PNAS
www.pnas.org

Supplementary Information for

Lgr5^{+} pericentral hepatocytes are self-maintained in normal liver regeneration and susceptible to hepatocarcinogenesis

Chow Hiang Ang[a], Shih Han Hsu[a,b], Fusheng Guo[a], Chong Teik Tan[c], Victor C Yu[c], Jane E Visvader[d,e], Pierce KH Chow[f,g,h] and Nai Yang Fu[a,b,1]

Corresponding author: Nai Yang FU
Email: naiyang.fu@duke-nus.edu.sg

This PDF file includes:

Supplementary MATERIALS AND METHODS
Figures S1 to S7
Table S1

www.pnas.org/cgi/doi/10.1073/pnas.1908099116

Supplementary MATERIALS AND METHODS

Doxycycline and BrdU administration

For labelling of Lgr5 descendant hepatocytes in adult mice, Lgr5-rtTA/TetO-cre/R26-tdTomato mice received both doxycycline feed and drinking water containing 1 mg/ml doxycycline and 10 mg/ml sucrose for seven continuous days. The mice were supplied with normal feed and drinking water for recovery after doxycycline treatment for at least three days prior to any procedures. For lineage tracing of $Lgr5^+$ hepatocytes under homeostasis, Lgr5-rtTA/TetO-cre/R26-tdTomato mice received doxycycline feed and drinking water at the age of 21 days for seven continuous days, and then raised with normal feed and drinking water. For DEN-induced liver injury models, male pups at postnatal day 7 received doxycycline through lactating dams provided with doxycycline feed and drinking water for five continuous days. Lgr5-rtTA/TetO-Neu mice received continuous doxycycline feed from the age of 21 days onwards. BrdU was administered to the mice via drinking water at 1 mg/ml.

Flow cytometry analysis and FACS sorting

For flow cytometry and gene expression analysis, the GFP^+ and $Ecad^+$ hepatocytes were isolated from the liver of 8-12 weeks old Lgr5-rtTA-GFP male mice using a modified two-step collagenase perfusion technique. Briefly, mice were anesthetized with 2% isoflurane inhalation. An incision was made through the abdominal skin and muscle layer to access the liver. Inferior vena cava and portal vein were exposed by gently moving the intestine to the left of the mouse. A winged cannula was inserted into the portal vein and secured with a needle. The inferior vena cava was cut, and the liver was perfused with perfusion medium for ten minutes at 3.5 ml/minute, followed by collagenase solution containing type I collagenase for additional ten minutes at 5 ml/minute. After adequate digestion, the liver was removed and hepatocytes were dispersed into DMEM medium with 5% FBS on a petri dish, and passed through a 70 μm cell strainer. The cell suspension was centrifuged for five minutes at 50 x*g*. Following resuspension of cell pellet in DMEM medium with 5% FBS, hepatocytes were purified by Percoll gradient centrifugation. Hepatocytes suspension was incubated with anti-Ecad antibody prior to flow cytometry analysis and fluorescent activated cell sorting (FACS).

qPCR analysis

For gene expression analysis, total RNA extraction was performed on FACS-purified GFP^+, GFP^- $Ecad^-$ and $Ecad^+$ hepatocytes according to manufacturer's protocol (Qiagen). Total RNA was converted to cDNA using GoScript™ Reverse Transcription Kit (Promega). SYBR green qPCR master mix was used for quantitative RT-PCR. Standard delta-delta Ct ($2^{-\Delta\Delta Ct}$) method was used to estimate the expression of target genes with reference to the house keeping gene *Actin*. For copy number estimation of the transgene in the Lgr5-rtTA-IRES-GFP line, SYBR green qPCR master mix

was used for qPCR performed on genomic DNA extracted from a small piece of toe tissue obtained from young pups. Copy number of the Lgr5-rtTA-IRES-GFP transgene in BAC transgenic mice was estimated by using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method. Briefly, Ct value obtained from amplification of the proximal promoter region of *Lgr5* was normalized to Ct value of an unrelated reference genomic locus in Lgr5-rtTA-IRES-GFP BAC transgenic and wild type control mice. Copy number was estimated by calculating the relative fold change of Lgr5-rtTA-IRES-GFP cassette copy numbers in the transgenic mice to wild-type mice ($2^{-\Delta\Delta Ct}$). Two endogenous copies of endogenous *Lgr5* were then subtracted for the estimation of copy number of transgene. The sequences of PCR primers used for all qPCR analyses are listed in SI Appendix Table S1.

Histopathology and immunofluorescent staining

Liver tissues were dissected and fixed in 4% paraformaldehyde (PFA) for 24 hours at room temperature, followed by another 24 hours at 4 °C. Tissues were embedded in paraffin and the sections were cut at 4 μm for immunofluorescent staining. Slides were dewaxed at 60 °C for an hour, rehydrated through a descending series of ethanol to water. Heat-induced epitope retrieval was performed in a pressure cooker for 10 minutes at the highest pressure with slides immersed in sodium citrate solution (pH 6). Slides were cooled down on ice and washed with tap water for several times. After two washes with PBS containing 0.1% Triton X-100, the slides were blocked in PBS containing 10% horse serum, 0.1% Triton X-100 and 2% mouse on mouse (M.O.M) blocking reagent for 30 minutes. The slides were washed with PBS containing 0.01% Triton X-100 for three times and then incubated overnight with primary antibodies diluted in PBS containing 0.01% Triton X-100 and 1% horse serum at 4°C. Slides were washed with PBS containing 0.01% Triton X-100 for three times, and incubated with secondary antibodies in PBS containing 0.01% Triton X-100 and 1% horse serum at room temperature for one hour. After three washes of PBS containing 0.01% Triton X-100, slides were stained with DAPI in PBS. mounted with anti-fade mounting medium and stored in the dark at 4 °C for image analysis. Images were acquired with a Leica TCS SP8 confocal microscope. The primary antibodies used in this study are listed in SI Appendix Table S1.

Quantification and statistical analysis

Data are shown as mean ± standard error of the mean (s.e.m). The Student's t-test was used where applicable, with $p<0.05$ considered significant.

