Supplementary Material for:

Suppression of YAP/TAZ-Notch1-NICD axis by bromodomain and extraterminal protein inhibition impairs liver regeneration

Running title: YAP/TAZ-Notch1-NICD axis regulates liver regeneration

Chen Liu[1¶], Xiawei Cheng[2¶], Juntao Chen[1], Yao Wang[3], Xiaoying Wu[4], Rui Tian[2], Baoqing Liu[5,1], Xianfeng Ding[3], Qiming Sun[2*], Weihua Gong[1*]

[1] Department of Surgery, Second Affiliated Hospital of School of Medicine, Zhejiang University, Hangzhou City, People's Republic of China

[2] Department of Biochemistry, and Department of Cardiology of Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310058, China.

[3]College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou 310018, Zhejiang, China

[4]Department of Thyroid and Breast Surgery, Tongde Hospital of Zhejiang Province, Hangzhou, 310000, China

[5]Department of general surgery, Zhejiang Hospital, Hangzhou, 310000, China.

[¶] Equal contribution

*Correspondence author: Weihua Gong, Department of Surgery, Second Affiliated Hospital of School of Medicine, Zhejiang University, Hangzhou 310058, China, E-mail: weihuagong@zju.edu.cn, Tel: +86 571-87783580, Fax: +86 571-87783581

or

Qiming Sun, Department of Biochemistry, and Department of Cardiology of Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310058, China, E-mail: qmsun@zju.edu.cn, Tel: +86 571-88208505, Fax: +86 571-88208505.

Weihua Gong is the Lead Contact.

Methods

Cell isolation and culture

Primary hepatocytes were isolated from male C57BL6/J mice performed 70% partial hepatectomy (PH) using solution containing 0.08% collagenase Ⅳ (Gibco, USA). The primary hepatocytes were collected by centrifuged at 70 g for 5 minutes. By performing Periodic Acid-Schiff (PAS) staining on the isolated primary hepatocytes, we confirmed the high purity of the isolated primary hepatocytes (**Figure S1**). Alpha mouse liver 12 (AML12) cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. AML12 cells were cultured in DMEM and Ham's F12 medium (Gibco, USA) with ITS Liquid Media Supplement (Sigma, UAS) 5.5 mL, 40 ng/mL dexamethasone and 10% fetal bovine serum (FBS, Gibco, USA). Cell cultures were maintained at 37°C in a humidified air containing 5% CO_2 for experiments. AML12 YAP shRNA knockdown cells and AML12 scrambled shRNA knockdown cells were obtained by adenovirus infection. The adenovirus contains shRNA to knockdown the expression of YAP. The sequence of shRNA was 5'-CTTGGAGGCGCTCTTCAATGTTC-3'. Adenovirus was produced following adenovirus packaging protocol. AML12 cells were plated in six-well plates for 24 hours. Then the cells were infected by YAP shRNA adenovirus or scrambled shRNA adenovirus. 48 hours after infection, the cells were treated with JQ1(500nM) or DMSO for 24 hours, then the protein levels of YAP, Jagged1, Notch1 and NICD were detected by western blot. For YAP overexpression AML12 cells, PAMD-YAP-Flag plasmids were transfected into AML12 cells by Lipofectamine 2000 (Life Technologies, Thermo Fisher Scientific) according to the manufacturers' instructions. 24 hours after transfection, the cells were treated with JQ1(500 nM) or DMSO for 24 hours, then the levels of YAP, Jagged1, Notch1 and NICD were detected.

Serum ALT and ALB Assay.

The serum levels of ALT and ALB were measured by detection kits based on the manufacturer's instructions, which were obtained from the Nanjing Jiancheng Institute

of Biotechnology (Nanjing, China).

