The Polyploid State Restricts Hepatocyte Proliferation and Liver Regeneration in Mice

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Supporting Information

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Supporting Experimental Procedures

Ploidy Analysis. For detection of ploidy populations, freshly isolated primary hepatocytes were washed twice in PBS, adjusted to a density of 1-2 million cells/ml and incubated on ice with 2 µl/ml Fixable Viability Dye (FVD) eFluor 780 (eBioscience, San Diego, CA). Following 2 washes with PBS, hepatocytes were fixed with 2% PFA, washed an additional 2 times with PBS, and incubated with permeabilization buffer (0.1% saponin and 0.5% BSA in PBS) + 15 µg/ml Hoechst 33342 (Life Technologies, Carlsbad, CA). Cells were washed twice and stored in PBS until flow cytometry analysis. We also detected Ki-67 in hepatic ploidy populations. Hepatocytes were prepared using the same methods as above except following FVD 780 incubation they were fixed with Foxp3 fixation buffer (eBioscience) at room temperature, washed twice with permeabilization buffer (eBioscience) and incubated in permeabilization buffer with mouse anti-Ki-67 eFluor 660 antibody (Invitrogen, Carlsbad, CA) + 15 µg/ml Hoechst 33342. Cells were washed twice with PBS and stored in permeabilization buffer until flow cytometry analysis.

Ploidy populations are indicated with *chromatid number* "c" (2c, 4c, 8c) or *chromosome number* "n" (2n, 4n, 8n). We used *chromatid number* in Fig. 1 and Supporting Fig. S3 since the gated cells contain a mixture of quiescent and proliferating cells, although >99% of adult hepatocytes are quiescent (e.g., a 4c cell is either a diploid in G2/M-phase or a tetraploid in G0/G1-phase). We used *chromosome number* in Fig. 5 and Supporting Figs. S5, S9 and S10 because quiescent cells are specifically gated (e.g., a 4n cell is a tetraploid).

Liver enzymes. Serum was collected from experimental animals and the levels of circulating serum enzymes, including alanine aminotransaminase (ALT), aspartate aminotransaminase (AST) and alkaline phosphatase (ALP), as well as direct bilirubin and total bilirubin were determined by the University of Pittsburgh Medical Center Clinical Laboratory.

Body weight and LW/BW. Livers from experimental animals were excised, washed in PBS and weighed. The percentage of the weight occupied by the liver was determined by dividing the liver weight by the total body weight of the mouse.

High Fat Diet treatment. LKO and control mice were placed on a high fat diet (HFD) for 8 weeks in which the chow was given *ad libitum* and replaced weekly. The mice were given an adjusted calorie diet in which 42% of the calories were from fat (TD.88137 Adjusted calories diet, Teklad Research Diets, Envigo, Indianapolis, IN). The mice were weighed regularly and after 8 weeks the mice were sacrificed following overnight fasting. The livers were isolated, washed in PBS, weighed to determine a final LW/BW and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA). To identify neutral lipids, sections were stained with Oil Red O by the McGowan Institute for Regenerative Medicine Histology Core Lab. The percentage of the liver tissue area that was stained with Oil Red O was quantified using ImageJ software (NIH).

β-galactosidase (β-gal) activity assay. β-galactosidase (β-gal) activity was detected in LKO and control liver tissue and cells. Livers were embedded in OCT compound (Sakura Finetek) and cryosectioned at a thickness of 10 µm. Frozen liver sections were fixed with 0.2% gluteraldehyde, 2% paraformaldehyde (PFA) and stained overnight with Xgal solution (2 mM Xgal in DMSO, 4 mM $K_3Fe(Cn)_6$, 4 mm $K_4Fe(Cn)_6$-H_2O, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% sodium deoxycholate, 10 mM HEPES, and PBS) in a 37°C humid incubator. The tissue was counterstained with Nuclear Fast Red and mounted with Permount (Fisher, Pittsburgh, PA). Cells were isolated via perfusion, seeded at 40,000 viable cells/well in 24-well Primaria Cell Culture plates (Corning) in seeding media and then fixed with 0.5% gluteraldehdye. The cells were stained overnight with Xgal solution (same as above) in a 37°C humid incubator.

General processing of paraffin embedded tissue. Livers from LKO and control mice were fixed in 10% neutral buffered formalin (NBF), embedded in paraffin, and sectioned at a thickness of 4 µm. Sections were deparaffinized using xylene and graded ethanol (100-95%) washes and incubated in citric-acid based antigen retrieval (Vector, Burlingame, CA).

Quantification of mono- and binucleate hepatocytes in liver sections. Following paraffin embedding, sectioning and antigen retrieval, liver sections were blocked with 2% BSA. For quantification of mono- and binucleate hepatocytes, sections were stained with mouse anti-β-catenin primary antibody (Santa Cruz, Dallas, TX) followed by goat anti-mouse AlexaFluor 594 secondary antibody (Invitrogen, Carlsbad, CA). The sections were counterstained with Hoechst 33342 and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL). A minimum of 700 hepatocytes/sample was scored.

Staining for proliferation markers in liver sections. To measure Ki-67 (G1/S/G2/M-phases) in postnatal development, tissue sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase and blocked with Avidin/Biotin blocking solution (Vector SP-2001). Sections were stained with rabbit anti-Ki-67 (Abcam, Cambridge, MA) followed by biotinylated goat anti-rabbit secondary antibody and Avidin-conjugated peroxidase (Vector ABC kit PK-6100); staining was visualized with ImmPACT DAB Peroxidase Substrate (Vector SK-4105). Sections were counterstained with hematoxylin, dehydrated with ethanol and xylene washes and mounted with Permount (Fisher).

To measure Ki-67 in regenerating livers following PH, liver sections were blocked with 2% BSA and stained with rabbit anti-Ki-67 primary antibody (Abcam) and mouse anti-E-cadherin primary antibody (BD Biosciences, San Jose, CA) followed by species-specific secondary antibodies: goat anti-rabbit AlexaFluor 594 and goat anti-mouse AlexaFluor 488 secondary antibody (both from Life Technologies). The sections were counterstained with Hoechst 33342 to visualize nuclei and mounted with Fluoromount-G (Southern Biotech). For PCNA (S-phase) and bright phospho-histone H3 (PHH3) (M-phase) staining, tissue sections were permeabilized with 0.1% Triton X-100 and blocked with 5% normal goat serum. The sections were then co-stained with rabbit anti-PHH3 primary antibody (Invitrogen), mouse anti-PCNA primary antibody (IgG2a, Santa Cruz) and mouse anti-β-catenin primary antibody (IgG1, Santa Cruz) followed by species and isotype specific secondary antibodies: goat anti-rabbit AlexaFluor 647, goat anti-mouse IgG2a AlexaFluor 488 and goat anti-mouse IgG1 AlexaFluor 594 (all from Life Technologies). The sections were counterstained with Hoechst 33342 to visualize nuclei and mounted with Fluoromount-G (Southern Biotech). A minimum of 600 hepatocytes/sample was scored to determine the percentage of Ki67+, PCNA+ and bright PHH3+ cells.

Liver zonation. Following paraffin embedding, sectioning and antigen retrieval, liver sections were permeablized using 1% Saponin followed by blocking in 2% BSA. Tissues were co-stained with primary antibodies mouse anti-glutamine synthetase (GS, Santa Cruz, Dallas, TX), goat anti-Cyp1A2 (Santa Cruz) and rabbit anti-β-catenin (Santa Cruz) followed by species-specific secondary antibodies: donkey anti-mouse AlexaFluor 647, donkey anti-rabbit AlexaFluor 488 and donkey anti-goat AlexaFluor 594 (all from Life Technologies). The sections were counterstained with Hoechst 33342 to visualize nuclei and mounted with Fluoromount-G (Southern Biotech). Pericentral (GS+ Cyp1A2+), mid-lobule (GS- Cyp1A2+) and periportal (GS- Cyp1A2-) regions were identified and the percentage of binucleate hepatocytes in each region was quantified. A minimum of 200 cells were counted in each region.

