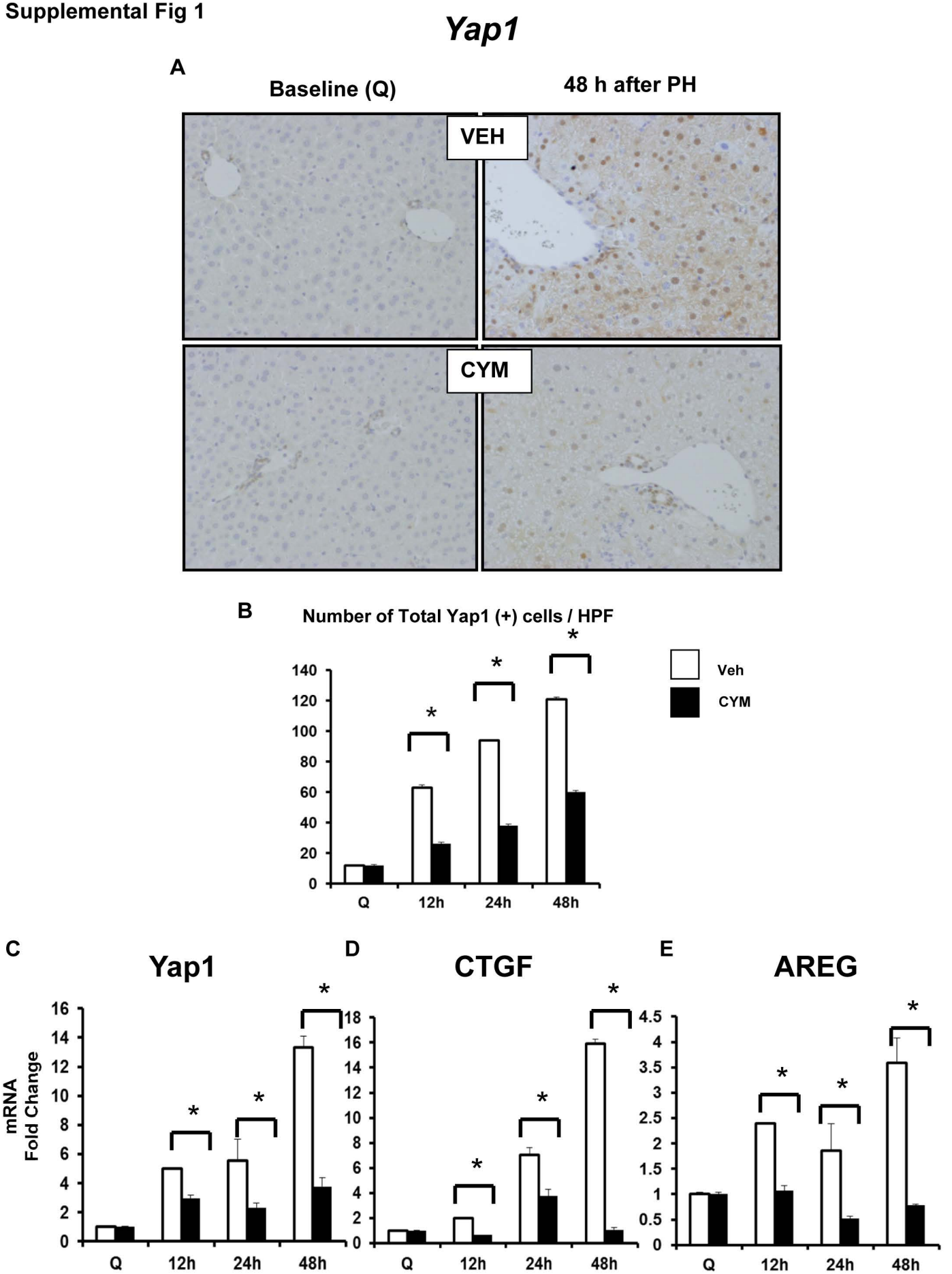
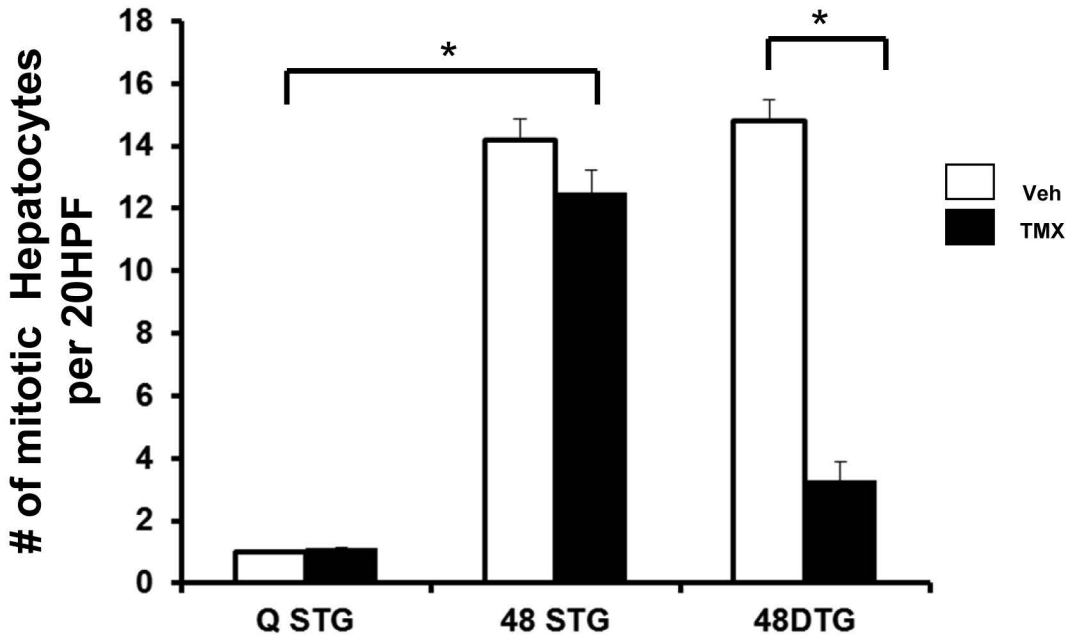
Supplemental Fig 1



