Supporting Information

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SI Results

Depletion of β-Catenin from Postnatal Mature Hepatocytes of Alb-Cre;Ctnnb1$^{flx/flx}$ Mice. To generate KO mice with β-catenin–free mature hepatocytes, we crossed *Ctnnb1*$^{flx/flx}$ mice with *Alb-Cre* mice. Tail DNA of the offspring was genotyped by using primer sets designed for detection of Tg *Alb-Cre* and for differentiating the WT, floxed, and floxdel alleles of *Ctnnb1* gene (Fig. S1*A*). In addition, the liver DNA was extracted from 2-mo-old mice to verify the recombination event, with the floxdel alleles clearly detected in mice Tg for *Alb-Cre* (Fig. S1*B*, lanes 2 and 3).

The extent of β-catenin deletion in mature hepatocytes was evaluated by Western blotting with the proteins extracted from the liver of 2-mo-old mice. The results showed an approximately 90% decrease of β-catenin level compared with that of age-matched WT mice (Fig. S1*C*). To test whether the residual β-catenin was derived from the nonparenchymal β-catenin(+) Kupffer or endothelial cells, we purified hepatocytes from 2-mo-old KO mice by collagenase perfusion of the liver, a technique able to enrich hepatocytes to more than 95% purity (1). β-Catenin was almost undetectable in the purified hepatocytes (Fig. S1*D*), validating the efficient depletion of β-catenin from mature hepatocytes in this mouse model.

Replacement Also Occurs in Liver of β-Catenin–KO Mice After PHx. To examine if the replacement event also occurs in the liver of β-catenin–KO mice that underwent PHx, we compared the regeneration of hepatocytes in two-thirds PHx livers from 2-mo-old WT mice and β-catenin–KO mice (in which β-catenin was absent from >99% of hepatocytes). The liver recovery rate was significantly lower in KO mice than in WT mice (Fig. S3*A*). The liver of WT mice recovered to its original size within 7 d of surgery, whereas recovery in the KO mice was only 25% of that in the WT mice 7 d after PHx. Of note, the ratio started to increase in the KO mice from 3 to 4 mo after PHx and was close to that of WT mice 7 mo after surgery (Fig. S3*A*).

The liver tissue was collected at various times after PHx for IHC staining with β-catenin antibody. β-Catenin(+) hepatocytes started to appear 2 mo after PHx (Fig. 3*B*, arrows) and the population gradually increased to approximately 90% of hepatocytes at 7 mo after PHx (Fig. S3 *B* and *C*), which was also supported by the Western blot results (Fig. S3*D*).

SI Materials and Methods

Protein Extraction and Western Blot Analysis. The protein lysate from the liver tissue was prepared by lysing 8M urea containing fresh complete EDTA-free protease inhibitor mixture (Roche) and Phosphatase Inhibitor Mixture Set II (Calbiochem). Approximately 20 to 100 μg of protein lysate was used for the 10% SDS/PAGE electrophoresis and subsequent Western blotting analysis by following the standard protocols described previously (2).

Antibodies. Primary antibodies used in this study are as follows: anti–β-catenin Ab was purchased from BD Bioscience; anti-CK19 (sc-33111) and anti-GFP Ab (sc-9996) were purchased from Santa Cruz Biotechnology; and anti-albumin Ab (ab19194) was purchased from Abcam. Anti-OV6 Ab was a gift from Chen Hui-Ling (National Taiwan University Hospital, Taipei, Taiwan); anti-Ep-CAM Ab (GTX61060) was purchased from GeneTex; anti-Ki67 Ab (550609) was purchased from BD Bioscience; and anti–β-actin Ab (A 5441) was purchased from Sigma-Aldrich. Horseradish peroxidase-conjugated secondary Abs were purchased from GeneTex; and FITC-conjugated or rhodamine red-conjugated secondary Abs were purchased from Invitrogen.

IHC Analysis. Liver tissues were freshly fixed in 10% formalin after being harvested from the mice, and then embedded in paraffin for subsequent tissue section preparation. Liver sections at 3 μm were prepared and used for IHC staining, with the signal detected by the N-Histofine Polymer Detection System Kit (Nichirei Bioscience) according to the manufacturer's instructions.

