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Supplemental Information

Lineage Tracing Reveals the Bipotency of SOX9^{+} Hepatocytes during Liver Regeneration

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SUPPLEMENTAL FIGURES AND LEGENDS

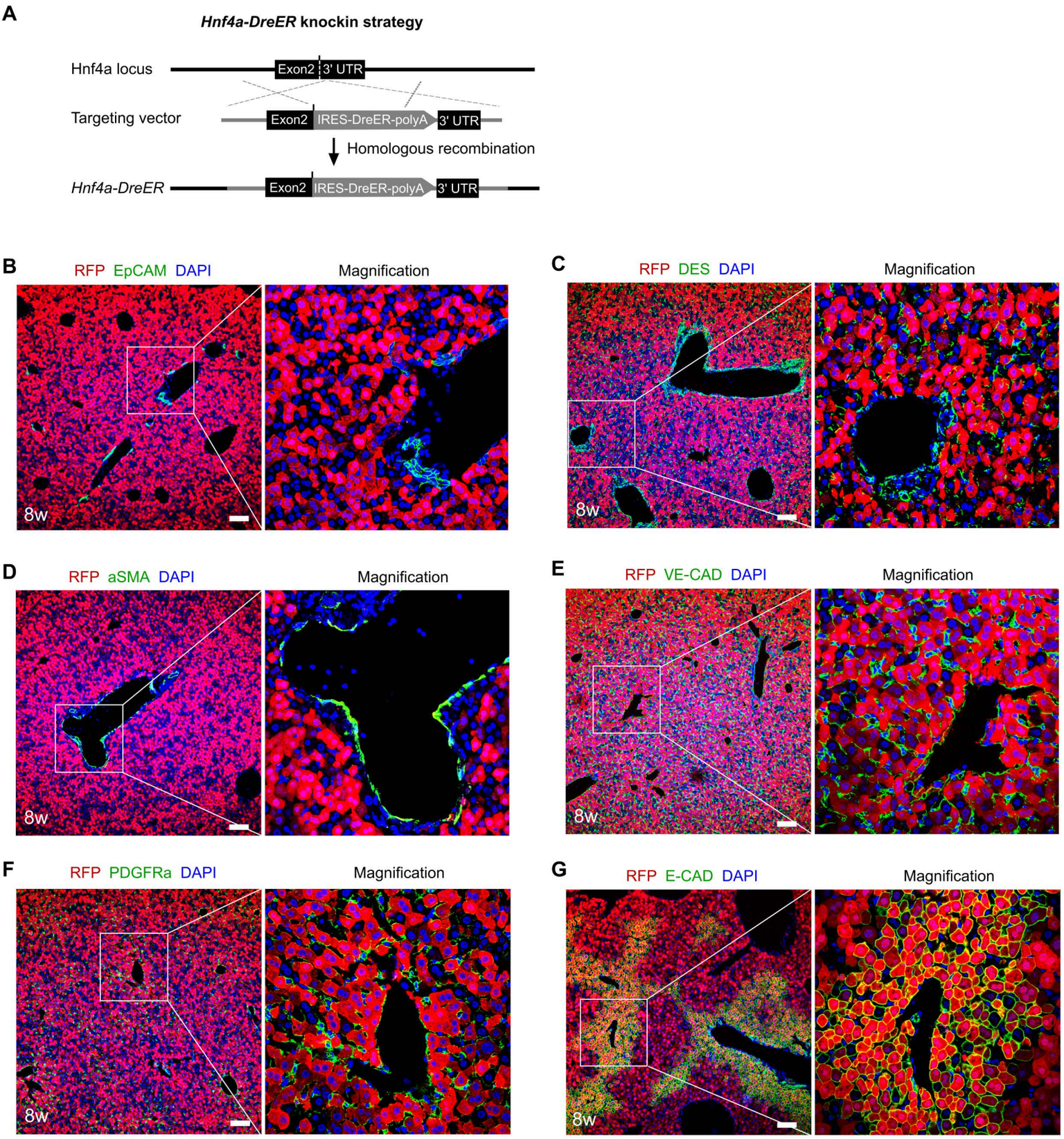


Figure S1: *Hnf4a-DreER* specifically label hepatocytes but not other lineages in adult liver, related to Figure 1
(A) Schematic figure showing knockin strategy for generation of *Hnf4a-DreER* allele.
(B-G) Immunostaining for RFP, EpCAM, DES, aSMA, VE-CAD, PDGFRa and E-CAD on liver sections. Scale bars, 100 μm. Each image is representative of 5 individual samples.

