Supplemental Information

Dysregulated Activation of Fetal Liver Program in Acute Liver Failure

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SUPPLEMENTAL MATERIALS AND METHODS

Isolation of primary hepatocytes

Primary hepatocytes were isolated from quiescent or regenerating livers of WT (n=38) or *Yap1*$^{flox/flox}$ mice (n=8) with AAV8-TBG-Luc or AAV8-TBG-Cre as described previously [1]. Briefly, mice were anaesthetized with isofluorane to immobilize in the recumbent position on a treatment table, and the inferior vena cava was cannulated under aseptic conditions. Livers were perfused *in situ* with EGTA and then collagenase (Roche, Indianapolis, IN) to disperse the cells. Primary hepatocytes were separated from non-parenchymal cells by gravity sedimentation. The purity of the isolated hepatocytes were evaluated with assessment of relative percentage of hepatocytic appearing cells using standard microscopy, and this is typically >96% regardless of the timing of hepatocyte isolation. Freshly isolated hepatocytes were immediately processed to obtain RNA and protein for evaluating gene expression.

Cell experiments

Hep3B, a human hepatoma cell line, was cultured in Eagle's Minimum Essential Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA), 1mM sodium pyruvate (Gibco), 1× non-essential amino acids (Gibco) and 1% penicillin/streptomycin (P/S) at 37 °C in a humidified atmosphere containing 5% CO_2. The expression of *IGF2BP3* was inhibited by transfecting Hep3B cells with 10 nM of *IGF2BP3* siRNA (ON-TARGETplus IGF2BP3 siRNA SMARTpool, GE Dharmacon, Lafayette, CO) or non-targeting siRNA (ON-TARGETplus Non-Targeting Pool, GE Dharmacon) as a negative control for 96 hours using Lipofectamine RNAi/MAX transfection reagent (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. To overexpress IGF2BP3, Hep3B cells were transfected with 0.5 µg/well of pDESTmycIGF2BP3 vector (a gift from Thomas Tuschl, Addgene plasmid #19879) [2] or empty vector as a negative control in 6 well plate for 48 hours using Lipofectamine 2000 (Invitrogen).

To evaluate the effect of YAP1 on stem cell-like characteristics, Hep3B cells (1.25×10^5 per well) were treated with Verteporfin (Sigma-Aldrich), an inhibitor of YAP1 activity, at two different doses (2 or 10 μM) or 0.1% DMSO as a vehicle for 24 or 48 hours. Cell proliferation/viability was assessed by cell counting kit-8 (CCK8, Dojindo Molecular Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. All cell experiments were repeated at least three times.

Sphere formation assay

Primary hepatocytes isolated from WT mice or *Yap1*$^{flox/flox}$ mice injected either AAV8-TBG-Luc or AAV8-TBG-Cre at pre-PH (0h) or 48h post-PH, and Hep3B manipulated either YAP1 or IGF2BP3 expression were cultured anchorage-independently to promote sphere formation. Cells were suspended in serum-free DMEM/F12 (Gibco) supplemented with 2% B27 (Gibco), 20 ng/ml EGF (Peprotech, Rocky Hill, NJ), 20 ng/ml FGF10 (Peprotech) and 4 μg/ml heparin (Sigma-Aldrich), and cultured in ultralow attachment 6-well plates (Corning Inc., Corning, NY) at a density of 5,000 cells/well. Fresh EGF, FGF10 and heparin were added every 3 days. The formation of spheres was observed and photographed using a phase microscope. The number of hepatocyte spheroids (≥ two-cell clumps) and Hep3B spheres (≥ 150 μm) was counted in all wells from each replicate (n=3/group), and the sizes of spheroids or spheres were measured automatically using Image J 1.6.0 software (http://imagej.nih.gov/ij/index.html).

RNA analysis

Total RNA was extracted from liver tissues or cells by using TRIzol reagent (Invitrogen). The concentration and purity of RNA were determined using NanoDrop 2000 (Thermo Scientific, Thermo Fisher Scientific). Template complementary DNA was synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) or miScript Reverse Transcriptase Kit (Qiagen, Valencia, CA) for mRNA or miRNA respectively, according to the manufacturer's protocols. The real-time quantitative polymerase chain reaction (qPCR) was performed by using Power SYBR Green Master Mix (Applied Biosystems) or miScript SYBR Green PCR Kit (Qiagen),

for mRNA or miRNA respectively, using the manufacturer's specifications (StepOnePlus™ Real-Time PCR System, Applied Biosystems). All reactions were in triplicate, and data were analyzed according to the $\Delta\Delta C_t$ method. 40S ribosomal proteins S9 (RPS9) mRNA for mRNA or U1A small nuclear RNA (RNU1A) for miRNA were used for normalization of the expression level. The sequences of all primers used in this study are summarized in Supplemental Table 2.

Western blot

Total protein was extracted from freshly isolated hepatocytes or cultured Hep3B cells using RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Homogenized samples were centrifuged at 13,000 × g for 15 minutes and the supernatants containing protein extracts were used for Western blotting. Protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amount of total protein lysates was separated by SDS-PAGE on 4-15% or 4-20% Mini-PROTEAN® TGX™ Precast Protein Gels (BIO-RAD, Hercules, CA) and then transferred onto a polyvinylidene difluoride membrane (Invitrogen). Primary antibodies used in this study were as follows: rabbit anti-IGF2BP3 antibody (Invitrogen or Millipore, Billerica, MA), rabbit anti-SOX9 antibody (Abcam, Cambridge, UK), rabbit anti-C/EBPα antibody (Santa Cruz, Dallas, TX), mouse anti-AFP antibody (Santa Cruz), rabbit anti-YAP1 antibody (Cell Signaling, Danvers, MA), rabbit anti-CCNE1 antibody (Abcam), mouse anti-CCNB1 antibody (Millipore) and rabbit anti-ESRP2 antibody (Abcam). Horseradish peroxidase (HRP)-conjugated Amersham ECL anti-rabbit or anti-mouse IgG (GE Healthcare, Little Chalfont, UK) were used as secondary antibodies and HRP-conjugated β-actin antibody (Genscript, Piscataway, NJ) was used as an internal control. Protein bands were developed using an Amercham ECL Western Blotting Detection Reagent (GE Healthcare) and detected using the ChemiDoc XRS+ (BIO-RAD). Densitometric analyses for protein quantification were conducted using Image J 1.6.0 software. Blots for each antibody were repeated at least three times.