DEN tumor induction model. Control and LKO (6-week-old) mice were injected intraperitoneally with 90 mg/kg DEN (Sigma-Aldrich). Three weeks following the injection, mice were fed a standard rodent diet containing 0.05% Phenobarbital-Sodium Salt (LabDiet) for an additional 3, 6 or 9 months. Following paraffin embedding, sectioning and antigen retrieval, liver sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase and blocked with Avidin/Biotin blocking solution (Vector SP-2001). Sections were stained with rabbit anti-glutamine synthetase primary antibody (Abcam) followed by biotinylated goat anti-rabbit secondary antibody and Avidin-conjugated peroxidase (Vector ABC kit PK-6100); staining was visualized with ImmPACT DAB Peroxidase Substrate (Vector SK-4105). Sections were counterstained with hematoxylin, dehydrated with ethanol and xylene washes, and mounted with Permount (Fisher). Hematoxylin & Eosin (H&E) staining was performed by the Development Laboratory in the University of Pittsburgh Department of Pathology. Glutamine synthetase positive and negative tumors were quantified and measured.

RNA isolation, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and PCR. Total RNA was isolated from liver tissue using Trizol (Life Technologies, Carlsbad, CA) or RNeasy Plus Mini Kit (Qiagen, Germantown, MD) and subjected to qRT-PCR to quantify the expression of protein-coding genes. Total RNA was reverse-transcribed into complementary DNA (cDNA) using M-MLV (Life Technologies) and quantitative PCR reaction was performed with SYBR Green PCR Master Mix (Life Technologies). Primer sequences are listed in supporting table. Reactions were performed using a StepOnePlus System (Life Technologies). Relative expression was calculated using the ΔΔCT method. To determine presence or absence of *E2f7/E2f8* a PCR reaction was performed using cDNA, and PCR products were analyzed on a 1% agarose gel.

Liver repopulation using LKO or control hepatocytes. Hepatocytes from 6-week-old LKO and control donor mice were isolated using a 2-step collagenase perfusion. 300,000 LKO or control donor (H-2K^{q}+) hepatocytes were intrasplenically injected into 6-8-week-old FRGN (H-2k^{q}-) recipients. Recipient mice were maintained on 8 mg/mL NTBC for 4 days following transplantation. The transplanted mice were cycled on/off NTBC to promote donor cell (H-2k^{q}+) proliferation. Following several cycles of NTBC withdrawal, mice were considered highly repopulated when body weights stabilized at ~100% of the initial weight. Hepatocytes were isolated from repopulated mice using a 2-step collagenase perfusion as previously described. The isolated hepatocytes were stained with FVD 780, anti-mouse H-2K^{q} AlexaFluor 647 antibody (Biolegend, San Diego, CA) for donor-derived cells and counterstained with 15 μg/ml Hoechst 33342 following permeabilization with 0.5% Saponin (Sigma-Aldrich) in PBS. The ploidy of the donor cells (H-2K^{q}) was determined using flow cytometry.

Competitive repopulation experiments. Hepatocytes from 6-week-old LKO and control mice were mixed in ~equal ratios (Group 1: LKO$^{60\%}$ and control$^{40\%}$) or skewed towards control hepatocytes (Group 2: LKO$^{17\%}$ and control$^{83\%}$). A combined total of 300,000 donor (Fah+) cells were intrasplenically injected into 6-8-week-old FRGN recipient mice. Recipient mice were maintained on 8 mg/mL NTBC for 4 days following transplantation. The transplanted mice were cycled on/off NTBC to promote donor cell proliferation. The mice were weighed regularly and re-started on NTBC when body weights decreased to 80% baseline to minimize morbidity and mortality. Following several cycles of NTBC withdrawal, mice were considered highly repopulated when body weights stabilized at ~100% of the initial weight when off NTBC. Hepatocytes were isolated from repopulated mice using 2-step collagenase digestion as previously described. To determine the extent of repopulation, hepatocytes were stained for Fah. Briefly, cells were seeded at 40,000 viable cells/well in 24-well Primaria Cell Culture plates (Corning) in seeding media, fixed in 4% PFA, and blocked with 5% normal donkey serum in 0.05% PBS-Tween 20. The cells were stained with custom rabbit anti-Fah primary antibody and donkey anti-rabbit AlexaFluor 555 secondary antibody (Life Technologies), counterstained with Hoechst 33342, and mounted with Fluoromount-G (Southern Biotech). To determine the percentage of donor cells in the repopulated liver, β-gal activity was assessed in the isolated hepatocytes. The cells were seeded at 40,000 viable cells/well in 24-well Primaria Cell Culture plates (Corning) in seeding media, fixed with 0.5% gluteraldehdye and stained overnight with Xgal solution in a 37°C humid incubator using methods previously described. The ratio of LKO (β-gal+) to control (β-gal-) donor hepatocytes in the repopulated liver was measured.

Partial hepatectomy. A 2/3 partial hepatectomy (PH) was performed on adult C57BL/6J WT, LKO and control mice using methods similar to those described previously (1). Briefly, a small vertical incision was made directly below the sternum of anesthetized mice. The left anterior liver lobe was removed after tying it with a ligature, followed by removal of the right and left segments of the median lobe. This effectively removes 2/3 of the liver mass. For tissue analysis, livers were harvested post-surgery, weighed, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 4 μm. The tissue sections were stained for Ki-67 and E-cadherin using previously described methods, and the number of proliferating (Ki-67+) hepatocytes quantified. For FACS analysis, cells from the livers of C57BL/6J WT mice that had undergone 2/3 PH were isolated via 2-step collagenase perfusion and prepared for Ki-67 FACS analysis using methods previously described. FACS analysis was used to quantify the total number of proliferating (Ki-67+) hepatocytes for each ploidy population.

In vitro proliferation using LKO and control hepatocytes. LKO or control hepatocytes from 14-day-old or adult mice were seeded at 40,000 viable cells/well in 24-well Primaria Cell Culture plates (Corning) in seeding media: DMEM-F12 with 15 mM HEPES (Corning), 5% FBS (Atlanta Biologicals, Atlanta, GA), and Antibiotic-Antimycotic Solution (Corning). After 4 hours, seeding media was replaced with growth media: DMEM-F12 with 15 mM HEPES (Corning), 0.5% FBS (Atlanta Biologicals), Antibiotic-Antimycotic Solution (Corning) and ITS Supplement (containing 1 µg/ml insulin, 0.55 µg/ml transferrin and 0.67 ng/ml sodium selenite; Gibco Life Technologies). For BrdU labeling, hepatocytes were cultured in growth media containing 1 mM BrdU (Sigma-Aldrich) for 12-24 hours prior to harvest (as indicated in each experiment), fixed in 4% PFA, washed in 1% Triton X-100 followed by 2 N HCl and 0.1 M Na_2B4O_7, and blocked with 0.5% skim milk. Next cells were incubated with mouse anti-BrdU primary antibody (BD Biosciences) and goat anti-mouse AlexaFluor 594 secondary antibody (Life Technologies), counterstained with Hoechst 33342, and mounted with Fluoromount-G (Southern Biotech). For phospho-histone H3 (PHH3) staining, cells were fixed in 4% PFA, blocked with 2% BSA in Normal Donkey Serum, stained with rabbit anti-PHH3 primary antibody (Invitrogen) and donkey anti-rabbit AlexaFluor 555 secondary antibody (Life Technologies), counterstained with Hoechst 33342 and mounted with Fluoromount-G (Southern Biotech). Nuclei with punctate PHH3 staining were considered to be in G2-phase. For measuring apoptosis, hepatocytes were cultured in growth media containing 0.5 µM NucView 488 substrate (a substrate for detecting active caspase-3, a marker of apoptosis; Biotium, Hayward, CA) for 30 minutes before harvest. Cells were fixed with 4% PFA, stained with Hoechst 33342, and mounted with Fluoromount-G (Southern Biotech). Approximately 100 cells/sample/time were scored for each stain. To measure molecular markers of proliferation, LKO and control hepatocytes from adult mice were seeded at $1x10^6$ viable cells/well in 10-cm Primaria Cell Culture dishes (Corning) in seeding media. After 4 hours, seeding media was replaced with growth media. At each time point the cells were washed, trypsinized, and harvested. RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and prepared for qRT-PCR using methods previously described.