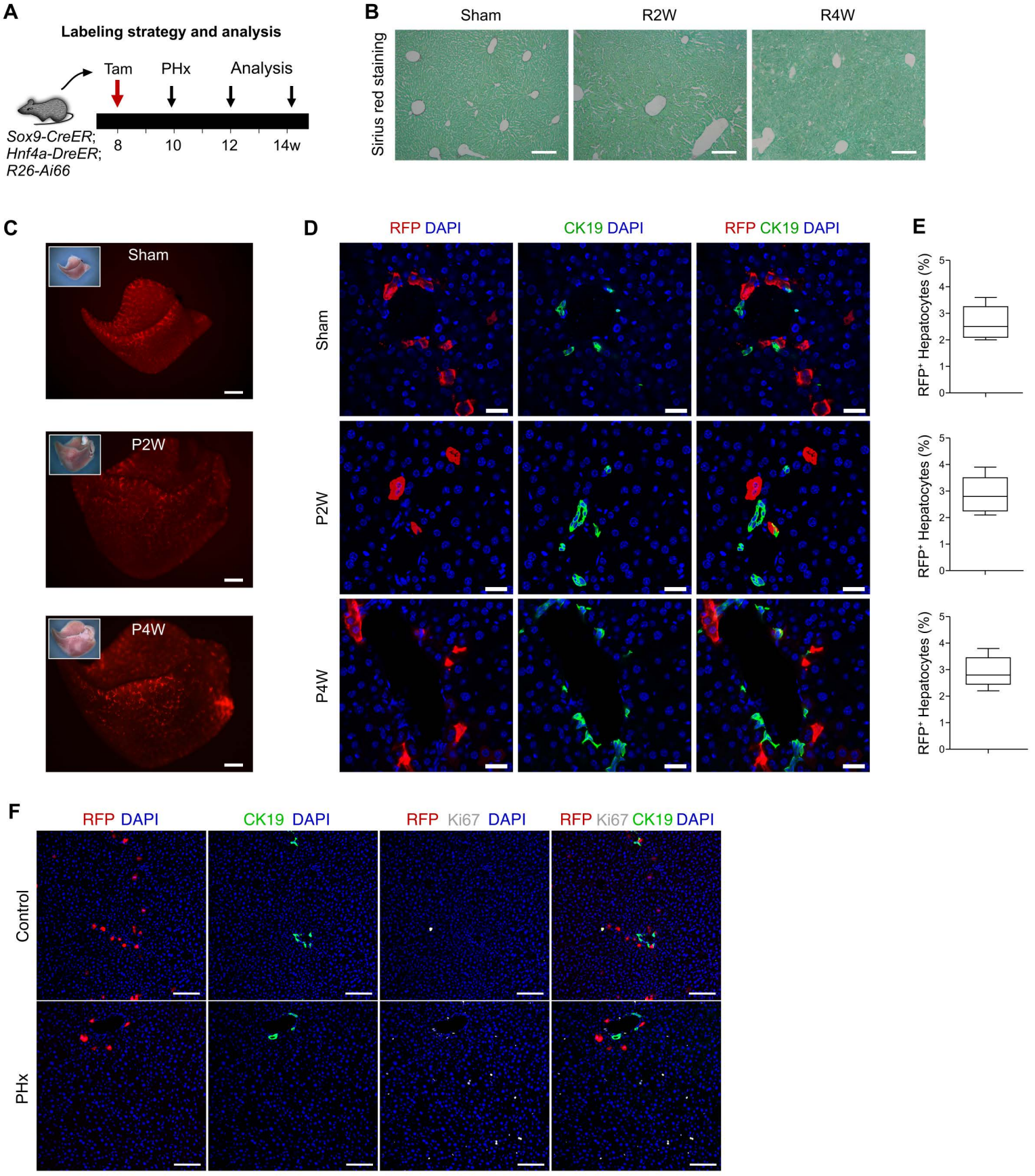


Figure S2: The percentage of RFP⁺ hepatocytes remained unchanged following partial hepatectomy (PHx), related to Figure 3
(**A**) Experimental strategy for tamoxifen treatment (Tam) and tissue analysis at different time points after PHx.
(**B**) Sirius red staining of liver sections from Sham or PHx-injured mice.
(**C**) Whole-mount fluorescence view of *Sox9-CreER;Hnf4a-DreER;R26-Ai66* livers. Inserts are bright-field images.
(**D**) Immunostaining for RFP and CK19 on liver sections shows no change of RFP⁺ cells following PHx injury.
(**E**) Quantification of the percentage of hepatocytes expressing RFP in the liver after PHx injury.
(**F**) Immunostaining for RFP, CK19 and Ki67 on liver sections of PHx or control groups. Scale bars, 200 μm in (**B**); 1 mm in (**C**); 50 μm in (**D**); 100 μm in (**F**). Each image is representative of 5 individual biological samples.

