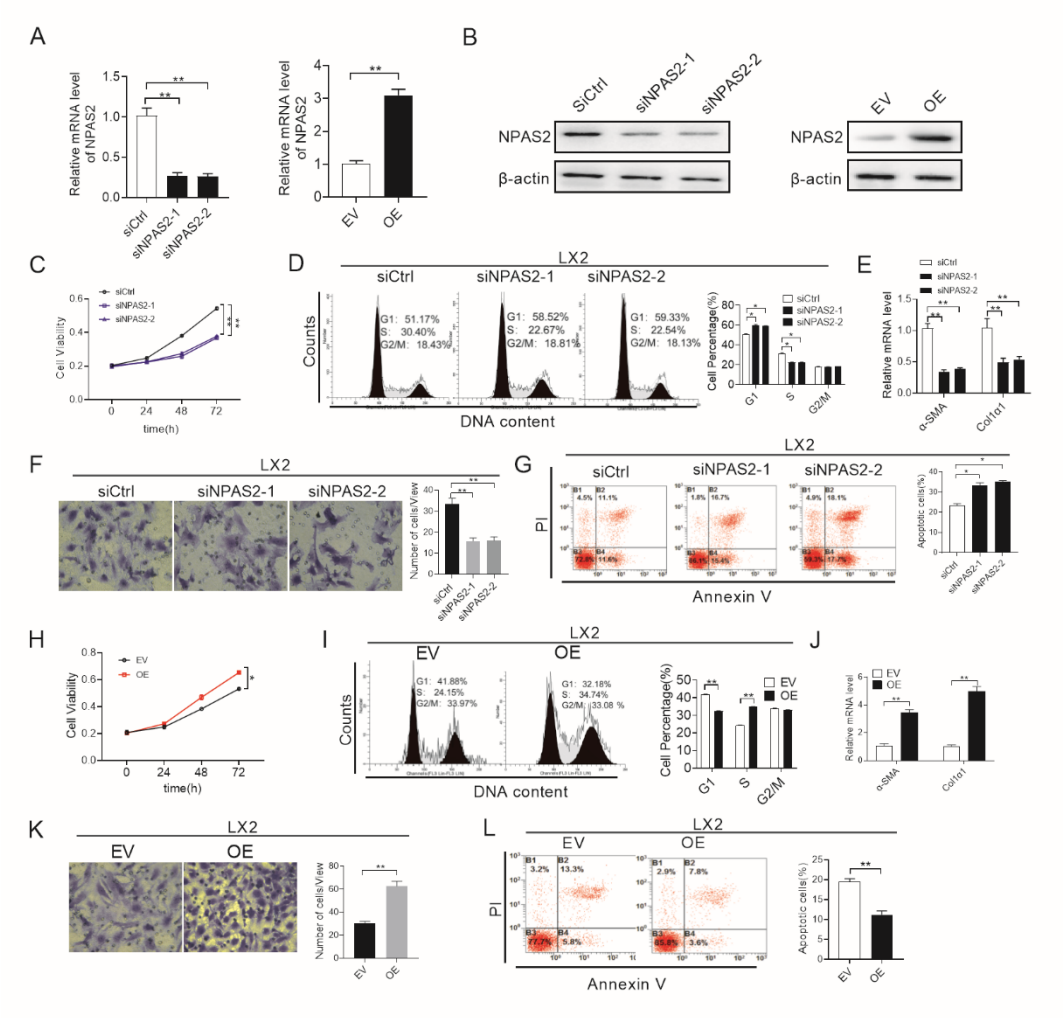

Figure S3. NPAS2 promotes HSCs activation

(A and B) qRT–PCR and western blot analyses for NPAS2 expression were performed in LX2 cells, which were transiently transfected with expression vector or siRNA as indicated. NPAS2, expression vector encoding NPAS2; EV, empty vector; siNPAS2-1 and siNPAS2-2, siRNAs against NPAS2; siCtrl, control siRNA. (C and H) MTS assays for LX2 cells proliferation with treatment as indicated. (D and I) Cell cycle analysis by flow cytometry in LX2 cells proliferation with treatment as indicated. (E and J) Col1α1 and α-SMA mRNA expression in LX2 cells with treatment as indicated. (F and K) Transwell matrigel invasion assay for cell invasion ability of LX2 cells with treatment as indicated. (G and L) Flow cytometry analysis of apoptosis by Annexin V and PI staining in LX2 cells with treatment as indicated. Data shown are the mean ± S.E.M. from three independent experiments. *P < 0.05; ** P< 0.01.

