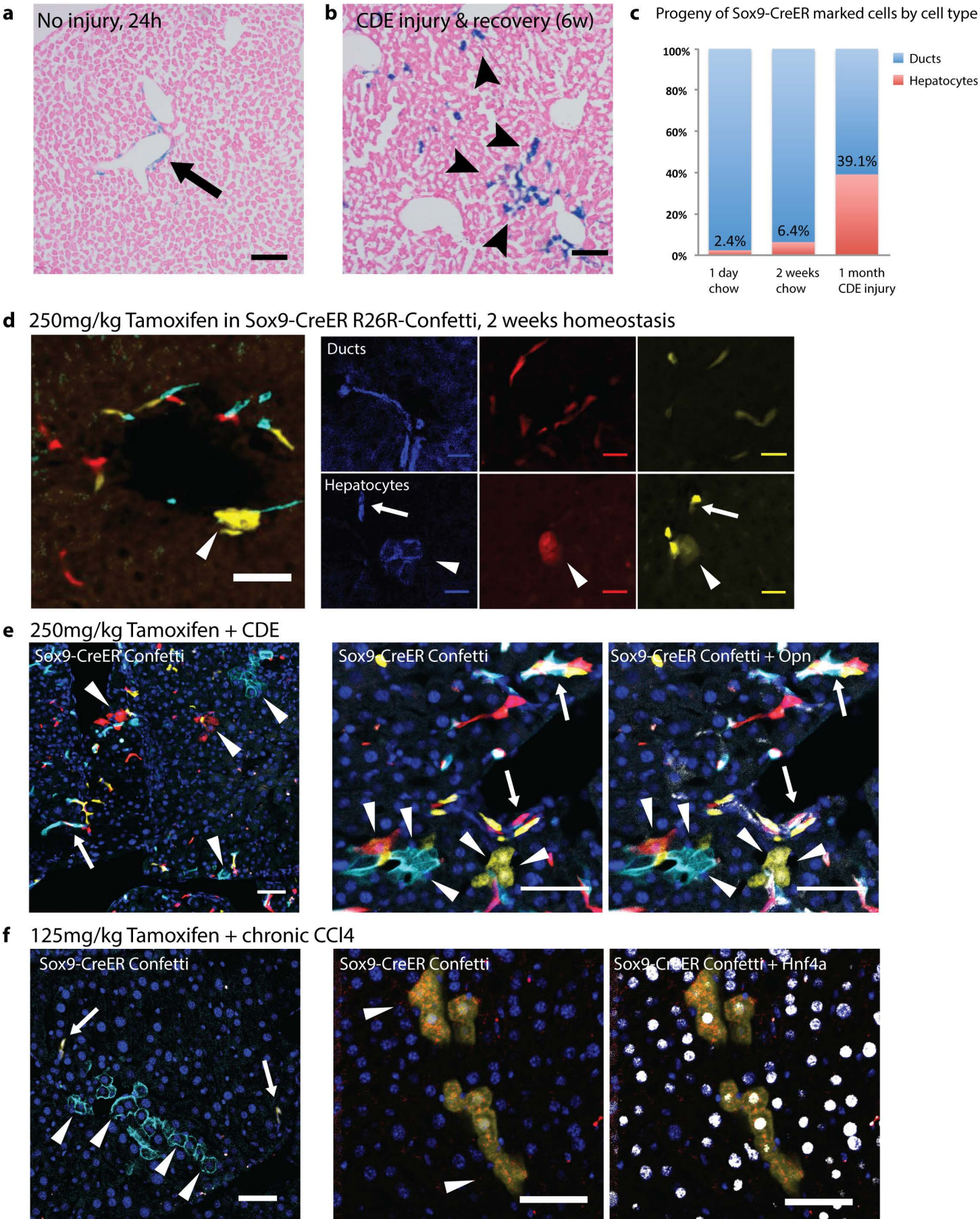
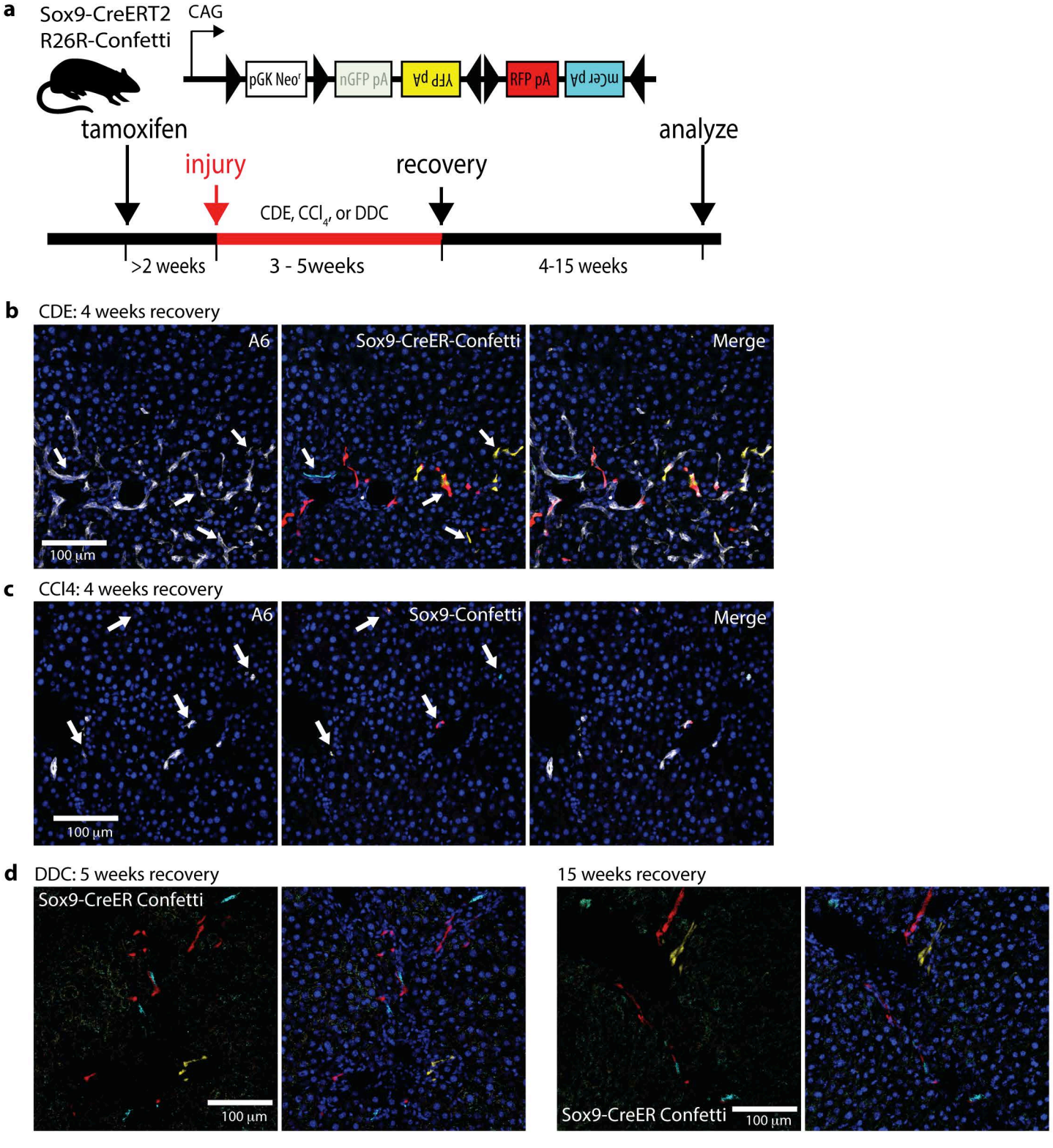
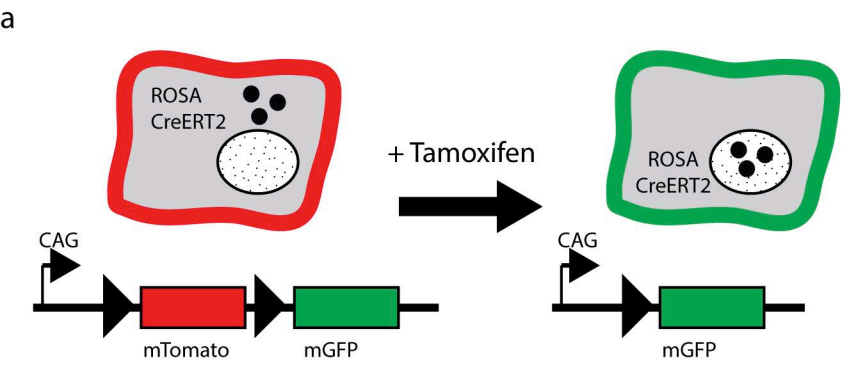
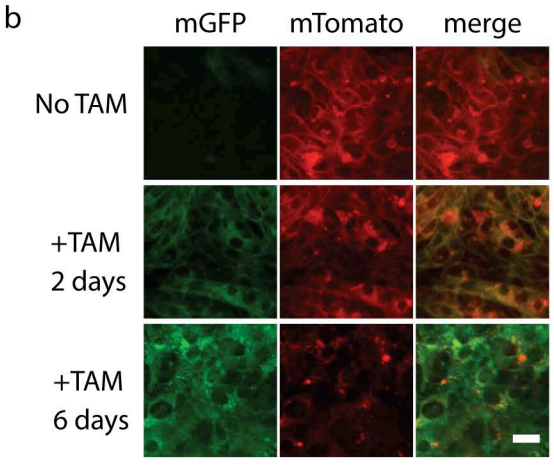
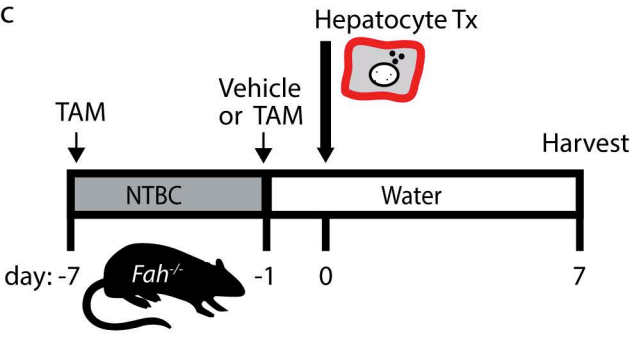
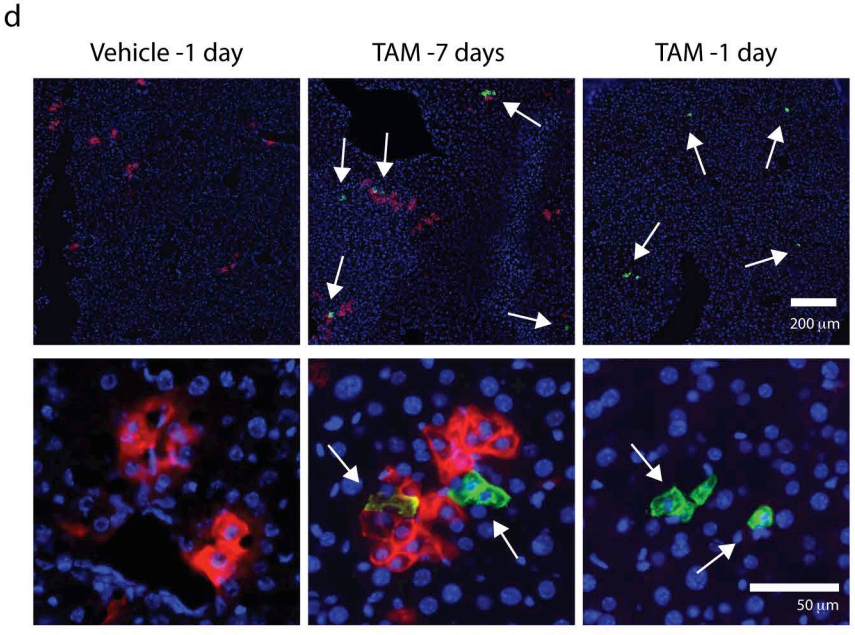

