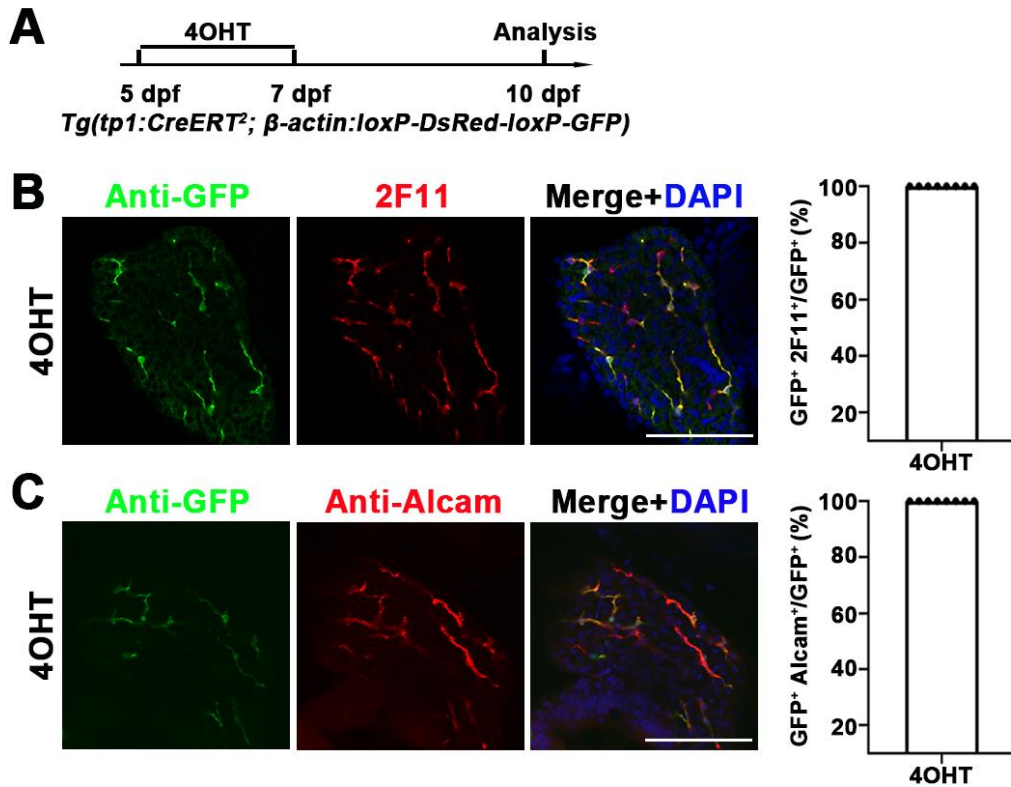# Supplemental Information

Contributions of biliary epithelial

cells to hepatocyte homeostasis

and regeneration in zebrafish

Wenfeng Zhang, Jingying Chen, Rui Ni, Qifen Yang, Lingfei Luo, and Jianbo He

SUPPLEMENTAL FIGURE AND LEGEND

He_Figure S1

Figure S1. Establishment of lineage tracing system of physiological BEC-to-hepatocyte conversion using the *tp1* promoter. Related to Figure 1 and Figure 2. (**A**) Experimental scheme illustrating the stage of 4OHT treatment to double transgenic line, *Tg(tp1:CreERT2; β-actin:loxP-DsRed-loxP-GFP)* from 5 dpf to 7 dpf and analysis at 10 dpf. (**B**) Immunostaining for 2F11 and