GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Hippo Cascade Controls Lineage Commitment of Liver Tumors in Mice and Humans

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Primary liver cancer consists mainly of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC). A subset of human HCCs expresses a ICC-like gene signature and is classified as ICC-like HCC. The Hippo pathway is a critical regulator of normal and malignant liver development. However, the precise function(s) of the Hippo cascade along liver carcinogenesis remain to be fully delineated. The role of the Hippo pathway in a murine mixed HCC/ICC model induced by activated forms of AKT and Ras oncogenes (AKT/Ras) was investigated. The authors demonstrated the inactivation of Hippo in AKT/Ras liver tumors leading to nuclear localization of Yap and TAZ. Coexpression of AKT/Ras with Lats2, which activates Hippo, or the dominant negative form of TEAD2 (dnTEAD2), which blocks Yap/TAZ activity, resulted in delayed hepatocarcinogenesis and elimination of ICC-like lesions in the liver. Mechanistically, Notch2 expression was found to be down-regulated by the Hippo pathway in liver tumors. Overexpression of Lats2 or dnTEAD2 in human HCC cell lines inhibited their growth and led to the decreased expression of ICC-like markers, as well as Notch2 expression. Altogether, this study supports the key role of the Hippo cascade in regulating the differentiation status of liver tumors. (*Am J Pathol* 2018, 188: 995–1006; <https://doi.org/10.1016/j.ajpath.2017.12.017>)

In the adult, primary liver cancer mainly consists of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC), both of which display an increasing incidence worldwide.^{1,2} A subtype of mixed HCC/ICC has also been identified.³ Diagnosis at advanced stages or the presence of chronic liver disease often precludes liver cancer patients from curative surgical resection or liver transplantation. HCC and ICC are well known chemoresistant tumors, making them among the most lethal cancers.^{4,5} Recent genomic analyses provide a global landscape of genetic variations occurring in HCC and ICC,^{6,7} thus supporting the development of personalized therapies against these deadly malignancies.

The Hippo pathway, an evolutionarily conserved signaling cascade, plays a critical role in organ size control. It was first

described in *Drosophila*, and later studies identified the mammalian homologs of the components of this pathway.⁸ Mammalian STE20-like protein kinase 1 (Mst1) and Mst2, mammalian homologs of Hippo (Hpo), phosphorylate and activate large tumor suppressor 1 (Lats1) and Lats2, with the support of the docking protein WW45 that potentiates the phosphorylation process.⁹ Lats1 and Lats2, together with the adaptor protein Mob, phosphorylate the main downstream

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effectors, Yes-associated protein (Yap) and transcriptional coactivator with PDZ-binding motif (TAZ; also known as WWTR1). Phosphorylated Yap and TAZ accumulate in the cytoplasm by binding to 14-3-3 and are subjected to ubiquitin-mediated proteolysis. Inactivation of Hippo signaling leads to translocation of Yap and TAZ into the nucleus, where they act as transcriptional coactivators and regulate the expression of target genes by stimulating the activity of several transcription factors, among which TEA domain (TEAD) family members play a key role, at least in the liver.^{10–14} Canonical Yap/TEAD target genes in the liver include *CTGF*,¹⁵ *CYR61*,¹⁶ *AXL*,¹⁷ and *Jag1*.¹⁸ Currently, the Hippo pathway is acknowledged as one of the fundamental checkpoints in monitoring organ size and restricting overgrowth. Because inactivation of the Hippo pathway could result in uncontrolled proliferation and diminished apoptosis, which is consistent with the major features of cancer cells, the role of the Hippo pathway in tumorigenesis has also drawn great attention. The deregulation of the Hippo pathway has been found to be associated with a wide variety of cancers, including leukemia, breast, lung, prostate, and ovarian tumors.^{19–25} Emerging evidence underlines the pivotal pathogenetic role of the Hippo cascade in liver cancer. Indeed, deficiency of both Mst1 and Mst2 was found to result in Yap activation, leading to HCC formation in mice.²⁶ Similarly, mixed HCC/ICC developed in mice depleted of *WW45*.²⁷ In 2006, Yap was reported as an oncogene in a mouse model of liver cancer.²⁸ Subsequently, as a main downstream effector of the Hippo pathway, Yap was found to play a pivotal function in liver carcinogenesis induced by elevated bile acid,²⁹ chemical carcinogens,³⁰ and Hepatitis B virus X protein.³¹ Frequent nuclear expression of Yap was found in HCC, and Yap could be considered as an independent prognostic marker for HCC patients.³² Despite this body of evidence, the exact role of the Hippo pathway and its regulatory mechanisms in HCC and ICC are far from being fully understood.

Concomitant activation of v-akt murine thymoma viral oncogene homolog (AKT)/mammalian target of Rapamycin (mTOR) and ras viral oncogene homolog (Ras)/mitogen-activated protein kinase (MAPK) cascades is a key oncogenic event in human liver cancer.³³ We established a mouse model of primary liver cancer by hydrodynamic injection of both activated forms of AKT and Ras oncogenes in our previous study, and found that concomitant activation of AKT/mTOR and Ras/MAPK cascades in this model leads to the formation of HCC/ICC within 3 to 4 weeks after injection.³⁴ In this study, the role of the Hippo pathway in AKT/Ras-driven hepatocarcinogenesis was investigated. The data demonstrate that the Hippo pathway regulates the differentiation status of liver tumors via Notch signaling.

Materials and Methods

Constructs and Reagents

The constructs used for mouse injection, including pT3-EF1 α , pT3-EF1 α -HA-myr-AKT (human), pT2CAGGS-NRasV12,

and pCMV/sleeping beauty transposase (SB), were described previously.^{33,35–37} Lats2 was kindly provided by Dr. Kungliang Guan (University of California, San Diego, CA), dominant negative TEAD2 (dnTEAD2) by Dr. Duoqia Pan (Johns Hopkins University, Baltimore, MD), and dominant negative RBPJ (RBPJ^{R218H}) (dnRBPJ) by Dr. Tasuku Honjo (Kyoto University, Kyoto, Japan). These plasmids were cloned into pT3-EF1 α via the Gateway PCR cloning strategy (Invitrogen, Carlsbad, CA). Plasmids were purified using the Endotoxin-Free Maxiprep Kit (Sigma-Aldrich, St. Louis, MO) before being injected into the mice.

Hydrodynamic Injection and Mouse Treatment

Female or male wild-type FVB/N mice were obtained from Charles River Laboratories (Wilmington, MA). All of the animal studies were conducted with 6- to 8-week-old mice. Hydrodynamic injection was performed as described previously.^{34,37} To generate AKT/Ras mice, 8 μ g of pT3-EF1 α -HA-myr-AKT, 8 μ g of NRasV12, and 40 μ g of pT3-EF1 α (empty vector control) along with 2.24 μ g of SB were delivered into FVB/N mouse liver. To activate the Hippo pathway, 40 μ g of Lats2 were injected in place of pT3-EF1 α . To block Yap/TAZ transcriptional activity, 40 μ g of dnTEAD2 were injected instead of pT3-EF1 α . Finally, to inhibit Notch2 signaling, 40 μ g of dnRBPJ were injected in place of pT3-EF1 α . Mice were monitored by daily abdominal palpation and were euthanized when they developed a high burden of liver tumors, ie, large abdominal masses. Mice were housed, fed, and monitored in accordance with protocols approved by the Committee for Animal Research at the University of California, San Francisco (San Francisco, CA).