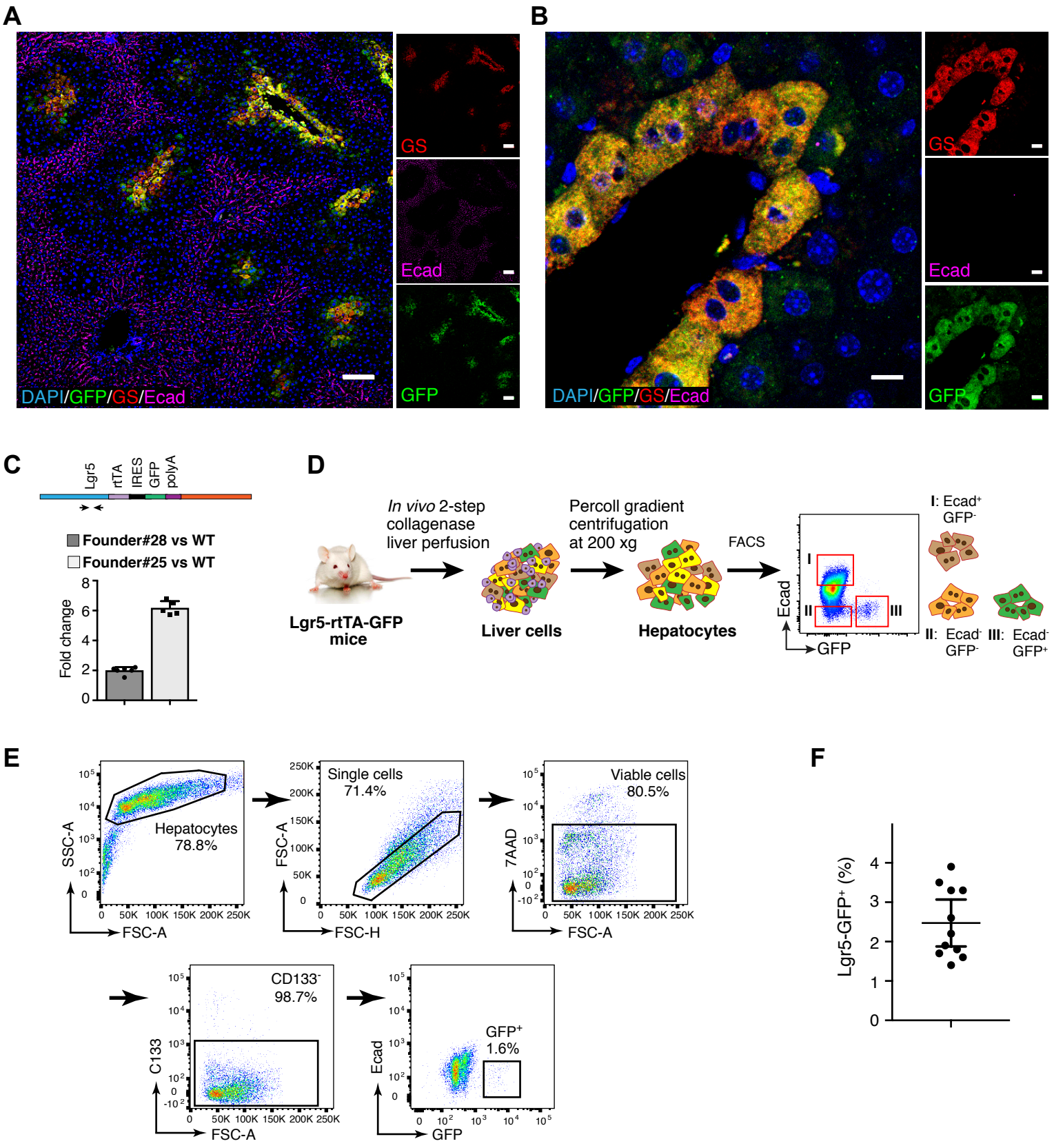

Fig. S1. Confocal microscopy, FACS, and transgene copy number analysis of Lgr5-rtTA-IRES-GFP mice

(A-B) Representative confocal images showing GFP expression in the liver of Lgr5-rtTA-IRES-GFP transgenic mice derived from the other independent founder. Compared with the data shown in Fig. 1, identical GFP expression patterns were observed in the two different Lgr5-rtTA-IRES-GFP BAC transgenic founders. Scale bars: 100 μm (A) and 10 μm (B). N = 4 mice.

(C) Estimation of transgene copy numbers in Lgr5-rtTA-IRES-GFP BAC transgenic mice from the two founders by qPCR. Diagram depicting amplification of the proximal promoter region in both the endogenous Lgr5 locus and BAC transgene by qPCR. The fold change corresponds to the total copy number of the proximal promoter region, which includes two copies for the endogenous Lgr5 locus. N = 5 mice. Error bars represent mean ± s.e.m.

(D) Graphic depiction of work flow for FACS isolation of three different hepatocyte subsets defined by the expression of Lgr5-GFP and Ecad from Lgr5-rtTA-IRES-GFP BAC transgenic mice.

(E) Representative FACS plot showing gating strategy to identify Lgr5-GFP$^+$ hepatocytes in Lgr5-rtTA-IRES-GFP transgenic mice. CD133 was used as a marker to exclude cholangiocytes contamination in the population of hepatocytes.

(F) Bar graph showing percentage of Lgr5-GFP$^+$ population detected by FACS. Error bars represent mean ± s.e.m. N = 11 mice.

