Loss of SPTBN1 Suppresses Autophagy Via SETD7-mediated YAP Methylation in Hepatocellular Carcinoma Initiation and Development

Shuyi Chen,1,* Huijie Wu,1,* Zhengyang Wang,1 Mengping Jia,1 Jieyu Guo,1 Jiayu Jin,1 Xiaobo Li,1 Dan Meng,1 Ling Lin,2 Aiwu Ruth He,2 Ping Zhou,1 and Xiuling Zhi1

1Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Fudan University, Shanghai, China; 2Department of Medicine and Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

SUMMARY

Spectrin beta, non-erythrocytic 1 cooperates with suppressor of variegation 3-9-enhancer of zeste-trithorax domain containing lysine methyltransferase 7 to promote Yes-associated protein methylation, enhancing autophagy of hepatic stem cells, hepatocellular carcinoma cells, and hepatocytes. Loss of spectrin beta, non-erythrocytic 1 promotes expansion and malignant transformation of hepatic stem cells via inhibiting autophagy during hepatocarcinogenesis.

BACKGROUND & AIMS: Loss of Spectrin beta, non-erythrocytic 1 (SPTBN1) plays an important role in the carcinogenesis of hepatocellular carcinoma (HCC); however, the mechanisms underlying its involvement remain poorly understood. Defects in autophagy contribute to hepatic tumor formation. Hence, in this study, we explored the role and mechanism of SPTBN1 in the autophagy of hepatic stem cells (HSCs) and HCC cells.

METHODS: Expansion, autophagy, and malignant transformation of HSCs were detected in the injured liver of Sptbn1+/− mice induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine treatment. Hippo pathway and Yes-associated protein (YAP) stabilization were examined in isolated HSCs, Huh-7, and PLC/PRF/5 HCC cells and hepatocytes with or without loss of SPTBN1.

RESULTS: We found that heterozygous SPTBN1 knockout accelerated liver tumor development with 3,5-diethoxycarbonyl-1,4-dihydrocollidine induction. Rapamycin promoted autophagy in murine HSCs and reversed the increased malignant transformation induced by heterozygous SPTBN1 deletion. Loss of SPTBN1 also decreased autophagy and increased YAP stability and nuclear localization in human HCC cells and tissues, whereas YAP inhibition attenuated the effects of SPTBN1 deficiency on autophagy. Finally, we found that SPTBN1 positively regulated the expression of suppressor of variegation 3-9-enhancer of zeste-trithorax domain containing lysine methyltransferase 7 to promote YAP methylation, which may lead to YAP degradation and inactivation.

CONCLUSIONS: Our findings provide the first demonstration that loss of SPTBN1 impairs autophagy of HSCs to promote expansion and malignant transformation during hepatocarcinogenesis. SPTBN1 also cooperates with suppressor of variegation 3-9-enhancer of zeste-trithorax domain containing lysine methyltransferase 7 to inactive YAP, resulting in enhanced autophagy of HCC cells. These results may open new avenues targeting SPTBN1 for the prevention and treatment of
Hepatocellular carcinoma (HCC) seriously threatens patient health, with incidence and mortality ranking fourth and second among malignant diseases in China and seventh and fourth among malignancies worldwide, respectively.\(^1\,^{12}\) Due to usually being diagnosed at late and advanced stages, HCC conveys poor overall prognosis and a low 5-year survival rate in patients, although great progress in treatment has been achieved in addition to surgical resection, the primary therapeutic strategy for HCC.\(^3\) Hence, it is of great significance to study the underlying mechanisms of HCC initiation and development for its early prevention and treatment.

Spectrin beta, non-erythrocytic 1 (SPTBN1), also known as ELF, βII spectrin or β2SP, is an important cytoskeletal protein belonging to the spectrin protein family that binds to ankyrin-B, ankyrin-G, and E-cadherin and participates in many cell processes, such as intracellular transport, cell diffusion and adhesion, cell polarity, cell cycle, and DNA repair.\(^4\,^{7}\) SPTBN1 recruits SMAD proteins to participate in transforming growth factor (TGF)-β signal transduction, activating downstream genes to influence cell growth.\(^8\,^{10}\) Sptbn1\(^-/-\) mice die during embryogenesis due to intestinal, liver, and cardiovascular diseases and neurological defects, most of which occur on day E11.5.\(^11\,^{12}\) Loss of SPTBN1 is linked to a variety of tumors, including pancreatic cancer, colon cancer, ovarian cancer, and liver cancer.\(^13\) It has been reported that 40% to 70% of Sptbn1\(^-/-\) mice spontaneously develop liver cancer at 15 months of age. Downregulation of SPTBN1 also promotes a stronger malignant and stemness phenotype in PLC/PRF/5 and SNU449 HCC cells\(^14\) and results in cell-cycle disruption with significant increases in cyclin D1, cyclin-dependent kinase 4, and hyperphosphorylated retinoblastoma protein.\(^15\,^{16}\) In addition, low expression of SPTBN1 in human HCC suggests that SPTBN1 might represent a new marker for the diagnosis and prognosis of patients.\(^11\,^{15}\,^{17}\) However, the detailed role of SPTBN1 in the regulation of HCC initiation and development remains poorly understood.

Hepatic stem cells (HSCs) are a group of progenitor cells that have the ability of self-renewal and bidirectional differentiation into mature hepatocytes and bile duct cells, which are usually present in the Hering duct of the hilar duct area, participating in physiological and pathological processes such as liver development, cell renewal and liver repair and regeneration.\(^18\) Of note, gene mutations, such as TP53 in the regulation of HCC initiation and development, cell renewal and liver repair and regeneration.\(^18\) Of note, gene mutations, such as PTEN and TP53, and/or dysregulation of signaling pathways, such as Wnt/β-catenin, Notch, or TGF-β, regulating cell renewal in HSCs can lead to malignant transformation of HSCs and HCC progression,\(^19\) providing a new direction for studying the molecular pathogenesis of liver cancer.

Many findings have demonstrated that inhibition of autophagy can induce hepatocarcinogenesis by activating HSCs. Autophagy is a dynamic process of “self-digestion” in eukaryotic cells, which acts as a “housekeeper” to cleave misfolded proteins.\(^20\,^{21}\) It is regulated by serine/threonine protein kinase and a series of autophagy-related genes (ATGs)\(^22\) and is involved in many pathological processes, including neurodegeneration, infectious disease, and cancer. Specific knockout of Atg7 in mouse hepatocytes promotes the expansion of HSCs and hepatocarcinogenesis.\(^23\) Autophagy can prevent malignant transformation of stem cells by maintaining their homeostasis.\(^24\,^{25}\)

The Hippo signaling pathway may be activated through the kinase cascade reaction primarily formed by mammalian ste20-like kinases 1/2 (MST1/2), NDR family kinases large tumor suppressor 1/2 (LATS1/2), and Yes-associated protein (YAP), exerting its anticancer function in mammals.\(^25\,^{26}\) In addition to phosphorylation by LATS1/2, acetylation, methylation, and glycosylation have been found to modify YAP and regulate its activity, which is closely related to autophagy. YAP liver-specific deficiency reversed HCC development induced by Atg7 knockout in mouse liver.\(^24\) On the other hand, Aurora A kinase improved YAP stability and protein expression by blocking autophagy to promote lung cancer progression.\(^27\) YAP is also degraded via the autophagy lysosome pathway, as shown by observing its colocalization with lysosomes in Atg7 knockout mouse liver.\(^23\)

It has been suggested that β-spectrin mutation leads to the decrease or loss of Hippo-YAP signal transduction activity, which might be caused by the destruction of the basic actin network.\(^28\) Similarly, Deng et al reported that spectrin mutation caused YAP accumulation in the nucleus and promoted cell proliferation in Drosophila;\(^29\) whereas loss of SPTBN1 reduced phosphorylation levels of LATS1 and YAP and promoted the translocation of YAP in the human colon adenocarcinoma cell line Caco-2.\(^30\) These reports indicate that SPTBN1 is likely to be involved in the regulation of the Hippo pathway, but its specific mechanism remains to be examined.

In this study, we explored the effect of SPTBN1 on hepatic cell autophagy in HCC initiation and development. We found that rapamycin (RAPA) promoted autophagy in eukaryotic cells, which acts as a “housekeeper” to cleave misfolded proteins.\(^20\,^{21}\) It is regulated by serine/threonine protein kinase and a series of autophagy-related genes (ATGs)\(^22\) and is involved in many pathological processes, including neurodegeneration, infectious disease, and cancer. Specific knockout of Atg7 in mouse hepatocytes promotes the expansion of HSCs and hepatocarcinogenesis.\(^23\) Autophagy can prevent malignant transformation of stem cells by maintaining their homeostasis.\(^24\,^{25}\)
murine HSCs and reversed the increased malignant transformation induced by heterozygous SPTBN1 deletion. SPTBN1 also cooperates with suppressor of variegation 3-9-enhancer of zeste-trithorax domain containing lysine methyltransferase 7 (SETD7) to promote methylation of YAP, resulting in YAP inactivation and enhanced autophagy of HCC cells. These findings demonstrate the functional significance of SPTBN1 in HCC, providing new targets for early diagnosis and treatment.

Results

The Kinetics of Carcinogenesis Induced by Sptbn1D/- are Drastically Accelerated by Cotreatment With 3,5 diethoxycarbonyl-1,4-dihydrocollidine

Because 40% to 70% of Sptbn1+/– mice spontaneously develop liver cancer at the age of 15 months, we utilized 6- to 8-week-old wild-type (WT) and Sptbn1+/– male mice administered a 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) supplemental diet to accelerate liver cancer formation. Our results showed that 40% of DDC-induced Sptbn1+/– mice developed HCC by the age of 3 months, and 100% developed HCC by the age of 5 months (Figure 1A-C). In contrast, no WT mice treated with DDC developed HCC at either 3 or 5 months old.