Hematoxylin and Eosin Staining (HE), Immunohistochemistry (IHC), Immunofluorescence (IF) and Periodic Acid-Schiff (PAS) Staining

To analyze histopathologic alteration of liver after PH, HE staining was routinely performed according to standard protocol. To evaluate hepatic cellular proliferation, immunohistochemical analysis for Ki-67 was undertaken in those harvested liver samples. Immunofluorescence (IF) with in vivo injection of BrdU was used to analyze hepatic cellular proliferation. With respect to immunohistochemistry (IHC), Liver samples embedded in paraffin were initially cut into 4 μm sections. These sections were then mounted on slides and heated at 60° C for 1 h. Afterwards, these sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. Deparaffinized and rehydrated sections were then heated in citrate buffer at 121°C for 30 min to retrieve antigenic activity. To inhibit endogenous peroxidase activity, these sections were subject to an incubation with 0.3% hydrogen peroxide in methanol for 20 min. Nonspecific reactions were blocked by 10% normal bovine serum. The sections were incubated with rabbit polyclonal antibodies specific to Ki-67 (1:100，Abcam) at 4°C overnight and then with horseradish peroxidase–conjugated secondary antibody at 37°C for 1 h. The sections were stained with hematoxylin for detection. With regard to immunofluorescence, frozen sections at 4 μm thickness were rehydrated, blocked with 5% normal goat serum (NGS) and incubated with rabbit polyclonal antibodies specific to BrdU (1:100, Abcam). Controls without primary antibodies were revealed with Alexa-597 goat-anti-mouse IgG (1:1000, Invitrogen). The sections were blindly analyzed by a fluorescence microscope (Nikon Eclipse E600, Kawasaki, Kanagawa, Japan). To evaluate the purity of the primary hepatocytes derived from mouse liver, we performed PAS staining. PAS Staining of primary hepatocytes was performed according to standard protocols of the manufacturer's (Solarbio. Co., Ltd., Beijing, China).

Real-time PCR (RT-PCR)

Total RNA was extracted from liver samples and primary hepatocytes using TRIZOL Reagent (Thermo Scientific, USA). Real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) by using standard SYBR Green RT-PCR kit (Vazyme Co., Ltd. Nanjing, China). The primers' sequences for RT-PCR were shown in **Table 1.** Level of target gene expression was normalized in comparison to the housekeeping gene β-actin by using the $^{\Delta\Delta}$-Ct method.

Protein Extraction and Western Blotting

The protein samples from liver tissues and cells were extracted according to standard protocols of the manufacturer's (Vazyme. Co., Ltd., Nanjing, China). The protein content was determined by the BCA protein assay kit (Bio-Rad, Hercules, USA). Proteins were separated on SDS polyacrylamide gel electrophoresis (8%–10%) and were then transferred to PVDF membranes (Millipore, USA). The membranes were washed and blocked. The membranes were incubated at 4°C overnight using the following antibodies, rabbit anti-YAP (1:1000, Cell Signaling), rabbit anti-phospho-YAP (Ser127) (1:1000, Cell Signaling), rabbit anti-TAZ (1:1000, Cell Signaling), rabbit anti-Phospho-TAZ (Ser89) (1:1000, Cell Signaling), rabbit anti-Notch1 (1:5000, Cell signaling), rabbit anti-NICD (1:1000, Cell Signaling), rabbit anti-Jagged1 (1:1000, Cell Signaling), mouse anti-Tubulin (1:1000, Sigma), and mouse anti-β-actin (1:1000, Sigma). The membranes were washed with PBS or TBST for three times. Afterwards, the membranes were incubated with a sheep anti-rabbit IgG or sheep anti-mouse IgG HRP-conjugated secondary antibody at 1:5000 dilution for 2 h at 4°C. The bands were visualized by enhanced chemiluminescence (ECL Plus Western Blotting Detection Reagents; GE Healthcare, Buckinghamshire, UK). The images were scanned and the relative density of immunoreactive bands was determined using ImageJ software.

Table 1. Real-time PCR Primer Sequences.

