Supporting Material

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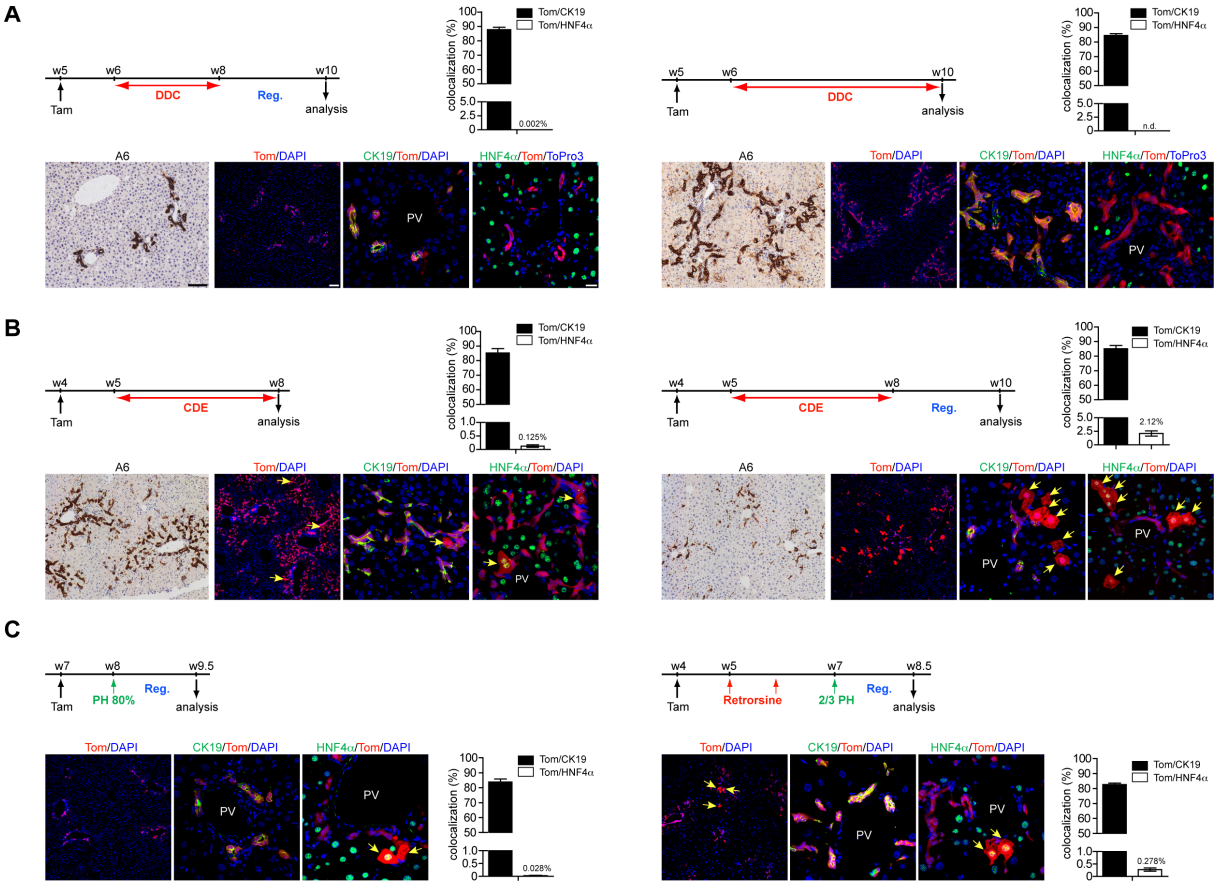
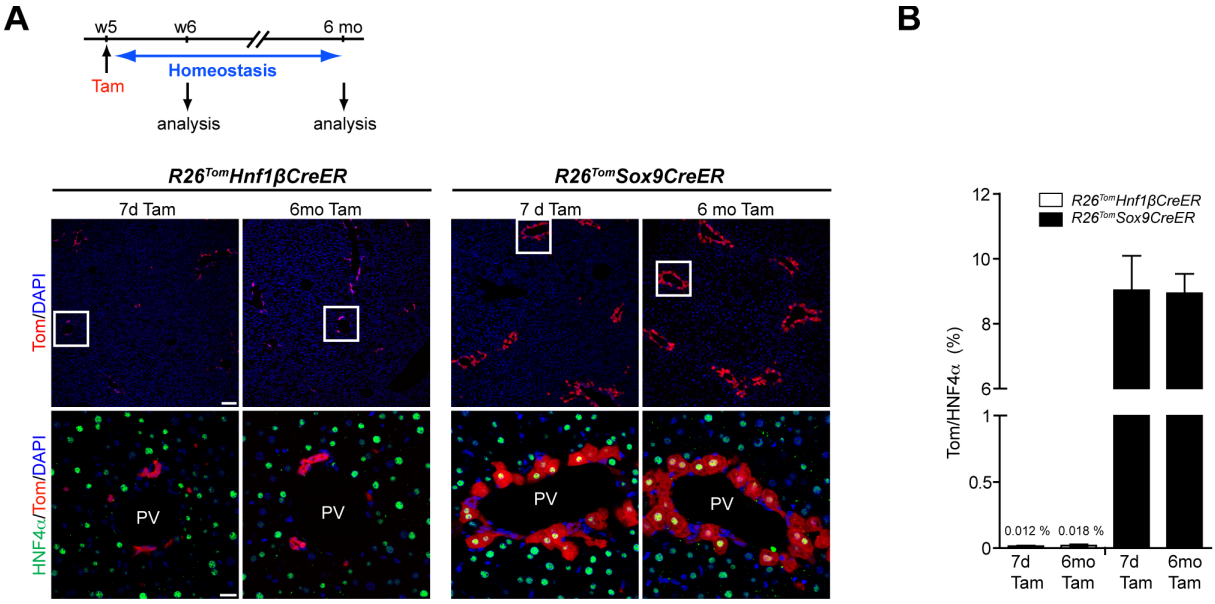
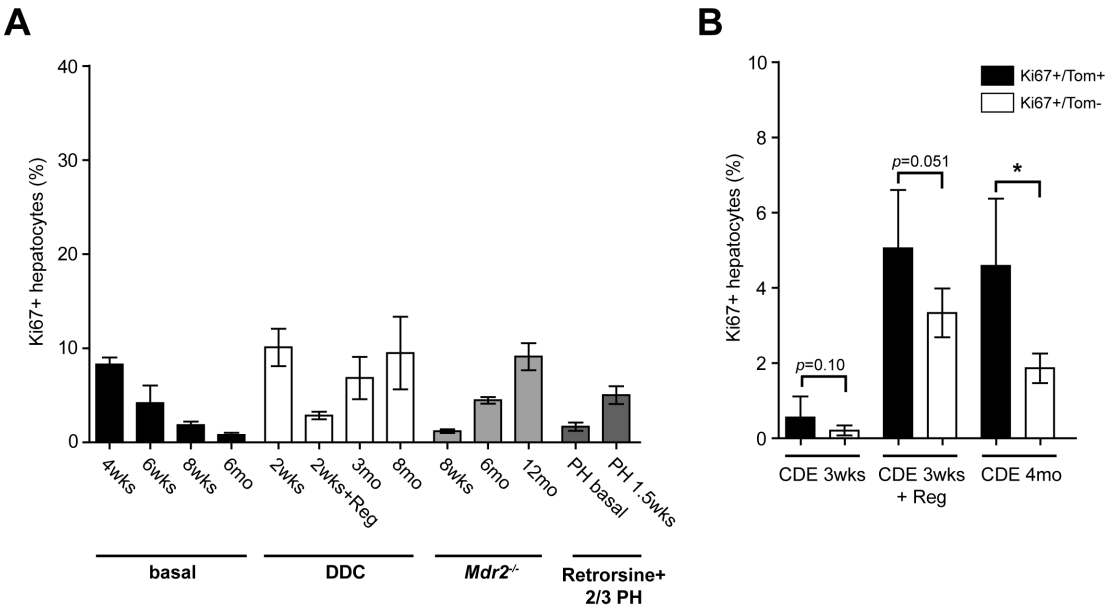