Supplemental Fig 2



Supplemental Figure 1: Pharmacologic inhibition of Smoothened represses expression of Yap1 and its target genes.

WT mice subjected to 70% PH received either olive oil (VEH) or Cyclopamine (CYM) i.p. daily. Mice were sacrificed 12 h (VEH: n = 5; CYM: n = 5), 24 h (VEH: n = 5; CYM: n = 5), and 48 h (VEH: n = 5; CYM: n = 5) after PH, and livers harvested for immunohistochemistry and qRT-PCR. (A) Representative photomicrographs showing Yap1 staining (brown) in VEH- (top panels) and CYM-(bottom panels) treated mice at baseline (Q) and at 48 h post-PH. (B) Yap1 quantification at time points after PH. Results were expressed as total number of Yap1(+) cells per HPF; mean ± SEM. White columns represent VEH-treated mice; solid columns represent CYM-treated mice. (C) Yap1 mRNA. (D) CTGF mRNA. (E) AREG mRNA. Results of genes were expressed as fold change relative to quiescent livers of VEH-mice; mean ± SEM were graphed. *$p<0.05$ compared with time-matched, VEH-treated mice.

Supplemental Figure 2: Livers with hedgehog signaling deficient HSC / MF contain significantly fewer mitotic hepatocytes after partial hepatectomy

αSMA-CreER^{t2}/SMO$^{flox/flox}$ double transgenic (DTG) and Smo$^{flox/flox}$ single transgenic (STG) mice were treated with either vehicle (VEH) or TMX, and then sacrificed at 48h post-PH. Livers were harvested and mitotic hepatocytes were counted in 20 random, high-powered fields. (A) Quantification of mitotic hepatocytes in VEH- and TMX-treated groups at time 0, and 48 h post-PH. Results were expressed as total number of mitotic hepatocytes per HPF; mean ± SEM; white columns represent VEH-treated DTG; black columns represent TMX-treated DTG. *$p<0.05$ compared with time-matched, VEH-treated mice, or otherwise indicated.

Supplemental Materials and Methods

Animals and experimental design

Animal care and surgical procedures were approved by the Duke University Medical Center Institutional Animal Care and Use Committee as set forth in the Guide for the Care and Use of Laboratory Animals published by the NIH.

All mice were housed in a facility with a 12-hour-light/dark cycle and allowed free access to food and water.