a
No injury, 24h
b
CDE injury & recovery (6w)
c
Progeny of Sox9-CreER marked cells by cell type
100%
80%
60%
40%
20%
0%
Ducts
Hepatocytes
2.4%
6.4%
39.1%
1 day chow
2 weeks chow
1 month CDE injury
d
250mg/kg Tamoxifen in Sox9-CreER R26R-Confetti, 2 weeks homeostasis
Ducts
Hepatocytes
e
250mg/kg Tamoxifen + CDE
Sox9-CreER Confetti
Sox9-CreER Confetti
Sox9-CreER Confetti + Opn
f
125mg/kg Tamoxifen + chronic CCl4
Sox9-CreER Confetti
Sox9-CreER Confetti
Sox9-CreER Confetti + Hnf4a

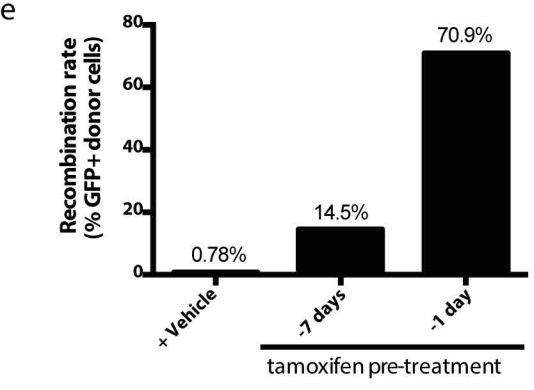

a
Sox9-CreERT2
R26R-Confetti
CAG
pGK Neo'
nGFP pA
YFP pA
RFP pA
mCer pA
tamoxifen
injury
recovery
analyze
CDE, CCl4, or DDC
>2 weeks
3 - 5weeks
4-15 weeks
b
CDE: 4 weeks recovery
A6
Sox9-CreER-Confetti
Merge
100 µm
c
CCl4: 4 weeks recovery
A6
Sox9-Confetti
Merge
100 µm
d
DDC: 5 weeks recovery
Sox9-CreER Confetti
100 µm
15 weeks recovery
Sox9-CreER Confetti
100 µm