GFP on livers (2D imaging) showing *tp1-CreER* labels the 2F11 positive cholangiocytes specifically after 4OHT treatment. Nuclei were stained with DAPI (blue). Quantification of the percentage of the GFP+2F11+ among the GFP+ cells in 4OHT (n=8) treated livers. (**C**) Immunostaining for Alcam and GFP on livers (2D imaging) showing *tp1-CreER* labels the Alcam positive cholangiocytes specifically after 4OHT treatment. Nuclei were stained with DAPI (blue). Quantification of the percentage of the GFP+Alcam+ among the GFP+ cells in 4OHT (n=8) treated livers. Scale bars: 100 μm. Data are represented as mean ±SEM.
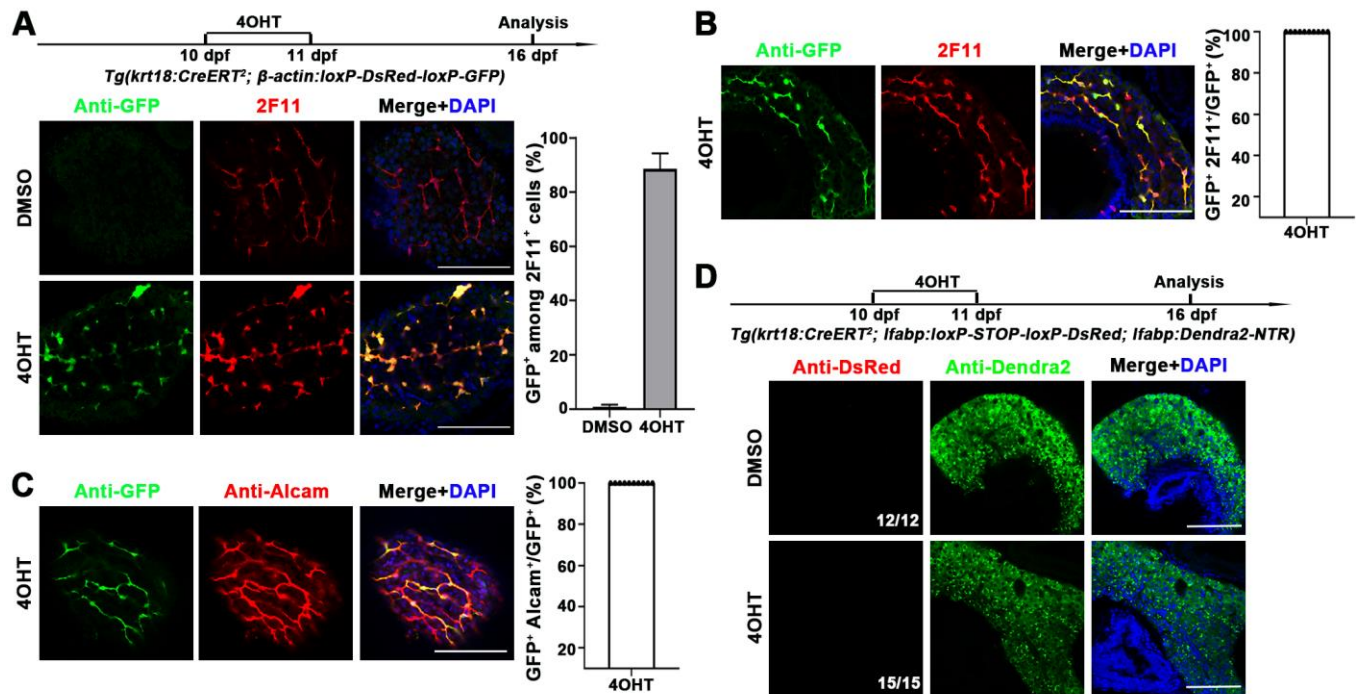
He_Figure S2