In vitro Studies

Hepatocyte Experiments: Hepatocytes were harvested from the regenerating liver remnants of $Smo^{flox/flox}$ (STG) mice (n = 10) and αSMA-Cre-ER^{t2}, $Smo^{flox/flox}$ (*Smo*^{tm2Amc}/J) (DTG) mice (n = 10) as previously described (1). Half the mice in each group (n = 5/group) had been treated with VEH and half with TMX as per the protocol specified above; hepatocytes were isolated at 48 h after PH. As controls, hepatocytes were similarly isolated from 5 VEH-treated and 5 TMX-treated non-hepatectomized STG mice. Freshly isolated hepatocytes from all mice were immediately processed to obtain RNA for qRT-PCR analysis.

Hepatic Stellate Cell Experiments: Primary HSCs were harvested from *Smo*^{tm2Amc}/J (SMO-flox) mice, and assessed for purity and viability, and seeded at a density of 3×10^2 cells per mm^2 in DMEM supplemented with 10% fetal bovine serum

and penicillin/streptomycin, as described previously (2). Isolated HSCs were then treated with adenoviral vectors bearing GFP (Ad-GFP, control) or Cre recombinase (Ad-Cre) on culture day 4 at a MOI of 50, as described previously (3). After 24 hours, virus-containing medium was aspirated and replaced with fresh medium. Viral efficiency of Ad-GFP infection was assessed by confocal microscopy, with >95% of cells found to be GFP positive. Cre expression of infected cells was confirmed by Western analysis of cellular whole cell extracts.

In separate studies a rat MF-HSC clonal line (8B cells) (4) was plated on 6-well plates at a density of 5×10^5 cells/well in DMEM with 10% FBS without antibiotics the day before transduction procedures. After cells reached 80% confluence, cultures were infected with Yap1 targeting lentivirus or non-targeting virus (see below for details) with polybrene (8 μg/mL) for 24 h. The transduction efficiency was determined visually by fluorescence microscopy and confirmed to be greater than 95%. All cells were then placed in fresh DMEM containing 10% FBS without antibiotics, cultured in this complete medium for 48 h, and then harvested to obtain RNA or protein for subsequent analysis. Two distinct siRNA sequences were used to target the rat Yap gene: ACCTCTTCTGGTCAGAGATACTTCTTAAA and ACTCAGGAATTGAGGACAATGACAACCAA. An siRNA not known to target any rat gene (non-targeting) was used as a negative control (GGGTGAACTCACGTCAGAA; Applied Biological Materials). The sequences were cloned into the piLenti-siRNA-GFP vector (Applied Biological Materials), which also contained enhanced green fluorescent protein (*EGFP*) gene driven by a CMV-driven GFP reporter. Plasmids were transfected

into HEK-293T cells using a packaging vector mix (Applied Biological Materials). Supernatants containing lentiviruses were harvested at 48 and 72 h after transfection, clarified by 0.22 uM filtration and either used immediately or stored at - 80°C until use.

Liver Architecture, Immunohistochemistry, and Quantification of Staining

Liver tissue was fixed in formalin and embedded in paraffin. Serial sections were stained with hematoxylin and eosin (H&E). Immunohistochemical staining to detect YAP1, αSMA, Desmin, Gli2 was performed using the DAKO Envision System (DAKO Corporation) according to the manufacturer's protocol. Immunostaining were performed as described previously (5). Briefly, formalin-fixed paraffin-embedded liver tissues were cut into 5-μm sections and placed on glass slides. Sections were de-paraffinized with xylene, dehydrated with ethanol, and then incubated with 3% hydrogen peroxide to block endogenous peroxidase. Antigen retrieval was performed by heating in 10 mM sodium citrate buffer (pH 6.0). Sections were blocked in DAKO protein block (X9090; DAKO), followed by incubation with primary antibodies. The primary antibodies used: YAP1 (Cell Signaling 4912S), αSMA (Abcam-32575), Desmin (Abcam6322) and Gli2 (GenWay Biotech EB-3B44). HRP-conjugated anti-rabbit (K4003; DAKO) secondary antibodies were used to visualize target proteins. DAB reagent (K3466; DAKO) was applied in the detection procedure. Tissue sections were counterstained with Aqua Hematoxylin-INNOVEX (Innovex Biosciences). Negative controls included liver specimens exposed to 1% bovine serum albumin instead of the respective primary antibodies.

The number of detected YAP1, αSMA, Desmin, and Gli2 immunoreactive cells were quantified by counting in 20 to 40 randomly chosen, 20x fields per section per mouse. The number of YAP1-Gli2 and YAP1-αSMA double(+) cells and hepatocyte mitoses were similarly quantified.