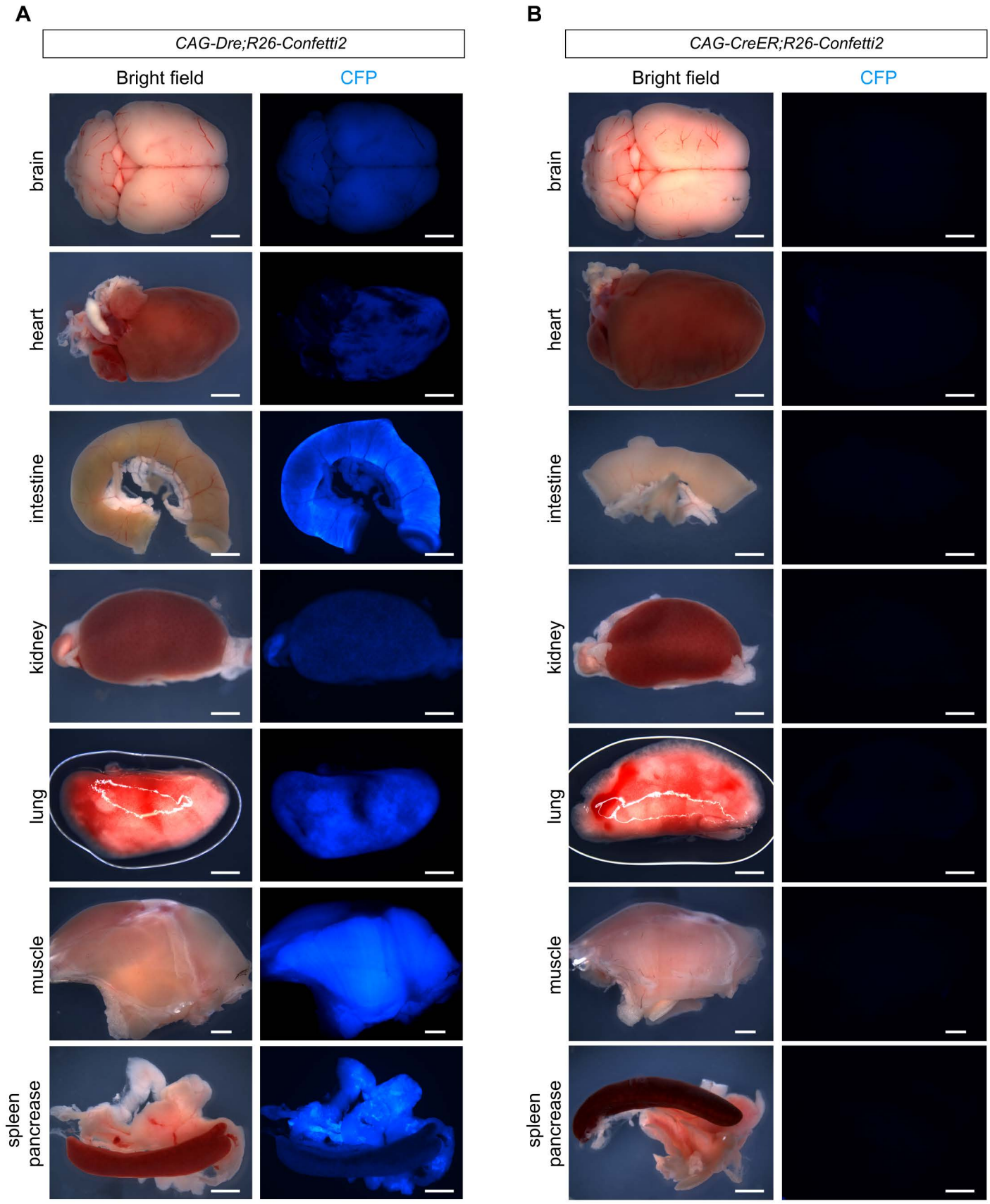
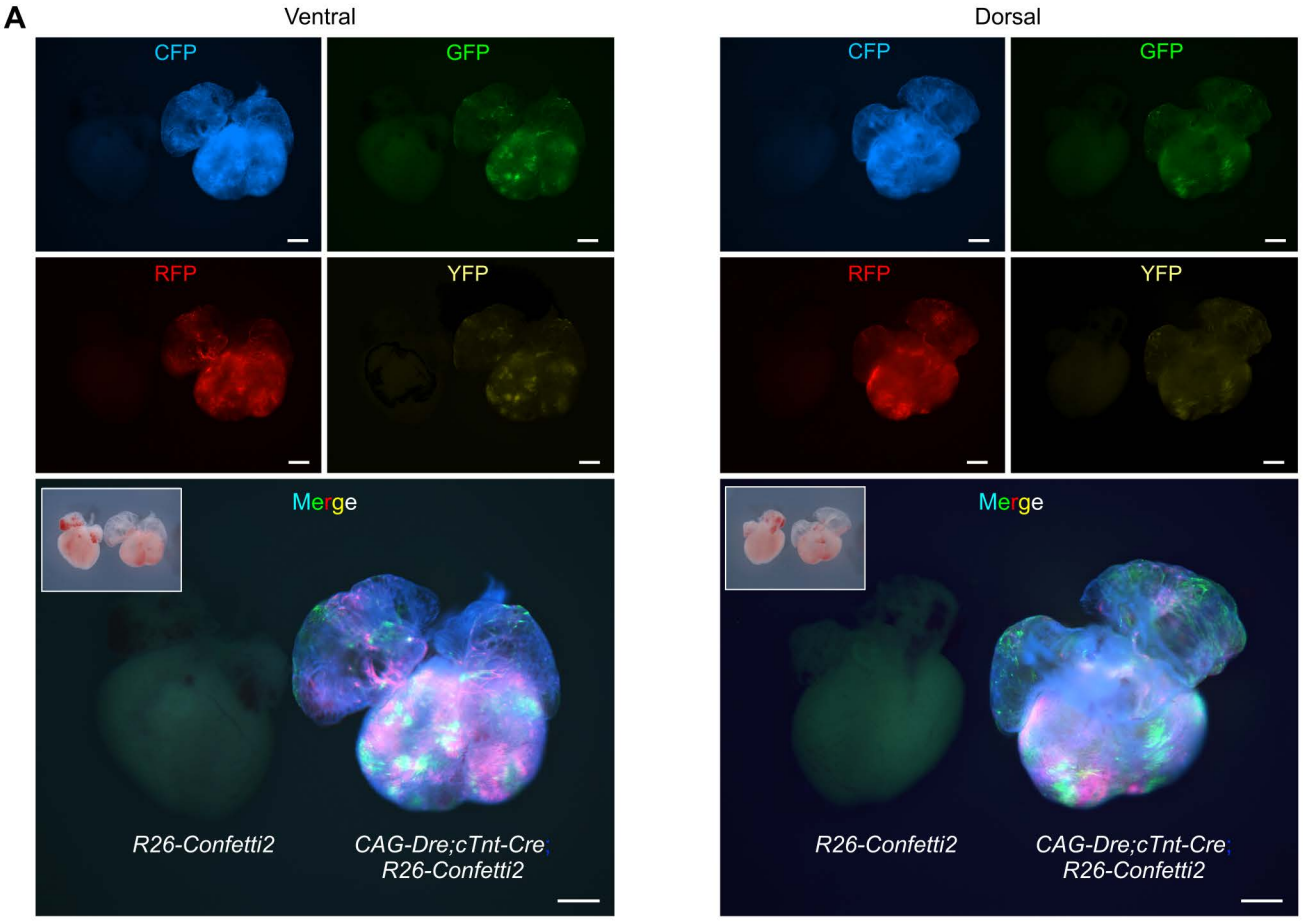


