Supplemental Information

Inflammation mediated by JNK in myeloid cells promotes the development of hepatitis and hepatocellular carcinoma

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Supplementary Experimental Procedures

Supplementary Figures S1-S4

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Genotype analysis

Genomic DNA was genotyped by using a PCR-based procedure. Amplimers 5'-AGGATTTATGCCCTCTGCTTGTC -3' and 5'- GAACCACTGTTCCAATTTCCATCC -3' were used to detect *Mapk8*$^{+}$ (540 bp) and *Mapk8*^{LoxP} (330 bp) alleles. Amplimers 5'-CCTCAGGAAGAAAGGGCTTATTTC -3' and 5'- GAACCACTGTTCCAATTTCCATCC -3' were used to detect *Mapk8*^{LoxP} (1,000 bp) and *Mapk8*$^{\Delta}$ (410 bp) alleles. Amplimers 5'-GTTTTGTAAAGGGAGCCGAC -3' and 5'- CCTGACTACTGAGCCTGGTTTCTC -3' were used to detect *Mapk9*$^{+}$ (224 bp) and *Mapk9*^{LoxP} (264 bp) alleles. Amplimers 5'-GGAATGTTTGGTCCTTTAG -3', 5'- GCTATTCAGAGTTAAGTG-3', and 5'-TTCATTCTAAGCTCAGACTC -3' were used to detect *Mapk9*^{LoxP} (560 bp) and *Mapk9*$^{\Delta}$ alleles (400 bp). Amplimers 5'- TTACTGACCGTACACCAAATTTGCCTGC -3' and 5'-CCTGGCAGCGATCGCTATTTTCCATGAGTG -3' were used to detect *Cre recombinase* (450 bp).

Blood analysis

Alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were measured by ALT and AST Reagent kit (Pointe Scientific). The concentration of blood cytokines and chemokines was measured by mulitplexed ELISA using a Luminex 200 machine (Millipore).

Quantitative RT-PCR analysis of mRNA

The expression of mRNA was examined by quantitative PCR analysis using a Quantstudio PCR machine (Life Technologies). TaqMan® assays were used to quantitate *Ccl2 (*Mm00441242_m1), *Ccl3 (*Mm00441259_g1), *Ccl4 (*Mm00443111_m1), *Ccl5 (*Mm01302427_m1), *Ccl7 (*Mm01308393_g1), *Ccl8 (*Mm01297183_m1), *Ccl17* (Mm00516136_m1), *Ccl22* (Mm00436439_m1), *Ccr2 (*Mm00438270_m1), *Ccr4* (Mm04207878_m1), *Ccr5 (*Mm01963251_s1), *Cd4* (Mm00442754_m1), *Cd8a* (Mm01182107_g1), *Cdk1* (Mm00772472_m1), *Cdkn1a (p15)* (Mm04205640_g1), *Cdkn2b (p21)* (Mm00483241_m1), *cMyc* (Mm00487804_m1), *Cxcr1* (Mm02766093_s1), *Cxcr2* (Mm99999117_s1), *Cxcl1* (Mm04207460_m1), *Cxcl2* (Mm00436450_m1), *Cxcl5* (Mm00436451_g1), *Emr1* (F4/80) (Mm00802530_m1), *Foxp3* (Mm00475162_m1), *Il1α* (Mm00439620_m1), *Il1β (*Mm00434228_m1), *Il6 (*Mm00446190_m1), *Socs3* (Mm00545913_s1), *Tgfβ1* (Mm03024053_m1), and *Tnfα* (Mm00443258_m1) (Applied Biosystems). The relative mRNA expression was normalized by measurement of the amount of *18S* RNA in each sample using Taqman© assays (catalog number 4308329; Life Technologies).

Isolation of hepatocytes and hepatic leukocytes

The livers of anesthetized mice were perfused (via the inferior vena cava) with 10 ml PBS. The liver was diced with scissors and gently pushed with a 10 ml plunger through a 100 μm cell strainer (Falcon). The cells were washed with 2% FBS-PBS and centrifuged at 1,700 rpm (6 min). The cells were re-suspended with 33.8 % Isotonic Percoll (GE Healthcare) and centrifuged at 2,000 rpm (12 min). The upper layer (hepatocytes) and the pellet (leukocytes) were collected. Red blood cells were removed with ACK lysing buffer (Life Technologies). The cells were then washed with PBS (4°C) and processed for FACS staining.