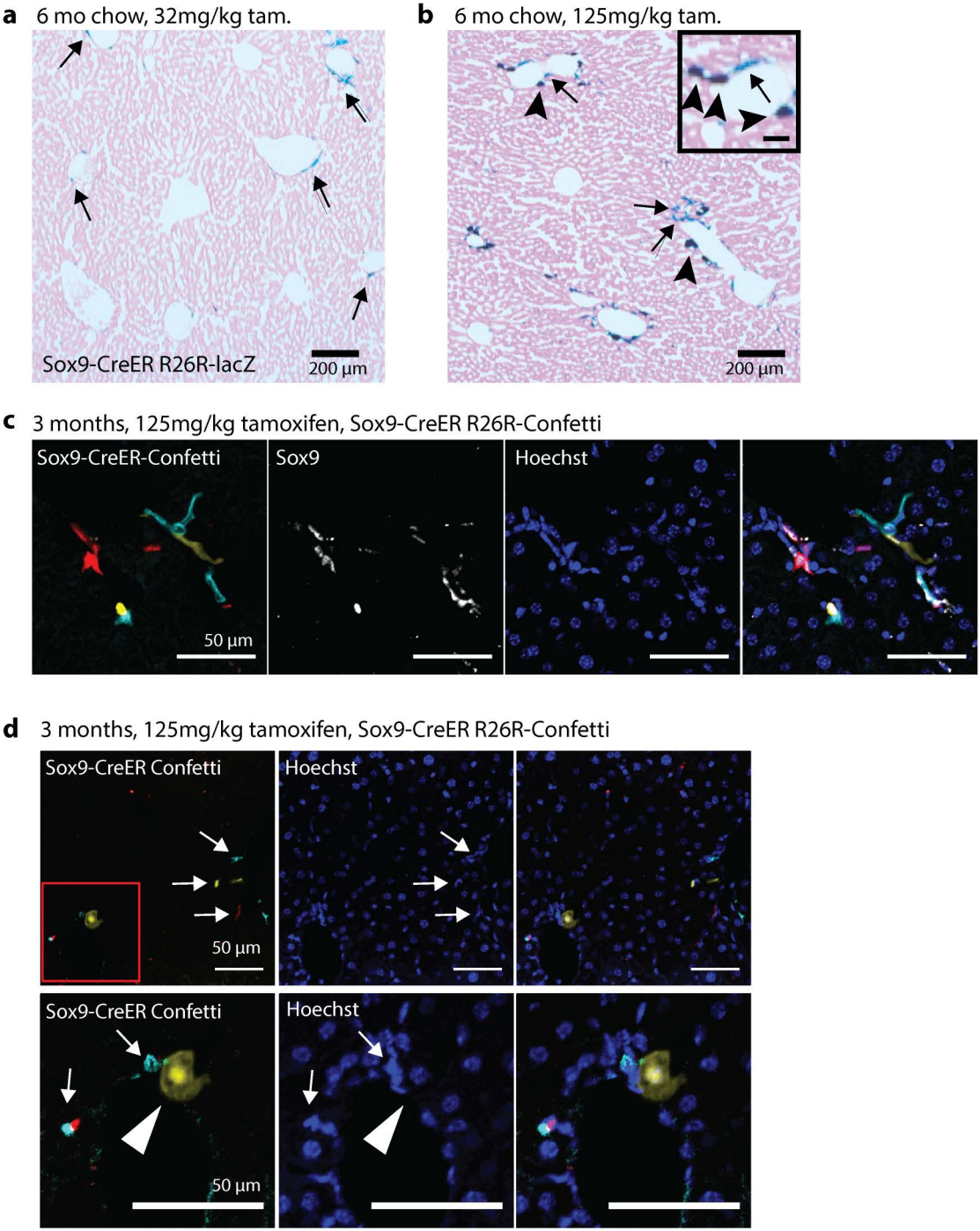

a
ROSA
CreERT2
+ Tamoxifen
ROSA
CreERT2
CAG
CAG
mTomato
mGFP
mGFP


b
mGFP
mTomato
merge
No TAM
+TAM
2 days
+TAM
6 days

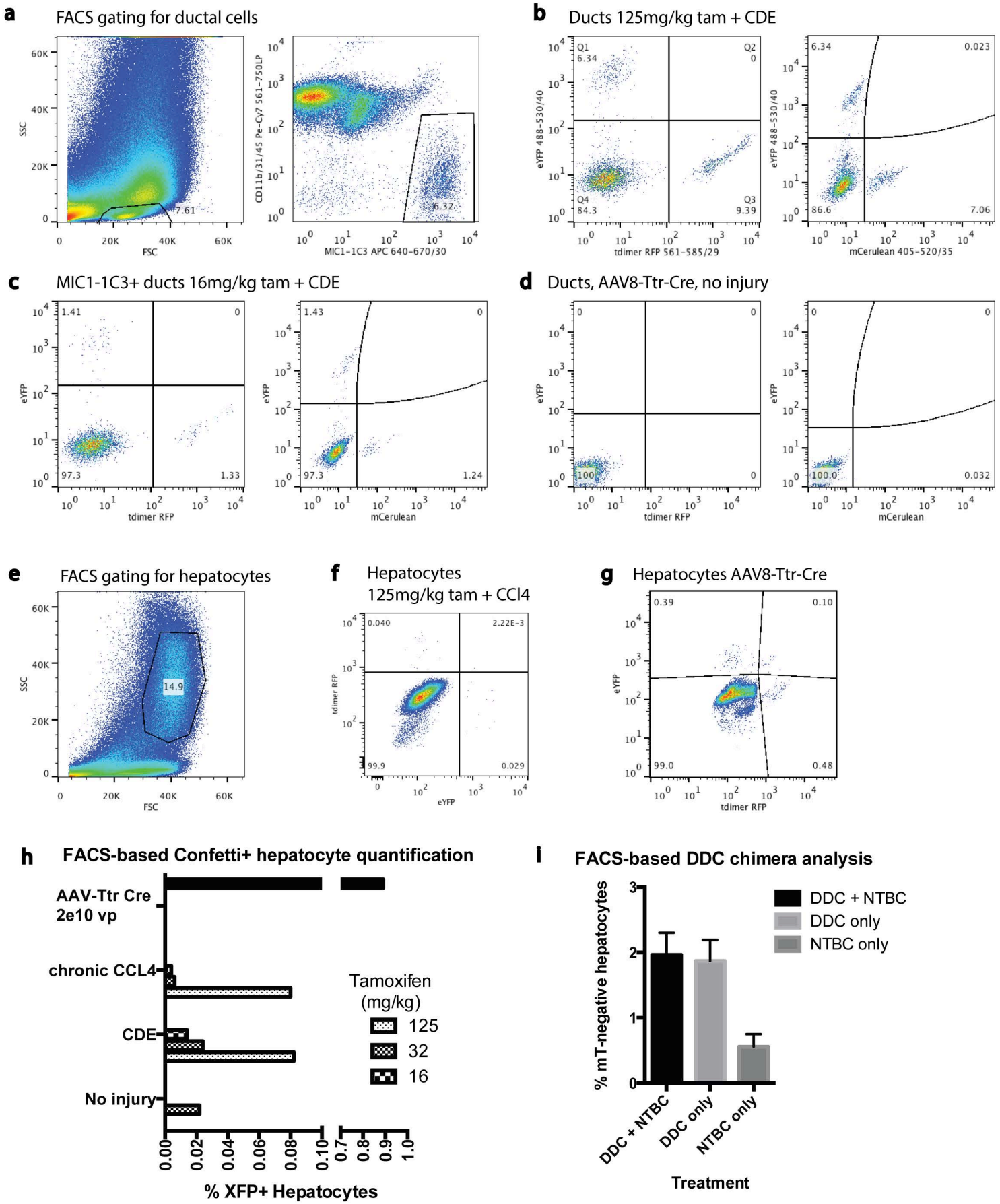

c
Hepatocyte Tx
TAM
Vehicle
or TAM
Harvest
NTBC
Water
day: -7
Fah-/-