Figure S3: Examination of *R26-roxRB* by *CAG-Dre* or *CAG-CreER*, related to Figure 5
(A) The leaky of *CAG-Dre;R26-Confetti2* and *CAG-CreER;R26-Confetti2* mice. As the data indicates, *CAG-Dre;R26-Confetti2* mice were CYP^{+}, but GFP^{-},RFP^{-} and YFP^{-}.
(B) While *CAG-CreER;R26-Confetti2* mice were no signal. Tamoxifen was induced two days before tissue collection. Scale bars, 2 mm. Each image is representative of 5 individual samples.



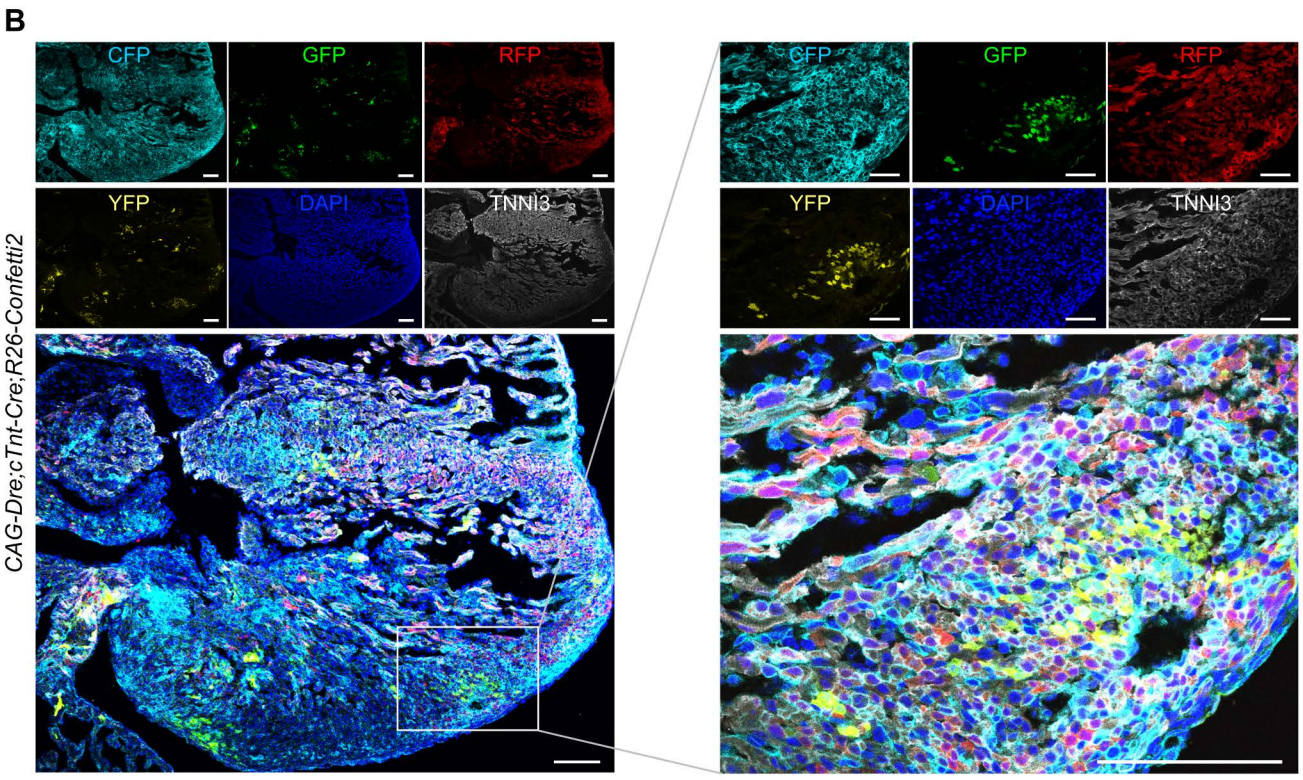


Figure S4: Characterization of *R26-Confetti2* in mouse heart, related to Figure 5
(A) Whole-mount fluorescence images of hearts collected from E18.5 *CAG-Dre;cTnt-Cre;R26-Confetti2* or littermate control *R26-Confetti2* embryos. Inserts are bright-field images.
(B) Fluorescent image of section stained with TNNI3 shows expression of GFP, RFP and YFP in TNNI3^{+} cardiomyocytes. Sclae bars, 500 μm in **(A)**; 100 μm in **(B)**.

