ZBTB20 regulates WNT/CTNNB1 signalling pathway by suppressing PPARG during hepatocellular carcinoma tumorigenesis

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Table of contents

Supplementary materials and methods

Hydrodynamic injection

Hydrodynamic injections were performed as previously described [1-5]. Briefly, male *Fah*-deficient mice (mixed genetic background C57BL/6 X 129) carrying the ubiquitously expressing *SB* transposase transgene knocked into the Rosa26 locus (*Rosa26*-SB11) were generated (*Fah*/SB11). Generation of empty vector control that contains bi-directional promoters driving *Fah* cDNA and luciferase reporter gene in one direction, and an empty cassette in the other, pKT2/GD-Empty, has been previously described [4]. Generation of pT2/GD-IRES-GFP vector that contains the destination cassette (DEST) for clonase reaction (Life Technologies) has been previously described [5]. *ZBTB20*, transcript variant 2 (isoform 2), was obtained from OriGene (NM_015642.2) in a pCMV6-XL5 vector. The *ZBTB20* insert was removed from pCMV6-XL5 by standard restriction enzyme digest and cloned into pENTR entry vector (Life Technologies) to get pENTR-ZBTB20. ZBTB20 component from pENTR-ZBTB20 was cloned into pT2/GD-IRES-GFP using LR clonase reaction to get pT2/GD-IRES-GFP-ZBTB20. Generation of transposon vector containing the short hairpin against *Trp53* gene (pT2/shp53) has been previously described [2-5]. All transgenes used for hydrodynamic tail vein injections were prepared using EndoFree Plasmid Maxi Kit (Qiagen). Twenty micrograms of each construct was hydrodynamically injected into 6-week old *Fah*/SB11 male mice using previously established conditions (n = 11) [1]. These mice were normally maintained with 7.5 μg/ml 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC, Swedish Orphan Biovitrum) drinking-water but replaced with normal drinking water after hydrodynamic injection of transposon vectors. Using tumor multiplicity as an output of carcinogenesis, 10 injected experimental animals should allow for adequate statistical power (>95%) with a confidence level of 95%, assuming no tumors will be obtained with the control vector (standard deviation ± 3 background tumors). All animals received human care and the

study protocols have been approved by the Animal Subjects Ethic Sub-Committee of The Hong Kong Polytechnic University, Hong Kong, SAR. The expression transposon vectors also co-express the *Fah* gene, which allows for selective repopulation of hepatocytes that co-express both transgene-of-interest and *Fah*. The liver is then observed for resulting phenotype of stably incorporated transgene overexpression after hydrodynamic delivery. Injected experimental animals were observed for weight changes and luciferase activity as previously described [2-5]. Some historical controls for pKT2/GD-Empty with pT2/shp53 were used in this study (n = 6).

Liver tumor analyses

The whole liver was carefully removed from the sacrificed animal, weighed, washed and placed in cold phosphate buffered saline (PBS). The lobes were separated and the number of surface liver tumor nodules was counted. All reasonably sized tumor nodules (>2 mm in diameter) were carefully removed from the liver lobes using fine forceps and placed in fresh cold PBS. Liver nodules that were large enough were then halved using a sterile razor blade and split into samples for RNA extraction and histopathological analysis. Tissue samples for RNA were stored at -80°C in RNAlater (Sigma) to prevent RNase contamination and degradation. Histological sections were only taken for larger tumor nodules (>2 mm in diameter). Formalin fixed-paraffin embedded sections from various tissues were sectioned at 5 microns using a standard microtome (Leica), mounted and heat-fixed onto glass slides. Tissue section slides were either processed and stained with hematoxylin-eosin (HE) using standard protocols, or used for immunohistochemical (IHC) analyses as described in the next section. Histopathological analyses were performed in part by board-certified human pathologists (M.A.L. and K.A.).

Cell number and size determination

For cell number determination, the number of cells in representative fixed area was counted for experimental and control animals from both genders. Five representative areas were counted for each animal and shown as an arbitrary cell number. As for cell size determination, ImageJ 1.40J software (NIH, Maryland, USA) was used to measure the shorter diameter of hepatocytes in experimental and control animals from both genders. Fourteen representative hepatocytes were randomly measured and shown as an arbitrary cell size.

Immunohistochemical (IHC) analyses

Glass tissue section slides were dewaxed and rehydrated through a gradual decrease in ethanol concentration. The antigen epitopes on the tissue sections were then unmasked using a commercially available unmasking solution (Vector Laboratories) according to the manufacturer's instructions. The tissue section slides were then treated with 3% hydrogen peroxide to remove any endogenous peroxidases. Blocking was performed at 4°C using a M.O.M. mouse immunoglobulin-blocking reagent (Vector Laboratories) in a humidified chamber for several hours. For IHC, sections were then incubated overnight at 4°C in a humidified chamber using the primary Ki67 antibody at 1:200 dilution (Abcam) or CTNNB1 primary antibody at 1:100 dilution (Cell Signaling Technology, CST). After primary incubation, sections were washed thoroughly in PBS before incubating with horseradish peroxidase-secondary antibody raised against the primary antibody initially used. After thorough washes with PBS, the sections were treated with freshly prepared DAB substrate (Vector Laboratories) and allowed for adequate signal to develop before stopping the reaction in water. Finally, sections were then lightly counter-stained with hematoxylin, dehydrated through gradual increase in ethanol concentration, cleared in xylene and mounted in Permount (Thermo Fisher Scientific).

RT-PCR

Extraction of RNA from tumor nodules was done using the Trizol reagent (Invitrogen) using protocols described by the manufacturer. First strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche) as described by the manufacturer using 1 µg total RNA as template. Both reactions with (RT+) and without (RT-) the reverse transcriptase were performed for all the samples. Subsequent PCR was performed using 1 µl of the cDNA as template with various primer pairs. Primer sequences for alpha-fetoprotein (*Afp*) were forward 5'-CCTGTGAACTCTGGTATCAG-3' and reverse 5'-GCTCACACCAAAGCGTCAAC-3' (amplicon 410 bp); secreted phosphoprotein 1 (*Spp1*) forward 5'-CTTTCACTCCAATCGTCCCTAC-3' and reverse 5'-GCTCTCTTTGGAATGCTCAAGT-3' (amplicon 305 bp); actin beta (*Actb*) forward 5'-GTGACGAGGCCCAGAGCAAGAG-3' and reverse 5'-AGGGGCCGGACTCATCGTACTC-3' (amplicon 938 bp); fumarylacetoacetate hydrolase (*Fah*) forward 5'-ATGAGCTTTATTCCAGTGGCC-3' and reverse 5'-ACCACAATGGAGGAAGCTCG-3' (amplicon 503 bp); Sleeping Beauty transposase (*SB11*) forward 5'-ATGGGAAAATCAAAAGAAATCAGCC-3' and reverse 5'-CGCACCAAAGTACGTTCATCTCTA-3' (amplicon 221 bp); zinc finger and BTB domain containing 20 (*Zbtb20*) forward 5'-ATGAGATTACTCAGCCGGGCGGATC-3' and reverse 5'-AGCTTGTCTTGGAAGAAGGGGCTGC-3' (amplicon 343 bp); human *ZBTB20* isoform 2 forward 5'-CGAGCGCATTCACAGCATCAACCTT-3' and reverse 5'-TCTCGATGTCGCTGTAGCCAAGCAG-3' (amplicon 200 bp). PCR conditions were 25 to 30 cycles to avoid amplicon saturation. Semi-quantitative analyses of non-saturated RT-PCR amplicons were performed using the ImageJ software. Briefly, intensity of non-saturated RT-

PCR amplicon bands was measured as an arbitrary value relative to *Actb* expression levels using ImageJ software.