Genes		Sequences
β-actin	Sense	CAACTGGGACGACATGGAGAAAAT
	Antisense	CCAGAGGCGTACAGGGATAGCAC
Yap	Sense	CCAGACGACTTCCTCAACAGTG
	Antisense	GCATCTCCTTCCAGTGTGCCAA
Taz	Sense	GACCAAGTACATGAACCACC
	Antisense	CCAACGCATCAACTTCAGGT
Cyr61	Sense	GTGCCGCCTGGTGAAAGAGA
	Antisense	GCTGCATTTCTTGCCCTTTTTTAG
Ctgf	Sense	ATGATGCGAGCCAACTGCCTG
	Antisense	CGGATGCACTTTTTGCCCTTCTTAATG
AmotL2	Sense	GACTCTTTCTGGAGATCGGA
	Antisense	ACTGATCCTCTGTCCTTCCT
Notch1	Sense	GCTGCCTCTTTGATGGCTTCGA
	Antisense	CACATTCGGCACTGTTACAGCC
Jagged1	Sense	AGAAGTCAGAGTTCAGAGGCGTCC
	Antisense	AGTAGAAGGCTGTCACCAAGCAAC
Hey1	Sense	CCGACGAGACCGAATCAATAAC
	Antisense	TCAGGTGATCCACAGTCATCT
Hes1	Sense	TCAACACGACACCGGACAAAC
	Antisense	ATGCCGGGAGCTATCTTTCTT
Sox9	Sense	CGACTACGCTGACCATCAGA
	Antisense	AGACTGGTTGTTCCCAGTGC
Hgf	Sense	CCGACGAGACCGAATCAATAAC
	Antisense	CGTCCCTTTATAGCTGCCTCC
Vegf	Sense	TGGCTCTACGACCTTAGACTG
	Antisense	CAGGTTTGACTTGTCTGAGGTT

Figure Legends

Figure S1. Periodic Acid-Schiff staining of primary hepatocytes derived from C57BL6/J mice. (A) PAS staining of primary hepatocytes (100× original magnification). (B) PAS staining of primary hepatocytes (200× original magnification). (C) PAS staining of primary hepatocytes (400× original magnification).

Figure S2. BET proteins inhibition down-regulated YAP/TAZ expression during liver regeneration in vivo. (A) Fold change in *Yap* mRNA expression in liver tissues from JQ1 treatment group and control group mice at day 2 after 70% PH. (B) Fold change in *Taz* mRNA expression in liver tissues from JQ1 treatment group and control group mice at day 2 after 70% PH. (C-D) Western blot analysis of YAP, p-YAP, TAZ and p-TAZ protein levels in liver tissues from JQ1 treatment group and control group mice at day 2 after 70% PH. (E-G) Fold change in YAP/TAZ target genes, *Cyr61, AmotL2* and *Ctgf* mRNA expression in liver tissues from JQ1 treatment group and control group mice. NS P≥0.05, *P<0.05, **P<0.01. Data are expressed as mean ± S.E.M.

Figure S3. BET proteins inhibition down-regulated the Notch signaling pathway in vivo. (A) Fold change in *Notch1* mRNA expression in liver tissues from JQ1 treatment group and control group mice at day 2 after 70% PH. (B) Fold change in *Jagged1* mRNA expression in liver tissues from JQ1 treatment group and control group mice at day 2 after 70% PH. (C-D) Western blot analysis of Notch1, Jagged1 and NICD protein levels in liver tissues from JQ1 treatment group and control group mice at day 2 after 70% PH. (E-G) Fold change in Notch signaling pathway target genes, *Hey1*, *Sox9* and *Hey1* mRNA expression in liver tissues from JQ1 treatment group and control group mice at day 2 after 70% PH. NS P≥0.05, *P<0.05, **P<0.01. Data are expressed as mean ± S.E.M.