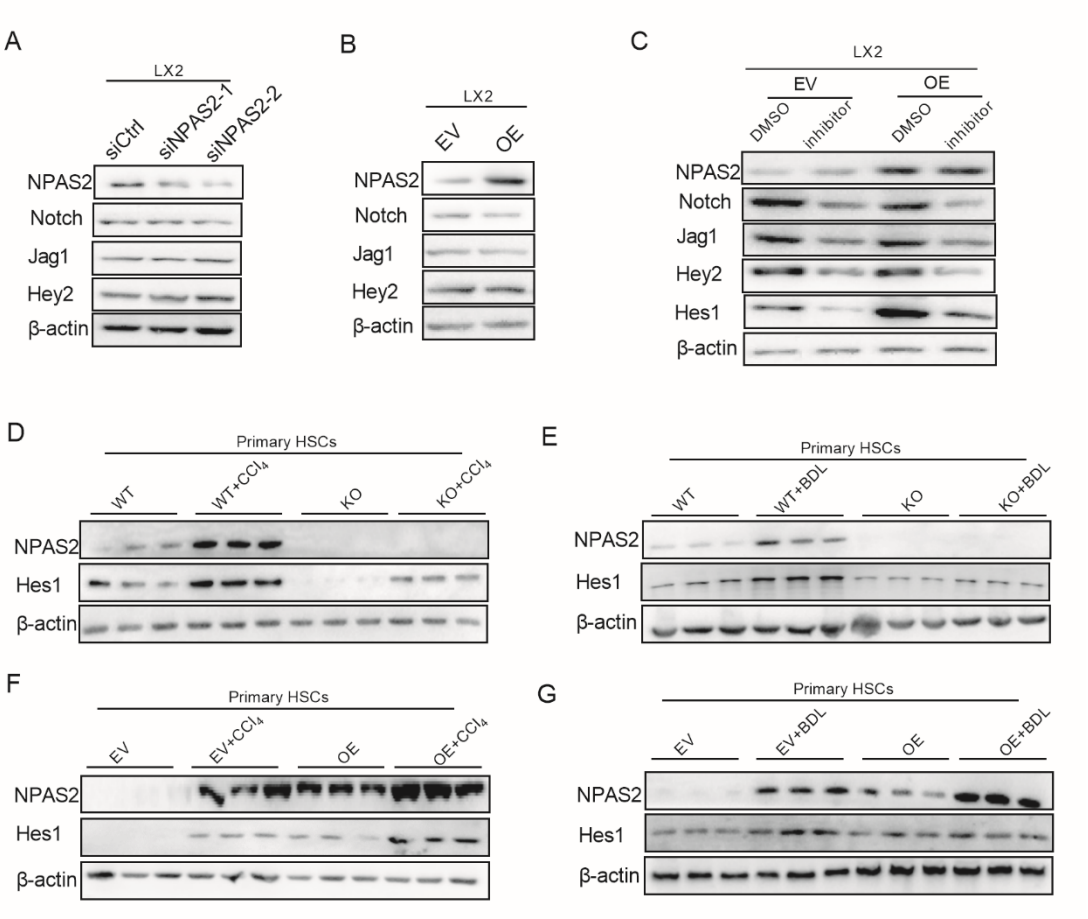

Figure S4. NPAS2 activates Hes1 to activate HSCs.

(A and B) western blot analysis for Notch signaling related genes were performed in LX2 cells, which were transiently transfected with expression vector or siRNA as indicated. NPAS2, expression vector encoding NPAS2; EV, empty vector; siNPAS2-1 and siNPAS2-2, siRNAs against NPAS2; siCtrl, control siRNA. (C) western blot analysis for Notch signaling related genes were performed in LX2 cells after inhibiting Notch signaling by GSI (inhibitor of Notch pathway). (D-F) western blot analysis for Hes1 were performed in primary HSCs with treatment as indicated.