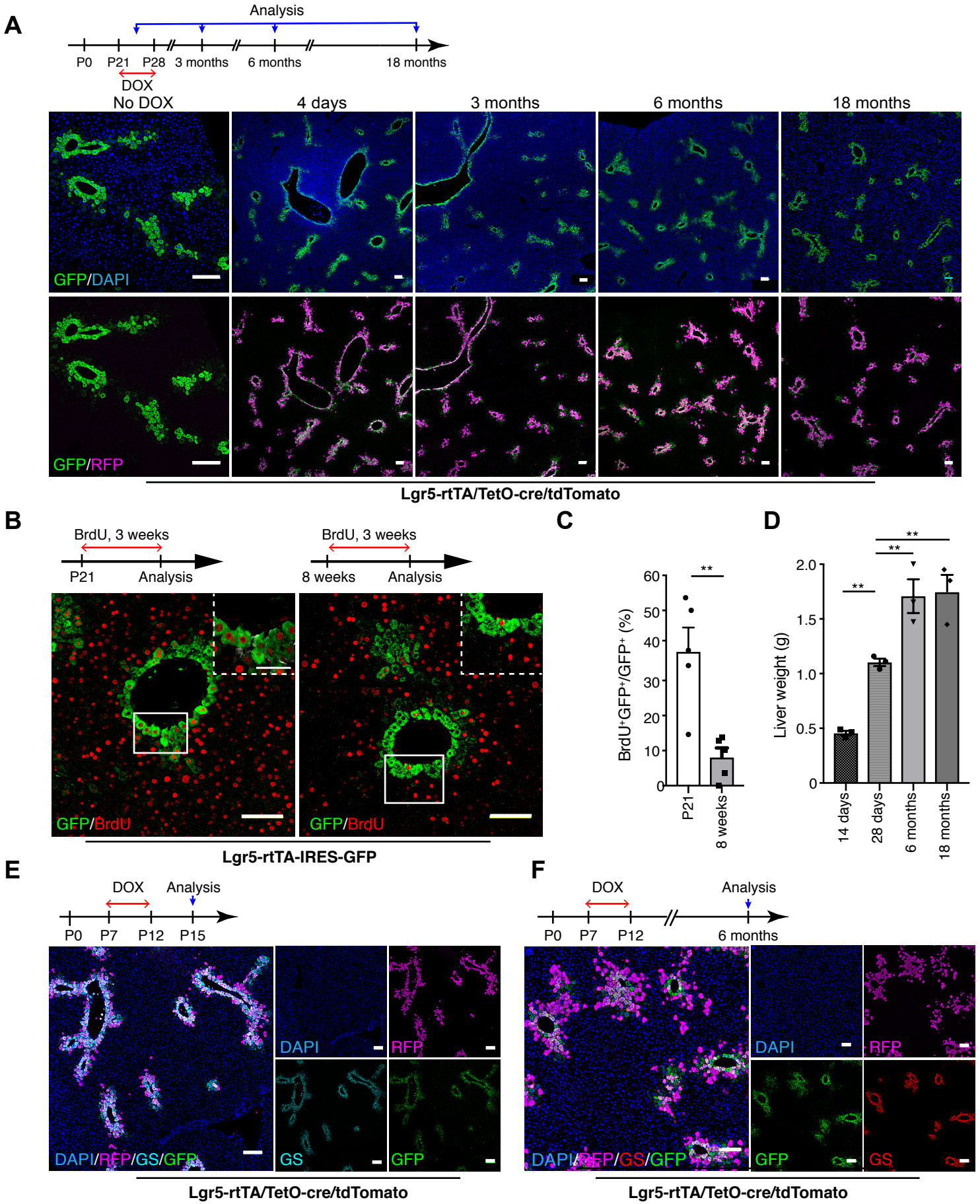

Fig. S2. Lgr5⁺ hepatocytes self-renew and replenish their own lineage during liver development and homeostasis

(A) Representative confocal images showing co-staining of GFP and RFP on liver sections of Lgr5-rtTA/TetO-cre/tdTomato mice at indicated analysis time point with low magnification for detection of larger liver area. Schematic illustration showing the experimental strategy for doxycycline induction and time points of analysis in lineage tracing of Lgr5-rtTA/TetO-Cre/R26-tdTomato mice. N=3 mice for each point. Scale bar: 100 μm.

(B) Representative confocal images showing the proliferation status of Lgr5-GFP hepatocytes in pubertal and adult mice. As depicted in the experimental schematic diagram, Lgr5-rtTA-IRES-GFP mice at P21 (N = 3) and 8 weeks old (N = 3) were treated with 1mg/ml BrdU in drinking water for 3 weeks. Lgr5-GFP⁺BrdU⁺ hepatocytes were evident in both group of mice. Scale bar: 100μm.

(C) Bar graph showing the percentage of BrdU+ hepatocytes in the Lgr5⁺ population in the P21 and 8-weeks old adult mice. Error bars represent mean ± s.e.m. N = 5. **P<0.01.

(D) Bar graph showing the liver weights of mice at 4 different ages. Liver mass increases more than 3 folds from 14 days old pups to adult mice (> 6 months old). Error bars represent mean ± s.e.m. N = 3 mice. **P<0.01.

(E-F) Representative confocal images of Lgr5-rtTA/TetO-Cre/R26-tdTomato mice pulsed with doxycycline (DOX) at P7 via lactating dams for 5 days and chased for 3 days (E) and 6 months (F). N = 3 mice for each time point. Scale bar: 100 μm.