Histology and Immunohistochemistry

Liver specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut at 5- μ m thickness. Preneoplastic and neoplastic liver lesions were assessed by two board-certified pathologists (S.R. and F.D.) in accordance with the criteria by Frith et al.³⁸ For immunohistochemistry, antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH 6.0) by placement in a microwave oven on high for 10 minutes, followed by a 20-minute cool down at room temperature. After a blocking step with the 5% goat serum and Avidin-Biotin blocking kit (Vector Laboratories, Burlingame, CA), the slides were incubated with primary antibodies overnight at 4°C. Slides were then subjected to 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity, and subsequently, the biotin-conjugated secondary antibody was applied at a 1:500 dilution for 30 minutes at room temperature. The primary antibodies are listed in Table 1. The immunoreactivity was visualized with the Vectastain Elite ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine as the chromogen. Slides were counterstained with hematoxylin. Proliferation index was determined by counting Ki67-positive

Table 1 Immunohistochemistry Antibody Information

Antibody	Company (location)	Catalog number	Dilution
CK19	Abcam (Cambridge, MA)	133496	1:150
SOX9	Abcam	185230	1:2000
HNF-4 α	Abcam	181604	1:2000
HA-Tag	Cell Signaling Technology (Danvers, MA)	3724	1:100
Phospho-AKT	Cell Signaling Technology	4060	1:100
Phospho-ERK	Cell Signaling Technology	4370	1:100
Ki67	Thermo Fisher Scientific (Rockford, IL)	MA5-14520	1:150
Yap	Cell Signaling Technology	14074	1:50

cells on at least 3000 tumor cells per sample for three samples in each group.

Protein Extraction and Western Blotting

Frozen mouse liver specimens and cultured cell lines were lysed in Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL) containing the Complete Protease Inhibitor Cocktail. Protein concentrations were determined with the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as standard. Aliquots of 40 μ g of lysate were denatured by boiling in Tris-Glycine SDS Sample Buffer (Invitrogen), separated by SDS-PAGE, and then transferred onto nitrocellulose membranes (Invitrogen, Grand Island, NY). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 hour and probed with specific antibodies listed in Table 2. Each primary antibody was followed by incubation with horseradish peroxidase-secondary antibody diluted 1:10,000 for 1 hour and then revealed with the Super Signal West Pico Chemiluminescent Substrate (Pierce Chemical Co., New York, NY). GAPDH, β -actin, and/or Histone H3 were assessed to ensure equal loading.

Quantitative Real-Time RT-PCR

Total mRNA was extracted from liver tissues and cells using the Quick RNA Miniprep Kit (Zymo Research, Irvine, CA). mRNA expression was determined by quantitative real-time RT-PCR (RT-qPCR) using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA) in a Mx4000 system (Stratagene, La Jolla, CA). Expression of each specific gene's mRNA was normalized with the 18S rRNA. Thermal cycling conditions included an initial hold period at 95°C for 10 minutes, which was followed by a three-step PCR program of 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 30 seconds for a total of 35 cycles. The sequences of the primers used in this study are listed in Table 3.

In Vitro Cell Culture and Colony Forming Assay

The SNU-475 and SK-HEP-1 human HCC cell lines were used for the *in vitro* studies. Cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and 100 U/mL penicillin, 100 g/mL streptomycin (Gibco). When cells reached 50% to 60% confluency in 60 $\times$ 15-mm culture dishes, EGFP, Lats2, or dnTEAD2 lentivirus was added to the culture medium. Forty-eight to 72 hours later, cells were trypsinized and cultured in 100 $\times$ 20-mm culture dishes in culture medium containing puromycin at the concentration of 1.5 μ g/mL for SNU-475 and 2 μ g/mL for SK-HEP-1. After 3 days of selection, cells were used for colony forming assay and Western blotting. For colony forming assay, SNU-475 and SK-HEP-1 cells transfected with corresponding lentivirus were plated in 6-well culture plates at a density of 0.5 $\times$ 10³ and 4 $\times$ 10³ cells per well, respectively, in triplicate. Two to 3 weeks later, colonies were stained with crystal violet and then counted for quantification.

Bioinformatics Analysis

The mRNA profiles of HCC, ICC, and ICC-like HCC (CLHCC) samples were downloaded from the GEO database (www.ncbi.nlm.nih.gov/geo; accession number GSE15765), and a list of up-/down-regulated genes was collected from the Hippo signature.^{39,40} Enrichment analysis of up-/down-regulated genes on each cancer sample was conducted using the ssGSEA method provided in the R package GSVA (R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org>).^{41,42} A higher ssGSEA score indicates a better enrichment of the gene set. An analysis of variance test was performed to estimate the difference of ssGSEA scores across multiple groups.

Table 2 Western Blotting Antibody Information

Antibody	Company (location)	Catalog number	Dilution
Yap-TAZ	Cell Signaling Technology	8418	1:1000
Histone H3	Cell Signaling Technology	4499	1:2000
β -Tubulin	Abcam	6046	1:1000
Mst1	Cell Signaling Technology	3682	1:1000
Mst2	Cell Signaling Technology	3952	1:1000
Sav1	Cell Signaling Technology	13301	1:1000
Lats1	Cell Signaling Technology	3477	1:1000
Mob1	Cell Signaling Technology	13730	1:1000
p-Mob1	Cell Signaling Technology	8699	1:1000
GAPDH	EMD Millipore (Hayward, CA)	MAB374	1:10000
β -Actin	Sigma-Aldrich	A5441	1:5000
Lats2	Bethyl Laboratories (Montgomery, TX)	A300-479A-T	1:1000
Jag1	Abcam	Ab109536	1:1000
Notch2	Cell Signaling Technology	5732	1:2000
CK19	Abcam	52625	1:20000
SOX9	Abcam	185230	1:1000
ERK	Cell Signaling Technology	9102	1:1000