Generally, development and architecture of liver tissues including the biliary epithelium were normal in Sptbn1+/– mice (Figure 1D). However, the liver volume of Sptbn1+/– mice was enlarged compared with that of WT mice treated with DDC for 1 month (Figure 1E). Therefore, we focused on the precancerous stage of HCC caused by loss of SPTBN1 after 1 month of DDC cotreatment.

To determine if the malignant transformation of HSCs could be the origin of HCC induced by loss of SPTBN1, murine HSCs were separated from the liver of Sptbn1+/– and WT mice (Figure 1F). The cultured HSCs showed about 32% epithelial cell adhesion molecule (EPCAM)-positive cell portion (Figure 1G). Stem markers were only detected in HSCs, compared with hepatocytes (Figure 1H). The expansion and malignant transformation of murine HSCs were evaluated during HCC development in Sptbn1+/– mice. As Figure 1I and 1J show, the mRNA and protein levels of EPCAM, a marker of liver progenitors, were elevated in the isolated and cultured Sptbn1+/– HSCs (Figure 1I-J). Meanwhile, EPCAM-positive cells increased and extended from the hilar duct area, indicating that HSCs expansion had occurred in DDC-induced Sptbn1+/– mouse livers (Figure 1K).

Epithelial-mesenchymal transition (EMT) is involved in carcinogenesis and conveys metastatic properties on cancer cells, such as enhanced mobility and invasion and stemness maintenance in tumor stem cells. It was reported that EMT was induced by SPTBN1 knockdown in HCC cell lines, as well as in HSCs from interleukin-6-induced Sptbn1+/– mice. To investigate whether enhanced EMT occurred in HSCs from DDC-induced Sptbn1+/– mice, mRNA and protein levels of EMT-related markers were assessed. We found that CDH1/E-cadherin, TJP1/20-1, and CLDN1/Claudin-1 expression decreased, and CDH2/N-cadherin, VIM/Vimentin, SNAIL1, SNAIL2, and TWIST1 expression increased (Figure 2A-B). Immunofluorescence staining of liver tissue showed that the number of double-positive cells of VIM and cytokeratin 19 (CK19) or EPCAM and CK19 in Sptbn1+/– mice was greater than in WT mice (Figure 2C). Furthermore, expression of the oncogene AFP, an important marker of liver cancer, was increased at the mRNA and protein levels in HSCs from Sptbn1+/– mice (Figure 2A-B). Our results suggest that SPTBN1 heterozygosity promotes malignant transformation of murine HSCs, ultimately resulting in HCC development.

SPTBN1 Heterozygous Deletion Promotes the Malignant Transformation of Murine HSCs by Inhibiting Autophagy

Many studies have proven that autophagy suppresses HCC progression at the initial stage. It was also reported that hepatocyte-specific knockout of Atg7 promotes expansion of HSCs and HCC development. To investigate whether SPTBN1 alters autophagy levels in HSCs, expression of autophagy-related genes was examined. We found that mRNA expression of Beclin1 (Atg6), Atg4b, Atg5, Atg7, and Atg10 was decreased in HSCs from Sptbn1+/– mice (Figure 3A), whereas Beclin1, Atg4b, and Atg10 were decreased in Sptbn1+/– embyronic fibroblast (MEF) cells (Figure 3B-C). Furthermore, the protein LC3B-II/I ratio and Beclin1/Beclin1-1 expression were downregulated in Sptbn1+/– HSCs (Figure 3D) and Sptbn1+/– MEFs (Figure 3E), whereas the number of EPCAM/SQSTM1 double-positive cells in the livers of DDC-treated Sptbn1+/– mice was higher compared with DDC-treated WT mice (Figure 3F). Our data suggest that the level of autophagy was decreased in HSCs and MEFs due to the loss of SPTBN1.

To further investigate whether SPTBN1 regulates the malignant transformation of murine HSCs through autophagy inhibition, the effect of the autophagy inducer RAPA was assessed. As shown in Figure 4A and 4B, mRNA level of autophagy related gene Beclin1 and Atg4b was decreased, with increased EMT-related gene Cdh1 and increased Vim in Sptbn1+/– HSCs, whereas RAPA reversed the effect of SPTBN1 heterozygosity, as well as the ratio LC3B-II/I at protein level. In DCC-treated Sptbn1+/– mice, the size and contour of extensive hyperplastic intrahepatic bile duct were irregular, with co-wall and "back-to-back" phenomenon. The bile duct epithelial was dysplasia, characterized by atypical, enlarged, and hyperchromatic nuclei, an increased nuclear/cytoplasmic ratio, and a loss of polarity, representing precancerous lesion incidences only in the Sptbn1+/– mice, whereas the morphological differences were rescued by RAPA injection (Figure 4C, upper). Immunofluorescence staining showed that the increased number of EPCAM/ SQSTM1 and VIM/CK19 double-positive cells in the livers of DDC-treated Sptbn1+/– mice were both reduced by RAPA (Figure 4C, lower). Our data indicate that loss of SPTBN1 attenuates autophagy of HCSs, leading to malignant transformation, whereas RAPA induced autophagy and rescued the precancerous phenotype induced by loss of SPTBN1.
To elucidate the mechanisms of SPTBN1 on HSCs autophagy and HCC development, we performed microarray analysis in EPCAM-positive HSCs from WT and Sptbn1+/− mice, and the Kyoto Encyclopedia of Genes and Genomes pathway was utilized with Omicsbean. The Hippo-YAP signaling pathway was one of the identified pathways, Figure 1. The kinetics of carcinogenesis induced by SPTBN1 heterozygous deletion were accelerated by cotreatment with DDC. A, Liver images of WT and Sptbn1+/− mice cotreated with DDC. Arrows point to HCC in DDC-treated Sptbn1+/− mice. Six- to eight-week-old male WT and Sptbn1+/− mice (n = 4 for each experiment with 3 experimental replicates) were fed a diet containing 0.1% DDC for 5 months. B, H&E staining of liver sections from WT and Sptbn1+/− mice induced by DDC. White bars represent 200 μm. C, Tumor incidence rate (n = 8). D, H&E staining of liver tissues from WT and Sptbn1+/− mice aged 6 to 8 weeks. The development and architecture of liver tissues are normal in Sptbn1+/− mice. White bars represent 100 μm. E, The ratio of liver weight to body weight. The liver and body weight of WT and Sptbn1+/− mice was measured 1 month after feeding a 0.1% DDC-containing diet (n = 4; **P < .01 vs WT). F, The morphology of cultured HSCs in 3, 7, 14, and 21 days. G, The number of EPCAM-positive cells in HSCs was quantitatively evaluated by flowcytometric analysis. H, The stemness nature of cultured HSCs were examined by RT-PCR following by DNA gel electrophoresis. I-J, mRNA and protein levels of EPCAM in HSCs of Sptbn1+/− mice detected by qRT-PCR (I) and Western blot (J). Data is representative of 3 independent experiments. Significance of the difference was evaluated using the Student t test (**P < .01 vs WT). K, Immunofluorescence staining. SPTBN1 heterozygous deletion enhanced expansion of EPCAM-positive (arrow) HSCs in DDC-treated livers. CV, Central veins; PV, portal veins. White bars represent 100 μm. Number of EPCAM positive cells were counted (n = 4, mean ± standard deviation). Significance of the difference was evaluated using the Student t test (**P < .01 vs WT).

Figure 2. The enhanced EMT occurred in HSCs from DDC-induced Sptbn1+/− mice. A, QRT-PCR. mRNA levels of Cdh1, Tjp1, and Cldn1 were decreased, whereas Snail1, Snail2, Vim, Cdh2, and Twist1 and the tumor marker Afp were increased in HSCs from DDC-treated Sptbn1+/− mice. B, Western blot. Protein levels of CDH1/E-cadherin were decreased, whereas SNAIL2, VIM/Vimentin, CDH2/N-cadherin, and AFP were increased. Data is representative of 3 independent experiments. Significance of the difference was evaluated using the Student t test (**P < .01 vs WT). C, Immunofluorescence staining. EPCAM/CK19 and VIM/CK19 double-stained cells were increased in the livers of DDC-treated Sptbn1+/− mice, and the arrow indicates double-stained cells. White bars represent 50 μm.
exhibiting 44 differential genes (Figure 4D and Supplementary Table 1). We then assessed the Hippo-YAP signaling pathway using Western blotting and immunofluorescence in HSCs, livers and MEFs from WT and Sptbn1+/− mice. Our results showed that expression of p-YAP5127 and p-LATS1 was decreased, whereas expression of total YAP and LATS1 was increased in HSCs from DDC-treated Sptbn1+/− mice (Figure 4E-F) and Sptbn1+/− MEFs (Figure 4G), indicating that loss of SPTBN1 elevates the protein level of YAP and inhibits the Hippo signaling pathway.

