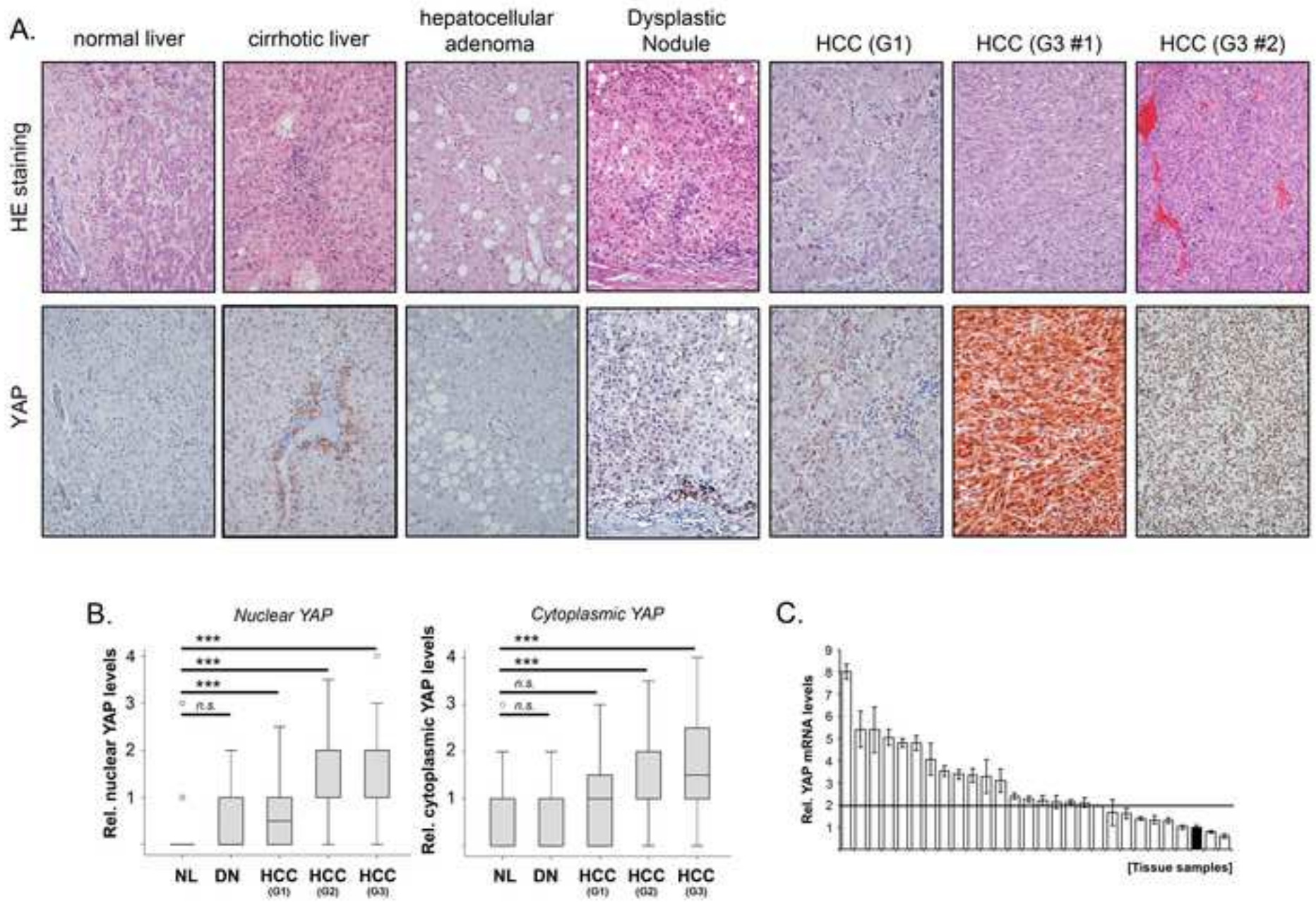
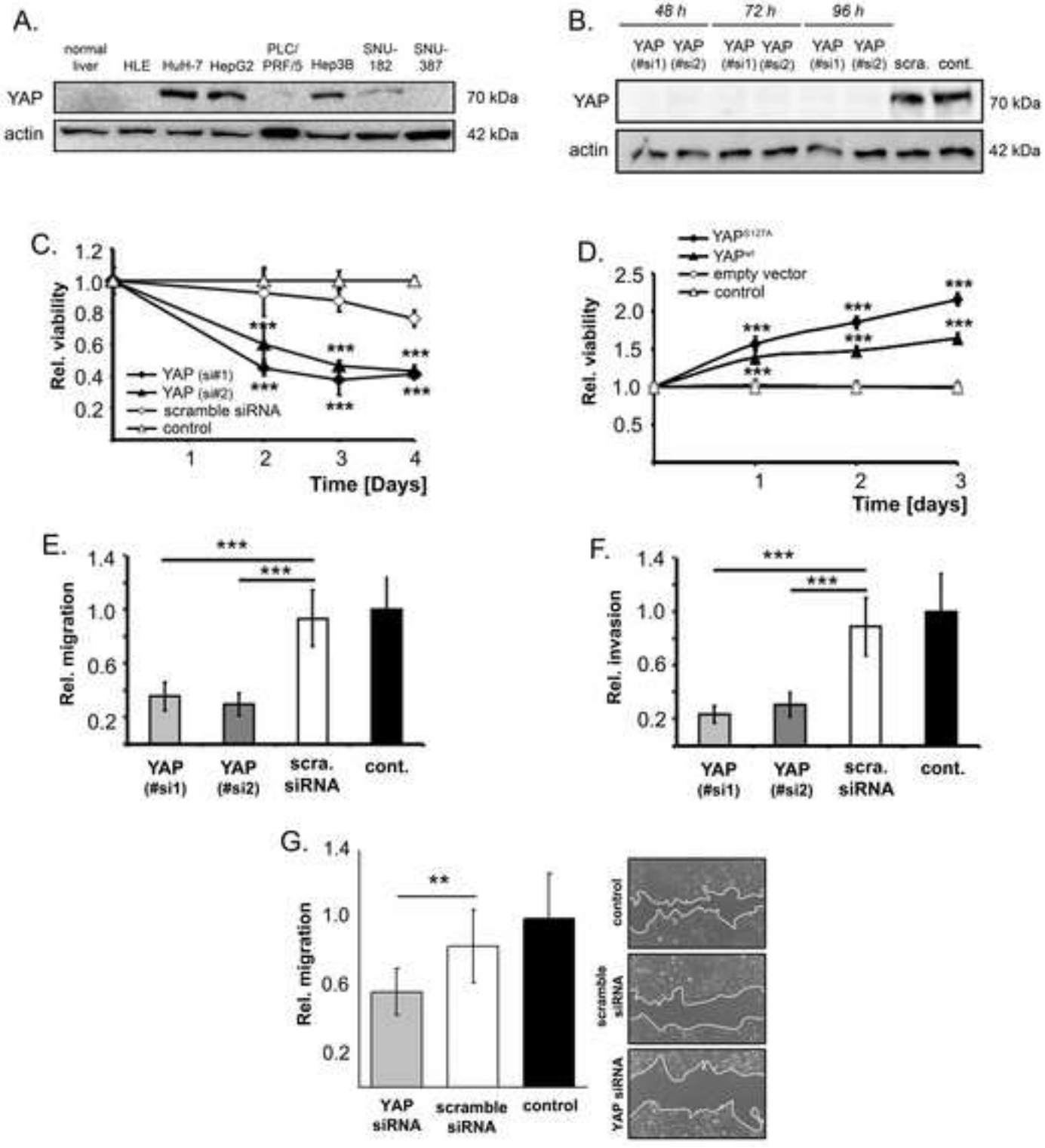