Immunostaining

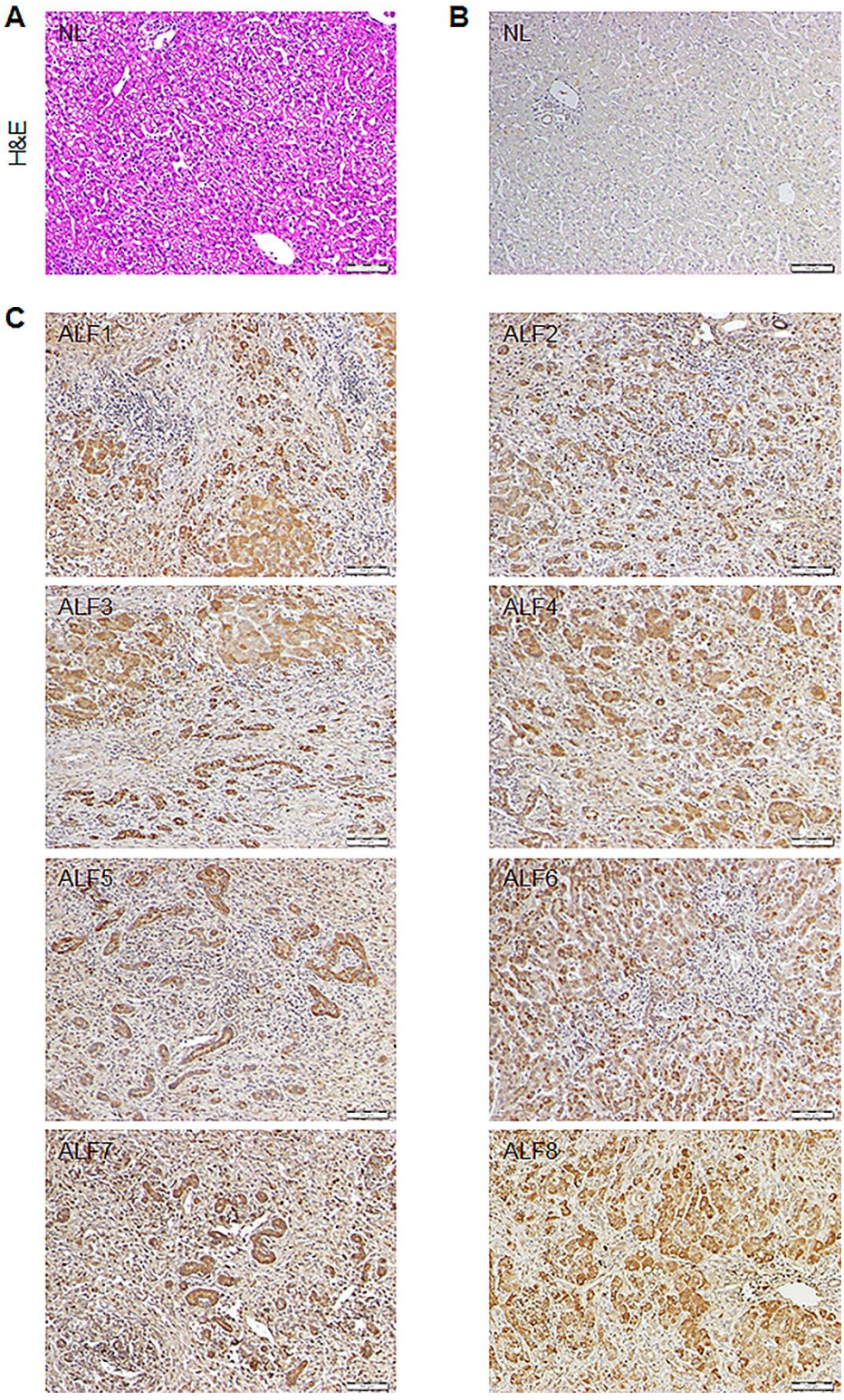
Liver specimens were fixed in 10% neutral buffered formalin, embedded in paraffin using standard methods and cut into 5 μm sections. For immunohistochemistry, liver sections were deparaffinized, hydrated and incubated in 3% hydrogen peroxide to block endogenous peroxidase. Antigen retrieval was performed by heating in 10 mM citrate buffer (pH 6.0) for 10 min using microwave or enzymatic method using pepsin. Specimens were blocked in Dako Protein Block solution (Agilent, Santa Clara, CA) for 30 min at room temperature followed by incubation with primary antibody at 4 °C overnight. Other sections were also incubated at 4 °C overnight in non-immune sera. Rabbit anti-IGF2BP3 antibody (Millipore), rabbit anti-SOX9 antibody (Millipore), rabbit anti-YAP1 antibody (Abcam or Cell Signaling), rabbit anti-phospho-SMAD2 antibody (Abcam), rabbit anti-Cytokeratin (pan-CK) antibody (Dako), rabbit anti-C/EBPα antibody (Santa Cruz), rabbit anti-Cre antibody (Cell signaling) and rabbit anti-ESRP2 antibody (Abcam) were used as primary antibodies and diluted in Antibody Diluent (Sigma-Aldrich). Polymer-HRP anti-rabbit (Dako) and polymer-HRP anti-mouse (Dako) were used as secondary antibodies and 3,3′-diaminobenzidine (DAB) as brown color was used to visualize the protein. Sections were counterstained with hematoxylin. To quantify IGF2BP3(+) hepatocytes after PH, 19 or 5 randomly chosen 10× fields/section of WT or *Yap1*$^{flox/flox}$ mice, respectively, were evaluated by counting the total number of IGF2BP3-stained cells/field for each mouse and proportion of IGF2BP3(+) mitotic figures was indicated separately by hatched line in **Fig 2B** or **Fig 5E**. Independently, the percentage of IGF2BP3(+) mitotic figures per total number of mitotic figures was calculated and shown in **Fig 2C**. Cells co-expressing IGF2BP3 with YAP, SOX9 or C/EBPα in liver tissues of ALF patients were identified by double IHC with DAB and Vina Green chromogens (Biocare Medical, Pacheco, CA), according to the manufacturer's recommendation. The denaturing solution (Biocare Medical) was used to elute the first antibody to prevent cross reactivity with subsequent reaction.