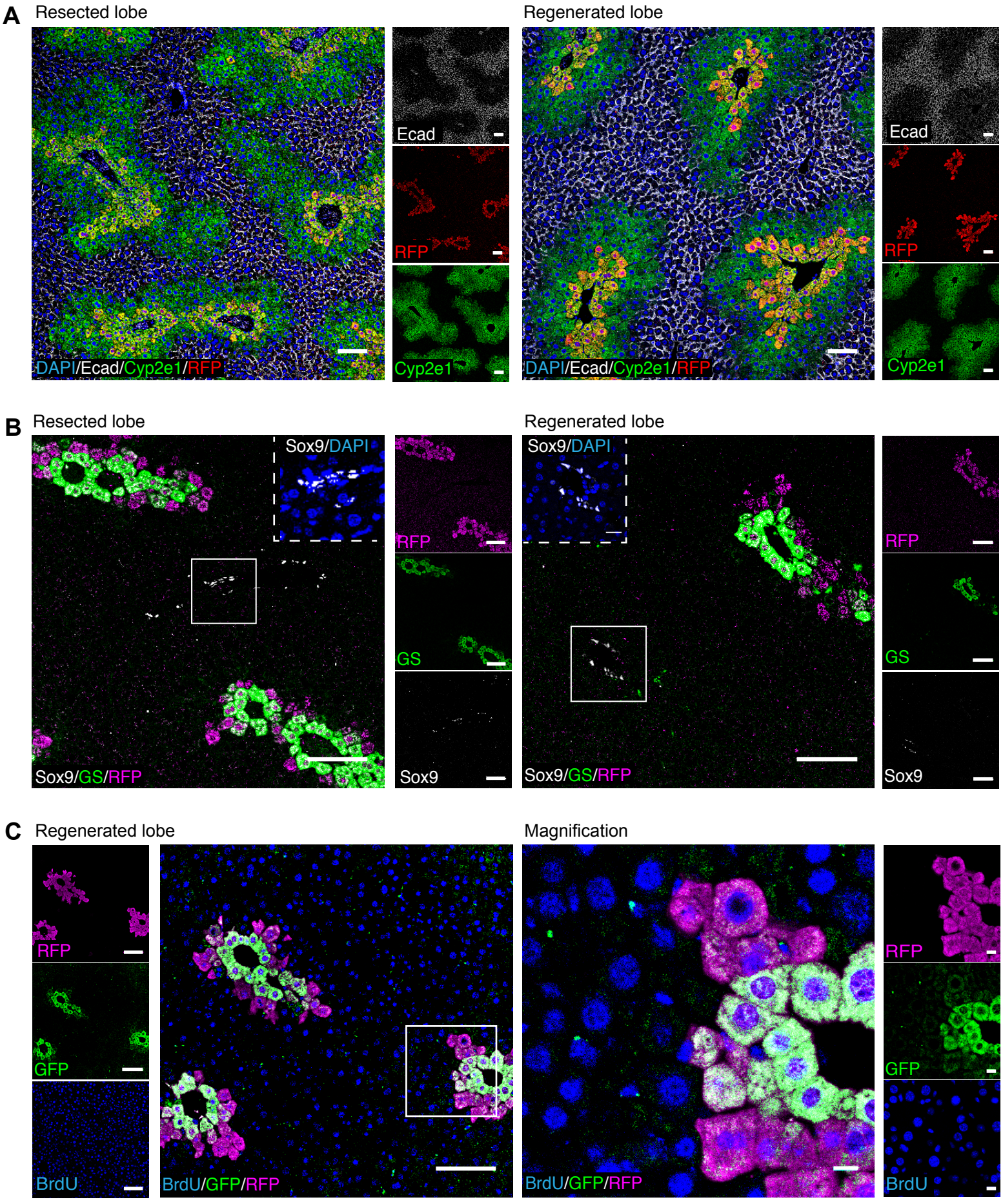

Fig. S3. Analysis of regenerated liver lobules of Lgr5-rtTA/TetO-Cre/tdTomato mice after 2/3 partial hepatectomy
(A) Representative confocal images showing identical staining patterns of zonation markers Cyp2e1and Ecad in resected and regenerated liver lobes after 2/3 partial hepatectomy. Partial hepatectomy was performed on the Lgr5-rtTA/TetO-Cre/R26-tdTomato mice with the same experimental strategy as depicted in Fig. 3. N = 3 mice. Scale bar: 100 μm.
(B) Confocal images showing expression of zonation markers GS and Sox9 remain the same in resected and regenerated lobes of Lgr5-rtTA/TetO-Cre/tdTomato mice. N = 3 mice. Scale bar: 100 μm.
(C) Confocal images showing that the majority of hepatocytes, including Lgr5-GFP^{+} hepatocytes, were BrdU^{+} and had undergone at least one round of proliferation during regeneration after 2/3 partial hepatectomy. Mice were treated with 1 mg/ml BrdU in drinking water for 5 continuous days immediately after partial hepatectomy. N = 3 mice. Scale bars: 100 μm (left panel); 10 μm (right panel).