Quantitative RT-PCR (qPCR) for in vivo liver tissues

Isolation of RNA from liver samples was performed using guanidine-isothiocyanate lysis, rotor stator homogenization, and column-based purification (PureLink RNA mini, Ambion) according to manufacturer protocols. For each sample, 10 µg of RNA was treated with DNase to remove contaminating genomic DNA (Turbo DNA-free, Ambion), and cDNA was subsequently synthesized from 1 µg of template using the SuperScript III First-Strand Synthesis System (Invitrogen) according to manufacturer protocols. qPCR was performed using FastStart Universal SYBR Green Master mix (Roche) according to manufacturer protocols, using 1µL of cDNA template in a 25 µL reaction volume. Control reactions were performed without cDNA template or using cDNA synthesized without reverse transcriptase. Reactions were run in a Bio-rad qPCR machine with the following conditions: 94°C for 5 min, 40 cycles of 94°C for 15 s, 58°C for 10 s, 72°C for 30 s, followed by melt-curve analysis. Target gene expression was quantified relative to *Actb* for each sample. Primer sequences were as follows: *Actb* forward 5'-TCCAGCCTTCCTTCTTGGGTATGGA-3' and reverse 5'-CGCAGCTCAGTAACAGTCCGCC-3' (amplicon 365 bp); axin 2 (*Axin2*) forward 5'-ATGAGTAGCGCCGTGTTAGTG-3' and reverse 5'-GGGCATAGGTTTGGTGGACT-3' (amplicon 150 bp); cyclin D1 (*Ccnd1*) forward 5'-CAGAAGTGCGAAGAGGAGGTC -3' and reverse 5'-TCATCTTAGAGGCCACGAACAT-3' (amplicon 130 bp); *Ctnnb1* forward 5'-ATGGAGCCGGACAGAAAAGC-3' and reverse 5'-CTTGCCACTCAGGGAAGGA-3' (amplicon 108 bp); lymphoid enhancer binding factor 1 (*Lef1*) forward 5'-TGTTTATCCCATCACGGGTGG-3' and reverse 5'-CATGGAAGTGTCGCCTGACAG-3' (amplicon 67 bp); myelocytomatosis oncogene (*Myc*) forward 5'-

ATGCCCCTCAACGTGAACTTC-3’ and reverse 5’-CGGAGTCGTAGTCGAGGTCATA-3’ (amplicon 55 bp); transcription factor 7, T cell specific (*Tcf7*) forward 5’-AGCTTTCTCCACTCTACGAACA-3’ and reverse 5'-AATCCAGAGAGATCGGGGGTC-3’ (amplicon 115 bp); glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) forward 5'-GTGTTCCTACCCCCAATGTGT-3' and reverse 5'- GAGACAACCTGGTCCTCAGTGT-3' (amplicon 148 bp). qPCRs were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.

qPCR for transfected cells

Transfected cell cDNA was synthesized from 500 ng of total RNA extracted from transfected cells using PrimeScript RT Master Mix (Takara) followed the protocol provided from the manufacturer. The cDNA was diluted in 1:10 ratio in nuclease-free water, and 4 μL of the diluted cDNA was used to perform qPCR using SYBR Green I containing qPCR master mix (GoTaq qPCR Master Mix, Promega) with specific primers (0.2 μM final concentration of each primer). Reactions were run on a QuantStudio7 Flex Real-time PCR Systems (Thermo Fisher) (University Research Facility in Life Sciences of The Hong Kong Polytechnic University, Hong Kong). Primer sequences were as follows: *ACTB* forward 5’-GCCGTCTTCCCCTCCATCGT-3’ and reverse 5’-TGCTCTGGGCCTCGTCGC-3’; *AXIN2* forward 5’-CTCCCCACCTTGAATGAAGA-3’ and reverse 5’-TGGCTGGTGCAAAGACATAG-3’; *AFP* forward 5’-CTTTGGGCTGCTCGCTATGA-3’ and reverse 5’-GCATGTTGATTTAACAAGCTGCT-3’; *PPARG* forward 5’-ACCAAAGTGCAATCAAAGTGGA-3’ and reverse 5’-ATGAGGGAGTTGGAAGGCTCT-3’; *CTNNB1* forward 5’-AAAGCGGCTGTTAGTCACTGG-3’ and reverse 5’-CGAGTCATTGCATACTGTCCAT-3’; *ZBTB20* forward 5’-CGAGCGCATTCACAGCATCAACCTT-3’ and reverse 5’-

TCTCGATGTCGCTGTAGCCAAGCAG-3'; *CCND1* forward 5'-GCTGCGAAGTGGAAACCATC-3' and reverse 5'-CCTCCTTCTGCACACATTTGAA-3'; glycogen synthase kinase 3 beta (*GSK3B*) forward 5'-TCGAGAGCTCCAGATCATGAGAA-3' and reverse 5'-CGGAACATAGTCCAGCACCAGA-3'; APC regulator of WNT signaling pathway (*APC*) forward 5'-AAAACGAGCACAGCGAAGAATAGC-3' and reverse 5'-TCGTGTAGTTGAACCCTGACCAT-3'; *GAPDH* forward 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse 5'-ACCACCCTGTTGCTGTAGCCAA-3'. qPCRs were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.

ZBTB20 overexpression in human liver cell lines

The piggyBac (*PB*) transposon system was used to stably integrate and overexpress both Flag-tagged variants of *ZBTB20* in immortalized human hepatic cell line (HHL7) [6], and human liver cancer cell lines – PLC/PRF/5 and Hep3B (both from ATCC). A Flag-tagged sequence, 5'-GACTACAAAGACGATGACGACAAG-3', was added right after the start codon of both *ZBTB20* transcript variant 1 (isoform 1) (OriGene, NM_001164342) and transcript variant 2 (isoform 2) (OriGene, NM_015642.2) cDNAs. These Flag-tagged *ZBTB20* variants was inserted into the pENTR entry vector using specific restriction enzymes to give pENTR-Flag-*ZBTB20* (isoform 1) or (isoform 2). Expression vector was obtained by introducing the Flag-tagged *ZBTB20* variants from the entry clone to the destination vector, pPB/SB-DEST-GFP [7], to yield pPB/SB-Flag-*ZBTB20* (isoform 1) or (isoform 2)-GFP. A control expression vector containing the orange fluorescent protein (OFP) was generated using the similar method to yield pPB/SB-Flag-OFP. One microgram of either expression or control vector was co-transfected with one microgram of *PB* transposase vector into the cell using ViaFect transfection reagent (Promega) [7]. Ratio of ViaFect Transfection Reagent to DNA equaled to

3:1 was applied to transfect the plasmids into 5 x 10^4 cell in 24-well plate as recommended by the manufacturer. Transfected cells were selected under 1 mg/mL of puromycin in medium. Stably transfected cell lines were obtained about 4-weeks post-transfection. The transfected cells were then collected for DNA, RNA and protein extraction; and for immunofluorescent (IF) staining.

Targeted disruption of ZBTB20 in human liver cancer cell lines

The CRISPR/Cas9 system was used as a gene editing tool to disrupt *ZBTB20*. Five different guide RNAs (named gRNA1 to gRNA5) were designed to target the BTB and DNA-binding zinc finger domains of *ZBTB20*. The gRNA sequences as shown with PAM sequences underlined in bold (**Table S1**). These gRNAs were ligated into Cas9 (pSpCas9(BB)-2A-Puro (PX459) V2.0 vector, which was a gift from Dr Feng Zhang (Addgene plasmid #62988; http://n2t.net/addgene:62988; RRID:Addgene_62988), to generate a knockout vector that carries both gRNA and Cas9 following its provided protocol [8]. Two hundred nanogram of each knockout vector was pooled and transfected into 15 x 10^4 PLC/PRF/5 and C3A (ATCC) cells in 6-well plates by ViaFect transfection reagent (Promega) as previously described. One microgram of pSpCas9-scramble vector containing non-targeting control gRNA sequence 5'-CATTTCTCAGTGCTATAGA-3' was also transfected as a negative control. Transfected cells were selected under 0.4 μg/mL of puromycin in medium for 5 days. Genomic DNA and RNA were extracted from the transfected cells for further analyses. To obtain single cell cultures, 50 cells were diluted in 10 mL medium and seeded into a 96-well plate at 100 μL/well.