For immunofluorescent staining, cytospin slides of primary hepatocytes or Hep3B cells cultured on chamber slide (Thermo Scientific) were fixed and permeabilized with cold acetone at -20°C. Slides were

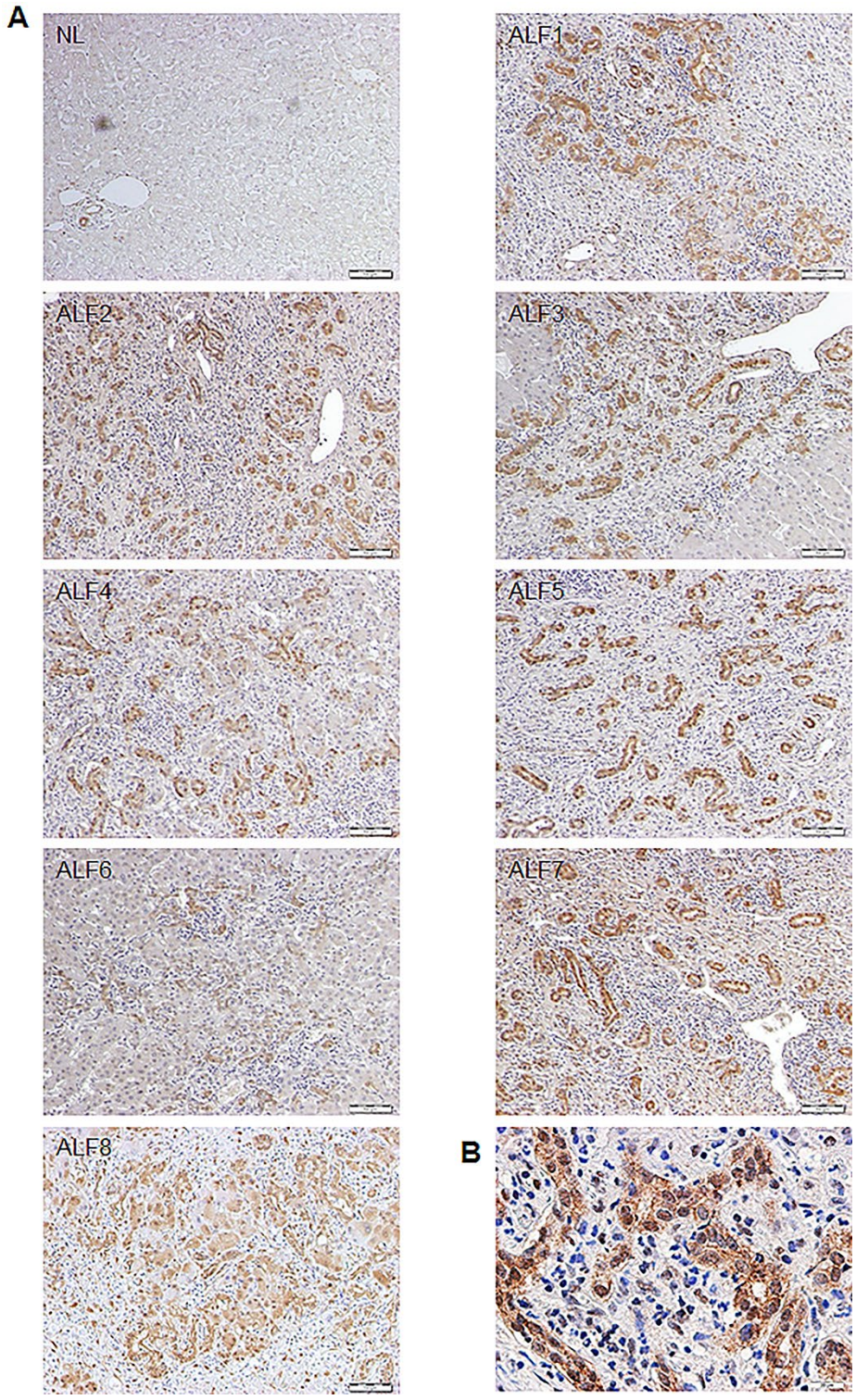
washed with TBS and incubated with Dako Protein Block for 30 minutes at room temperature followed by administration of primary antibody or mixed primary antibodies from different origin, at 4 °C overnight. Cells were washed with TBS and incubated with fluorescein-labelled secondary antibodies for 1 hour at room temperature. Rabbit anti-IGF2BP3 antibody (Millipore), rat anti-Ki67 antibody (Dako), goat anti-CD133 antibody (Santa Cruz), rabbit anti-ESRP2 antibody (Abcam) and rabbit anti-YAP1 antibody (Abcam) were used as primary antibodies and Alexa Fluor 488 chicken anti-rabbit IgG, Alexa Fluor 647 donkey anti-goat IgG or Alexa Fluor 647 goat anti-rat IgG (Invitrogen) were used as secondary antibodies. Slides were covered by antifade mounting medium with 4′,6-diamidno-2-phenylinole (DAPI, VectaShield, Burlingame, CA) for nuclear counter staining.

Prediction of binding sites of IGF2BP3 protein in ESRP2 mRNA

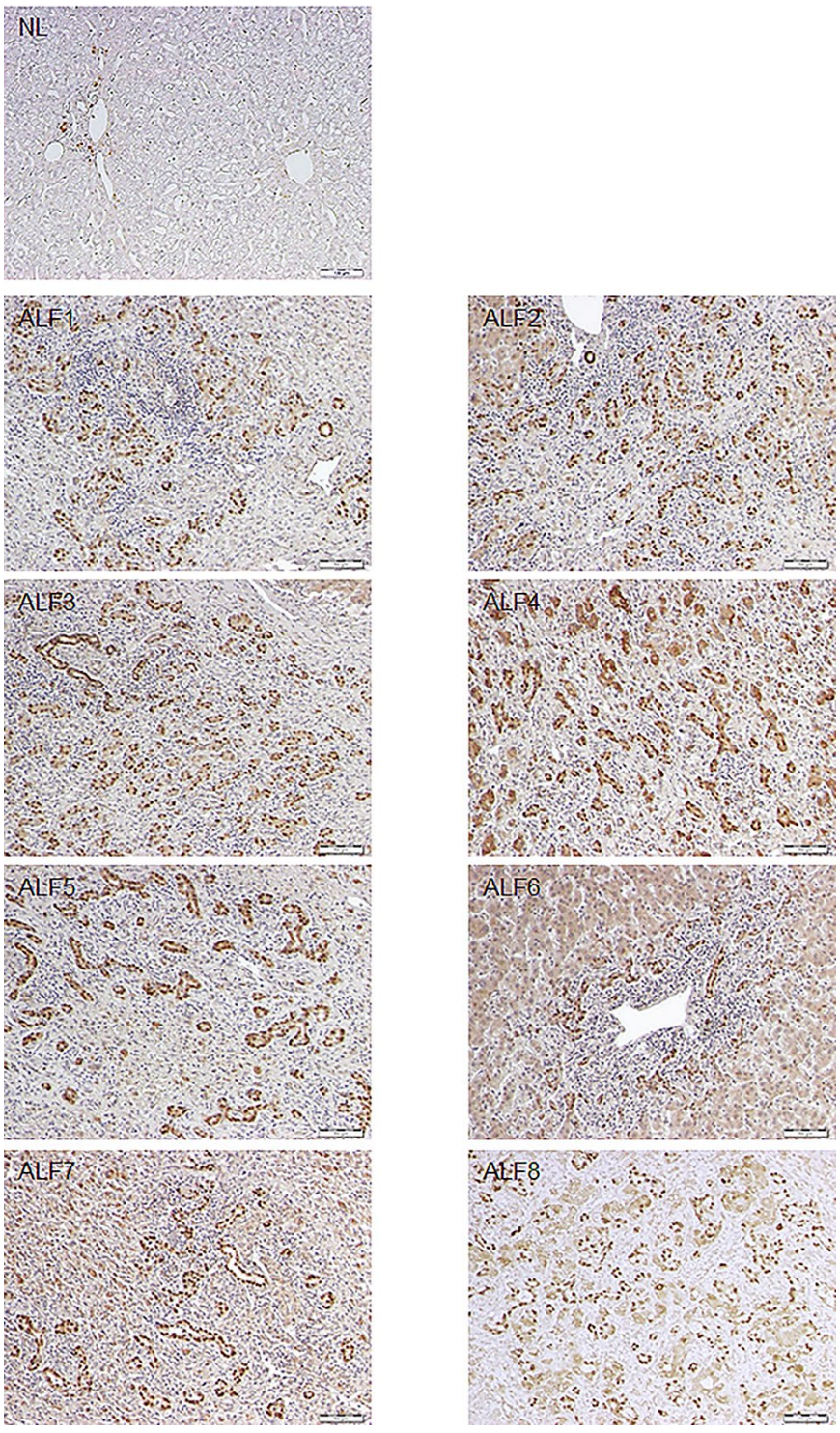
The PRIdictor v3.3 [3] and RAID v2.0 [4] databases were used to identify protein-RNA interactions. A list of protein-RNA interactions was downloaded from RAID v2.0, and binding sites of IGF2BP3 protein in ESRP2 mRNAs were predicted using PRIdictor web application v3.3.