Isolation of Kupffer cells

Kupffer cells ($F4/80^+$) were purified using MACS streptavidin microbeads (Miltenyi Biotec) and biotinylated F4/80 antibody (Biolegend; #123106; 2.5 μl/10^7 cells).

Isolation of B cells, T cells, neutrophils and macrophages

B cells ($B220^+$), T cells ($CD3^+$), neutrophils ($Gr\text{-}1^+$) and macrophages ($F4/80^+$) were purified using MACS streptavidin microbeads (Miltenyi Biotec) and biotinylated CD45R/B220 antibody (BD Biosciences; #553086; 2.5μl/10^7 cells), CD3e antibody (Biolegend; #100304; 2.5μl/10^7 cells), Gr-1 antibody (BD Pharmigen; #553125; 2.5 μl/10^7 cells) or F4/80 antibody (Biolegend; #123106; 2.5 μl/10^7 cells), respectively.

Flow cytometry

Cell surface antigens were examined using cells stained with live/dead fixable blue dead cell stain kit (Life Technologies), 2% FBS-PBS plus FcBlock (BD Biosciences; #553142; 1:100), and conjugated antibodies including CD45-APC/Cy7 (Biolegend; #103116; 1:100), F4/80-PE (Biolegend; #123110; 1:100), CD11b-PE/Cy5 (Biolegend; #101210; 1:1,000), CD11b-APC (BD Biosciences; #553312; 1:100), Gr-1-FITC (BD Biosciences; #553127; 1:100), CD8α-PercP (BD Pharmigen; #552878; 1:200), CD4-PE (BD Pharmigen; #553049; 1:200), NK1.1-PE/Cy7 (BD Pharmigen; #552878; 1:200) CD3ε-APC (BD Pharmigen; #553066; 1:100) B220-FITC (BD Pharmigen; #553038; 1:100), and CD11c-AF700 (eBioscience; #56-0114-82; 1:50). The cells were washed and subsequently fixed with 2% paraformaldehyde.

Intracellular staining for TNFα was performed using cells pre-treated with 1:1,000 GolgiPlug (Stock 1,000X; BD Biosciences) and 1 μg/ml LPS for 2 h at 37°C. After cell surface staining, the cells were incubated with Cytofix/Cytoperm buffer (BD Biosciences) for 20 min and then stained with TNFα-PE/Cy7 (BD Biosciences; #557644; 1:100).

Flow cytometry analysis was performed using a LSR-II cytometer (Becton Dickenson). Data were processed using FlowJo Software (Tree Star).

Immunoblot analysis

Tissue extracts were prepared using Triton lysis buffer (20 mM Tris at pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL of aprotinin and leupeptin). Extracts (20–50 μg of protein) were examined by protein immunoblot analysis by probing with antibodies to cleaved Caspase 3 (Cell Signaling; #9661; 1:1,000), Caspase 3 (Cell Signaling; #9662; 1:1,000), GAPDH (Santa Cruz; sc-25778; 1:2,000); JNK1/2 (BD Biosciences; #554285; 1:2,000), cleaved PARP (Cell Signaling; #9548; 1:1,000), PARP (Cell Signaling; #9542; 1:1,000), Bad (Santa Cruz; sc-942; 1:500), and α-Tubulin (Sigma; #T-5168; 1:5,000). Immune complexes were detected using the Odyssey infrared imaging system (LI-COR Biosciences).

Analysis of tissue sections

Histology was performed using tissue fixed in 10% formalin for 24 h, dehydrated, and embedded in paraffin. Sections (5 μm) were cut, stained using hematoxylin & eosin (American Master Tech Scientific), and examined by a Board-certified pathologist (Dr. David Garlick, Department of Molecular Cell and Cancer Biology, University of Massachusetts Medical School) blinded to group allocation. Specimens were imaged with a Carl Zeiss Axiovert 200M microscope equipped with a Plan-Neofluar 10X/0.3 objective and an AxioCamMR2 camera system with Axiovision software (Carl Zeiss Microimaging, Inc.).