Supporting Figure 1: DRCs do not significantly contribute to hepatocyte neogenesis after short-term liver injury (additional data related to Fig. 2).
Tamoxifen-treated (i.p., 100 µg/g BW) *R26*^{Tom}*Hnf1βCreER* animals were subjected to various short-term injury protocols (± recovery period) of **(A)** DDC diet (n=4 for each group), **(B)** CDE diet (n=5 and n=7), or were subjected to **(C)** 80% partial hepatectomy (PH) (n=4) or 2/3 PH after retrorsine pretreatment (n=5) (experimental design for each protocol is indicated in the figure). Representative A6 IHC stainings in (A) and (B) show typical DR morphology for each experimental setting. The proportion of CK19+ DRCs that co-express tdTom remained >80% in all models as assessed by co-IF analysis (black bars in graphs). A small proportion of HNF4α+ hepatocytes co-expressed tdTom in the CDE models and after 2/3-PH with retrorsine pretreatment, indicating their biliary origin (yellow arrows indicate tdTom+ hepatocytes, for quantification see white bars in graphs). Graphs show mean values ± SEM expressed in %. PV, portal vein; scale bars in (A) = 100 µm and 20 µm for IF magnifications.



Supporting Figure 2: Lack of evidence for a 'streaming liver': Neither HNF1β+ ductal cells nor periportal zone 1 hepatocytes provide homeostatic renewal for the liver parenchyma by 'streaming' to the pericentral zone (additional data related to Fig. 2).
(A) 4-5 week-old *R26^{Tom}Hnf1βCreER* and *R26^{Tom}Sox9CreER* animals were injected with a single dose of tamoxifen (100 µg/g BW) and expression of tdTom was analyzed after 7 days and 6 months. In *R26^{Tom}Hnf1βCreER* animals, *Cre* expression remains highly restricted to the biliary compartment with extremely rare tdTom labeling of HNF4α hepatocytes as assessed by tdTom/HNF4α