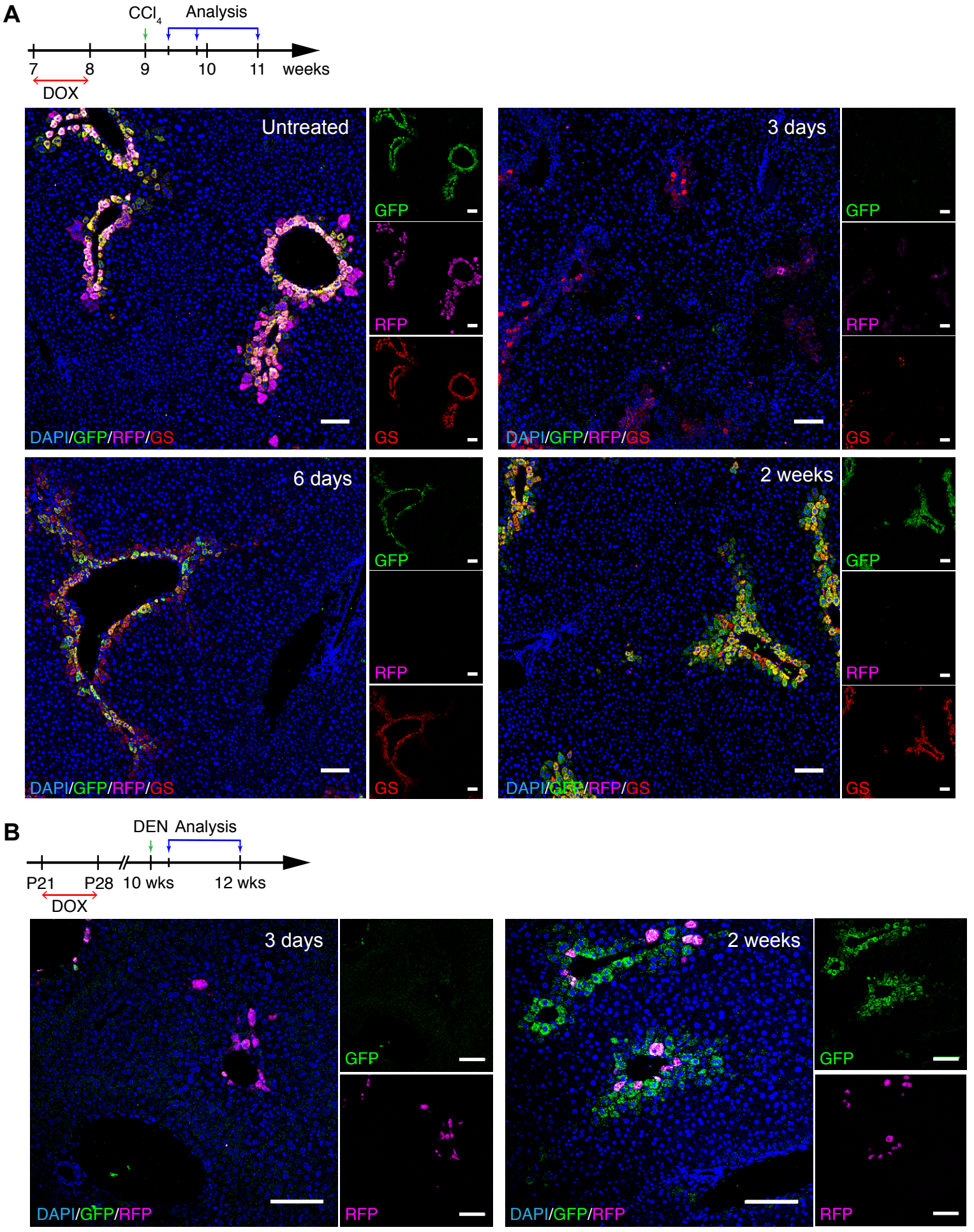

Fig. S4. Acute depletion of Lgr5^{+} hepatocytes by CCl_4 and DEN damage, and replenishment of these cells by Lgr5^{-} cells
(A) Representative confocal images showing staining of GFP, RFP, and GS in liver sections of untreated control mice or mice at 3 days, 6 days and 2 weeks after CCl_4 administration. N = 2 mice for each experimental condition. Scale bar: 100 μm.
(B) Confocal images showing co-staining of GFP and RFP on liver sections of mice at 3 days and 2 weeks after DEN administration. As depicted in the experimental schematic diagram, adult Lgr5-rtTA/TetO-cre/R26-tdTomato mice were treated with high dose of DEN to induce acute pericentral liver damage. Scale bar: 100 μm.

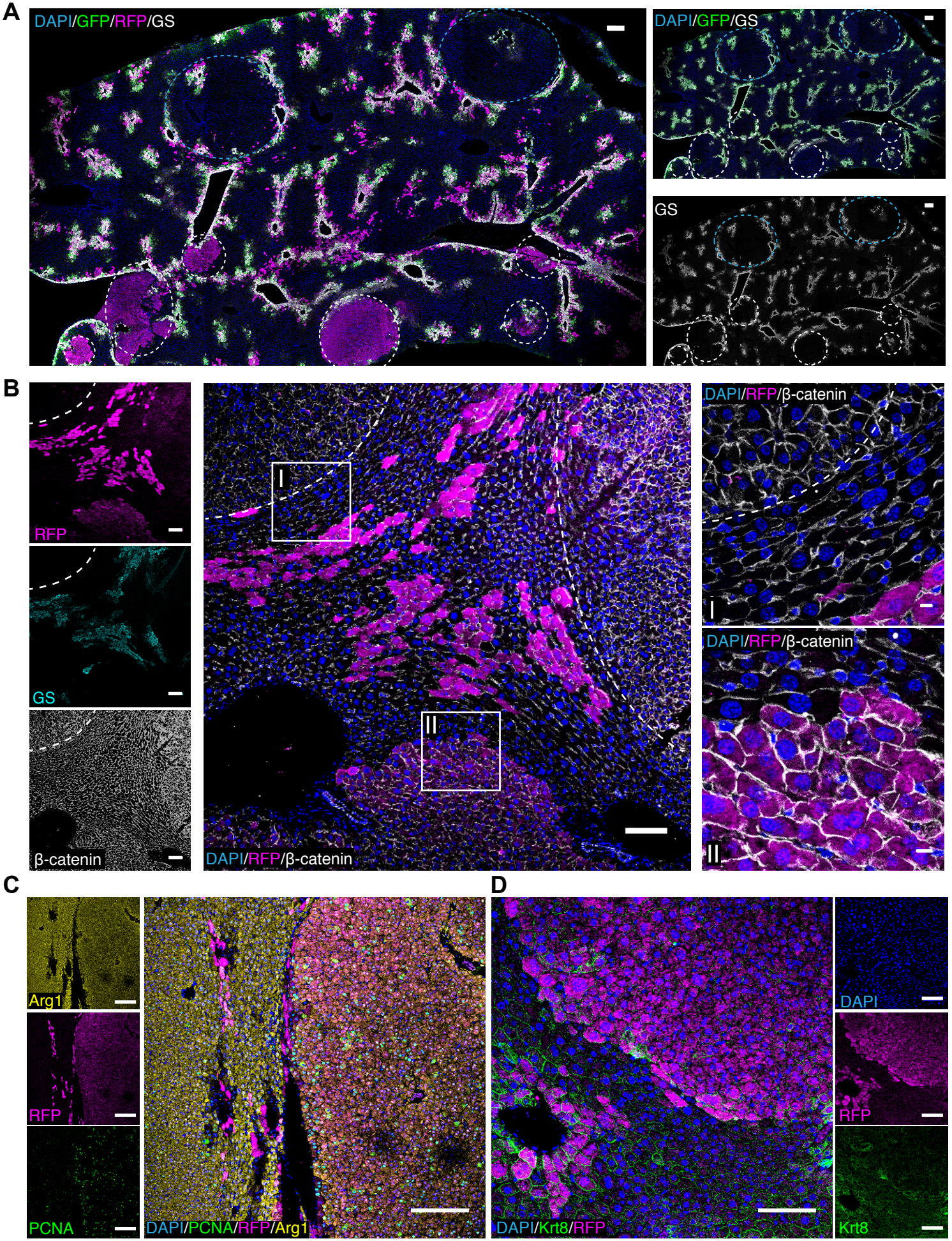

Fig. S5. Characterization of Lgr5⁺ hepatocytes-derived liver tumors in Lgr5-rtTA/TetO-Cre/tdTomato mice treated with DEN

(A) Confocal tile scan images showing that both tdTomato⁺ (white circles) and tdTomato⁻ (blue circles) tumors were negative for GS. tdTomato⁺ tumors were derived from Lgr5⁺ hepatocytes. Lgr5-rtTA/TetO-Cre/tdTomato mice were pulsed with DOX and treated with DEN to induce hepatocellular carcinoma as described in Fig. 4. N = 3 mice. Scale bar: 200 μm.

(B) Representative confocal images showing that β-catenin was exclusively expressed in the plasma membrane of tumor cells in tdTomato⁺ and tdTomato⁻ tumors (indicated by white dotted lines). Lgr5-rtTA/TetO-Cre/tdTomato mice were pulsed with DOX and treated with DEN as in (A). N = 3 mice. Scale bar: 100 μm.