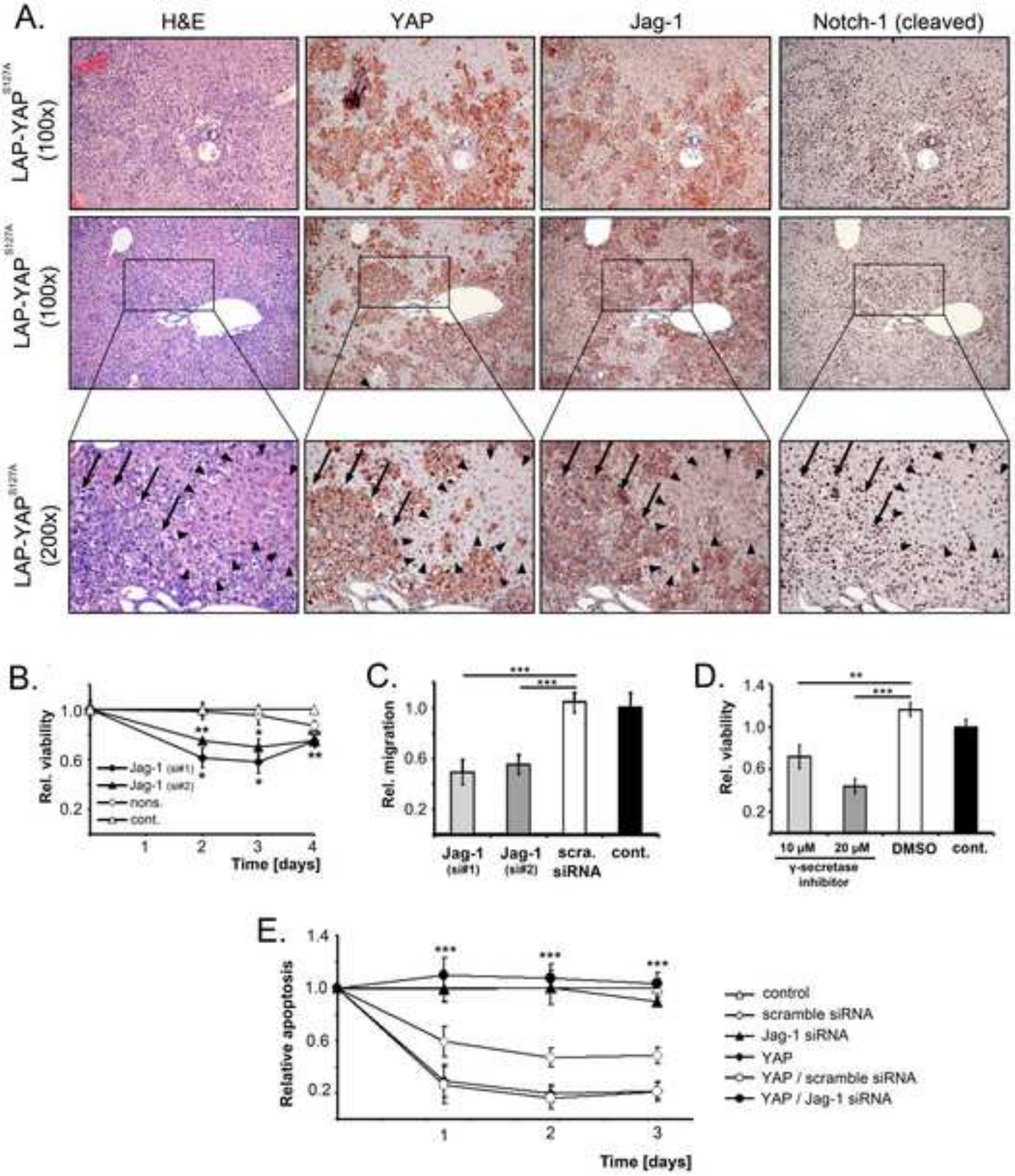
Supporting Figure S1