In vitro proliferation using WT *C57BL/6 hepatocytes, with and without mitogens.* Hepatocytes from 20-day-old WT C57BL/6 mice were seeded at 100,000 viable cells/well in 6-well Primaria Cell Culture plates (Corning) in seeding media before switching to growth media 4 hours later. The cells were grown in growth media containing 1 mM BrdU for 12-24 hours prior to harvest, fixed in 4% PFA and stained for BrdU, PHH3, and active caspase-3 using methods stated previously. For growth factor stimulation, cells were seeded, switched to serum-free media and growth factors added 4 hours later. Growth factors included mouse HGF, human EGF, mouse FGF-acidic (FGF1), mouse FGF-basic (FGF2), human PDGF-AA, human TGFα (all 40 ng/mL: R&D Biosystems, Minneapolis, MN), and insulin (1 µg/ml). The cells were incubated with 1 mM BrdU for 12-24hr prior to harvest, fixed in 4% PFA, and stained for BrdU using methods described previously. The number of positively stained mono- and binucleate hepatocytes was quantified by overlaying phase-contrast and fluorescent images. Approximately 350 cells/sample/time/growth condition were scored.

Flow cytometry. All FACS analyses were performed with a FACS Aria II-SORP (BD Biosciences) equipped with a 130 µm nozzle and running DiVa v8.0.2 software. FACS plots were generated using FlowJo 9.9.6 (Treestar, Ashland, OR).

Microscopy. Fluorescent images were captured with a Nikon TiU fluorescent microscope equipped with a monochrome camera. Non-fluorescent images were captured with a Nikon TS100 microscope equipped with a color camera. Gross morphology images were taken using a Nikon D3100 DSLR Digital Camera with 18-55mm f/3.5-5.6 Auto Focus-S Nikkor Zoom Lens. Images were processed with Nikon NIS Elements Basic Research software, NIH ImageJ software and Adobe Photoshop.

Detailed information for select reagents

Primer pairs

Gene	*Forward primer (5' to 3')*	*Reverse primer (5' to 3')*
Ccna2	TGTCAACCCCGAAAAACTGG	ATGAATGGTGAAGGCAGGCT
Ccnb1	GGCTTTCTCTGATGTAATCCTTGC	TCTTCTTGGGCACACAACTGTTC
Ccnd1	TCAAGACGGAGGAGACCTGT	GGAAGCGGTCCAGGTAGTTC
Ccne1	TGAGTTCCAAGCCCAAGTCC	GGATGAAAGAGCAGGGGTCC
Cyp2e1	TTTCTGCAGGAAAGCGCG	CTGCCAAAGCCAATTGTAACAG
Cyp2b10	CACACGGAGTTCCATCACCA	GCGGTCATCAAGGGTTGGTA
Car	TCAACACGTTTATGGTGCAA	CTGCGTCCTCCATCTTGTAG
E2f7	TCTCCCTAGATGAGGTCGCC	GACCGTGCCAACCATACTGA
E2f8	GAGAAATCCCAGCCGAGTC	CATAAATCCGCCGACGTT
Gapdh	TCCTGCACCACCAACTGCTTAG	TGCTTCACCACCTTCTTGATGTC

Primary Antibodies

Epitope	*Antibody with species reactivity*	*Vendor*	*Catalog #*
β-catenin	Rabbit anti-β-catenin	Santa Cruz	sc-7199
β-catenin	Mouse anti-β-catenin	Santa Cruz	sc-7963
BrdU	Mouse anti-BrdU	BD Biosciences	347580
Cyp1A2	Goat anti-Cyp1a2	Santa Cruz	sc-9835
E-cadherin	Mouse anti-E-cadherin	BD Biosciences	610181
FAH	Rabbit anti-FAH	Custom, Grompe Lab	n/a
Glutamine Synthetase	Mouse anti-Glutamine Synthetase	Santa Cruz	sc-74430
Glutamine Synthetase	Rabbit anti-Glutamine Synthetase	Abcam	ab49873
$H2K^q$	Mouse anti-$H2K^q$ AlexaFluor 647	Biolegend	115106
Ki-67	Mouse anti-Ki-67 eFluor660	Invitrogen	50569882
Ki-67	Rabbit anti-Ki-67	Abcam	ab15580
PCNA	Mouse anti-PCNA IgG2a	Santa Cruz	sc-25280
Phospho-histone H3	Rabbit anti-Phospho-histone H3	Invitrogen	PA5-17869

Secondary Antibodies

Species reactivity	*Antibody with conjugate*	*Vendor*	*Catalog #*
Goat	Donkey anti-Goat/AlexaFluor 594	Life Technologies	A11058
Mouse	Donkey anti-Mouse/AlexaFluor 647	Life Technologies	A31571
Mouse	Goat anti-Mouse/AlexaFluor 488	Life Technologies	A11029
Mouse	Goat anti-Mouse/AlexaFluor 488 (specific for IgG2a)	Life Technologies	A21131
Mouse	Goat anti-Mouse/AlexaFluor 594	Life Technologies	A11055
Mouse	Goat anti-Mouse/AlexaFluor 594 (specific for IgG1)	Life Technologies	A21125
Rabbit	Donkey anti-Rabbit/AlexaFluor 488	Life Technologies	A11055
Rabbit	Donkey anti-Rabbit/AlexaFluor 555	Life Technologies	A31572
Rabbit	Goat anti-Rabbit/AlexaFluor 594	Life Technologies	A11012
Rabbit	Goat anti-Rabbit/AlexaFluor 647	Life Technologies	A21244
Rabbit	Goat anti-Rabbit/Biotinylated	Vector Laboratories	BA-1000