SPTBN1 Knockdown Decreases Autophagy Levels in HCC Cells

We further examined malignancy and autophagy levels in HCC cells in response to SPTBN1 knockdown. Expression of the stemness marker NANOG and the oncogene
ASCL1 was increased in Huh-7 and PLC/PRF/5 cells transfected with SPTBN1 small interfering RNAs (siRNAs) (Figure 5A-D), implying that HCC cells obtained a more malignant phenotype in response to SPTBN1 deficiency. In addition, expression of BECN1, ATG4B, and ATG10 at the mRNA level (Figure 6A), as well as the LC3B-II/I ratio and expression of BECN1 at the protein level, were significantly reduced in Lentivirus (LV)-SPTBN1-sh stably infected Huh-7 and PLC/PRF/5 cells, whereas protein levels of SQSTM1 were increased in LC3B-II/I in Huh-7 and PLC/PRF/5 cells after 24 hours of starvation, whereas YAP was decreased in Lentivirus (LV)-SPTBN1-sh stably infected Huh-7 and PLC/PRF/5 cells, whereas protein levels of SQSTM1 were increased in HCC cells (Figure 6B) and human HCC tissues with reduced SPTBN1 expression (Figure 6C-D), indicating that SPTBN1 knockdown decreased autophagy levels of HCC cells, as well as HSCs. Higher expression of SPTBN1 and lower expression of SQSTM1 were both significantly correlated with increased survival in patients with HCC by performing Kaplan-Meier (K-M) plotter survival analysis (website: http://kmplot.com/analysis/). Interestingly, expression of SPTBN1 was not correlated with prognosis in female patients with HCC (Figure 6E-H). Moreover, SPTBN1 overexpression increased autophagy levels of Huh-7 and PLC/PRF/5 cells (Figure 7A-C). By electron microscopy, we found that autophagosomes were more enriched in Huh-7 and PLC/PRF/5 cells transfected with CON-sh plasmids than in those transfected with SPTBN1-sh after 24-hour incubation in Hanks’ balanced salt solution (HBSS) (Figure 7D). To examine the effect of SPTBN1 on the formation of autophagosomes, Huh-7 and PLC/PRF/5 cells with SPTBN1 knockdown were infected with the dual labeled lentivirus GFP-RFP-LC3, which combines an acid-sensitive GFP with an acid-insensitive RFP, monitoring the change from autophagosome (with a neutral pH) to autolysosome (acidic pH). As shown in Figure 7E, both autophagosomes and autolysosomes were decreased in response to SPTBN1 knockdown after 24 hours of starvation. Taken together, these data suggest that SPTBN1 knockdown in HCC cells blocks autophagy.

**Loss of SPTBN1 Increases Protein Levels and Nuclear Translocation of YAP**

As SPTBN1 heterozygosity elevates the protein level of YAP and inhibits the Hippo signaling pathway in Sptbn1+/− HSCs, we further verified the effects of SPTBN1 on the Hippo pathway in human HCC cells. We found that mRNA expression of YAP and LATS1 remained unchanged in response to either SPTBN1 knockdown or overexpression (Figure 8A-B). In contrast, protein levels of total YAP and LATS1 were increased, whereas the p-YAP5127 and p-LATS1 levels were decreased in LV-SPTBN1-sh stably transfected Huh-7 and PLC/PRF/5 cells (Figure 8C), which is quite the contrary to those cells with SPTBN1 overexpression (Figure 8D). Moreover, YAP nuclear localization was increased in Huh-7 and PLC/PRF/5 cells without SPTBN1 (Figure 8E-F). As a result, knockdown of SPTBN1 promoted mRNA expression of YAP targeted gene Cysteine-rich 61 (CYR61) and connective tissue growth factor (CTGF) (Figure 9A). The facts indicated that downregulation of SPTBN1 promotes YAP activation and inactivates the Hippo pathway. Conversely, nuclear YAP levels were decreased (Figure 9B), and YAP nuclear translocation was inhibited (Figure 9C) by SPTBN1 overexpression.

Consistent with the inhibition of YAP activity by SPTBN1 in Huh-7 and PLC/PRF/5 cells, human HCC tissues with reduced SPTBN1 expression in Figure 6C displayed stronger staining of total YAP and weaker staining of p-YAP5127 compared with adjacent normal tissues (Figure 9D).

**Loss of SPTBN1 Inhibits Autophagy by Activating YAP in HCC Cells**

We found that SPTBN1 promoted autophagy and inactivated YAP in HCC cells and tissues. Next, we explored whether SPTBN1-induced autophagy was mediated by YAP inactivation. As shown in Figure 10, siRNA targeting YAP reversed mRNA levels of BECN1, ATG4B and ATG10 (Figure 10A-B) and the ratio of protein LC3B-II/I (Figure 10C-D), which was decreased by SPTBN1 knockdown in Huh-7 and PLC/PRF/5 cells. The YAP inhibitor verteporfin had the same effects as YAP siRNA (Figure 10E-F). Notably, SPTBN1 knockdown decreased the number of both autophagosomes and autolysosomes in Huh-7 and PLC/PRF/5 cells after 24 hours of starvation, whereas YAP siRNA abrogated this reduction (Figure 10G).
SPTBN1 Promotes YAP Degradation and Methylation

Given that SPTBN1 overexpression decreased YAP protein levels, whereas YAP mRNA expression was unchanged in Huh-7 and PLC/PRF/5 cells, we reasoned that SPTBN1 would decrease the protein stability of YAP. As shown in Figure 6, blocking proteasome activity with MG132 rescued the decreased levels of YAP induced by SPTBN1 (Figure 11A) and enhanced the increase in YAP caused by SPTBN1 knockdown (Figure 11B). We also tested the level of p-YAP5127 and found that MG132 reversed the decreased ratio of YAP/p-YAP5127 induced by SPTBN1 overexpression (Figure 11A). Correspondingly, MG132 enhanced the increase of YAP/p-YAP5127 caused by SPTBN1 knockdown (Figure 11B). Our results demonstrate that loss of SPTBN1 promotes total YAP stabilization by decreasing proteasome degradation. Moreover, besides phosphorylation of YAP, it is possible that other modifications, such as methylation of YAP, may be involved in YAP stabilization induced by loss of SPTBN1.

In the presence of cycloheximide (CHX), an inhibitor of protein translation, SPTBN1 overexpression accelerated the degradation of YAP in Huh-7 cells (Figure 11C). This evidence suggests that SPTBN1 promotes YAP degradation through the ubiquitin proteasome degradation system.

As an epigenetic marker, methylation modification of nonhistone proteins can regulate protein stability and nucleoplasm localization.34 YAP stability is primarily regulated by p-YAP triggered ubiquitination, which eventually leads to YAP degradation under high cell density.35,36 However, whether methylation-dependent ubiquitination of YAP occurs is currently unknown. We next investigated whether SPTBN1 modulates YAP methylation and promotes YAP ubiquitination and degradation. It was found that methylated YAP protein was increased, whereas expression of total YAP protein was reduced in HCC cells with SPTBN1 overexpression (Figure 11D-E), indicating that the reduction in YAP stability caused by SPTBN1 might be related to YAP methylation. In addition, the levels of monomethylation of histone 3 at the lysine 4 site (H3K4me) were increased in response to SPTBN1 overexpression, demonstrating that SPTBN1 also promotes histone methylation.

SPTBN1 Transcriptionally Regulates SETD7 Expression

Next, we performed microarray analysis to identify lysine methyltransferases regulated by SPTBN1. We compared 2 microarrays performed using EPCAM-positive HSCs from WT and Sptbn1+/− mice and SNU449 cells with SPTBN1 knockdown and found that expression of the methyltransferase SETD7 was 2.5-fold lower in HSCs and SNU449 cells lacking SPTBN1 (Figure 12A-B and Supplementary Table 2). In addition, a positive correlation between SPTBN1 and SETD7 was shown by Gene Expression Profiling Interactive Analysis using HCC retrieved from The Cancer Genome Atlas data (Figure 12C). By performing K-M plotter survival analysis (website: http://kmplot.com/analysis/), we observed that higher expression of SETD7 was significantly correlated with increased survival in
patients with HCC (Figure 12D). We further verified the correlation between SPTBN1 and SETD7 in Huh-7 and PLC/PRF/5 cells. Quantitative real-time-polymerase chain reaction (qRT-PCR) showed that mRNA levels of SETD7 were significantly decreased in SPTBN1 siRNA-transfected HCC cells (Figure 12E). SPTBN1 knockdown in PLC/PRF/5 cells noticeably inhibited luciferase activity of the SETD7 promoter but did not decrease luciferase activity of the SETD7
promoter when the SPTBN1 binding site was mutated (Figure 12F). In addition, chromatin (Ch)-immunoprecipitation (IP)-PCR showed that SPTBN1 bound to the SETD7 promoter (Figure 12G). Our data demonstrated that SPTBN1 transcriptionally regulates SETD7. Protein levels of SETD7 were also significantly decreased in Huh-7 and PLC/PRF/5 cells in response to SPTBN1 knockdown (Figure 12H) but were markedly increased by SPTBN1 overexpression (Figure 12I).

**YAP Degradation Promoted by SPTBN1 is Mediated by SETD7**

The lysine methyltransferase SETD7 is localized both in the cytoplasm and nucleus, modifying histone and nonhistone proteins alike. It has been reported that methylation at lysine (K) 494 of YAP is induced by SETD7, leading to cytoplasmic retention.37We previously found that SPTBN1 increases methylation of YAP and promotes degradation of YAP. We further explored whether this effect of SPTBN1 was mediated by SETD7.

We first tested whether SPTBN1 bound to YAP and SETD7. Co-IP assay revealed an interaction between endogenous YAP and SPTBN1, as well as YAP and SETD7, in Huh-7 cells (Figure 13A). YAP and SPTBN1, as well as YAP and SETD7, also coprecipitated from the lysate of HEK293 cells engineered to express V5-SPTBN1, Flag-SETD7, and GFP-YAP, suggesting that SPTBN1, SETD7, and YAP combined with one another exogenously (Figure 13B).