Serum Biochemical Test. The serum samples collected from the mice were processed for evaluation of ALT, total bilirubin, and albumin by DRI-CHEM 3500s (Fujifilm).

RNA Extraction and Quantitative RT-PCR. Total RNA was isolated from liver tissues with TRIzol reagent (Rezol C and PROtech). Two micrograms of RNA was reverse-transcribed to cDNA by using the SuperScript III One-Step RT-PCR System (SuperScript III First-Strand Synthesis System; Invitrogen). Quantitative real-time PCR was conducted by LightCycler FastStart DNA Master SYBR Green I Kit (Roche), using a LightCycler PCR machine (Roche), with details as described previously (3). The primer sets used for the quantitative PCR in the current study are summarized in Table S1.

PHx. To induce liver regeneration, the mice were processed for two-thirds PHx by removal of left lateral and medium lobe of liver with details as described previously (4). The recovering liver tissues were then harvested at different time points after PHx as indicated, with the serum samples collected at the same time for further analysis.

1. Klaunig JE, et al. (1981) Mouse liver cell culture. I. Hepatocyte isolation. *In Vitro* 17: 913–925.
2. Yang WJ, et al. (2009) Hepatitis B virus X protein enhances the transcriptional activity of the androgen receptor through c-Src and glycogen synthase kinase-3beta kinase pathways. *Hepatology* 49:1515–1524.
3. Wang SH, et al. (2009) Identification of androgen response elements in the enhancer I of hepatitis B virus: a mechanism for sex disparity in chronic hepatitis B. *Hepatology* 50: 1392–1402.
4. Wu BK, et al. (2006) Blocking of G1/S transition and cell death in the regenerating liver of Hepatitis B virus X protein transgenic mice. *Biochem Biophys Res Commun* 340: 916–928.

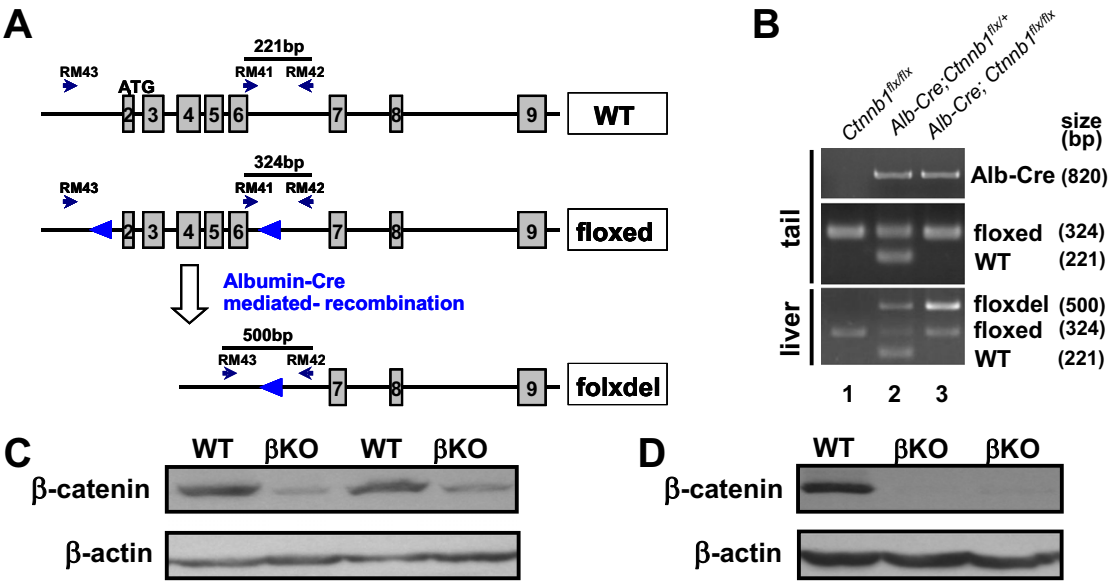

Fig. S1. Efficient deletion of β-catenin from mature hepatocytes of β-catenin–KO mice. (*A*) The floxed sites in the floxed and the floxdel alleles are indicated by arrowheads. The primer mixtures of RM41, RM42, and RM43 were used for genotyping (as indicated by small arrows), with the amplicon size indicated above the primer sets. The successful deletion of β-catenin in the liver was verified by genotyping (*B*) and Western blot analysis (*C*). (*D*) The hepatocytes purified by liver perfusion technology were processed for Western blotting by probing with antibodies as indicated.