co-IF 7 days (0.012 ± 0.003%, n=10) and 6 months (0.018 ± 0.010%, n=3) after tamoxifen treatment (see figures at the right). In contrast, in *R26^{Tom}Sox9CreER* animals, tamoxifen treatment resulted in additional and robust tdTom labeling of periportal zone 1 HNF4α+ hepatocytes after 7 days of tamoxifen treatment (9.0 ± 1.1%, n=5), which, however, remained unchanged after 6 months (8.9 ± 0.6%, n=4) (right figures). **(B)** Quantification of tdTom/HNF4α co-IF data obtained from tamoxifen-treated *R26^{Tom}Hnf1βCreER* and *R26^{Tom}Sox9CreER* (mean values ± SEM). PV, portal vein; scale bars in (A) = 100 µm and 20 µm for magnified IF.



Supporting Figure 3: Hepatocyte proliferation in liver injury models (additional data related to Fig. 2, 3 and Supp. Fig. 1).

(A) Ki67 IHC was performed to determine hepatocyte proliferation in *R26^{Tom}Hnf1βCreER* animals after various liver injury models or in *Mdr2$^{-/-}$R26^{Tom}Hnf1βCreER* animals and quantified in % Ki67+ hepatocytes (please refer to Fig. 2, Fig. 3 and Supp. Fig. 1 for details of experimental design; basal = tamoxifen-only-treated *R26^{Tom}Hnf1βCreER* controls where age of analysis is indicated). Bar graph shows mean values ± SEM expressed in % obtained from n=3-5 animals per group. **(B)** For CDE models, selective quantification of Ki67 staining for tdTom+ and tdTom- hepatocytes was performed by co-IF for tdTom and Ki67 (hepatocytes were identified by morphology, bar graph shows mean values ± SEM expressed in % obtained from n=3 (CDE 3wks), n=7 (CDE 3wks+Reg), and n=4 (CDE 4mo); ∗, p< 0.05; Student's *t*-test, unpaired, two-tailed).

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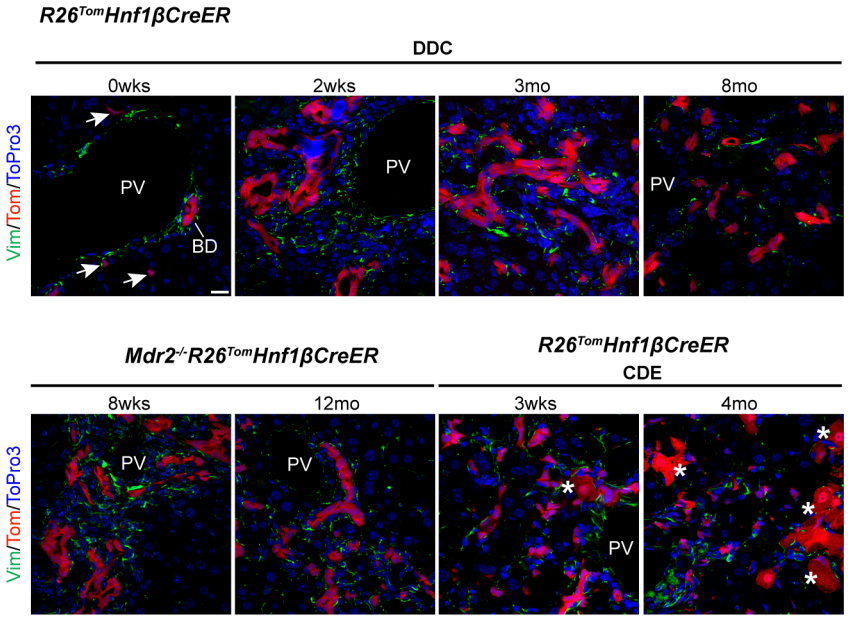
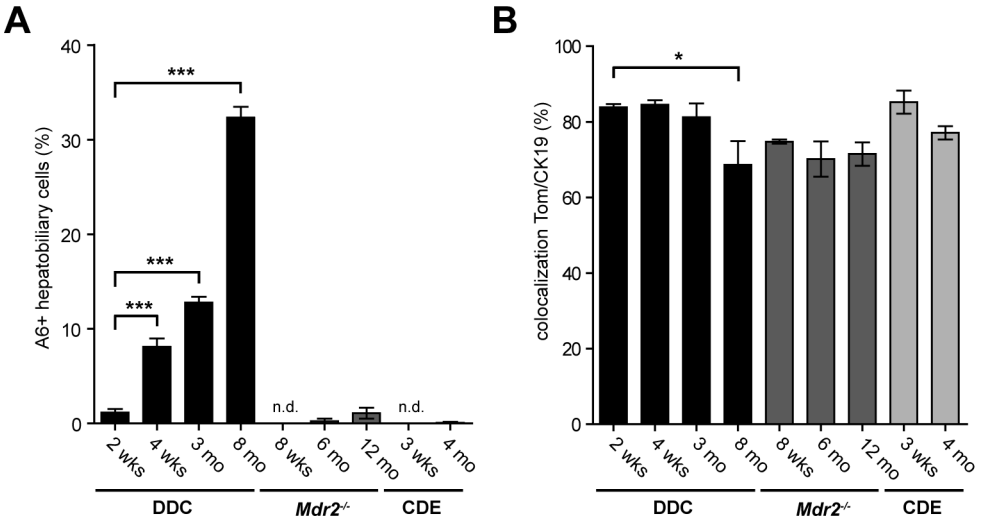
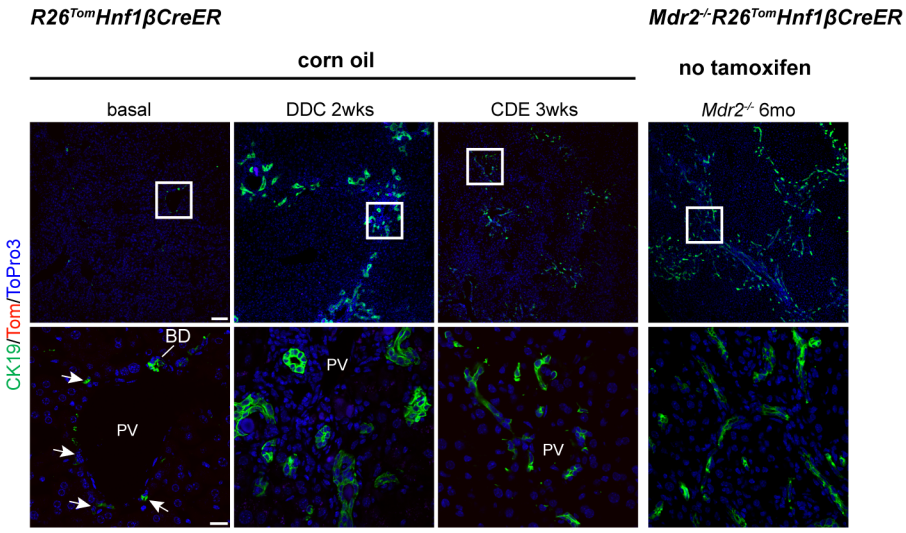


