

Supplementary Figure 1

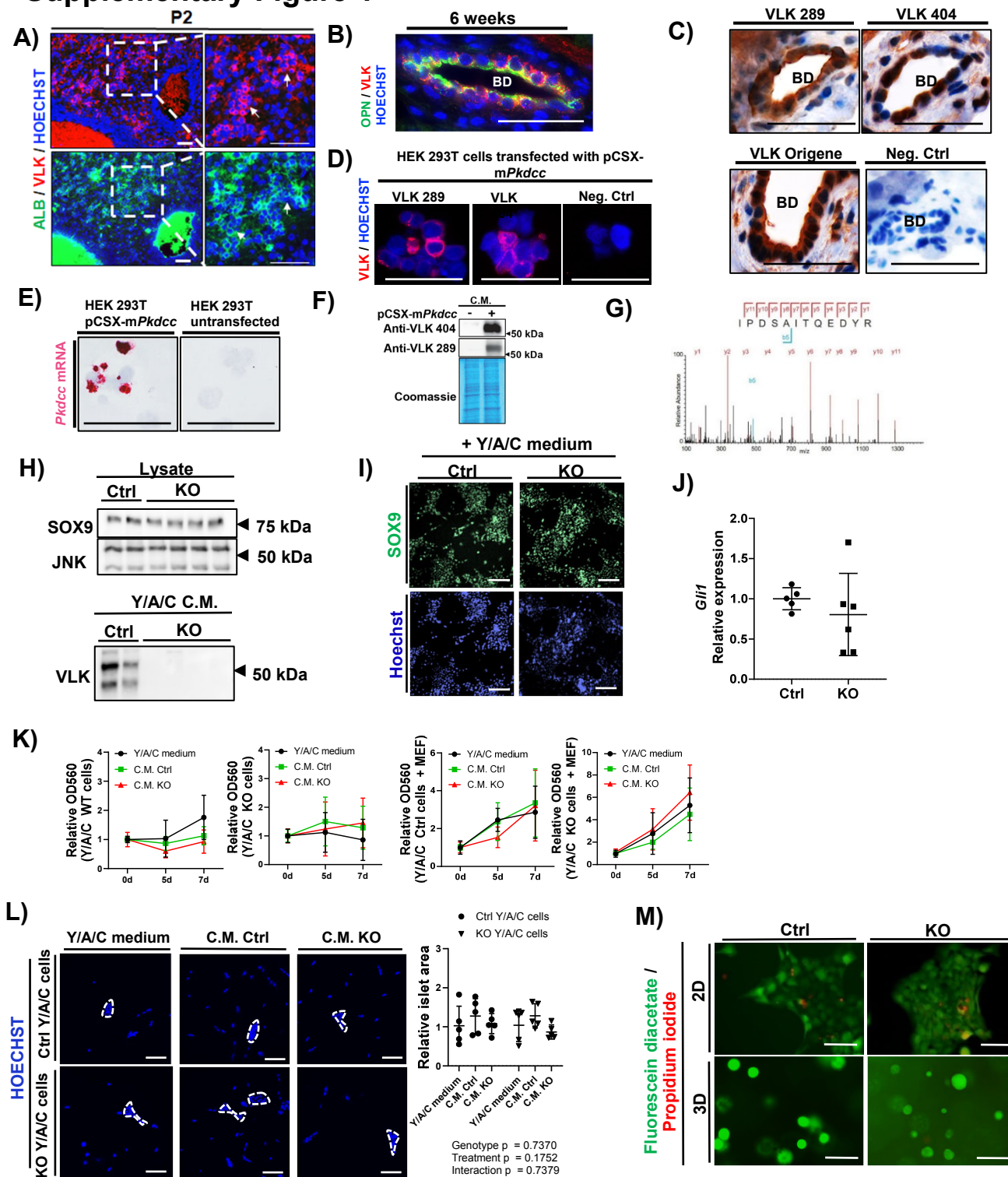


Fig. S1. Validation of the specificity of VLK immunostaining and characterization of primary liver progenitor-like cells that secrete VLK

(A) Serial mouse liver sections at P2 were analyzed by immunofluorescence for VLK (red) and albumin (Alb; green). Nuclei were counterstained with Hoechst (blue). The area indicated by the white rectangle in the left panel is shown at higher magnification in the right panel. Arrows point to ALB/VLK-positive hepatocytes.

(B) Mouse liver sections were analyzed by immunofluorescence for VLK (red) and osteopontin (OPN; green). Nuclei were counterstained with Hoechst (blue). BD: Bile duct.

(C) Liver sections were analyzed by immunohistochemistry for VLK using three different antibodies (VLK 289, VLK 404, VLK Origene). Sections were counterstained with hematoxylin. BD = Bile duct.

(D) HEK 293T cells were transfected with a VLK expression vector (pCSX-*mPkdcc*) and analyzed by immunofluorescence using two different VLK antibodies (red) (VLK 289 and VLK 404) or no first antibody (negative control). Nuclei were counterstained with Hoechst (blue).

(E) HEK 293T cells, transfected or not with a VLK expression vector (pCSX-*mPkdcc*), were analyzed by RNAscope for *Pkdcc* mRNA (red).