Analyses of ZBTB20-disrupted cells

To determine the disruption of *ZBTB20* in transfected PLC/PRF/5 and C3A cells, different primer pairs were used to identify the indels in genomic DNA and cDNA of *ZBTB20* disrupted

cells (**Table S2**). For genomic DNA, the primer pairs 1 and 2 were used to amplify the target BTB domain – 515 bp and 446 bp for wild-type (WT) and deletion allele, respectively. Primer pairs 3 and 4 were used to amplify the target zinc finger domain – 275 bp and 203 bp for WT and deletion allele, respectively. For cDNA, the primer pairs 1 and 2 were used to amplify the target BTB domain – 380 bp and 309 bp for WT and deletion allele, respectively. Primer pairs 4 and 5 were used to amplify the target zinc finger domain – 501 bp and 441 bp for WT and deletion allele, respectively.

Immunofluorescent (IF) staining

Stably transfected cells were seeded onto round-cover slides in a 24-well plate overnight. Medium was discarded and cells were washed twice with cold-PBS (1X). Next, cells were fixed by incubating with 4% formaldehyde for 10 minutes at room-temperature and blocked with 5% BSA for 1 hour at room-temperature. Cells were then incubated with primary antibodies at 4°C overnight. Primary antibodies used were Flag antibody (Sigma-Aldrich), active CTNNB1 (CST) or total CTNNB1 (CST) followed the dilution provided by the manufacturers. Then, the cells were rinsed twice with cold PBS and incubated with specific secondary antibodies for 1 hour at room-temperature. Finally, a 5-minute incubation with DAPI at dilution of 1:5000 was used to stain the nucleus of the cells. Cells were then mounted onto a glass slides using Anti-Faded mounting media. IF was visualized using the Leica TCS SP8 MP Multiphoton Microscope (The Hong Kong Polytechnic University, University Research Facility in Life Sciences).

Western blot analyses of transfected cells

Protein was extracted from transfected cells using SDS protein lysis buffer. Protein concentration was detected using standard protein assay (Bio-Rad) and 30 μg of protein was

loaded into the SDS-PAGE and transferred onto PVDF membrane. Primary antibodies active CTNNB1 (CST), total CTNNB1 (CST), PPARG (CST), ACTB (TransGen Biotech) and Flag (Sigma-Aldrich) were diluted in 5% BSA at 1:1000 concentration. Secondary antibodies (anti-mouse or anti-rabbit) were diluted in 5% BSA at 1:2000 concentration. Membrane was blocked with 5% non-fat milk, then incubated with primary antibody at 4°C overnight followed by secondary antibody incubation at room temperature for an hour. The membrane was then washed with 1X TBST for 3 times in 10 minutes' interval. Finally, membrane was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). Semi-quantitative analyses of protein bands were measured using ImageJ software. Intensity of bands was calculated as an arbitrary value relative to ACTB expression level.

Promoter activity analyses

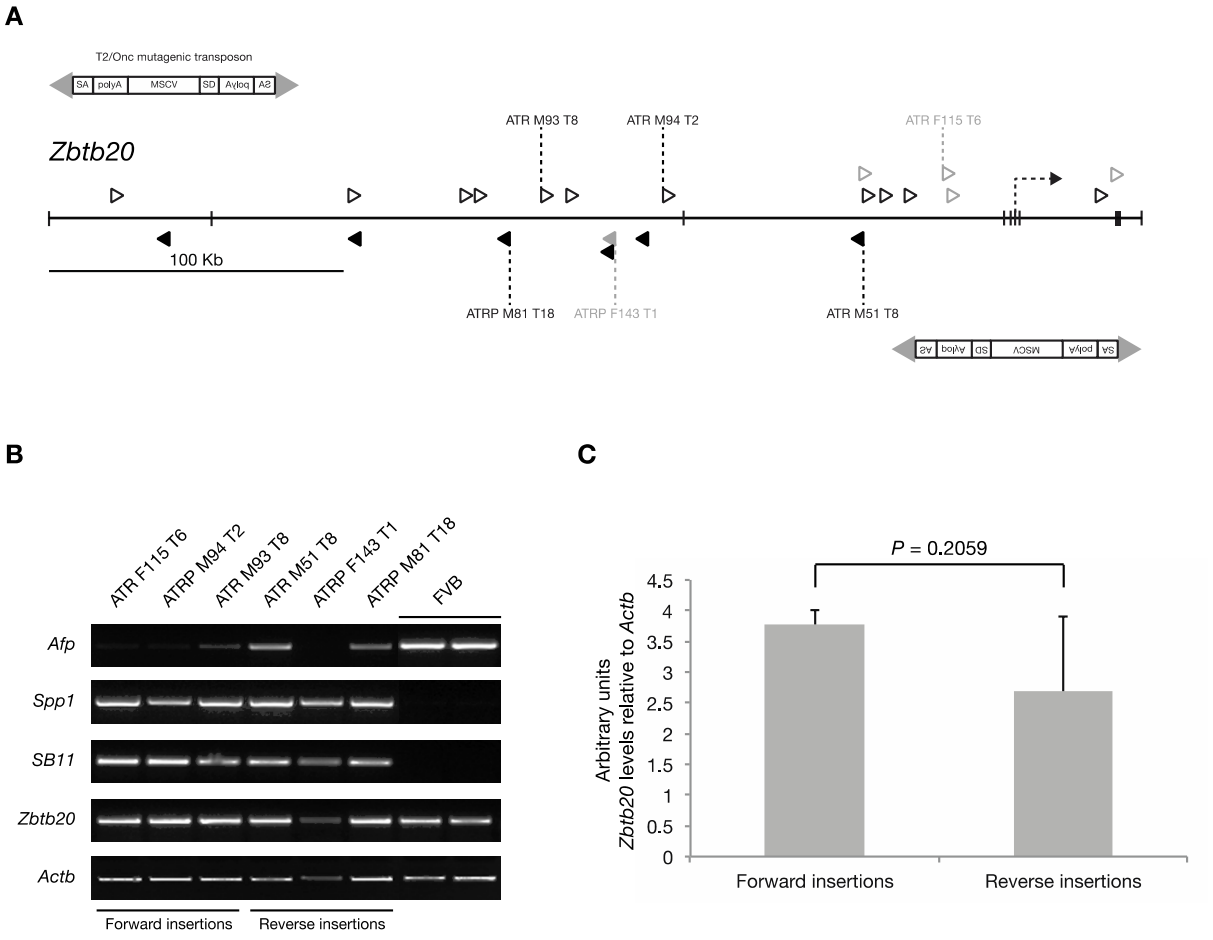
The pGL3-PPARG reporter plasmid was constructed to investigate the transcriptional role of ZBTB20 on the *PPARG* promoter. The putative binding motif for ZBTB20 has been previously identified as the following sequence – GATGTATA [9]. Interestingly, this binding motif is also found in the promoter region of *PPARG*. Therefore, 1038 bp promoter region of *PPARG* containing the putative binding site for ZBTB20 [10], was PCR amplified by high fidelity *Taq* polymerase from HHL7 genomic DNA using the following primer pair: forward 5'- GGTACCCACTCATGTGACAAGACCTGCTCC-3' and reverse 5'-GCTAGCAGCATGGAATAGGGGTTTGCTGTAATTC-3'. The 1038 bp promoter fragment was then directionally cloned into the *Kpn*I and *Nhe*I sites of pGL3 basic vector (Promega) to obtain the expression vector pGL3-PPARG. Sanger sequencing was performed to ensure no mutations were introduced during PCR amplification. HHL7 cells previously transfected with either *ZBTB20* isoforms or OFP control were seeded (6 X 10^4 cells) onto 12-well plates overnight, before being transfected with 1 μg of pGL3-PPARG together with 0.1 μg of pRL-

TK (Promega) as an internal control for the normalization of transfection efficiency. After 24 hours post-transfection, luciferase activity was measured by a dual-luciferase reporter assay according to the manufacturer's instructions using the Glomax 20/20 Luminometer (Promega).

