Supplementary Figure Legends

Supplementary Figure 1: (A) Disruption of liver macrophage Notch1 increases M1 while reducing M2 phenotype in liver IRI. Liver macrophages (Kupffer cells) were isolated from Notch1$^{M\text{-}KO}$ and Notch1$^{FL/FL}$ mice at 6h of reperfusion after 90min of ischemia. The mRNA levels coding for S1009A, CD206, and Arg-1 was analyzed by quantitative RT-PCR. Each column represents mean±SD (n=3-4 samples/group). *p<0.05. Notch1$^{FL/FL}$（□）, Notch1$^{M\text{-}KO}$（■）. (B) Knockdown of liver macrophage ROCK1 in Notch1$^{M\text{-}KO}$ mice diminishes M1 while augmenting M2 phenotype in liver IRI. Liver macrophages (Kupffer cells) were isolated from ROCK1 siRNA or non-specific (NS) siRNA-treated Notch1$^{M\text{-}KO}$ mice at 6h of reperfusion after 90min of ischemia. The mRNA levels coding for S1009A, CD206, and Arg-1 was analyzed by quantitative RT-PCR. Each column represents mean±SD (n=3-4 samples/group). *p<0.05. Notch1$^{M\text{-}KO}$ Sham（□）, Notch1$^{M\text{-}KO}$ + NS siRNA（■）, Notch1$^{M\text{-}KO}$ + ROCK1 siRNA （■）.

Supplementary Figure 2: Myeloid Notch1 deficiency increases serum levels of ALP and DBIL during liver IRI. Mice were subjected to 90min of partial liver warm ischemia, followed by 6h and 24h of reperfusion. Serum levels of alkaline phosphatase (ALP) and direct bilirubin (DBIL) were measured by an automatic biochemical analyzer (Olympus-AU5400, Japan). (A) Serum levels of ALP (U/L) in WT mice after liver IRI. (B) Serum levels of DBIL (umol/L) in WT mice after liver IRI. (C) Serum levels of ALP (U/L) in Notch1$^{FL/FL}$ and Notch1$^{M\text{-}KO}$ mice after liver IRI. (D) Serum levels of DBIL (umol/L) in Notch1$^{FL/FL}$ and Notch1$^{M\text{-}KO}$ mice after liver IRI. Each column represents the mean±SD (n=4-6 samples/group). *p<0.05, **p<0.01. WT Sham （□）, WT IR （▨）, Notch1$^{FL/FL}$（▩）, Notch1$^{M\text{-}KO}$ （■）.

Supplementary Figure 3: Schematic illustration of generation of myeloid-specific Notch1 knockout mice. Mouse with Cre transgene under the control of a myeloid-specific Lyz2 promoter is crossed with a mouse in which the Notch1 gene is placed between loxP sites. In the resulting bitransgenic mouse Cre is expressed in myeloid leading to crossing over of the lox sites

and inactivation of the Notch1 gene in myeloid cells. In all other cell types no crossing over takes places and the Notch1 gene remains active