(F) HEK 293T cells were transfected with a VLK expression plasmid (pCSX-*mPkdcc*) or left untransfected. Conditioned media (C.M.) produced in the presence of serum-free Opti-MEM were analyzed for VLK by Western blot using two different VLK antibodies. Coomassie staining was used to verify equal loading.

(G) Representative spectrum of a unique VLK peptide identified by mass spectrometry analysis of the secretome of cells grown in Y/A/C-supplemented medium. Spectrum is representative for 3 independent batches of C.M. VLK peptide was not detected in C.M. of KO cells.

(H) Primary hepatocytes from Ctrl or KO mice were cultured in Y/A/C-supplemented medium for 14 days. C.M. were analyzed by Western blot for VLK, and lysates were analyzed for SOX9 and JNK. Representative of 3 independent experiments.

(I) SOX9 expression (green) detected by immunofluorescence staining of primary hepatocytes from Ctrl or KO mice grown in Y/A/C-supplemented medium. Nuclei were stained with Hoechst (blue). Representative images of 3 independent isolations are shown.

(J) RNA samples from Y/A/C Ctrl or KO cells cultured in Y/A/C-supplemented medium for 14 days were analyzed by RT-qPCR for *Gli1* relative to *Rps18*. Ctrl: N=5, KO: N=6. Mean expression levels in Ctrl cells were set to 1.

(K) Primary hepatocytes from Ctrl and KO mice were cultured for 7 days in the indicated medium in the absence (left two panels) or presence of immortalized mouse embryonic fibroblasts (MEF; right two panels). N=6-12 cultures from three independent experiments. Values obtained at day 0 were set to 1.

(L) Representative photomicrographs of primary hepatocytes from Ctrl or KO mice incubated in the presence of the indicated culture medium and analyzed by staining of nuclei with Hoechst. Graph shows average area of “liver progenitor” islets. Results of three independent cell culture experiments are shown (right panel), each dot represents

a culture from a different mouse. N=5 per group. Mean islet area of Ctrl cells cultured in Y/A/C medium was set to 1.

(M) Representative immunofluorescence images of primary hepatocytes from Ctrl or KO mice grown in 2D or 3D incubated with Y/A/C medium for 10 days. Cell viability was controlled by addition of fluorescein diacetate (green) and propidium iodide (red). N=3.

Graphs show mean \pm SD. Statistical significance was assessed by unpaired nonparametric Mann-Whitney test (J), ordinary one-way ANOVA (Tukey's multiple comparisons test) (K) or two-way ANOVA (L). Magnification bars: 100 μ m.