TOP/FOP-Flash luciferase reporter assay

The *CTNNB1* reporter plasmids, consisting of M50 Super 8x TOP-Flash (Addgene plasmid #12456; http://n2t.net/addgene:12456; RRID:Addgene_12456) and its mutant control, M51 Super 8x FOP-Flash (Addgene plasmid #12457; http://n2t.net/addgene:12457; RRID:Addgene_12457) were gifts from Dr Randall Moon [11]. The TOP/FOP-Flash reporters, pRL-TK (Promega) and pPB/SB-Flag-*ZBTB20* were co-transfected into PLC/PRF/5 and Hep3B cells, at a density of 6 X 10^4 cells onto 12-well plates. After transfection for 24 to 48 hours, the cells were harvested for luciferase measurements using Dual-Luciferase Reporter Assay System (Promega) and the luciferase activity was measured by Glomax 20/20 Luminometer (Promega). Relative luciferase activities were expressed as the ratio of TOP-flash/FOP-flash luciferase activity and normalized against pRL-TK luciferase activity.

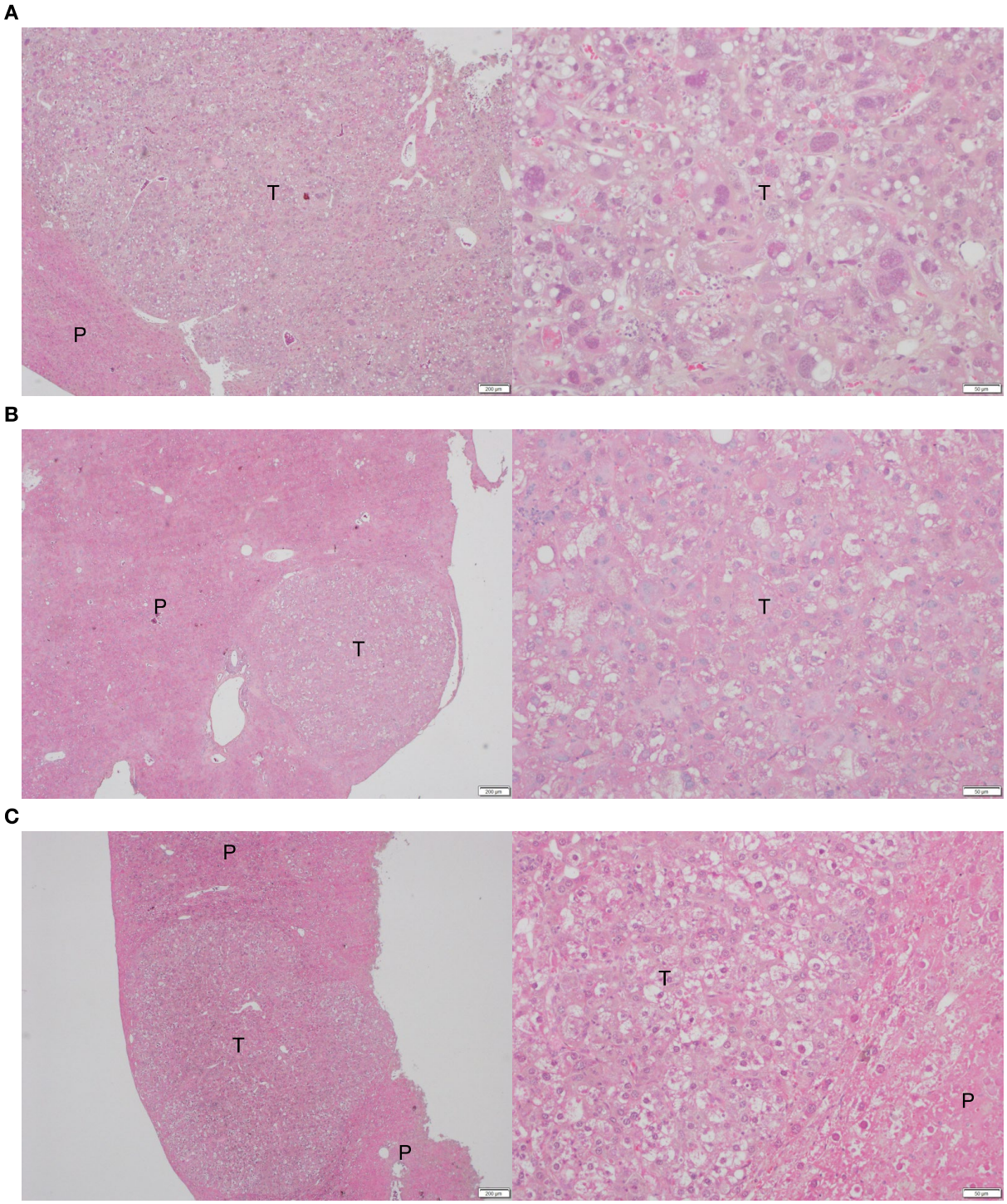
Fig. S1



Supplementary Fig. 1 Identification of *Zbtb20* as an oncogene involved in HCC tumorigenesis. (**A**) Diagrammatic representation of mutagenic transposon (T2/Onc) insertions into the *Zbtb20* gene. Transposon insertion profile indicates that *Zbtb20* functions as a putative oncogene in HCC tumorigenesis. Schematic representation of the T2/Onc mutagenic transposon is shown. Grey triangles, inverted repeats/direct repeats (IR/DR) transposon sequences flanking the cassette cargo containing gain-of-funtion and loss-of function DNA components; SA, splice acceptor; polyA, polyadenalytion signal; MSCV, LTR of the murine stem cell virus; SD, splice donor; open arrowhead, sense orientated insertion of the T2/Onc relative to the *Zbtb20* gene; arrowhead, anti-sense orientation insertion of the T2/Onc relative to the *Zbtb20* gene; black and grey arrowheads indicate transposon insertions

in either male or female animals, respectively. (**B**) Representatibe RT-PCR for various genes in livers taken from male and female liver tumors with transposon insertions in *Zbtb20*. *Afp*, alpha fetoprotein; *Spp1*, secreted phosphoprotein 1; *SB11*, Sleeping Beauty transposase; *Zbtb20*, endogenous *Zbtb20*; *Actb*, actin beta; FVB, normal livers taken from 12-day old wild-type FVB/N mice. (**C**) Arbitary expression level of *Zbtb20* relative to *Actb* was obtained using the ImageJ showed a non-significant trend towards higher expression in tumors with transposon insertions in a forward configuration (forward insertions) relative to the *Zbtb20* translational start site compared to transposon insertions ina reverse configuration (reverse insertions).