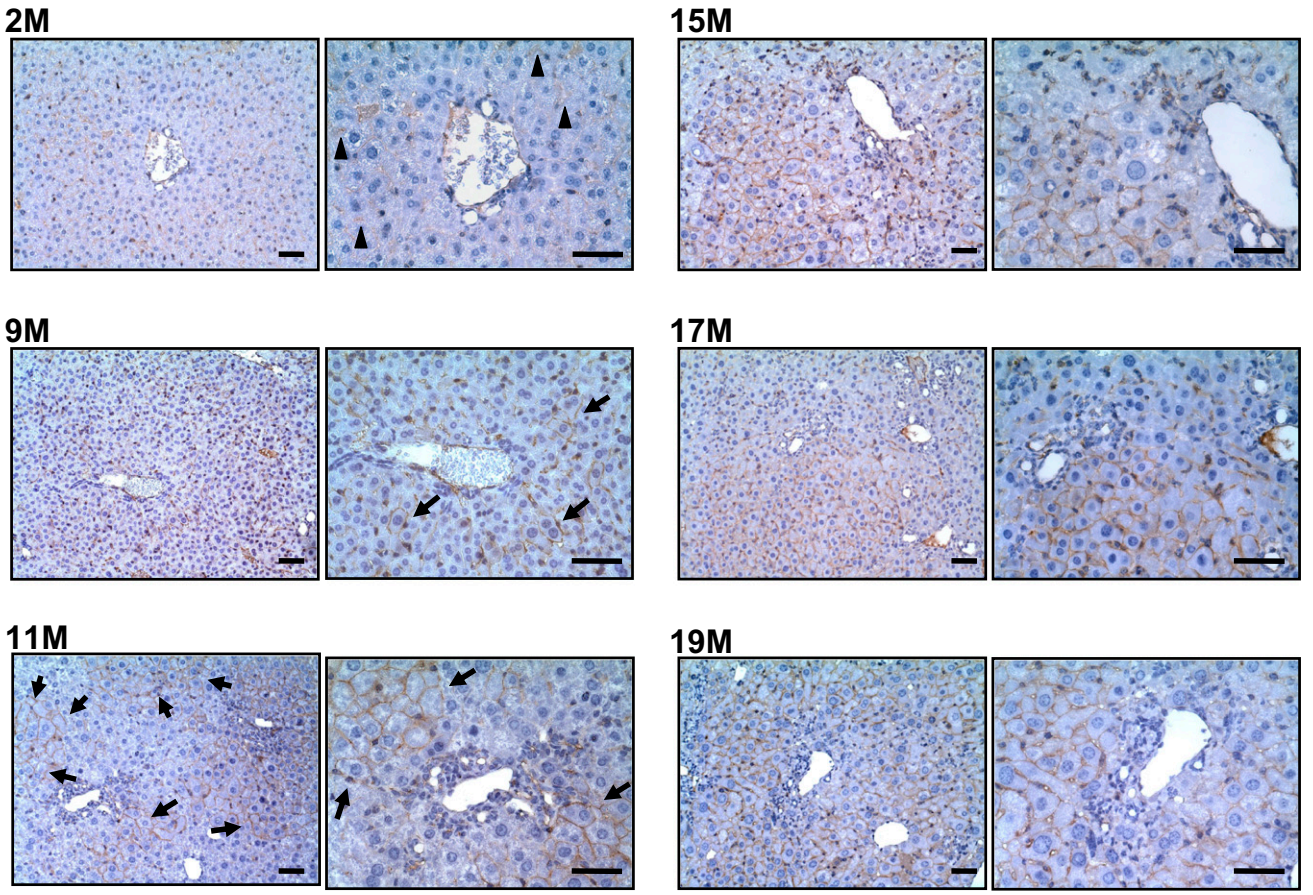

Fig. S2. The replacement of β-catenin(–) hepatocytes by β-catenin(+) hepatocytes in β-catenin–KO liver by IHC staining. Liver tissues collected from the KO mice at different ages as indicated were processed for IHC staining with β-catenin antibody. The β-catenin was successfully deleted in hepatocytes at 2 mo (2M) of age, with only some β-catenin(+) nonparenchymal cells (arrowhead). Some β-catenin(+) hepatocytes started to appear in the liver, from the age of 9 mo (9M), as marked by arrows. After that, the percentage of the β-catenin(+) hepatocytes gradually increased, and reached greater than 90% at the end of follow up. (Scale bar: 50 μM.)