We next examined whether SETD7 mediated the methylation and degradation of YAP induced by SPTBN1 in PLC/PRF/5 cells. As shown in Figure 13C, left, YAP protein levels were increased, whereas YAP methylation (Me-YAP) was decreased, after SPTBN1 knockdown, which was reversed by SETD7 overexpression. Methylation levels of H3K4, a substrate for SETD7, were consistent with Me-YAP.40 In addition, SETD7 knockdown ameliorated the effect of SPTBN1 overexpression, resulting in greater levels of YAP protein and reduced levels of Me-YAP and H3K4me (Figure 13C, right). These findings demonstrate that the methylation and degradation of YAP promoted by SPTBN1 is mediated by SETD7, although the specific mechanisms for the ubiquitination-dependent degradation of YAP require further study.

**Loss of SPTBN1 Inhibits Autophagy of Normal Hepatocyte LO2 by Inhibiting YAP Methylation**

HCC also possibly originates from differentiated hepatocytes. Therefore, we studied the effect of loss of SPTBN1 on normal hepatocytes using LO2 cell lines. Our results showed that the LC3B-II/I ratio and BECN1 protein level were downregulated; meanwhile SQSTM1 expression was upregulated in LO2 with SPTBN1 knockdown. However, the protein level of EMT-related markers including CDH1, VIM, and SNAIL2 was not changed in LO2 cells after SPTBN1 knockdown (Figure 14A). As in HCC cells, YAP mRNA expression was unchanged (Figure 14B), but the protein level of YAP was increased by SPTBN1 knockdown in LO2 cells (Figure 14C), with upregulated mRNA expression of YAP targeted gene CYR61 and CTGF (Figure 14B).

Next, we investigated if SPTBN1 modulated SETD7-mediated YAP methylation. Our data showed that the mRNA and protein expression of SETD7 was decreased by SPTBN1 knockdown in LO2 cells as well with decreased Me-YAP, which was reversed by SETD7 overexpression in LO2 cells (Figure 14B and C, left). SETD7 knockdown ameliorated the effect of SPTBN1 overexpression, resulting in greater levels of YAP protein and reduced levels of Me-YAP (Figure 14C, right). Moreover, siRNA targeting YAP reversed the ratio of protein LC3B-II/I, which was decreased by SPTBN1 knockdown in LO2 cells (Figure 14D). Comparing the results with those in HSCs, our data suggest that it may be universal that loss of SPTBN1 inhibits autophagy by inhibiting YAP methylation, at least in HCC cell lines, HSCs, and hepatocytes.

**Discussion**

In addition to the dedifferentiation of mature hepatocytes, accumulating evidence has shown that the abnormal differentiation of HSCs can lead to the development of liver cancer.19,39 For example, the Hippo-Salvador pathway restrains hepatic oval cell proliferation, liver size, and liver tumorigenesis.40 Similarly, liver-specific deletion of the neurofibromatosis type 2 tumor suppressor gene in developing or adult mice leads to expansion of stem cells without affecting differentiated hepatocytes; these mice develop HCC and intrahepatic cholangiocarcinoma.41 Furthermore, any mouse hepatic cells, including hepatic progenitors,
Figure 7. SPTBN1 overexpression increased autophagy level in the HCC cells. A and B, QRT-PCR. The mRNA level of autophagy-related genes BECN1 and ATG10 was increased in the SPTBN1 overexpression Huh-7 (A) and PLC/PRF/5 (B) cells. C, Western blot. The ratio of LC3BII/LC3B I and the protein level of BECN1 were increased, whereas SQSTM1 was decreased in the SPTBN1 overexpressed Huh-7 and PLC/PRF/5 HCC cells (n = 3; *P < .05, **P < .01 vs V5-CON). D, Transmission electron microscope analysis. Huh-7 (upper) and PLC/PRF/5 (lower) cells were transfected with CON-sh or SPTBN1-sh plasmids for 48 hours, starved for 24 hours by HBSS, and then examined. Autophagosomes (white arrow) and autophagolysosomes (red arrow) in HCC cells were reduced in response to SPTBN1 knockdown. Black bars represent 500 nm. E, Autophagic flux detected by fluorescence microscope. Huh-7 (upper) and PLC/PRF/5 (lower) cells were infected with GFP-RFP-LC3 LV for 48 hours and then transiently transfected with control siRNA or siRNA to SPTBN1 for another 24 hours. White bars represent 10 μm.
Loss of SPTBN1 Suppresses Autophagy in HCC Initiation

A

B

C

D

E

F

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The diagram depicts the effects of SPTBN1 knockdown on SPTBN1, LATS1, p-LATS1, p-YAP5127, and YAP expression and autophagy levels in HCC cells. The images show protein expression levels normalized by β-actin. The bar graphs compare LV-CNV-sh and LV-SPTBN1-sh conditions, indicating a significant suppression of SPTBN1 and YAP expression and an increase in autophagy markers in LV-SPTBN1-sh cells compared to LV-CNV-sh in Huh-7 and PLC/PRF/5 cells.
hepatoblasts, that express activated oncogenes (such as H-RAS or SV40LT) can undergo transformation to develop into intrahepatic cholangiocarcinoma or HCC. Dysregulation of liver developmental miRNAs in EPCAM-positive HSCs contribute to liver carcinogenesis by promoting the transformation of HSCs to cancer stem cells (CSCs) of HCC. Ring1 promotes the transformation of HSCs into CSCs, then developing into HCC, through the Wnt/β-catenin signaling pathway. Based on above evidence, HSCs are possibly one of the origins of HCC.

Autophagy is a double-edged sword for HCC that plays different roles during different stages of cancer development. Increasing studies have proven that basal autophagy acts as a tumor suppressor by removing damaged organelles and reactive oxygen species, maintaining genome stability, and preventing malignant transformation of cells. It has been reported that Becn1−/− mice spontaneously develop HCC at the age of 18 to 22 months. Hepatocyte-specific knockout of Atg5 and Atg7 in mice stimulates hepatomegaly, hepatic adenoma, and even HCC formation. Hence, defects in autophagy promote the occurrence of HCC.

Furthermore, heterozygous deletion of SPTBN1 constitutively activates the NF-κB signaling pathway in the inflammatory environment mediated by interleukin-6, and subsequently, the crosstalk between the activated NF-κB pathway and inactivated TGF-β pathway promote the transformation of HSCs into CSCs, leading to the development of liver cancer. However, it remains unclear if autophagy was involved in loss of SPTBN1-induced malignant transformation of HSCs.

In our study, we demonstrated that EPCAM-positive HSCs expanded in the injured liver of Sptbn1−/− mice induced by DDC treatment, also enhancing EMT and increasing expression of the oncogene AFP. Consistent with above reports, our results indicated that HSCs might be reprogrammed into CSCs in the persistent inflammatory microenvironment in the liver of Sptbn1−/− mice. Meanwhile, our data confirmed the role of the loss of SPTBN1 in suppressing HSC autophagy both in vitro and in vivo and that autophagy activation by RAPA blocks the malignant transformation of HSCs caused by SPTBN1 heterozygosity.

The Hippo-YAP signaling pathway plays an important role in regulating stem cell self-renewal, tissue regeneration, and organ size, and its disruption leads to malignant transformation of HSCs and tumor development. Knockout of MST1/2, Sav family WW domain-containing protein 1 and neurofibromatosis type 2 (mammalian homolog of Merlin), or overexpression of YAP in mouse liver causes expansion of HSCs or dedifferentiation of hepatocytes, leading to hepatomegaly and hepatocarcinogenesis. Moreover, the Hippo-YAP signaling pathway has been reported to regulate autophagy levels. In mammalian cells, MST1/2 promotes autophagy by directly phosphorylating LC3 at threonine whereas silencing of YAP promotes autophagy in glioblastoma and thyroid cancer. In our study, we further observed that loss of SPTBN1 contributes to YAP activation in vitro and in vivo. Downregulation of SPTBN1 in Huh-7 and PLC/PRF/5 cells inhibited autophagy by increasing YAP expression or activity. Lee et al also reported that loss of autophagy promotes the expansion of HSCs and hepatocarcinogenesis by inhibiting YAP degradation, whereas deletion of YAP reversed the promoted hepatocarcinogenesis by loss of ATG7. So, we speculate that autophagy and YAP is possibly negatively correlated with each other.

Phosphorylation of YAP has great significance for its nuclear localization and degradation. It was found that YAP has 5 common serine motifs (HRXXS) that are phosphorylated by LATS1. The phosphorylation sites include S61, S109, S127, S164, and S381. YAP binds to 14-3-3 protein and remains in the cytoplasm after phosphorylation at the S127 site. YAP phosphorylation at the S381 site leads to instability and degradation. In addition to the autophagy lysosomal degradation pathway, YAP is primarily degraded through the ubiquitin proteasome pathway by recruiting the E3 ubiquitin ligases SCF3, FBXW7, and SOCS5/6. Our results demonstrated that SPTBN1 reduced protein levels of LATS1 and YAP and increased levels of p-LATS1 and p-YAP, leading to decreased YAP nuclear localization in Huh-7 and PLC/PRF/5 HCC cells. Furthermore, MG132 enhanced the increase of YAP/p-YAP caused by SPTBN1 knockdown, indicating that loss of SPTBN1 promoted total YAP stabilization by decreasing proteasome degradation. In addition, it is possible that other modifications besides phosphorylation of YAP may be involved in YAP stabilization induced by loss of SPTBN1.