Table 3 Quantitative Real-Time RT-PCR Primer Sequence Information

Gene	Forward sequence	Reverse sequence
<i>SOX9</i>	5'-AGTACCCGCATCTGCACAAC-3'	5'-ACGAAGGGTCTCTTCTCGCT-3'
<i>EPCAM</i>	5'-CTGGCGTCTAAATGCTTGGC-3'	5'-CCTTGTCGGTTCTTCGGACTC-3'
<i>Notch1</i>	5'-CCCTTGCTCTGCCTAACGC-3'	5'-GGAGTCCTGGCATCGTTGG-3'
<i>Notch2</i>	5'-ATGTGGACGAGTGTCTGTTGC-3'	5'-GGAAGCATAGGCACAGTCATC-3'
<i>Hes1</i>	5'-AAAGCCTATCATGGAGAAGAGGCG-3'	5'-GGAATGCCGGGAGCTATCTTTCTT-3'
<i>Hey1</i>	5'-GCGCGGACGAGAATGGAAA-3'	5'-TCAGGTGATCCACAGTCATCTG-3'
<i>HeyL</i>	5'-CAGCCCTTCGCAGATGCAA-3'	5'-CCAATCGTCGCAATTCAGAAAAG-3'
<i>18S rRNA</i>	5'-CGGCTACCACATCCAAGGAA-3'	5'-GCTGGAATTACCGCGGCT-3'
<i>CTGF</i>	5'-GGGCCTCTTCTGCGATTTC-3'	5'-ATCCAGGCAAGTGCATTGGTA-3'
<i>Cyr61</i>	5'-CTGCGCTAAACAACCTCAACGA-3'	5'-GCAGATCCCTTTCAGAGCGG-3'

Statistical Analysis

Data were analyzed with Prism software version 6 (GraphPad Software, San Diego, CA). Data are presented as means ± SEM. Comparisons between two groups were performed with two-tailed unpaired *t*-test. Survival curves were constructed using the Kaplan-Meier method and compared by log-rank test. *P* < 0.05 was considered statistically significant.

Results

Hippo Cascade Is Inactivated in AKT/Ras-Driven Mouse Liver Tumors

First, it was assessed whether the Hippo pathway is dysregulated in AKT/Ras liver tumors. Compared with wild-type normal mouse livers, the expression of total TAZ protein was higher in AKT/Ras tumors, whereas no increase in Yap

expression was observed (Figure 1A). Because activated Yap and TAZ translocate from the cytoplasm into nucleus to exert their oncogenic activity,^{10–14} the expression of nuclear Yap and TAZ was then determined by Western blotting on nuclear protein extracts. Noticeably, the levels of nuclear Yap and TAZ were elevated in AKT/Ras liver tumors (Figure 1A). The results were confirmed by immunohistochemistry (IHC), showing nuclear Yap accumulation in AKT/Ras liver tumor lesions, but not in normal livers (Figure 1B). Next, the protein levels of the key components of the Hippo kinase cascade, including Mst1/2, Sav1, Lats1, Lats2, Mob1, and p-Mob1 were determined. Western blot analysis showed that the levels of Last1 and Lats2 were significantly decreased in AKT/Ras tumors compared with normal liver tissues (Figure 1C). These findings indicate that the Hippo pathway is inactivated in AKT/Ras liver tumors, leading to Yap/TAZ nuclear localization, which might be attributed to reduced Lats1 and Lats2 expression.

Figure 1 Hippo cascade is inactivated in AKT/Ras-driven mouse liver tumors. **A:** Western blotting was performed to detect the expression of total and nuclear Yap/TAZ in WT and AKT/Ras liver tissues. Loading controls include: β-actin for total cell lysate; Histone H3 for nuclear fractions; and β-tubulin for cytoplasm fractions. **B:** Representative immunohistochemistry of Yap in WT and AKT/Ras mouse livers. **Insets** show Yap nuclear accumulation in AKT/Ras tumor cells, but not in WT livers. **C:** Western blotting was performed to detect the expression of key kinase components in Hippo cascade in total cell lysate in WT and AKT/Ras liver tissues. GAPDH served as loading control. Scale bars = 100 μm. Original magnification: ×200 (**B**, main images); ×940 (**B**, insets). WT, wild type.

Figure 2 Activation of the Hippo pathway or blockade of Yap/TAZ transcriptional activity delays AKT/Ras-induced liver tumor development in mice. **A:** Study design. **B:** Survival curve of AKT/Ras/pT3, AKT/Ras/Lats2, and AKT/Ras/dnTEAD2 mice. **C:** Gross images and hematoxylin and eosin staining of AKT/Ras/pT3, AKT/Ras/Lats2, and AKT/Ras/dnTEAD2 mouse livers at 8 weeks after hydrodynamic injection. $n = 9$ AKT/Ras/pT3 and AKT/Ras/Lats2 (**B**); $n = 7$ AKT/Ras/dnTEAD2 (**B**). Survival curves were constructed using the Kaplan-Meier method and compared by log-rank test. $***P < 0.001$ versus AKT/Ras/pT3 control mice. Scale bars = 200 μ m. w.p.i., weeks after injection.

Activation of the Hippo Pathway or Blockade of Yap/TAZ Transcriptional Activity Delays AKT/Ras-Induced Liver Tumor Development in Mice

To investigate the role of Hippo pathway in AKT/Ras-dependent hepatocarcinogenesis, the Hippo cascade was activated via coexpressing Lats2⁴³ with AKT and Ras oncogenes in mice (AKT/Ras/Lats2). As an alternative approach, AKT/Ras were coexpressed with the dominant negative form of TEAD2 (dnTEAD2), which blocks Yap/TAZ transcriptional activity⁴⁴ (AKT/Ras/dnTEAD2). AKT/Ras plasmids were coexpressed with pT3-EF1 α as empty vector control (AKT/Ras/pT3) (Figure 2A). Noticeably, overexpression of either Lats2 or dnTEAD2 substantially delayed liver tumor development induced by AKT/Ras expression in mice and prolonged survival (Figure 2B). Although AKT/Ras/pT3 control mice developed lethal tumor burden at around 8 weeks after injection, only small tumor nodules were observed on the liver tissues of AKT/Ras/Lats2 and AKT/Ras/dnTEAD2 mice at this time point (Figure 2C). All AKT/Ras/Lats2 and AKT/Ras/dnTEAD2 mice eventually

developed high tumor burden and were all euthanized by approximately 16 weeks after injection (Figure 2B). IHC of HA tag for ectopically expressed AKT, p-AKT, and p-ERK indicated equal AKT transfection as well as activation of AKT/mTOR and Ras/MAPK pathways in AKT/Ras/pT3, AKT/Ras/Lats2, and AKT/Ras/dnTEAD2 liver tissues (Supplemental Figure S1). Ki67 IHC showed that tumor cell proliferation was significantly suppressed when the Hippo pathway was forcedly induced (Supplemental Figure S1). Altogether, the present data indicate that activation of the Hippo pathway or impairment of Yap/TAZ transcriptional activity delays AKT/Ras-induced liver tumor development in mice and represses tumor cell proliferation, without affecting AKT/mTOR and Ras/MAPK cascades.