Supporting Fig. 4: tdTom+ DRs are not derived from vimentin-expressing mesenchymal cells (additional data related to Figure 3).

Co-IF analysis of liver sections from *Mdr2*$^{-/-}$*R26*^{Tom}*Hnf1βCreER* animals (tamoxifen treatment at week 3-4) or *R26*^{Tom}*Hnf1βCreER* animals subjected to DDC or CDE diet (started 7d after tamoxifen treatment at week 4-5) was performed for expression of tdTomato and the mesenchymal marker vimentin at various time points as indicated in the figure (please refer to Figure 2A and 3A for details of the experimental protocols). At basal (upper panel, "DDC 0wks"), tdTom-labeled cells do not co-stain for vimentin (green). After short-term or long-term injury (DDC model, upper panel; *MDR2*$^{-/-}$ and CDE model, lower panel) vimentin-positive cells typically surround or intermingle tdTom+ DRs and DR-derived hepatocytes (asterisks) but tdTom+/vimentin+ cells are never observed. Representative IF sections are shown obtained from n≥3 animals analyzed per group. PV, portal vein; BD, bile duct; arrows indicate CoH; Scale bar = 20 μm.

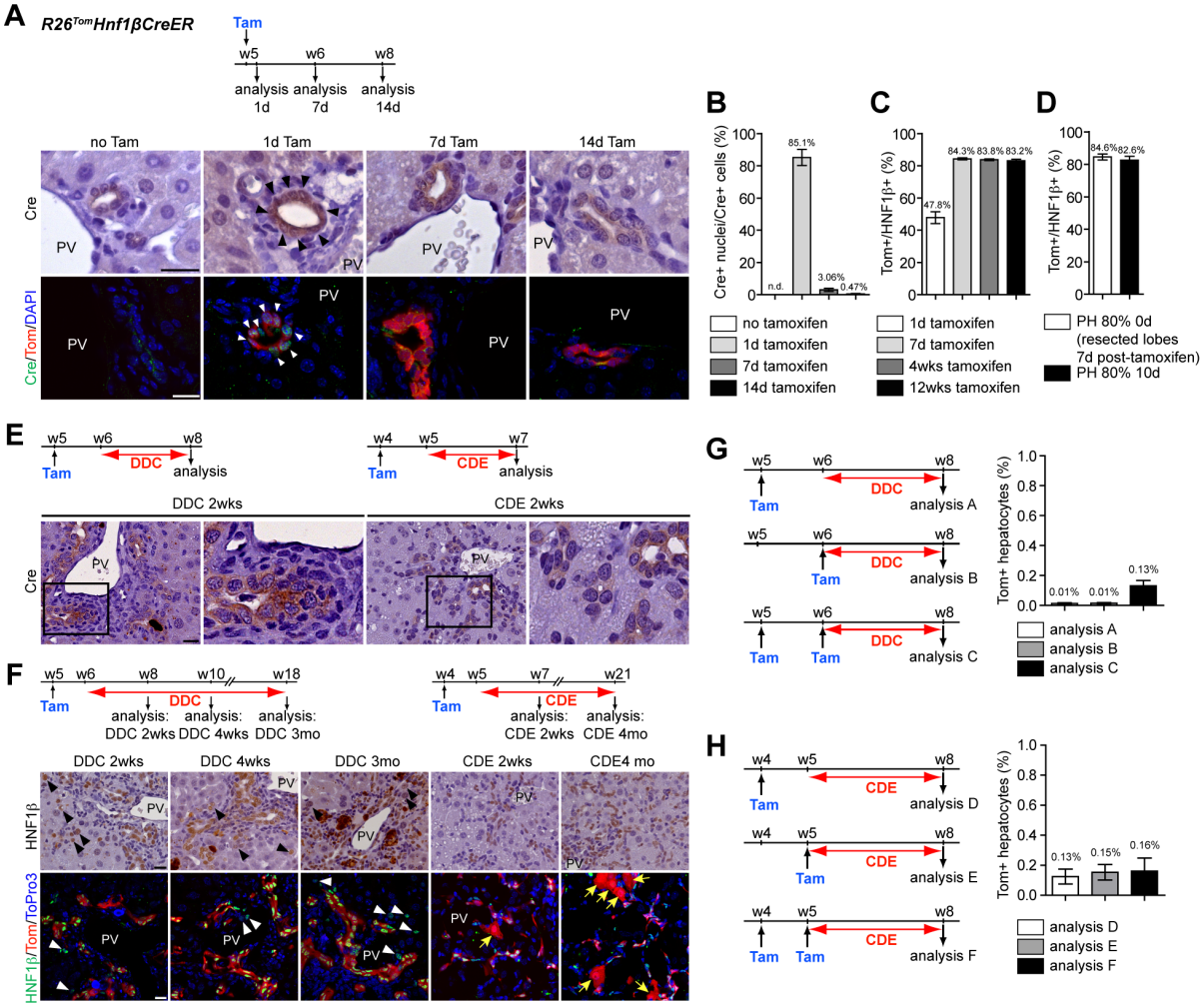


Supporting Figure 5: **Long-term DDC diet induces widespread appearance of A6+ hepatocytes/hepatobiliary cells of hepatocyte origin (additional data related to Fig. 3C).**