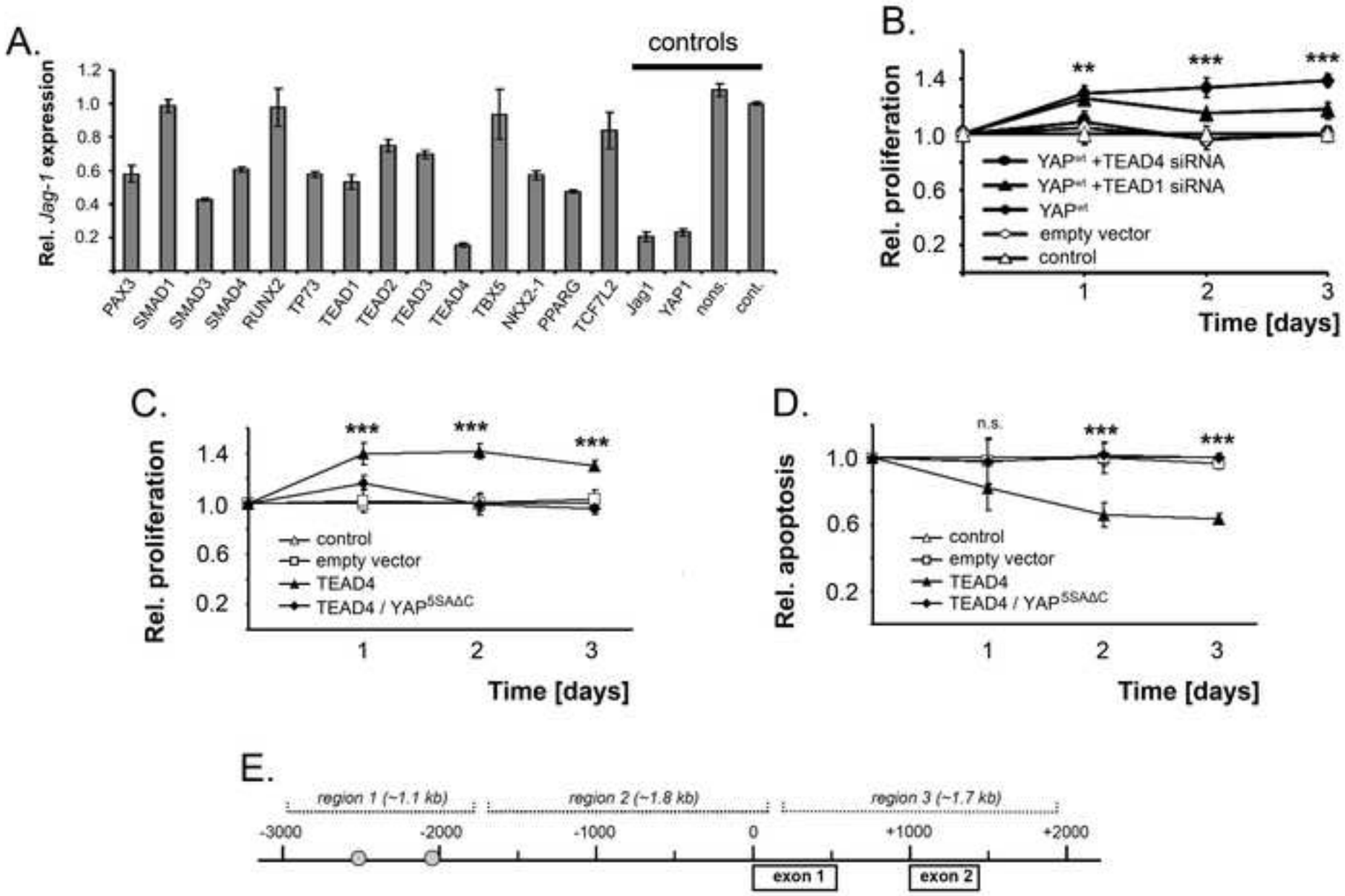
Supporting Figure S2



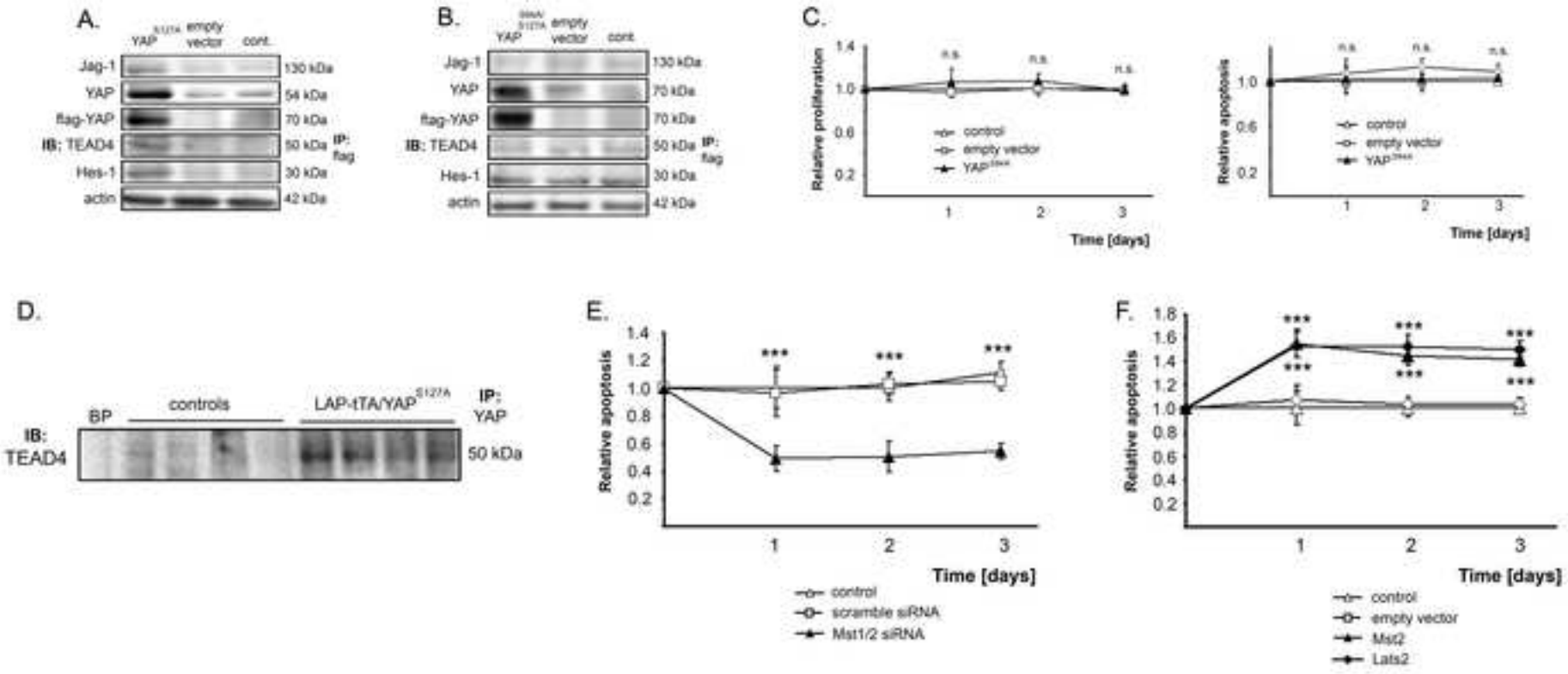
Supporting Figure S3



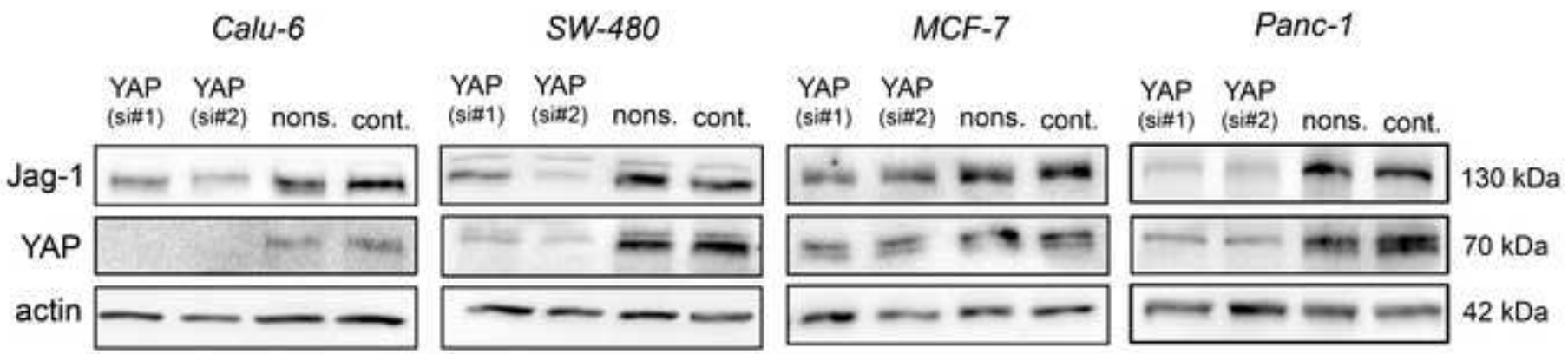
Supporting Figure S4



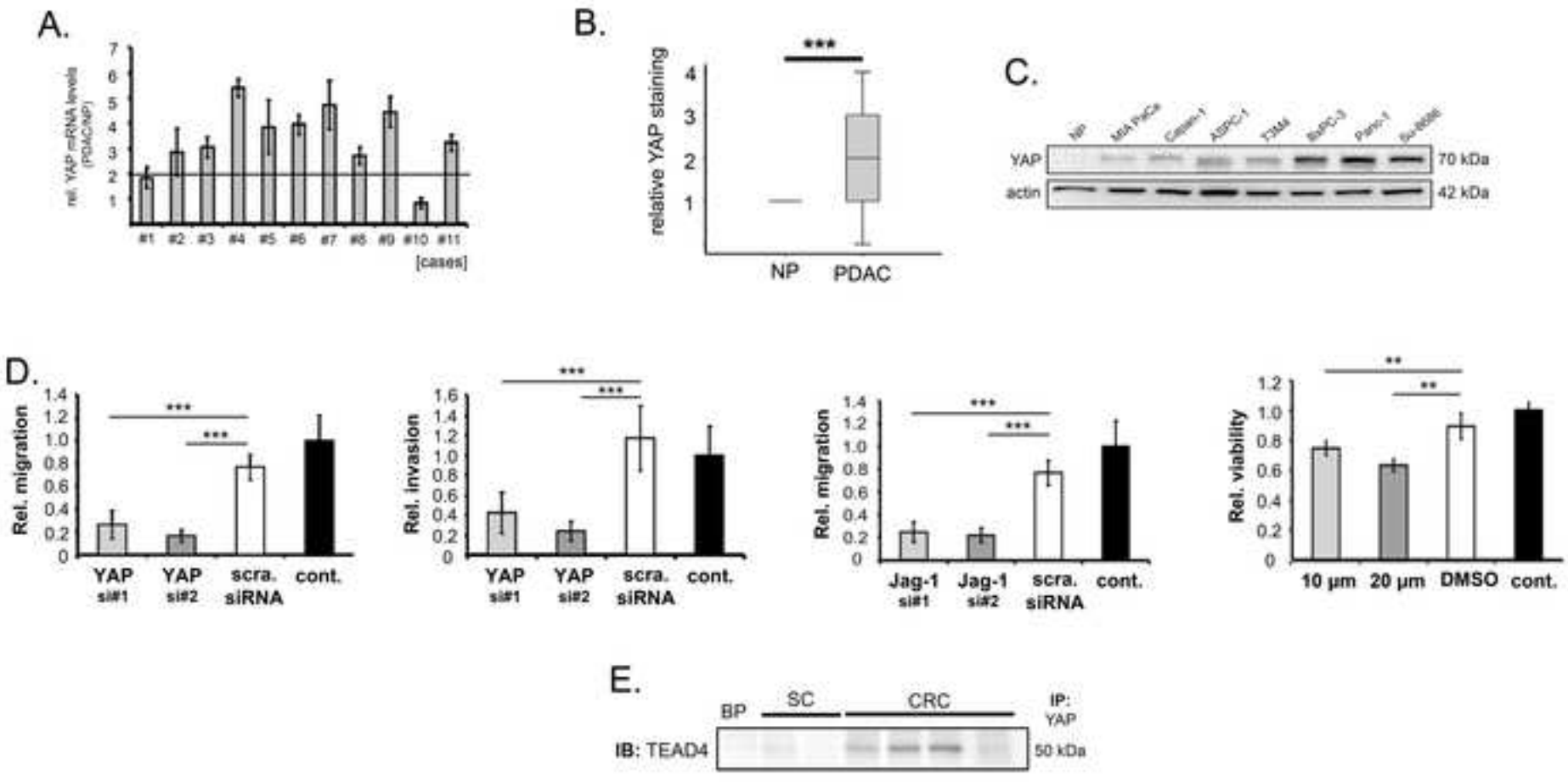
Supporting Figure S5



Supporting Figure S6



Supporting Figure S7



Primers and siRNAs

Type of oligonucleotide	Gene	Sequence (5`--> 3`)
siRNA1	huYAP (sense)	CCA CCA AGC UAG AUA AAG A-dT-dT
siRNA2	huYAP (sense)	GGU CAG AGA UAC UUC UUA A-dT-dT
siRNA1	huJAG1 (sense)	CCA GGA AGU UUC AGG GAG A-dT-dT
siRNA2	huJAG1 (sense)	CGA CAA GGC UGC AGU CCU A-dT-dT
siRNA1	huTEAD4 (sense)	GGG CAG ACC UCA ACA CCA A-dT-dT
siRNA2	huTEAD4 (sense)	CCG CCA AAU CUA UGA CAA A-dT-dT
siRNA	huMST1 (Santa Cruz)	Cat. N. sc-39249
siRNA	huMST2 (Santa Cruz)	Cat. N. sc-39247
TaqMan primer	huYAP (for)	CCT GCG TAG CCA GTT ACC AA
TaqMan primer	huYAP (rev)	CCA TCT CAT CCA CAC TGT TC
TaqMan primer	huJAG1 (for)	GAG ACA TCG ATG AAT GTG CC
TaqMan primer	huJAG1 (rev)	GAG CAG TTC TTG CCC TCA TA
TaqMan primer	muJAG1 (for)	GCT GTA TCT GTC CAC CTG GCT A
TaqMan primer	muJAG1 (rev)	GTC ACT GGC ACG ATT GTA GCA
TaqMan primer	huHes1 (for)	ACA CGA CAC CGG ATA AAC
TaqMan primer	huHes1 (rev)	TCA GCT GGC TCA GAC TTT C
TaqMan primer	muHes1 (for)	CCA GCT GAT ATA ATG GAG A