Supporting References

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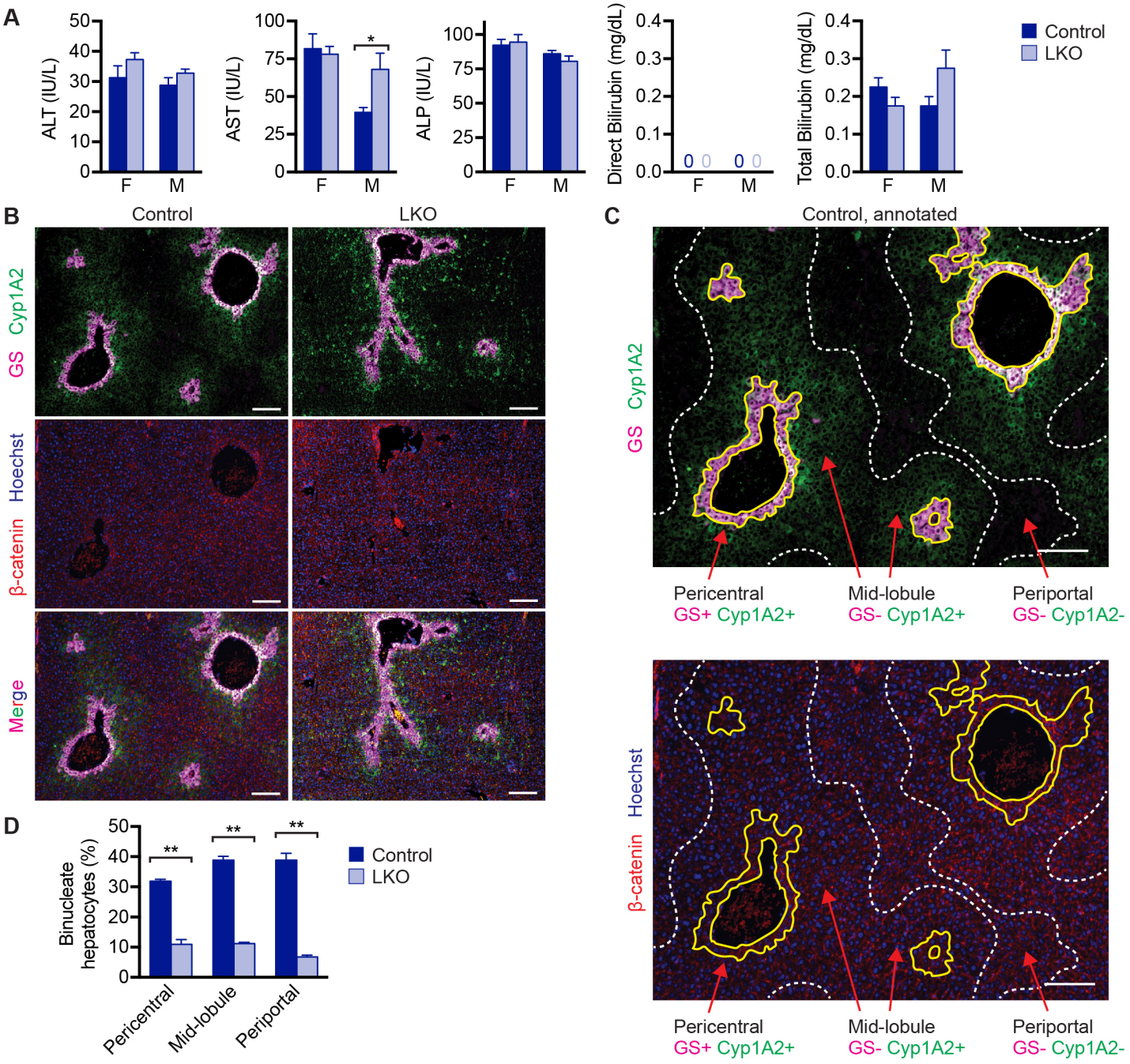
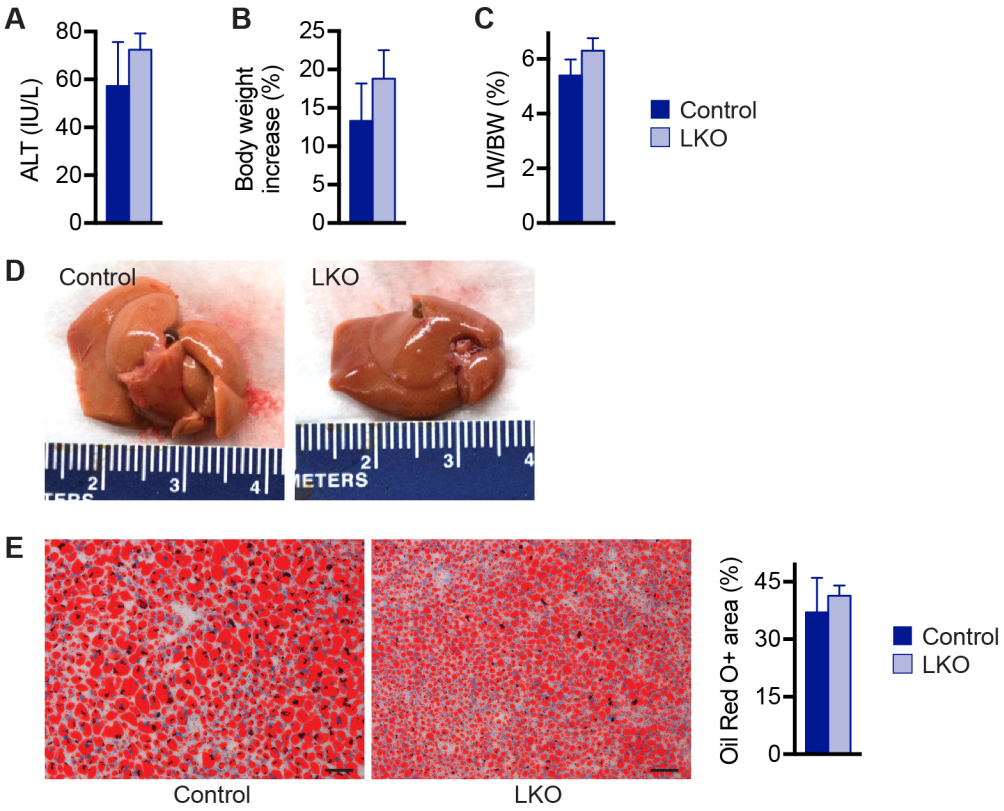
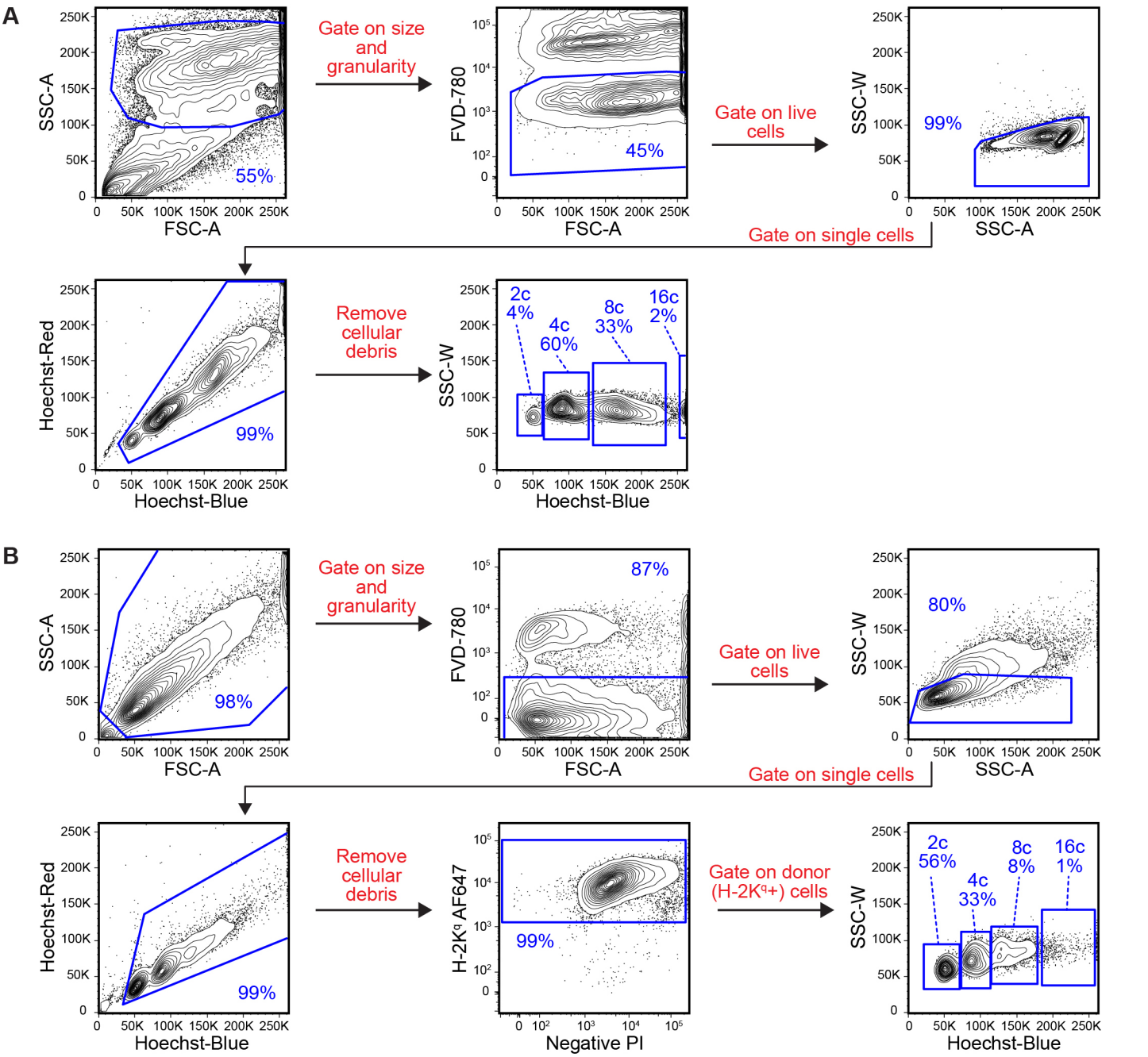
Author names in bold designate shared co-first authorship.