Activation of the Hippo Pathway or Yap/TAZ Transcriptional Activity Blockade Impedes the Formation of ICC-Like Lesions in AKT/Ras Mice

Histological analysis of AKT/Ras/pT3, AKT/Ras/Lats2, and AKT/Ras/dnTEAD2 liver tissues showed the prevalence of

Figure 3 Activation of the Hippo pathway or blockade of Yap/TAZ transcriptional activity hampers the formation of ICC lesions in AKT/Ras mice. **A:** Representative immunohistochemistry of bile duct markers CK19, SOX9, and hepatocyte marker HNF-4α in late stage of AKT/Ras/pT3, AKT/Ras/Lats2, and AKT/Ras/dnTEAD2 mouse liver tumor development. **B:** CK19 immunoreactivity was quantified by using the ImageJ software version 1.51 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) and demonstrated as the percentage of positive staining area of the whole area. **C:** Western blotting was performed to detect the expression of Yap/TAZ, CK19, SOX9, and t-ERK in indicated groups. GAPDH was used as loading control. **D:** Quantitative real-time RT-PCR was performed to determine the gene expression levels of *CTGF* and *Cyr61* (canonical Yap targets) as well as *SOX9* and *EPCAM* bile duct markers. Data are expressed as means ± SEM (**B**) or as relative expression (−ΔΔct) ± SEM (**D**). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars: 200 μm (**A**, top two rows); 100 μm (**A**, bottom row). Original magnification: ×100 (**A**, top two rows); ×200 (**A**, bottom row). t, total; WT, wild type.

adenomas over carcinomas in the liver of AKT/Ras mice upon modulation of the Hippo pathway (Figure 2C and Supplemental Figure S2). Furthermore, similar to what we reported previously,⁴⁵ AKT/Ras/pT3 control liver tumors consisted of poorly differentiated HCC and ICC. By contrast, AKT/Ras/Lats2 and AKT/Ras/dnTEAD2 tumors displayed a hepatocellular phenotype, mostly consisting of adenoma and well-differentiated HCC, whereas ICC-like lesions could not be observed (Figure 2C and Supplemental Figure S2). Western

blot analysis and IHC staining data showed that when compared with AKT/Ras/pT3 control mice, the expression level of biliary epithelial cell (BEC) marker Sry-box containing gene 9 (SOX9) was significantly decreased in late-stage AKT/Ras/Lats2 and AKT/Ras/dnTEAD2 liver tumors (Figure 3, A and B), whereas more prominently, cytokeratin 19 (CK19) expression was almost abolished (Figure 3, A–C, and Supplemental Figure S2). By contrast, the expression of the hepatocyte-specific marker HNF-4α was absent in ICC-like

Figure 4 Hippo pathway negatively regulates Notch2 expression in AKT/Ras-induced liver tumors. **A:** Western blotting was performed to detect the expression of Notch2 and Jag1 in AKT/Ras/pT3, AKT/Ras/Lats2, and AKT/Ras/dnTEAD2 liver tumor tissues. GAPDH was used as loading control. **B:** The mRNA expression of *Notch2*, *Hes1*, *Hey1*, and *HeyL* in normal liver, AKT/Ras/pT3-, AKT/Ras/Lats2-, and AKT/Ras/dnTEAD2-induced tumor tissues was tested by quantitative real-time RT-PCR. Data are expressed as relative expression ($-\Delta\Delta\text{ct}$) $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

lesions from AKT/Ras/pT3 mice, whereas HNF-4 α expression could be detected throughout the tumor lesions in AKT/Ras/Lats2 and AKT/Ras/dnTEAD2 mice (Figure 3A). Consistently, mRNA expression levels of canonical Yap target genes, including *CTGF* and *CYR61* as well as BEC markers *SOX9* and *EPCAM*, were significantly lower in AKT/Ras/Lats2 and AKT/Ras/dnTEAD2 tumors than in AKT/Ras/pT3 tumors, as assessed by RT-qPCR analysis (Figure 3D). These data indicate that activation of the Hippo pathway or blockade of the Yap/TAZ transcriptional activity inhibits the formation of ICC-like lesions in AKT/Ras mice.

The Canonical Notch Pathway Is Regulated by the Hippo Cascade in AKT/Ras-Driven Liver Carcinogenesis

In a previous study, we showed that blocking Notch2 with an anti-Notch2-specific antibody delays AKT/Ras-driven

liver tumor development and eliminates ICC-like lesions.⁴⁶ Of note, the histologic phenotype of AKT/Ras lesions subjected to treatment with the Notch2 antibody is highly similar to what is described here as a consequence of Hippo induction (Figures 2 and 3). Subsequently, the levels of some Notch pathway members were evaluated in the three cohorts of mice. Among them, Notch2 and its ligand Jag1 have been recently identified as a direct target of Yap.^{47,48} Western blot analysis revealed that the levels of Notch2 and Jag1 proteins were significantly decreased in AKT/Ras/Lats2 and AKT/Ras/dnTEAD2 tumors when compared with AKT/Ras/pT3 control mice (Figure 4A). In addition, mRNA expression of *Notch2* as well as downstream targets of the canonical Notch pathway, including *Hes1*, *Hey1*, and *HeyL*, was reduced in AKT/Ras/Lats2 and AKT/Ras/dnTEAD2 liver tumors (Figure 4B).

In our previous study using the anti-Notch2-specific antibody,⁴⁶ it could not be determined whether Notch2 functions in a cell autonomous or nonautonomous manner because the antibody blocks Notch2 indistinctly in all cell types of the liver. Here, it was hypothesized that the canonical Notch signaling functions downstream of Hippo in a cell autonomous fashion to regulate AKT/Ras-driven liver carcinogenesis *in vivo*. To validate this hypothesis, dnRBPJ, the dominant negative form of RBP-J, was coexpressed with AKT/Ras oncogenes (AKT/Ras/dnRBPJ). dnRBPJ has been shown to effectively block the canonical Notch cascade both *in vitro* and *in vivo*.^{49–51} Again, AKT/Ras/pT3 mice were used as control (Figure 5, A and B). Histologic analysis showed that hepatocellular adenomas were prevalent over carcinomas in AKT/Ras/dnRBPJ livers (Figure 5, B and C). Importantly, ICC-like lesions could not be observed upon dnRBPJ coexpression (Figure 5, B and C), which was confirmed by CK19 IHC (Figure 5, B and D). The hepatocellular adenoma lesions were characterized by low proliferative activity, as underlined by Ki-67 staining (Figure 5B). As concerns mouse survival, both mouse cohorts were sacrificed at the same time (data not shown), suggesting that inhibition of the Notch pathway by overexpression of dnRBPJ does not significantly prolong the survival length of AKT/Ras/dnRBPJ mice over AKT/Ras/pT3 mice. Nonetheless, it should be underlined that AKT/Ras/dnRBPJ mice were euthanized when they exhibited large abdominal masses based on our Institutional Animal Care and Use Committee protocol. Although AKT/Ras/dnRBPJ tumors were less malignant, their volume was high because these lesions were composed of large tumor cells due to extensive cytoplasmic accumulation of lipids (Figure 5B). These cells differ from the highly malignant tumor cells found in AKT/Ras livers, which are instead characterized by low cytoplasmic/nuclear ratio and are very small in size overall.⁴⁵ This is the main reason why the two tumor cohorts were sacrificed simultaneously and, apparently, did not show differences in survival length.