Terminal deoxynucleotidyltransferase-mediated nick and lableling (TUNEL) staining of deparafinized liver sections was performed using the In Situ Cell Death Detection kit Fluorescein (Roche) following antigen retrieval (Antigen Unmasking Solution, Vector Laboratories). Immunohistochemistry was performed using de-paraffinized tissue sections that were incubated in blocking buffer for 1 h (1% BSA, 10 % normal goat serum in PBS) and permeabilized with 0.1% Triton X-100. The sections were incubated in blocking buffer containing a biotin-conjugated antibody to PCNA (Life Technologies) that was detected by incubation with Streptavidin conjugated to Alexa Fluor 488 (Life Technologies). DNA was detected by staining with DAPI (Life Technologies). Fluorescence was visualized using a Leica TCS SP2 confocal microscope (10X objective) equipped with a 405-nm diode laser and images were acquired using Leica confocal software (Version 2.61).

Stereomicroscopy was performed with a Zeiss Stereo Discovery V12 (EMS3/SyCoP3) microscope equipped with an AxioCamMR3 camera system with ZEN 2012 software (Carl Zeiss Microscopy GmbH). Achromat S 0.3X/0.043 and 1X/0.0144 objectives were used to image the liver for studies of hepatitis and HCC, respectively.

Figure S1

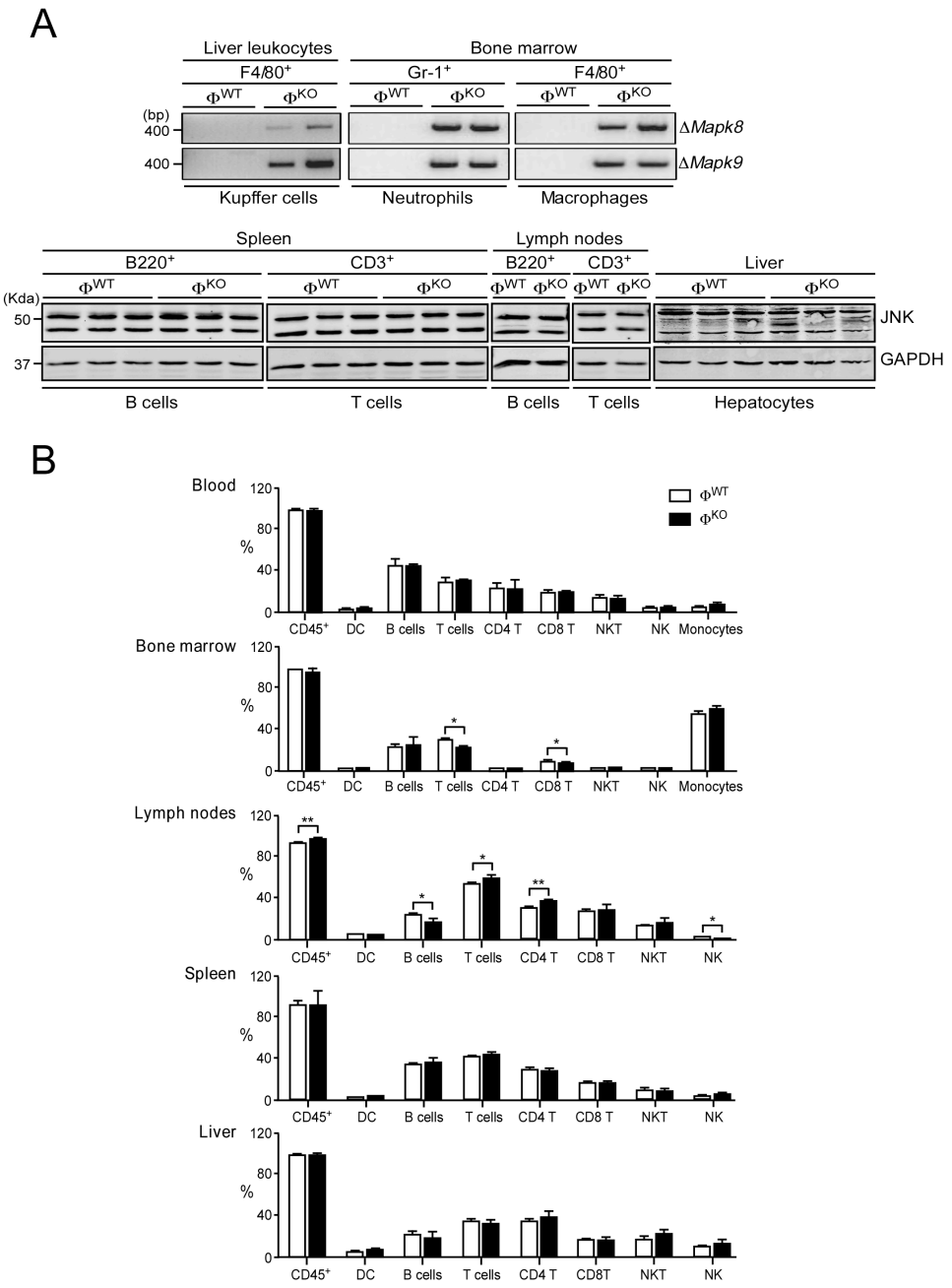

Figure S1. Immunophenotyping of Ø^{WT} and Ø^{KO} mice, Related to Figure 1.

(**A**) Genomic DNA from Kupffer cells, neutrophils, and macrophages was examined by PCR analysis using amplimers designed to detect *Cre*-mediated ablation of the *Mapk8* and *Mapk9* genes (*upper panel*). The expression of JNK and GAPDH by B cells, T cells, and hepatocytes was examined by immunoblot analysis (*lower panel*).

(**B**) Total leukocytes (CD45$^+$), dendritic cells (CD45$^+$ CD11c$^+$ CD11b$^-$), B cells (CD45$^+$ B220$^+$ CD11c$^-$), T cells (CD45$^+$ CD3ε$^+$ NK1.1$^-$), CD4 T cells (CD45$^+$ CD4$^+$), CD8 T cells (CD45$^+$ CD8$^+$),

NKT cells ($CD45^+$ $CD3\varepsilon^+$ $NK1.1^+$), NK cells ($CD45^+$ $CD3\varepsilon^-$ $NK1.1^+$), and monocytes ($CD45^+$ $CD11b^+$) in peripheral blood, bone marrow, lymph nodes, spleen, and liver of $Ø^{WT}$ and $Ø^{KO}$ mice was examined by flow cytometry (mean ± SEM; n = 3). Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (*, $p<0.05$; **, $p<0.01$).