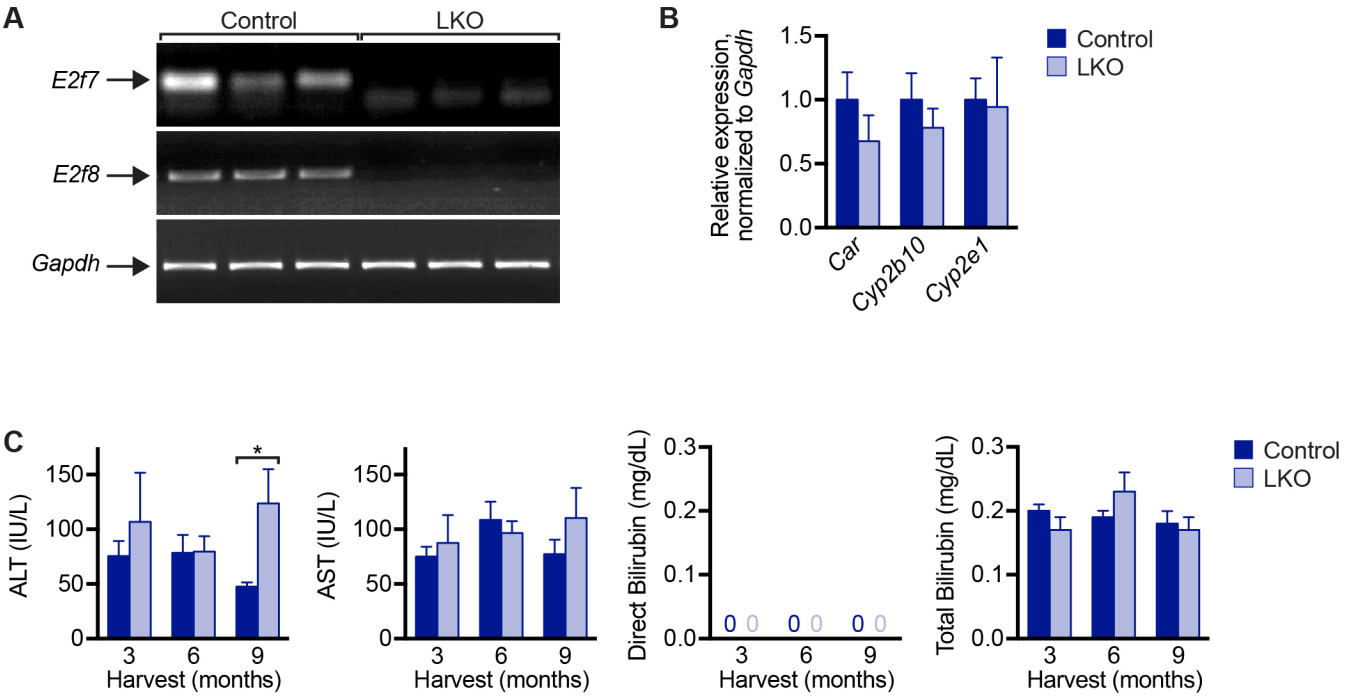
Supporting Fig. S1. LKO and control mice have comparable liver enzyme levels and liver architecture. (A) LKO and control mice at 2.5 months have similar levels of ALT, ALP and direct/total bilirubin. AST levels are slightly elevated in male LKO (n = 4/genotype/age/gender; F, female; M, male). (B) Livers from 2.5-month-old male LKO and control mice were stained for Glutamine Synthetase (GS, a pericentral marker, pink), Cytochrome P450 1A2 (Cyp1A2, a pericentral and mid-lobule marker, green), β-catenin (cell membrane marker, red) and Hoechst dye (nuclear marker, blue). Representative images are shown as 2-color panels and as a 4-color merge. (C) The GS and Cyp1A2 expression pattern was used to define pericentral, mid-lobule and periportal regions. Within each region, mono- and binucleate hepatocytes were identified using β-catenin and Hoechst staining. Representative images are shown for a control mouse. Solid yellow lines indicate the pericentral region and dashed white lines highlight the mid-lobule area. (D) Mono- and binucleate hepatocytes were quantified in each zone. Overall, the percentage of binucleate hepatocytes is reduced in LKO livers, which is consistent with Fig. 1C. However, similar to control livers, binucleate hepatocytes in LKO livers are distributed evenly in all zones. * $P < 0.05$, ** $P < 0.001$. Graphs show mean ± sem. Scale bars are 100 μm.



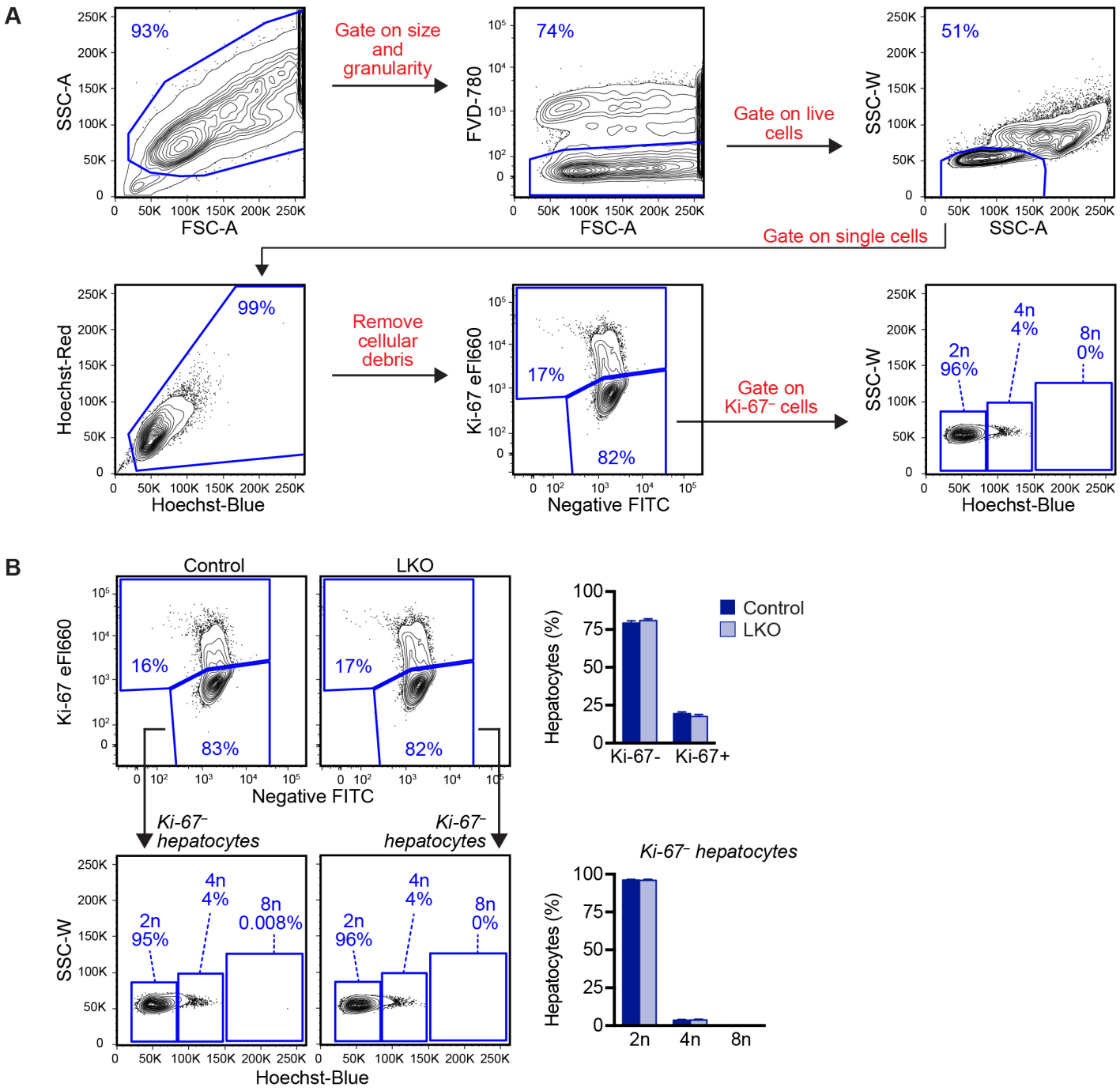
Supporting Fig. S2. LKO and control mice respond to HFD equivalently. (A-C) Approximately 3-month-old LKO (n = 8) and control mice (n = 3) were maintained on HFD for 8 weeks to model fatty liver disease. ALT levels (A), body weight increase (B) and LW/BW ratios (C) were the same. (D,E) Livers harvested after 8 weeks were similar in terms of gross morphology (D) and accumulation of neutral lipids, as seen by Oil Red O staining (E). Representative images are shown, and the percentage of Oil Red O+ tissue quantified. Graphs show mean ± sem. Scale bar is 100 μm.