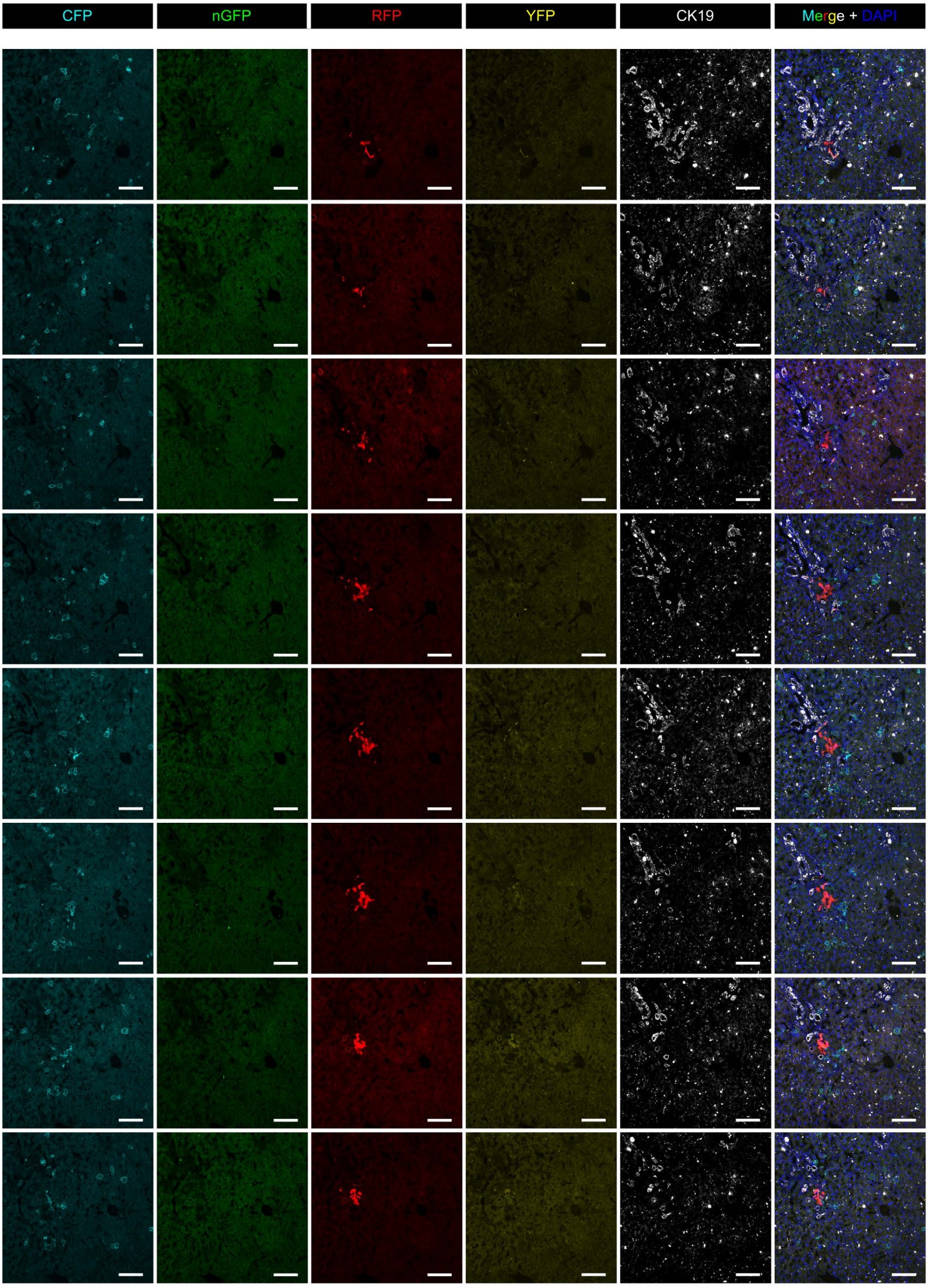


Figure S5: Zoom out fluorescent sections of Figure 6 stained with BEC marker CK19. Slide 1-8 are serial sections. Scale bars, 100 µm, related to Figure 6