Supplementary Figure 2

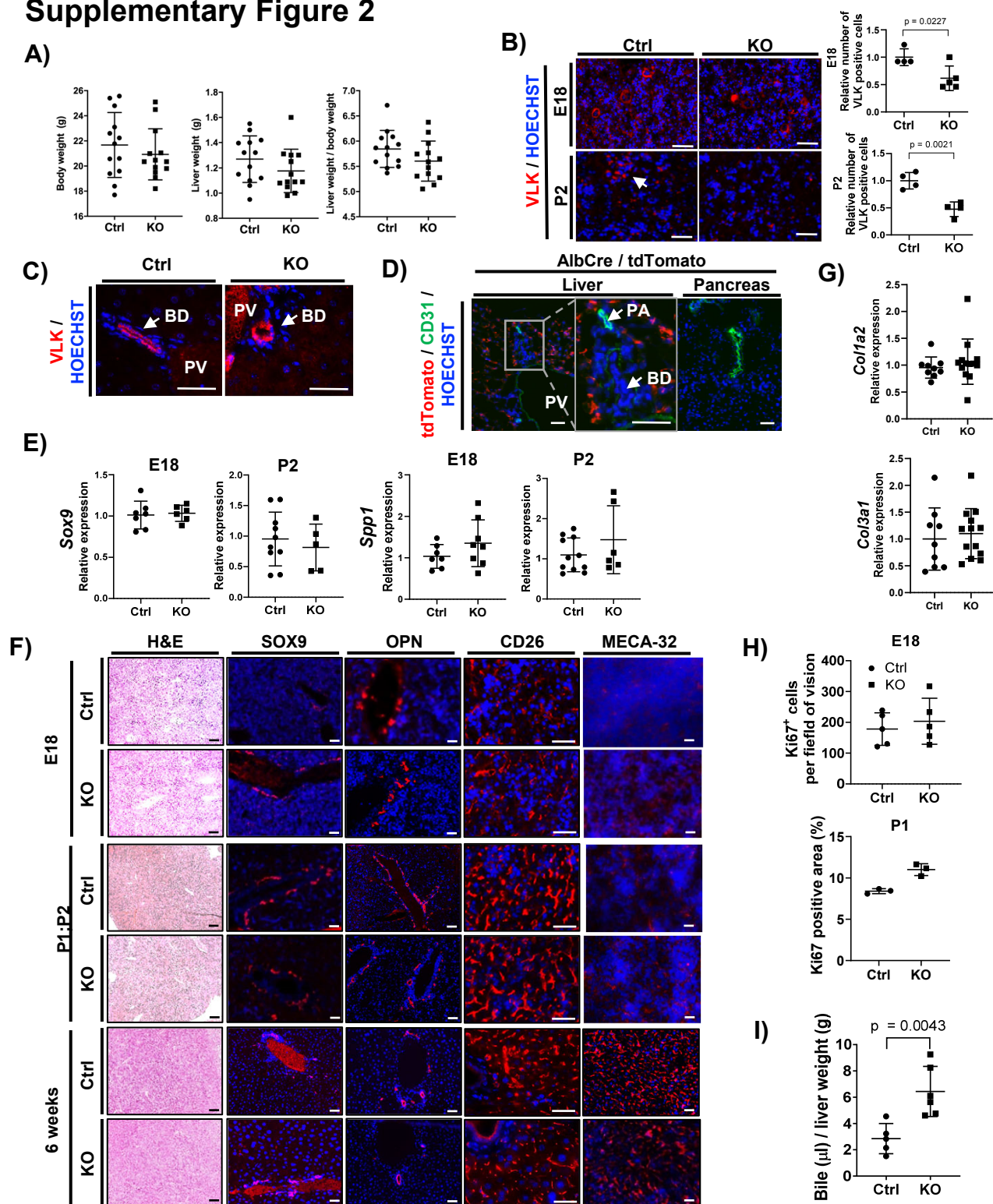


Fig. S2. Characterization of liver morphology and function in mice lacking VLK in hepatocytes

- (A) Body weight, liver weight and liver-to-body weight ratio in 6 week-old Ctrl and KO mice.
- (B) Immunofluorescence detection of VLK (red) in liver sections of Ctrl and KO mice and counterstaining of nuclei with Hoechst (blue). Graph shows quantification of VLK-positive cells at the indicated developmental stage. N = 4-5 per genotype.
- (C) Immunofluorescence staining of liver sections from adult mice for VLK (red) and counterstaining of nuclei with Hoechst (blue). BD: bile duct, PV: portal vein.
- (D) Fluorescence detection of tdTomato combined with immunofluorescence staining for CD31 in liver and pancreas sections of double-transgenic mice expressing Cre under the control of the albumin promoter and harboring a tdTomato reporter gene preceded by a floxed STOP cassette. Note the specific deletion of the cassette in hepatocytes, but not in cholangiocytes. PA: portal artery.
- (E) RNA from liver tissue of Ctrl or KO mice at the indicated age was analyzed by RT-qPCR for *Sox9* (upper panels) and *Spp1* (lower panels) relative to *Rps18*. E18: N=6-7, P2: N=5-10. Mean expression levels in Ctrl mice were set to 1.
- (F) Liver sections from Ctrl or KO mice at the indicated age were stained with H&E or analyzed by immunofluorescence for SOX9, OPN, CD26 and MECA-32 (red). Nuclei were counterstained with Hoechst (blue).
- (G) RNA from liver tissue of adult Ctrl or KO mice was analyzed by RT-qPCR for *Col1a2* and *Col3a1* relative to *Rps18*. N = 9-12. Mean expression levels in Ctrl mice were set to 1.
- (H) Quantification of Ki67-positive cells in the liver of E18 and P1 old mice. E18: N=5, P1: N=3.
- (I) Volume of bile per gram liver weight in the gallbladders of Ctrl or KO mice. N = 5-6. Graphs show mean \pm SD. Statistical significance was assessed in all panels of this figure by unpaired nonparametric Mann-Whitney test. Magnification bars: 100 μ m.