Figure S2. Establishment of lineage tracing system of physiological BEC-to-hepatocyte conversion using the *krt18* promoter. Related to Figure 3. (**A**) Experimental scheme illustrating the stage of 4OHT treatment to double transgenic line, *Tg(krt18:CreERT2; β-actin:loxP-DsRed-loxP-GFP)* from 10 dpf to 11 dpf and analysis at 16 dpf. Immunostaining for 2F11 and GFP on livers (2D imaging) showing *krt18-CreER* labels the 2F11 positive cholangiocytes specifically after 4OHT treatment. Nuclei were stained with DAPI (blue). Quantification of the percentage of the GFP$^+$ among the 2F11$^+$ cells in DMSO (n=6) and 4OHT (n=6) treated livers. (**B**) Immunostaining for 2F11 and GFP on livers (2D imaging) showing *krt18-CreER* labels the 2F11 positive cholangiocytes specifically after 4OHT treatment. Nuclei were stained with DAPI (blue). Quantification of the percentage of the GFP$^+$2F11$^+$ among the GFP$^+$ cells in 4OHT (n=10) treated livers; (**C**) Immunostaining for Alcam and GFP on livers (2D imaging) showing *krt18-CreER* labels the Alcam positive cholangiocytes specifically after 4OHT treatment. Nuclei were stained with DAPI (blue). Quantification of the percentage of the GFP$^+$Alcam$^+$ among the GFP$^+$ cells in 4OHT (n=10) treated livers. (**D**) Experimental scheme illustrating the stage of 4OHT treatment to triple transgenic line *Tg(krt18:CreERT2; lfabp: loxP-STOP-loxP-DsRed; lfabp:Dendra2-NTR)* from 10 dpf to 11 dpf and analysis at 16 dpf. Immunostaining for DsRed and Dendra2 in livers (2D imaging) showing no DsRed$^+$Dendra$^+$ cells. Nuclei were stained with DAPI (blue). Scale bars: 100 μm. Data are represented as mean ±SEM.
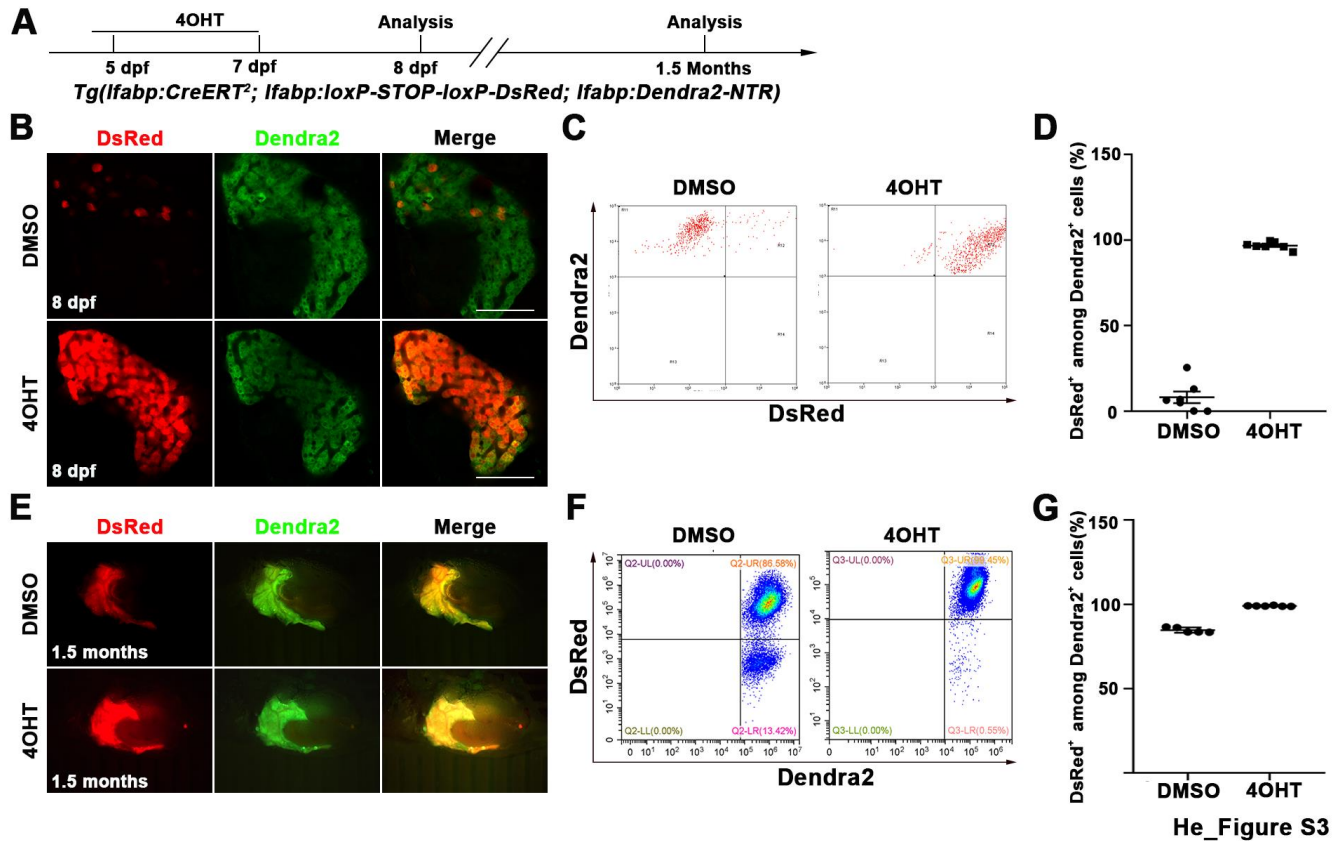