TaqMan primer	muHes1 (rev)	GGC CTC TTC TCC ATG ATA G
TaqMan primer	muTubulin (for)	CTA CTG TGC CTG AAC TTA CC
TaqMan primer	muTubulin (rev)	GGA ACA TAG CCG TAA ACT GC
TaqMan primer	hu18S (for)	AAA CGG CTA CCA CAT CCA AG
TaqMan primer	hu18S (rev)	CCT CCA ATG GAT CCT CGT TA
TaqMan primer	huCTGF (for)	GCT GCG AGG AGT GGG TGT
TaqMan primer	huCTGF (rev)	GGA CCA GGC AGT TGG CTC TA
TaqMan primer	huCXCR4 (for)	ATC TGG AGA ACC AGC GGT TA
TaqMan primer	huCXCR4 (rev)	TCT TCA CGG AAA CAG GGT TC

Antibodies

Antibody	Dilution	Source
anti-YAP	WB: 1:300 IHC: 1:25	Cell Signalling
anti-YAP	IP: 1:100	Santa Cruz
anti-pYAP	WB: 1:500	Cell Signalling
anti-MST1	WB: 1:200	Cell Signalling
anti-MST2	WB: 1:300	Cell Signalling
anti-LATS2	WB: 1:500	Abcam
anti-Jag1	WB: 1:500 IHC: 1:50	Santa Cruz
anti-Jag1	IHC: 1:50	Atlas Antibodies
anti-CK19	IHC: 1:100	Abcam
anti-SMA	ICH: 1:200	Abcam
anti-TEAD4	WB: 1:500	Abcam
anti-TEAD4	WB: 1:100	Santa Cruz
anti-TEAD1	WB: 1:200	Santa Cruz
anti-Notch1	WB: 1:500	Santa Cruz
anti-Notch1	IHC: 1:50	Cell Signaling
anti-actin	WB: 1:10.000	MP-Biomedicals
anti-Ki67	IHC: 1:2000	Bethyl
anti-HES1	WB: 1:500	Abcam
anti-β-catenin	IHC: 1:600	BD Biosciences
Histone H3	WB: 1:500	Santa Cruz
DYKDDDDK tag (anti-FLAG)	WB: 1:1000	Cell Signaling
Myc-Tag	WB: 1:1000	Cell Signaling