(C) Representative confocal image showing that Arg1 and PCNA were expressed in DEN tumors. Lgr5-rtTA/TetO-Cre/tdTomato mice were pulsed with DOX and treated with DEN as in (A). tdTomato⁺ tumors were derived from Lgr5⁺ hepatocytes. N = 3 mice. Scale bar: 100 μm.

(D) Representative confocal image showing that cytokeratin-8 (Krt8) was weakly expressed in DEN tumors. Lgr5-rtTA/TetO-Cre/tdTomato mice were pulsed with DOX and treated with DEN as in (A). tdTomato⁺ tumors were derived from Lgr5⁺ hepatocytes. N = 3 mice. Scale bar: 100 μm.

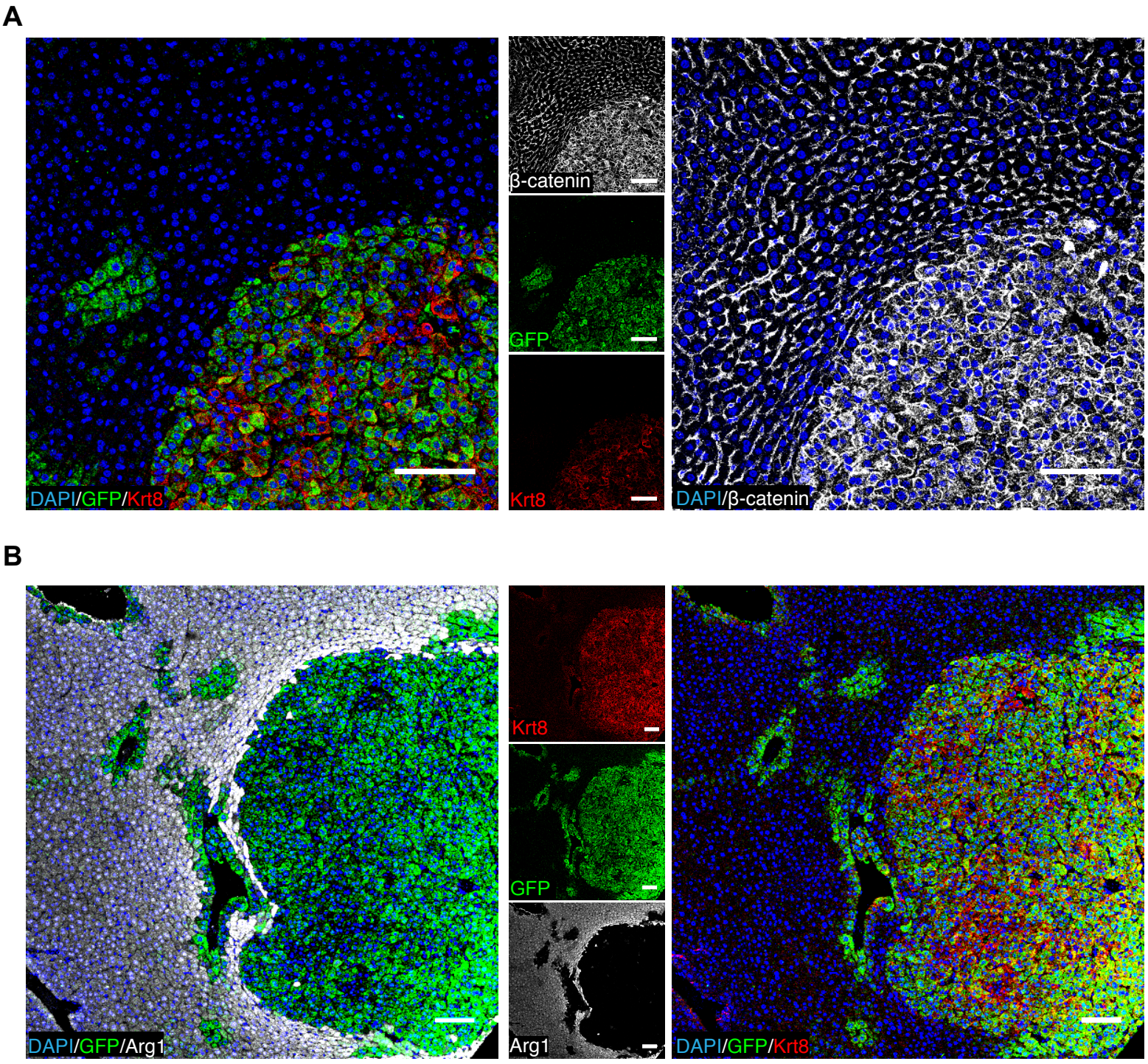

Fig. S6. Characterization of tumors in the Lgr5-rtTA/TetO-Neu hepatocellular carcinoma model
(A) Representative confocal image showing that cytosolic and nuclear expression of β-catenin could be detected in tumor cells of the Lgr5-rtTA/TetO-Neu model. Tumor cells showed higher expression of Krt8 than that in the surrounding normal hepatocytes. N = 3 mice. Scale bar: 100 μm.
(B) Representative confocal image showing that Lgr5-rtTA/TetO-Neu tumors are negative for Arg1. N = 3 mice. Scale bar: 100 μm.