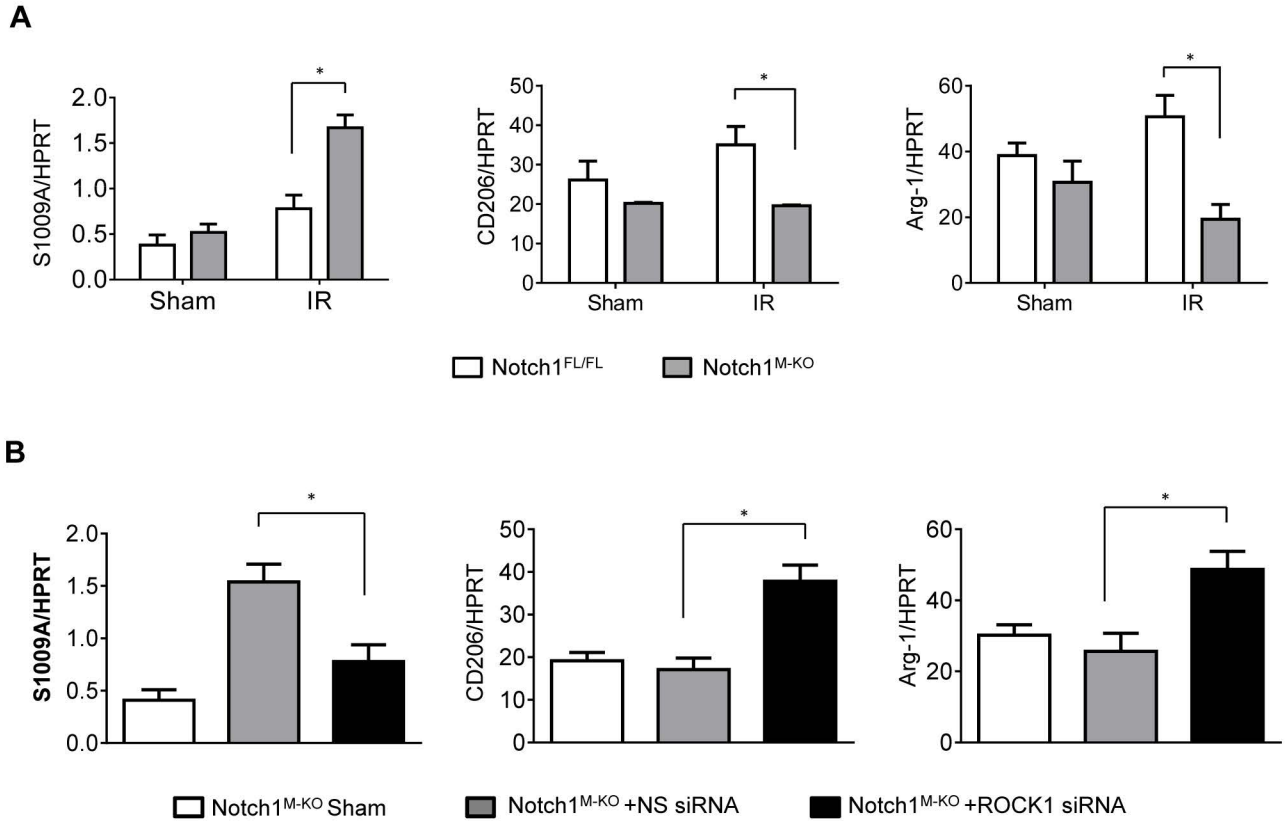
Supplementary Figure 4: Schematic illustration of information on the backcrossing of the transgenic strains. Two steps were used to generate myeloid specific Notch1 KO mice. First, a homozygous loxP flanked Notch1 mouse is mated with a homozygous Lyz2-Cre mouse to generate the F1 mice that are heterozygous for a loxP-flanked Notch1 allele and heterozygous for the Lyz2-cre. Next, these F1 mice were backcrossed to the homozygous loxP-flanked Notch1 mice, resulting in generation of myeloid specific Notch1 KO mice ($Notch1^{M\text{-}KO}$, 25% of the offspring), which were homozygous for the loxP-flanked Notch1 allele and heterozygous for the Lyz2-Cre allele.

Supplementary Figure 5: Liver IR affects Notch expression. Wild-type (WT) mice were subjected to 90min of partial liver warm ischemia, followed by 6h of reperfusion. (A) The mRNA levels coding for Notch1 was analyzed by quantitative RT-PCR. Each column represents mean±SD (n=3-4 samples/group). *p<0.05. Sham（□）, IR（■）. (B) Western-assisted analysis of Notch1 intracellular domain (NICD). Representative of three experiments.

Supplementary Figure 6: Myeloid Notch1 deficiency upregulated JNK activation in both liver macrophages (Kupffer cells) and hepatocytes during liver IRI. The $Notch1^{FL/FL}$ and $Notch1^{M\text{-}KO}$ mice were subjected to 90 min of partial liver warm ischemia, followed by 6h of reperfusion. The primary hepatocytes and Kupffer cells were isolated from $Notch1^{FL/FL}$ and $Notch1^{M\text{-}KO}$ mice. (A) Western-assisted analysis of p-JNK and JNK in liver macrophages (Kupffer cells). Representative of three experiments. (B) Western-assisted analysis of p-JNK and JNK in primary hepatocytes. Representative of three experiments.

Supplementary Table 1. Primers used in qRT-PCR studies.