Flexiplate

NCBI gene symbol	mRNA Accession No.	siRNA Target Sequence
PAX3	NM_000438	CAGCCGCATCCTGAGAAGTAA
SMAD1	NM_005900	TTAGCTCAGTTCCGTAACTTA
SMAD3	NM_005902	AAGAGATTCGAATGACGGTAA
SMAD4	NM_005359	CCCTGTTAAACAGTAGTTGTA
RUNX2	NM_004348	CAGAAGCTTGATGACTCTAAA
TP73	NM_005427	CCCGCTCTTGAAGAAACTCTA
TEAD1	NM_021961	CGCCGCTTCATTGCACATTCA
TEAD2	NM_003598	CTCCAGGTGGTGACAAACAGA
TEAD3	NM_003214	AAGGTCCTCACTGTTTGCATA
TEAD4	NM_003213	CTGCAGGTGGTCACCAACAGA
TBX5	NM_000192	AAGGCGGATGTTTCCCAGTTA
NKX2-1	NM_003317	CCGCGTTTAGACCAAGGAACA
PPARG	NM_005037	CAGACAAATCACCATTCGTTA
JAG1	NM_000214	CTGCATTTAGGGAGTATTCTA
YAP1	NM_006106	CACATTAACGACTAGATTAAA
TCF7L2	NM_030756	GAGCGACAGCTTCATATGCAA

Supplemental Information for Manuscript GASTRO-D-12-00769

Yes-Associated Protein Upregulates Jagged-1 and Activates the NOTCH Pathway in Human Hepatocellular Carcinoma

DF Tschaharganeh, X Chen, P Latzko, M Malz, MM Gaida, K Felix, S Ladu, S Singer, F Pinna, N Gretz, C Sticht, ML Tomasi, S Delogu, M Evert, B Fan, S Ribback, L Jiang, S Brozzetti, F Bergmann, F Dombrowski, P Schirmacher, DF Calvisi, K Breuhahn

Inventory of Supplemental Information

1. Supplemental Experimental Procedures

2. Supplemental References

1. Supplemental Experimental Procedures

1.1 Cells and culture conditions

HCC cell lines Hep3B, PLC/PRF-5, and HepG2 were obtained from ATCC; HuH-7 was obtained from JCRB; HuH-1 and SNU-449 were a kind gift of Dr. Snorri Thorgeirsson (NCI, Bethesda, MD). The pancreatic cancer cell line Panc-1 was a kind gift of the European Pancreas Center Heidelberg. Cell lines were cultured at 37°C in a 5% CO_2 atmosphere in DMEM (HuH-7, PLC/PRF/5, HuH-1), RPMI (HepG2, Panc-1) or MEM (Hep3B) as described.[1] Primary murine hepatocytes were isolated as previously described.[2] Hepatocytes were seeded on collagen-coated 6 cm plates (800,000 cells) in pre-starvation medium (Williams Medium Plus containing 1% penicillin/streptomycin, 100 nM dexamethasone, 2 mM glutamate). The pre-starvation medium was removed after 24 h and cells were cultured in complete medium (Williams Medium Plus containing 10% FCS, 1% penicillin/streptomycin, 0.1% insulin, 100 nM dexamethasone, 2 mM glutamate) either with or without doxycycline (2 µg/µl, Sigma Aldrich, Taufkirchen, Germany).

1.2 Gene silencing, vectors and transfection, γ*-secretase and SAHM1 treatment*

Gene-specific small interfering siRNAs (siRNAs) for human YAP and Jag-1 were obtained from Eurofins MWG Operon (Ebersberg, Germany). siRNAs specific for human Mst1 and Mst2 genes were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Final concentrations for all siRNAs were 20 nM. Untreated cells and nonsense siRNA-transfected cells were used as controls. AllStars siRNA was used as nonsense control (Qiagen, Hilden, Germany). For the FlexiPlate approach, predesigned siRNAs were selected using the Qiagen FlexiPlate design tool (www.qiagen.com). HuH-7 cells were transfected with respective siRNAs using

HiPerFect transfection reagent according to the manufacturer's instructions (Qiagen). Total RNA was isolated 24 h after transfection. Sequences of siRNAs and primers used in this study are listed in Supplementary Table S1. For siRNA transfection, cells were seeded without FCS one day before transfection. The carrier Oligofectamine (Invitrogen/Gibco, Karlsruhe, Germany) was used as previously described.[1]

Transient transfection of expression vectors using Lipofectamine 2000 was performed following the manufacturer's protocol (Invitrogen). Wild-type YAP and TEAD4 cDNA in pCMV6-XL4 vector and Mst2 cDNA in pCMV6-XL5 vector were purchased from OriGene Technologies (Rockville, MD). YAP^{S127A} and $YAP^{5SA\Delta C}$ constructs including FLAG- and Myc-tag, and Lats2 wild-type cDNA were kindly provided by Dr. Kunliang Guan (University of California, San Diego), and were subcloned in a pT3-EF1a vector. FLAG-tagged $YAP^{S94A/S127A}$ and HA-tagged Jag-1 were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA) and subcloned in a pT3-EF1a vector. For stable transfection of FLAG-tagged YAP^{S127A}, stable transfectants were selected with cloning cylinders after 3–4 weeks in medium containing Geneticin (600 µg/ml).

For γ-secretase inhibitor treatment, cells were seeded in 96-well plates in standard medium (8000 cells/well) and treated with the γ-secretase inhibitor at a final concentration of 10 µM or 20 µM (InSolution gamma X; Calbiochem, Darmstadt, Germany). DMSO was used as control. Cell viability was analyzed after 24 h.

The Notch transcription factor inhibitor SAHM1 was obtained from Millipore (Meck, Darmstadt, Germany).[3] Cells were treated with 20 µM SAHM1 for the indicated timepoints.

1.3 Mouse Work

All experiments were performed with the approval of the German Regional Council of Baden-Wuerttemberg (Karlsruhe, Germany) and in accordance with the institutional regulations of the IBF (Interfakultäre Biomedizinische Forschungseinrichtung, University Heidelberg, Germany). Generation of Col1A1-YAP^{S127A} transgenic mice was described previously.[4] For liver-specific transgene expression, mice were crossed with animals expressing the tTA tetracycline transactivator under the control of the LAP promoter (LAP-tTA; Jackson Laboratory, Bar Harbor; stock number: 003563). To suppress transgene expression for breeding, water was supplemented with 2 mg/ml doxycycline and 10 mg/ml sucrose. Six weeks after birth, doxycycline was withdrawn to induce hepatic YAP^{S127A} expression. Liver tissues were obtained 4 or 8 weeks after doxycycline deprivation. Mice that received doxycycline served as controls.

1.4 Human Tissue Samples

Fresh frozen samples of human livers/HCCs (n=75), paired pancreas/PDACs (n=11), and paired colon/colon cancers (n=40) for isolation of mRNA and protein fractions were either collected at the University Hospitals of Heidelberg and Rome or kindly provided by Dr. Snorri S. Thorgeirsson (NCI, Bethesda, MD). Institutional Review Board approval was obtained at participating hospitals and the NIH. All specimens were classified according to established criteria (WHO, UICC) by experienced pathologists (PS, DFT, MMG, FB, FD, ME). Liver tumors were divided in HCC with shorter/poor (HCC-P) and longer/better (HCC-B) survival, characterized by <3 and >3 years survival following partial liver resection, respectively.[5]

1.5 Sample preparation, real-time PCR, Western blotting, immunoprecipitation, and expression profiling

For isolation of total RNA and proteins from cultured cells, the NucleoSpin RNA II kit (Machery-Nagel, Düren, Germany) and Cell Lysis Buffer (Cell Signalling/New England Biolabs, Frankfurt, Germany) were used, respectively. Isolation of RNA and protein extracts from HCCs, PDAC, and CRCs was performed as described elsewhere. [6-8] Nuclear extracts were prepared with the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Rockford, IL) following the manufacturer's recommendations.