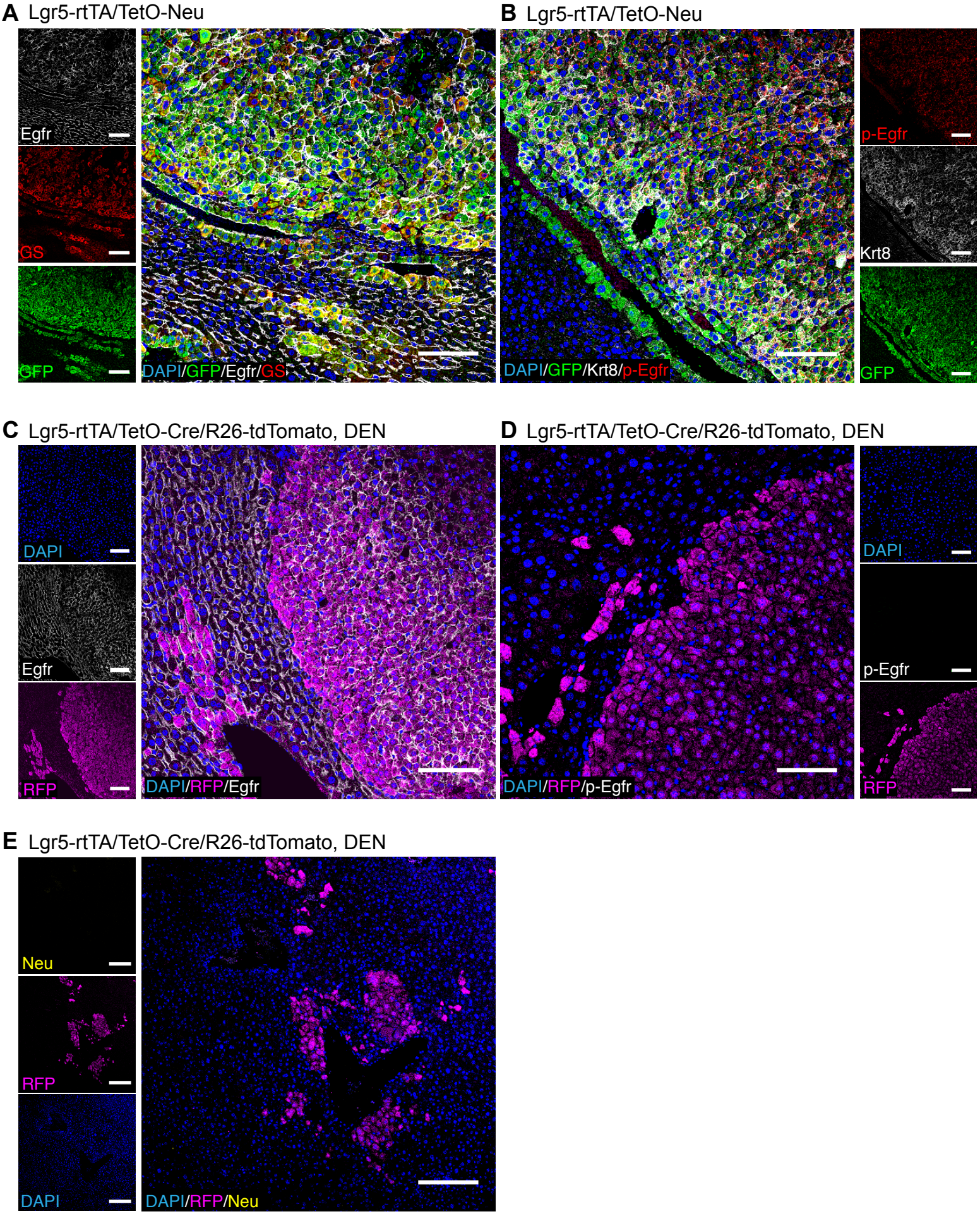

Fig. S7. Analysis of Erbb pathway activation in tumors derived from Lgr5-rtTA/TetO-Neu model and Lgr5-rtTA/TetO-Cre/tdTomato mice treated with DEN
(A-B) Representative confocal images of tumors in the Lgr5-rtTA/TetO-Neu model showing membrane expression of total Egfr (A) and activation of the Erbb pathway evident by detection of phospho-Egfr (p-Egfr) (B). High expression of GS and Krt8 was also observed in the tumors. N = 3 mice. Scale bar: 100 μm.
(C–D) Representative confocal images showing that DEN-induced tumors in Lgr5-rtTA/TetO-Cre/tdTomato mice expressed membrane Egfr (C), but it was in an inactive form (negative for p-Egfr) (D). Lgr5-rtTA/TetO-Cre/tdTomato mice were pulsed with DOX and treated with DEN to induce hepatocellular carcinoma as described in Fig. 4. tdTomato$^+$ tumors were derived from Lgr5$^+$ hepatocytes. N = 3 mice. Scale bar: 100 μm.
(E) Representative confocal images showing DEN tumors were negative for Neu/Her2. Lgr5-rtTA/TetO-Cre/tdTomato mice were pulsed with DOX and treated with DEN to induce hepatocellular carcinoma as described in (C-D). tdTomato$^+$ tumors were derived from Lgr5$^+$ hepatocytes. N = 3 mice. Scale bar: 100 μm.

Table S1. List of reagents and resources used in the study.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-E-cadherin antibody	BD Biosciences	Cat# 610182; RRID: AB_397581
Mouse purified anti-glutamine synthetase antibody	BD Biosciences	Cat# 610517; RRID: AB_397879
Rabbit polyclonal anti-RFP antibody	Rockland	Cat# 600-401-379; RRID: AB_2209751
Chicken polyclonal anti-GFP antibody	Abcam	Cat# ab13970; RRID: AB_300798
Rat monoclonal anti-cytokeratin-19 (Clone TROMA-III-C)	DSHB	Deposited to the DSHB by Kemler, R. (DSHB Hybridoma Product TROMA-III); RRID: AB_2133570
Rabbit polyclonal anti-Cyp2e1 antibody	Sigma	Cat# HPA009128; RRID: AB_1078613
Mouse purified anti-BrdU antibody (Clone B44)	BD Biosciences	Cat# 347580; RRID: AB_400326
Mouse anti-HNF4alpha antibody	Invitrogen	Cat# MA1-199; RRID: AB_2633309
Rabbit anti-HER2/Neu antibody	Cell Signaling	Cat# 09/2016; RRID: AB_10692490
Rabbit polyclonal anti-albumin antibody	Proteintech	Cat# 16475-AP; RRID: AB_2242567
Rat monoclonal anti-cytokeratin-8 (Clone TROMA-I-C)	DSHB	Deposited to the DSHB by Brulet, P. / Kemler, R. (DSHB Hybridoma Product TROMA-I); RRID AB_531826
Rabbit monoclonal anti-Arginase-1 (D4E3M™) antibody	Cell Signaling	Cat# 93668;
Rabbit monoclonal anti-phospho-epidermal growth factor (EGF) receptor (Tyr1068) (D7A5) XP® antibody	Cell Signaling	Cat# 3777; RRID AB_2096270
Rabbit monoclonal anti-epidermal growth factor (EGF) receptor (D1P9C) antibody	Cell Signaling	Cat# 71655; RRID AB_2799807
Purified anti-mouse CD16/32 antibody (Clone 93)	Biolegend	Cat# 101302; RRID: AB_312801
Alexa Fluor 647 anti-mouse/human CD324 (E-cadherin) antibody (Clone DECMA-1)	Biolegend	Cat# 147308; RRID AB_2563955
PE/Cy7 anti-mouse CD133 antibody (Clone 315-2C11)	Biolegend	Cat# 141210; RRID: AB_2564069
Chemicals, Peptides, and Recombinant Proteins		
Carbon tetrachloride (CCl4), 99+%, Spectrophotometric grade	ACROS Organics	Cat# AC167725000; Kind gift from Prof. Go Mei Lin
Sunflower seed oil from *Helianthus annuus*	Sigma Aldrich	Cat# S5007-1L CAS Number: 8001-21-6
N-Nitrosodiethylamine (Diethylnitrosamine, DEN)	Sigma	Cat# NO258-1G; Lot number: MKCB0796V
Mouse IgG Blocking Reagent	Vector Laboratories	Cat# MKB-2213; Lot number: ZE1226
Triton™ X-100	Sigma	Cat# T8787; Lot number: MKBW8386V
Horse serum	Thermo Fisher Scientific	Cat# 16050130
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Invitrogen	Cat# D9542 CAS Number: 28718-90-3
7-Aminoactinomycin D (7AAD)	Sigma Aldrich	Cat#A9400 CAS Number: 7240-37-1
Deoxyribonuclease I (DNAse I)	Worthington Biochemical Corp	Cat# LS002140 CAS Number: 9003-98-9
5-Bromo-2´-Deoxyuridine (BrdU)	Sigma	Cat# 10280879001 CAS Number: 59-14-3