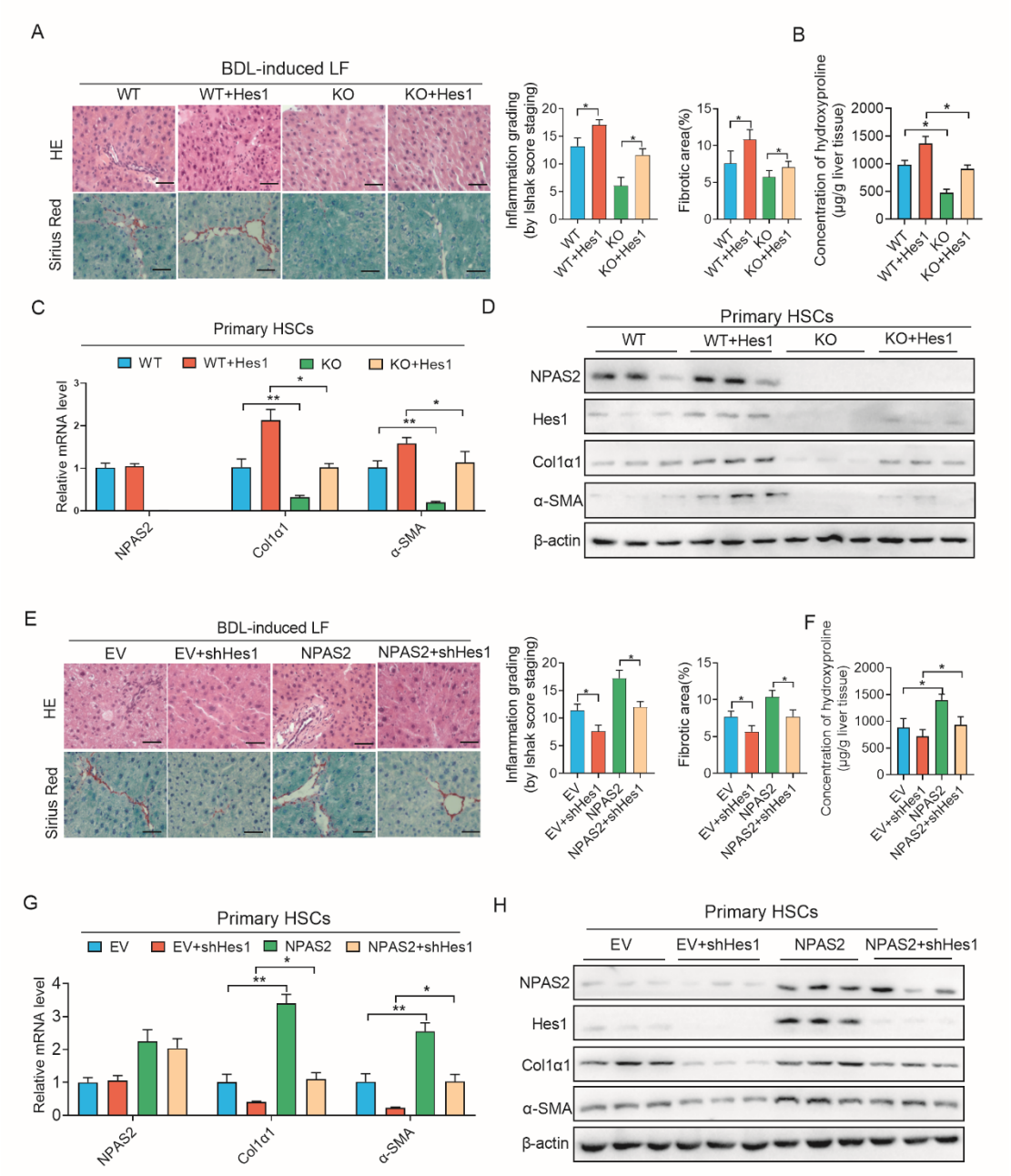

Figure S5. Profibrotic effects of NPAS2 require Hes1 indicated by BDL-induced LF model.