Suppl. Fig. 1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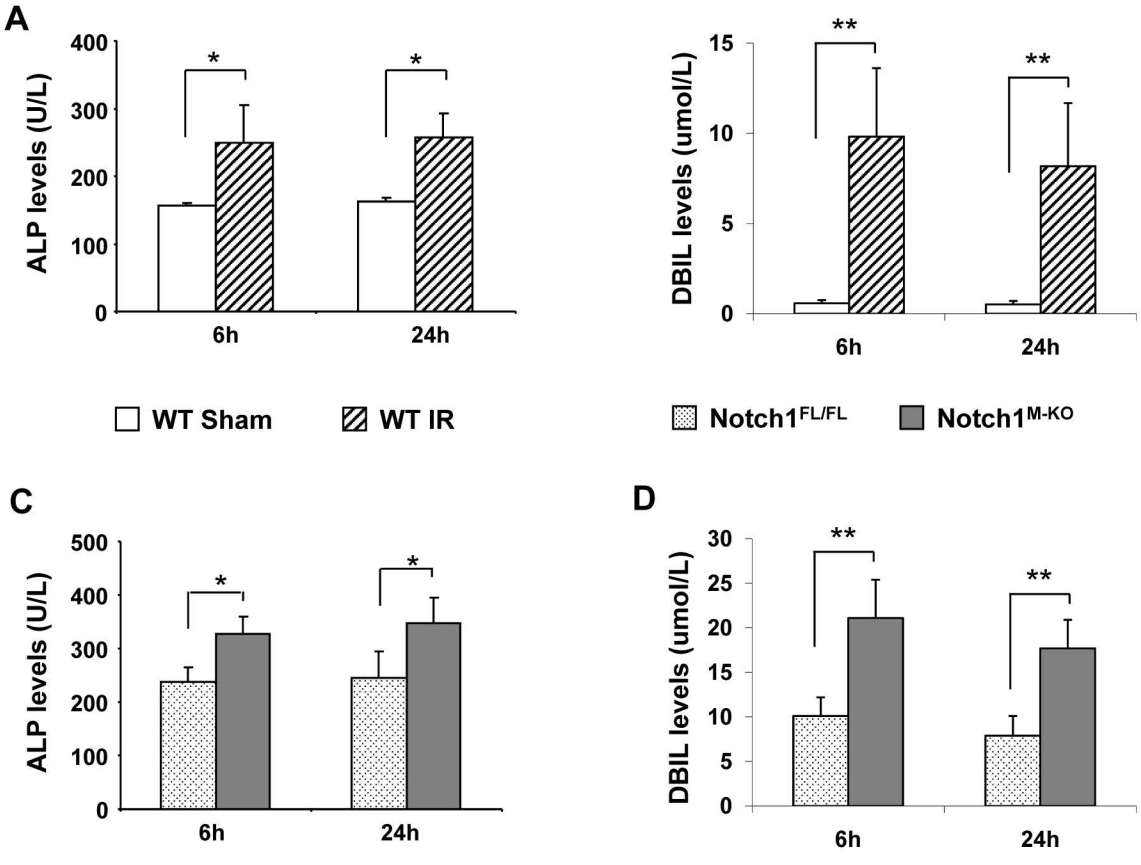
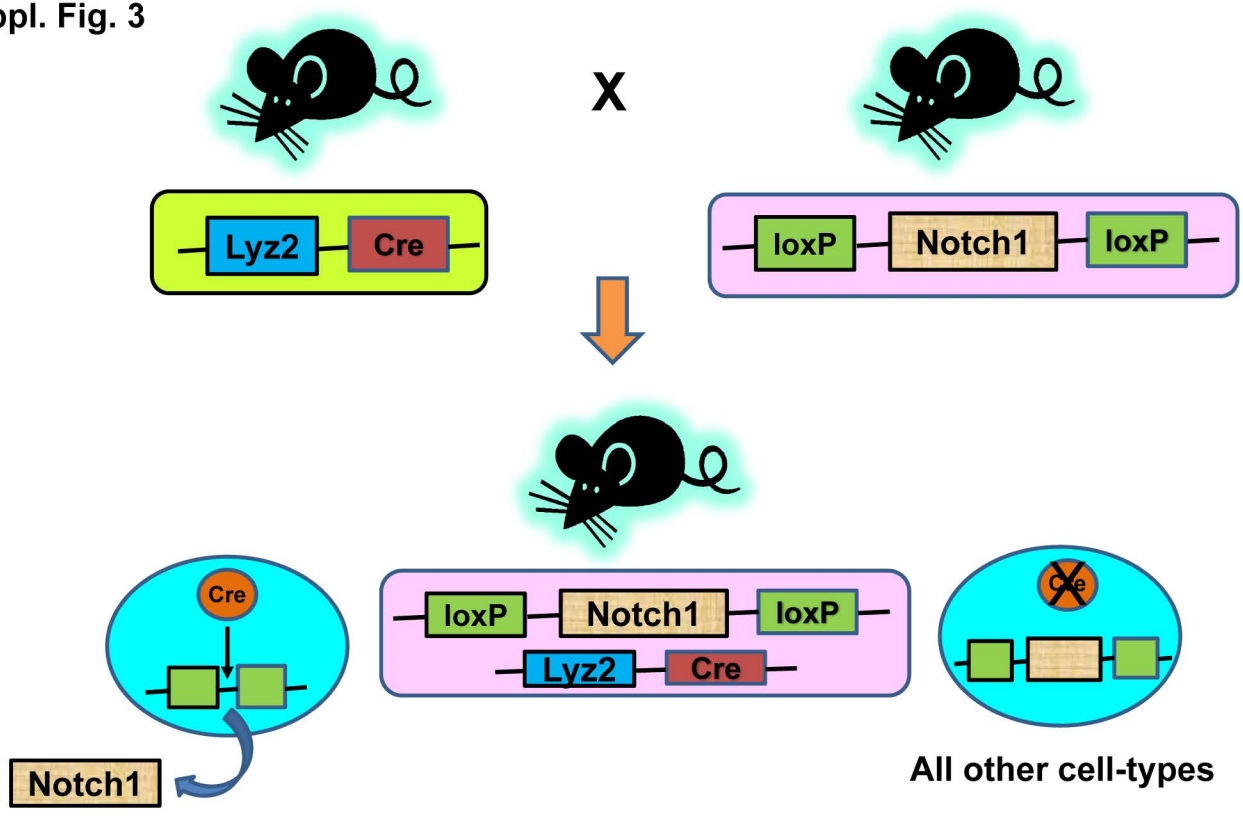
1.5
1.0
0.5
0.0
Sham
IR
*
CD206/HPRT
50
40
30
20
10
0
Sham
IR
*
Arg-1/HPRT
60
40
20
0
Sham
IR
*
Notch1FL/FL
Notch1M-KO
B
S1009A/HPRT
2.0
1.5
1.0
0.5
0.0
*
CD206/HPRT
50
40
30
20
10
0
*
Arg-1/HPRT
60
40
20
0
*
Notch1M-KO Sham
Notch1M-KO +NS siRNA
Notch1M-KO +ROCK1 siRNA