Figure S3. Transgenic line *Tg(lfabp:CreERT²)* shows high leakiness in long term lineage tracing. Related to Figure 2 and Figure 3. (**A**) Experimental scheme illustrating the stage of 4OHT treatment to triple transgenic line *Tg(lfabp:CreERT²; lfabp:loxP-STOP-loxP-DsRed; lfabp:Dendra2-NTR)* and analysis at 8 dpf and 1.5 months, respectively. (**B**) Single-optical images showing a low level of "leaky" (low level of DsRed⁺ in Dendra2⁺ cells) in early development (8 dpf) in DMSO treatment. (**C**) Fluorescence Activating Cell Sorter (FACS) analysis showing the ratio of DsRed⁺ among Dendra2⁺ cells in DMSO and 4OHT treated livers. (**D**) Quantification of the percentages of DsRed⁺ among Dendra2⁺ hepatocytes (DMSO, n=7; 4OHT, n=7). (**E**) Live images showing strong DsRed expression in livers at 1.5 months in DMSO and 4OHT treatment. (**F**) FACS analysis showing the ratio of DsRed⁺ among Dendra2⁺ cells in DMSO and 4OHT treated livers at 1.5 months. (**G**) Quantification of the percentages of DsRed⁺ among Dendra2⁺ hepatocytes (DMSO, n=5; 4OHT, n=6). Scale bars: 100 μm. Data are represented as mean ±SEM.
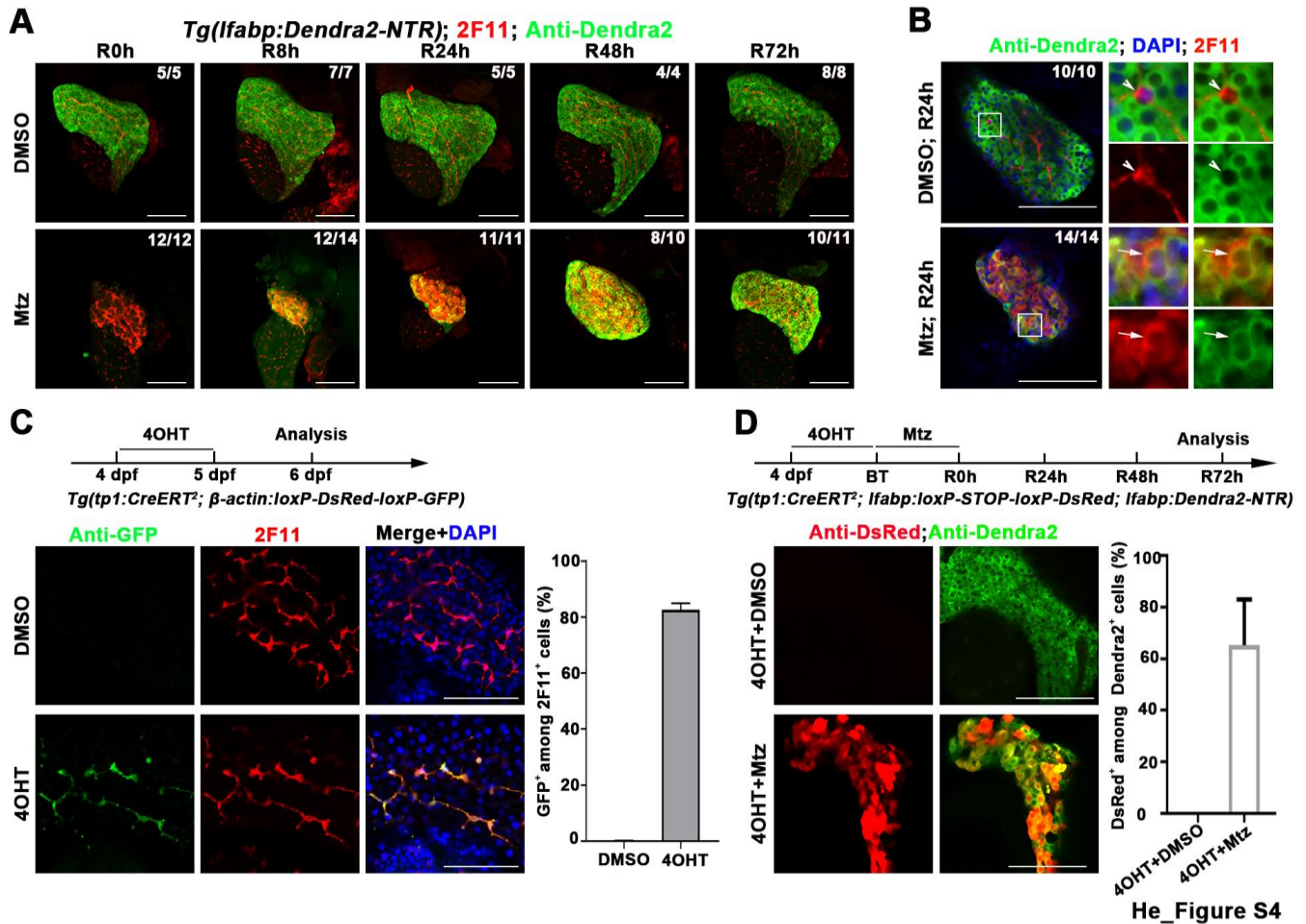
Figure S4. Lineage tracing of BECs during hepatocyte regeneration after extreme injury to healthy liver. Related to Figure 4. (**A**) Confocal projection images (3D imaging) showing the co-immunostaining for 2F11 and Dendra2 in liver regeneration after Mtz treatment. (**B**) Single-optical images showing the expression of 2F11 and Dendra2 at R24h in DMSO and Mtz treatment. There are no 2F11 and Dendra2 double-positive cells in DMSO treated liver (arrowheads); most of the new regenerating hepatocytes are 2F11 