It was also analyzed whether the canonical Notch pathway affects Yap activity in AKT/Ras/dnRBPJ mice. By

Figure 5 Inhibiting Notch2 signaling by dnRBPJ coinjection eliminates ICC lesions in AKT/Ras-induced liver tumors. **A:** Study design. **B:** Gross images, H&E staining, and immunohistochemistry of CK19, Yap, and Ki67 in AKT/Ras/pT3 and AKT/Ras/dnRBPJ mouse livers. **Insets** show that nuclear Yap accumulation was preserved in AKT/Ras/dnRBPJ mice liver tumors. **C:** Histological quantification of tumor lesions in AKT/Ras/pT3 and AKT/Ras/dnRBPJ mouse liver tissues. **D:** CK19 immunolabeling was quantified by using the ImageJ software version 1.51 and demonstrated as the percentage of positive staining area of the whole area. Data are expressed as means $\pm$ SEM. *** $P < 0.001$. Scale bars: 200 μ m (**B**, H&E and CK19 IHC); 100 μ m (**B**, Yap IHC and Ki67 IHC). Original magnification: $\times 100$ (**B**, H&E and CK19 IHC); $\times 200$ (**B**, Yap IHC and Ki67 IHC); $\times 940$ (**B**, insets). H&E, hematoxylin and eosin; WT, wild type.

using IHC, nuclear Yap accumulation was found to be preserved in AKT/Ras/dnRBPJ liver tumors despite inactivation of the Notch signaling (Figure 5C), thus indicating that Notch functions downstream of Yap in AKT/Ras mice.

Altogether, our study indicates that the Hippo cascade regulates the development of ICC-like lesions in AKT/Ras mice, at least in part, via modulating the canonical Notch signaling.

Figure 6 Hippo pathway suppresses the expression of bile duct markers and inhibits clonogenic capacity of human HCC cell lines. SNU-475 and SK-HEP-1 human HCC cell lines were transfected to overexpress EGFP (control), Lats2, or dnTEAD2 via lentivirus. **A:** Representative Western blot analysis in SNU-475 and SK-HEP-1 cells. GAPDH was used as loading control. **B:** Colony forming assay was performed in triplicate in SNU-475 and SK-HEP-1 cells. Representative images are shown. Data are expressed as means $\pm$ SEM. *** $P < 0.001$.

Hippo Pathway Regulates BEC Marker Expression and Proliferation in Human HCC Cell Lines

Next, the role of the Hippo pathway was investigated in regulating human liver cancer potential, as well as differentiation, using human HCC cell lines. SNU-475 and SK-HEP-1 HCC cells were chosen, because both cell lines demonstrated high expression of SOX9 and CK19 biliary markers. EGFP, Lats2, or dnTEAD2 was overexpressed in SNU-475 and SK-Hep1 cells. As expected, Yap and TAZ protein expression was effectively inhibited by Lats2 overexpression, but not affected by dnTEAD2. Importantly, overexpression of Lats2 or dnTEAD2 led to decreased expression of BEC markers CK19 and SOX9 in SNU-475 and SK-Hep1 cells (Figure 6A). Consistently, Notch2 expression was also decreased when Lats2 or dnTEAD2 was overexpressed (Figure 6A). Activation of the Hippo pathway or blocking Yap/TAZ transcriptional activity also tremendously suppressed the clonogenic capacity of both cell lines, and only very few clones could be formed in cells transfected with Lats2 or dnTEAD2 (Figure 6B). Furthermore, the modulation of the Hippo pathway also resulted in reduced proliferation and increased apoptosis in human HCC cells (Supplemental Figure S3). Altogether, these data suggest that the Hippo pathway suppresses the expression of BEC markers and inhibits human HCC cell growth.

Discussion

Mounting evidence indicates that the Hippo pathway is a crucial player in tumor biology.⁵² Hippo pathway inactivation has been frequently observed in human liver cancer.⁵³

In particular, nuclear accumulation of Yap, which is used as a biomarker for Hippo inactivation, was found in the majority of human HCC, mixed HCC/ICC, and almost all ICC samples.^{31,32,53,54} However, the precise mechanisms by which Hippo is deregulated leading to activation of Yap during liver tumor development remain unclear. In the AKT/Ras murine model, we demonstrate the increased nuclear localization of Yap and TAZ in liver tumor cells. The protein expression of genes involved in the Hippo cascade was assayed; levels of Lats1 and Lats2, but not of Mst1/2, were significantly decreased in AKT/Ras liver tumor samples. The results are consistent with a previous study showing that Lats inactivation, but not loss of Mst1/2, is associated with Yap induction in human HCC.³¹ These results suggest that down-regulation of Lats proteins may be the major mechanism leading to activated Yap/TAZ in human and mouse liver tumors. Because somatic mutations in genes of the Hippo pathway are uncommon,⁵⁵ Lats dysregulation might be attributed to hypermethylation via epigenetic regulation,^{56,57} crosstalk with other oncogenic signaling pathways such as the IGF1-AKT cascade,⁵⁸ and possibly, modulation by miRNAs.⁵⁹ Clearly, additional studies are required to define the molecular mechanisms responsible for the inactivation of the Hippo cascade in human liver tumors.

In the current study, coexpression of dnTEAD2 along with AKT/Ras resulted in similar impairment of liver cancer development as coexpressing Lats2. This observation suggests that the tumor-suppressive activity of the Hippo cascade is achieved through YAP/TAZ-TEAD-dependent transcriptional regulation. It has been shown that Yap functions as an oncogene and an early driving force in liver tumorigenesis.⁶⁰ Yap and its paralog TAZ can regulate

proliferation, anchorage-independent growth, migration, invasion, as well as cancer stem cell–like properties in liver tumor cells.^{17,61–65} Yap overexpression led to dedifferentiation of hepatocytes into hepatic progenitor-like cells.⁴⁷ Our results, therefore, add additional evidence strengthening the role of Yap/TAZ as major signaling molecules downstream of the Hippo kinases in liver tumor development. However, multiple questions remain unanswered. In particular, it is not clear whether Yap and TAZ share redundant or distinct roles in AKT/Yap-driven liver tumor development in humans and mice. Also, it remains to be clarified whether Yap and TAZ regulate the same set of genes. In addition, there are multiple TEAD genes expressed in the liver, and it would be of pivotal importance to identify the specific members of the TEAD family that interact with Yap and TAZ.