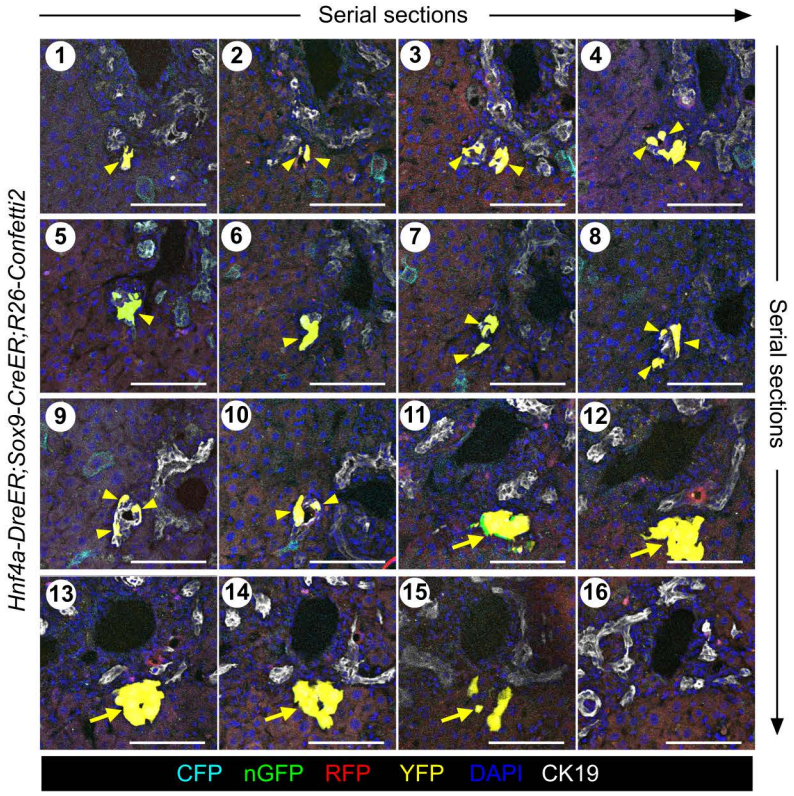


Figure S6: Additional bi-potent clone in serial sections after liver injury , related to Figure 6
Another fluorescent sections of a bi-potent clone that contains both BECs (arrowheads) and hepatocytes (arrows) stained with BEC marker CK19. Slide 1-16 are serial sections. Scale bars, 100 μm.