positive in Mtz treatment (arrows). Nuclei were stained with DAPI (blue). (**C**) Experimental scheme illustrating the stage of 4OHT treatment to double transgenic line, *Tg(tp1:CreERT²; β-actin:loxP-DsRed-loxP-GFP)* from 4 dpf to 5 dpf and analysis at 6 dpf. Immunostaining for 2F11 and GFP on livers (2D imaging) showing *tp1-CreER* labels the 2F11 positive cholangiocytes specifically after 4OHT treatment. Nuclei were stained with DAPI (blue). Quantification of the percentages of GFP⁺ among 2F11⁺ hepatocytes (DMSO, n=6; 4OHT, n=6). (**D**) Experimental scheme illustrating the stage of 4OHT and Mtz treatment and analysis at R72h. Single-optical images showing the expression of Dendra2 and DsRed in regenerating livers at R72h after 4OHT and Mtz treatment and control. Most of the new regenerating hepatocytes are DsRed positive. Quantification of the percentage of the DsRed⁺ among Dendra2⁺ cells (DMSO, n=10; 4OHT, n=10). Numbers indicate the proportion of larvae exhibiting the expression shown. Scale bars: 100 µm. Data are represented as mean ±SEM.
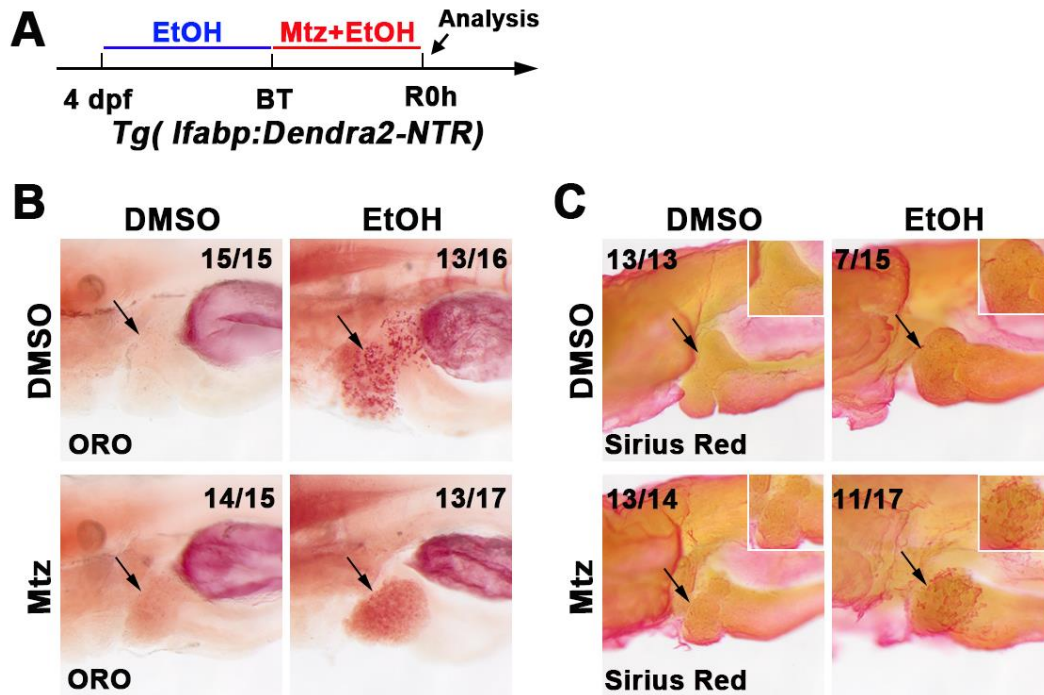
Figure S5. Establishing an Ethanol-induced Fibrotic Liver Model in Zebrafish. Related to Figure 4. (**A**) Experimental scheme illustrating the stage of EtOH and Mtz treatment in transgenic line *Tg(lfabp:Dendra2-NTR)*. (**B**) ORO staining shows fat deposits in livers after DMSO, EtOH, MTZ, and MTZ plus EtOH treatment at 6 dpf and R0h. The black arrowhead indicates the liver. (**C**) Sirius Red staining shows liver fibrosis after DMSO, EtOH, MTZ, and MTZ plus EtOH treatment at 6 dpf and R0h. The black arrowhead indicates the liver and the magnified liver regions were in a white box.