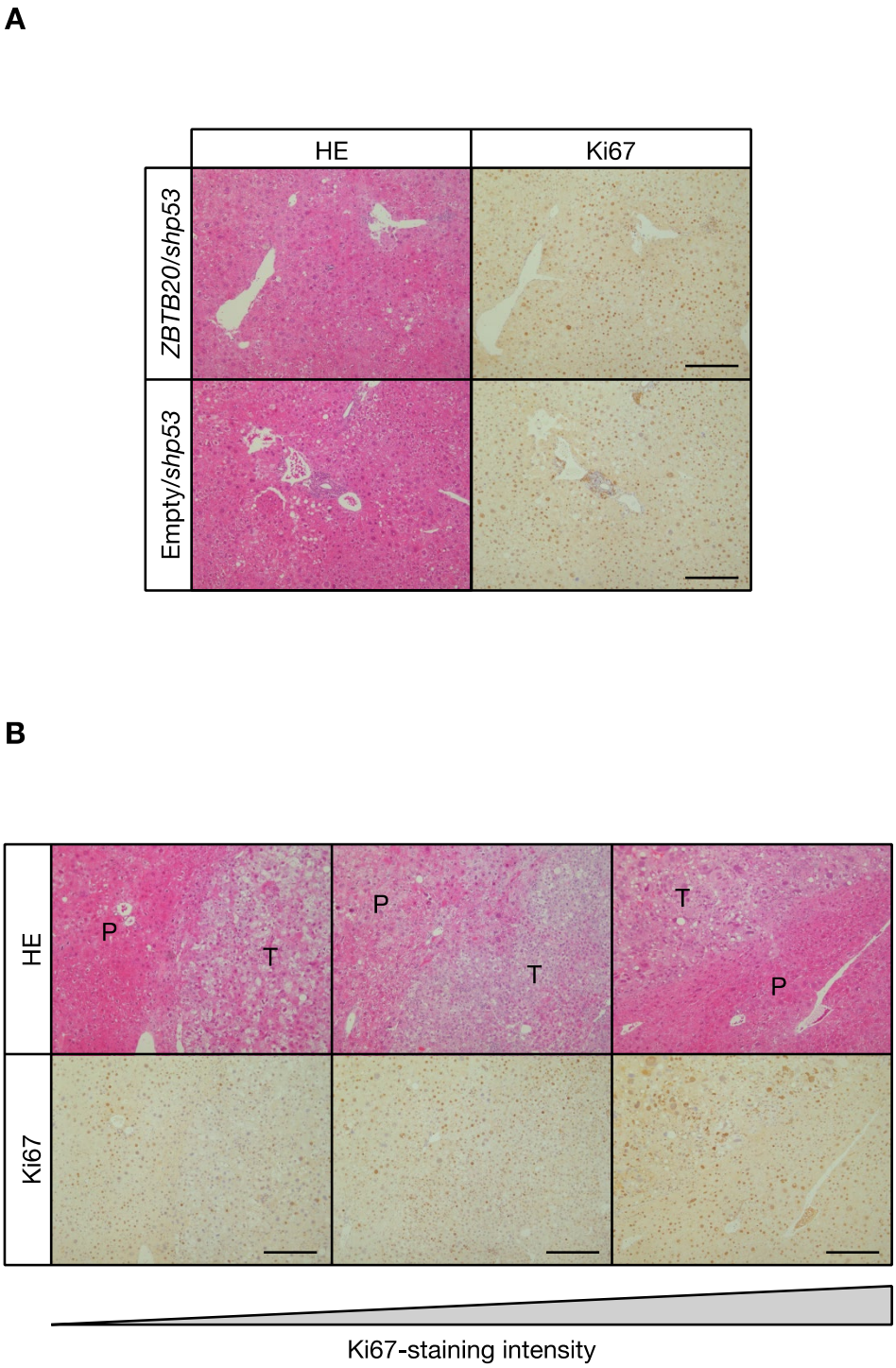
Fig. S2



Supplementary Fig. 2 Representative liver tumors from *ZBTB20/shp53 Fah*/SB11 mice at 120-days post-hydrodynamic tail vein injection. (**A**) Liver tumor nodule from

Fah/SB11 M1582 at 4X (left) and 20X (right) magnification. Histologically, the liver tumor can be classified as HCC. (**B**) Liver tumor nodule from *Fah*/SB11 M1584 at 4X (left) and 20X (right) magnification. Histologically, the liver tumor can be classified as well-differentiated hepatic neoplasm. (**C**) Liver tumor nodule from *Fah*/SB11 M1752 at 4X (left) and 20X (right) magnification. Histologically, the liver tumor can be classified as well-differentiated hepatic neoplasm. T, tumor nodule; P, parenchymal liver cells; scale bars, 200 μm for left panels and 50 μm for right panels.

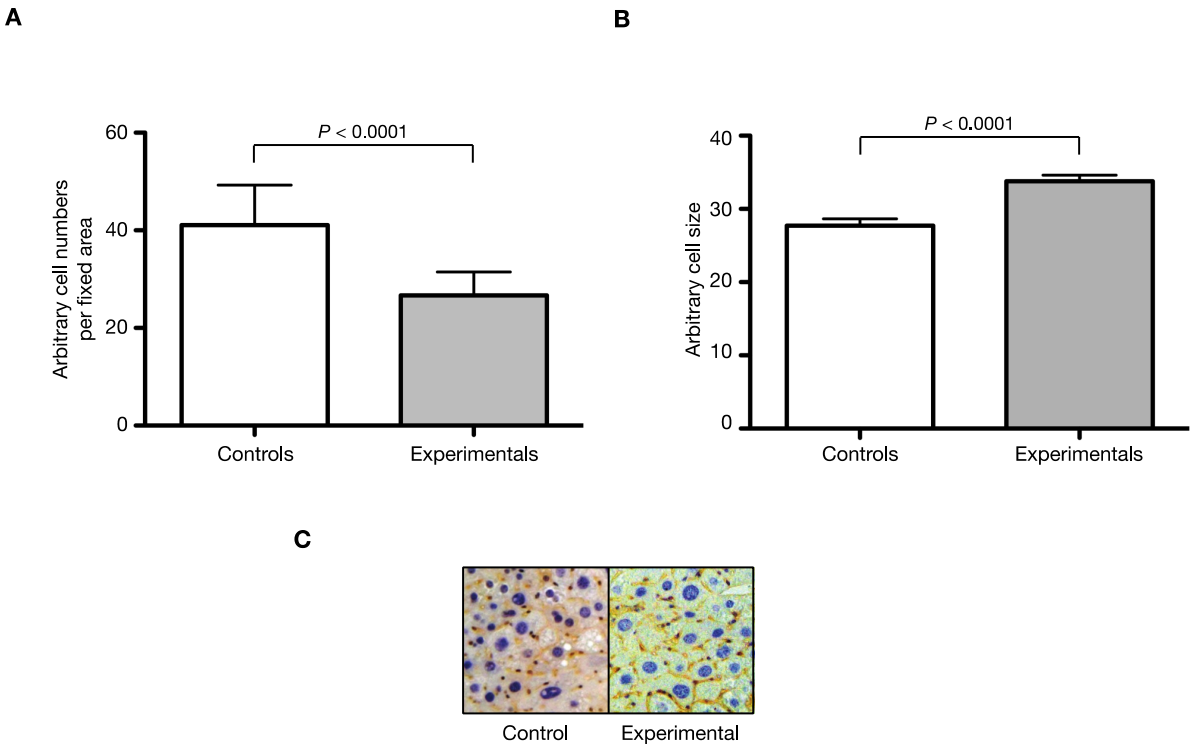
Fig. S3



Supplementary Fig. 3 Cellular proliferation and liver tumorigenesis in *ZBTB20*/*shp53* injected *Fah*/SB11 mice. (**A**) Representative serial HE (left panels) and Ki67