Supplementary Figure 3

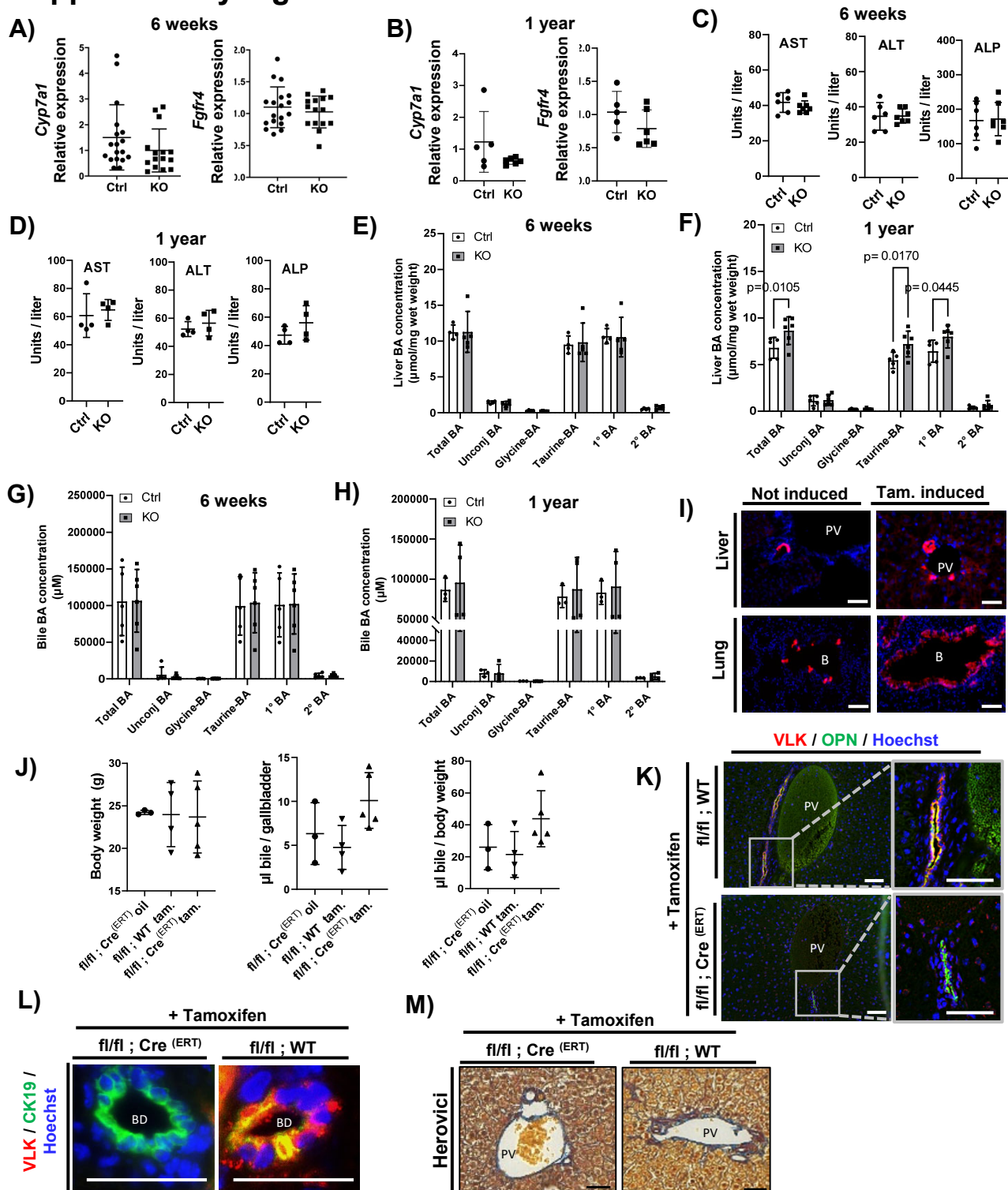


Fig. S3. Hepatocytes but not cholangiocyte-derived VLK is important for liver homeostasis

(A, B) RNA from liver tissue of 6-week (A) or 1-year old (B) Ctrl or KO mice was analyzed by RT-qPCR for *Cyp7a1* and *Fgfr4* relative to *Rps18*. N=15-18. Mean expression levels in Ctrl mice were set to 1.

(C, D) AST, ALT and ALP enzyme levels in the plasma of 6-week (C) or 1 year-old (D) Ctrl and KO mice. N=6-7.

(E, F) Liver total bile-acid (BA), unconjugated (unconj) BA, glycine-conjugated BA, taurine-conjugated BA, primary (1°) BA and secondary (2°) BA concentrations were determined in 6 week-old liver (E) or 1 year-old liver (F). White bars represent Ctrl, grey bars KO mice. Graphs show mean \pm SD, N=3-6. Statistical analysis was performed using 2-way ANOVA, Sidak's multiple comparisons test.

(G, H) Bile from the gallbladder of 6 week-old mice (G) and 1 year-old mice (H) was analyzed by liquid chromatography-tandem mass spectrometry. White bars represent Ctrl, grey bars KO mice. Data show mean \pm SD, N=3-6. Statistical analysis was performed using 2-way ANOVA, Sidak's multiple comparisons test.

(I) Representative photomicrographs of sections from mouse liver carrying a tdTomato Cre reporter and expressing a CK19Cre^(ERT) transgene. Nuclei were counterstained with Hoechst (blue). Mice were injected with tamoxifen to activate the Cre^(ERT) promoter (Tam. induced) or oil (vehicle) (not induced). PV = portal vein, B = bronchiole.

(J) Body weight and bile volume in fl/fl;Cre^(ERT) or fl/fl;Wt mice injected with tamoxifen or oil. N=3-5.

(K) Representative photomicrographs of sections from fl/fl;Cre^(ERT) or fl/fl;Wt mice injected with tamoxifen and stained for VLK (red) and osteopontin (OPN) (green). Nuclei were counterstained with Hoechst (blue). N=4. PV = portal vein.

(L) Representative photomicrographs of liver bile ducts from fl/fl;Cre^(ER) or fl/fl;Wt mice injected with tamoxifen and stained for VLK (red) and cytokeratin 19 (CK19) (green). Nuclei were counterstained with Hoechst (blue). N=4. BD = bile duct.

(M) Representative liver sections from fl/fl;Cre^(ERT) or fl/fl;Wt mice injected with tamoxifen, stained with Herovici. N=4. PV = portal vein.