TRANSPARENT METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Jianbo He (hejianbo@swu.edu.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal Strains

The zebrafish facility and study were approved by the Institutional Review Board of Southwest University (Chongqing, China). Zebrafish were maintained in accordance with the Guidelines of Experimental Animal Welfare from Ministry of Science and Technology of People's Republic of China (2006) and the Institutional Animal Care and Use Committee protocols from Southwest University (2007). A complete list of the zebrafish strains is provided in the Key Resources Table. For the study both sexes were used.

METHOD DETAILS

Cell Sorting and Analysis

The *Tg(tp1:CreERT²; lfabp:DenNTR; lfabp: loxP-STOP-loxP-DsRed)*, *Tg(krt18:CreERT²; lfabp:DenNTR; lfabp:loxP-STOP-loxP-DsRed),* and *Tg(lfabp:CreERT²; lfabp:DenNTR; lfabp:loxP-STOP-loxP-DsRed)* transgenic larval and adult livers were dissected and dissociated in 0.5% Trypsin solution (0.5% trypsin and 1 mM EDTA in PBS, pH 8.0) for 5-10 minutes. Dissociated cells were collected by centrifugation at 3000×g for 2 minutes, resuspended in 1 ml PBS, and filtered using 40 μm cell strainers (Falcon). Then, cells were Analyzed using a Moflo XDP Fluorescence-Activated Cell Sorter (Beckman), Dendra2 and DsRed positive cells were analyzed.

Generation of Transgenic Lines

Lfabp-CreERT² construct was generated by replacing the *Dendra2-NTR* with *CreERT²* in the *pBluescript2(+) lfabp:Dendra2-NTR* vector. For the generation of *Tg(lfabp:CreERT²)* line, the construct *lfabp-CreERT²* was digested with I-sce1 and injected into zebrafish embryos of AB background at the 1-cell stage. For the generation of *Tg(tp1:CreERT²)* line, the construct *tp1-CreERT²* (a gift from Michael Parsons) was co-injected with *tol2* mRNA into zebrafish embryos of AB (wide type) background at the 1-cell stage.

Antibody Staining and Imaging

The process was performed according to previously described (Chen et al., 2019; Lu et al., 2013). Briefly, larvae and adult liver were fixed with 2% formaldehyde in 0.1 M PIPES, 1 mM MgSO4, 2 mM EGTA, pH 7.0 at 4°C overnight. For the larval experiments, the skin was manually removed, and the larvae were washed three times with PBS. For the adult liver, the whole liver was embedded into 4% low-melting agarose, sectioned in 50 μm, and followed by incubation with acetone at -20°C for 30 minutes. After being washed three times with the washing solution (1% Triton X-100 in PBS) and blocked in the blocking solution (4% BSA, 0.02% NaN3, 1% Triton X-100 in PBS) for an hour at 4°C, larvae were incubated with antibodies against Dendra2 ((1:1000; AB821, Evrogen,

Moscow, Russia), GFP (1:1000; ab6658, Abcam, Cambridge, MA), 2F11 (1:1000; ab71826, Abcam, Cambridge, MA), Anti-Alcam (1:50; ZN-5, ZIRC), Anti-collagen 1 (1:100; ab23730, Abcam, Cambridge, MA) and DsRed2 (1:1000; sc-101526, Santa Cruz, Dallas, TX) diluted in the blocking solution at 4°C overnight. Then, larvae were washed five times with the washing solution for 40 minutes each and incubated with Alexa fluorescent-conjugated secondary antibodies (1:1000; Invitrogen, Grand Island, NY) diluted in the blocking solution at 4°C overnight. After washed five times with the washing solution, larvae were proceeded for mounting and imaging.

Whole-mount *in situ* hybridization

The process was performed according to previously described (Liu et al., 2016). Briefly, larvae were fixed with 4% paraformaldehyde (PFA) in PBS at 4°C overnight, followed by incubation in 100% methanol at -20°C for at least 24 hours. Larvae were serially transferred into 75%, 50%, 25%, and 0% methanol in PBT (0.1% Tween in PBS). Then the larvae were digested with Proteinase K (5 µg/ml in PBT, 1:2000 dilution) for 40min. Refixation in 4% PFA in PBS, 30 min at room temperature, the larvae were washed in PBT four times. Then the larvae pre-hybridized in HYB (50% formamide, 5×SSC, 0.1% Tween20, 5 mg/ml torula yeast RNA, 50 mg/ml heparin) at 65°C for 2 hours. Digoxigenin-labeled probes were then administrated, and hybridizations were carried out at 68.5°C overnight. After removal of probes, the larvae were serially washed at 65°C with 100%, 75%, 50%, 25% HYB in 2×SSCT, and finally with 0.2×SSCT. Afterwards, the larvae were serially incubated with 25%, 50%, 75% and 100% MABT (150 mM maleic acid, 100 mM NaCl, 0.1% Tween-20, pH 7.5) in 0.2×SSCT, then blocked for 2 hour with 2% Block Reagent (Roche, 11096176001) in MAB. Anti-Dig AP (Roche, 11093274910) were administrated at a 1:2000 dilution and incubated at 4°C overnight. The larvae were serially washed with MABT for eight times, After the larvae were washed with NTMT (100 mM Tris HCl pH 9.5, 50 mM MgCl$_2$, 100 mM NaCl, 0.1% Tween 20) for three times. During last wash, transfer embryos to a 24 well plate. Then incubated embryos in BCIP/ NBT staining solution (Roche, 11681451001, 1:50) at room temp or 4°C in the dark, without shaking.. Stop the reaction by removing the staining solution and washing the embryos 3x in PBT, then washed and stored in STOP solution (0.05M phosphate buffer pH 5.8, 1mM EDTA 0.1% Tween). The Whole-mount *in situ* hybridization (WISH) images were captured using the SteREO Discovery V20 microscope (Carl Zeiss, Germany). The template for *cp* antisense probes were amplified with the following primer, forward primer: 5'-CTGCGGAGGAGGACGACACGG-3', reverse primer: 5′-ATTGTAATACGACTCACTATAGGGGCATGTCGACACCGTCAGCC-3′. The template for *gc* antisense probes were amplified with the following primer, forward primer 5'-CCTCCAAGTCATTGGAATTG-3', reverse primer: 5′-ATTGTAATACGACTCACTATAGGGCGGAATGGGTACGACTGGAC -3′. Digoxigenin-labeled probes were generated by in vitro transcription (DIG RNA Labeling Kit, Roche).