(A) Quantification of A6-expressing hepatocytes/hepatobiliary cells in long-term DDC- or CDE-treated *R26^{Tom}Hnf1βCreER* animals and in *Mdr2$^{-/-}$R26^{Tom}Hnf1βCreER* mice (refer to Fig. 3 for study design). The proportion (%) of hepatocytes and hepatobiliary cells (identified by morphology in A6 IHC) expressing the biliary marker A6 is indicated (mean values ± SEM expressed in % obtained from n=4-6 animals per group; ∗∗∗, p<0.001 [one-way ANOVA followed by Tukey's post test], n.d., not detected.) and shows widespread appearance of A6+ hepatocytes/hepatobiliary cells only in DDC-treated animals over time. **(B)** Quantification of CK19+ cells co-expressing tdTom (mean values ± SEM expressed in % obtained from n=4-6 animals per group; ∗, p<0.05 [one-way ANOVA followed by Tukey's post test]) indicate that the proportion of CK19+ DRs co-expressing tdTom remains high throughout all models indicating their biliary origin. A 15% decrease of CK19/tdTom co-expressing DRCs in 8 months DDC-treated animals to 68.6 ± 6.3% suggests a small fraction of non-biliary-derived DRCs after long-term DDC treatment.



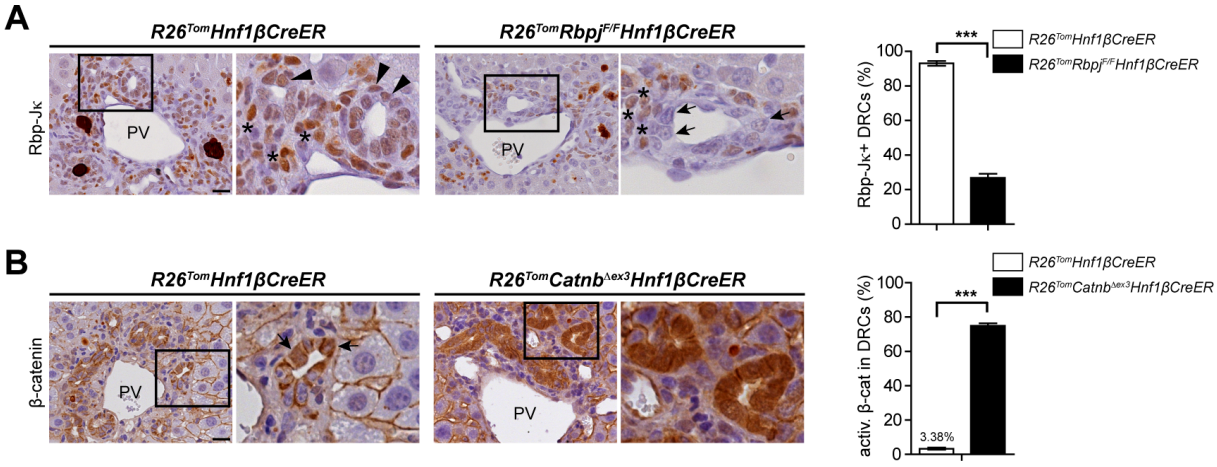
Supporting Figure 6: Tamoxifen-independent expression of the reporter tdTomato does not occur at basal or during injury in *R26^{Tom}Hnf1βCreER* animals (additional data related to Fig. 2 and 3):

