ONLINE SUPPLEMENT:

EXPERIMENTAL PROCEDURES (cont.)

Morphometric Analysis. All experiments on mice were performed under the strict guidelines of the National Institutes of Health and the Institutional Animal Use and Care Committee at the University of Pittsburgh. Age- and sex-matched conditional KO and *Con* littermates were weighed and killed (n=3–5). Livers were isolated and the wet weights were recorded to calculate the liver weight to body weight ratio (LW/BW X100). The means for the 2 groups were compared for statistically significant differences by Student t test.

Acetaminophen Liver Toxicity and morbidity. Twelve-week-old *Lrp*-LKO mice (n=5) and Con mice (n=5) received an intraperitoneal injection of acetaminophen (600 mg/kg dissolved in 0.45% NaCl). Animals were sacrificed 24 hours after acetaminophen administration. Blood samples were collected from the orbital sinus, and serum alanine aminotransferases (ALT) and aspartate aminotransferase (AST) levels were determined by automated methods at the University of Pittsburgh Medical Center Clinical Chemistry laboratory.

Protein Extraction and Western Blots. Whole-cell lysate preparation was performed using RIPA buffer (9.1mmol/L dibasic sodium phosphate, 1.7mmol/L monobasic sodium phosphate, 150mmol/L sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [pH adjusted to 7.4]) containing fresh protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO) as described previously (3). 40-50 mg of protein was resolved by sodium

dodecyl sulfate–polyacrylamide gel electrophoresis analysis using the mini-PROTEIN 3 electrophoresis module assembly (Biorad, Hercules, CA) and transferred to immobilon-polyvinylidene difluoride membranes (Millipore, Billerica, MA). Proteins were detected by Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized by autoradiography.

Primary antibodies used in this study were against β-catenin (BD Transduction); TCF4 (Cell Signaling), Lrp5 (Cell Signaling), Lrp6 (Cell Signaling), Cyclin-D1 (Thermo Marker); g-catenin (Cell Signaling), E-cadherin (Santa Cruz Biotechnology), Regucalcin (Santa Cruz Biotechnology), Glutamine Synthetase (Santa Cruz Biotechnology), Cyp1a2 (Santa Cruz Biotechnology), Cyp2e1 (Millipore), Wls (Millipore), Actin (Chemicon); and GAPDH (Millipore) at 1:1000. Horseradish-peroxidase-conjugated secondary antibodies (Chemicon) were used at 1:20,000 to 1:50,000 dilutions.

Histology and Immunohistohemistry. Liver sections from the age- and sex-matched KO and *Con* mice were also analyzed by immunohistochemistry (IHC) for β-catenin (Santa Cruz Biotechnology), Glutamine Synthetase (Santa Cruz Biotechnology), Cyp1a2 (Santa Cruz Biotechnology), Cyp2e1 (Millipore) and PCNA (Santa Cruz Biotechnology) to determine their expression and/or localization using the indirect immunoperoxidase technique as described previously (4). Briefly, 4 mm paraffin sections were passed through xylene, graded ethanol, and rinsed in phosphate-buffered saline (PBS). 3% hydrogen peroxide (Sigma) was used to inactivated endogenous peroxide. Slides were microwaved in citrate buffer for 8 minutes and blocked in super block (ScyTek

Laboratories) for 10 minutes followed by 1-hour incubation at room temperature in the primary antibody. After 3 washes with PBS, the sections were incubated in the secondary horseradish-peroxidase-conjugated antibody (Chemicon) for 30 min at room temperature and signal was detected using the ABC Elite kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. Sections were counterstained with Shandon (Thermo Scientific) for 1 minute and passed through the dehydration process followed by cover slipping and mounting using DPX (Fluka Labs, St. Louis, MO). Slides were viewed under an Axioskop 40 (Zeiss) upright research microscope and digital images were obtained by Nikon Coolpix camera.

Livers from age- and sex-matched KO and *Con* mice after PH were assessed for histological changes by Hematoxylin and Eosin (H&E) staining. Mitotic figures were assessed by examination of H&E sections at various time points after PH from various KO and Con. Number of mitotic figures were counted in at least five representative 200X fields from at least 3 KO and Con per time point. The average numbers of mitotic figures were compared for statistical assessment by the Student t test. P value of less than 0.05 was considered significant. For assessing hepatocytes in S-phase of cell cycle, indirect IHC was performed for PCNA that recognizes cells in the cell cycle. The positive cells were counted in 3 low-power fields (200X) in 3 sections from 3 different knockout or control livers. The average numbers of positive cells were compared and statistical assessment was made by the Student t test. P value of less than 0.05 was considered significant.

Immunoprecipitation. Co-precipitation studies were performed with 500 µg of protein as described elsewhere, using A/G agarose beads (Santa Cruz Biotechnology) (2). Antibodies used for immunoprecipitation were mouse anti-β-catenin (BD Transduction) and rabbit anti-TCF4 (Cell Signaling). Respective input controls were also assessed for each immunoprecipitation reaction.