Ethanol and Metronidazole Treatment

The *Tg(lfabp:Dendra2-NTR)^{cq1}* transgenic larvae at 5 dpf was incubated with 10 mM Mtz (Sigma-Aldrich) in 0.2% DMSO for 24 hours. Then, larvae were washed three times and recovered in egg water marking the 0 hour-post treatment (hpt) point. For ethanol (EtOH) treatment, the larvae were pretreated with 1.5% EtOH (vol/vol) (Sigma-Aldrich) from 4 dpf and from 5 to 6 dpf treated with 10 mM MTZ in 1.5% EtOH for 24 hours (Huang et al., 2014). After washing the MTZ, the larvae were incubated in 1.5% EtOH.

Temporal Control of CreERT^2 Activities

4-hydroxytamoxifen (4OHT; Sigma) was dissolved in 100% DMSO to prepare a stock concentration of 10 mM. Working concentrations were titrated, and 2-5 µM (pre-warm at 60°C for 20 minutes and shake at 37°C for 1 hour) was found to be optimal to induce the Cre-mediated recombination without leading to physiologically deleterious defects. For *Tg(tp1:CreERT^2; lfabp:DenNTR; lfabp: loxP-STOP-loxP-DsRed)*, larvae were incubated with 2 µM 4OHT in egg water at 28°C from 5 dpf to 7 dpf for 48 hours and 5 µM 4OHT in egg water at 28°C from 4 dpf to 5 dpf for 24 hours, respectively. For *Tg(tp1:CreERT^2; β-actin:loxP-DsRed-loxP-GFP)*, larvae were incubated with 2 µM 4OHT in egg water at 28°C from 5 dpf to 7 dpf for 48 hours, and 5 µM 4OHT in egg water at 28°C from 4 dpf to 5 dpf for 24 hours, respectively. For *Tg(tp1:CreERT^2; β-actin:loxP-DsRed-loxP-GFP)* and *Tg(tp1:CreERT^2; lfabp:DenNTR; lfabp: loxP-STOP-loxP-DsRed)*, larvae were incubated with 5 µM 4OHT and 1.5% EtOH (vol/vol) in egg water at 28°C from 4 dpf to 5 dpf for 24 hours. For *Tg(krt18:CreERT^2; lfabp:DenNTR; lfabp:loxP-STOP-loxP-DsRed)* and *Tg(krt18:CreERT^2; β-actin:loxP-DsRed-loxP-GFP)*, larvae were incubated with 2 µM 4OHT in egg water at 28°C from 10 dpf to 11 dpf for 24 hours. For *Tg(lfabp:CreERT^2; lfabp:DenNTR; lfabp:loxP-STOP-loxP-DsRed)* larvae were incubated with 2 µM 4OHT in egg water at 28°C from 5 dpf to 7 dpf for 48 hours. Then, larvae were washed three times and recovered in egg water.

Hematoxylin and Eosin Staining

For liver histology analysis, the embryos at 7 dpf were fixed in 4% PFA at 4°C overnight, serially dehydrated in ethanol, xylene and embedded in paraffin. The liver paraffin-sections (7 µm) were stained with hematoxylin and eosin according to the manufacturer's instruction.