Semiquantitative real-time PCR, cDNA synthesis, and Western blot analysis were performed as previously described.[1] For immunoprecipitation studies, 200 µg nuclear extract was precipitated with 5 µg agarose-conjugated antibodies. The complexes of YAP with TEAD4 were determined by immunoprecipitating either endogenous YAP using a rabbit polyclonal anti-YAP antibody (Santa Cruz Biotechnology) or exogenous YAP using a rabbit anti-FLAG antibody (Cell Signalling Technology). As negative controls, antibodies used for immunoprecipitation were blocked prior to IP by preincubation with the respective peptide at RT for 2 h (1:40 w/w). Antibodies and dilutions as well as primer sequences used in this study are listed in Supplementary Table S1.

Gene expression profiling was performed using Human Gene 1.0 ST Arrays from Affymetrix (Affymetrix, High Wycombe, UK). Biotin-labeled cRNA was prepared according to the Affymetrix standard labeling protocol. cRNA was purified, fragmented, and hybridized using a GeneChip Hybridization oven 640, stained using a GeneChip Fluidics Station 450, and scanned with a GeneChip Scanner 3000 (Affymetrix). Raw fluorescence intensity values were normalized by quantile

normalization, and differential gene expression was calculated by ANOVA using the software package SAS JMP7 Genomics, version 4 (SAS Institute, Cary, NC, USA). A false positive rate of a=0.05 with FDR correction was taken as the level of significance. The data were filtered for genes whose expression changed 1.5-fold as compared to nonsense siRNA-transfected cells. Raw and normalized data were deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; accession No. GSE-35004).

1.6 Functional in vitro assays

Cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide) assay. After adding the MTT solution (0.5 mg/mL in medium), the supernatant was removed after 2 h incubation and the tetrazolium salt was redissolved in 100 µL of DMSO/ethanol solution (1:2). Colorimetric measurement was performed at 570 nm on an ELISA reader.

Proliferation was assessed with the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology, Danvers, MA) by measuring the absorbance at 450 nm following the manufacturer's protocol.

To measure apoptosis, genetically manipulated cells were plated at a concentration of 2.0 x 10^3 cells/well in 96-well plates, cultured for 12 h, and subjected to serum deprivation for 24 h. Subsequently, apoptosis was assessed at four time points: 0, 24, 48, and 72 h using the Cell Death Detection Elisa Plus Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol.

Migratory activity and invasiveness were analyzed using Transwell Permeable Supports (Corning, Corning NY) and BD Biocoat Matrigel invasion chambers (BD Bioscience, Heidelberg, Germany), respectively. Cells were transfected in 6-well

plates with the indicated siRNAs, and quantified 24 h after transfection. Transwell inserts were pretreated according to the manufacturer's instructions, and defined cell numbers (HCC cells: 25,000 cells/insert; Panc-1: 30,000 cells/insert) were seeded in serum free medium. After 24 h, non-migrated cells were removed using Q-tips, and serum free medium was applied. Migrated/invaded cells in the lower part of the membranes were fixed in ice-cold methanol for 10 minutes, stained in toluidine blue solution (200 mg toluidine blue in 200 ml 0.05 M sodium tetraborate) for 45 minutes, and dried overnight. Membranes were digitally documented and cells of ten randomly chosen membrane images were counted visually.

The cell population migratory activity (scratch assay) was analyzed three days after transfection of siRNAs. Cells were treated with mitomycin-c (5 µg/ml) for three h to repress proliferation. Subsequently, the cell monolayer was damaged with a 'scratch' using a pipette tip. Cells were treated with HGF (10 ng/ml) and incubated for further 18 h. Scratches were digitally documented and relative migratory activity was ascertained by calculating the cell free areas.

All experiments were performed in triplicate, and were repeated at least 2 times.

1.7 Tissue Microarrays (TMAs)

HCC TMAs contained 2 representative areas (diameter: 0.6 mm) of 42 histologically normal livers, 9 Dysplastic Nodules (DNs), and 85 HCCs (grading: 13 x G1, 45 x G2, 27 x G3). PDAC TMAs contained 2 representative areas of 10 normal pancreatic tissue samples (diameter: 1 mm) from gender-matched patients and 73 PDACs (grading: 3 x G1, 47 x G2, 23 x G3). In addition, a separate collection of livers/HCC (n=48) as well as colon/colon cancers (n=51) and 14 hepatocellular adenomas were

used for immunohistochemistry. Staining procedure, signal evaluation, and statistical data analysis were performed as described.[9]

Immunohistochemical staining on human and mouse liver tissue specimens was performed on 10% formalin-fixed, paraffin-embedded sections. Deparaffinized sections were incubated in 3% H_2O_2 dissolved in phosphate-buffered saline (PBS) 1X for 30 minutes to quench the endogenous peroxidase. For antigen retrieval, slides were microwaved in 10 mmol/L citrate buffer (pH 6.0) for 12 minutes. The following antibodies were applied: rabbit monoclonal anti-Notch1 (Cell Signaling Technology, Danvers, MA; 1:50), rabbit polyclonal anti-YAP (Cell Signaling Technology; 1:50), anti-CK19 (Abcam, Cambridge, United Kingdom; 1:100), anti-smooth muscle actin (Abcam; 1:200), anti-Ki-67 (Bethyl Laboratories Inc., Montgomery, TX; 1:2000), goat polyclonal anti-Jag-1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50). The immunoreactivity was visualized with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine as the chromogen. Slides were counterstained with hematoxylin.

1.8 Luciferase Reporter Gene Assay

Luciferase reporter gene assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Three constructs containing three distinct regions of the human Jag-1 promoter were generated and subcloned in a pGL4.11 vector (Promega Corporation, Madison, WI). PLC/PRF-5 cells were transferred into 24-well plates at 3×10^4 cells per well. After 24 h, 0.2 µg of each of the three pGL4.11-Jag-1 reporter vectors were individually transfected together with either the YAP^{S127A} or TEAD4 expression vector (or the respective empty control). A Renilla expressing vector was used to normalize the

data. Luminescence was measured 48 h later. Verteporfin (VWR International, Radnor, PA) was dissolved in DMSO and added at 10 µM and 20 µM concentration 24 h after transfection and administered for 48 h to a group of transfected and untransfected cells. All experiments were performed at least two times.

1.9 Statistical analysis and software

Data are presented as mean +/- standard deviation. The Spearman's rank coefficient was used as a statistical measure of association. The statistical comparison between two groups (unpaired and paired values) was accomplished with the non-parametric Mann-Whitney U test. The significance levels were defined as *$p<0.05$, **$p<0.01$, and ***$p<0.001$ (SPSS software). n.s.: not significant. For identification of potential TEAD binding sites in the human Jag-1 promoter, TESS (transcriptional element search tool was used (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME).[10]

For all functional assays shown in this study, nonsense siRNA or mock-transfected cells were used for statistical comparison.