(A) H&E and picrosirius red staining revealed the reversed liver bridging fibrosis and collagen deposition in NPAS2-KO together with Lenti-Hes1 mice in BDL-induced LF model. (B) Hydroxyproline content in the BDL-induced liver tissues. (C and D) qPCR and western blot for Col1α1 and α-SMA expression in the liver tissues with treatment as indicated in BDL-induced LF models. (E) H&E and picrosirius red staining revealed Lenti-shHes1 injection inhibits the aggravated liver bridging fibrosis and

collagen deposition in NPAS2-OE mice in BDL-induced LF model. (F) Hydroxyproline content in the BDL-induced liver tissues. (G and H) qPCR and western blot for Col1α1 and α-SMA expression in the liver tissues with treatment as indicated in BDL-induced LF models. *P < 0.05; ** P< 0.01.

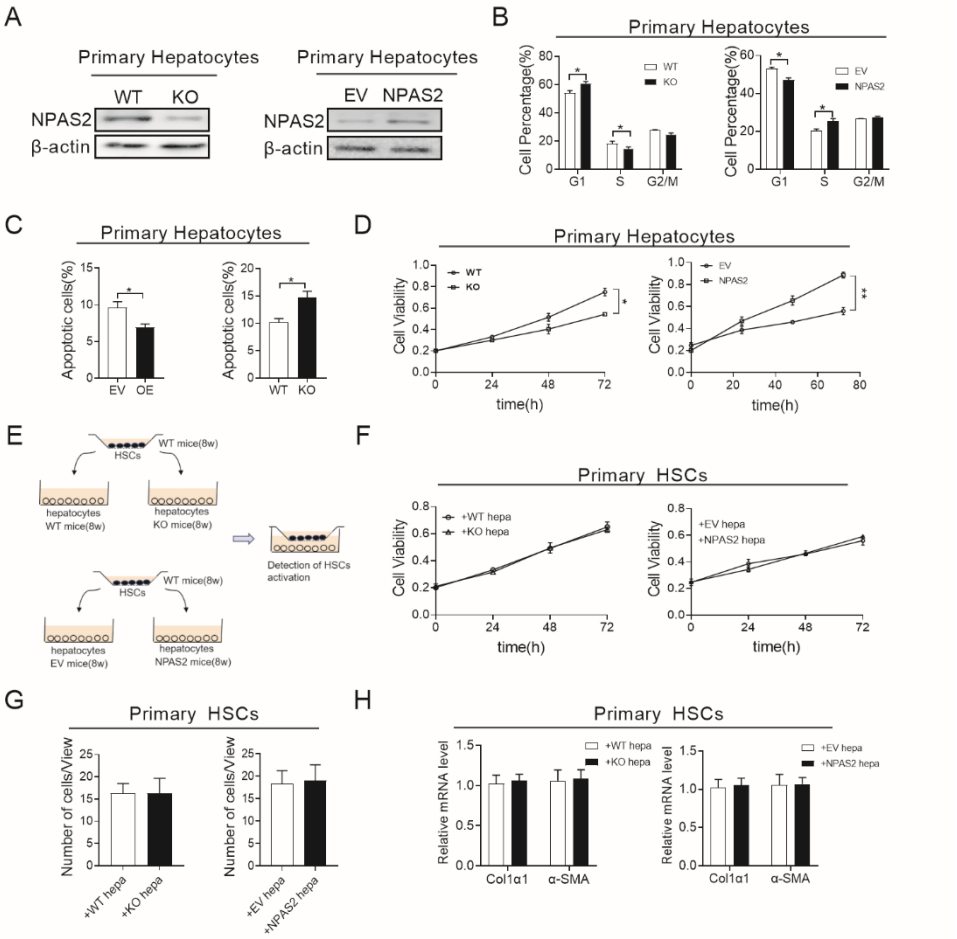

Figure S6. Hepatic function detected by ALT/AST levels.

(A, B, C, and D) ALT and AST levels in serum of CCl4- and BDL-induced liver fibrosis models. （E, F, G, and H) ALT and AST levels in serum of liver fibrosis mice with treatments as indicated in Fig6 and FigS5. *P < 0.05; ** P< 0.01.