-1
0
7

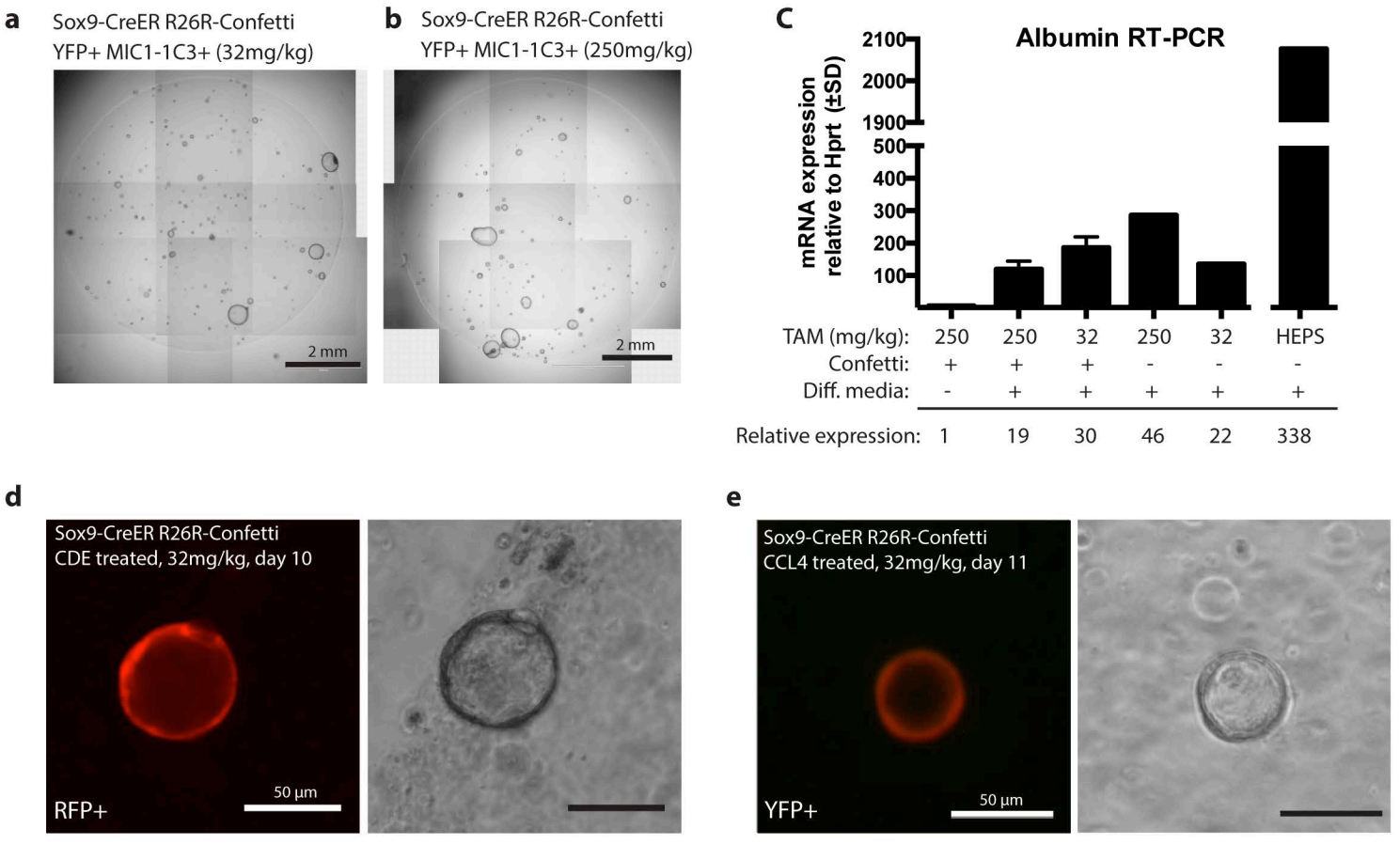

d
Vehicle -1 day
TAM -7 days
TAM -1 day
200 µm
50 µm

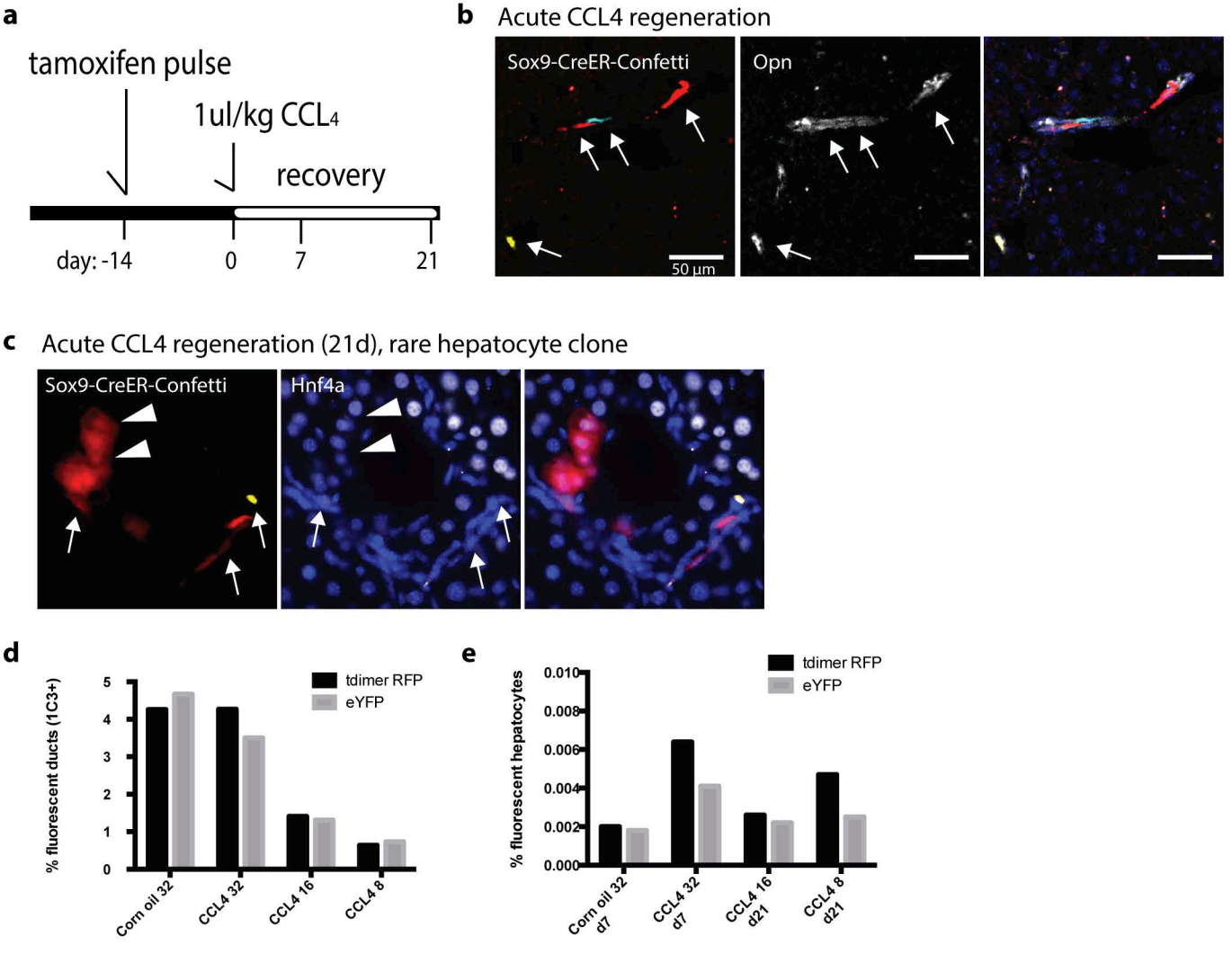

e
Recombination rate
(% GFP+ donor cells)
80
60
40
20
0
0.78%
14.5%
70.9%
+ Vehicle
-7 days
-1 day
tamoxifen pre-treatment