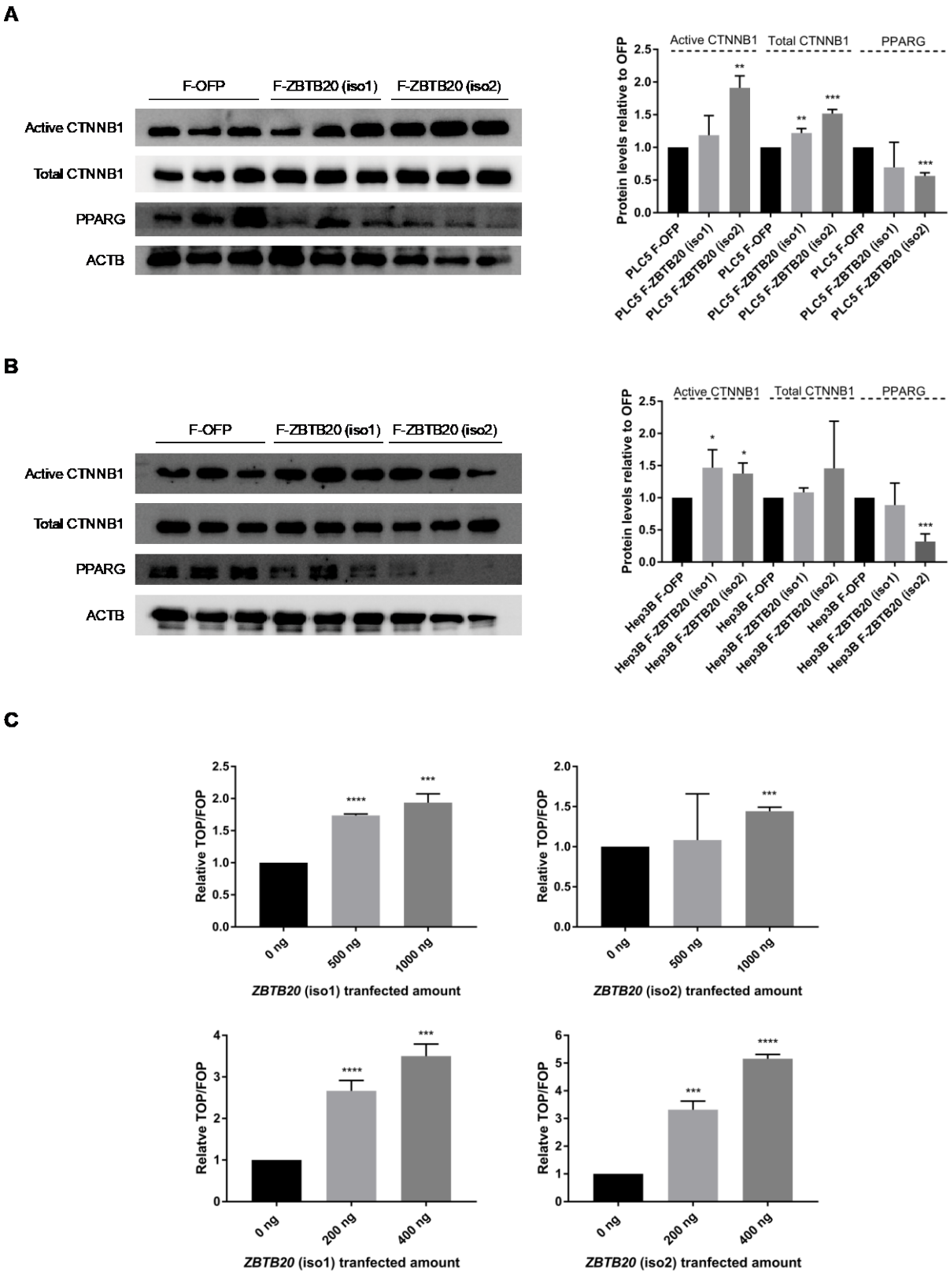
(right panels) staining showing similar high mitotic index were observed in both *ZBTB20*/*shp53* and Empty/*shp53 Fah*/SB11 injected livers. (**B**) Representative serial HE (top panels) and Ki67 (bottom panels) staining showing various levels of Ki67 immunoreactivity were observed in *Fah*/SB11 liver tumors injected with *ZBTB20*/*shp53*. P, parenchymal liver cells; T, tumor nodule. HE, hematoxylin-eosin staining; Ki67, cellular marker for proliferation; all scale bars, 250 μm.

Fig. S4



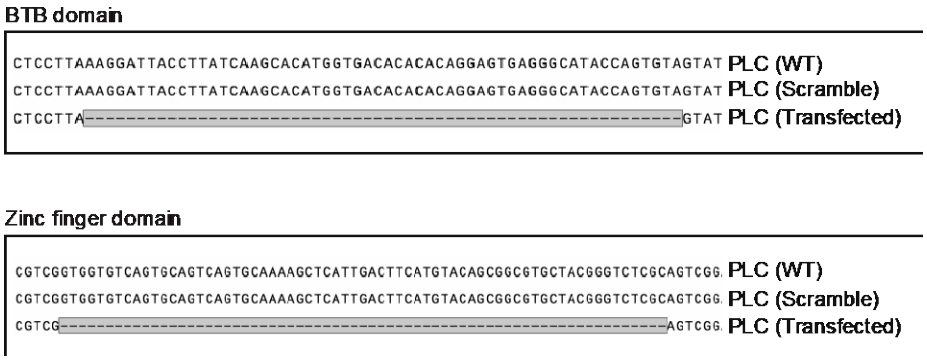
Supplementary Fig. 4 Hepatic hypertrophy observed in *ZBTB20*/*shp53* injected animals. (**A**) Evidence of hypertrophy seen in *ZBTB20*/*shp53* injected experimental animals with less cells counted per field of view than Empty/*shp53* injected control cohorts. *P*, unpaired *t*-test. (**B**) Enlarged hepatocytes confirmed in *ZBTB20*/*shp53* injected experimental animals. *P*, unpaired *t*-test. (**C**) Representative enlarged images of *ZBTB20*/*shp53* and Empty/*shp53* injected cohorts, showing fewer but enlarged cells in *ZBTB20*/*shp53* animals. Each individual image grid is 190 µm square.

Fig. S5


A
F-OFP
F-ZBTB20 (iso1)
F-ZBTB20 (iso2)
Active CTNNB1
Total CTNNB1
PPARG
ACTB
Active CTNNB1
Total CTNNB1
PPARG
Protein levels relative to OFP
PLC5 F-OFP
PLC5 F-ZBTB20 (iso1)
PLC5 F-ZBTB20 (iso2)
B
F-OFP
F-ZBTB20 (iso1)
F-ZBTB20 (iso2)
Active CTNNB1
Total CTNNB1
PPARG
ACTB
Active CTNNB1
Total CTNNB1
PPARG
Protein levels relative to OFP
Hep3B F-OFP
Hep3B F-ZBTB20 (iso1)
Hep3B F-ZBTB20 (iso2)
C
Relative TOP/FOP
0 ng
500 ng
1000 ng
ZBTB20 (iso1) tranfected amount
ZBTB20 (iso2) tranfected amount
Relative TOP/FOP
0 ng
200 ng
400 ng
ZBTB20 (iso1) tranfected amount
ZBTB20 (iso2) tranfected amount