Left, co-IF analysis of CK19 and tdTom revealed completely absent tdTom expression in CK19+ biliary (arrows) or other liver cells in 6 week-old *R26^{Tom}Hnf1βCreER* mice (7d after corn oil injection, n=3). Reporter expression also remained undetectable when *R26^{Tom}Hnf1βCreER* mice were subsequently subjected to DDC (2wks; n=3) or CDE diet (3wks; n=5) (middle panels). Likewise, no tdTom+ cells were detected in 6 month-old *Mdr2$^{-/-}$R26^{Tom}Hnf1βCreER* animals without tamoxifen treatment (n=2). BD, bile duct; PV, portal vein; scale bars = 100 μm and 20 μm for magnification.

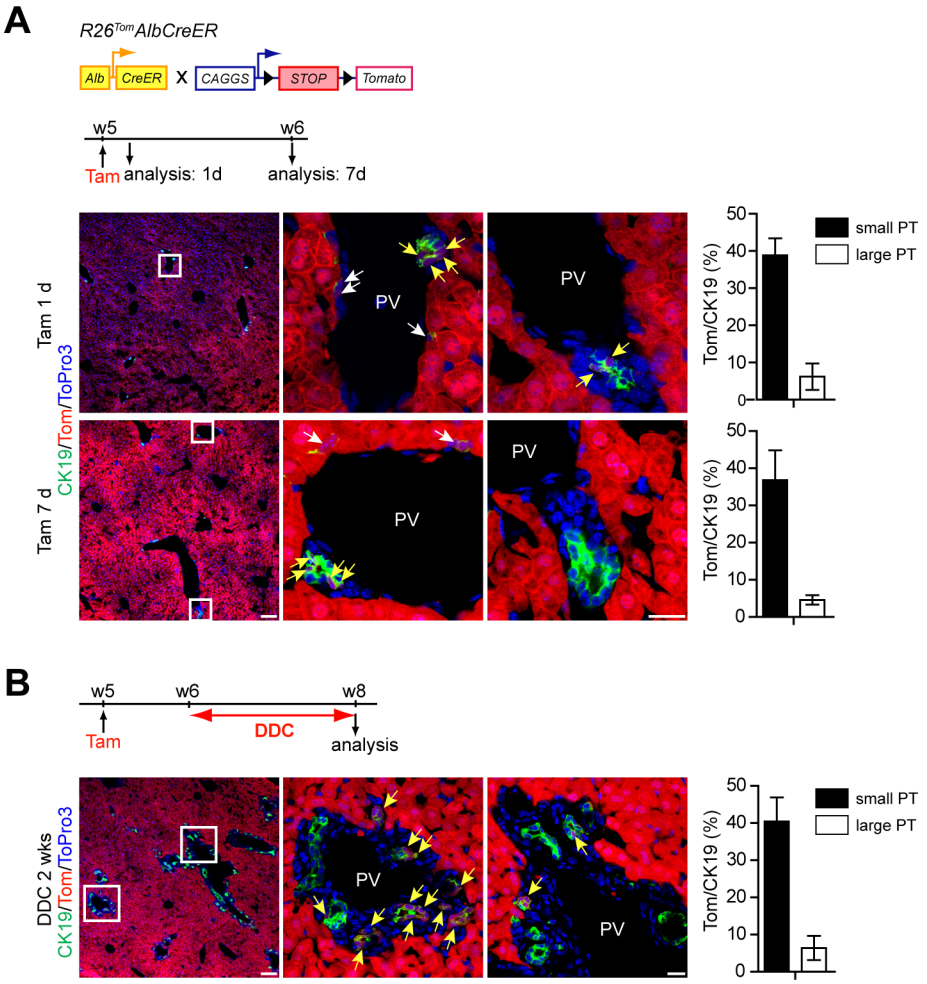


Supporting Figure 7: Potentially remnant levels of tamoxifen do not suffice to perpetuate de novo reporter labeling of HNF1β-expressing cells beyond 7 days of administration in our model.