Figure S4. YAP/TAZ inhibition significantly suppressed liver regeneration after partial hepatectomy and down-regulated the Notch signaling pathway in vivo. (A) Liver regenerative index of LW/BW was analyzed for both experimental group

(Verteporfin intraperitoneal injection at the dose of 50 mg/kg, n=5) and control group (vehicle solution, n=5) at day 4 after 70% PH. (B-C) Western blot analysis of YAP, TAZ, Notch1, Jagged1 and NICD protein levels in liver tissues from Verteporfin treatment group and control group mice at day 4 after 70% PH. (D) Ki-67 staining of liver tissues from Verteporfin treatment group and control group mice at day 4 after 70% PH (200× original magnification). Scale bars, 200μm. (E) Ki-67-positive cell count at day 4 after 70% PH. *P<0.05, **P<0.01. Data are expressed as mean ± S.E.M.

Figure S5. The liver regeneration injury caused by the inhibition of BET proteins can be rescued by *Yap* overexpression in mouse liver. (A) LW/BW ratio was analyzed for AAV-Vector+Vehicle group, AAV-Vector+JQ1 group, AAV-*Yap*+Vector group and AAV-*Yap*+JQ1 group mice at day 4 after 70% PH. (B-C) Western blot analysis of YAP, Notch1, Jagged1 and NICD protein levels in liver tissues from AAV-Vector+Vehicle group, AAV-Vector+JQ1 group, AAV-*Yap*+Vector group and AAV-*Yap*+JQ1 group mice at day 4 after 70% PH. (F) Ki-67 staining of liver tissues from AAV-Vector+Vehicle group, AAV-Vector+JQ1 group, AAV-*Yap*+Vector group and AAV-*Yap*+JQ1 group mice at day 4 after 70% PH (200× original magnification). Scale bars, 200μm. (G) Ki-67-positive cell count at day 4 after 70% PH. NS P≥0.05, *P<0.05. Data are expressed as mean ± S.E.M.