Supplementary Figure 4

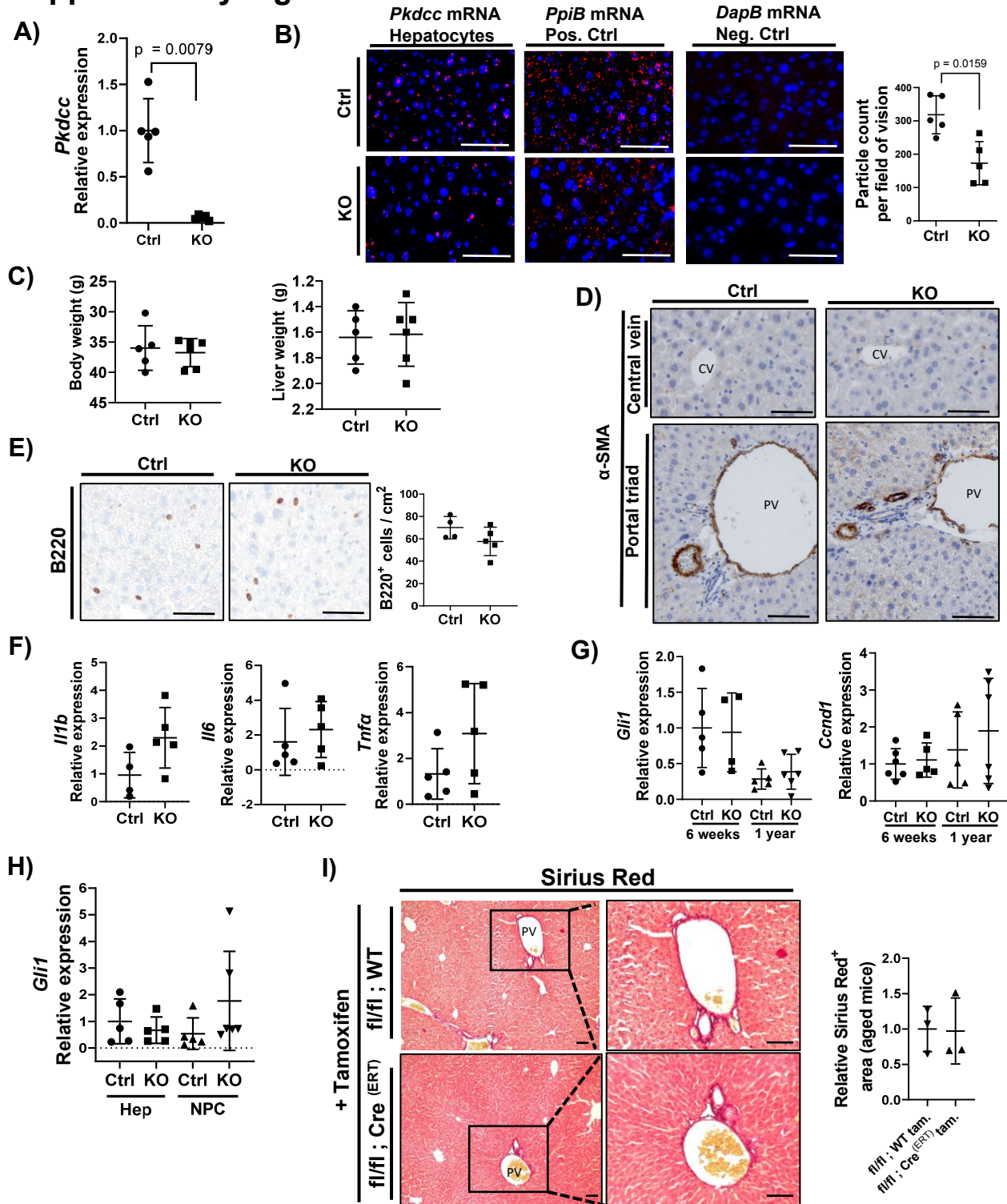


Fig. S4. Characterization of liver function in aged mice lacking VLK in hepatocytes.

(A) RNA from total liver of 56 week-old Ctrl and KO mice was analyzed by RT-qPCR for *Pkdcc* relative to *Rps18*. N= 5-6. Mean expression levels in Ctrl mice were set to 1. (B) *Pkdcc* mRNA in liver of 56 week-old Ctrl and KO mice visualized by RNA-Scope *in situ* hybridization (red). *PpiB* and *DapB* mRNAs were used as positive and negative controls, respectively. Nuclei were counterstained with Hoechst. Graph shows quantification of *Pkdcc* RNA positive particles with N=5.

(C) Body and liver weight of 1 year-old Ctrl and KO mice. N=5-6.

(D) Liver sections were analyzed by immunohistochemistry for α -SMA. Central vein (CV) in the upper panels and portal vein (PV) in the lower panels are depicted. Sections were counterstained with hematoxylin.

(E) Representative photomicrographs of liver sections analyzed by immunohistochemistry for B220 and quantification of B220-positive cells. N=4-5; n=3 pictures per mouse. Mean in Ctrl mice was set to 1.

(F) RNA samples from whole liver of wild-type mice at 56 weeks of age were analyzed for *Il1b*, *Il6*, and *Tnfa* relative to *Rps18* expression by RT-qPCR. N=4-5 mice per time point.

(G) RNA from total liver of 6-week-old and 56 week-old Ctrl and KO mice was analyzed by RT-qPCR for *Gli1* and *Cnnd1* relative to *Rps18*. N= 4-6 mice per genotype and age. Mean expression levels in Ctrl mice were set to 1.