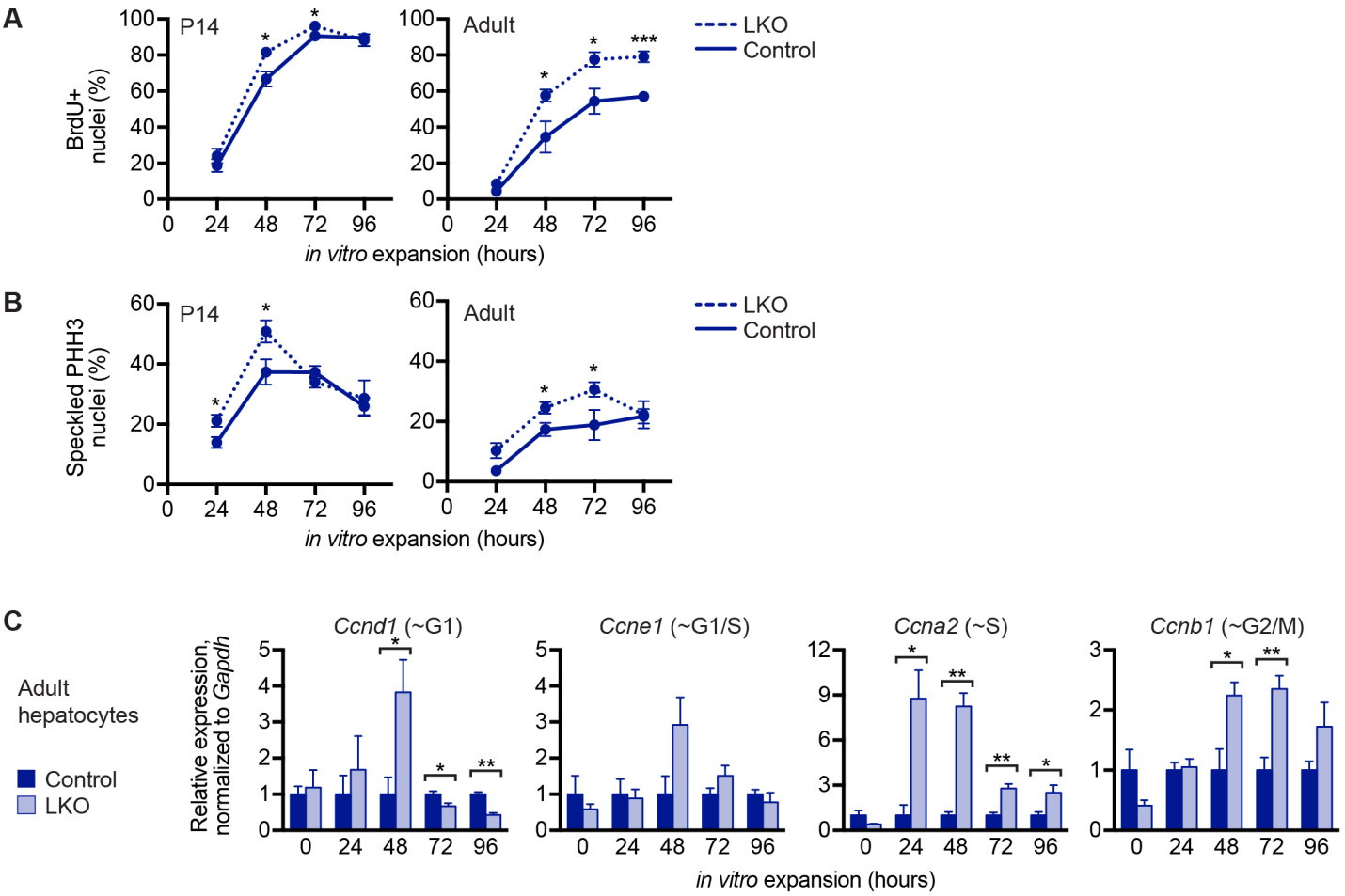
Supporting Fig. S3. FACS gating strategy corresponding to Fig. 1. (A) Single cell suspensions of LKO and control hepatocytes were stained with FVD-780 (a viability dye) + Hoechst dye and analyzed by flow cytometry. Cells were gated on the basis of size/granularity and viability. Subsequently, single cells were selected and cellular debris removed. Hepatic ploidy populations were determined by separating the cells based on incorporation of Hoechst using the Hoechst-blue channel. Ploidy populations are marked with chromatid number "c" since they contain a mixture of cycling and quiescent cells (although >99% are quiescent). Representative FACS plots are shown for a control mouse. (B) Hepatocytes isolated from FRGN livers repopulated by LKO or control hepatocytes were stained with FVD-780 + H-2K^q-AF647 (a donor marker) + Hoechst dye. Cells were gated on the basis of size/granularity and viability. Single cells were then selected and cellular debris removed. Donor-derived cells (H-2K^q+) were selected and hepatic ploidy populations determined. Representative FACS plots are shown for an FRGN mouse repopulated by LKO hepatocytes.



Supporting Fig. S4. Characterization of LKO and control mice in the DEN/PB tumor model. (A,B) Hepatocytes were isolated from 6-week-old LKO and control mice prior to DEN injection, as described in Fig. 2A (n = 3-4; males). As expected, *E2f7* and *E2f8* were expressed by control hepatocytes but not by LKO hepatocytes (A). Genes involved in DEN metabolism (*Cyp2b10*) and PB-induced proliferation (*Car* and *Cyp2e1*) were equivalently expressed. Gene expression was normalized to *Gapdh* (C). Liver enzyme levels were determined after 3, 6 and 9 months of PB treatment (n = 7-13/genotype/time point). ALT levels were equivalent at 3 and 6 months, and they were 2-3-fold elevated at 9 months. AST and total/direct bilirubin were equivalent. * $P = 0.02$. Graphs show mean ± sem.



Supporting Fig. S5. Hepatic ploidy is equivalent in 14-day-old LKO and control mice. (A,B) Single cell suspensions of freshly isolated LKO and control hepatocytes were stained with FVD-780, fixed/permeabilized, stained with Ki-67-AF647 + Hoechst dye and analyzed by flow cytometry. Cells were gated on the basis of size/granularity and viability. Next, single cells were selected and cellular debris removed. Finally, cells were separated based on Ki-67 expression and ploidy was determined for the Ki-67- subset. Here, ploidy populations are marked with chromosome number "n" since they contain quiescent cells only (e.g., 2n, 4n, 8n). The gating strategy is shown for a representative LKO mouse (A). Ki-67 expression and ploidy are comparable in LKO and control mice (n = 3-4 mice/genotype) (B). Representative FACS plots are shown and the data summarized. Graphs show mean ± sem.



Supporting Fig. S6. LKO hepatocytes cycle faster than control hepatocytes, as measured by nuclear proliferation markers and mRNA expression of cyclin family members. (A,B) BrdU and PHH3 stained cells from Fig. 3 were evaluated for BrdU+ nuclei (A) and speckled PHH3 nuclei (B). The percentage of positive nuclei at each time point correlates with the percentage of positive cells in Fig. 3. (C) LKO and control hepatocytes from 2.5-month-old adults were cultured as described in Fig. 3A and harvested at each time point to measure mRNA expression of cyclin family members (n = 3-4/genotype/time point; male). The approximate phase of the cell cycle corresponding to each gene is indicated. CyclinD1 (*Ccnd1*) is enriched in G1-phase; CyclinE1 (*Ccne1*) is enriched at G1/S transition; CyclinA2 (*Ccna2*) is enriched at S/G2 transition; and CyclinB1 (*Ccnb1*) is enriched in G2/M (2-4). Cyclin expression was normalized to *Gapdh* and control values were set to 1.0 at each time point. * $P < 0.05$, ** $P < 0.008$, *** $P = 0.0006$. Graphs show mean ± sem.