2. Supplemental References

1. Nussbaum T, Samarin J, Ehemann V, et al. Autocrine insulin-like growth factor-II stimulation of tumor cell migration is a progression step in human hepatocarcinogenesis. Hepatology 2008;48:146-56.
2. Tschaharganeh DF, Kaldenbach M, Erschfeld S, et al. Glycoprotein 130-dependent pathways in host hepatocytes are important for liver repopulation in mice. Liver Transpl 2010;16:23-32.
3. Moellering RE, Cornejo M, Davis TN, et al. Direct inhibition of the NOTCH transcription factor complex. Nature 2009;462:182-8.
4. Camargo FD, Gokhale S, Johnnidis JB, et al. YAP1 increases organ size and expands undifferentiated progenitor cells. Curr Biol 2007;17:2054-60.
5. Lee JS, Chu IS, Heo J, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. Hepatology 2004;40:667-76.
6. Calvisi DF, Wang C, Ho C, et al. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. Gastroenterology 2011;140:1071-83.

7. Malz M, Weber A, Singer S, et al. Overexpression of far upstream element binding proteins: a mechanism regulating proliferation and migration in liver cancer cells. Hepatology 2009;50:1130-9.
8. Wente MN, Gaida MM, Mayer C, et al. Expression and potential function of the CXC chemokine CXCL16 in pancreatic ductal adenocarcinoma. Int J Oncol 2008;33:297-308.
9. Singer S, Ehemann V, Brauckhoff A, et al. Protumorigenic overexpression of stathmin/Op18 by gain-of-function mutation in p53 in human hepatocarcinogenesis. Hepatology 2007;46:759-68.
10. Schug J. Using TESS to predict transcription factor binding sites in DNA sequence. Curr Protoc Bioinformatics 2008;Chapter 2:Unit 2 6.

Yes-Associated Protein Upregulates Jagged-1 and Activates the NOTCH Pathway in Human Hepatocellular Carcinoma

DF Tschaharganeh, X Chen, P Latzko, M Malz, MM Gaida, K Felix, S Ladu, S Singer, F Pinna, N Gretz, C Sticht, ML Tomasi, S Delogu, M Evert, B Fan, S Ribback, L Jiang, S Brozzetti, F Bergmann, F Dombrowski, P Schirmacher, DF Calvisi, K Breuhahn

Supplementary Figure Legends

Supplementary Figure S1: Stepwise increase of YAP levels in hepatocarcinogenesis

(A) Representative immunohistochemical analysis for YAP protein in tissue microarrays (TMAs) of human liver tissues and lesions. Absent or very weak YAP immunoreactivity is detected in hepatocytes from normal liver, while biliary cells exhibit nuclear accumulation of YAP. In surrounding cirrhotic liver, most hepatocytes show a similar faint nuclear positivity; however, a strong cytoplasmic and nuclear immunoreactivity characterizes hepatocytes, located in close proximity to septae. In hepatocellular adenomas and dysplastic nodules (DNs), absent or very weak YAP staining is detected. Well differentiated hepatocellular carcinomas (*HCC G1*) displays weak to moderate nuclear immunoreactivity. In contrast, poorly-differentiated HCCs (*HCC G3 #1* and *#2*) exhibit strong homogeneous YAP staining in both cytoplasms and nuclei (*HCC G3 #1*) or almost exclusively in the nuclei (*HCC G3 #2*) of tumor cells. Original magnification: x200. **(B)** Boxplot illustration and statistical analyses of cytoplasmic and nuclear YAP expression in DNs and in groups of HCCs with different grades of dedifferentiation compared to normal livers (NL). Open circles: outliers. The statistical comparison between 2 groups was accomplished with the nonparametric Mann-Whitney U test. n.s. - not significant; significance levels: ***$p<0.001$. **(C)** Relative YAP transcript levels were analyzed in 27 primary human HCCs (white bars) and a mixture of 3 normal liver tissues (black bar). The horizontal line represents the threshold for a 2-fold induction (defined as overexpressed) compared to normal liver tissue.

Supplementary Figure S2: High-level expression of YAP supports tumor cell growth and migration/invasion of human HCC cells

(A) Total YAP levels in cell lysates from normal liver tissue and HCC cell lines (HLE, HuH-7, HepG2, PLC/PRF/5, Hep3B, SNU-182, SNU-387) were analyzed using immunoblot analysis. For further studies, HuH-7 and Hep3B were used as examples of HCC cells with high-level expression of YAP, and PLC/PRC/5 served as an example of cells with low-level YAP expression. **(B)** Transient transfection of two independent YAP-specific siRNAs (si#1 and si#2, final concentration: 20 nM) for 48, 72, or 96 h abrogated YAP expression in HuH-7 cells. Untreated (cont.) and scramble (scra.) siRNA-transfected cells served as controls. **(C)** Cell viability of HuH-7 cells after inhibition of YAP was measured by MTT-assay 48, 72, and 96 h after transfection of siRNAs. **(D)** Cell proliferation of PLC/PRF/5 after overexpression of YAP^{wt} or constitutively active YAP^{S127A} was determined 24, 48, and 72 h after transient transfection of the respective vectors. **(E)** Migratory activity of HuH-7 after silencing of YAP was measured using a transwell-assay 24 h after siRNA transfection. **(F)** Invasive capacity of HuH-7 was defined using matrigel invasion chambers 24 h after transfection. **(G)** Migration of HuH-7 cell at the cell population level was measured using the 'scratch-assay' method. Cell-free areas were digitally documented immediately after scratching and 24 h after administration of the motogen hepatocyte growth factor (HGF). For (A) and (B) actin was used as loading control. For functional assays in (C), (D), (E), (F), and (G) untreated (cont.) and scramble siRNA- or empty vector-transfected cells served as controls and for statistical comparison. For (C), (E), and (F) similar functional data were generated using an independent cell line (Hep3B, data not shown). Untreated cells were used for calibration. Statistical test: Mann-Whitney U. Values represent means ± SD. **p<0.01, ***p<0.001.

Figure S3: Inhibition of Jag-1/Notch signaling reduces viability and migration of HCC cells

(A) Serial sections, stained by H&E and immunohistochemically for YAP, Jag-1, and cleaved Notch-1 derived from YAP^{S127A}-expressing mice liver. Two different areas are shown (100x magnification). Higher magnification (boxed area) reveals in more detail the expression of YAP, Jag-1, and cleaved Notch1 in the same group of altered small basophilic hepatocytes (arrows), located near the portal tract at the bottom (200x magnification). In contrast, unaltered hepatocytes (arrowheads) do not stain positive for YAP or Jag-1 and show very faint or absent nuclear

immunoreactivity for Notch1. **(B)** Cell viability of HuH-7 cells after inhibition of Jag-1 was measured by MTT-assay 48, 72, and 96 h after transfection of different gene-specific siRNAs (si#1 and si#2). Untreated (cont.) and scramble-transfected cells served as controls. **(C)** Migratory capacity of HuH-7 cells after inhibition of Jag-1 (si#1 and si#2) was measured using a transwell-assay 24 h after siRNA transfection. **(D)** Relative cell viability of HuH-7 cells after administration of a γ-secretase inhibitor (10 and 20 µM) for 24 h. **(E)** Tumor cell apoptosis of PLC/PRF/5 cells after overexpression YAP^{S127A} and simultaneous Jag-1 inhibition using RNAi. Statistical testing compares Jag-1 siRNA and YAP^{S127A}/Jag-1 siRNA. For (A), (B), (C), and (D) untreated cells were used for calibration and scramble-transfected cells served for statistical comparison. Values represent means ± SD. Statistical test: Mann-Whitney U. p*<0.05, **p<0.01, ***p<0.001. All functional results for (A), (B), and (C) were confirmed in an independent HCC cell line (Hep3B, data not shown).