(H) RNA from total primary hepatocytes and non-parenchymal cells (NPC) of 6 week-old Ctrl and KO mice was analyzed by RT-qPCR for *Gli1* relative to *Rps18*. N= 5-6 mice per genotype. Mean expression levels in Ctrl mice were set to 1.

(I) Representative liver sections from fl/fl;Cre^(ERT) or fl/fl;Wt mice injected with tamoxifen over 8 weeks and analyzed at the age of 42-weeks. Sections were stained with Sirius Red. Graph shows quantification of relative Sirius Red positive area per field of vision. fl/fl;Wt aged: N=3, fl/fl;Cre^(ERT) aged: N=3. PV = portal vein.

Graphs represent mean \pm SD. Statistical significance was assessed by unpaired non-parametric Mann-Whitney test. Magnification bars: 100 μ m.

Supplementary Figure 5

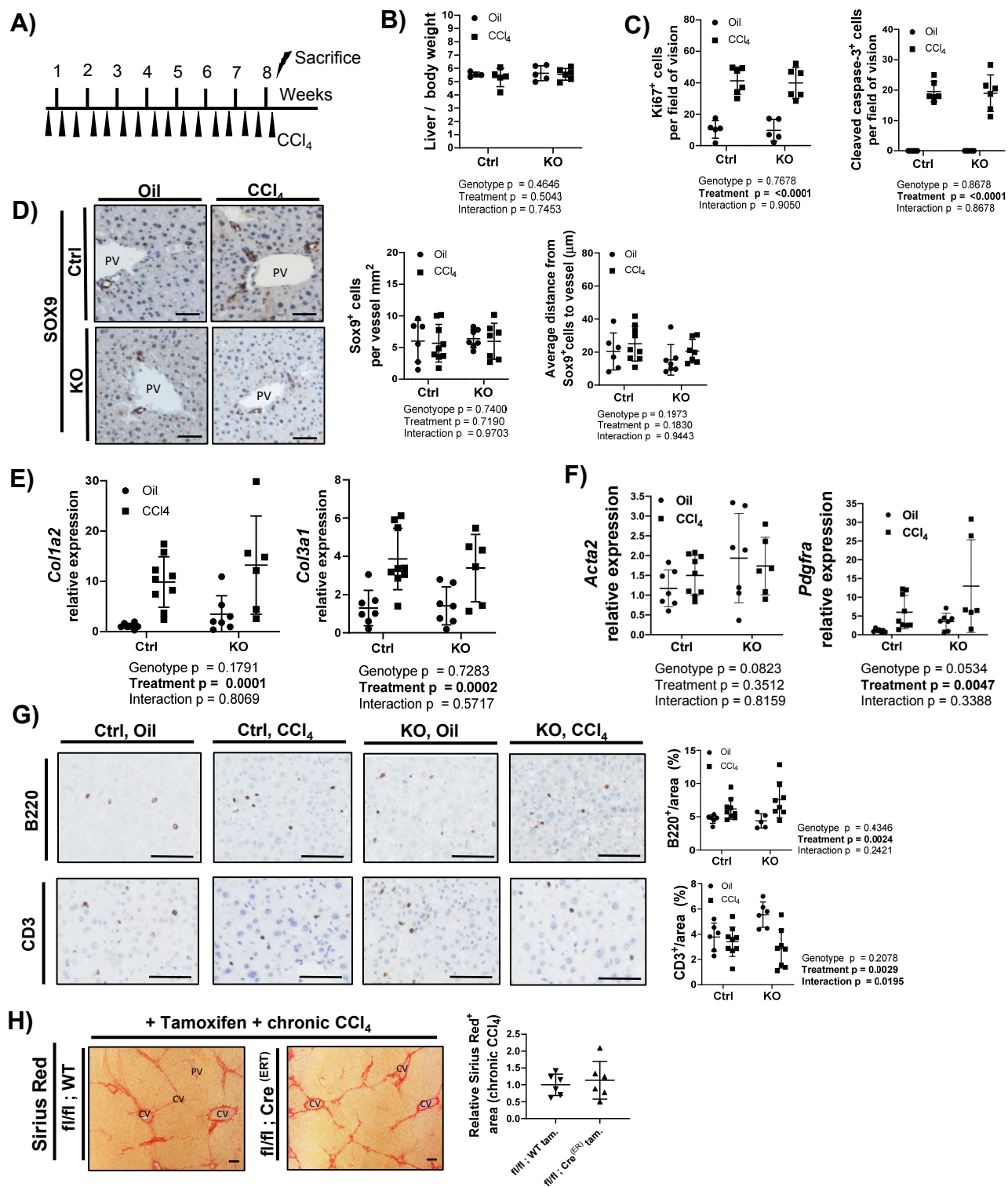


Fig. S5. Analysis of liver fibrosis in Ctrl and KO mice chronically treated with CCl₄

(A) Timeline of CCl₄ injections. Injection with vehicle (olive oil) was used as control.

(B) Liver to body weight ratios in Ctrl and KO mice following chronic oil or CCl₄ treatment.