Suppl. Fig. 2

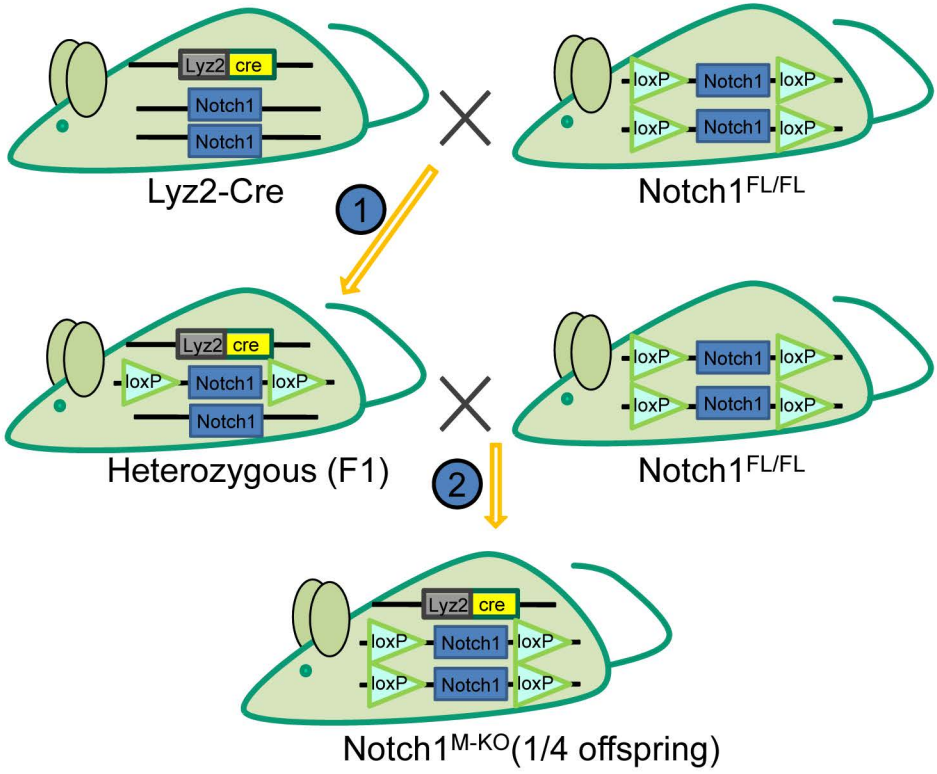

A
ALP levels (U/L)
400
300
200
100
0
*
*
6h
24h
WT Sham
WT IR
DBIL levels (umol/L)
15
10
5
0
**
**
6h
24h
Notch1FL/FL
Notch1M-KO
C
ALP levels (U/L)
500
400
300
200
100
0
*
*
6h
24h
D
DBIL levels (umol/L)
30
25
20
15
10
5
0
**
**
6h
24h

Suppl. Fig. 3

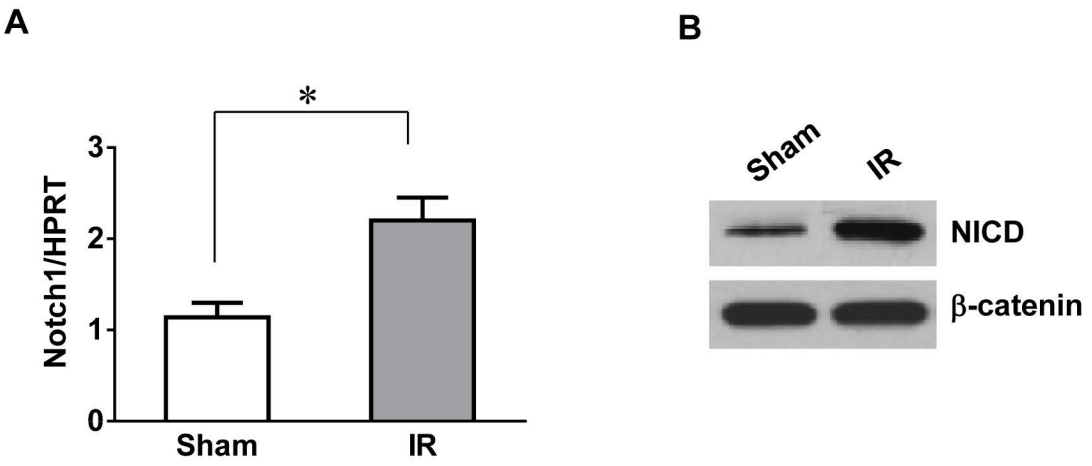


Myeloid specific Notch1 deletion

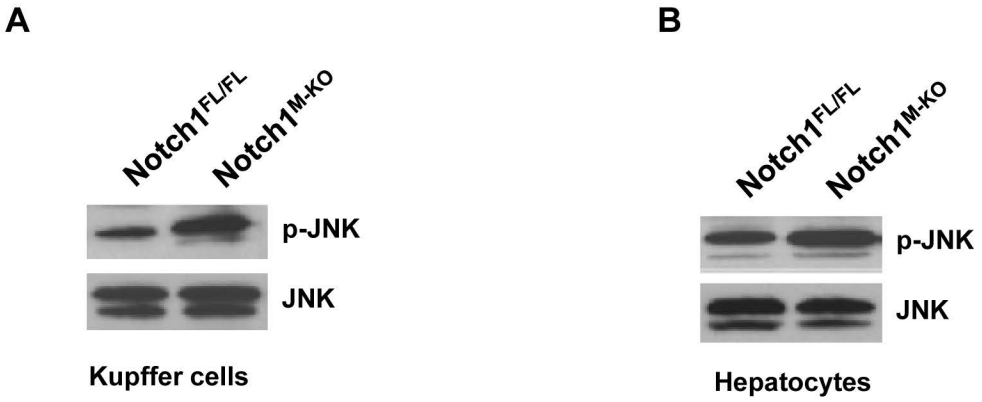
Suppl. Fig. 4


Lyz2
cre
Notch1
Notch1
Lyz2-Cre
loxP
Notch1
loxP
loxP
Notch1
loxP
Notch1FL/FL
1
Heterozygous (F1)
2
Notch1FL/FL
Notch1M-KO(1/4 offspring)