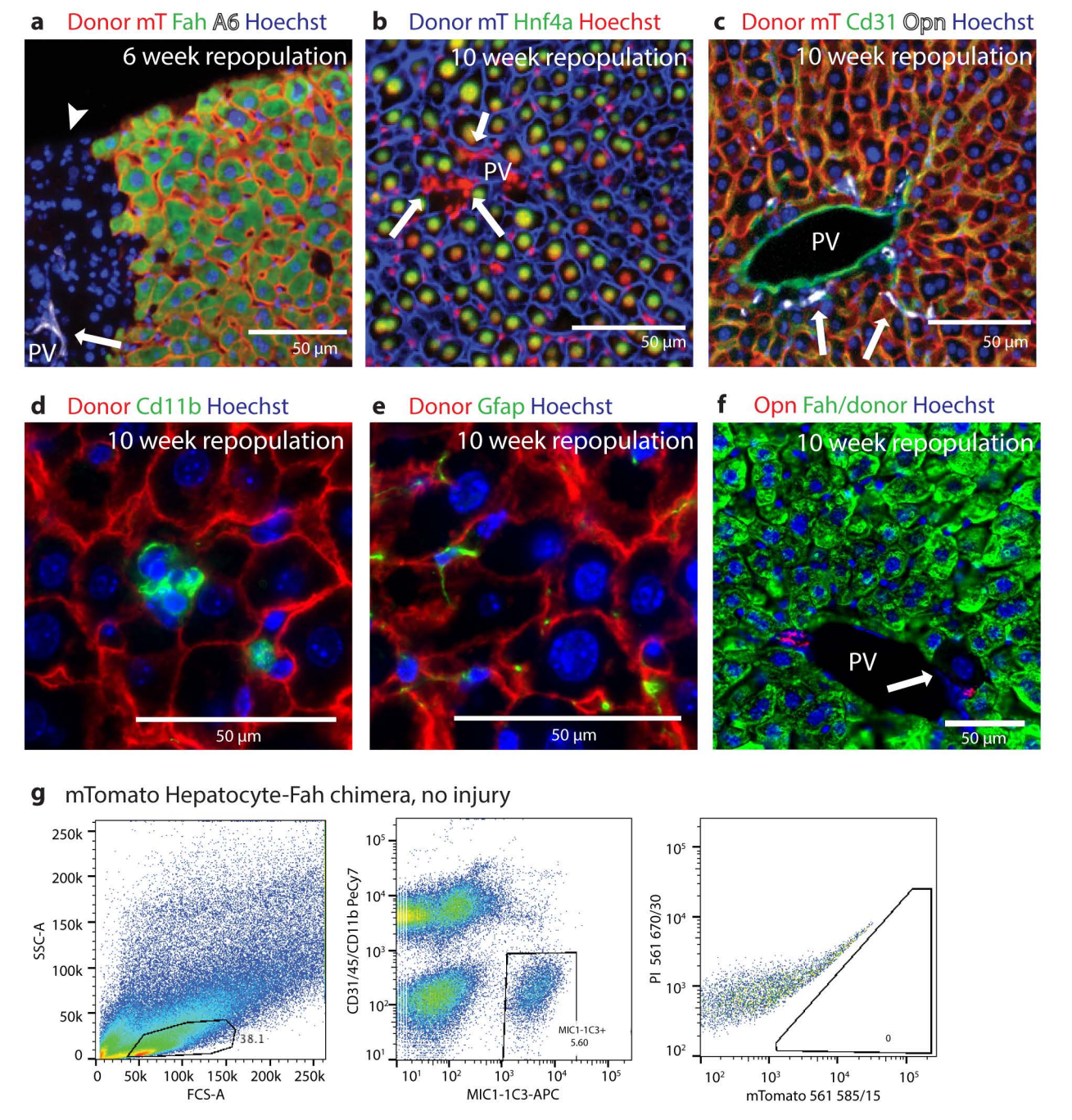

a
6 mo chow, 32mg/kg tam.
Sox9-CreER R26R-lacZ
200 µm
b
6 mo chow, 125mg/kg tam.
200 µm
c
3 months, 125mg/kg tamoxifen, Sox9-CreER R26R-Confetti
Sox9-CreER-Confetti
Sox9
Hoechst
50 µm
d
3 months, 125mg/kg tamoxifen, Sox9-CreER R26R-Confetti
Sox9-CreER Confetti
Hoechst
50 µm
Sox9-CreER Confetti
Hoechst
50 µm

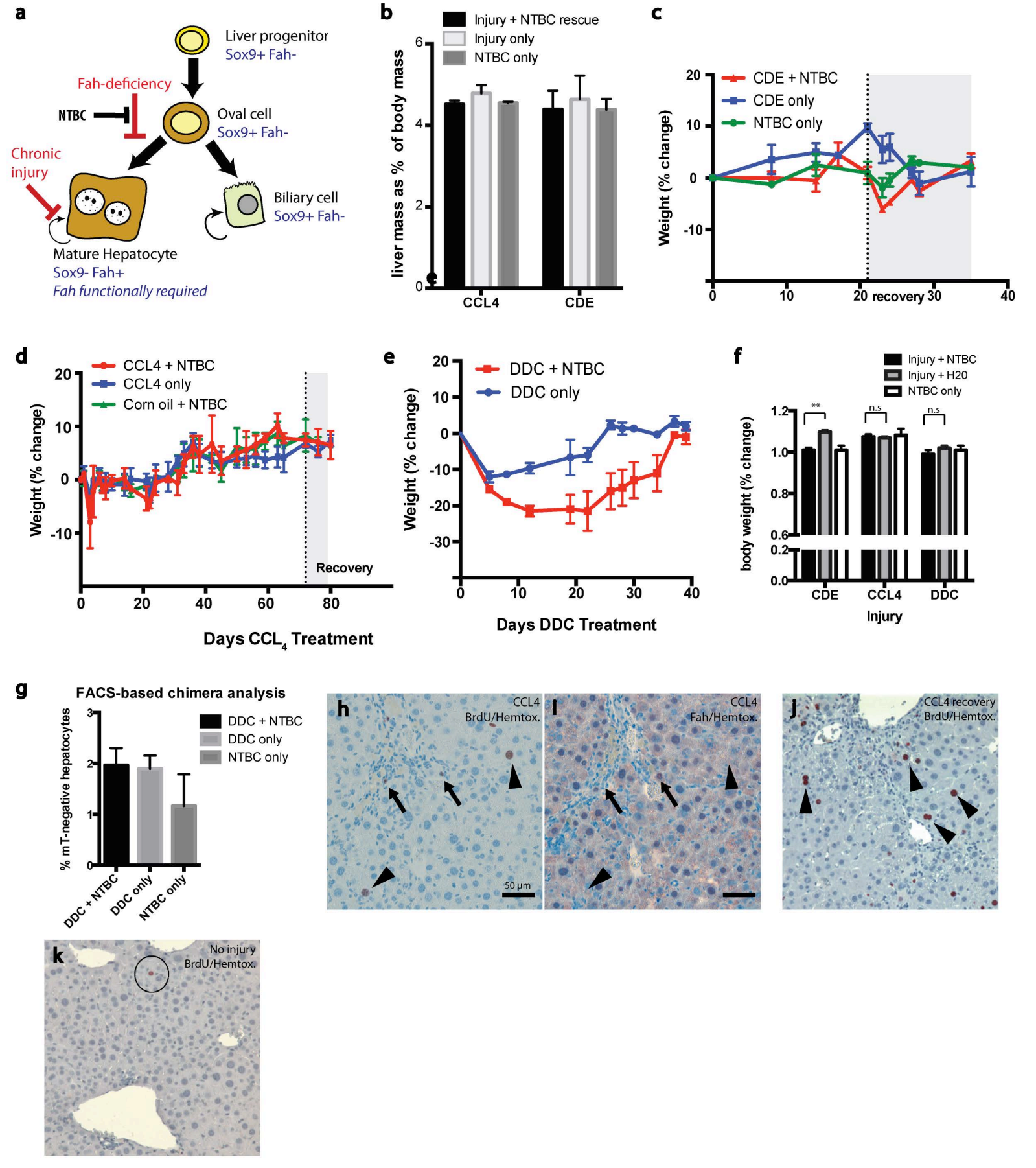
Supplemental Figure 3: FACS-based analysis of chimeric and Sox9-CreER marked liver cells