Figure S4: Functional relevance of YAP/TEAD4 interaction on HCC cells

(A) Real-time PCR of Jag-1 mRNA after inhibition of 14 YAP-interacting transcription factors (FlexiPlate approach). Untreated (contr.) and scramble siRNA-transfected cells served as negative controls; Jag-1 and YAP inhibition were used as positive controls. Untreated cell samples were used for calibration. **(B)** Cell proliferation kinetics of PLC/PRF/5 cells after overexpression YAP^{wt} and concomitant inhibition of TEAD1 or TEAD4. Statistical test compares YAP/TEAD1 siRNA and YAP/TEAD4 siRNA. **(C)** Cell proliferation of PLC/PRF/5 cells after overexpression of TEAD4 and simultaneous expression of TEAD4 and dominant negative $YAP^{5SA\Delta C}$. **(D)** Cell apoptosis of PLC/PRF/5 cells after overexpression of TEAD4 and simultaneous expression of TEAD4 and dominant negative $YAP^{5SA\Delta C}$. For (C) and (D) the statistical test compares TEAD4 siRNA and TEAD4/ $YAP^{5SA\Delta C}$. **(E)** Scheme of the Jag-1 promoter indicating two putative TEAD binding sites in region 1 (circles). The TESS web tool was used for identification of potential TEAD binding sites. For (B), (C), and (D) untreated (control) and empty vector-transfected cells served as controls. Untreated cells were used for calibration. Statistical test: Mann-Whitney U. Values represent means ± SD. n.s. - not significant. **p<0.01; ***p<0.001.

Figure S5: YAP/TEAD4 complex formation is essential for induction of Jag-1/Notch signaling

(A) Protein analyses of Jag-1, YAP, flag-tagged YAP, TEAD4, and Hes-1 in PLC/PRF/5 cell protein extracts after vector-based overexpression of YAP. For analyzing the specific interaction between YAP and TEAD4, flag-tagged YAP was immunoprecipitated (IP) and TEAD4 was detected by immunoblotting (IB). Note that YAP^{S127A} was flag-tagged to prove expression of exogenous YAP and for precipitation. **(B)** Immunoblot analysis of Jag-1, YAP, flag-tagged YAP, TEAD4, and Hes-1 in PLC/PRF/5 cells after overexpression of $YAP^{S94A/S127A}$ lacking TEAD4 binding capacity. For detection of the potential interaction between $YAP^{S94A/S127A}$ and TEAD4, flag-tagged YAP was immunoprecipitated and TEAD4 was analyzed by immunoblotting. Note that $YAP^{S94A/S127A}$ was flag-tagged to prove expression of exogenous YAP and for precipitation. **(C)** Proliferation and apoptosis of PLC/PRF/5 cells after transfection of YAP^{S94A}, lacking TEAD4 binding capacity. **(D)** Protein analyses of TEAD4 after immunoprecipitation of endogenous murine YAP from cell lysates isolated from LAP-tTA/YAP^{S127A} liver samples with (control) and without doxycycline. BP: negative control for IP. Antibodies used for immunoprecipitation were neutralized prior to IP by preincubation with the respective immunogenic peptide (1:40 w/w). **(E)** Apoptosis of SNU-449 cells (with low-level expression of Mst1/2) after combined inhibition of Mst1 and Mst2. Statistical testing compares scramble siRNA and Mst1/2 siRNA. **(F)** Tumor cell apoptosis of HuH-1 cells (showing low-levels of Mst2 and Lats2) after expression of Lats2 or Mst2. Statistical testing compares empty vector and Mst2 or Lats2, respectively. For all immunoblots untreated (cont.), scramble siRNA-transfected, and empty vector-transfected cells were used as controls. Actin was used as a loading control. Values represent means ± SD.Statistical test: Mann-Whitney U. Values represent means ± SD. n.s. - not significant. ***$p<0.001$.

Figure S6: YAP regulates Jag-1 expression in cell lines derived from different cancer entities.

Transient transfection of two YAP-specific siRNAs (si#1 and si#2, final concentration: 20nM) reduces YAP expression in NSCLC (Calu-6), colon adenocarcinoma (SW-480), breast cancer (MCF-7), and PDAC cells (Panc-1). Respective untreated (cont.)

and scramble-transfected cells served as controls. Actin was used as a loading control.

Figure S7: Oncogenic activation of YAP-induced Jag-1/Notch signaling in tumor samples of PDAC and CRC patients

(A) Relative transcript levels of YAP in 11 PDAC tissue samples. Each tumor sample was compared to the respective mRNA derived from non-tumorous pancreatic tissue. The horizontal line indicates the threshold for a 2-fold induction of mRNA. For normalization, 18S-rRNA mRNA was determined. **(B)** Boxplot illustration and statistical analyses of YAP expression, as detected bby immunohistochemistry, in normal pancreatic tissues (NP) and PDAC tissue samples. Statistical test: Mann-Whitney U, ***p<0.01. Open circles indicate outliers. **(C)** Immunoblot analysis of YAP levels in normal pancreatic tissue (NP) and different PDAC cell lines (MIA PaCa, Capan-1, ASPC-1, T3M4, BxPC-3, Panc-1, and Su-8686). Actin served as a leading control. Based on these results, Panc-1 cells with high-level expression of YAP were selected for use in functional assays. **(D)** Migratory capacity of PDAC cells after inhibition of YAP was measured using a transwell-assay 24 h after siRNA transfection. Invasive capacity of Panc-1 cells was defined using matrigel invasion chambers 24 h after siRNA transfection. Migration of PDAC cells after inhibition of Jag-1 was measured using a transwell-assay 24 h after siRNA transfection. Viability of Panc-1 cells after inhibition of the Notch pathway using 10 or 20 µM of a γ-secretase inhibitor. DMSO was used as solvent control. **(E)** Boxplot illustration and statistical analyses of nuclear YAP, Jag-1, TEAD4, NICD, and Hes-1 in all SCs and CRCs analyzed after densitometric protein quantification. Actin and histone H3 were used for normalization of total protein (Jag-1, NICD, TEAD4, and Hes-1) and nuclear protein fractions (YAP), respectively. Statistical test: Mann-Whitney U; ***p<0.001. Open circles indicate outliers. **(F)** For analyzing the interaction between YAP and TEAD4 in primary tissue samples, YAP was immunoprecipitated (IP) and TEAD4 was detected by immunoblotting (IB). BP: negative control for IP. Antibodies used for immunoprecipitation were neutralized prior to IP by preincubation with the respective immunogenic peptide (1:40 w/w). For functional assays untreated (cont.) and scramble siRNA-transfected cells served as controls. Untreated cells were used for calibration and scramble siRNA- or empty vector-transfected cells were used for

statistical comparison. Statistical test: Mann-Whitney U. Values represent means ± SD. **p<0.01, ***p<0.001.