Suppl. Fig. 5



Suppl. Fig. 6


A
Notch1FL/FL
Notch1M-KO
p-JNK
JNK
Kupffer cells
B
Notch1FL/FL
Notch1M-KO
p-JNK
JNK
Hepatocytes

Supplementary Materials

MYELOID NOTCH1 DEFICIENCY ACTIVATES RHOA/ROCK PATHWAY AND AGGRAVATES HEPATOCELLULAR DAMAGE IN MOUSE ISCHEMIC LIVERS

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Materials and Methods

Animals. The floxed Notch1 (Notch1$^{FL/FL}$, catalog number 007181) mice (The Jackson Laboratory, Bar Harbor, ME) and the mice expressing Cre recombinase under the control of the Lysozyme 2 (Lyz2) promoter (LysM-Cre; catalog number 004781, The Jackson Laboratory) were used to generate myeloid-specific Notch1 knockout (Notch1$^{M\text{-}KO}$) mice (Supplementary Figure 3). Two steps were used to generate Notch1$^{M\text{-}KO}$ mice. First, a homozygous loxP-flanked Notch1 mouse was mated with a homozygous Lyz2-Cre mouse to generate the F1 mice that were heterozygous for a loxP-flanked Notch1 allele and heterozygous for the Lyz2-Cre. Next, these F1

mice were backcrossed to the homozygous loxP-flanked Notch1 mice, resulting in generation of Notch1$^{M\text{-}KO}$ (25% of the offspring), which were homozygous for the loxP-flanked Notch1 allele and heterozygous for the Lyz2-Cre allele (Supplementary Figure 4). Mouse genotyping was performed by using a standard protocol with primers described in the JAX Genotyping protocols database. Male C57BL/6 wild-type (WT) mice were obtained from The Jackson Laboratory. Animals at 6-8 weeks of age were used in all experiments. This study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. The study protocols were approved by the Institutional Animal Care and Use Committee of The University of California at Los Angeles, University of Southern California, and Nanjing Medical University in China.

Mouse liver IRI model. We used an established mouse model of warm hepatic ischemia followed by reperfusion (24). Mice were injected with heparin (100U/kg) and an atraumatic clip was used to interrupt the arterial/portal venous blood supply to the cephalad liver lobes. After 90min of ischemia, the clip was removed, and mice were sacrificed at 6h or 24h of reperfusion. Some animals were injected via tail vein with JSAP1 siRNAs, ROCK1 siRNA or non-specific (NS) control siRNA (2 mg/kg) (Santa Cruz Biotechnology) mixed with mannose-conjugated polymers (Polyplus transfection™, Illkirch, France) at a ratio according to the manufacturer's instructions 4h prior to ischemia as described (8).

Hepatocellular function assay. Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured by IDEXX Laboratories (Westbrook, ME).

Histology, immunohistochemistry, immunofluorescence staining. Liver sections (5-μm) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's criteria (25) on a scale from 0-4. Liver macrophages were detected using primary rat anti-mouse CD11b^{+} Alexa Fluor 488 (M1/70, eBiosciences, San Diego, CA) mAb for immunofluorescence staining. DAPI was used for nuclear counterstaining. Liver neutrophils were

detected using primary rat anti-mouse Ly6G (BD Biosciences) mAb. The secondary, biotinylated goat anti-rat IgG (Vector, Burlingame, CA) was incubated with immunoperoxidase (ABC Kit, Vector), according to the manufacturer's instructions. Positive cells were counted blindly in 10 HPF/section (x200).

TUNEL assay. The Klenow-FragEL DNA Fragmentation Detection Kit (EMD Chemicals, Gibbstown, NJ) was used to detect the DNA fragmentation characteristic of oncotic necrosis/apoptosis in formalin-fixed paraffin-embedded liver sections (7). Results were scored semi-quantitatively by averaging number of apoptotic cells/microscopic field at 200× magnification. Ten fields were evaluated/sample.

Caspase-3 activity assay. Caspase-3 activity was determined by an assay kit (Calbiochem, La Jolla, CA) as described (26). Protein (30 μg/sample) was incubated with 200μM of enzyme-specific colorimetric caspase-3 substrate at 37°C for 2h. Caspase-3 activity was assessed by measuring the absorbance at a wavelength of 405nm with a plate reader. To determine cellular activity, the inhibitor-treated protein extracts and the purified caspase-3 (as a standard) were used.