A Group 1: LKO$^{60\%}$ + Control$^{40\%}$

Transplant Recipient	INPUT		OUTPUT	RATIO
	Control β-gal- (%)	LKO β-gal+ (%)	LKO β-gal+ (%)	β-gal+ Output/Input
1	40	60	96.1	1.6
2	40	60	90.9	1.5
3	40	60	93.7	1.6
4	40	60	92.7	1.5
5	40	60	89.2	1.5
mean ± sem	*40*	*60*	*92.5 ± 1.2*	*1.5 ± 0.02*

B Group 2: LKO$^{17\%}$ + Control$^{83\%}$

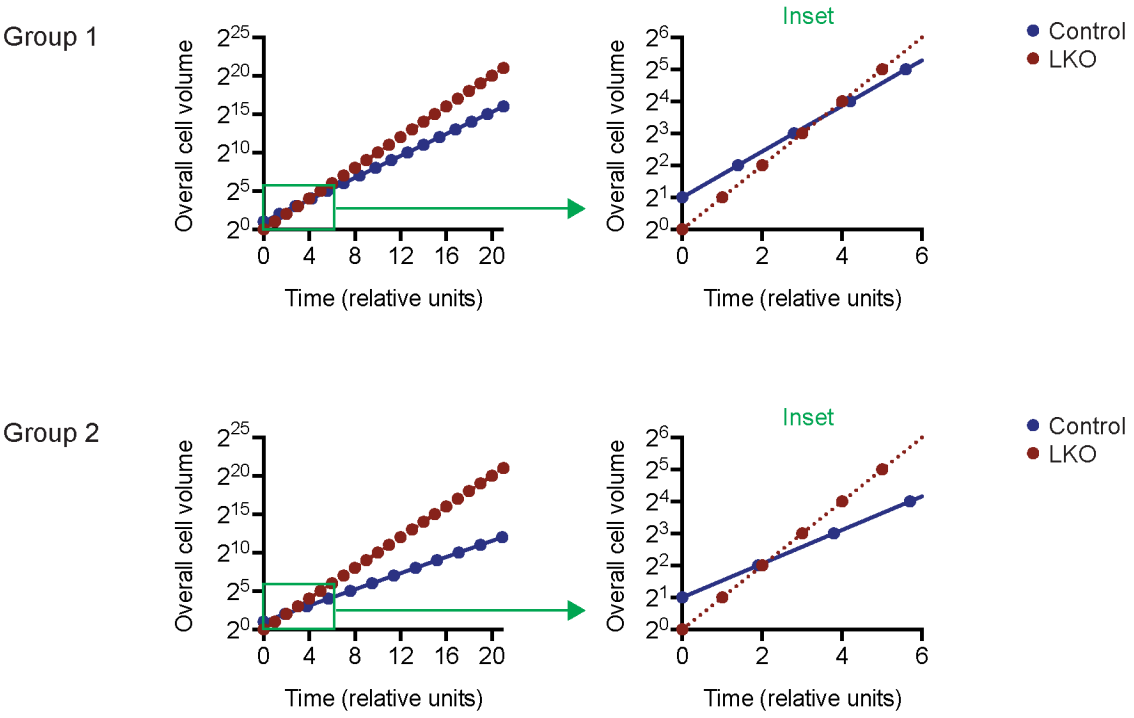
Transplant Recipient	INPUT		OUTPUT	RATIO
	Control β-gal- (%)	LKO β-gal+ (%)	LKO β-gal+ (%)	β-gal+ Output/Input
1	84	16	94.0	5.9
2	84	16	93.6	5.9
3	84	16	91.4	5.7
4	84	16	92.5	5.8
5	84	16	97.2	6.1
6	80	20	90.1	4.5
7	80	20	85.9	4.3
mean ± sem	*82.9 ± 0.7*	*17.1 ± 0.7*	*92.1 ± 1.3*	*5.4 ± 0.3*

Supporting Fig. S7. LKO hepatocytes out-compete controls during competitive liver repopulation. (A,B) LKO (β-gal+) and control (β-gal-) hepatocytes were mixed in defined ratios and transplanted into FRGN recipients, as described in Fig. 4D. Group 1 contained 60% LKO and 40% control (n = 5 recipients); Group 2 contained 17% LKO and 83% control (n = 7 recipients). After complete liver repopulation, the extent of donor chimerism was determined by β-gal activity. Note that the contribution of β-gal- FRGN hepatocytes was considered negligible because these cells represented <10% of the hepatocyte pool after liver repopulation, as illustrated in Fig. 4E. Tables show the percentage of β-gal- and β-gal+ hepatocytes at the time of transplant (marked as “input”) and after liver repopulation (marked as “output”) for each transplant recipient. The ratio of “output to input” β-gal+ cells is reported to highlight the fold expansion by LKO cells during liver repopulation. These data are summarized in Fig. 4F.