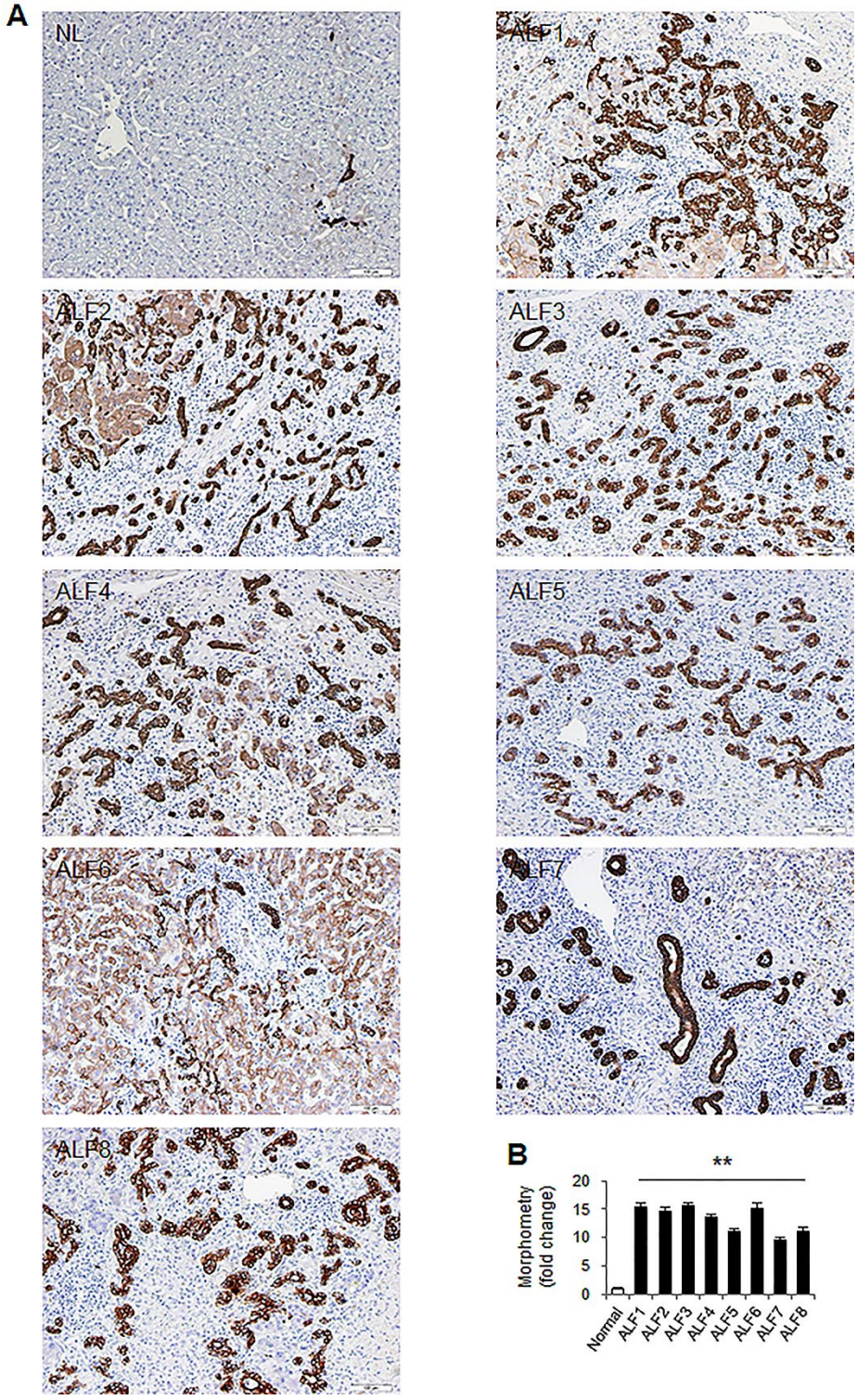
Supplemental figure 1. IGF2BP3 expression in human livers of patients with acute liver failure. (A) Representative hematoxylin and eosin (H&E) staining in healthy human liver (NL). **(B and C)** Immunohistochemistry (IHC) for IGF2BP3 in NL **(B)** or explanted liver tissues of patients with acute liver failure (ALF) **(C)**. Representative images are shown. Scale bar=100μm.