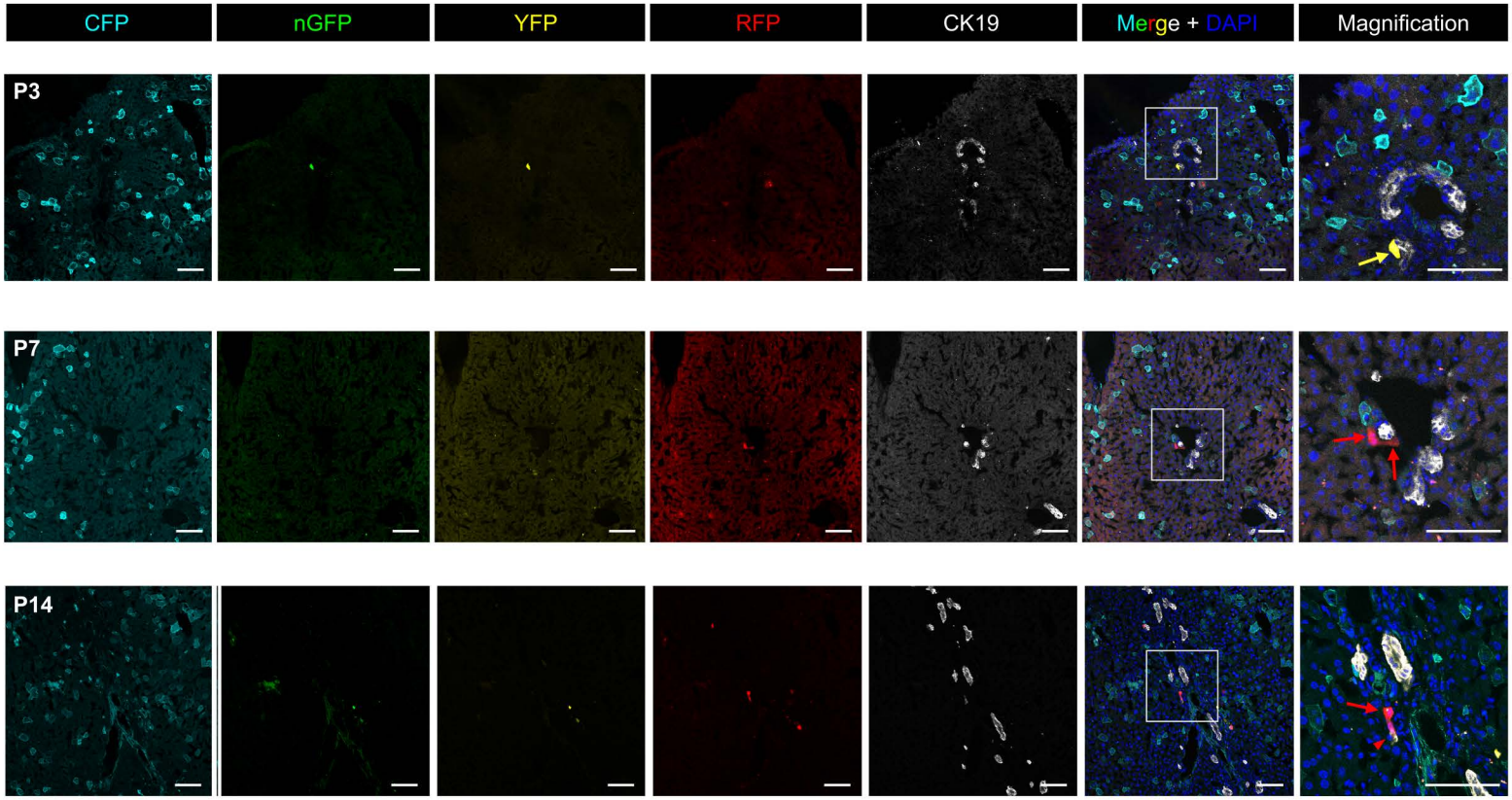


Figure S7: Clonal analysis of SOX9⁺ hepatocyte at early stages after BDL, related to Figure 6
Fluorescent sections stained with BEC marker CK19 shows only hepatocytes (arrows) at post-BDL day 3 (P3) or P7. Double hepatocyte could be detected at P7 (arrowheads). Only at P14 could we detect both hepatocyte (arrow) and BEC (arrowhead) in clone. Scale bars, 100 μm.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

All mouse studies were carried out strictly according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Institute of Biochemistry and Cell Biology and the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. *Hnf4a-DreER* was generated by CRISPR/Cas9 through homologous recombination. A complementary cDNA encoding IRES-DreER^{T2} was inserted in-frame with the translation codon of the *Hnf4a* gene. The chimeric mice positive for targeted ES cells were germ line transferred to F1 generation and bred on a C57BL/6 x ICR background. The *Sox9-CreER^{T2}* line was generated by the National Institute for Biological Sciences, Beijing, China. *R26-Ai66* (*Rosa26-rox-Stop-rox-loxP-Stop-loxP-tdTomato*) as reported previously (Madisen et al., 2015; Zhang et al., 2016). The *Rosa26-Rox-Stop-Rox-tdTomato* (*R26-RSR-RFP*) mouse line was generated by crossing *ACTB-Cre* with *R26-Ai66* to excise the second loxP-flanked Stop cassette, and *ACTB-Cre* was not passaged to the subsequent mouse breeding. *R26-RSR-RFP* was responsive to Dre but not Cre recombinase. The *Rosa26-rox-Stop-rox-Confetti* (*R26-Confetti2)* was generated by targeting CAG-rox-stop-rox-Confetti cassette into the Rosa26 gene locus by homologous recombination (Figure 5A). All experimental mice were maintained on a C57BL6/ICR mixed background. Tamoxifen (Sigma, T5648) was dissolved in corn oil (20 mg/ml) and administered by gavage at the indicated time points. We treated *R26-RFP* and *R26-Confetti2* mice with 0.4 mg tamoxifen/g of mouse body weight (mg/g) and 0.15 mg/g tamoxifen respectively.

Genomic PCR

Genomic DNA was prepared from mice tail. Tails were lysed by incubation with Proteinase K overnight at 55 °C, followed by centrifugation at maximum speed for 8 min, to obtain supernatant with genomic DNA. DNA was precipitated by adding isopropanol and washed in 70% ethanol, and was finally dissolved in distilled H2O. All mice were genotyped with specific primers that distinguish knock-in allele from wild-type allele. For the *R26-RSR-RFP* line, primers 5'-ACGGGTGTTGGGTCGTTTGTTC-3' and 5'-TTCTTGTAATCGGGGATGTCGGCG-3 were used to detect the tdTomato positive allele, and 5'-AAGGGAGCTGCAGTGGAGTA-3'and 5'-CCGAAAATCT- GTGGGAAGTC-3' were used to detect the wild-type allele. For the *Hnf4a-DreER* line, primes 5'- GCTCTGGTGGTCTGCTCTGA-3' and 5'-CCCTTGTTGAATACGCTTGA-3' were used to detect the DreERT2 positive allele, and 5'-GCTCTGGTGGTCTGCTCTGA-3' and 5'- CTGGCCTTGAATGTCTGGAA-3' were used to detect the wild-type allele. For the *Sox9-CreERT2* line, primers 5'-GCAAAAGTATTACATCACGGGGG-3' and 5'-ACAAAGTCCAAACAGGCAGGG-3' were used to detect the CreER positive allele, and 5'-GTAAAGGAAGGTAACGATTGCTGG-3' and 5'- ACAAAGTCCAAACAGGCAGGG-3' were used to detect the wild-type allele. For the *R26-LSL-RFP* line, primers 5'-GGCATTAAAGCAGCGTATCC-3' and 5'-CTGTTCCTGTACGGCATGG-3' were used to detect the tdTomato positive allele, and 5'-AAGGGAGCTGCAGTGGAGTA-3' and 5'-CCGAAAATCTGTGGGAAGTC-3' were used to detect the wild type allele. For the *R26-Confetti2* line, primes 5'- TGCTGTGCTGCATCAGAAGAAC-3' and 5'-TGCTTGTCGGCGGTGATATAG-3' were used to detect the CFP positive allele, and 5'-TTGGAGGCAGGAAGCACTTG-3' and 5'- CCGACAAAACCGAAAATCTGTG-3' were used to detect the wild-type allele.