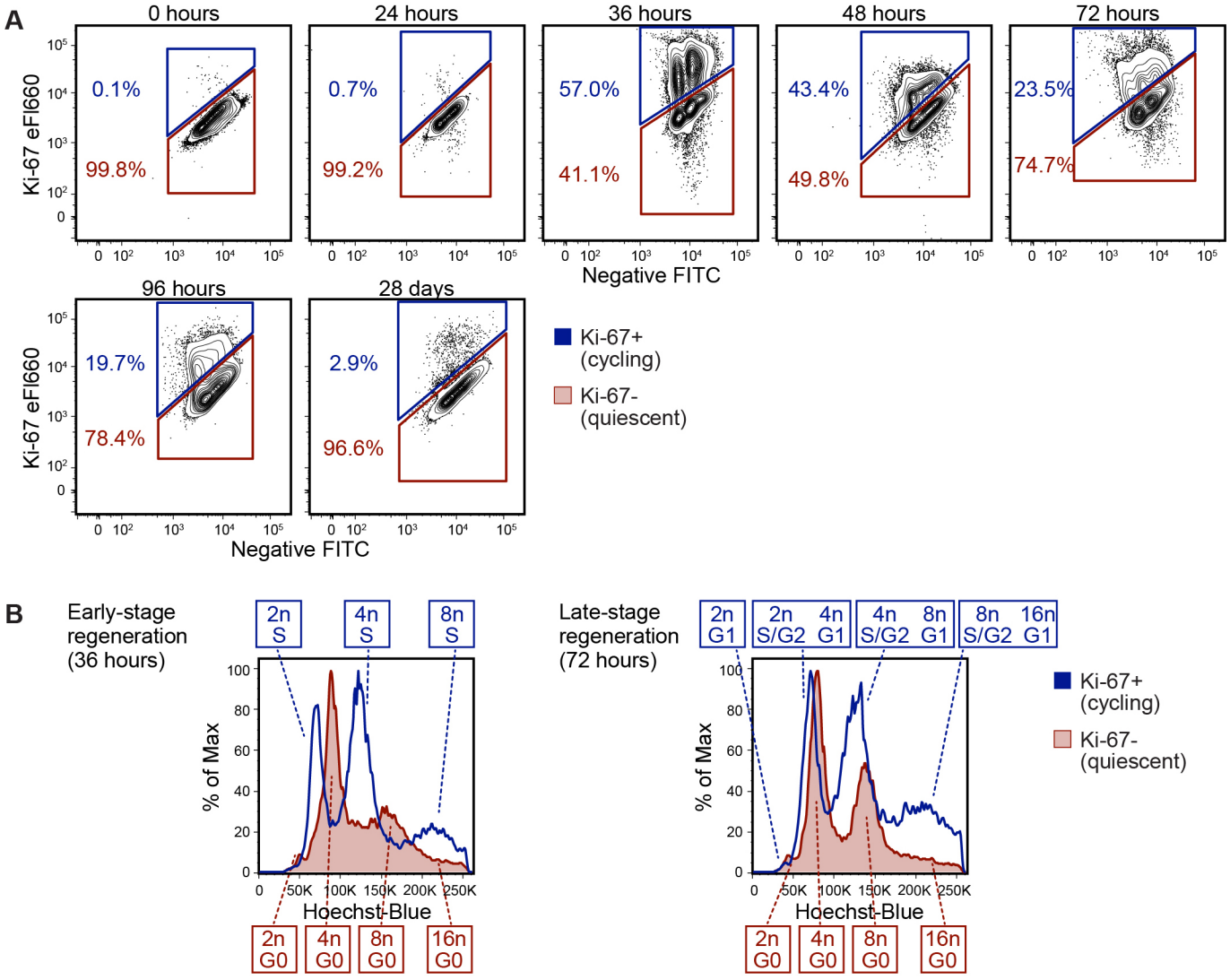
A

Calculations	Group 1 (40:60)		Group 2 (83:17)	
	Control	LKO	Control	LKO
i. Transplanted cells (300K total)	40% (120K)	60% (180K)	83% (249K)	17% (51K)
ii. Estimated number of cells engrafted (10% = 30K)	12K	18K	24.9K	5.1K
iii. Donor-derived cells in repopulated livers	7.5% (2.25M)	92.5% (27.75M)	7.9% (2.37M)	92.1% (27.63M)
iv. Fold expansion	187.5	1542	95.2	5420
v. Population doublings	7.6	10.6	6.6	12.4
vi. Population doubling time (relative units)	13.2	9.4	15.2	8.1
vii. Relative proliferation rate	**1.0**	**1.4**	**1.0**	**1.9**

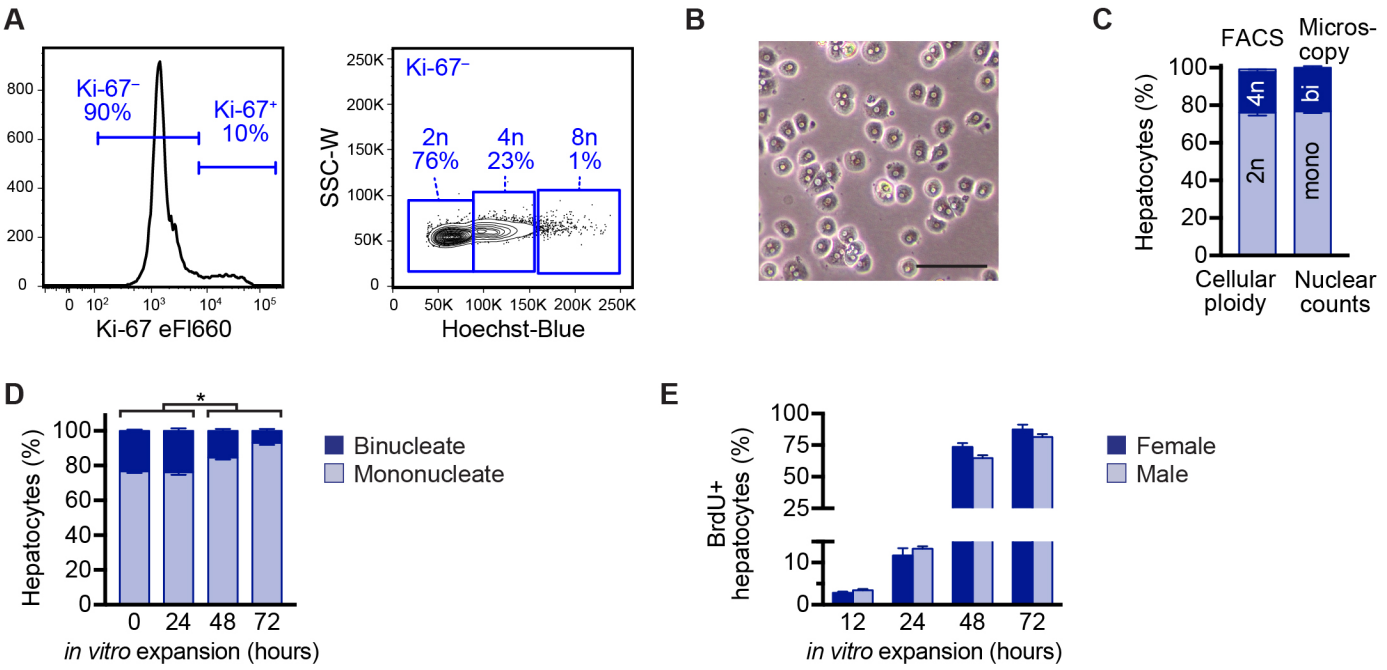
B



Supporting Fig. S8. Impact of cell size and cell doubling time during control and LKO hepatocyte proliferation. (A) Calculation to determine relative population doubling times. (i) In competitive repopulation experiments (Fig. 4D), a total of 300,000 hepatocytes from LKO and control mice were mixed and transplanted into FRGN recipients in defined ratios (Groups 1 and 2). (ii) Approximately 10% of transplanted cells engraft into liver parenchyma (5). (iii) Following complete liver repopulation, engrafted donor cells generate ~30 million hepatocytes. Percentages are derived from Supporting Fig. S7 (iv) *Fold expansion* represents the fold increase of each cell type during repopulation and was calculated as follows: [# cells in repopulated liver / # engrafted cells]. (v) *Population doublings* refers to the number of doublings required to achieve the indicated fold expansion; calculated as [$\log_2$ (fold expansion)]. (vi) If time to complete repopulation is "100 relative units" and cells proliferate continuously, the *population doubling time* is calculated by [100 / # population doublings]. (vii) *Relative proliferation rate,* calculated as [1 / (doubling time for LKO / doubling time for control)], indicates that LKO population doublings are 1.4 (Group 1) or 1.9 (Group 2) times faster than population doublings by control hepatocytes. (B) Combined effect of proliferative capacity and cell size on production of liver mass. Diploid hepatocytes are smaller than polyploids, and, for simplicity, it was assumed that LKO hepatocytes (mostly diploid) are half the size of control hepatocytes (mostly polyploid). Two population doubling times are considered, based on observations that population doublings by LKO hepatocytes are 1.4 (Group 1) or 1.9 (Group 2) times faster than population doublings by control hepatocytes. Graphs show the cumulative increase in cell volume over time. Together, the data show that slower cycling and larger control hepatocytes contribute greater cell volume after 1 or 2 cell divisions, but after 3 cell divisions, faster cycling and smaller LKO hepatocytes are more productive, generating increased cumulative volume with each successive division.



Supporting Fig. S9. Supporting figure corresponding to Fig. 5 (A) Representative FACS plots show the percentage of Ki-67+ cycling and Ki-67- quiescent hepatocytes during liver regeneration induced by 2/3 PH (24-96 hours) and after complete liver regeneration (28 days) (n = 3-6/time point). (B) Representative histograms show ploidy overlays of Ki-67+ cycling and Ki-67- quiescent hepatocytes 36 and 72 hours after PH (n = 3-5). The cell population within each peak is annotated.



Supporting Fig. S10. In 20-day-old WT livers, most mononucleate hepatocytes are diploid, and most binucleate hepatocytes are tetraploid. (A-C) Hepatocytes isolated from 20-day-old mice were analyzed for Ki-67 expression and ploidy using flow cytometry (n = 6). Representative FACS plots are shown (A) and percentage of hepatic ploidy populations is graphed (C). See Supporting Fig. S5A for the gating strategy. Hepatocytes were seeded on cell culture dishes and mono- and binucleate hepatocytes were quantified 3-4 hours after attachment (n = 6). Representative micrograph is shown (B), and the percentage of mono- and binucleate hepatocytes is graphed (C). Together, the data show that 76% of quiescent hepatocytes are diploid and 22% are tetraploid; similarly, in terms of nucleation, 77% of hepatocytes are mononucleate and 23% are binucleate. (D) The percentage of mono- and binucleate hepatocytes is indicated throughout the culture period (n = 6). (E) The proliferative index is independent of gender. Hepatocytes were isolated from female (n = 3) and male (n = 6) 20-day-old WT mice and pulsed with BrdU, as outlined in Fig. 6A. The percentage of BrdU+ hepatocytes is indicated. * $P < 0.0005$. Graphs show mean ± sem. Scale bar is 100 μm.