A major finding of the current study is that the Hippo cascade regulates liver tumor cell differentiation. Specifically, it was found that either *Lats2* or *dnTEAD2* overexpression led to the development of well-differentiated HCC or hepatic adenoma and inhibition of ICC-like lesions in AKT/Ras mice. In human HCC cell lines, activation of the Hippo pathway suppressed the expression of BEC markers. In a recent study, Sohn et al⁴⁰ identified the gene expression signature associated with Silence of Hippo (SOH) in the liver. It was investigated whether the SOH signature was enriched in human HCC, CLHCC, and/or ICC samples. No enrichment of genes that are up-regulated in SOH signature in HCC, CLHCC, or ICC samples, was found. However, as concerns the genes that are down-regulated in SOH, ie, those that are induced by the Hippo pathway, they are statistically enriched in HCC compared with CLHCC or ICC (Supplemental Figure S4). The results in humans are consistent with the current study, showing that either activation of Hippo or blocking the transcriptional activity of Yap/TAZ promotes the development of hepatocellular lesions while inhibiting the formation of ICC-like lesions in mice. Thus, we hypothesize that the Hippo pathway and Yap/TAZ might be major determinants for the development of CLHCC and hepatocyte-derived ICC in human carcinogenesis. Obviously, additional studies are required to address this question.

What are the major signaling molecules downstream of Hippo in the liver? This study and others suggest that Notch2 is a key target of Hippo in liver tumor development.^{66,67} Specifically, we found that Notch2 expression is down-regulated in AKT/Ras liver tumors when coexpressed with *Lats2* or *dnTEAD2*. Similar results were observed in human HCC cell lines. Importantly, blocking canonical Notch signaling (Figure 5) or treatment with the anti-Notch2 antibody⁴⁶ in AKT/Ras mice led to the development of well-differentiated HCC and loss of ICC-like lesions, recapitulating the phenotypes of reactivation of Hippo. Although Hippo cascade may be difficult to be targeted, Notch signaling has been demonstrated to be druggable. Specifically, anti-Notch–specific antibodies as well as small

molecules targeting Notch, such as γ -secretases inhibitors and staple peptides, have been tested in various human cancers.⁶⁸ It is possible that human HCC and ICCs with loss of Hippo cascade may benefit from anti-Notch–based therapy, and nuclear Yap/TAZ expression might represent a useful biomarker to stratify liver cancer patients for anti-Notch therapies.

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Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.12.017>.

References

1. Bridgewater J, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ: Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *J Hepatol* 2014, 60:1268–1289
2. Ghouri YA, Mian I, Rowe JH: Review of hepatocellular carcinoma: epidemiology, etiology, and carcinogenesis. *J Carcinog* 2017, 16:1
3. Moeini A, Sia D, Zhang Z, Camprecios G, Stueck A, Dong H, Montal R, Torrens L, Martinez-Quetglas I, Fiel MI, Hao K, Villanueva A, Thung SN, Schwartz ME, Llovet JM: Mixed hepatocellular cholangiocarcinoma tumors: cholangiolocellular carcinoma is a distinct molecular entity. *J Hepatol* 2017, 66:952–961
4. Bodzin AS, Busuttil RW: Hepatocellular carcinoma: advances in diagnosis, management, and long term outcome. *World J Hepatol* 2015, 7:1157–1167
5. Razumilava N, Gores GJ: Cholangiocarcinoma. *Lancet* 2014, 383: 2168–2179
6. Farshidfar F, Zheng S, Gingras MC, Newton Y, Shih J, Robertson AG, et al; Cancer Genome Atlas Research Network: Integrative genomic analysis of cholangiocarcinoma identifies distinct IDH-mutant molecular profiles. *Cell Rep* 2017, 19:2878–2880
7. Cancer Genome Atlas Research Network: Comprehensive and integrative genomic characterization of hepatocellular carcinoma. *Cell* 2017, 169:1327–1341.e1323
8. Pan D: Hippo signaling in organ size control. *Genes Dev* 2007, 21: 886–897
9. Chan EH, Nousiainen M, Chalamalasetty RB, Schafer A, Nigg EA, Sillje HH: The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene* 2005, 24:2076–2086
10. Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders RA, Maitra A, Pan D: Elucidation of a universal size-control mechanism in *Drosophila* and mammals. *Cell* 2007, 130: 1120–1133
11. Edgar BA: From cell structure to transcription: hippo forges a new path. *Cell* 2006, 124:267–273
12. Yu FX, Guan KL: The Hippo pathway: regulators and regulations. *Genes Dev* 2013, 27:355–371
13. Piccolo S, Cordenonsi M, Dupont S: Molecular pathways: YAP and TAZ take center stage in organ growth and tumorigenesis. *Clin Cancer Res* 2013, 19:4925–4930