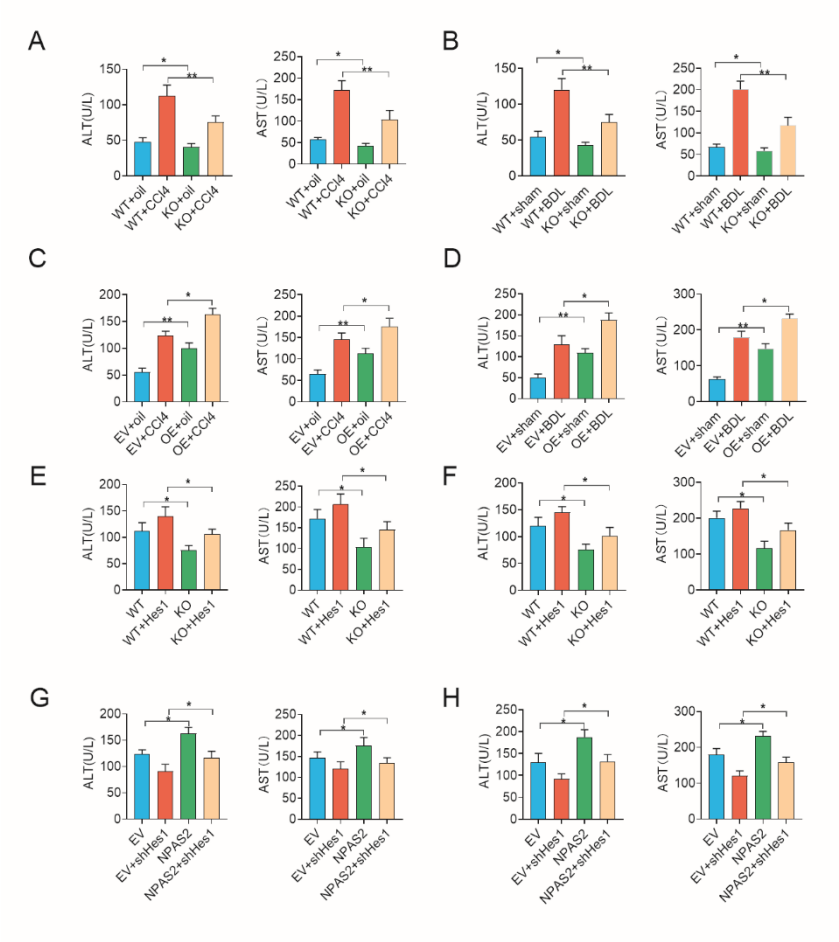

Figure S7. The effects of NPAS2 in primary hepatocytes on activation of primary HSCs.

(A) Western blot analysis for expression levels of NPAS2 in primary hepatocytes with treatments as indicated.（B) Cell cycle analysis by flow cytometry in primary hepatocytes proliferation from mice with treatments as indicated. (C) Flow cytometry analysis of apoptosis by Annexin V and PI staining in primary hepatocytes proliferation from mice with treatments as indicated. (D) MTS assay for primary hepatocytes proliferation from mice with treatments as indicated. (E) A schematic representation of co-culture experiments with primary HSCs and hepatocytes isolated from mice. (F, G and H) The effects of NPAS2 in primary hepatocytes on activation of primary HSCs. F) Cell cycle analyses by flow cytometr, G) Flow cytometry analyses of apoptosis by Annexin V and PI staining, H) MTS assays. *P < 0.05; ** P< 0.01.