REFERENCES

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2. Wickline ED, Awuah PK, Behari J, Ross M, Stolz DB, Monga SP. Hepatocyte gamma-catenin compensates for conditionally deleted beta-catenin at adherens junctions. J Hepatol 2011;55:1256-1262.
3. Monga SP, Pediaditakis P, Mule K, Stolz DB, Michalopoulos GK. Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration. Hepatology 2001;33:1098-1109.
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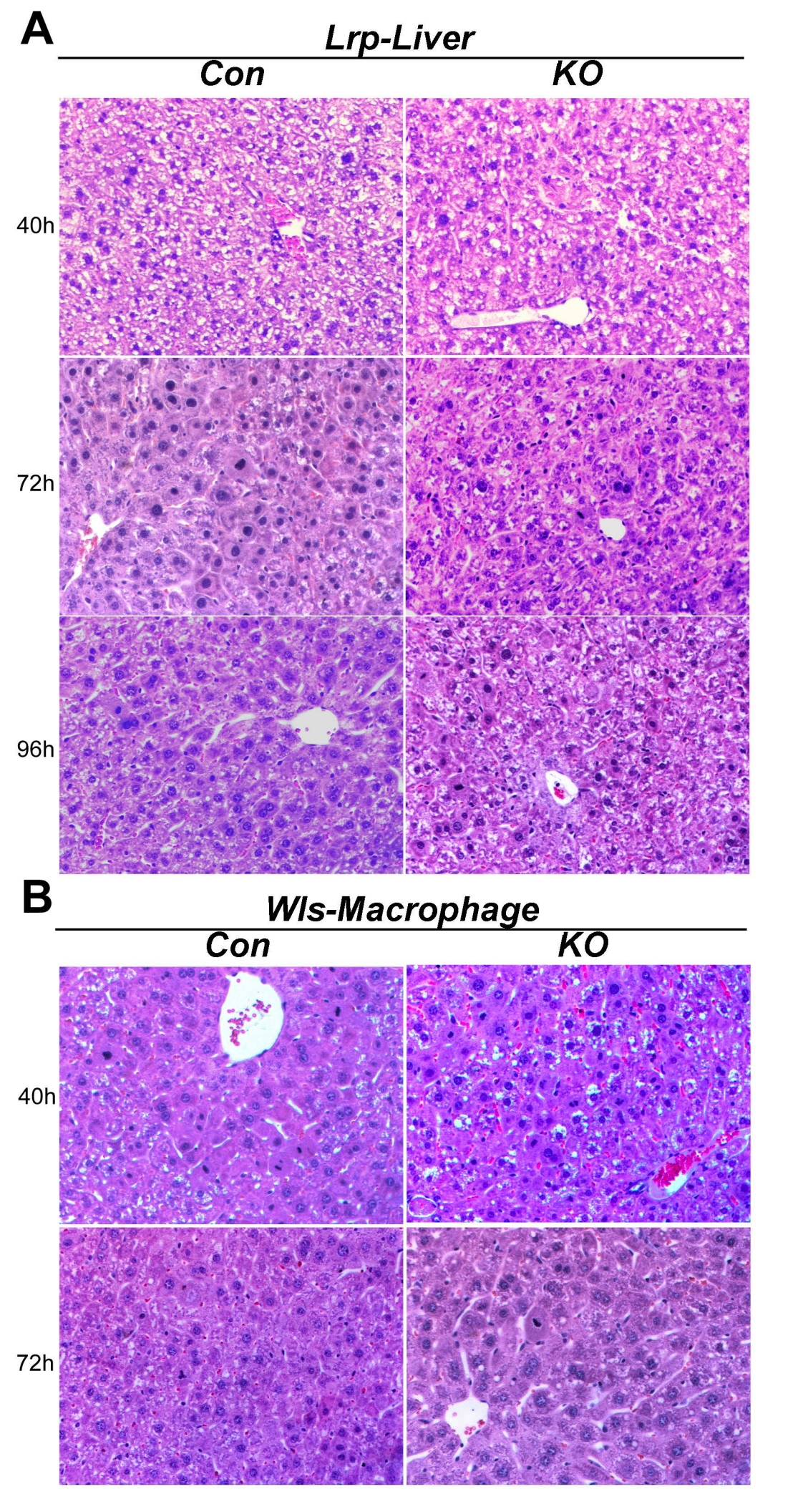
ONLINE FIGURE LEGEND

A. Representative H&E images from regenerating livers of *Lrp*-LKO and *Con* at 40 hours, 72 hours and 96 hours after PH.
B. Representative H&E images from regenerating livers of *Wls*-MKO and *Con* at 40 hours and 72 hours after PH.


A
Lrp-Liver
Con
KO
40h
72h
96h
B
Wls-Macrophage
Con
KO
40h
72h