Figure S2

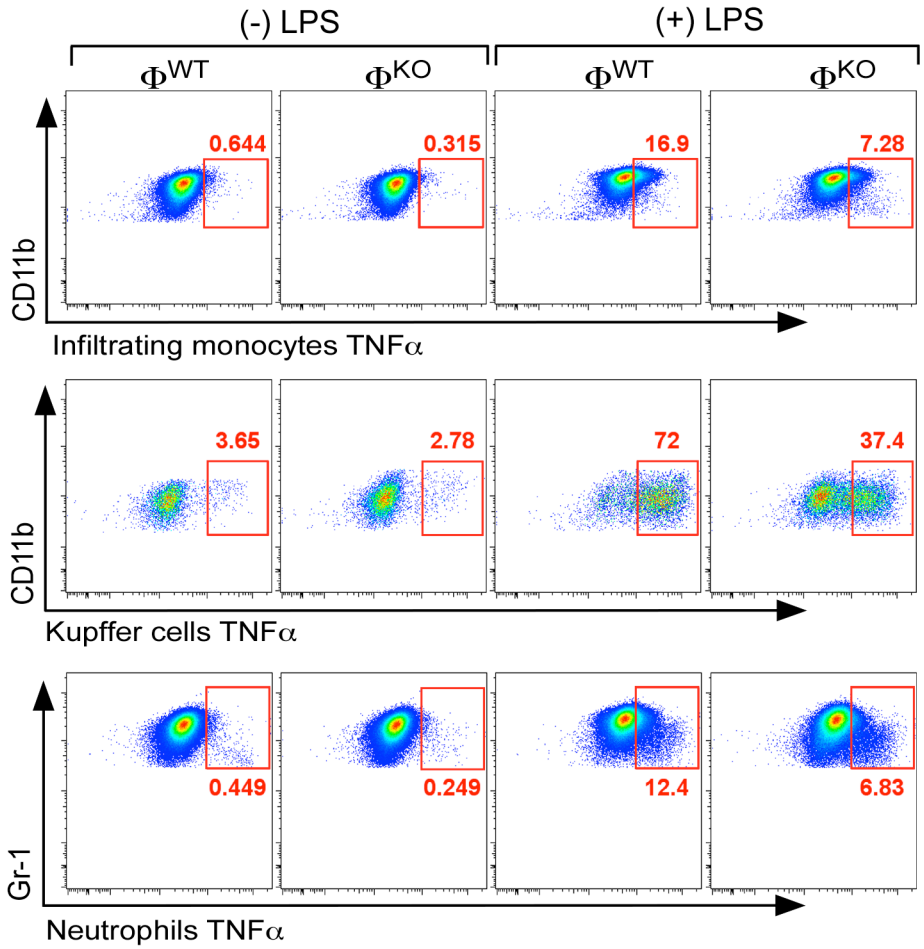

Figure S2. JNK in myeloid cells promotes TNFα expression, Related to Figure 2.

The expression of TNFα by hepatic myeloid cells (infiltrating monocytes (*upper panels*), Kupffer cells (*middle panels*), and neutrophils (*lower panels*)) was measured by intracellular staining. Representative flow cytometry data are presented.