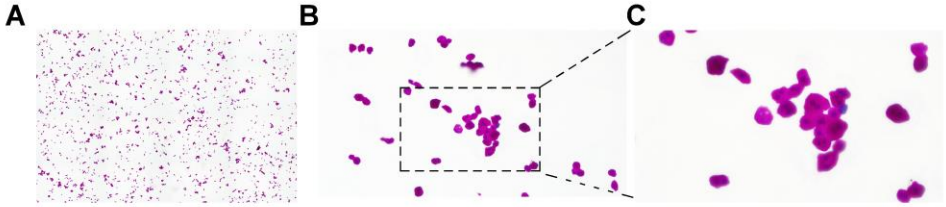

Figure S1

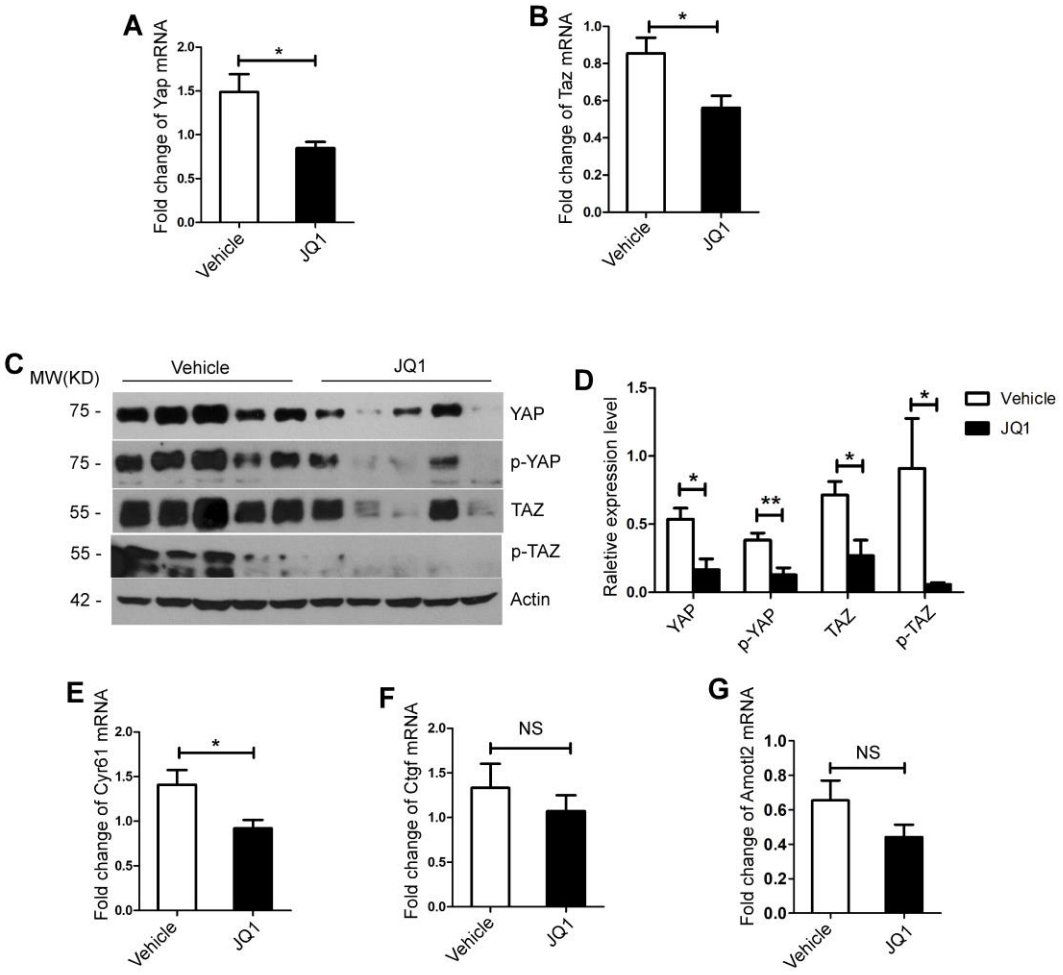
A
Fold change of Yap mRNA
2.0
1.5
1.0
0.5
0.0
*
Vehicle
JQ1
B
Fold change of Taz mRNA
1.0
0.8
0.6
0.4
0.2
0.0
*
Vehicle
JQ1
C
MW(KD)
Vehicle
JQ1
75 -
YAP
75 -
p-YAP
55 -
TAZ
55 -
p-TAZ
42 -
Actin
D
Raletive expression level
1.5
1.0
0.5
0.0
*
**
*
*
YAP
p-YAP
TAZ
p-TAZ
Vehicle
JQ1
E
Fold change of Cyr61 mRNA
2.0
1.5
1.0
0.5
0.0
*
Vehicle
JQ1
F
Fold change of Ctgf mRNA
2.0
1.5
1.0
0.5
0.0
NS
Vehicle
JQ1
G
Fold change of Amotl2 mRNA
1.0
0.8
0.6
0.4
0.2
0.0
NS
Vehicle
JQ1