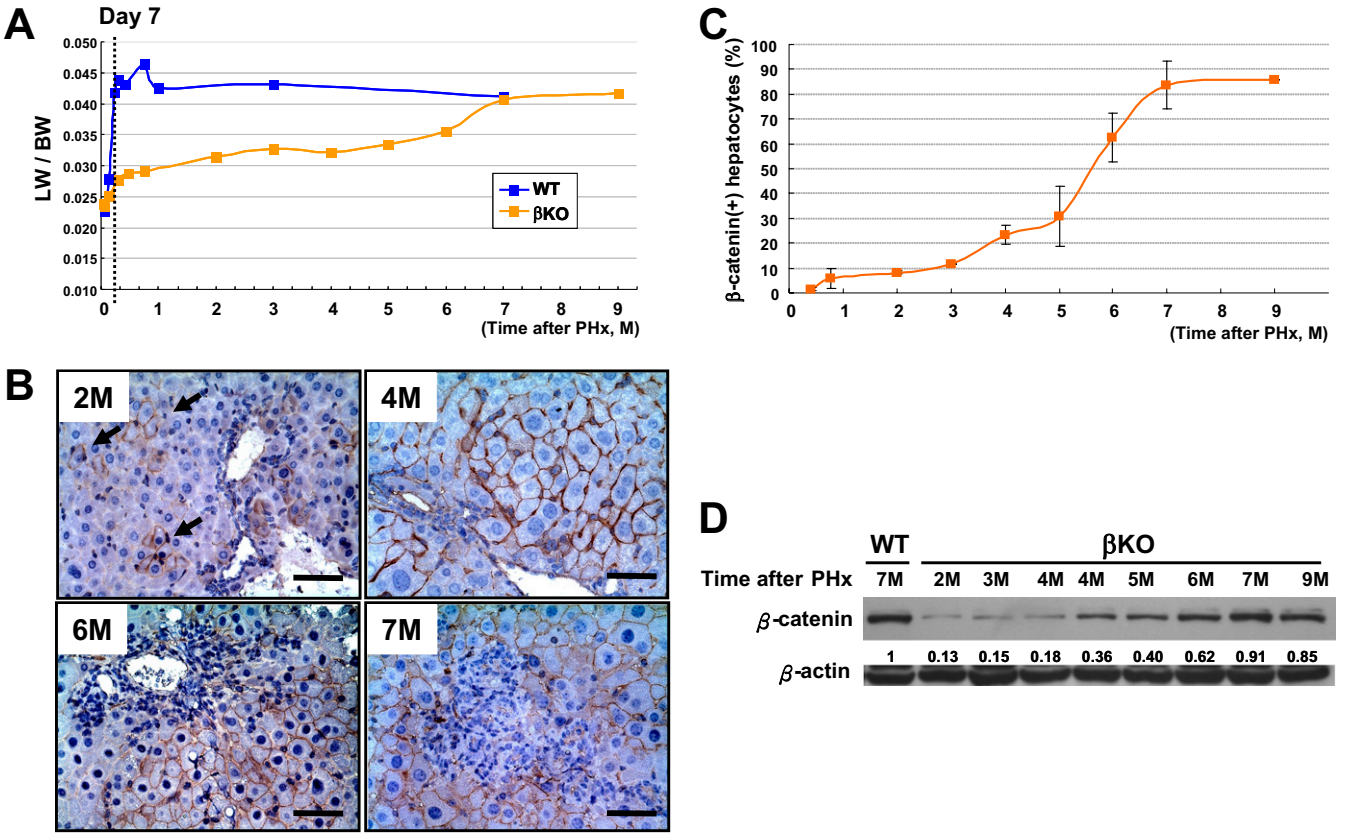

Fig. S3. Replacement of β-catenin(−) hepatocytes with β-catenin(+) hepatocytes occurred in hepatectomized livers of β-catenin–KO mice. (*A*) The LW/BW ratios of WT and β-catenin–KO mice at various times after PHx. (*B*) Serial liver sections at various times after PHx were stained with β-catenin antibody. (*C*) The percentage of β-catenin(+) hepatocytes at various times after PHx is illustrated in a quantitative manner. (*D*) Protein lysates extracted from the hepatectomized livers of WT (7 mo after PHx) and β-catenin–KO mice (at various times after PHx) were processed for Western blot analysis by probing with antibodies as indicated. (Scale bar: 50 μM.)

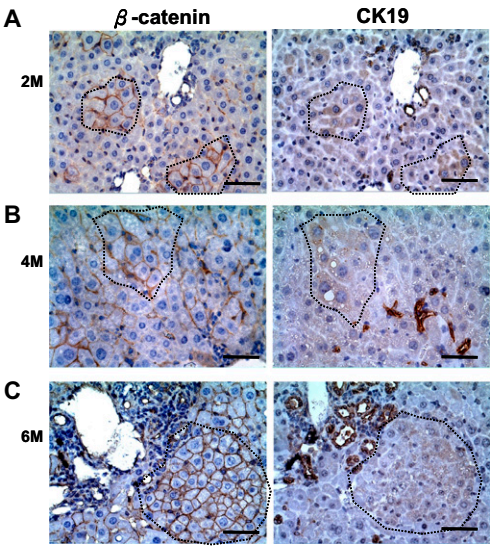

Fig. S4. The β-catenin(+) immature hepatocytes in the β-catenin–KO mice are CK19(+) and in close proximity to the bile duct/ductule cells in the regenerative liver. Two-month-old β-catenin–KO mice underwent a PHx and liver samples were collected after 2 mo (*A*), 4 mo (*B*), and 6 mo (*C*). Continuous liver tissue sections from each liver were stained with antibodies against β-catenin (*Left*) and CK19 (*Right*).