Figure 8. (See previous page) Loss of SPTBN1 increased protein levels and nuclear translocation of YAP. A and B, QRT-PCR, A, mRNA levels of LATS1 and YAP were not affected by SPTBN1 knockdown in Huh7 (left) or PLC/PRF/5 (right) cells (n = 3; **P < .01 vs LV-CON-sh). B, The mRNA level of LATS1 and YAP was not altered by SPTBN1 overexpression in the Huh-7 and PLC/PRF/5 cells (n = 3; *P < .05, **P < .01 vs V5-CON). C and D, Western blot. C, Protein levels of LATS1 and YAP were increased, whereas phosphorylated LATS1 and YAP were decreased in Huh7 (left) and PLC/PRF/5 (right) cells in response to SPTBN1 knockdown. Significance of the mean value difference was determined using a Student t test (*P < .05; **P < .01 compared with the LV-CON-sh group). D, The protein level of LATS1 and YAP was decreased, whereas the phosphorylation of LATS1 and YAP were increased by SPTBN1 overexpression (n = 3; *P < .05, **P < .01 vs V5-CON). E, Huh-7 (upper) and PLC/PRF/5 (lower) cells were transiently transfected with control siRNA or siRNA to SPTBN1 for 48 hours. Nucleus and cytoplasm proteins were extracted, respectively, for Western blot analysis. Significance of the mean value difference was determined using a Student t test (**P < .01 compared with the siCON group). F, Immunocytofluorescence staining. Loss of SPTBN1 promoted nuclear translocation of YAP in Huh7 (upper) and PLC/PRF/5 (lower) cells. White bars represent 20 μm.
Figure 9. SPTBN1 overexpression inhibited YAP nuclear translocation. A, Huh-7 (left) and PLC/PRF/5 (right) cells were transiently transfected with siRNAs for 48 hours before mRNA levels of CYR61 and CTGF, downstream target genes of YAP, were detected (n = 3; **P < .01 vs siCON). B, Western blot. The protein levels of YAP were reduced in both the nucleus and cytoplasm, whereas the p-YAPs127 level was elevated in the cytoplasm by SPTBN1 overexpression in the Huh-7 (upper) and PLC/PRF/5 (lower) HCC cells (**P < .01 vs V5-CON group). C, Immunocytofluorescence staining. YAP was decreased in the nucleus of the Huh-7 (upper) and PLC/PRF/5 (lower) HCC cells with SPTBN1 overexpression. White bars represent 20 μm. D, Immunohistochemistry of YAP in human HCC tissues. Expression of total YAP was greater, and p-YAPs127 was lower in HCC tissues from patients compared with adjacent noncancerous tissues (left) with corresponding statistical results of immunohistochemistry (right). Black bars represent 50 μm (*P < .05, **P < .01 compared with adjacent normal).
Figure 10. Loss of SPTBN1 inhibited autophagy by activating YAP in HCC cells. A and B, QRT-PCR. Huh-7 (A) and PLC/PRF/5 (B) cells were transiently transfected with siRNA to SPTBN1 or YAP for 48 hours and then analyzed. YAP siRNA reversed the expression of autophagy-related genes BECN1 and ATG4B that were downregulated by SPTBN1 knockdown (n = 3; *P < .05, **P < .01 vs siCON, ***P < .05, ****P < .01 vs siSPTBN1). C and D, Western blot. The decreased ratio of LC3BII/LC3B I protein was reversed by YAP siRNA in Huh-7 (C) and PLC/PRF/5 (D) cells. Significance of the mean value difference was determined using a Student t test (**P < .01 vs siCON, ###P < .01 vs siSPTBN1). E, Analysis of autophagy level by Western blot. The decreased ratio of protein LC3BII/LC3B I was reversed by YAP inhibitor verteporfin in the Huh-7 (upper) and PLC/PRF/5 (lower) HCC cells. F, The statistical analysis of Western blot in Figure E (n = 3; ***P < .01 vs siCON, ###P < .05, ####P < .01 vs siSPTBN1). G, Autophagy and autophagic flux detection by fluorescence microscopy after GFP-RFP-LC3 LV transfection and HBSS induction for 24 hours. The reduced autophagy spots in response to SPTBN1 knockdown were reversed by YAP siRNA in Huh-7 (left) and PLC/PRF/5 (right) cells. White bars represent 10 μm.
Figure 11. SPTBN1 promoted YAP degradation and methylation. A and B, Western blot. For overexpression of SPTBN1 (A), PLC/PRF/5 cells were transfected with V5-SPTBN1 plasmid (V5-CON plasmid as blank vector) for 48 hours. For suppression of SPTBN1 (B), PLC/PRF/5 cells were transfected with siRNAs to SPTBN1 for 48 hours. Cells then were treated without or with 20 μM MG132 for 3 hours before analyzed by Western blot. The intensities of SPTBN1, YAP, p-YAPS127, and β-actin in 3 independent Western blotting were measured by ImageJ software. A, **P < .01 vs V5-CON, ##P < .01 vs V5-SPTBN1. B, *P < .05, **P < .01 vs siCON, #P < .05, ##P < .01 vs siSPTBN1. C, CHX, a protein synthesis inhibitor, was used to detect SPTBN1-regulated YAP stability by Western blot (left) in Huh-7 cells. YAP protein had a higher decay rate in cells with SPTBN1 overexpression after CHX pretreatment (right) (n = 3; *P < .05, **P < .01, ***P < .001 vs V5-CON). D and E, The lysis of Huh-7 (D) and PLC/PRF/5 (E) cells transfected with V5-SPTBN1 plasmids were immunoprecipitated with antibody to YAP, and the immunoprecipitates were then immunoblotted with anti-methylated lysine antibody to detect methylation of endogenous YAP (n = 3; **P < .01 vs V5-CON).
cytoplasm. YAP is methylated by SETD7 at K494, causing its retention in the cytoplasm, but the mechanism whereby this occurs is unclear. Our results demonstrated that SPTBN1 enhanced YAP methylation and promoted its degradation by the ubiquitin proteasome pathway in Huh-7 and PLC/PRF/5 cells. Consistent with this observation, SPTBN1 can interact with SETD7 and transcriptionally regulate SETD7 expression. As a result, SETD7 exhibited lower expression in HSCs of Sptbn1−/− mice and in HCC cells with suppressed expression of SPTBN1, whereas SETD7 overexpression rescued decreased YAP methylation in response to SPTBN1 knockdown. Our study suggests that YAP activity is regulated not only by the Hippo pathway kinase cascade but also by Hippo-independent mechanisms, such as YAP methylation induction via methyltransferase.

Interestingly, our data suggest that it may be universal that loss of SPTBN1 inhibits autophagy by inhibiting YAP methylation, at least in HCC cell lines, HSCs, and hepatocytes. However, we could not demonstrate malignant transformation of normal hepatocytes in vitro by loss of SPTBN1, and it should be studied further whether hepatocytes are another possibility origin of HCC induced by SPTBN1.

In summary, our results suggested that loss of SPTBN1 inhibited HSC autophagy and Hippo pathway and promoted HSC expansion and malignant transformation, leading to hepatocarcinogenesis. SPTBN1 also inhibited autophagy in HCC cells. Loss of SPTBN1 suppresses hepatic cell autophagy via SETD7-mediated YAP methylation, promoting HCC initiation and development. Our study demonstrates new mechanisms of SPTBN1 in inhibiting HCC and provides new targets for HCC treatment.

Methods

Animal Care and Genotyping

SPTBN1 heterozygous (Sptbn1+/−) mice were generated and amplified as reported by the Shanghai Research Center for Model Organisms (Shanghai, China). Mice were housed in the Experimental Animal Department of Fudan University. All procedures were performed following the approval of the Institutional Animal Care and Use Committee of Fudan University. For mouse genotyping, genomic DNA PCR was employed.

Construction of the Sptbn1+/− Liver Injury Model to Induce Activation of HSCs and Liver Cancer

To construct the Sptbn1+/− liver injury model, 6- to 8-week-old male WT and Sptbn1+/− mice (n = 4, per experiment with 3 experimental replicates) were fed a diet containing 0.1% DDC for 1, 3, and 5 months. At the end of the experiment, mice were weighed and sacrificed to observe liver tumor incidence, and liver tissues were harvested and subjected to histological staining.

To elucidate the mechanisms for the malignant transformation of activated HSCs, 6- to 8-week-old male WT and Sptbn1+/− mice were randomly treated with RAPA (2 mg/kg, APExBIO, Houston, TX) by intraperitoneal injection every other day, and the other one-half of the mice were injected with vehicle. One month later, mice were sacrificed, and liver tissues were harvested.

HSC Isolation and Cell Line Culture

Murine HSCs were separated from the liver of Sptbn1+/− and WT mice after 1 month of DDC treatment as previously described. In brief, the collected cells were washed and then cultured in Dulbecco’s Modified Eagle Medium/F12 medium containing 10% fetal bovine serum (FBS), 20 ng/ml stem cell factor (PeproTech), 10 ng/mL leukemia inhibitory factor (Millipore), 10 ng/mL epidermal growth factor (PeproTech), and 10 ng/mL hepatocyte growth factor (PeproTech). After 7 days, the cells formed a cloned cell cluster. The stemness nature and EPCAM positive