Figure S2

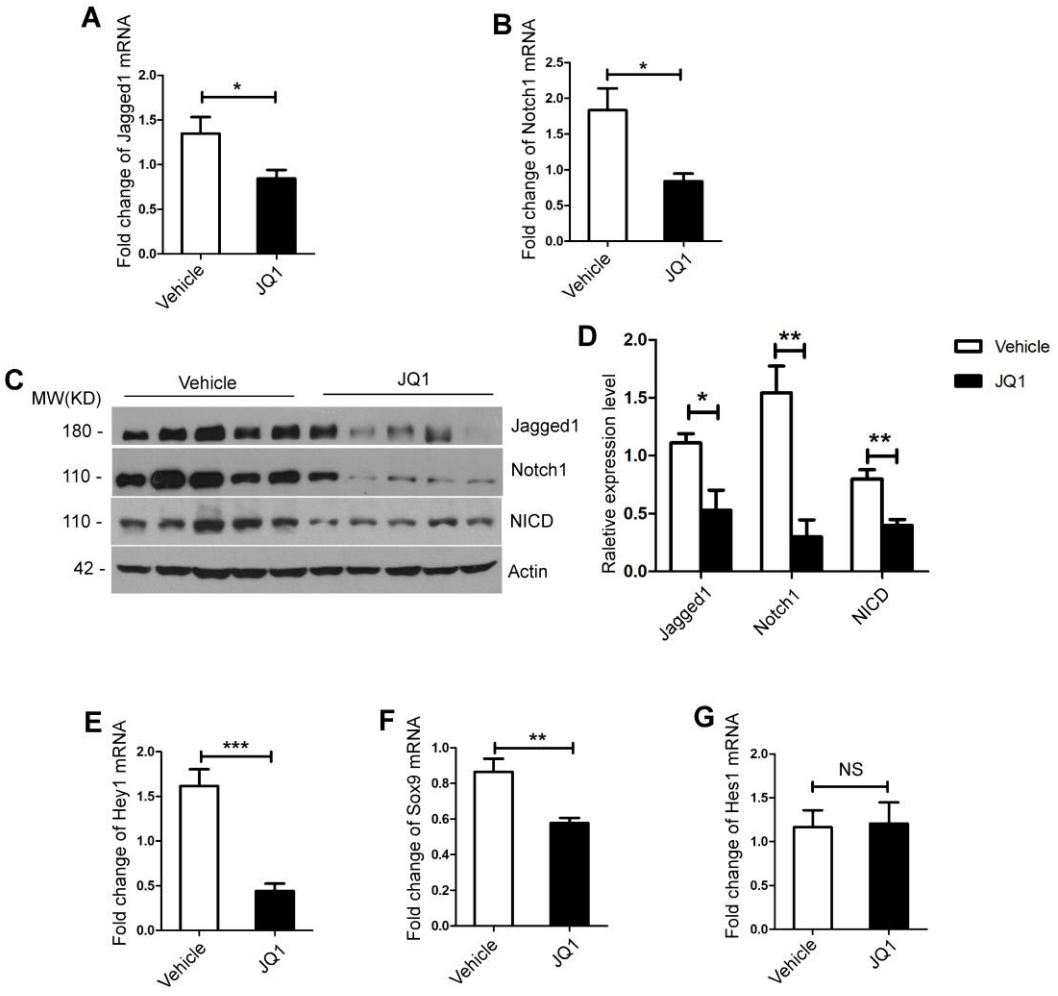
A
Fold change of Jagged1 mRNA
2.0
1.5
1.0
0.5
0.0
*
Vehicle
JQ1
B
Fold change of Notch1 mRNA
2.5
2.0
1.5
1.0
0.5
0.0
*
Vehicle
JQ1
C
MW(KD)
Vehicle
JQ1
180 -
110 -
110 -
42 -
Jagged1
Notch1
NICD
Actin
D
Raletive expression level
2.0
1.5
1.0
0.5
0.0
*
**
**
Jagged1
Notch1
NICD
Vehicle
JQ1
E
Fold change of Hey1 mRNA
2.0
1.5
1.0
0.5
0.0
***
Vehicle
JQ1
F
Fold change of Sox9 mRNA
1.0
0.8
0.6
0.4
0.2
0.0
**
Vehicle
JQ1
G
Fold change of Hes1 mRNA
2.0
1.5
1.0
0.5
0.0
NS
Vehicle
JQ1