14. Hong W, Guan KL: The YAP and TAZ transcription co-activators: key downstream effectors of the mammalian Hippo pathway. *Semin Cell Dev Biol* 2012, 23:785–793
15. Zhao B, Ye X, Yu J, Li L, Li W, Li S, Lin JD, Wang CY, Chinnaiyan AM, Lai ZC, Guan KL: TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* 2008, 22:1962–1971
16. Zhang J, Smolen GA, Haber DA: Negative regulation of YAP by LATS1 underscores evolutionary conservation of the Drosophila Hippo pathway. *Cancer Res* 2008, 68:2789–2794
17. Xu MZ, Chan SW, Liu AM, Wong KF, Fan ST, Chen J, Poon RT, Zender L, Lowe SW, Hong W, Luk JM: AXL receptor kinase is a mediator of YAP-dependent oncogenic functions in hepatocellular carcinoma. *Oncogene* 2011, 30:1229–1240
18. Lanigan DT, Hey JH, West RA: Major vascular complications of orthognathic surgery: hemorrhage associated with Le Fort I osteotomies. *J Oral Maxillofac Surg* 1990, 48:561–573
19. Li W, Wang L, Katoh H, Liu R, Zheng P, Liu Y: Identification of a tumor suppressor relay between the FOXP3 and the Hippo pathways in breast and prostate cancers. *Cancer Res* 2011, 71:2162–2171
20. Tapon N, Harvey KF, Bell DW, Wahrer DC, Schiripo TA, Haber D, Hariharan IK: Salvador promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* 2002, 110:467–478
21. Zhou X, Wang S, Wang Z, Feng X, Liu P, Lv XB, Li F, Yu FX, Sun Y, Yuan H, Zhu H, Xiong Y, Lei QY, Guan KL: Estrogen regulates Hippo signaling via GPER in breast cancer. *J Clin Invest* 2015, 125:2123–2135
22. Wang X, Su L, Ou Q: Yes-associated protein promotes tumour development in luminal epithelial derived breast cancer. *Eur J Cancer* 2012, 48:1227–1234
23. Wang Y, Dong Q, Zhang Q, Li Z, Wang E, Qiu X: Overexpression of yes-associated protein contributes to progression and poor prognosis of non-small-cell lung cancer. *Cancer Sci* 2010, 101:1279–1285
24. Zhang X, George J, Deb S, Degoutin JL, Takano EA, Fox SB, Bowtell DD, Harvey KF: The Hippo pathway transcriptional co-activator, YAP, is an ovarian cancer oncogene. *Oncogene* 2011, 30:2810–2822
25. Jimenez-Velasco A, Roman-Gomez J, Agirre X, Barrios M, Navarro G, Vazquez I, Prosper F, Torres A, Heiniger A: Down-regulation of the large tumor suppressor 2 (LATS2/KPM) gene is associated with poor prognosis in acute lymphoblastic leukemia. *Leukemia* 2005, 19:2347–2350
26. Zhou D, Conrad C, Xia F, Park JS, Payer B, Yin Y, Lauwers GY, Thasler W, Lee JT, Avruch J, Bardeesy N: Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene. *Cancer Cell* 2009, 16:425–438
27. Lee KP, Lee JH, Kim TS, Kim TH, Park HD, Byun JS, Kim MC, Jeong WI, Calvisi DF, Kim JM, Lim DS: The Hippo-Salvador pathway restrains hepatic oval cell proliferation, liver size, and liver tumorigenesis. *Proc Natl Acad Sci U S A* 2010, 107:8248–8253
28. Zender L, Spector MS, Xue W, Flemming P, Cordon-Cardo C, Silke J, Fan ST, Luk JM, Wigler M, Hannon GJ, Mu D, Lucito R, Powers S, Lowe SW: Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* 2006, 125:1253–1267
29. Anakk S, Bhosale M, Schmidt VA, Johnson RL, Finegold MJ, Moore DD: Bile acids activate YAP to promote liver carcinogenesis. *Cell Rep* 2013, 5:1060–1069
30. Kowalik MA, Saliba C, Pibiri M, Perra A, Ledda-Columbano GM, Sarotto I, Ghiso E, Giordano S, Columbano A: Yes-associated protein regulation of adaptive liver enlargement and hepatocellular carcinoma development in mice. *Hepatology* 2011, 53:2086–2096
31. Zhang T, Zhang J, You X, Liu Q, Du Y, Gao Y, Shan C, Kong G, Wang Y, Yang X, Ye L, Zhang X: Hepatitis B virus X protein modulates oncogene Yes-associated protein by CREB to promote growth of hepatoma cells. *Hepatology* 2012, 56:2051–2059
32. Xu MZ, Yao TJ, Lee NP, Ng IO, Chan YT, Zender L, Lowe SW, Poon RT, Luk JM: Yes-associated protein is an independent prognostic marker in hepatocellular carcinoma. *Cancer* 2009, 115:4576–4585
33. Avruch J, Zhou D, Fitamant J, Bardeesy N, Mou F, Barrufet LR: Protein kinases of the Hippo pathway: regulation and substrates. *Semin Cell Dev Biol* 2012, 23:770–784
34. Chen X, Calvisi DF: Hydrodynamic transfection for generation of novel mouse models for liver cancer research. *Am J Pathol* 2014, 184:912–923
35. Tao J, Calvisi DF, Ranganathan S, Cigliano A, Zhou L, Singh S, Jiang L, Fan B, Terracciano L, Armeanu-Ebinger S, Ribback S, Dombrowski F, Evert M, Chen X, Monga SPS: Activation of beta-catenin and Yap1 in human hepatoblastoma and induction of hepatocarcinogenesis in mice. *Gastroenterology* 2014, 147:690–701
36. Marti P, Stein C, Blumer T, Abraham Y, Dill MT, Pikiolek M, Orsini V, Jurisic G, Megel P, Makowska Z, Agarinis C, Tornillo L, Bouwmeester T, Ruffner H, Bauer A, Parker CN, Schmelzle T, Terracciano LM, Heim MH, Tchorz JS: YAP promotes proliferation, chemoresistance, and angiogenesis in human cholangiocarcinoma through TEAD transcription factors. *Hepatology* 2015, 62:1497–1510
37. Hu J, Che L, Li L, Pilo MG, Cigliano A, Ribback S, Li X, Latte G, Mela M, Evert M, Dombrowski F, Zheng G, Chen X, Calvisi DF: Co-activation of AKT and c-Met triggers rapid hepatocellular carcinoma development via the mTORC1/FASN pathway in mice. *Sci Rep* 2016, 6:20484
38. Frith CH, Ward JM, Turusov VS: Tumours of the liver. *IARC Sci Publ* 1994, 111:223–269
39. Woo HG, Lee JH, Yoon JH, Kim CY, Lee HS, Jang JJ, Yi NJ, Suh KS, Lee KU, Park ES, Thorgeirsson SS, Kim YJ: Identification of a cholangiocarcinoma-like gene expression trait in hepatocellular carcinoma. *Cancer Res* 2010, 70:3034–3041
40. Sohn BH, Shim JJ, Kim SB, Jang KY, Kim SM, Kim JH, Hwang JE, Jang HJ, Lee HS, Kim SC, Jeong W, Kim SS, Park ES, Heo J, Kim YJ, Kim DG, Leem SH, Kaseb A, Hassan MM, Cha M, Chu IS, Johnson RL, Park YY, Lee JS: Inactivation of Hippo pathway is significantly associated with poor prognosis in hepatocellular carcinoma. *Clin Cancer Res* 2016, 22:1256–1264
41. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, Schinzel AC, Sandy P, Meylan E, Scholl C, Frohling S, Chan EM, Sos ML, Michel K, Mermel C, Silver SJ, Weir BA, Reiling JH, Sheng Q, Gupta PB, Wadlow RC, Le H, Hoersch S, Wittner BS, Ramaswamy S, Livingston DM, Sabatini DM, Meyerson M, Thomas RK, Lander ES, Mesirov JP, Root DE, Gilliland DG, Jacks T, Hahn WC: Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* 2009, 462:108–112
42. Hanzelmann S, Castelo R, Guinney J: GSEA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* 2013, 14:7
43. Furth N, Aylon Y: The LATS1 and LATS2 tumor suppressors: beyond the Hippo pathway. *Cell Death Differ* 2017, 24:1488–1501
44. Liu-Chittenden Y, Huang B, Shim JS, Chen Q, Lee SJ, Anders RA, Liu JO, Pan D: Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes Dev* 2012, 26:1300–1305
45. Ho C, Wang C, Mattu S, Destefanis G, Ladu S, Delogu S, Armbruster J, Fan L, Lee SA, Jiang L, Dombrowski F, Evert M, Chen X, Calvisi DF: AKT (v-akt murine thymoma viral oncogene homolog 1) and N-Ras (neuroblastoma ras viral oncogene homolog) coactivation in the mouse liver promotes rapid carcinogenesis by way of mTOR (mammalian target of rapamycin complex 1), FOXM1 (forkhead box M1)/SKP2, and c-Myc pathways. *Hepatology* 2012, 55:833–845
46. Huntzicker EG, Hotzel K, Choy L, Che L, Ross J, Pau G, Sharma N, Siebel CW, Chen X, French DM: Differential effects of targeting Notch receptors in a mouse model of liver cancer. *Hepatology* 2015, 61:942–952