Links	Gene Symbol	Uniprot	Pseudogene	microRNA	Integrated Score	Number of Sources	Score Class
iID GC UP	NPAS2	Q99743		hsa-miR-17-5p	0.953174880764457	21	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-106b-5p	0.949287842598554	21	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-19a-3p	0.947454338565796	20	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-19b-3p	0.946721171167091	20	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-199b-5p	0.92790341482925	23	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-218-5p	0.925841335645154	20	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-106a-5p	0.924150085150512	20	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-20a-5p	0.916984223757973	19	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-93-5p	0.912459050350389	17	Very High
iID GC UP	NPAS2	Q99743		hsa-miR-20b-5p	0.894406446503852	20	Very High

Figure S8. Top ten predicted miRNAs targeting NPAS2 based on microRNA Data Integration Portal (mirDIP)-based target prediction programs

Supplemental materials and methods

Construction of reporter plasmids and site-directed mutagenesis

Promoter sequences of Hes1 were obtained from UCSC Genome Browser. Then pGL3-Basic vectors (Promega, Madison, WI) inserted by truncated portions of Hes1 promoter was generally constructed by PCR amplification of selected regions with primers listed in Supplementary Table 3. This construct corresponds to the sequence from nt-2177 to nt-+67 (relative to the transcriptional start site) of the 5'-flanking region of the human Hes1 gene. Site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis Kit (NEB, E0552S) according to the manufacturer's instructions. The first E-box was mutated (underlined) to ACCGGA (wt, CACGTG). The sequences of PCR products were confirmed by sequencing (Sangon, Shanghai, China).

Co-culture of primary hepatocytes and primary HSCs

To examine the effects of NPAS2 knockout or overexpression in hepatocytes on HSCs, primary hepatocytes were isolated from mice with treatments of knockout and upregulation in vivo. After thorough rinsing with PBS, the hepatocytes were co-cultured with primary HSCs from wild-type mice for 24 h in the upper-chamber of 0.4 μm trans-well plates (3450, Corning). Antibodies against TGFβ1 (sc-146, Santa Cruz) or control IgG (rabbit) was simultaneously added into the medium to neutralize the cytokine. Total RNA of HSCs was harvested 48-72 h later. The indicators of HSCs activation were used as discribed above.

Supplementary Tables

Supplementary Table 1. Sequences of primers and siRNAs.

Primer name		Sequences
1. Primers for real-time PCR:		
NPAS2(human)	forward	TCTGGATCACAGAGCACCTC
	reverse	CAGGAGCTCCAGGTCATCA
HES1(human)	forward	AAGAAAGATAGCTCGCGGCA
	reverse	TACTTCCCCAGCACACTTGG
COL1A1(human)	forward	CGGTGTGACTCGTGCAGC
	reverse	ACAGCCGCTTCACCTACAGC
ACTB(human)	forward	AAAGCAAGTCCTCCAGCGTT
	reverse	CAGGATTCCCGTCTTAGTCCC
CCND1(human)	forward	ATCAAGTGTGACCCGGACTG
	reverse	CTTGGGGTCCATGTTCTGCT
PERP(human)	forward	TGTGGTGGAAATGCTCCCAA
	reverse	TACCCCACGCGTACTCCAT
KLF10(human)	forward	CGCTGTCCATTGCAGCTTAC
	reverse	TGCATGATGCCTTCGTGTTG
ITGB5(human)	forward	GGAGCCAGAGTGTGGAAACA
	reverse	AGATAGCCAGGAGTGCAAGC
RASL11B(human)	forward	CCGGTTCCTCACCAAACGAT
	reverse	GGCTGTTCTCATGGACCTGAA
ITGA8(human)	forward	CTCAGGAAACTGGCAGGAGAA
	reverse	CCAGCAACCAATTCAAGGTAAC
GATA4(human)	forward	AAGACACCAGCAGCTCCTTC
	reverse	CCCGTAGTGAGATGACAGGC
NKIRAS1(human)	forward	AGAAGATGGGAAAGGGCTGC
	reverse	TCGCAATCTTCCATTCCAATAGT
PAK1(human)	forward	GGGAGTTTACGGGAATGCCA
	reverse	CCTGCGGGTTTTTCTTCTGC
miR-19a-3p(human)	forward	GGGGGGGTGTGCAAATCT
	reverse	GTGCGTGTCGTGGAGTCG
miR-19b-3p(human)	forward	CACTGTTCTATGGTTAG
	reverse	CACTACCACAGTCAGTT