Figure S3

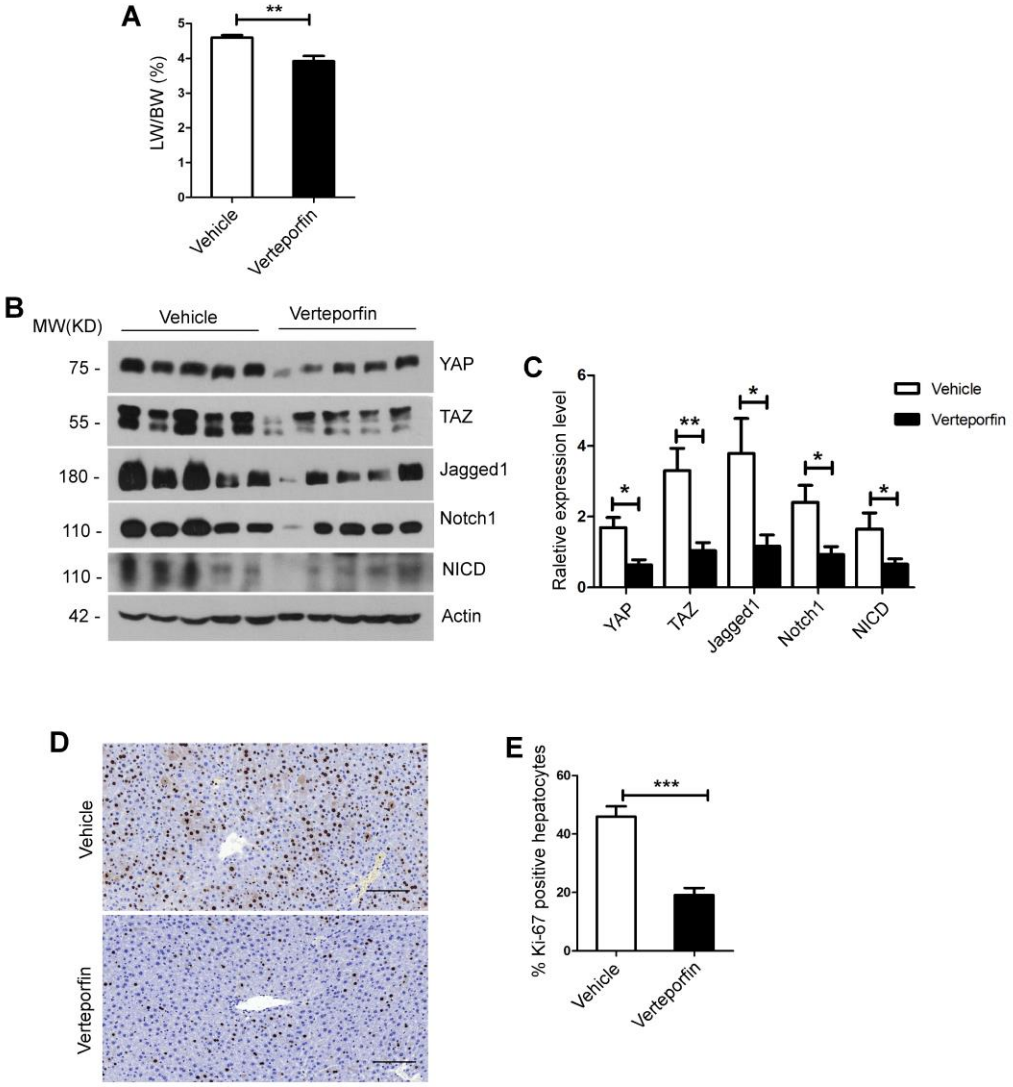
A
**
LW/BW (%)
0
1
2
3
4
5
Vehicle
Verteporfin
B
MW(KD)
Vehicle
Verteporfin
75 -
55 -
180 -
110 -
110 -
42 -
YAP
TAZ
Jagged1
Notch1
NICD
Actin
C
Raletive expression level
0
2
4
6
*
**
*
*
*
Vehicle
Verteporfin
YAP
TAZ
Jagged1
Notch1
NICD
D
Vehicle
Verteporfin
E
% Ki-67 positive hepatocytes
0
20
40
60
***
Vehicle
Verteporfin