Ctrl oil: N=4; Ctrl CCl₄: N=5; KO oil: N=5; KO CCl₄: N=6.

(C) Graph showing quantification of Ki67- or cleaved caspase 3-positive cells per field of vision in mice chronically treated with CCl₄ or oil, respectively. N=5-6 per group.

(D) Representative photomicrographs of liver sections analyzed by immunohistochemistry for SOX9 and quantification (right panels) of SOX9⁺ cells per vessel area and of average distance of SOX9⁺ cells from vessels. N=6-9 mice, = 3 pictures per mouse. PV = portal vein.

(E, F) RNA samples from total liver of Ctrl and KO mice, chronically treated with CCl₄ or vehicle, were analyzed by RT-qPCR for *Col1a2* and *Col3a1* (E) or *Acta2* and *Pdgfra* (F) relative to *Rps18*. Ctrl oil: N=3-7; Ctrl CCl₄: N=5-9; KO oil: N=5-7; KO CCl₄: N=5-6. Mean expression levels in Ctrl mice were set to 1.

(G) Representative liver sections from Ctrl and KO mice chronically treated with CCl₄ or vehicle (oil), analyzed by immunohistochemistry for B220 or CD3, co-stained with hematoxylin, and quantification (right panels) of the B220⁺ and CD3⁺ cells per area. Ctrl oil: N=6-7; Ctrl CCl₄: N=5-9; KO oil: N=6; KO CCl₄: N=6.

H) Representative liver sections from fl/fl;Cre^(ERT) or fl/fl;Wt mice injected with tamoxifen and chronically treated with CCl₄ or oil (vehicle) over 8 weeks stained with Sirius Red. Graph shows quantification of relative Sirius Red positive area per field of vision. fl/fl;Wt + CCl₄: N=6, fl/fl;Cre^(ERT) + CCl₄: N=6. PV = portal vein, CV = central vein.

Graphs show mean \pm SD. Statistical analysis was performed using two-way ANOVA (B, C, D, E, F, G) or unpaired non-parametric Mann-Whitney test (H). Representative results from two independent experiments are shown. Magnification bars: 100 μ m.