Figure 12. (See previous page). SPTBN1 transcriptionally regulated SETD7 expression. A, Differential expression of methyltransferase in HSCs by microarray analysis. EPCAM-positive HSCs were isolated by flow cytometry from WT and Sptbn1+/− mouse livers (n = 3). Then the collected cells were examined by microarray analysis. B, Differential expression of methyltransferase in the HCC cells SNU449 by microarray analysis. SNU449 cells were transfected with control siRNA or siRNA to SPTBN1 for 48 hours and screened by microarray analysis. C, Correlation analysis between SPTBN1 and SETD7 in patients with HCC was analyzed using The Cancer Genome Atlas database by Gene Expression Profiling Interactive Analysis (r = 0.35; P < .01). D, The OS was assessed and compared between the low-SETD7 and high-SETD7 groups based on extracted clinical data from The Cancer Genome Atlas in all of 364 patients with HCC. E, QRT-PCR. Huh-7 (left) and PLC/PRF/5 (right) cells were transiently transfected with siRNAs as indicted and then analyzed. Data is representative of 3 independent experiments. Significance of the mean value difference was determined using a Student t test (*P < .05, **P < .01 compared with the siCON group). F, Analysis of SETD7 promoter activity. PLC/PRF/5 cells were transfected with SPTBN1 siRNA or control siRNA for 24 hours and then transfected with a plasmid containing the SETD7 promoter sequence or a mutated sequence for 24 hours. Then cells were lysed, and luciferase activity was measured (n = 3; **P < .01 vs siCON for SETD7-WT group). G, ChIP-qPCR. PLC/PRF/5 cells were infected with LV-CON-sh or LV-SPTBN1-sh for 96 hours and then analyzed. IgG antibody was used as a negative control, and H3 antibody was used as a positive control. Homo-RPL30 primers were used as the positive control for ChIP-qPCR detection. SETD7 promoter region sequences were enriched by SPTBN1 in the obtained ChIP DNA (n = 4; **P < .01 vs IgG). H, Western blot. The intensities of SPTBN1, SETD7, and β-actin were measured by ImageJ software, and the ratio of SPTBN1/β-actin and SETD7/β-actin were analyzed by Student t test. (*P < .05, **P < .01 vs siCON, and the data are representative of 3 independent experiments). I, Western blot. The protein level of SETD7 was upregulated in the SPTBN1 overexpressed Huh-7 (left) and PLC/PRF/5 (right) cells (n = 3; *P < .05, **P < .01 vs V5-CON).
populations of HSCs fractions were examined by RT-PCR and flowcytometric analysis.

The human HCC cell lines Huh-7 and PLC/PRF/5 and normal hepatocytes LO2 were cultured with Dulbecco’s Modified Eagle Medium supplemented with 10% FBS and antibiotics. Human embryonic kidney (HEK293T) cells were maintained in Roswell Park Memorial Institute 1640/10% FBS with antibiotics. All cells were cultured in a humidified atmosphere with 5% CO2 at 37°C.

**Plasmid and Small Interfering RNA Transfection**

For silencing or overexpression of target genes, SPTBN1-sh, V5-SPTBN1, Flag-SETD7, and GFP-YAP were transiently transfected into cells using jetPRIME transfection reagent according to the manufacturer’s instructions (Polyplus Transfection SA, France). The siRNAs listed in Supplementary Table 3 were designed and chemically synthesized by GenePharma (Shanghai, China). Cells were also treated with the YAP inhibitor verteporfin (10 μM, APExBIO, Houston, TX) after SPTBN1 siRNA transfection. In one set of experiments in the study, Huh-7 and PLC/PRF/5 cells were infected with LV-SPTBN1-sh purchased from Shanghai Genechem (Shanghai, China). Seventy-two hours after infection, stably transduced cells were selected.

To assess autophagic flux, cells were transfected with SPTBN1 siRNA for 72 hours and then infected with GFP-
RFP-LC3 lentivirus purchased from Shanghai Genechem (Shanghai, China). After 24 hours of HBSS-induced autophagy, cells were imaged with an Olympus microscope. The CON-sh- and SPTBN1-sh-transfected Huh-7 and PLC/PRF/5 cells were incubated with HBSS for 24 hours, fixed in 2.5% glutaric dialdehyde solution, and then observed with a transmission electron microscope.

**Western Blot and Coimmunoprecipitation**

Whole cell extracts were prepared using RIPA buffer. Nuclear and cytoplasmic fractions were separated by a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, Beijing, China). For each sample, an equal amount (40 μg–100 μg) of protein was loaded onto sodium dodecyl sulphate–polyacrylamide gel electrophoresis gels, electrotransferred onto Immobilon-P membranes (Millipore, Bedford, MA), blocked with 5% skim milk in TBST, and incubated with specific primary and the corresponding horseradish peroxidase-conjugated secondary antibodies. Blots were detected using an ECL kit (Catalog#180-501, Tanon, Shanghai, China). Densitometric quantification was performed using ImageJ software.

To detect the methylation of YAP, cell supernatants were incubated with anti-YAP antibody at 4 °C overnight. The immune complexes were recovered with protein G magnetic beads (Bio-Red, Hercules, CA), which were then washed 4 times with lysis buffer; the immunoprecipitants were then subjected to Western blot analysis. Rabbit polyclonal antibody to Methylated Lysine (Anti-Methylated Lysine, Abcam, MA) was used to incubate the blot.
To detect the interaction between proteins, the supernatants of whole cell extracts were incubated with antibodies at 4°C overnight. Immune complexes were subsequently pulled down using protein G magnetic beads (Bio-Red, Hercules, CA). The complexes and whole cell extracts were then analyzed by Western blot analysis. The antibodies used in Western blot and Co-IP are listed in Supplementary Table 4.

**Luciferase Assay**

To investigate the *SETD7* promoter activity in SPTBN1 knockdown PLC/PRF/5 cells, pGL3-luciferase reporter plasmids comprising the *SETD7* promoter fragment (Supplementary Table 5) were generated using the Hieff Clone Plus One Step Cloning Kit (YEASEN, Shanghai, China). PLC/PRF/5 cells transfected with SPTBN1 siRNA or CON siRNA were assessed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) after transfection with SETD7 pGL3-luciferase reporter plasmids or mutants for 24 hours.

**Chromatin IP Quantitative PCR**

In brief, chromatin fragments derived from PLC/PRF/5 cells were immunoprecipitated with 5 µg of the antibody against SPTBN1, Ig G (3900S, CST, Danvers, MA, USA) or H3 (4620S, CST, Danvers, MA, USA). DNA extraction was purified using a Qiagen Purification kit. Real-time PCR analysis was performed with primers amplifying the promoters of *SETD7* (Forward 5′ to 3′ AGAATGGACAGGAGCTTCTAA, Reversed 5′ to 3′ TATCCCCTGGGGTACATGCT). The antibodies used in the above experiments are listed in Supplementary Table 4.

**RNA Extraction and qRT-PCR**

Total RNA was extracted from cells using TRIzol-trichloromethane-isopropanol reagent and reverse transcribed into cDNA using the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). The 10 µl real-time PCR system contained 10 ng/µl cDNA, 5 µl SYBR Green, 4 µl ddH2O and 1 µl mixed primers. The primer sequences for targeted genes are listed in Supplementary Table 6A and 6B.

**Histological Staining**

Hematoxylin and eosin (H&E) staining of liver tissues was used to identify liver cancers that developed in *Sptbn1*+/− mice. For immunohistochemistry staining, sections were dewaxed, rehydrated, blocked, incubated with primary antibodies for SPTBN1 (1:300), YAP (1:500), p-YAPS127 (1:600), and SQSTM1 (1:500) and corresponding horseradish peroxidase-conjugated secondary antibodies, and finally incubated with hematoxylin for nuclear staining. Information from patients for the tissue microarray (Ribiology Company, Wuhan, Hubei, China) is presented in Supplementary Table 7. For immunofluorescence staining, after incubation with primary antibodies, sections were incubated with a corresponding fluorescently conjugated secondary antibody. Nuclei were stained with 4′-diamidino-2-phenylindole.

For immunocytochemistry, cells were fixed in 4% paraformaldehyde for 15 minutes and then permeabilized with 0.1% Triton X-100 for 20 minutes. After blocking with 5% goat serum for 2 hours, cells were incubated with primary antibodies overnight at 4°C. Then, cells were washed and incubated with secondary antibodies and finally incubated with 4′-diamidino-2-phenylindole for nuclear staining. Epifluorescent images were acquired with an Olympus microscope.

**Analysis of The Cancer Genome Atlas Database and Gene Microarrays**

The correlation analysis of SPTBN1 and SETD7 was analyzed by Gene Expression Profiling Interactive Analysis (website: http://gepia.cancer-pku.cn/detail.php?cicktag=correlation) using hepatocellular carcinoma clinicopathological data retrieved from The Cancer Genome Atlas database. Overall survival (OS, n = 364) was determined using the K-M method (website: http://kmplot.com/analysis/index.php?p=service&cancer=liver_rnaseq) by retrieving RNA-seq data on hepatocellular carcinoma provided by Menyhart et al.54 Gene microarrays of EPCAM+ HSCs from WT and *Sptbn1*+/− mice and SNU449 cells transfected with SPTBN1 siRNA or control siRNA were performed by Affymetrix, Inc. (Santa Clara, CA). The Kyoto Encyclopedia of Genes and Genomes pathways and differentially expressed genes were analyzed using OmicsBean.

**Statistical Analyses**

Data are presented as the mean ± standard deviation. The Student t test was used for 2-sample comparisons. Immunohistochemical scores were nonnormally distributed and are presented as the median with range, which was analyzed by rank-sum test for comparison. Pearson correlation test was performed on the correlation analysis. All statistical analyses were completed using GraphPad Prism 8.0 software, and *P < .05* was accepted as statistically significant.

**Supplementary Material**

Note: To access the supplementary material accompanying this article, visit the online version of Cellular and Molecular Gastroenterology and Hepatology at www.cmghjournal.org, and at http://doi.org/10.1016/j.jcmgh.2021.10.012.