47. Yimlamai D, Christodoulou C, Galli GG, Yanger K, Pepe-Mooney B, Gurung B, Shrestha K, Cahan P, Stanger BZ, Camargo FD: Hippo pathway activity influences liver cell fate. *Cell* 2014, 157:1324–1338
48. Tschaharganeh DF, Chen X, Latzko P, Malz M, Gaida MM, Felix K, Ladu S, Singer S, Pinna F, Gretz N, Sticht C, Tomasi ML, Delogu S, Evert M, Fan B, Ribback S, Jiang L, Brozzetti S, Bergmann F, Dombrowski F, Schirmacher P, Calvisi DF, Breuhahn K: Yes-associated protein up-regulates Jagged-1 and activates the Notch pathway in human hepatocellular carcinoma. *Gastroenterology* 2013, 144:1530–1542.e1512
49. Kato H, Taniguchi Y, Kurooka H, Minoguchi S, Sakai T, Nomura-Okazaki S, Tamura K, Honjo T: Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. *Development* 1997, 124:4133–4141
50. Blanpain C, Lowry WE, Pasolli HA, Fuchs E: Canonical notch signaling functions as a commitment switch in the epidermal lineage. *Genes Dev* 2006, 20:3022–3035
51. Che L, Fan B, Pilo MG, Xu Z, Liu Y, Cigliano A, Cossu A, Palmieri G, Pascale RM, Porcu A, Vidili G, Serra M, Dombrowski F, Ribback S, Calvisi DF, Chen X: Jagged 1 is a major Notch ligand along cholangiocarcinoma development in mice and humans. *Oncogenesis* 2016, 5:e274
52. Zanconato F, Cordenonsi M, Piccolo S: YAP/TAZ at the roots of cancer. *Cancer Cell* 2016, 29:783–803
53. Avruch J, Zhou D, Fitamant J, Bardeesy N: Mst1/2 signalling to Yap: gatekeeper for liver size and tumour development. *Br J Cancer* 2011, 104:24–32
54. Nishio M, Sugimachi K, Goto H, Wang J, Morikawa T, Miyachi Y, Takano Y, Hikasa H, Itoh T, Suzuki SO, Kurihara H, Aishima S, Leask A, Sasaki T, Nakano T, Nishina H, Nishikawa Y, Sekido Y, Nakao K, Shin-Ya K, Mimori K, Suzuki A: Dysregulated YAP1/TAZ and TGF-beta signaling mediate hepatocarcinogenesis in Mob1a/1b-deficient mice. *Proc Natl Acad Sci U S A* 2016, 113:E71–E80
55. Harvey KF, Zhang X, Thomas DM: The Hippo pathway and human cancer. *Nat Rev Cancer* 2013, 13:246–257
56. Takahashi Y, Miyoshi Y, Takahata C, Irahara N, Taguchi T, Tamaki Y, Noguchi S: Down-regulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers. *Clin Cancer Res* 2005, 11:1380–1385
57. Jiang Z, Li X, Hu J, Zhou W, Jiang Y, Li G, Lu D: Promoter hypermethylation-mediated down-regulation of LATS1 and LATS2 in human astrocytoma. *Neurosci Res* 2006, 56:450–458
58. Kim D, Shu S, Coppola MD, Kaneko S, Yuan ZQ, Cheng JQ: Regulation of proapoptotic mammalian ste20-like kinase MST2 by the IGF1-Akt pathway. *PLoS One* 2010, 5:e9616
59. Yang X, Yu J, Yin J, Xiang Q, Tang H, Lei X: MiR-195 regulates cell apoptosis of human hepatocellular carcinoma cells by targeting LATS2. *Pharmazie* 2012, 67:645–651
60. Perra A, Kowalik MA, Ghiso E, Ledda-Columbano GM, Di Tommaso L, Angioni MM, Raschioni C, Testore E, Roncalli M, Giordano S, Columbano A: YAP activation is an early event and a potential therapeutic target in liver cancer development. *J Hepatol* 2014, 61:1088–1096
61. Hayashi H, Higashi T, Yokoyama N, Kaida T, Sakamoto K, Fukushima Y, Ishimoto T, Kuroki H, Nitta H, Hashimoto D, Chikamoto A, Oki E, Beppu T, Baba H: An imbalance in TAZ and YAP expression in hepatocellular carcinoma confers cancer stem cell-like behaviors contributing to disease progression. *Cancer Res* 2015, 75:4985–4997
62. Mao B, Hu F, Cheng J, Wang P, Xu M, Yuan F, Meng S, Wang Y, Yuan Z, Bi W: SIRT1 regulates YAP2-mediated cell proliferation and chemoresistance in hepatocellular carcinoma. *Oncogene* 2014, 33:1468–1474
63. Higashi T, Hayashi H, Ishimoto T, Takeyama H, Kaida T, Arima K, Taki K, Sakamoto K, Kuroki H, Okabe H, Nitta H, Hashimoto D, Chikamoto A, Beppu T, Baba H: miR-9-3p plays a tumour-suppressor role by targeting TAZ (WWTR1) in hepatocellular carcinoma cells. *Br J Cancer* 2015, 113:252–258
64. Liu P, Zhang H, Liang X, Ma H, Luan F, Wang B, Bai F, Gao L, Ma C: HBV preS2 promotes the expression of TAZ via miRNA-338-3p to enhance the tumorigenesis of hepatocellular carcinoma. *Oncotarget* 2015, 6:29048–29059
65. Guo Y, Pan Q, Zhang J, Xu X, Liu X, Wang Q, Yi R, Xie X, Yao L, Liu W, Shen L: Functional and clinical evidence that TAZ is a candidate oncogene in hepatocellular carcinoma. *J Cell Biochem* 2015, 116:2465–2475
66. Li X, Tao J, Cigliano A, Sini M, Calderaro J, Azoulay D, Wang C, Liu Y, Jiang L, Evert K, Demartis MI, Ribback S, Utpatel K, Dombrowski F, Evert M, Calvisi DF, Chen X: Co-activation of PIK3CA and Yap promotes development of hepatocellular and cholangiocellular tumors in mouse and human liver. *Oncotarget* 2015, 6:10102–10115
67. Kim W, Khan SK, Gvozdenovic-Jeremic J, Kim Y, Dahlman J, Kim H, Park O, Ishitani T, Jho EH, Gao B, Yang Y: Hippo signaling interactions with Wnt/beta-catenin and Notch signaling repress liver tumorigenesis. *J Clin Invest* 2017, 127:137–152
68. Kumar R, Juillerat-Jeanneret L, Golshayan D: Notch antagonists: potential modulators of cancer and inflammatory diseases. *J Med Chem* 2016, 59:7719–7737