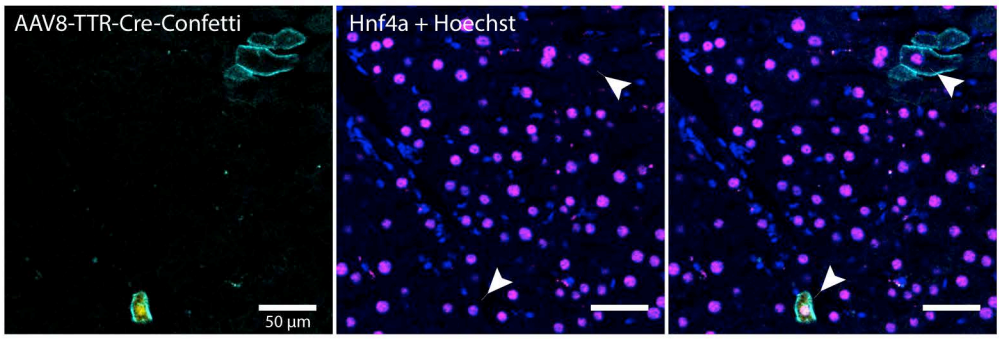


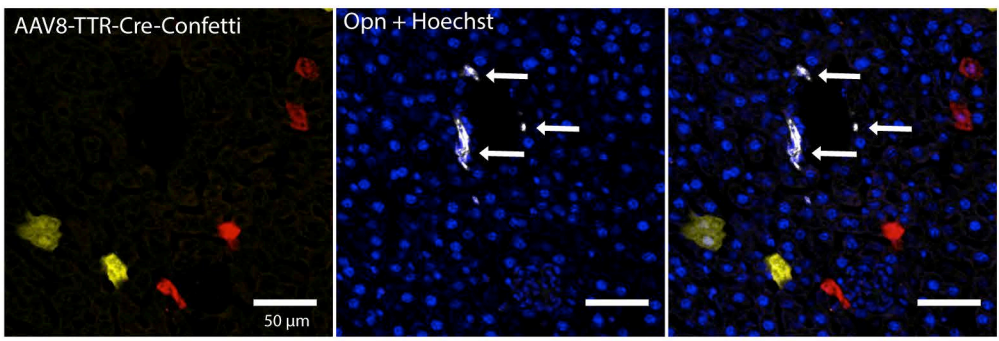

a
Sox9-CreER R26R-Confetti
YFP+ MIC1-1C3+ (32mg/kg)
2 mm
b
Sox9-CreER R26R-Confetti
YFP+ MIC1-1C3+ (250mg/kg)
2 mm
c
Albumin RT-PCR
mRNA expression relative to Hprt (±SD)
2100
2000
1900
500
400
300
200
100
TAM (mg/kg): 250 250 32 250 32 HEPS
Confetti: + + + - - -
Diff. media: - + + + + +
Relative expression: 1 19 30 46 22 338
d
Sox9-CreER R26R-Confetti
CDE treated, 32mg/kg, day 10
RFP+
50 µm
e
Sox9-CreER R26R-Confetti
CCL4 treated, 32mg/kg, day 11
YFP+
50 µm

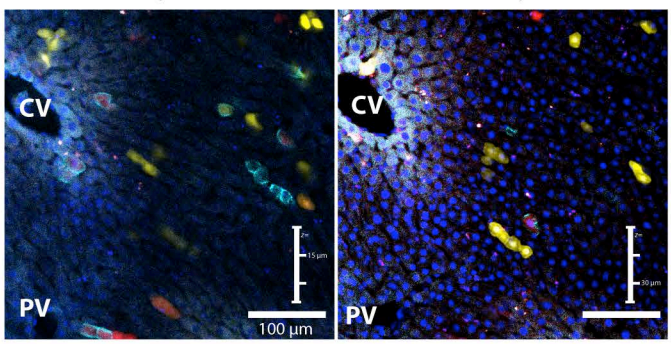

a
tamoxifen pulse
1ul/kg CCL4
recovery
day: -14
0
7
21
b
Acute CCL4 regeneration
Sox9-CreER-Confetti
Opn
50 µm
c
Acute CCL4 regeneration (21d), rare hepatocyte clone
Sox9-CreER-Confetti
Hnf4a
d
% fluorescent ducts (1C3+)
tdimer RFP
eYFP
Corn oil 32
CCL4 32
CCL4 16
CCL4 8
e
% fluorescent hepatocytes
tdimer RFP
eYFP
Corn oil 32 d7
CCL4 32 d7
CCL4 16 d21
CCL4 8 d21

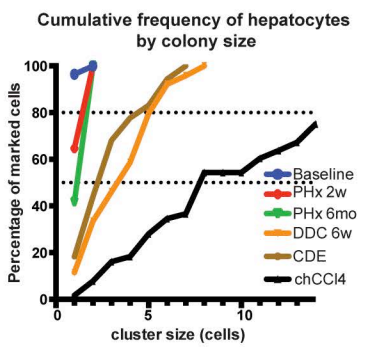

a Donor mT Fah A6 Hoechst
6 week repopulation
PV
50 µm
b Donor mT Hnf4a Hoechst
10 week repopulation
PV
50 µm
c Donor mT Cd31 Opn Hoechst
10 week repopulation
PV
50 µm
d Donor Cd11b Hoechst
10 week repopulation
50 µm
e Donor Gfap Hoechst
10 week repopulation
50 µm
f Opn Fah/donor Hoechst
10 week repopulation
PV
50 µm
g mTomato Hepatocyte-Fah chimera, no injury
SSC-A
FCS-A
38.1
CD31/45/CD11b PeCy7
MIC1-1C3-APC
MIC1-1C3+
5.60
PI 561 670/30
mTomato 561 585/15
0

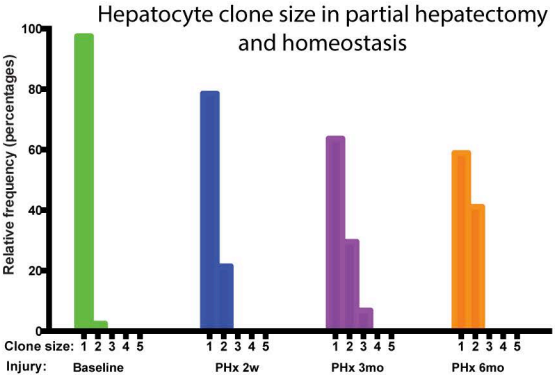

a
Liver progenitor
Sox9+ Fah-
Fah-deficiency
NTBC
Oval cell
Sox9+ Fah-
Chronic injury
Biliary cell
Sox9+ Fah-
Mature Hepatocyte
Sox9- Fah+
Fah functionally required
b
Injury + NTBC rescue
Injury only
NTBC only
liver mass as % of body mass
CCL4
CDE
c
CDE + NTBC
CDE only
NTBC only
Weight (% change)
recovery
d
CCL4 + NTBC
CCL4 only
Corn oil + NTBC
Weight (% change)
Recovery
Days CCL4 Treatment
e
DDC + NTBC
DDC only
Weight (% change)
Days DDC Treatment
f
Injury + NTBC
Injury + H20
NTBC only
body weight (% change)
**
n.s
n.s
CDE
CCL4
DDC
Injury
g
FACS-based chimera analysis
DDC + NTBC
DDC only
NTBC only
% mT-negative hepatocytes
DDC + NTBC
DDC only
NTBC only
h
CCL4
BrdU/Hemtox.
50 µm
i
CCL4
Fah/Hemtox.
j
CCL4 recovery
BrdU/Hemtox.
k
No injury
BrdU/Hemtox.