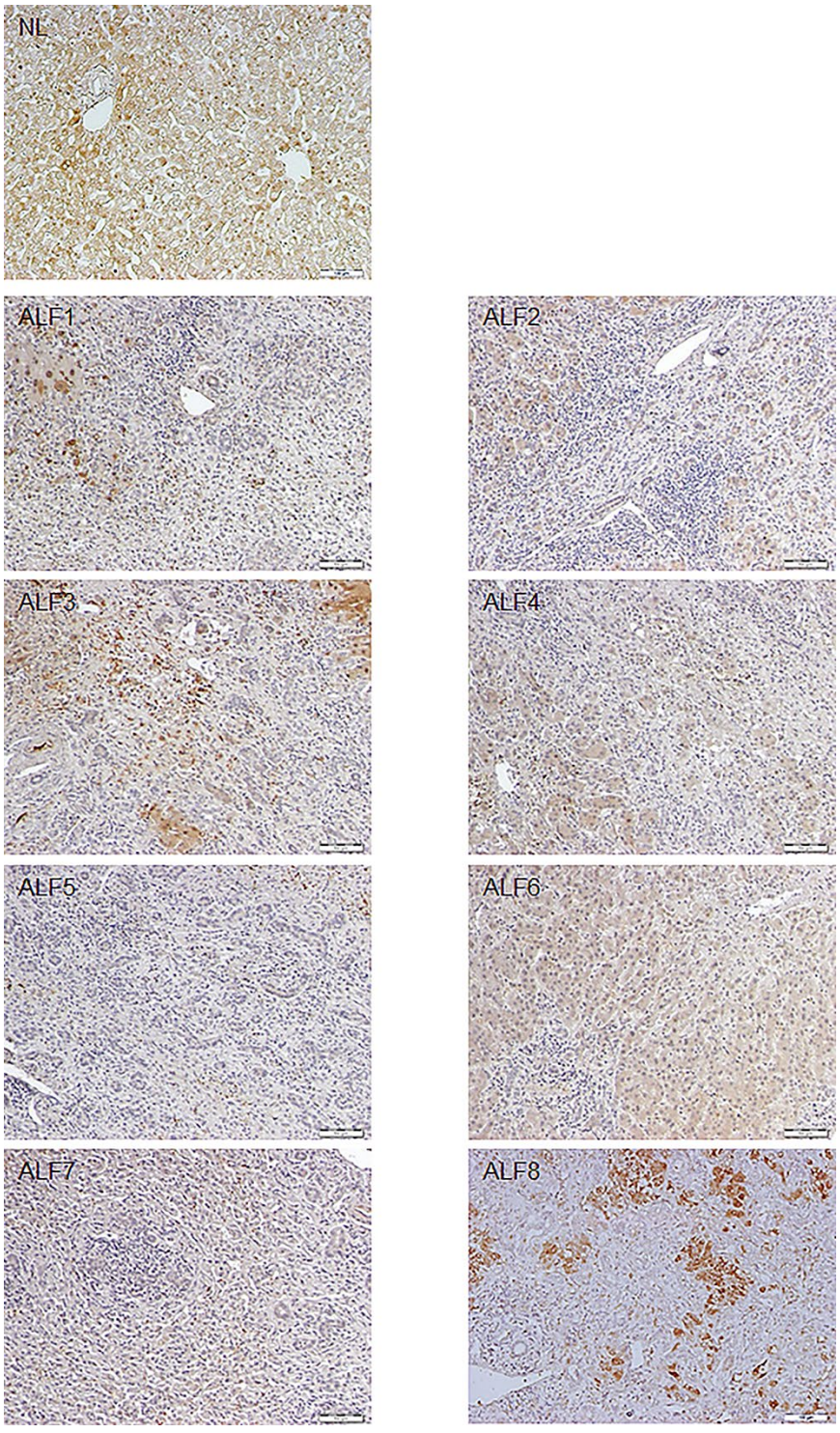
Supplemental figure 2. Expression of YAP1 and p-SMAD2 in human livers with acute liver failure. **(A)** IHC for YAP1 in healthy human liver (NL) or explanted liver tissues of 8 patients with acute liver failure (ALF). Representative images are shown. Scale bar=100μm. **(B)** IHC for p-SMAD2 in a representative liver tissue of ALF patients. Scale bar=20μm.



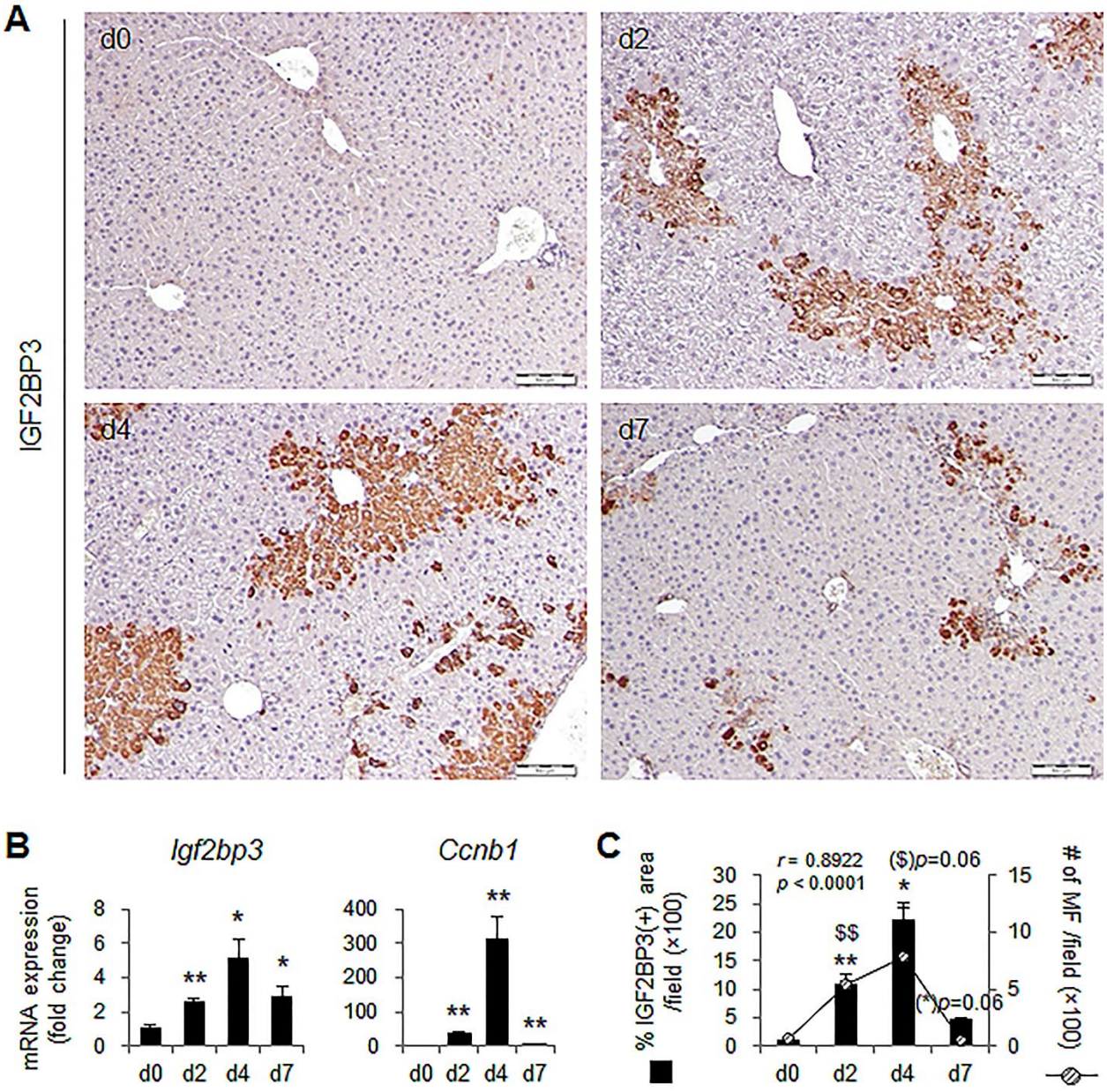
Supplemental figure 3. SOX9 expression in human livers with acute liver failure. IHC for SOX9 (a ductal progenitor marker) in healthy human liver (NL) or explanted liver tissues of 8 patients with acute liver failure (ALF). Representative images are shown. Scale bar=100μm.