Supplementary Figure 6

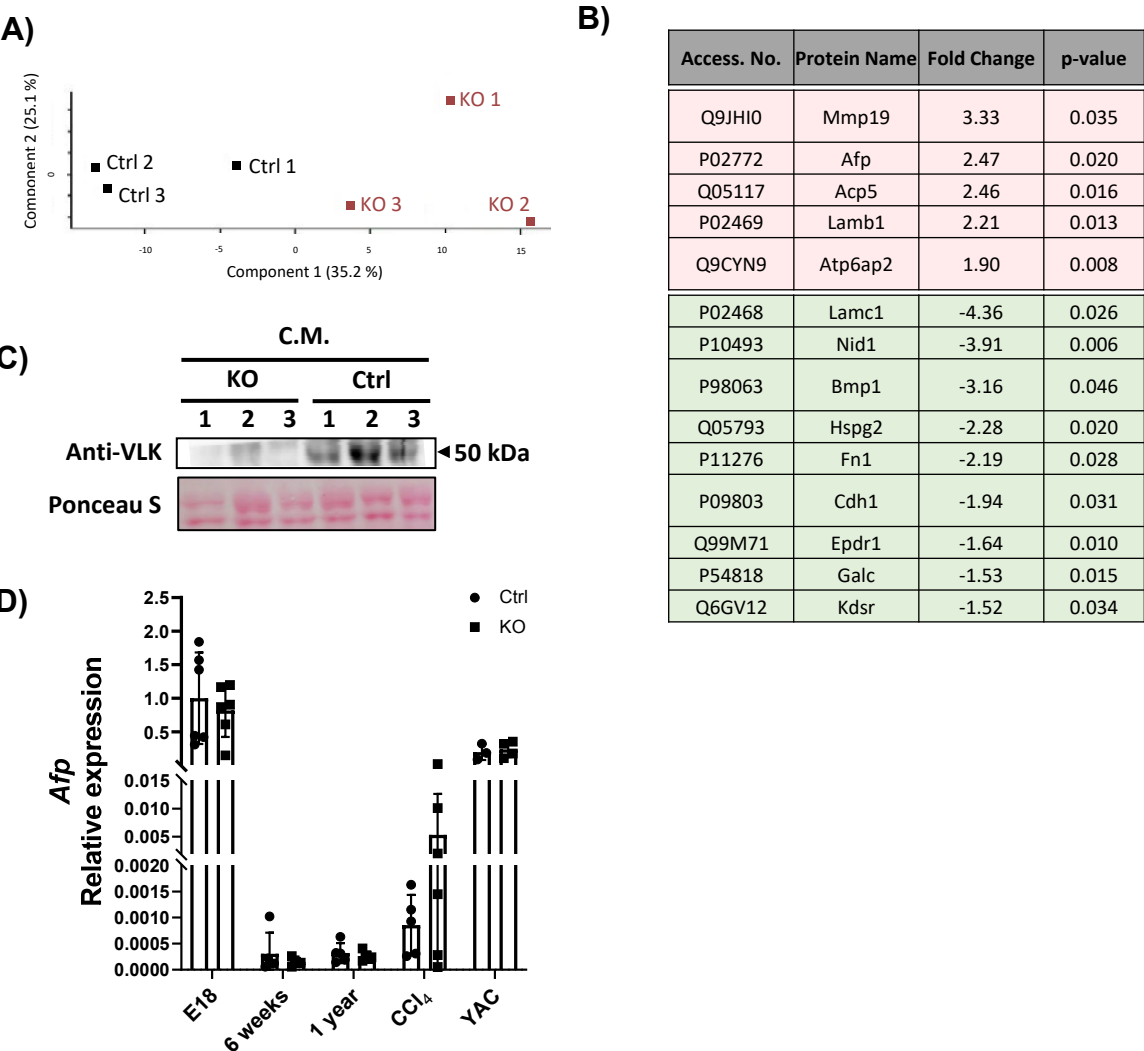


Fig. S6. Increased AFP levels in the conditioned medium (C.M.) and whole cell lysate (WCL) of Y/A/C treated hepatocytes

(A) Principle component analysis (PCA) to identify variability in the dataset. Individuals are represented by black squares (Ctrl) or red squares (KO).

(B) LC-MS/MS list of proteins with a signal peptide that are significantly differentially abundant (FC>1.5, p<0.05) in WCL of Y/A/C-treated hepatocytes. Red: more abundant in the KO cells; green; less abundant in the KO cells. N=3 per genotype.

(C) Representative Western blot of conditioned media (C.M.) of Y/A/C-supplemented hepatocytes analyzed for VLK. Ponceau S was used to ensure equal loading. Every line represents a cell isolation from an individual mouse.

(D) NA from total liver of E18, 6-week-old and 1-year-old Ctrl and KO mice and from CCl₄-treated Ctrl and KO mice, or from Y/A/C treated hepatocytes of Ctrl and KO mice were analyzed by RT-qPCR for *Afp* relative to *Rps18*. N= 5-6 mice per genotype. Mean expression levels in Ctrl mice at E18 were set to 1.

Graphs show mean \pm SD. Statistical significance was assessed by unpaired non-parametric Mann-Whitney test.

Table S1. Genotyping

The following primers were used:

Primer	Sequence forward primer	Sequence reverse primer
<i>mPkdcc</i>	CAC ACG CTC AAT CAT ACC ACA	GGT CAT TAG GTC ACA GGG TAG
<i>mCre</i>	CGA CCA GGT TCG TTC ACT CA	CGA GTT GAT AGC TGG CTG GT
<i>mR26 loc wt</i>	AAG GGA GCT GCA GTG GAG TA	CCG AAA ATC TGT GGG AAG TC
<i>mtdTomato</i>	CTG TTC CTG TAC GGC ATG G	GGC ATT AAA GCA GCG TAT
<i>mCK19</i>	CAG AAT GCG CAG GAA TTG AC	mCK19wt CGG AAA AAA CCC CCT GA mCk19mut AGG CAA ATT TTG TGT AGG G

Table S2. Primer list RT-qPCR

Primer	Sequence forward primer	Sequence reverse primer
<i>mPkdcc</i>	CACACGCTCAATCATACCACA	GGTCATTAGGTCACAGGGTAG
<i>mAlb</i>	CGACCAGGTTCTGTTCACTCA	CGAGTTGATAGCTGGCTGGT
<i>mSox9</i>	GCTTGTCCGTTCTTCACCGA	TCTGGAGGCTGCTGAACGAG
<i>mAdgre1</i>	GACAAACACTTGGTGGTGTGA	CCAGAATCCAGTCTTTCCCA
<i>mCol1a</i>	TGTTCACTTTGACCTCCGGC	TCTCCCTTGGGTCCCTCG
<i>mCol3a1</i>	TCCCCTGGAATCTGTGAATC	TGAGTCGAATTGGGGAGAAT
<i>mPdgfra</i>	GTGCGACCTCCAACCTGA	GGCTCATCTCACCTCACATCT
<i>mActa2</i>	CTGCCGAGCGTGAGATTG	ATAGGTGGTTTCGTGGATGC
<i>mCyp7a1</i>	GGGATTGCTGTGGTAGTGAGC	GGTATGGAATCAACCCGTTGTC
<i>mFgfr4</i>	TTGGCCCTGTTGAGCATCT	GCCCTCTTTGTACCAGTGACG
<i>mSpp1</i>	CTTTCACCGGGAGGGAGGA	TGCAGTTCTCCTGGCTGAAT
<i>mTgfb</i>	AAAGCCCTGTATTCCGTCTCC	CGCAACAACGCCATCTATGA
<i>m18s</i>	GATCCATTGGAGGGCAAGTCT	CCAAGATCCAACCTACGAGCTTTT
<i>mGli1</i>	GTATGAGACAGACTGCCGCT	T GCTCACTGTTGATGTGGTG
<i>mCcnD1</i>	ACTGCCGAGAAGTTGTGCAT	AAGCAGTTCCATTTGCAGCAG
<i>mAfp</i>	TGCTTCCAGACAAAGAGAGCAT	GTTGTTGCCTGGAGGTTTCG