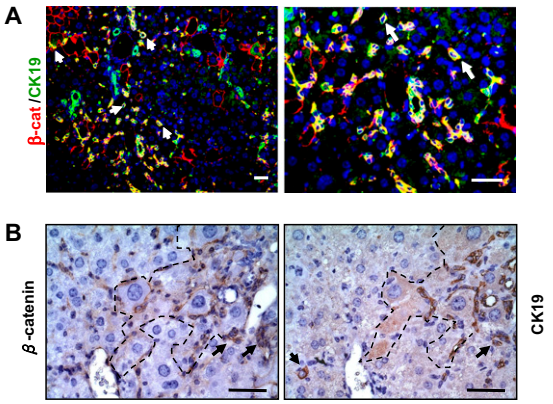

Fig. S5. Some hepatic oval cells in β-catenin–KO mice were in close proximity to the β-catenin(+) immature hepatocytes and the proliferating bile duct/ductule cells. (*A*) Representative results for double staining of liver tissues from one 15-mo-old β-catenin–KO mouse using β-catenin antibody (red) in combination with CK19 (green) antibody. The small ovoid cells with scant cytoplasm and a strong yellow signal indicating positivity for β-catenin and CK19 could be hepatic oval cells (indicated by white arrows). (*B*) Continuous liver tissue sections from the same mouse as in *A* were processed for IHC staining with antibodies against β-catenin (*Left*) and CK19 (*Right*). Some hepatic oval cells (black arrows) are located close to the cluster of immature hepatic progenitor cells (marked by dotted lines with membranous β-catenin and a weak CK19 signal). (Scale bar: 50 μM.)