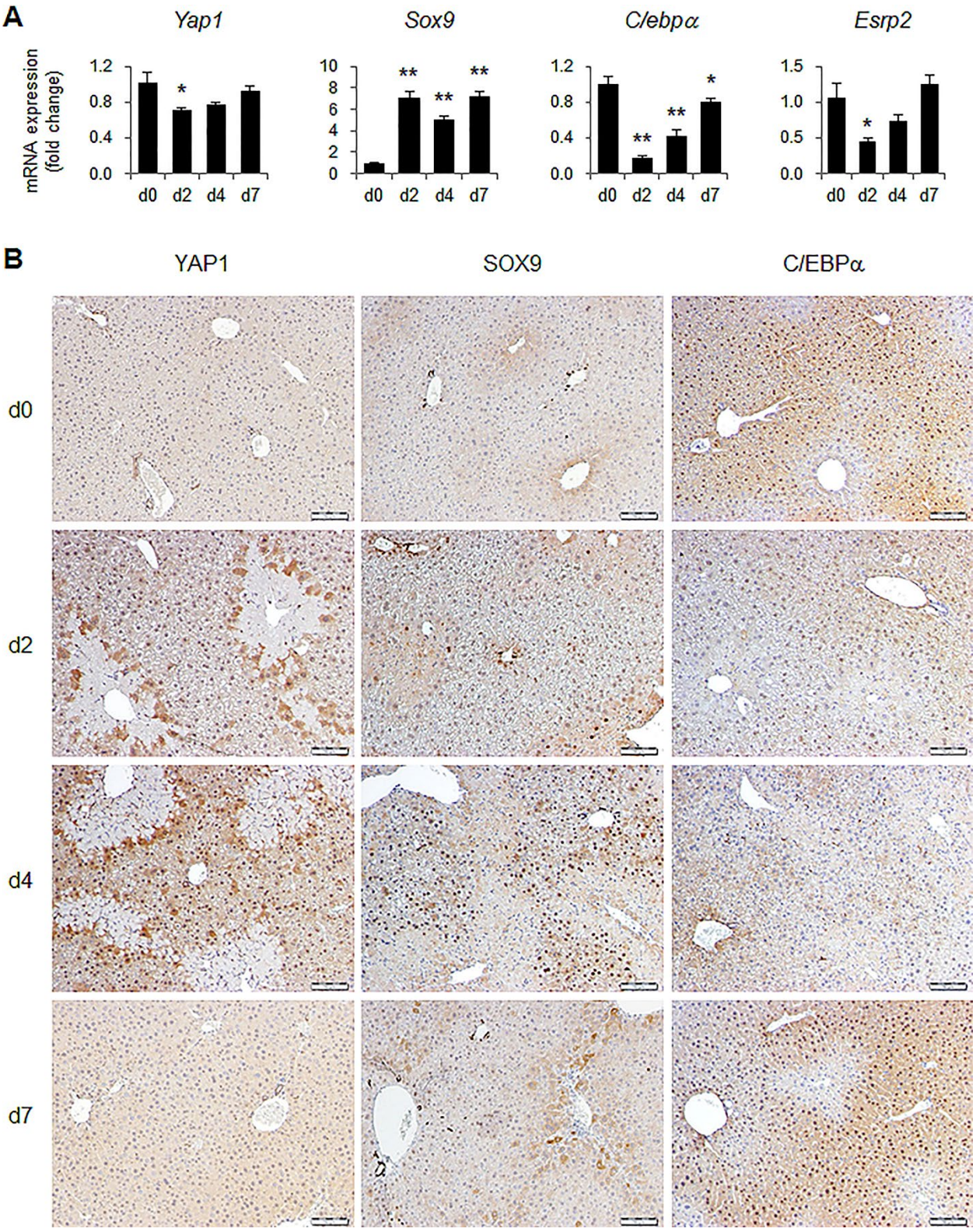
Supplemental figure 4. Expression of pan-CK in human livers with acute liver failure. (A) IHC for pan-CK (another ductal progenitor marker) in healthy human liver (NL) or explanted liver tissues of 8 patients with acute liver failure (ALF). Representative images are shown. Scale bar=100μm. **(B)** Mean±s.e.m. result from morphometric analysis is graphed and statistical analysis was performed using two-tailed student *t* test compared to NL (n=10 10× fields/section, $^{**}p<0.005$).



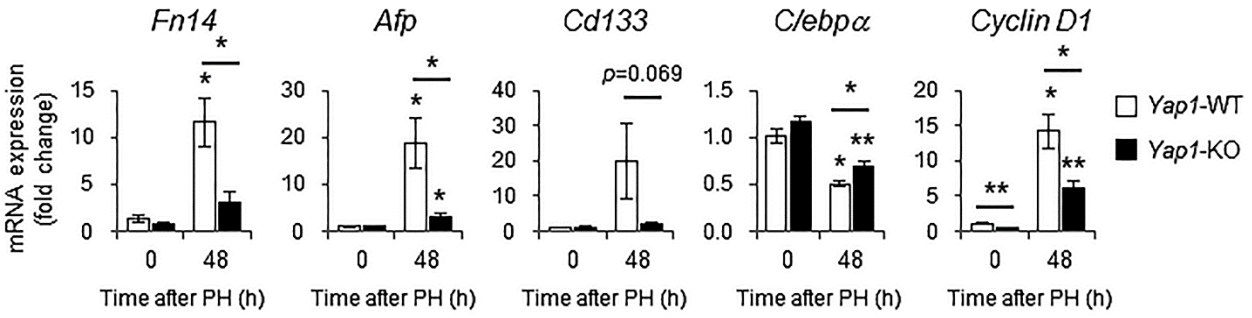
Supplemental figure 5. Mature hepatocytes are rare in human livers with acute liver failure. IHC for C/EBPα (a marker of mature hepatocytes) in healthy human liver (NL) or explanted liver tissues of 8 patients with acute liver failure (ALF). Representative images are shown. Scale bar=100μm.