Two-step real-time RT-PCR and conventional RT-PCR

Total RNA was extracted from primary hepatocytes or whole livers using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction, followed by RNase-free DNase I treatment (Qiagen). RNA was reverse transcribed to cDNA templates using random hexamer primer and SuperScript RNase H-Reverse Transcriptase (Invitrogen). For semiquantitative qRT-PCR, 1.5% of the first-strand reaction was amplified using StepOne Plus Real-Time PCR Platform (ABI/Life Technologies) and specific oligonucleotide primers for target sequences as well as the ribosomal S9 housekeeping gene as control. qRT-PCR parameters were as follows: denaturating at 95°C for 3 minutes, followed by 40 cycles of denaturing at 95°C for 10 seconds and annealing/extension at the optimal primer temperature for 60 seconds. Threshold cycles (*C*t) were automatically calculated by the StepOne Plus Real-Time Detection System. Target gene levels in the cells are presented as a ratio to levels detected in the corresponding control cells according to the 2–ΔΔCt method. Primer sequences are listed in ***Supplemental Table 1.***

Western Blot Analysis

Protein extracts were prepared by homogenization of liver tissue in RIPA buffer (R0278; Sigma) and quantified by Pierce BCA kit. Proteins were visualized by western analysis using the following primary antibodies; YAP1 (CS-4912S Cell Signaling), pYAP1-S127 (CS-4911S Cell Signaling), Lats1 (CS-9153 Cell Signaling), pLats1-Thr1079 (DS7D3 Cell Signaling) and β-actin (sc-47778, Santa Cruz Biotechnology).

Statistics

All data were expressed as mean ± SEM. Statistical analysis was performed using Student's *t* test or one-way ANOVA as indicated. All analysis was conducted using Graph-Pad Prism 4 software (GraphPad Software Inc.). Differences with $P \leq 0.05$ were considered to be statistically significant.

References

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2. Choi SS, Witek RP, Yang L, Omenetti A, Syn WK, Moylan CA, et al. Activation of Rac1 promotes hedgehog-mediated acquisition of the myofibroblastic phenotype in rat and human hepatic stellate cells. Hepatology 2010; 52:278-90.
3. Choi SS, Syn WK, Karaca GF, Omenetti A, Moylan CA, Witek RP, et al. Leptin promotes the myofibroblastic phenotype in hepatic stellate cells by activating the hedgehog pathway. J Biol Chem 2010;285:36551-60
4. Greenwel P, Schwartz M, Rosas M, Peyrol S, Grimaud JA, Rojkind M. Characterization of fat-storing cell lines derived from normal and CCl4-cirrhotic livers. Differences in the production of interleukin-6. Lab Invest 1991; 65:644-53.
5. Michelotti GA, Xie G, Swiderska M, et al. Smoothened is a master regulator of adult liver repair. J Clin Invest 2013; 123:2380-94.

Supplemental Table 1. Primer list for RT-PCR

Gene	Forward	Reverse
Gli 1	ATCTCCGGGCGGTTCCTAC	CTTCAGCTGGCAGGTTGCAC
Gli 2	ACCATGCCTACCCAACTCAG	CTGCTCCTGTGTCAGTCCAA
YAP1	AGCAGCAGCAAATACAGCAG	AGCATTTGCTGTGCTGGGATTGT
Smo	TCACCGAGGCCGAGCAC	CCACGAAGAAACACGCATTGAC
Desmin	TACACCTGCGAGATTGATGC	ACATCCAAGGCCATCTTCA
aSma	GATGAAGCCCAGAGCAAGAG	CTTTTCCATGTCGTCCCAGT
CTGF	TCAAGCTGCCTGGGAAATG	TCTGGGCCAAATGTGTCTTC
Collagen	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
AREG	GCCATCATCCTCGCAGCTAT	CCCGTTTTCTTGTCGAAGCC
SNAIL	GAGGACAGTGGCAAAAGCTC	TCGGATGTGCATCTTCAGAG
FOXM1	CCCAAAGCCCAAGAAGTCCT	ATCGGCTCACCTCCCTTTTC
CCND1	TAGGCCCTCAGCCTCACT	CCACCCCTGGGATAAAGCAC
HNF4a	TCAACGACCGGCAGTACGAC	CTGGCAGACCCTCCGAGAAG
S9	GACTCCGGAACAAACGTGAGGT	CTTCATCTTGCCCTCGTCCA