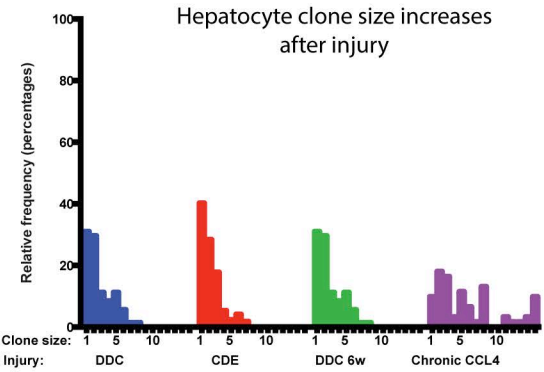

a
AAV8-TTR-Cre-Confetti
Hnf4a + Hoechst
50 µm

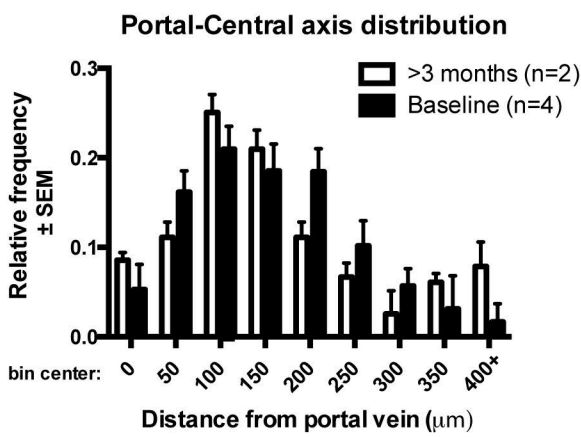

b
AAV8-TTR-Cre-Confetti
Opn + Hoechst
50 µm

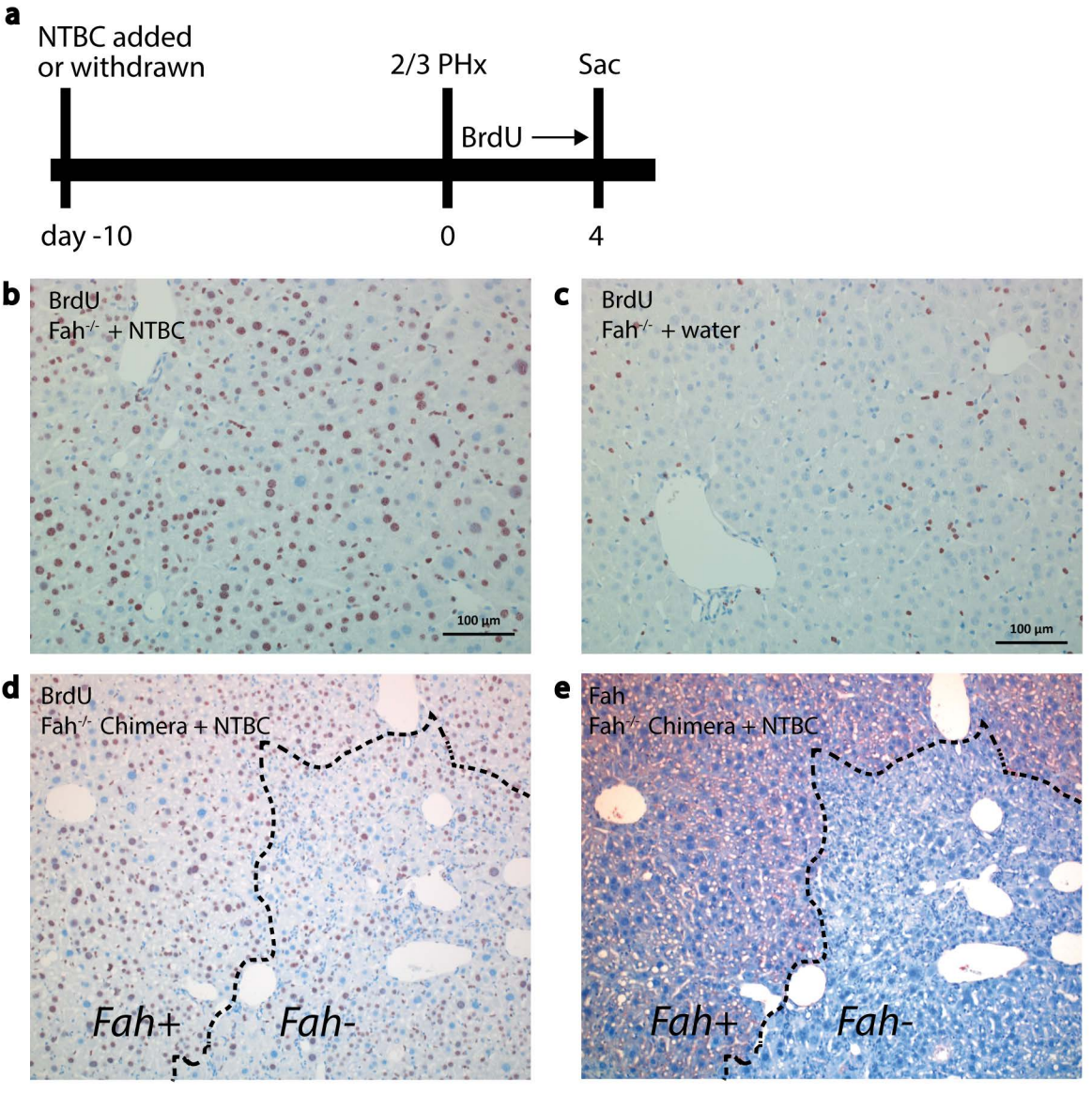

c 1x acute CCl4 (AAV8-Ttr-Cre Confetti + Hoechst)
CV
PV
100 µm
CV
PV


d
Cumulative frequency of hepatocytes by colony size
Percentage of marked cells
cluster size (cells)
Baseline
PHx 2w
PHx 6mo
DDC 6w
CDE
chCCl4


e
Hepatocyte clone size in partial hepatectomy and homeostasis
Relative frequency (percentages)
Clone size:
Injury:
Baseline
PHx 2w
PHx 3mo
PHx 6mo


Hepatocyte clone size increases after injury
Relative frequency (percentages)
Clone size:
Injury:
DDC
CDE
DDC 6w
Chronic CCL4


f
Portal-Central axis distribution
>3 months (n=2)
Baseline (n=4)
Relative frequency ± SEM
bin center:
Distance from portal vein (µm)


a
NTBC added
or withdrawn
2/3 PHx
Sac
BrdU
day -10
0
4
b
BrdU
Fah-/- + NTBC
100 µm
c
BrdU
Fah-/- + water
100 µm
d
BrdU
Fah-/- Chimera + NTBC
Fah+
Fah-
e
Fah
Fah-/- Chimera + NTBC
Fah+
Fah-

FACS sorting and analysis

Hepatocytes and NPCs were isolated with a multiple-step collagenase dissociation and gravity centrifugation protocol as described previously(12). Cells were immunostained for FACS with antibodies listed in the supplementary table. PI was used as a cell viability indicator. Cells were analyzed and sorted with a Cytopeia inFluxV-GS equipped with 405, 488, 561, and 640nm excitation lasers.