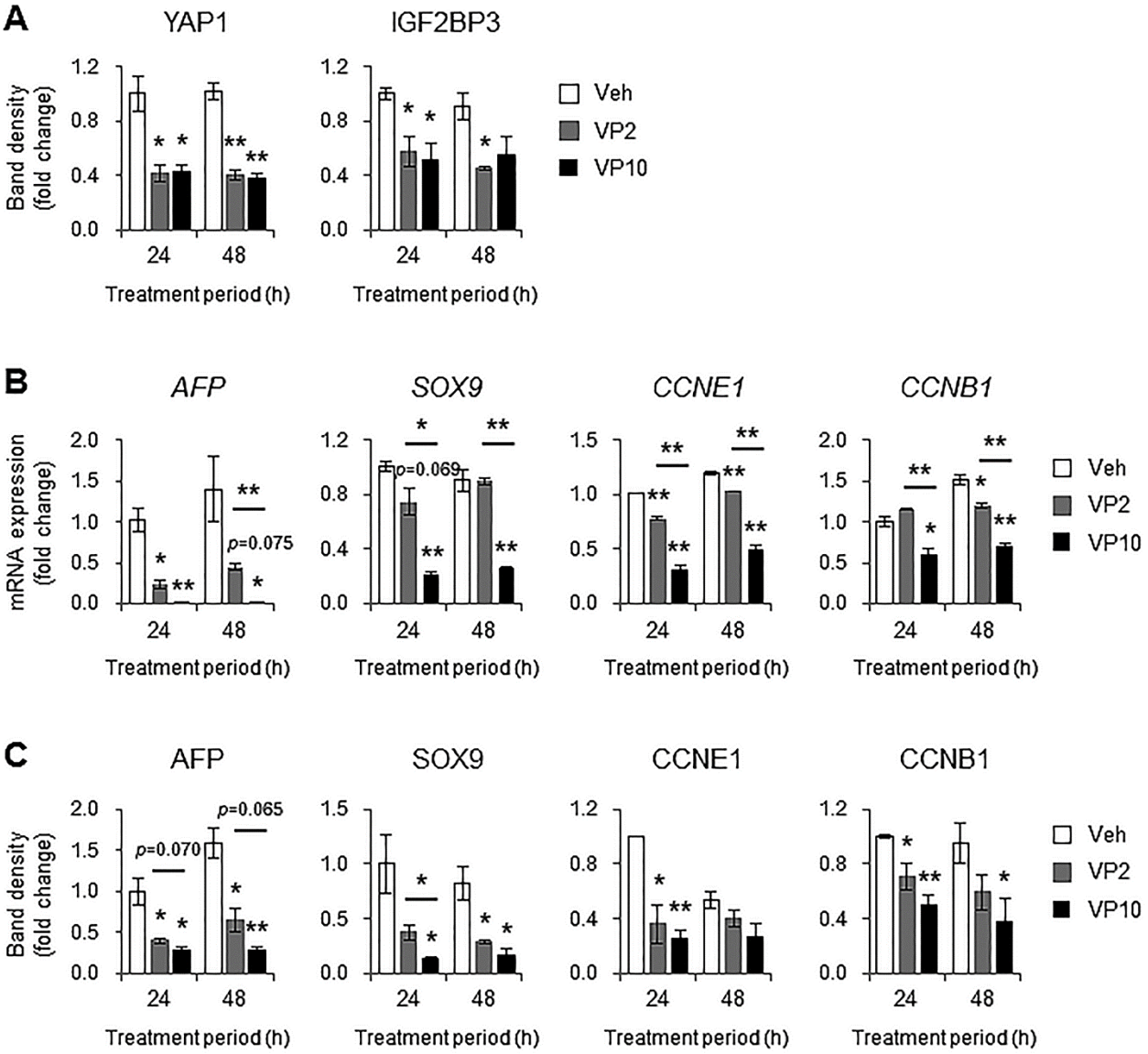
Supplemental figure 6. IGF2BP3-positive hepatocytes transiently accumulate in regenerating mouse livers after acute toxic injury. (A) IHC for IGF2BP3 (brown) in liver sections of mice sacrificed at 0, 2, 4 or 7 days after acute carbon tetrachloride (CCl_4) treatment (one injection). Representative images are shown. Scale bar=100µm. **(B)** qRT-PCR analysis for *Igf2bp3* and *Cyclin B1* (*Ccnb1*) in liver tissues of mice sacrificed before (0) or at 2, 4 or 7 days after CCl_4 injection. The mean±s.e.m. results are graphed, and statistical analysis was assessed using two-tailed student *t* test (n=3 mice/time, **p*<0.05, ***p*<0.005). **(C)** Bar graph shows the mean±s.e.m. results of morphometric analysis for IGF2BP3 IHC. Significance was analyzed by using two-tailed student *t* test compared to baseline (0 h) (n=3 mice/time, 10 randomly-selected 10× fields/section, **p*<0.05, ***p*<0.005). Line graph shows the mean±s.e.m. of the number of mitotic figures at different time points after CCl_4 treatment (n=3 mice/time, 10 randomly-selected 10× fields/section, $$*p*<0.005). The correlation between the average % area of IGF2BP3 positivity and the number of mitotic figures was analyzed using Pearson's r (*r*, correlation coefficient; *p*, *p* value).


A
Yap1
Sox9
C/ebpα
Esrp2
mRNA expression (fold change)
d0 d2 d4 d7
B
YAP1
SOX9
C/EBPα
d0
d2
d4
d7

Supplemental figure 7. YAP1- and SOX9-positive, but C/EBPα-negative, immature hepatocytes transiently accumulate in regenerating mouse livers after acute toxic injury. (A) qRT-PCR analysis for a reprogramming factor (*Yap1*), a progenitor marker (*Sox9*) and a marker of mature hepatocytes (*C/ebpα* and *Esrp2*) in liver tissues of mice sacrificed at 0, 2, 4 or 7 days after acute CCl_4 treatment. The mean±s.e.m. results are graphed, and statistical analysis was assessed using two-tailed student *t* test (n=3 mice/time, **p*<0.05, ***p*<0.005). **(B)** IHC for YAP1, SOX9 and C/EBPα (brown) in liver sections of CCl_4-treated mice at different time points. Representative images are shown. Scale bar=100μm.



Supplemental figure 8. Depleting *Yap1* suppresses induction of progenitor gene in regenerating livers after PH. qRT-PCR analysis for fetal liver/progenitor cell markers, including *Fn14*, *Afp* and *Cd133*, a hepatocyte differentiation marker, *C/ebpα*, and a cell cycle progression marker, *Cyclin D1*. The mean±s.e.m. results are graphed, and significance was analyzed by using two-tailed student *t* test compared to each baseline (0h) or between AAV8-TBG-Luc and AAV8-TBG-Cre group at each time point (n=4-7 mice/group/time, **p*<0.05, ***p*<0.005).