(A) IHC and IF analysis of *R26^{Tom}Hnf1βCreER* livers demonstrate a rapid decline of nuclear Cre-translocation (arrowheads) beyond 24h after tamoxifen application (once i.p., 100 µg/g BW), quantified in **(B)**, n=3-5 animals per group. **(C)** Analyzing the time course of reporter labeling of the HNF1β+ compartment does not indicate a further increase of the tdTom+/HNF1β+ labeling index (%) beyond 7d after tamoxifen as assessed by tdTom/HNF1β co-IF, n=3-5 animals per group. **(D)** To control for interindividual variability, reporter labeling was further analyzed over time within identical animals using tissue from the PH model. Direct comparison of resected lobes at 7d post tamoxifen with remnant liver lobes at 17d post tamoxifen also revealed no increase in the tdTom+/HNF1β+ labeling index, indicating that there is no perpetuating de novo cell labeling beyond 7d after tamoxifen treatment (n=3). **(E)** Cre was exclusively detected cytoplasmic (inactive) during injury in 2wks DDC- or CDE-treated animals (n=3 animals per group). **(F)** Injury-induced 'ectopic' HNF1β-expressing hepatocytes were sporadically detected in DDC-treated animals (arrowheads in IHC and IF) but never co-stained for tdTom while HNF1β+ hepatocytes were not detected in the CDE model; particularly, tdTom+ hepatocytes never co-stained for HNF1β (yellow arrows) after short- or long-term CDE. **(G)** DDC-induced (injury-induced) 'ectopically' HNF1β-expressing hepatocytes are susceptive to tamoxifen-induced reporter expression but require an additional tamoxifen dose without washout period (tamoxifen applied twice at 7d before + at start of DDC diet = analysis C) when a small fraction of tdTom+ hepatocytes was detected (0.13%), n=9 (analysis A), n=5 (analysis B), and n=3 (analysis C). **(H)** In the 3wk CDE model, additional application of tamoxifen without washout period (analysis F) did not further increase the small number of duct-derived tdTom+ hepatocytes clearly indicating that 'ectopic' HNF1β expression in hepatocytes with subsequent non-specific hepatocyte reporter labeling is not of concern in the CDE model under the experimental conditions used throughout our study, n=5 (analysis D), n=3 (analysis E), and n=3 (analysis F). Bar graphs indicate quantification of IHC/IF data (mean values ± SEM expressed in %). PV, portal vein; scale bars in (A), (E), and (F) = 20 µm; n.d., not detected.



Supporting Figure 8: **Conditional inactivation of canonical Notch or activation of Wnt/β-catenin signaling specifically in DDC-induced DRCs (additional data related to Fig. 4).**
(A) *R26*^{Tom}*Rbpj*$^{F/F}$*Hnf1βCreER* and *R26*^{Tom}*Hnf1βCreER* control animals were injected with tamoxifen (i.p. once at 100 µg/g BW at week 5), subjected to 2 weeks of DDC diet (week 6-8), and IHC for the Notch adaptor protein RBP-Jκ was performed. In control animals, nuclear staining can be observed in the vast majority of DRCs (arrowheads) and in periportal immune cells (asterisks). In *R26*^{Tom}*Rbpj*$^{F/F}$*Hnf1βCreER* animals, RBP-Jκ expression is largely absent in DDC-induced DRCs (arrows) but maintained in immune cells (asterisks) indicating effective genetic deletion of *Rbpj* specifically in DRCs. Bar graphs show quantification of IHC, expressed in % of DRCs (identified by morphology) with positive nuclear staining for nuclear RBP-Jκ (93.13 ± 1.38% vs. 26.85 ± 2.35%, n=4), ***, p<0.001 (Student's *t*-test, unpaired, two-tailed). **(B)** Likewise, IHC for β-catenin was performed in DDC-treated *R26*^{Tom}*Catnb*$^{\Delta ex3}$*Hnf1βCreER* and *R26*^{Tom}*Hnf1βCreER* control animals. In DDC-treated control animals, β-catenin is localized to membranes of hepatocytes and DRCs with few DRCs displaying nuclear and cytoplasmic β-catenin (arrows in left figures; 3.38% ± 0.58, n=3). In equally treated *R26*^{Tom}*Catnb*$^{\Delta ex3}$*Hnf1βCreER* animals, the majority of DRCs (but not hepatocytes) display nuclear and cytoplasmic β-catenin staining (75.0 ± 1.29%, n=5), indicating activation of Wnt/β-catenin signaling specifically within DRCs. Bar graphs show quantification of IHC, expressed in % of DRCs (identified by morphology) with nuclear and cytoplasmic β-catenin staining (mean values ± SEM expressed in %; >10-15 periportal areas per mouse were quantified; ***, p<0.001, Student's *t*-test, unpaired, two-tailed). PV, portal vein; scale bars = 20 µm.