Quantitative RT-PCR analysis. Quantitative real-time PCR was performed as described (27). Total RNA was purified from liver tissue or cell cultures using RNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Reverse transcription to cDNA was performed by using SuperScript III First Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25μl, the following were added: 1× SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen, San Diego, CA) cDNA and 10μM of each primer. Amplification conditions were: 50°C (2min), 95°C (5min), followed by 40 cycles of 95°C (15sec) and 60°C (30sec). Primer sequences used for the amplification of TNF-α, IL-1β, MCP-1, RhoA, Hes1, and

HPRT are shown in Supplementary Table 1. Target gene expressions were calculated by their ratios to the housekeeping gene HPRT.

Western blot analysis. Protein was extracted from liver tissue or cell cultures as described (27). Protein was extracted from liver tissue or cell cultures with ice-cold protein lysis buffer (50mM Tris, 150mM Nacl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 1% Triton-100). The buffer contains 1% proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich). Proteins (30 μg/sample) in SDS-loading buffer (50mM Tris, pH 7.6, 10% glycerol, 1% SDS) were subjected to 4-20% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% dry milk and 0.1% Tween 20 (USB, Cleveland, OH). The monoclonal rabbit anti-mouse NICD, Hes1, p-JNK, JNK, ROCK1, PTEN, p-Akt, Akt, TLR4, cleaved caspase-3, p-IκBα, and β-actin Abs (Cell Signaling Technology, MA) and mouse monoclonal antibody JSAP1 (Santa Cruz Biotechnology, CA) were used. The membranes were incubated with Abs, and then developed according to the Pierce SuperSignal West Pico Chemiluminescent Substrate protocol (Pierce Biotechnology, Rockford, IL). Relative quantities of protein were determined and expressed in absorbance units (AU) comparing to β-actin expression using a densitometer (Kodak Digital Science 1D Analysis Soft-ware, Rochester, NY).

Lentiviral vector construction. The DNA fragment containing the murine NICD coding sequence from pLPCX-NICD (Addgene, MA) was cloned into the pSin-EF2-IRES-Pur vector (Addgene), which expresses the NICD that contains EF2 promoter and puromycin gene (pSin-NICD). The LentiCRISPRv2 is a 3rd generation lentiviral vector containing CMV-driven GFP (Addgene). pSPAX2 and pCMV-VSV-G are lentiviral packaging plasmids (Addgene). The 293T cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS. Cells were seeded in six-well plate and transfected at 60%-70% confluence at the time of transfection using a modified lipofectamine LTX with Plus reagent transfection protocol (Life

Technologies, NY). The following amount of plasmid DNA was used per well: 1μg of pSin-NICD (or control vector), 0.625μg of pSPAX2, and 0.375μg of pCMV-VSV-G. Forty-eight hours after transfection, viral vector-containing supernatant was collected and filtered in 0.45μm filter. The virus was either used immediately or snap-frozen at -80ºC for later use.

The lentiviral CRISPR Hes1 or JSAP1 knockout vector was constructed by the first cloning of Hes1 or JSAP1 single guide RNA (sgRNA) sequences into the site of *BsmB*I of LentiCRISPRv2 vector, as described (28). The Hes1 and JSAP1 sgRNA sequences used for the cloning were as follows: Hes1: 5'-CACCGTGGGGTAGCAGCCACCGGGG-3' (forward); 5'-AAACCCCCGGTGGCTGCTACCCCAC-3' (reverse). JSAP1: 5'-CACCGTGAGTTTCAGACACGCCAGC-3' (forward); 5'-AAACGCTGGCGTGTCTGAAACTCAC-3' (reverse). For lentiviral vector production, 293T cells were transfected as described above. The following amount of plasmid DNA was used per well: 2μg of LentiCRISPRv2-Hes1 KO (LV-Hes1 KO) or Lenti-CRISPRv2-JSAP1 KO (LV-JSAP1 KO), 1.5μg of psPAX2, and 0.5μg of pCMV-VSV-G. LentiCRISPRv2 vector without gRNA sequence (LV-control) was used as a control.