Supplemental figure 9. YAP1 inactivation reduces expression of progenitor cell markers and Cyclins. (A and C) Band densitometry of immunoblots (shown in **Fig 6B and 6E**) for (A) YAP, IGF2BP3, (C) AFP, SOX9, CCNE1 and CCNB1 normalized to β-actin expression in Hep3B treated with 2 or 10 μM of YAP1 inhibitor, Verteporfin (VP), or 0.1% DMSO as a vehicle (Veh) for 24 or 48 hours. **(B)** qRT-PCR analysis for *AFP*, *SOX9*, *CCNE1* and *CCNB1* in Hep3B treated with either VP (2 or 10 μM) or Veh for 24 or 48 hours. All results are graphed as mean±s.e.m. of triplicate experiments; significance was analyzed using two-tailed student *t* test compared (n=3 repeats/group/time, **p*<0.05, ***p*<0.005).

A

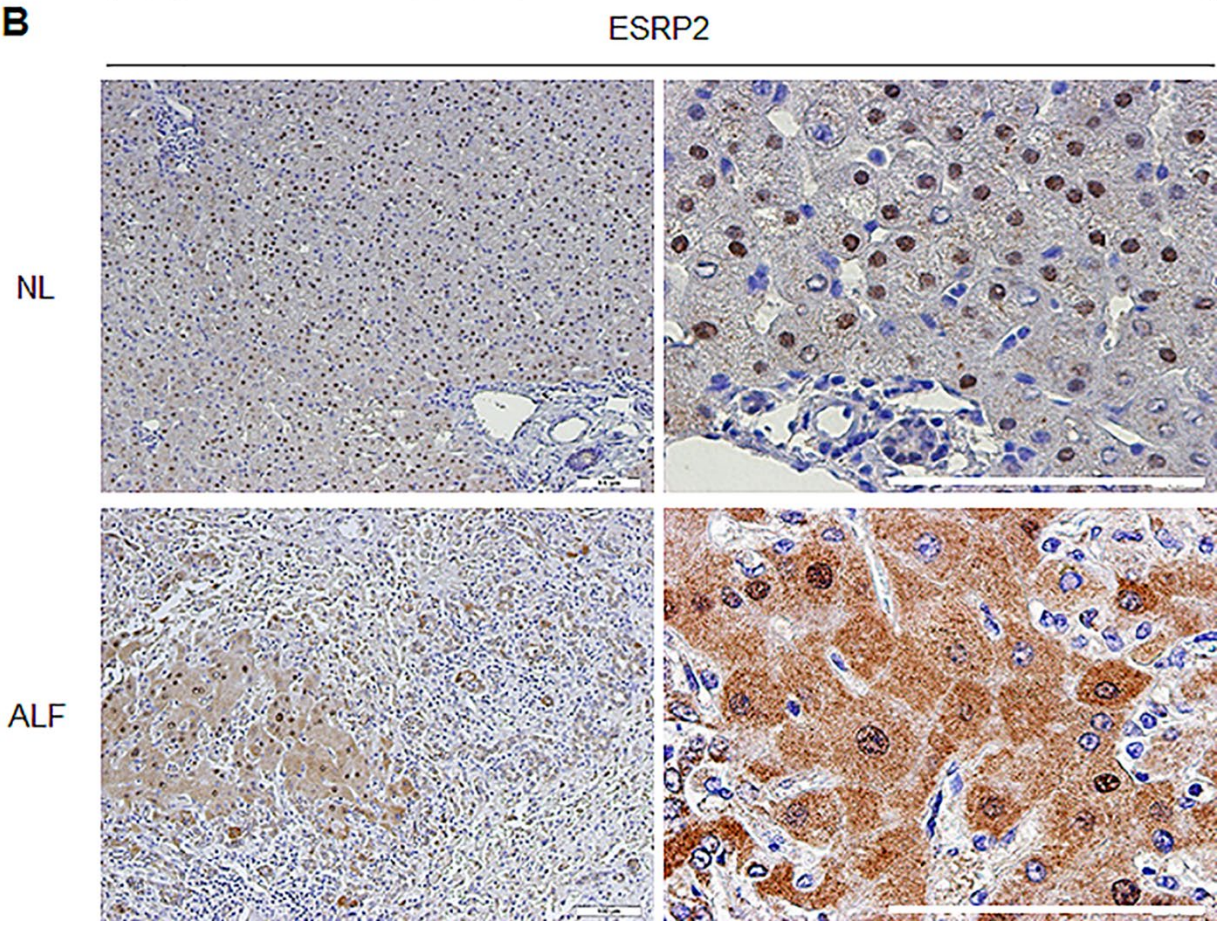
in silico IGF2BP3 protein – ESRP2 RNA interaction

RAID_id	mRNA	Entrez ID	Protein	Entrez ID	Methods	Score
RAID04348638	ESRP2	80004	IGF2BP3	10643	CLIP-seq	0.5483

Predicted binding sites of IGF2BP3 protein (NP_006538.2) in ESRP2 RNA

ESRP2 mRNAs	NCBI Reference	Binding nucleotides (bp)	Sequence length (bp)
pre-mRNA	bAug10 (Aceview)	386	8104
mRNA isoform 1	NM_024939.3	162	3429
mRNA isoform 2	NM_001365264.1	167	3459
mRNA isoform 3	NM_001365264.1	155	3263

B



Supplemental figure 10. IGF2BP3 interacts with ESRP2, an RNA splicing factor, which is suppressed in ALF. (A) *in silico* analyses of interaction between IGF2BP3 protein and ESRP2 mRNA, and predicted number of binding sites of IGF2BP3 protein in ESRP2 mRNA sequences (base pair, bp). **(B)** IHC for ESRP2 in healthy human liver (NL) or explanted liver tissues of patients with acute liver failure (ALF). Representative images are shown. Scale bar=100μm.

REFERENCES

1 Ochoa B, Syn WK, Delgado I, Karaca GF, Jung Y, Wang J*, et al.* Hedgehog signaling is critical for normal liver regeneration after partial hepatectomy in mice. Hepatology 2010;**51**:1712-23.

2 Landthaler M, Gaidatzis D, Rothballer A, Chen PY, Soll SJ, Dinic L*, et al.* Molecular characterization of human Argonaute-containing ribonucleoprotein complexes and their bound target mRNAs. RNA 2008;**14**:2580-96.

3 Tuvshinjargal N, Lee W, Park B, Han K. PRIdictor: Protein–RNA interaction predictor. Biosystems 2016;**139**:17-22.

4 Yi Y, Zhao Y, Li C, Zhang L, Huang H, Li Y*, et al.* RAID v2. 0: an updated resource of RNA-associated interactions across organisms. Nucleic Acids Res 2016;**45**:D115-D8.