Figure S4

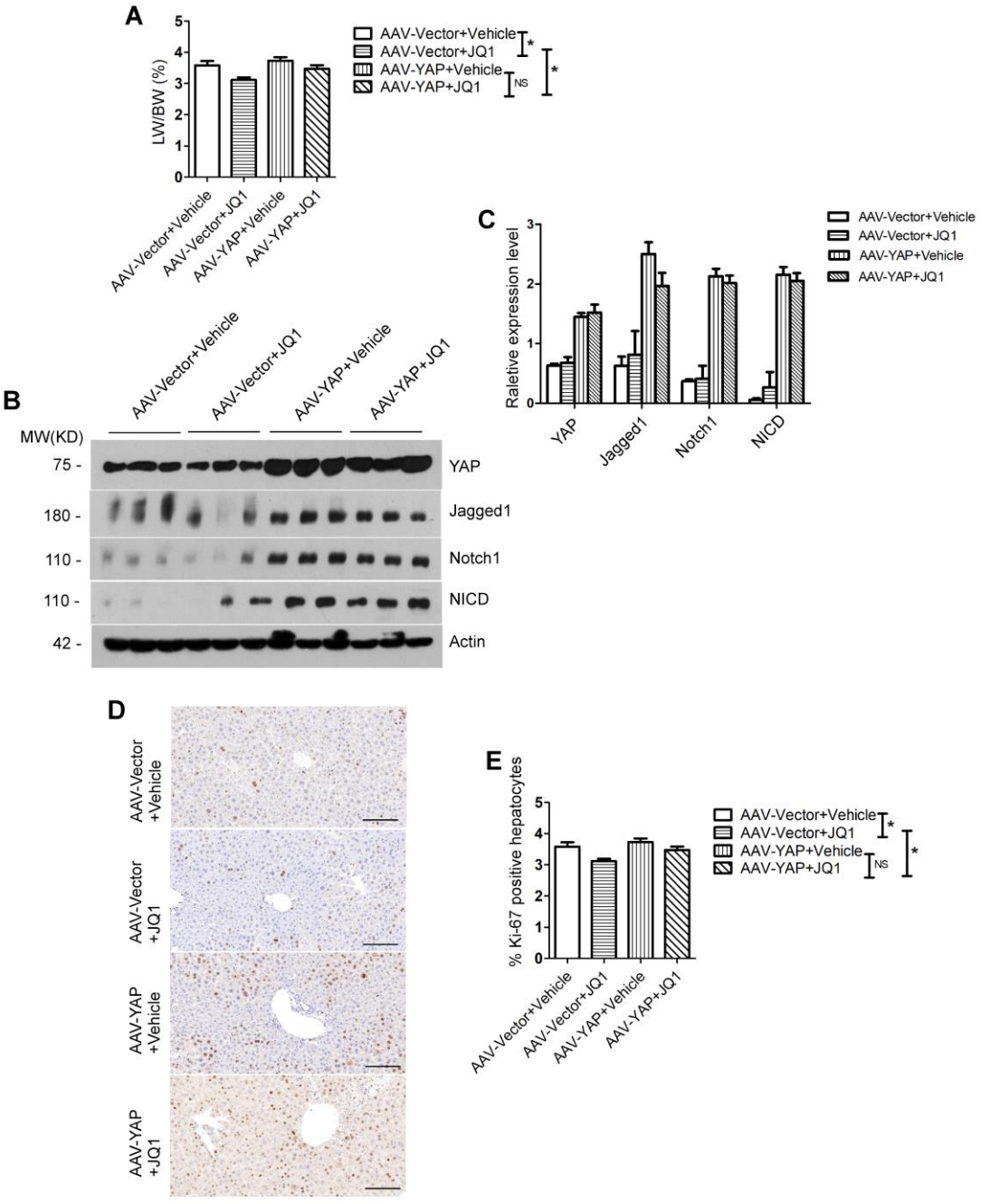
A
LW/BW (%)
AAV-Vector+Vehicle
AAV-Vector+JQ1
AAV-YAP+Vehicle
AAV-YAP+JQ1
NS
*
*
B
MW(KD)
75 -
180 -
110 -
110 -
42 -
YAP
Jagged1
Notch1
NICD
Actin
C
Raletive expression level
YAP
Jagged1
Notch1
NICD
D
AAV-Vector +Vehicle
AAV-Vector +JQ1
AAV-YAP +Vehicle
AAV-YAP +JQ1
E
% Ki-67 positive hepatocytes

Figure S5