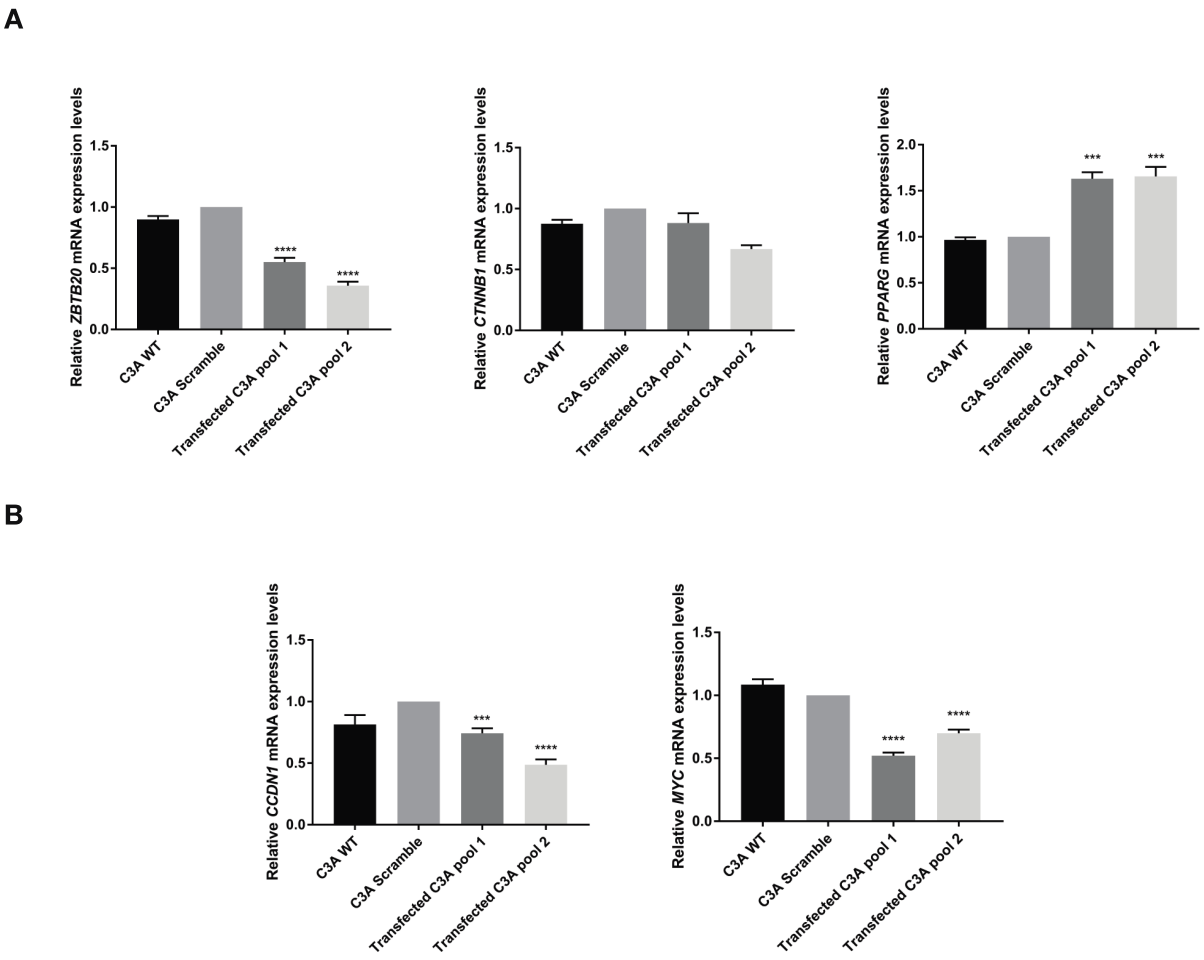
Supplementary Fig. 5 ***In vitro*** **overexpression of** ***ZBTB20*** **and its effect on the WNT/CTNNB1 pathway**. (**A**) Representative Western blot and relative protein levels of active CTNNB1, total CTNNB1, PPARG and ACTB in PLC/PRF/5 (PLC5) transfected cells. (**B**) Representative Western blot and relative protein levels of active CTNNB1, total CTNNB1, PPARG and ACTB in Hep3B transfected cells. ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$. (**C**) Representative TOP/FOP-Flash assay for WNT/CTNNB1 signaling showing dose-dependent increase in TOP/FOP activity in both Hep3B (top) and PLC/PRF/5 (bottom) cells transfected with indicated concentrations of ZBTB20 isoforms. ****, $P < 0.00005$; ***, $P < 0.0005$.

Fig. S6



Supplementary Fig. 6 ***In vitro*** **disruption of endogenous *ZBTB20* in human liver cancer cell line PLC/PRF/5 (PLC)**. Detection of knockout regions in PLC genomic DNA using specific primer pairs. Sequencing results indicated deletion of BTB (top) and zinc finger (bottom) domains in *ZBTB20* by gRNA1 and gRNA2, as well as gRNA4 and gRNA5, respectively. WT, wild-type PLC cells; Scramble, PLC cells transfected with pSpCas9-scramble vector containing non-targeting control gRNA sequence; Transfected, PLC cells transfected with pSpCas9 vectors containing the relevant gRNAs designed to either target the BTB or DNA-binding zinc finger domains of *ZBTB20*.

Fig. S7

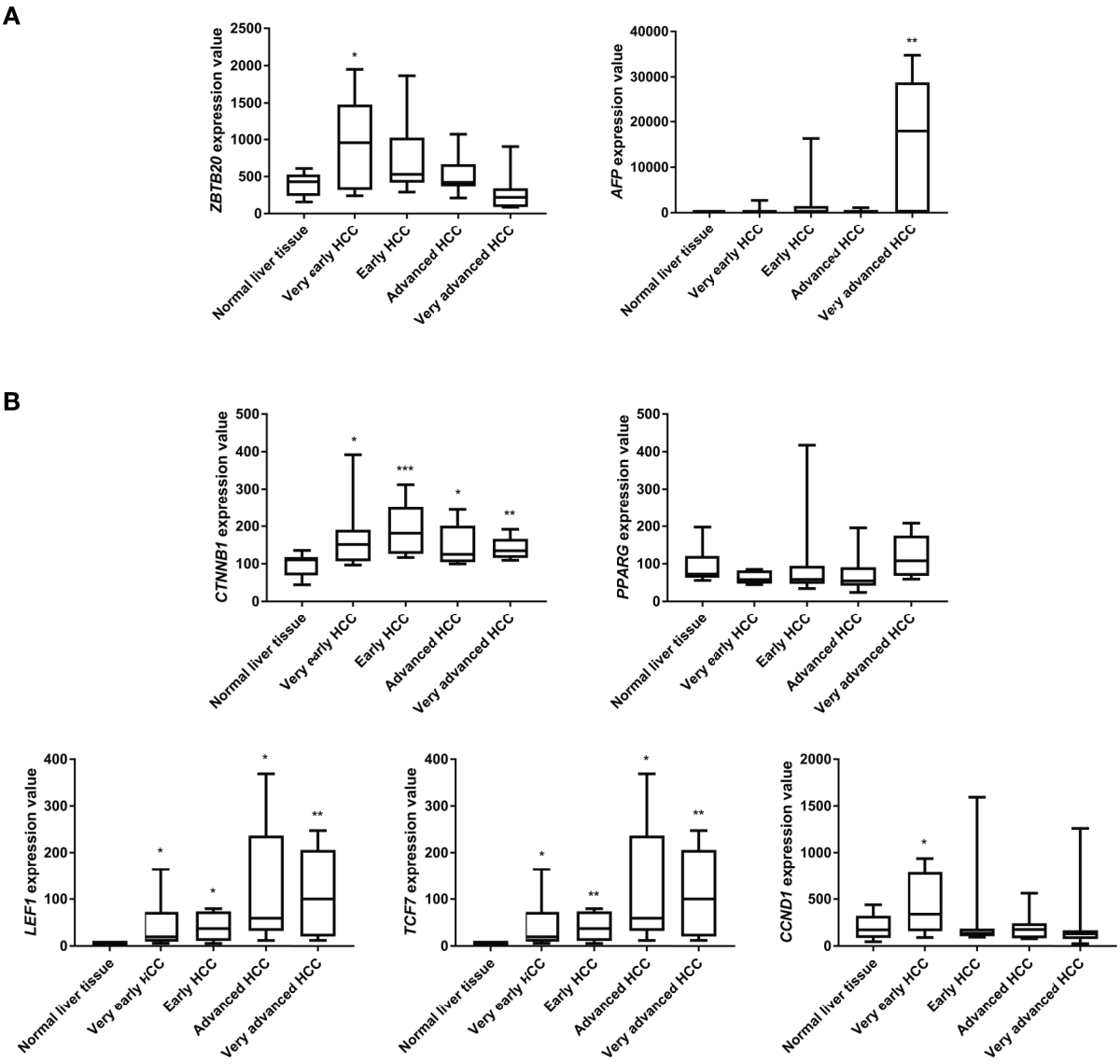


Supplementary Fig. 7 ***In vitro*** **disruption of *ZBTB20* in C3A human liver cancer line and its effect on the WNT/CTNNB1 signaling pathway**. (**A**) Relative mRNA expression levels of *ZBTB20*, *PPARG* and *CTNNB1* in *ZBTB20* disrupted C3A cells. (**B**) Relative mRNA expression levels of downstream target genes of the WNT/CTNNB1 signaling pathway in *ZBTB20* disrupted C3A cells. ****, $P < 0.00005$; ***, $P < 0.0005$.