Figure S3

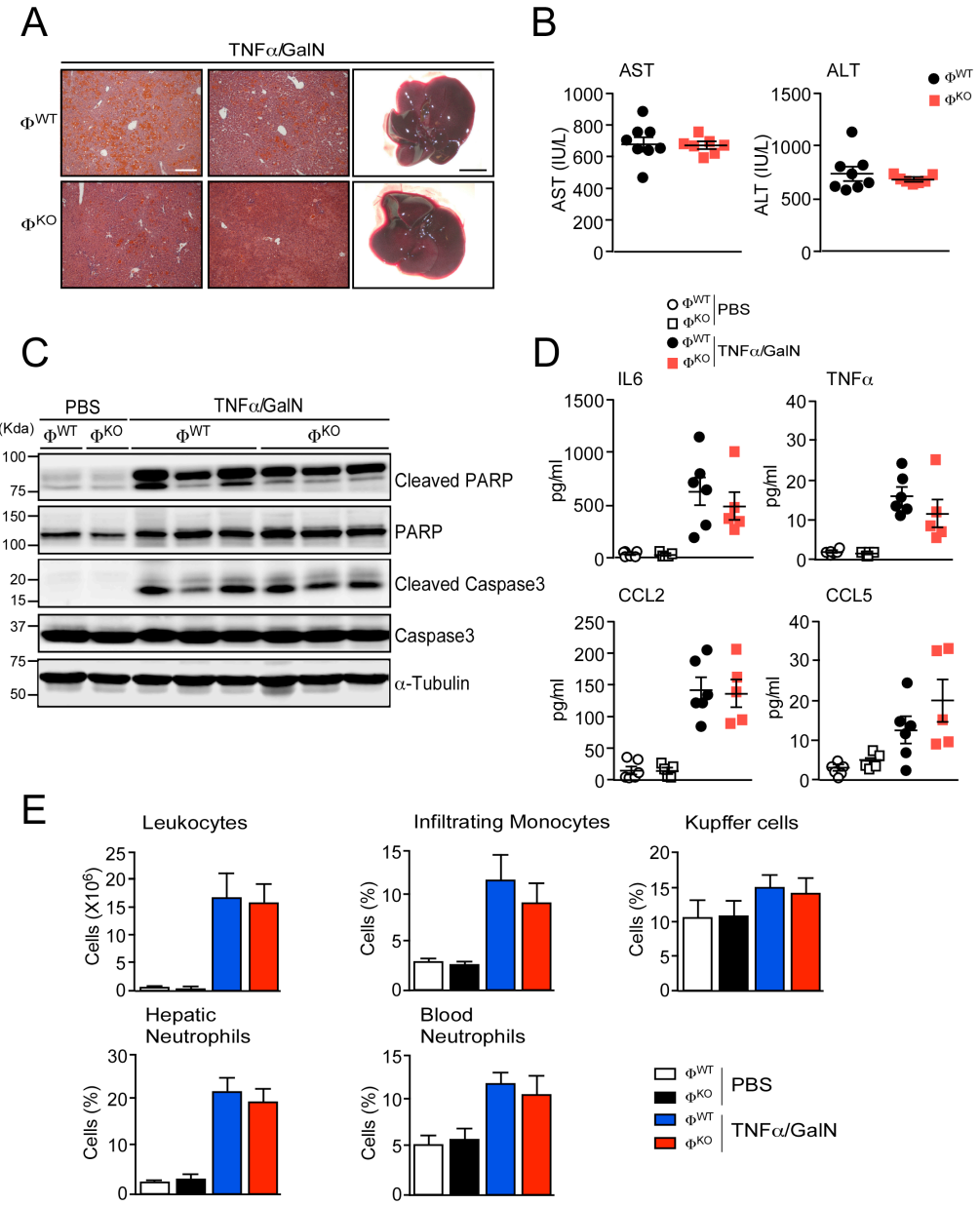

Figure S3. Myeloid JNK-deficiency does not prevent TNFα-induced hepatitis, Related to Figure 3.

(**A**) Mice ($Ø^{WT}$ and $Ø^{KO}$) were treated with TNFα/GalN (6 h). Representative images of the dissected liver (scale bar, 5 mm) and hematoxylin & eosin-stained liver sections (scale bar, 100 μm) are presented.

(**B**) The accumulation of hepatic aminotransferases (ALT and AST) in the blood of $Ø^{WT}$ and $Ø^{KO}$ mice treated with TNFα/GalN (6 h) was measured (mean ± SEM; n = 7 ~ 8). No statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice were detected ($p > 0.05$).

(**C**) Liver extracts prepared from mice treated with PBS or TNFα/GalN (6 h) were examined by immunoblot analysis using antibodies to cleaved PARP, PARP, cleaved Caspase 3, and Caspase 3, and α−Tubulin.

(**D**) $Ø^{WT}$ and $Ø^{KO}$ mice were treated with PBS or TNFα/GalN (6 h). The concentration of inflammatory cytokines (IL6 and TNFα) and chemokines (CCL2 and CCL5) in the blood was measured by multiplexed ELISA (mean ± SEM; n = 5 ~ 6). No statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice were detected ($p > 0.05$).

(**E**) $Ø^{WT}$ and $Ø^{KO}$ mice were treated with PBS or TNFα/GalN (5.5 h). The total number of hepatic leukocytes is presented (mean ± SEM; PBS n = 4; TNFα/GalN n = 6). The percentage of total hepatic leukocytes corresponding to infiltrating monocytes, infiltrating neutrophils, and Kupffer cells is shown (mean ± SEM; PBS n = 4; TNFα/GalN n = 6). Blood neutrophils (% of total blood leukocytes) were also quantitated (mean ± SEM; PBS n = 2; TNFα/GalN n = 5).