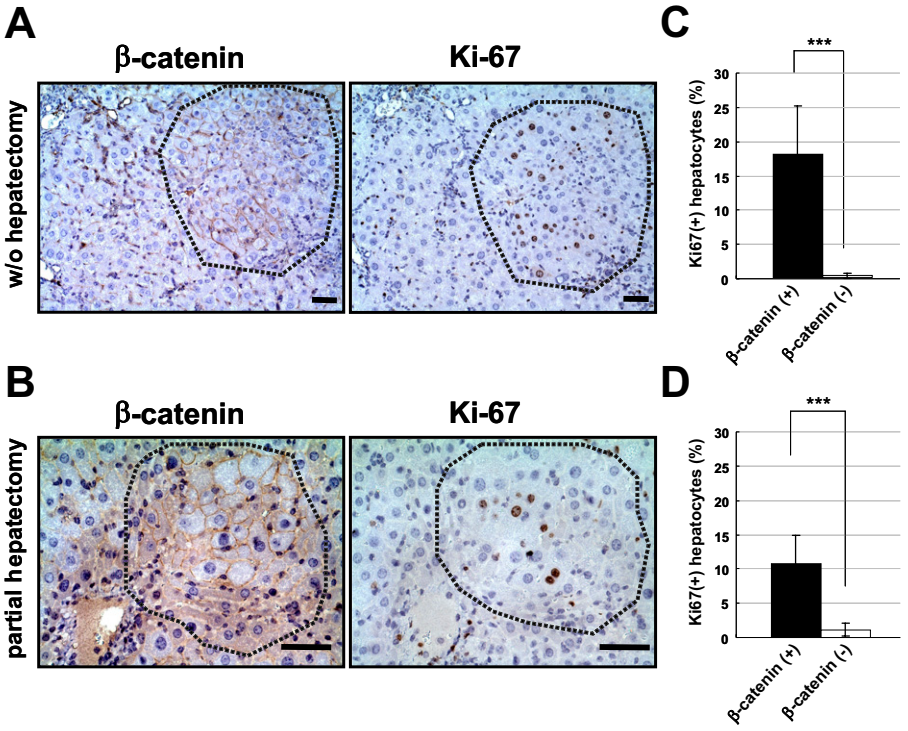

Fig. S6. The replication activity of β-catenin(–) hepatocytes is significantly lower than that of β-catenin(+) hepatocytes as revealed by IHC staining. Continuous liver tissue sections collected from one 14-mo-old β-catenin–KO mouse (*A*) and from one KO mouse that received PHx (6 mo after surgery) (*B*) were processed for IHC staining by using β-catenin and Ki-67 antibodies. The regions containing β-catenin(+) hepatocytes are indicated by dotted lines. (Scale bar: 50 μM.) Quantification results for the percentage of Ki67(+) hepatocytes in six β-catenin(+) vs. six β-catenin(+) clusters of cells from (*C*) a 14-mo-old β-catenin–KO mouse and (*D*) a KO mouse 6 mo after PHx. The mean percentage of each group of samples with SE bars are illustrated, and were statistically analyzed with a *t* test (***$P < 0.001$).