Liver Perfusion Medium (1x)	Gibco	Cat# 17701-038; Lot number 1960305
Fetal Bovine Serum	Sigma Aldrich	Cat# 12003C
Collagenase, Type 1	Worthington	Cat# LS004196
IgG from rat serum	Sigma	Cat#I4131; Lot number SLBF6029V
GoScript™ Reverse Transcription Kit	Promega	Cat# A5000
SYBR™ Green PCR Master Mix	Thermo Fisher Scientific	Cat# 4309155
RNeasy Micro Kit	Qiagen	Cat# 74004
Experimental Models: Organisms/Strains		
Mouse: FVB/NJ	The Jackson Laboratory	ISMR Cat# JAX:001800, RRID:IMSR_JAX:001800
Mouse: C57BL/6J	The Jackson Laboratory	IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664
Mouse: Lgr5-rtTA-IRES-GFP	This paper	N/A
Mouse: B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J	The Jackson Laboratory	ISMR Cat# JAX:007909, RRID:IMSR_JAX:007909
Mouse: B6;SJL-Tg(tetO-Erbb2*)8-4Jek/J	The Jackson Laboratory	ISMR Cat# JAX:010577, RRID:IMSR_JAX:010577
Mouse: B6;C-Tg(tetO-cre)LC1Bjd/BjdCnrm	Heidelberg University, Germany	A gift from Dr. K. Schönig
Oligonucleotides		
Genotyping primers of the Lgr5-rtTA-IRES-GFP line: (1). 5'-tctgctcccagtctcgggcaccatgg-3' (2). 5'-tggtaggtgtctctctrtcctctt-3'	Geneworks	N/A
Genotyping primers of the TetO-cre mouse line: (1). 5'-gcggtctggcagtaaaaactatc-3' (2). 5'-gtgaaacagcattgctgtcactt-3'	Geneworks	N/A
Genotyping primers of the TetO-Neu mouse line: (1). 5'-aagaagagcccaagctgga-3' (2). 5'-gtgtacggtgggaggcctat-3'	Geneworks	N/A
Genotyping primers of the Rosa26-tdTomato mouse line: (1). 5'-aagggagctgcagtggagta-3' (2). 5'-ccgaaaatctgtgggaagtc-3' (3). 5'-ggcattaaagcagcgtatcc-3' (4). 5'-ctgttcctgtacggcatgg-3'	Geneworks	N/A
qPCR primer set 1 for *Lgr5*: (1). 5'-cctactcgaagacttacccagt-3' (2). 5'-gcattggggtgaatgatagca-3'	Geneworks	N/A
qPCR primer set 2 for *Lgr5*: (1). 5'-acattcccaagggagcgttc-3' (2). 5'-atgtggttggcatctaggcg-3'	Geneworks	N/A
qPCR primers for *Glul*: (1). 5'-tgaacaaaggcatcaagcaaatg-3' (2). 5'-cagtccagggtacgggtctt-3'	Integrated DNA Technologies	N/A
qPCR primers for *Slc1a2*: (1). 5'-cagaagttggaagccagtgc-3' (2). 5'-caggtttcggtgctttgg-3'	Integrated DNA Technologies	N/A

qPCR primers for *Axin2*: (1). 5'-gacccagtcaatccttatcacg-3' (2). 5'-tgtttcttactccccatgcg-3'	Integrated DNA Technologies	N/A
qPCR primers for *Arg1*: (1). 5'-ctccaagccaaagtccttagag-3' (2). 5'-aggagctgtcattagggacatc -3'	Integrated DNA Technologies	N/A
qPCR primers for *Cdh1*: (1). 5'-caggtctcctcatggctttgc-3' (2). 5'-cttccgaaaagaaggctgtcc-3'	Integrated DNA Technologies	N/A
qPCR primers of *Lgr5* gene for copy number assay of the Lgr5-rtTA-IRES-GFP line: (1). 5'-cgcgactgagatgtgaaagctaaa-3' (2). 5'-gaggctttgatagttgagcaagagg-3'	Integrated DNA Technologies	N/A
qPCR primers of *p53* gene as reference for copy number assay of the Lgr5-rtTA-IRES-GFP line: (1). 5'-ggttaaacccagcttgacca-3' (2). 5'-ggaggcagagacagttggag-3'	Integrated DNA Technologies	N/A
Software and Algorithms		
Image J	Fiji	RRID: SCR_003070 URL: https://imagej.net/
Leica Application Suite X	Leica Microsystems GmbH	RRID:SCR_013673 URL:https://www.leica-microsystems.com/products/microscope-software/details/product/leica-las-x-ls/
FlowJo	Tree Star	RRID:SCR_008520 URL: https://www.flowjo.com/solutions/flowjo
GraphPad Prism 7	GraphPad Software	RRID:SCR_002798
Mus musculus gene information	NCBI	ftp://ftp.ncbi.nlm.nih.gov