Figure S4

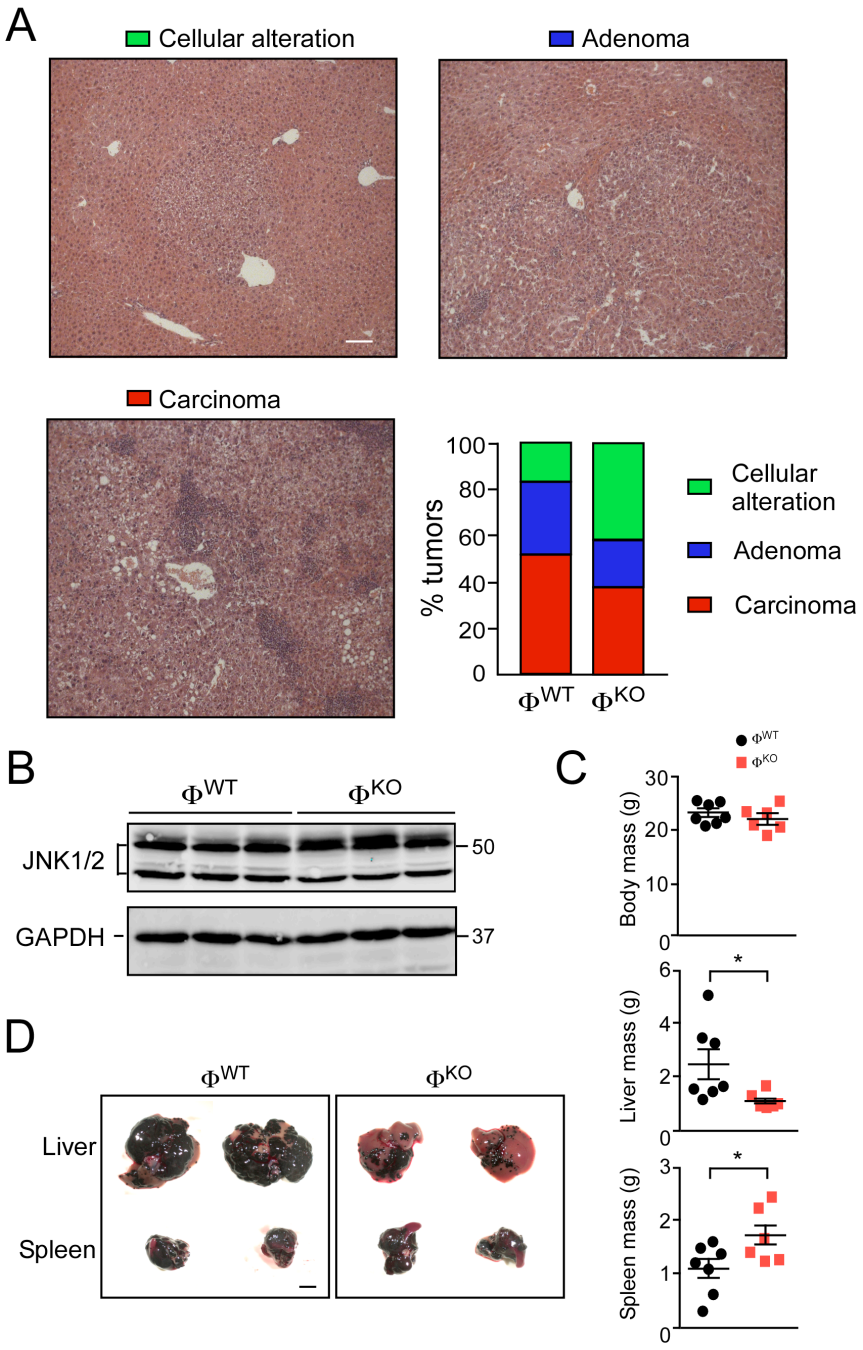

Figure S4. Comparison of tumor grade in Ø^{WT} and Ø^{KO} mice, Related to Figure 4.

(**A**) Hematoxylin & eosin-stained liver sections were examined by a Board-certified pathologist to assess tumor grade (n = 14 ~ 21). Tumor grade is presented as % of tumors examined.

(**B**) Tumors were examined by immunoblot analysis by probing with antibodies to JNK and GAPDH.

(**C,D**) Liver tumor formation in $Ø^{WT}$ and $Ø^{KO}$ mice was examined by intrasplenic injection of B16 melanoma cells. Total body mass, liver mass, and spleen mass were measured (mean ± SEM; $Ø^{WT}$ n = 7; $Ø^{KO}$ n = 6). Statistically significant differences between $Ø^{WT}$ and $Ø^{KO}$ mice are indicated (*, $p<0.05$) (C). Representative images of livers and spleens are presented (scale bar = 5 mm) (D).