Fig. S8

Supplementary Fig. 8 *PPARG* promoter sequence (chromosome 3p25.2, 12392051 to 12393088) with the putative binding motif for ZBTB20 (12392366 to 12392374) highlighted (UCSC Genome Browser, GRCh37/hg19).

Fig. S9



Supplementary Fig. 9 Expression levels of *ZBTB20*, *PPARG* and *CTNNB1* target genes in human HCC specimens using the online database Gene Expression Omnibus (GEO) for comparison. The GSE6764 dataset contains gene expression profiles of 75 tissue samples covering four neoplastic stages (very early HCC, early HCC, advanced HCC and very advanced HCC) from HCC patients comparing with normal liver as control. (**A**) Higher expression of *ZBTB20* was detected at very early stages of HCC while no significant changes in *AFP* was detected. (**B**) Activation of WNT signaling pathway at very early stages of the

disease via the upregulation of *CTNNB1* and its targets genes. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ were determined the two-tailed unpaired *t*-tests compared with the normal liver tissue control.

Table S1 Guide RNAs (gRNAs) designed to target *ZBTB20* functional domains.

gRNA	**Sequences (5' to 3')**	**Target domain**
gRNA1	CAGCGACATCGATCCCGT**CGG**	BTB
gRNA2	CGTGCTACGGGTCTCGCAGT**CGG**	
gRNA3	AACAGAACTACGTCAAGCA**TGG**	Zinc finger
gRNA4	AACCGCGAGGAAGAGGAATT**AGG**	
gRNA5	GTGTAGTATCTGCAACAAGC**TGG**	

PAM sequences are underlined in bold.

Table S2 Primers used to identify indels in *ZBTB20*-disrupted cells.

Primers	**Target domain**	**Sequence (5' to 3')**
Primer 1	BTB	CGAGCGCATTCACAGCATCAACCTT
Primer 2		GAACACATCGCCCACGTTCT
Primer 3	Zinc finger	CCGCCAAACAGAACTACGTC
Primer 4		CCAGGAGGGTCTTGTGAGAGA
Primer 5		CAGCCCTCTTCACTACCCAG

Table S3 Tumor multiplicity and liver weight percentages of injected cohorts.

ZBTB20*/*shp53	**PHI**	**Liver weight (%)**	**No. of nodules**
Fah/SB11 M1582	120	7.2	5
Fah/SB11 M1584	120	6.4	7
Fah/SB11 M1751	120	6.5	5
Fah/SB11 M1752	120	7	5
Fah/SB11 M1753	120	6.4	4
Fah/SB11 M1761	120	6.4	3
Fah/SB11 M1762	120	6.6	5
Fah/SB11 M1764	120	6.5	2
ZBTB20	**PHI**	**Liver weight (%)**	**No. of nodules**
Fah/SB11 M1971	122	8.7	0
Fah/SB11 M1982	143	6.3	0
Fah/SB11 M1983	143	6.1	1
Empty/*shp53*	**PHI**	**Liver weight (%)**	**No. of nodules**
Fah/SB11 M982	130	5.2	0
Fah/SB11 M983	130	5.5	0
Fah/SB11 M984	131	4.7	0
Fah/SB11 M1991	157	5.6	0
Fah/SB11 M1992	157	4.9	0
Fah/SB11 M1993	160	5.5	0

PHI, days post-hydrodynamic injection; liver weight (%), percentage weight of liver to total weight of mouse; no. of nodules, total number of liver tumor nodules counted in whole liver.

Table S4 CTNNB1 immunohistochemical staining of experimental and control injected cohorts.

ZBTB20*/*shp53	**PHI**	**Nuclear staining**	**Cytoplasmic staining**
Fah/SB11 M1582	120	Pos	Neg
Fah/SB11 M1584	120	Pos	Neg
Fah/SB11 M1751	120	Pos	Neg
Fah/SB11 M1752	120	Pos	Neg
Fah/SB11 M1753	120	Pos	Neg
Fah/SB11 M1761	120	Pos	Neg
Fah/SB11 M1762	120	Pos	Neg
Fah/SB11 M1764	120	Pos	Pos
Empty/*shp53*	**PHI**	**Nuclear staining**	**Cytoplasmic staining**
Fah/SB11 M1991	157	Neg	Pos
Fah/SB11 M1992	157	Neg	Pos
Fah/SB11 M1993	160	Neg	Pos

Neg, no detectable staining in several fields of view; Pos, positive staining detectable in several fields of view.

Supplementary References

[1] Bell JB, Podetz-Pedersen KM, Aronovich EL, Belur LR, McIvor RS, Hackett PB. Preferential delivery of the Sleeping Beauty transposon system to livers of mice by hydrodynamic injection. Nature protocols 2007;2:3153-3165.

[2] Wangensteen KJ, Wilber A, Keng VW, He Z, Matise I, Wangensteen L, et al. A facile method for somatic, lifelong manipulation of multiple genes in the mouse liver. Hepatology 2008;47:1714-1724.

[3] Keng VW, Villanueva A, Chiang DY, Dupuy AJ, Ryan BJ, Matise I, et al. A conditional transposon-based insertional mutagenesis screen for genes associated with mouse hepatocellular carcinoma. Nat Biotechnol 2009;27:264-274.

[4] Keng VW, Tschida BR, Bell JB, Largaespada DA. Modeling hepatitis B virus X-induced hepatocellular carcinoma in mice with the Sleeping Beauty transposon system. Hepatology 2011;53:781-790.

[5] Keng VW, Sia D, Sarver AL, Tschida BR, Fan D, Alsinet C, et al. Sex bias occurrence of hepatocellular carcinoma in Poly7 molecular subclass is associated with EGFR. Hepatology 2013;57:120-130.

[6] Clayton RF, Rinaldi A, Kandyba EE, Edward M, Willberg C, Klenerman P, et al. Liver cell lines for the study of hepatocyte functions and immunological response. Liver international : official journal of the International Association for the Study of the Liver 2005;25:389-402.

[7] Chiu AP, Tschida BR, Sham TT, Lo LH, Moriarity BS, Li XX, et al. HBx-K130M/V131I Promotes Liver Cancer in Transgenic Mice via AKT/FOXO1 Signaling Pathway and Arachidonic Acid Metabolism. Mol Cancer Res 2019;17:1582-1593.

[8] Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc 2013;8:2281-2308.

[9] Yin Y, Morgunova E, Jolma A, Kaasinen E, Sahu B, Khund-Sayeed S, et al. Impact of cytosine methylation on DNA binding specificities of human transcription factors. Science 2017;356.

[10] Fajas L, Auboeuf D, Raspe E, Schoonjans K, Lefebvre AM, Saladin R, et al. The organization, promoter analysis, and expression of the human PPARgamma gene. J Biol Chem 1997;272:18779-18789.

[11] Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Current biology : CB 2003;13:680-685.