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## Supplementary Table 1. Differential Expressed Genes for Hippo Pathway, PI3K-Akt/mTOR Pathways (Microarray Analysis of WT vs Sptbn1<sup>−/−</sup> HSCs)

| PI3K-Akt signaling pathway | Count = 120 | mTOR signaling pathway | Count = 44 | Hippo signaling pathway | Count = 44 |
|----------------------------|-------------|------------------------|-------------|-------------------------|------------|
| Gene | Fold change | Gene | Fold change | Gene | Fold change |
| Pck1 | 7.588402 | Prkaa2 | 3.313512 | Patj | 7.447174 |
| Pitr | 6.205024 | Igif1 | 2.966077 | Rassf6 | 5.249817 |
| Fgf2 | 5.559759 | Chuk | 2.715469 | Pard3 | 6.18582 |
| Gnt1 | 5.548901 | Strad | 2.704452 | Wwc1 | 3.548835 |
| Ghr | 4.054142 | Ir1 | 2.654457 | Bmpr1a | 3.347636 |
| Fgfr4 | 3.485656 | Rps6kb1 | 2.625695 | Fgf1 | 2.807672 |
| Sgk2 | 3.462673 | Prkca | 2.589811 | Pard6b | 2.377636 |
| Creb3l3 | 3.398698 | Cab39l | 2.292622 | Bmp4 | 2.257083 |
| Prkaa2 | 3.313512 | Caf152 | 2.41339 | Cdh1 | 2.339999 |
| lkb1 | 3.115047 | Tbc1d7 | 2.244674 | Prkcz | 2.132192 |
| Met | 3.068178 | Pten | 2.066916 | Dig3 | 2.115372 |
| Igf1 | 2.966077 | Mapk3 | 2.06035 | Ywhab | 2.02804 |
| Bdnf | 2.83152 | Pragd | 2.07648 | Ccnd3 | 2.0925 |
| Fgf1 | 2.807672 | Fln | 2.07943 | Id2 | 2.12387 |
| Chuk | 2.715469 | Grb2 | 2.09558 | Ctnna1 | 2.13969 |
| Irs1 | 2.654457 | Castor2 | 2.16875 | Nkd1 | 2.15902 |
| Rps6kb1 | 2.625695 | Atp6v1b2 | 2.16939 | Gsk3b | 2.1746 |
| Prkca | 2.589811 | Gsk3b | 2.1746 | Lgl1 | 2.21941 |
| Gys2 | 2.574207 | Igif1 | 2.30931 | Pps2r2a | 2.25611 |
| Rbl2 | 2.570327 | Wdr59 | 2.36484 | Bmp5 | 2.32131 |
| G6pc | 2.485964 | Tnfsf1a | 2.37198 | Ywhah | 2.34549 |
| Vtn | 2.479085 | Nras | 2.40049 | Apc | 2.50835 |
| Fn1 | 2.43188 | Rps6ka1 | 2.40625 | Serpine1 | 2.53345 |
| Rora | 2.42612 | Akt1 | 2.52956 | Wwtr1 | 2.53556 |
| Vegfa | 2.356407 | Rictor | 2.64083 | Pard6g | 2.8463 |
| Lamb3 | 2.197116 | Strada | 2.76802 | Fzd8 | 2.88065 |
| Them4 | 2.142961 | Lamtor3 | 2.79703 | Tcfl1 | 2.97074 |
| Col4a5 | 2.072375 | Atp6v1h | 2.82706 | Ccnd1 | 3.07245 |
| Pten | 2.066916 | Fzd8 | 2.88065 | Rassf1 | 3.13521 |
| Ywhab | 2.02804 | Rps6ka3 | 2.93579 | Csnk1e | 3.17693 |
| Itga4 | 2.04317 | Ptk3d3 | 3.7123 | Lats2 | 3.35052 |
| Creb3 | 2.04986 | Castor1 | 3.77316 | Itgb2 | 3.35182 |
| Mapk3 | 2.06035 | Fzd6 | 3.94144 | Bmp2 | 3.52212 |
| Lamb1 | 2.06601 | Wnt9b | 4.09329 | Smad7 | 3.62828 |
| Itgb7 | 2.08704 | Slec3a2 | 4.21566 | Tgfb2 | 3.69774 |
| Thbs2 | 2.08887 | Prkcb | 4.22162 | Fzd6 | 3.94144 |
| Ccnd3 | 2.0935 | Ptk3d3 | 4.38551 | Wnt9b | 4.09329 |
| Gb2 | 2.09558 | Sgk1 | 4.70343 | Bmp2 | 5.35349 |
| Crtc2 | 2.11723 | Grb10 | 5.50352 | Wt1p | 5.54228 |
| Gnb2 | 2.12402 | Elf4e | 5.64502 | Ccnd2 | 5.72462 |
| Lama1 | 2.12786 | Akt3 | 6.68715 | Id1 | 6.23852 |
| Pdgfrb | 2.166 | Wnt2 | 8.29182 | Wnt2 | 8.29182 |
| Gsk3b | 2.1746 | Rps6ka2 | 9.14494 | Bmp6 | 8.77418 |
| Jak2 | 2.17781 | Tnf | 9.36789 | Ccn2 | 11.8758 |
| Gene       | Fold change | Gene       | Fold change | Gene       | Fold change |
|------------|-------------|------------|-------------|------------|-------------|
| Ppp2r2a    | -2.25611    | Sgk3       | -2.28178    | Igf1r      | -2.30931    |
| Lama4      | -2.32106    | Co4a3      | -2.33219    | Ywhah      | -2.34549    |
| Cdk4       | -2.37386    | Pik3cg     | -2.3803     | Nras       | -2.40049    |
| Lamic1     | -2.41417    | Hsp90aa1   | -2.45632    | Reln       | -2.49724    |
| Mcl1       | -2.52081    | Akt1       | -2.52956    | Gnb4       | -2.53501    |
| Gnb1       | -2.53936    | Lpar4      | -2.56111    | Erbb2      | -2.58512    |
| Ptk2       | -2.61244    | Bcl2       | -2.66664    | Ppp2r5c    | -2.7578     |
| Col6a3     | -2.8139     | Nkx1b1     | -2.81441    | Col4a4     | -2.89443    |
| Pck2       | -2.93795    | Csf1       | -2.95712    | Pdgfra     | -2.96851    |
| Pdgfc      | -3.01045    | Pik3r5     | -3.03443    | Ccnd1      | -3.07245    |
| Lpar6      | -3.11387    | Il4r       | -3.24021    | F2r        | -3.33094    |
| Itga1      | -3.40374    | Csf1r      | -3.49223    | Kit        | -3.58355    |
| Bcl2i11    | -3.61937    | Pik3r3     | -3.7123     | Jak1       | -3.74938    |
| Nr4a1      | -3.78545    | Angpt2     | -3.79157    | Etna2      | -3.83325    |
| Nos3       | -4.00918    | Ifna2      | -4.04876    | Tnxb       | -4.14256    |
| If33ra     | -4.29327    | Etna2      | -4.36264    | Pik3cd     | -4.38551    |
| Gene  | Fold change | Gene  | Fold change | Gene  | Fold change |
|-------|-------------|-------|-------------|-------|-------------|
| Il6   | 4.41769     | Itga6 | 4.52203     | Pdgfb | 4.56128     |
| Pik3r6| 4.68722     | Sgk1  | 4.70343     | Ntf3  | 4.87067     |
| Il2rg | 5.10068     | Tlr4  | 5.61008     | Eif4e | 5.64502     |
| Gngt2 | 5.67983     | Ccnd2 | 5.72462     | Flt1  | 5.74386     |
| Col4a1| 5.80492     | Col4a2| 6.55273     | Aktr3 | 6.68715     |
| Itga9 | 6.71574     | Flt4  | 6.78615     | Tlr2  | 7.05292     |
| Itga5 | 7.12998     | Osmr  | 7.34988     | Gng11 | 8.18049     |
| Kdr   | 8.25627     | Pdgfd | 8.29916     | Lamb2 | 10.7514    |
| Its8  | 11.4952     | Vwf   | 12.4518     | Cdkn1a| 12.8695     |
| Tek   | 16.4338     |       |             |       |             |
### Supplementary Table 2: Differential Expressed Methylation-related Genes in WT-HSC and Sptnb1+/−-HSC

| Gene       | Fold change |
|------------|-------------|
| Nnmt       | 5.7         |
| Shmt1      | 4.5         |
| Anubl1     | 4.07        |
| Ube2k      | 4.04        |
| Tpmt       | 3.8         |
| Ube2u      | 3.77        |
| Mettl1     | 3.3         |
| Shmt2      | 3           |
| Pemt       | 2.9         |
| Rnmt1      | 2.6         |
| Ube2v2     | 2.58        |
| Ube2l6     | 2.52        |
| Uba6       | 2.46        |
| Setd6      | 2.4         |
| Mettl5     | 2.3         |
| Usp2       | 2.27        |
| Uba3       | 2.26        |
| Usp24      | 2.22        |
| Setd4      | 2.2         |
| Setd5      | 2.1         |
| Ufc1       | 2.02        |
| Trmt5      | 2           |
| Comtd1     | 2           |
| Uba2       | 2.03        |
| Ubap2l     | 2.05        |
| G2e3       | 2.14        |
| Usp12      | 2.17        |
| Anih1      | 2.18        |
| Usp7       | 2.44        |
| Uhrf2      | 2.47        |
| Setd7      | 2.5         |
| Dnmt1      | 2.9         |
| Usp27x     | 3.39        |
| Uba7       | 3.48        |
| Dnmt3a     | 3.7         |
| Mtr        | 3.8         |
| Ubash3b    | 3.86        |
| Prmt2      | 4.3         |
| Sptbn1     | 4.8         |
| Hecw2      | 19.47       |
### Supplementary Table 3. The Sequences of siRNAs for Target Genes

| Name  | Sense (5’ to 3’)          | Antisense (5’ to 3’)          |
|-------|---------------------------|-------------------------------|
| SPTBN1-1 | GGAUUGCGAAGGAGCUAGUAUC     | GAUACUAGACGCUCCUCUGCAAUCC     |
| SPTBN1-2 | ACCUCGAGAUGGAGGAUAGCUAU    | AUGGCAUCCUGCAUCCUCCAGAGU     |
| NC     | GGAACGUGAGUGCAGAUCUAUAC    | GAUAAGAUCUAGCACUCCAGUCC       |
| YAP-1  | GABAUCUCUGUCAAGATT        | UCUCGAGCAAAAGAUGUCC          |
| YAP-2  | ACAGUGACUAGGAACUUUTT      | AAAGUUCCUAAGUACGUTT           |
| NC     | AACGAUGAUCGACAGCAGAG      | CUGGUGGUAAGUACUGG            |
| SETD7-1 | GGCGACCUGGAGUUGCGGAdTdT    | UCCGUCAGUCCUGGCCdTdT         |
| SETD7-2 | GGGGUGUGCUGGUAUAUdTdT     | AAUAUAUCCAGCACACUCdTdT       |
| SETD7-3 | CAAACUCAUACGAUCUAdTdT     | AAUAUGAUGUAGUAdTdT           |
| NC     | AACAGCGUCACCCAAUAAGA      | UCUAUGUGGUGUGUCGCGU          |