Isolation of hepatocyte and liver macrophages. Primary hepatocytes and liver macrophages (Kupffer cells) from Notch1$^{FL/FL}$ and Notch1$^{M\text{-}KO}$ mice were isolated as described (27, 29). In brief, livers were perfused in situ with warmed (37°C) HBSS solution, followed by a collagenase-buffer (collagenase type IV, Sigma, St Louis, MO). Perfused livers were dissected and teased through 70-μm nylon mesh cell strainers (BD Biosciences). Nonparenchymal cells (NPCs) were separated from hepatocytes by centrifuging at 50 × *g* for 2min three times. NPCs were suspended in HBSS and layered onto a 50%/25% two-step Percoll gradient (Sigma) in a 50-ml conical centrifuge tube and centrifuged at 1800 × *g* at 4°C for 15min. KCs in the middle layer were collected and allowed to attach onto cell culture plates in DMEM with 10% FBS, 10mM HEPES, 2mM GlutaMax, 100 U/ml penicillin, and 100 μg/ml streptomycin for 15min at 37°C. Nonadherent cells were removed by replacing the culture medium. The purity of macrophages in

ischemic livers was 80% as assessed by immunofluorescence staining for $CD11b^+$ (data not shown).

BMM isolation and in vitro transfection. Murine bone-derived macrophages (BMMs) were generated as described (26). In brief, bone marrow cells were removed from the femurs and tibias of wild type (WT), $Notch1^{FL/FL}$ and $Notch1^{M\text{-}KO}$ mice and cultured in DMEM supplemented with 10% FCS and 15% L929-conditioned medium. Cells ($1x10^6$/well) were cultured for 7 days and then transduced with lentivirus-expressing NICD, CRISPR/Cas9-Hes1 KO, CRISPR/Cas9-JSAP1 KO, or control vector. After 24-48h, cells were supplemented with 100 ng/ml of LPS for additional 6h.

ELISA assay. Murine serum and BMM culture supernatants were harvested for cytokine analysis. ELISA kits were used to measure TNF-α, IL-1β, MCP-1, and IL-6 levels (eBiosciences).

Reactive oxygen species assay. ROS production in BMMs was measured using the 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (Carboxy-H2DFFDA, Life Technologies). In brief, BMMs from WT, $Notch1^{FL/FL}$, and $Notch1^{M\text{-}KO}$ mice were cultured on collagen-coated cover slips after LPS stimulation. After washing with PBS, cells were then incubated with 10μM of Carboxy-H2DFFDA. The Carboxy-H2DFFDA was converted to a green-fluorescent form when it was hydrolyzed by intracellular esterase and oxidized in the cells. Cells were fixed with 2% paraformaldehyde and stained with Hoechst dye. ROS produced by BMMs were analyzed and quantified by fluorescence microscopy. Positive green fluorescent-labeled cells were counted blindly in 10 HPF/section (x200).

Statistical analysis. Data are expressed as mean±SD and analyzed by Permutation *t* test and Pearson correlation. Per comparison two-sided *p* values less than 0.05 were considered statistically significant. Multiple group comparisons were made using one-way ANOVA followed by Bonferroni's post hoc test. When groups showed unequal variances, we applied Welch's

ANOVA to make multiple group comparisons. All analyses were used by SAS/STAT software, version 9.4.

Supplementary Table 1: Primer sequences for the amplification

Target genes	Forward primers	Reverse primers
HPRT	5’-TCAACGGGGGACATAAAAGT-3’	5’-TGCATTGTTTTACCAGTGTCAA-3’
TNF-α	5’-GCCTCTTCTCATTCCTGCTTGT-3’	5’-GATGATCTGAGTGTGAGGGTCTG-3’
IL-1β	5’-TGTAATGAAAGACGGCACACC-3’	5’-TCTTCTTTGGGTATTGCTTGG-3’
MCP-1	5'-GAAGGAATGGGTCCAGACAT-3'	5'-ACGGGTCAACTTCACATTCA-3'
Notch1	5’-TGCCAGGACCGTGACAACTC-3’	5’-CACAGGCACATTCGTAGCCATC-3’
Hes1	5’-AAAGACGGCCTCTGAGCAC-3’	5’-GGTGCTTCACAGTCATTTCCA-3’
RhoA	5′-GCAGGTAGAGTTGGCTTTATGG-3′	5′-CTTGTGTGCTCATCATT CCGA-3′
S1009A	5′-TGGAGGACCTGGACACAAATG-3′	5’-TCGTCACCCTCGTGCATCTT-3’
CD206	5’-GGTCTATGGAACCACGGATG-3’	5’-TGCCCAGTAAGGAGTACATGG-3’
Arginase-1	5’-GGAAAGCCAATGAAGAGCTG-3’	5’-GATGCTTCCAACTGCCAGAC-3’