Supporting Figure 9: ***Cre* expression in adult *R26^{Tom}AlbCreER* animals is sensitive to trace amounts of tamoxifen and lacks hepatocyte compartment specificity (data related to discussion section).**

(A) *Cre*-induced expression of the fluorescent dye tdTom in *AlbCreER* mouse strain was determined in 5-6 week-old *R26^{Tom}AlbCreER* reporter mice 1 and 7 days after a single injection of low-dose tamoxifen (i.p., 0.1 µg/g BW). IF analysis for tdTom and CK19 revealed widespread tdTom expression not only in hepatocytes but also in a significant portion of CK19+ bile ducts (yellow arrows) and small ductules (white arrows), predominantly in smaller, more peripheral located portal tracts (PT). Tom+/CK19+ cells (%) were quantified in small (PV diameter ≤150 µm) and larger (PV diameter >150 µm) portal tracts. Shown are mean values ± SEM obtained from n=3 animals per group. Notably, tdTom expression was completely absent in all liver cells in non tamoxifen-treated 5 week-old *R26^{Tom}AlbCreER* animals (data not shown). **(B)** One week after a single injection of low-dose tamoxifen *R26^{Tom}AlbCreER* animals were subjected to 2 weeks of DDC diet. IF analysis and quantification (right graph, shown are mean values ± SEM obtained from n=3 animals) revealed that the proportion of CK19+ biliary cells co-expressing tdTom (yellow arrows) in smaller (left inset, enlarged in middle figure) and larger tracts (right inset, enlarged in right figure) remained unchanged after DDC, suggesting their biliary origin. PV, portal vein; scale bars in (A) and (B) = 100 µm and 20 µm.

Genotyping PCR		
Primer	**Forward**	**Reverse**
CreERT2	CCTGGAAAATGCTTCTGTCCG	CAGGGTGTTATAAGCAATCCC
β-catenin	CTGAATGAACTGCAGGACGA	TTCCCAGTCCTTCACGCAAG
Rbpj-loxP	AGTTTAGGCTTTCCAAAAGGC	GTATTGCTAAGAACTTGTTGC
Mdr2-Neo	TGTCAAGACCGACCTGTCCG	TATTCGGCAAGCAGGCATCG
tdTom-loxP	CTGTTCCTGTACGGCATGG	GGCATTAAAGCAGCGTATCC

Supporting Table 1. PCR primers (5'-3') used.

Primary antibody	Species	Source	Dilution/Application	Catalog number
Acetylated-Tubulin (acTub)	mouse	Sigma	1:250 (IF)	T6793
AFP	goat	R&D Systems	1:100 (IHC)	AF5369
A6	rat	kindly provided by Valentina Factor	1:250 (IHC, IF)	Engelhardt et al., Differentiation. 1990; 45(1):29-37
β-catenin	mouse	BD Bioscience	1:100 (IHC)	610154
CD44	rat	eBioscience	1:500 (IHC)	BMS145
CD133 (13A4)	rat	Affymetrix	1:100 (IF)	14-1331
Collagen Type IV (Col IV)	rabbit	Biozol	1:100 (IHC,IF)	CED-CL-50451AP-1
Cre	rabbit	Biolegend	1:100 (IHC, IF)	PRB-106P
Cytokeratin 19 (CK19)	rat	Dev. Studies Hybrid. Bank	1:250 (IHC, IF)	TROMA III
GP73	goat	Santa Cruz	1:50 (IHC)	sc-48011
HNF1β (H85)	rabbit	Santa Cruz	1:100 (IHC, IF)	sc-22840
HNF1β (C20)	goat	Santa Cruz	1:100 (IF)	sc-7411
HNF4α (H171)	rabbit	Santa Cruz	1:100 (IF)	sc-8987
HNF4α (C19)	goat	Santa Cruz	1:100 (IF)	sc-6556
Ki67	rabbit	Abcam	1:250 (IHC, IF)	ab-15580
Rbp-Jκ (D10A4)	rabbit	Cell signaling	1:100 (IHC)	5313
SOX9	rabbit	Millipore	1:250 (IHC, IF)	ab-5535
Vimentin	rabbit	Cell signaling	1:200 (IF)	5741

Secondary antibody	Dilution/Application	Source	Catalog number
Biotinylated-α-rabbit	1:400 (IHC)	Vector Laboratories	BA-1000
Biotinylated-α-goat	1:400 (IHC)	Vector Laboratories	BA-5000
Biotinylated-α-mouse	1:400 (IHC)	Vector Laboratories	BA-9200
Biotinylated-α-rat	1:400 (IHC)	Vector Laboratories	BA-9400
α-rabbit-Alexa-405	1:200 (IF)	Invitrogen	A-31556
α-rat-CF405M	1:200 (IF)	Biotium	20347
α-rat-Alexa-488	1:500 (IF)	Invitrogen	A-11006
α-rabbit-Alexa-488	1:500 (IF)	Invitrogen	A-11034
α-mouse-Alexa-488	1:500 (IF)	Invitrogen	A-11029
α-mouse-Alexa-647	1:200 (IF)	Invitrogen	A-21235
α-rabbit-Alexa-555	1:500 (IF)	Invitrogen	A-21429
α-goat-Alexa-647	1:200 (IF)	Invitrogen	A-21447
α-rat-Alexa-647	1:200 (IF)	Invitrogen	A-21247
α-rabbit-Alexa-647	1:200 (IF)	Invitrogen	A-21244

Supporting Table 2. Antibodies used.