Injury Model

For the CCl4 induced chronic injury model, CCl4 was dissolved at 1:3 in corn oil and injected intraperitoneally at a dose of 4 μl/g body weight every 3 days for 10 times. Partial hepatectomy (PHx) was generated by removing two thirds of the liver to induce the injury. For details, 8 week old mice were anaesthetized with 2% isoflurane and oxygen flow in a plexiglass chamber. The abdominal fur was removed and the skin was disinfected. The mice were transferred onto a 37 °C heat pad and maintained anesthesia by inhalation of isoflurane with oxygen. A midline abdominal skin and muscle incision was made to expose the liver. The left lobe and the median lobe were tied and cut. The peritoneum was closed with a 5–0 suture and skin was closed afterwards. After closing the abdomen, the skin surrounding the suture was disinfected with betadine and the animal was placed on a warming pad for recovery. BDL injury model was carried out according to established protocols described previously (Pu et al., 2016). Anesthesia procedure was the same as PH. A midline abdominal incision was made to expose the liver. The common bile duct was ligated twice with 4–0 silk sutures. Sham-operated animals were used as controls. For DDC induced chronic injury model, mice received mouse diet (Harlan Teklad, 5015) containing 0.1% DDC (Sigma-Aldrich).

Whole-mount Fluorescence Microscopy

Collected mouse liver was washed in PBS and placed on agar for the whole mount bright field and fluorescence imaging using the Zeiss (AxioZoom V16). To determine magnification of specific regions, we used the automated Z-stack images acquired by the Zeiss stereo-scope (AxioZoom V16).

Immunostaining

Immunostaining was performed according to the standard protocols described previously (Tian et al., 2013). For details, tissues were dissected in PBS and fixed in 4% paraformaldehyde (PFA Sigma) at 4 °C for 45-60 min. Afterwards, tissues were washed in PBS 5 min for three times, dehydrated in 30% sucrose overnight at 4 °C. Following an hour of immersion in optimum cutting temperature (O.C.T., Sakura) at 4 °C, the liver was embedded in blocks and frozen at −80 °C. Sections of 8-10 μm thickness were collected on slides and air dried afterwards at room temperature at least 2 hours. For immunostaining, dried sections were washed in PBS 5 min for three times and then blocked with 5% normal donkey serum (Jackson Immunoresearch) and 0.1% Triton X-100 in PBS for 30 min at room temperature. Sections were incubated with the primary antibodies overnight at 4 °C. The following antibodies were used: RFP (Rockland, 600-401-379, 1:200), HNF4a (Santa Cruz, sc-6556, 1:100), cytokeratin 19 (CK19, Developmental Studies Hybridoma Bank, TROMA-III, 1:100), VE-cadherin (R&D, AF1002, 1:100), Desmin (R & D, AF3844, 1:100), PDGFR (R&D, AF1062, 1:100), E-cadherin (Cell Signaling, 3195, 1:100), EpCAM (Abcam, ab92383, 1:100), SMA (Sigma, F3777, 1:100), Ki67 (Thermo scientific, RM-9106 - S0, 1:100). Signals were developed with Alexa fluorescence antibodies (Invitrogen), and nuclei were counterstained with 4'6-diamidino-2-phenylindole (DAPI, Vector lab). For clonal analysis, CK19 was stained on the liver sections in far red channel. Totally 246 clones were analyzed: 820 cholangiocytes in cholangiocyte clones; 412 hepatocytes in hepatocyte clones; 156 cholangiocytes and 66 hepatocytes in mixed clones. And there was bleed-through of fluorescence for YFP and GFP, as this was due to YFP signal could also be detected in the GFP channel. In addition, our GFP is nGFP (nuclear), so pure GFP signal should be in the nucleus; while bleed-through signal in the GFP channel from YFP should not be nuclear signal. Immunostaining images were acquired by Olympus fluorescence microscope (BX53), Zeiss stereomicroscope (AXIO Zoom, V16), Zeiss confocal laser scanning microscope (LSM510) and Olympus confocal microscope (FV1200).

Sirius Red Staining

Sirius red staining was aimed to assess fibrotic tissue after chronic injury models. For details, cryosections were washed in PBS for 15 min and then fixed in 4% paraformaldehyde (PFA Sigma) for 15 min. Slides were then washed with PBS for 15 min and fixed overnight in Bouins solution (5% acetic acid, 9% formaldehyde and 0.9% picric acid) at room temperature or 1 hour in 55°. Subsequently, the slides were washed in running tap water until the yellow color developed. Slides were stained with 0.1% Fast Green (Fisher) for 2.5-3 min and incubated in 1% acetic acid for 1 min followed by incubation with 0.1% Sirius red (Sigma) for 1-1.5 min; followed by rinsing with tap water before incubation into the staining solution. Finally, slides were dehydrated in 100% ethanol twice, cleared in xylene and covered with resinous medium. Images were obtained on an Olympus microscope (BX53).

Statistics

All data were collected from at least five independent experiments as indicated. Data for two groups were analyzed by a two-sided unpaired Student's t-test, whereas comparison between more than two groups was performed using an analysis of variance followed by Tukey's multiple comparison tests. Significance was accepted when $P < 0.05$. All data were presented as mean value ± SEM.

Data Availability

Data supporting the findings of this study are available within the article and its Supplementary Information files, and from the corresponding author upon reasonable request.

SUPPLEMENTAL REFERENCES

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