Cell Counting and Image analysis

Fluorescent and brightfield mages were captured with Zeiss AxioImager.M2 with Xen Blue 2011 software and a computer controlled motorized stage (Zeiss Inc.). Images were analyzed and scored with ImageJ/FIJI (www.fiji.sc). For analysis of injured chimeric liver tissue, sections were immunostained for Fah and nuclear counterstained with hemotoxylin or Hoechst 33258. mTomato direct fluorescence was also captured. Multiple high-resolution images were collected and the total numbers of positive and negative hepatocytes were scored from multiple liver lobes and sections from each animal.

For clonal AAV8-Ttr-Cre analysis in the R26R-Confetti mouse, direct fluorescence from fluorescent proteins was collected on a LSM780 laser scanning confocal microscope. Stitched z-stack images were collected and colony size was scored from a single 6um optical plane in all treatment groups for consistency. The thin section is may underestimate of hepatocyte cluster size. For hepatocyte clonal analysis, single clones were defined as cells of an identical color combination that were in direct contact with each other.

To evaluate the position of AAV8-Ttr-Cre-marked hepatocytes on the portal-central axis liver, 20uM thick fixed-frozen sections were first immunostained for osteopontin (AF647) and counterstained with Hoeschst 33258 to identify portal tracts. Multiple immunofluorescent images were collected from RFP, Opn-647, and Hoeschst channels using a Zeiss Apotome upright microscope outfitted with a (Xen Blue 2011, Carl Zeiss Inc). Images from adjacent fields were stitched together (ImageJ) to approximately 3300 x 2500 micron dimensions. Pixels were converted to micron measurements based on established calibration (1.550px/micron). The distance from the center of the marked cell to the nearest portal vein was measured for each Cre-marked clone (ImageJ).

For Sox9-CreER lineage tracing, 3-color direct fluorescence was collected from 50-100μm thick sections or 10μm sections co-labeled with a ductal marker (Opn or A6/AF647). After injury clones were scored as single color ductal cells that were at least 5 cells away from another marked cell of the same color.

Assumptions for clonal marking

Based on FACS based analysis of Sox9-CreER R26R-confetti$^{+/-}$ mice treated with 32mg/kg tamoxifen we assumed that 3% cholangiocytes were marked with mCerulean, eYFP, or tdimer-RFP. We did not observe double positive Sox9+ cells when tamoxifen was allowed to washout. We assumed that 20 cholangiocytes were present at each portal triad in a single section in a normal liver at the time of marking. Given our assumptions, we calculated there was greater than an 88% chance that single color cell in a portal triad represented a distinct clone. For hepatocyte clonal analysis, single clones were defined as cells of an identical color that were in direct contact with each other.

Extended tamoxifen recombination

Adult male *Fah*$^{-/-}$ mice were given IP tamoxifen 100mg/kg (dissolved in sesame oil) 7 days or 24 hours prior to intrasplenic hepatocyte transplantation. Sesame oil vehicle was given 24 hours prior. *Fah*$^{-/-}$ mice were maintained on 8mg/L NTBC water until 24 hours prior to transplant when NTBC was permanently discontinued. Then, hepatocytes were isolated from a *ROSA*-$^{CreERT2/mTmG}$ mouse (1,2) by standard collagenase perfusion and gravity centrifugation. The donor mouse also harbored a Scx-GFP (cytoplasmic) transgene that is not expressed in the liver(3). Donor cells were directly injected into the spleen of pre-treated recipient *Fah*$^{-/-}$ mice. Transplanted mice were sacrificed after 7 days and livers, fixed in 4% paraformaldehyde for 3 hours, cryopreserved in 30% sucrose, and frozen in OCT. 8 μm frozen sections were counterstained with Hoechst 33342 and analyzed on a fluorescent microscope.

For in vitro experiments, cells were plated in DMEM/F12 supplemented with 10% FBS, pen/strep, and 10mM Hepes. After cell attachment, tamoxifen citrate (dissolve in ethanol) was added to media overnight at a final concentration of 400nM. Media was then replaced every 2 days thereafter. Cells were imaged 2 or 6 days after tamoxifen administration on a flourescent microscope (Evos, Life Technologies) and images were identically processed with ImageJ.

Statistical analysis

Image quantification graphs and statistical tests results were generated with Prism 6.0b (Graphpad Software, Inc, La Jolla, CA, including histograms, bar graphs, standard error and confidence intervals, and unpaired parametric t-test.

Supplementary Table 1 - Antibodies for FACS and IF/IHC

Antibody /Antigen	Species	Dilution factor	Use	Product	Source
MIC1-1C3	Rat mAB	100	FACS	NBP1-18961	Novus Biologics
OC2-2F8	Rat mAB	20	FACS	Gift	Craig Dorrell, OHSU
Cd31	Rat mAB	100	FACS	561410	BD Biosciences
Cd45	Rat mAB	100	FACS, IF	552848	BD Biosciences
Cd11b	Rat mAB	100	FACS, IF	552850	BD Biosciences
Fah	Rabbit pAB	500	IF/IHC	Custom	Grompe Lab
Brdu	Rat mAB	50-250	IF/IHC	ab6326	Abcam
Ostepontin	Goat pAB	100	IF	AF808	R&D systems
A6	Rat mAB	50	IF	Gift	Valentina Factor, NCI
Ck19	Rabbit pAB	500	IF	Gift	Xin Wang, U. Minnesota
Sox9	Rabbit pAB	500	IF	AB5535	Millipore, Inc
Hnf4a	Rabbit pAB	100	IF	sc-8987	Santa Cruz Bio

Supplementary Table 2 - Transgenic mice in study

Short name	Scientific name	Jax Stock #	Reference
ROSA-lacZ	B6.129S7-*Gt(ROSA)26Sor*/J	002192	Friedrich G 1991
R26R-lacZ	B6.129S4-Gt(ROSA)26Sortm1Sor/J	003474	Soriano P, 1999
ROSA-mTomato	B6.129(Cg)-*Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo*/J	007676	Muzumdar M, 2007
R26R-Confetti	B6.Cg-Gt(ROSA)26Sortm1(CAG-Brainbow2.1)Cle/J	017492	Snippert H, 2010
Sox9-CreERT2	Tg(Sox9-cre/ERT2)1Msan/J	018829	Kopp J, 2011
Fah-/-	Fah tm1Mgo	N/A	Grompe M, 1993
ROSA-CreERT2	Gt(ROSA)26Sortm2(cre/ERT2)Brn	N/A	Hameyer D, 2007

Supplementary references:

1. Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. A global double-fluorescent Cre reporter mouse. Genesis. 2007;45:593–605.

2. Hameyer D, Loonstra A, Eshkind L, Schmitt S, Antunes C, Groen A, et al. Toxicity of ligand-dependent Cre recombinases and generation of a conditional Cre deleter mouse allowing mosaic recombination in peripheral tissues. Physiological Genomics. 2007;31:32–41.

3. Pryce BA, Brent AE, Murchison ND, Tabin CJ, Schweitzer R. Generation of transgenic tendon reporters, ScxGFP and ScxAP, using regulatory elements of the scleraxis gene. Dev. Dyn. 2007;236:1677–1682.