miR-106a-5p(human)	forward	GATGCTCAAAAAGTGCTTACAGTGCA
	reverse	TATGGTTGTTCTGCTCTCTGTCTC
miR-106b-5p(human)	forward	CTGGAGTAAAGTGCTGACAGTG
	reverse	GTGCAGGGTCCGAGGT
miR-218-5p(human)	forward	TTGTGCTTGATCTAACCATGT
	reverse	CAGTGCGTGTCGTGGAGT
GAPDH(human)	forward	AATGGGCAGCCGTTAGGAAA
	reverse	GCCCAATACGACCAAATCAGAG
U6(human)	forward	GCTTCGGCAGCACATATACTAAAAT
	reverse	CGCTTCACGAATTTGCGTGTCAT
NPAS2(mouse)	forward	TCCATGCTCCCTGGTAACACT
	reverse	TCTGCAAGAATCCGATGACCTT
HES1(mouse)	forward	CACCGGACAAACCAAAGACG
	reverse	GGAATGCCGGGAGCTATCTT
COL1A1(mouse)	forward	ACTGCAACATGGAGACAGGTCAGA
	reverse	ATCGGTCATGCTCTCTCCAAACCA
ACTB(mouse)	forward	GGGGTGATGGTGGGAATG
	reverse	GCAGGGTGGGATGCTCTT
GAPDH(mouse)	forward	TCAACAGCAACTCCCACTCTTCCA
	reverse	TTGTCATTGAGAGCAATGCCAGCC

2.Primers for *HES1* promoter construct

(-2177/+67) HES1	forward	AAGGAATGAAGTGTCTGAGAACCGT
(-997/+42) HES1	forward	TGCGTGCGTGTGTGTGTAGGAGGGC
	reverse	AGGTCCTGGTTCCTCTCTCCATCTG

3. Primers for *HES1* promoter site-directed mutagenesis

(-2177/+67) HES1 mutation	CGGCGCCCGCacCGgaGCACCCGGCGGCG
(-2177/+67) HES1 mutation	CAGTGTGTTAATCTGTATTAATTC

4. Primers used for ChIP in the *HES1* promoter

HES1	forward primer:	TAGGGGGTCCCTCCGGCGGCGGTC
	reverse primer:	CCCGCCGACGCACGACGGACGACC

5. siRNAs

NPAS2 siRNA 1	sense:	CGUCGGAUGUCAUGGAUCA
	antisense:	UGAUCCAUGACAUCCGACG
NPAS2 siRNA 2	sense:	UCAAAGAGCUCAGUUCCAU
	antisense:	AUGGAACUGAGCUCUUUGA
Hes1 siRNA	sense:	ACGGTTCCTAGCGAGGTCC
	antisense:	AAUGGCUCCUCUUCAGAGC

Control siRNA	sense:	UUCUCCGAACGUGUCACGU
	antisense:	ACGUGACACGUUCGGAGAA

6. mimic and inhibitor of miR-19b-3p

miR-19b-3p mimic	forward	UGUGCAAAUCCAUGCAAAACUGA
	reverse	AGUUUUGCAUGGAUUUGCACAUU
mimic control	forward	UUCUCCGAACGUGUCACGUTT
	reverse	ACGUGACACGUUCGGAGAATT
miR-19b-3p inhibitor		UCAGUUUUGCAUGGAUUUGCACA
inhibitor control		CAGUACUUUUGUGUAGUACAA

Supplementary Table 2. Primary antibodies used for Western blotting and Immunohistochemistry analysis.

Antibody	Company (Cat. No.)	Working dilutions
NPAS2	NOVUS (NBP1-31363)	WB: 1/1000，IHC:1/200
Hes1	Abcam (ab71559)	WB: 1/1000，IHC:1/200
Colα1	Abcam(ab34710)	WB: 1/2000，IHC:1/400
α-SMA	Proteintech (14395-1-AP)	WB: 1/800， IHC:1/400
Notch2	Cell Signaling (#5732)	WB: 1/1000
Notch3	Cell Signaling (#4053)	WB: 1/1000
Jag1	Proteintech (66890-1-Ig)	WB: 1/5000
Hey2	Proteintech (10597-1-AP)	WB: 1/1000
β-actin	Beijing TDY (TDY051C)	WB: 1/3000