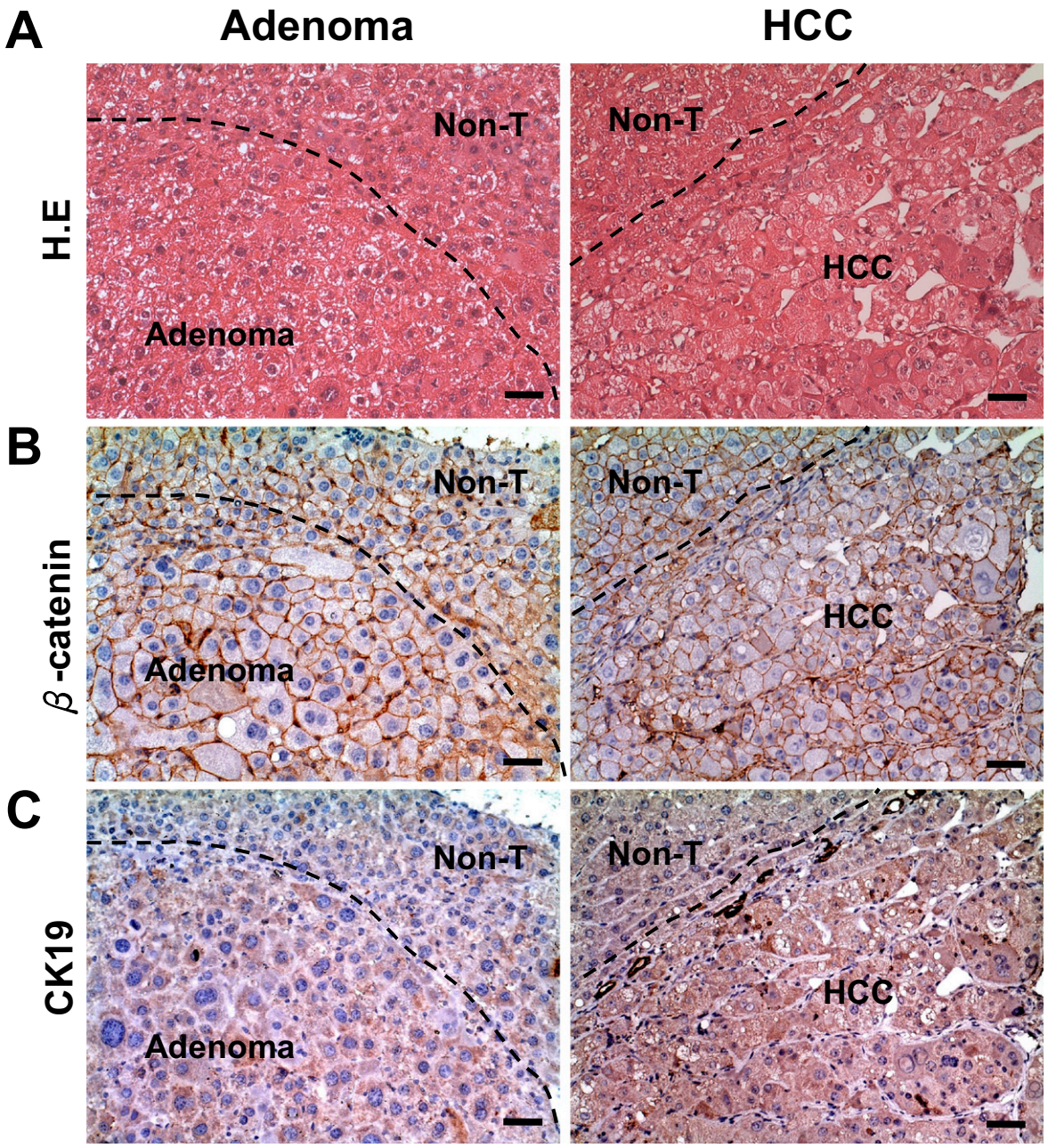

Fig. S7. Adenomas and HCCs developed in the livers of β-catenin–KO mice. Representative liver tissues collected from an adenoma from a 17-mo-old β-catenin–KO mouse and from an HCC from a 19-mo-old β-catenin–KO mouse were processed for H&E staining (*A*), and for IHC staining with antibodies against β-catenin (*B*) and CK19 (*C*). (Scale bar: 50 μM.)

Table S1. Primer sets used for quantitative PCR analysis

Name	Forward	Reverse
Mouse PBGD	5′-TGAAGGATGTGCCTACCATACTA-3′	5′-TCAAGAGTATTCGGGGAAACCTC-3′
Cre	5′-CGTACTGACGGTGGGAGAAT-3′	5′-CCCGGCAAAACAGGTAGTTA-3′
Mouse EpCAM	5′-TGGACCTGAGAGTGAACGGAG-3′	5′-ACCCATCTCCTTTATCTCAGCC-3′
Mouse H19	5′-TCCAGCCTTCTTGAACACCATG-3′	5′-TCAGAACGAGACGGACTTAAAG-3′
Mouse Prom1	5′-ATAAGAGCCATCCACCAGCATC-3′	5′-ACAGAGTCCAAAGAGGCAAGG-3′
Mouse CK19	5′-AGGTCAGTGTGGAGGTGGATTC-3′	5′-TCGGTCTTGCTTATCTGGATCTGC-3′
Mouse CK7	5′-TGGAGACGGAATGGGACCTGT-3′	5′-CCTGCGGGTAGTAGATGTGGTC-3′
Mouse CD44	5′-AGACTCATCCAAGGACTCCAGGG-3′	5′-AGGAGAGATGCCAAGATGATGAGC-3′
Mouse AFP	5′-ATGAAACCTATGCCCCTCCC-3′	5′-TCAGGCTTTTGCTTCACCAGG-3′