Oil Red O Staining

Add 0.5 g Oil Red O (ORO) (BBI Life Sciences) to 100 ml 100% isopropyl alcohol as ORO stock solution. Larvae were fixed with 4% PFA at 4°C overnight. The larvae were washed with PBS, then washed with 60% isopropanol for 1 hour, stained with filtered ORO working solution (3 part ORO stock solution to 2 part H$_2$O) (for 15 min at room temperature, and then washed twice with H$_2$O for 15 min.

Sirius Red Staining

Larvae were fixed with 4% PFA at 4°C overnight. The larvae were washed with PBS, removed the skins, then stained with 10% Sirius Red staining solution (SenBeiJia Life Sciences) for 10min at room temperature. Then washed twice with H$_2$O for 15 min.

Partial Hepatectomy of Zebrafish larvae

Larvae from 5 dpf were embedded in 1% low melting agarose (Life Science), and the liver was dissected and resected with sharp tweezers (55#, WPI) under the fluorescent microscope at 50 x amplification. After PH, the larvae were bred in the egg water at 28.5°C.

Quantification and Statistical Analysis

zebrafish were imaged using a SteREO Discovery V2.0 microscope equipped with AxioVision Rel 4.8.2 software (Carl Zeiss). Antibody stained and imaged using ZEN 2010 software equipped on an LSM780 and LSM880 confocal microscope (Carl Zeiss). All figures, labels, arrows, scale bars, and outlines were drawn using the Adobe Photoshop software. Unpaired two-tailed Student's t-test was used for statistical analysis; P<0.05 was considered

statistically significant. Quantitative data were shown as means ±SEM. The exact sample number (n), *P* value for each experimental group and statistical tests were indicated in the figure legends.

SUPPLEMENTAL TABLE S1–KEY RESOURCES

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-Dendra2 (1:1000)	Evrogen	Cat# AB821
Rabbit anti-Collagen 1 (1:100)	Abcam	Cat#ab23730
Mouse anti-Alcam (1:50)	Zebrafish International Resource Center	Cat# zn-5
Goat anti-GFP (1:1000)	Abcam	Cat#ab6658
Mouse 2F11 (1:1000)	Abcam	Cat#ab71826
Mouse anti-DsRed (1:1000)	Santa Cruz	Cat#SC-101526
Donkey anti-goat IgG Alexa fluor 488-conjugated (1:1000)	Invitrogen	Cat#A11055
Donkey anti-goat IgG Alexa fluor 633-conjugated (1:1000)	Invitrogen	Cat#A11057
Donkey anti-mouse IgG Alexa fluor 568-conjugated (1:1000)	Invitrogen	Cat#A10073
Donkey anti-mouse IgG Alexa fluor 647-conjugated (1:1000)	Invitrogen	Cat#A31571
Donkey anti-rabbit IgG Alexa fluor 488-conjugated (1:1000)	Invitrogen	Cat#A10042
Anti-Dig-AP (1:2000)	Roche	Cat#11093274910
Chemicals, Peptides, and Recombinant Proteins		
NBT/BCIP Stock Solution	Roche	11681451001
Ethanol (EtOH)	Sigma-Aldrich	P7023
Low-melting agarose	BBI Life Sciences	A600015-0025
4-Hydroxytamoxifen (4OHT)	Sigma-Aldrich	H7904
Metronidazole (Mtz)	Sigma-Aldrich	M3761
Oil Red O	BBI Life Sciences	A600395-0050
Critical Commercial Assays		
Sirius Red staining solution	SenBeiJia Life Sciences	BP-DL030
Hematoxylin staining solution	BBI Life Sciences	E607317-0100
Eosin staining solution	BBI Life Sciences	E607321-0100
Experimental Models: Organisms/Strains		
Zebrafish: *Tg(lfabp:DenNTR)*[cq1]	He et al., 2014	ZFIN:ZDB-ALT-150922-3
Zebrafish: *Tg(krt18:CreERT2)*[cq74]	He et al., 2019	N/A

Zebrafish:*Tg(lfabp:loxP-STOP-loxP-DsRed2)^{cq4}*	He et al., 2014	ZFIN:ZDB-TGCONSTRCT-150922-5
Zebrafish: *Tg(tp1:CreERT^2)*	This study	N/A
Zebrafish: *Tg(lfabp:CreERT^2)*	This study	N/A
Recombinant DNA		
pBluescript-lfabp-CreERT^2	This study	N/A
Tol2-tp1-CreERT^2	Wang et al., 2011	N/A
Software and Algorithms		
ZEN2010 Imaging software	Carl Zeiss	https://www.zeiss.com
AxioVision Rel 4.8.2 software	Carl Zeiss	https://www.zeiss.com
GraphPad Prism	GraphPad	https://www.graphpad.com
Moflo XDP Fluorescence-Activated Cell Sorter	Beckman	https://www.beckmancoulter.com

SUPPLEMENTAL REFERENCES

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