### Supplementary Table 4. Antibodies Used in Western Blot, IP, and Immunohistochemistry Staining

| Antibody       | Catalog No. | Company     | Country |
|----------------|-------------|-------------|---------|
| SPTBN1         | SC1180      | Genscript   | China   |
| Epcam          | 21050-1-AP  | Proteintech | USA     |
| β-actin        | 66009-1-Ig  | Proteintech | USA     |
| EMT antibody sampler kit | 9782 | CST | USA |
| CK-19          | sc-376126   | Santa Cruz  | USA     |
| AFP            | 14550-1-AP  | Proteintech | USA     |
| LC3BI/II       | 3868        | CST         | USA     |
| Beclin1        | 3495        | CST         | USA     |
| SQSTM1/p62     | ab56416     | Abcam       | UK      |
| YAP1           | 4912/66900-1-Ig | CST/Proteintech | USA |
| p-YAP1         | 4911        | CST         | USA     |
| LATS1          | 9157        | CST         | USA     |
| p-LATS1        | 9153        | CST         | USA     |
| ASCL1          | ab223229    | Abcam       | UK      |
| NANOG          | sc-134218   | Santa Cruz  | USA     |
| SETD7          | ab124708    | Abcam       | UK      |
| H3K4 me        | 5326        | CST         | USA     |
| H3             | ab176842    | Abcam       | UK      |
| Anti-Methylated Lysine | ab23366 | Abcam       | UK      |
| V5             | sc-271926   | Santa Cruz  | USA     |
| GFP            | sc-53882    | Santa Cruz  | USA     |
| Flag           | F3165       | Sigma-Aldrich | USA |
| Goat anti-Rabbit HRP IgG | A0208 | Beyotime | China |
| Goat anti-Mouse HRP IgG | A0216 | Beyotime | China |
| Alexa Fluor 488-labeled goat anti-mouse IgG (H+L) | A0428 | Beyotime | China |
| Cy3-labeled goat anti-rabbit IgG (H+L) | A0521 | Beyotime | China |
### Supplementary Table 5. Primer Sequences of the Promotor of SETD7

| Primer | Sequence (5' to 3') |
|--------|---------------------|
| Forward | CGAGCTTTACGCGTGCTAGCCACCTCTCGTTGGCCAAAACT |
| Reversed | CAGTACCGGAATGCAAAGCTTATGTGCATTCCCCAGCTCATT |

### Supplementary Table 6A. The Murine Primer Sequences Used in the qRT-PCR Analysis

| Gene name | Forward primer (5' to 3') | Reverse primer (5' to 3') |
|-----------|---------------------------|---------------------------|
| Sptbn1    | CGTGAGGACCTACTGGATCAT    | GTCCACGTCGTCTTGTGTTTT    |
| Epcam     | CGTGAGGACCTACTGGATCAT    | GTCCACGTCGTCTTGTGTTTT    |
| Atg4b     | TATGATCCTCCTGGTTTCTGA    | GTTCCCCCAATAGCTGGAAAG    |
| Atg5      | TGTGCTTTGAGATGTTTTGTT    | GTCAATAGCTGACTTGGCAA     |
| Atg7      | GCTGACCCCTTTAATAGTGCC    | TGAACCTCAAACGTCAAGCGG    |
| Atg10     | GATGTTACAATGGCTGGTTTTC   | AGCATAACGGCTCCCATCTAAA   |
| Becn1     | ATGGAGGGGTCTAAGGCTGCT    | TCCTCTCGTAGTTAGCTCCT     |
| Snail2    | TGGTCAAGAACATTTCACGCCC   | GGTGAGGATGTTCTGGTTTTGTA  |
| Snail     | CACAGGCGTCGCTTTGTGTTT    | GTTCAAGCAAAGGACAGGTT    |
| Vim       | CGGCCCCACGACCTAGCTACAG   | GGGGGTGAAGGATAGAGGCT     |
| Cdhl      | CAGGTCTCTCTAGGTGGTTTGC   | CTGCCGAAAGGCTGTCC       |
| Cdhl2     | AGCCAGGTCTCAGGCAGGAGG   | TGCTCTGCTGCTGCTTGGTGG   |
| Clchn1    | GGGGACAACATCGTGACCCG    | AGAAGTCAGGACATTTCAGCT   |
| Cltnb1    | ATGGAGGACCAACAGAAGG     | CTTGCCACGCTAGGAGGGA      |
| Tjp1      | GCCGCCTAGAGACAGACAGA    | TCCCCACTCGGAAATAGGAGA    |
| Zeb1      | GCTGGCAAGAACAGCAAGCTGG   | GCCTCAAGATAATGACCGG     |
| Twist1    | GGAACAGCTGAGAAGATTCA    | CGGAGAGGCGTGGTGGAG      |
| Afp       | AGGCTGGATGATGATGCTGCC   | ACAAAGTGGTTAAGGTTAGG     |
| Actn, beta| GGGTGTATCTCCTCGCCATCG   | CCAAGTTGAAACATGCAATG    |
| Pkm       | CGCAACACTGCGATCATTTT    | TGCTCCACGCGACACTCCT     |
| Krt19     | GCCGCCTAGAGACAGAGATGGA  | CGAAGTGACCTGAGACAGGAGG  |
| Thy1      | GAACCAAAACCCTTCCGCT    | GCCTCAGAAAGTACTGCC      |
| Gja1      | CCCATCGAAAGACTGCGG     | CGCTGGCTTGCTTGGTTA      |
| Kit       | GCCAGCTTGACCGCATCTG     | GTCCGCCAGCTCTAACTTTAATT |
| Rplp0     | AGATTGCGGATATGCTGGTGC  | TGCGGTCCTAGACAGATTGG    |
### Supplementary Table 6B. The Human Primer Sequences Used in the qRT-PCR Analysis

| Gene name | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|-----------|---------------------------|--------------------------|
| SPTBN1    | ATCTAACGCACACTACAACCTG    | TCAAGCACCTTTCCAATTCTG    |
| ASCL1     | CCCAAGCAAGTCAAGCGACA      | AAGCCGCTGAAGTTGAGCC      |
| NANOG     | TTTGTGGGGCTGAAGAAAACT     | AGGGCTGTCTCTGATAAGCAG    |
| BECN1     | GGTGTCTCTGCAGATTCATC      | TCAGTCTTGGCTGAGTTCT      |
| ATG4B     | GGTGTGGACAGATGATCTTTTC   | CCAACTCCCATTTCGCTATC     |
| ATG5      | AGAAGCTGTTTCGTCTGTGG      | AGGTGTTTCACATTTGCTC      |
| ATG7      | ATGATCCCTGTAACCTAGCCA     | CACGGAAGCAAAAACACTCCAAC  |
| ATG10     | ATAGATGCGACTGCTACAGG      | CAAATGCTCAGCATGTATGTAT   |
| LAST1     | CCACCTACCCAAAAACATCTG     | CGCTGCTGATGAGTTTGAGTAC   |
| YAP       | TGGGACTCAAAATCAGTGTC      | CCATCTCCTTCCAGTTCTCC     |
| CTGF      | AAAAGTGCAATCCGTCCTCCCCA   | CCGCAGGTACATCCACACAG     |
| CYR61     | GTGCTGTACGGTCTCACC       | CCGGCAAGTTGCACTTCCAG     |
| SETD7     | GGCAGGGGATTTACACTTAC     | CGTATCAGGGTACACATAGCTA   |
| ACTIN, beta | CATGTACGTTGCTACCCAGC    | CCTCTTATGTCAGCCACGAT     |

### Supplementary Table 7. Clinical Characteristics of the 24 Patients Used for the Tissue Microarray

| Item                             | Classification | Amount | Item                             | Classification | Amount |
|----------------------------------|----------------|--------|----------------------------------|----------------|--------|
| Gender                           | Male           | 18     | Age, years                       | >50            | 10     |
|                                  | Female         | 6      | <50                              | 14             |        |
| Maximum diameter of mass, cm     | >5             | 13     | Hepatitis B virus infection      | Yes            | 23     |
|                                  | <5             | 11     | No                               | 1              |        |
| Intrahepatic metastasis          | Yes            | 7      | Cirrhosis                        | Yes            | 10     |
|                                  | No             | 17     | No                               | 14             |        |
| Lymph node metastasis            | Yes            | 0      | Extrahepatic metastasis          | Yes            | 1      |
|                                  | No             | 24     | No                               | 23             |        |
| Differentiation                  | Low            | 10     |                                  |                |        |
|                                  | Middle         | 11     |                                  |                |        |
|                                  | High           | 3      |                                  |                |        |