SUPPLEMENTARY INFORMATION

Combined activities of JNK1 and JNK2 in hepatocytes protect against toxic liver injury

Short Title: **Redundancy of JNK1 and JNK1 in hepatocytes**

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SUPPLEMENTARY MATERIAL AND METHODS

Isolation and culture of primary hepatocytes

Primary mouse hepatocytes were isolated from 7-8 week-old mice by collagenase perfusion. Living cells were plated on collagen-precoated petri dishes at a density of 1.5 X 10^4/cm^2 in DMEM medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with L-glutamine, high glucose (4.5 g/l), 10% FBS and 100 U/ml penicillin/streptomycin. After 4 h incubation (37°C, 5% CO_2) and every 2 days, medium was renewed. Hepatocytes were cultured up to 4 days.

Quantitative real-time PCR (qPCR)

Total RNA from liver tissues or cultured cells was isolated using Trizol reagent (Invitrogen, Karlsruhe, Germany). Due to low RNA amount in cultured cells, RNeasy Lipid Tissue Mini Kit was used to collect and purify RNA. Reverse-transcription was

performed using an Omniscript RT Kit (Qiagen). Relative quantitative gene expression was measured via real-time PCR using a 7300 Real Time PCR System with SDS software 1.3.1 (Applied Biosystems, Foster City, CA) and a SYBR Green PCR Kit (Invitrogen, Carlsbad, CA). GAPDH expression was used as internal standard. Primer sequences can be provided upon request.

Histological evaluation and immunofluorescence staining

Hepatic tissue were fixed in 4% paraformaldehyde (PFA) immediately after extraction, embedded in paraffin, sectioned and stained for H&E or Sirius red. Samples were reviewed by a blinded pathologist who analyzed the degree of liver injury. The percentage of Sirius red area fraction in all animals was quantified on 10 or 20 low-power (magnification, X10) fields per slide, using the NIH ImageJ® software (http://rsbweb.nih.gov/). Immunohistochemistry for CK-19 (DAKO, Hamburg, Germany), αSMA (Sigma, Steinheim, Germany), phospho-JNK (Cell Signaling, Danvers, MA) and F4/80 (Serotec, Dusseldorf, Germany) on paraffin sections, was performed using a Leica automatic stainer (Wetzlar, Germany). For the immunofluorescence staining, frozen cryosections were incubated with Ki-67 (Santa Cruz, Heidelberg, Germany), Collagen IA1 (Bio trend, Cologne, Germany) or *in situ* cell death detection kit (Roche, Mannheim, Germany) and incubated with fluorescence labelled secondary antibodies (AlexaFluor 488 and 564, Invitrogen, Carlsbad, CA, USA). All fluorescence-labelled cryosections were analyzed and documented using an Imager Z1 fluorescence microscope together with Axiovision software (Carl Zeiss, Jena, Germany).

Immunoblot analysis

Liver tissues were homogenized in ice cold NP40-Buffer containing 50 mM Tri- HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40 and 50 mM NaF freshly supplemented with Complete Mini (Roche), PhosSTOP (Roche), 1 mM orthovanadate and 1 mM pefablock. Protein concentrations were determined by BIO-RAD protein assay (BIORAD). Samples were separated by SDS-PAGE and transferred to a cellulose membrane and probed with antibodies for αSMA (Sigma), COLLAGEN 1AI (Monosan, Beutelsbach, Germany), PCNA (Dianova GmbH, Hamburg, Germany), CYCLIN A (Santa Cruz), CYCLIN D1, BCLXL and PKCα (Santa Cruz), JNK1, JNK2, pJNK (pT183/Y185), P38, CMYC, STAT3, pAMPK, AMPK and MAPK/CDK Substrates (PXP*P or S*PXR/K) (34B2) (Cell Signaling), and GAPDH (Biorad, Munich, Germany). As secondary antibodies, anti-rabbit-HRP (Cell Signaling) and anti-mouse-HRP (Santa Cruz) were used.

Microarray Analysis

Liver and hepatocyte RNA using TRIzol reagent and purified with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions was isolated from 8-10 weeks-old male control *Jnk2*$^{-/-}$ and *Jnk*$^{\Delta hepa}$ mice. Concentrations and purity of RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, The Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips. RNA samples from 3 mice per experimental group were used for microarray analysis. Samples were hybridized on Affymetrix GeneChip Mouse Genome 430 2.0 arrays in the microarray core laboratory of the Nutrigenomics Consortium at Wageningen University, The Netherlands. Hybridization, washing, and scanning of the arrays were performed according to standard Affymetrix protocols.

Array images were processed using packages from the Bioconductor project[1]. Probe sets were redefined according to Dai[2]. Probes were assigned to unique gene identifiers, in this case Entrez IDs. Arrays were normalized with the Robust Multi-array Average (RMA) method. Differentially expressed probe sets were identified using intensity-based moderated paired t-statistics. *P* values were corrected for multiple testing using a false discovery rate (FDR) method. Detailed descriptions of the applied methods are available on request.

Peptide lysis, dimethyl-labelling, phosphopeptide enrichment and LC-MS/MS analysis

Primary hepatocytes were scraped off the cell culture dishes after APAP/DMSO treatment, washed three times in ice-cold PBS and lysed in 8M urea (in 50mM ammonium bicarbonate), containing protease and phosphatase inhibitor cocktails (Serva, Heidelberg, Germany) for 30 min on ice and cleared by centrifugation at 20000 g, for 15 min at 4°C. After determination of protein concentration using a BCA assay (Thermo, Waltham, MA) the individual lysates (500 µg each) are reduced with DTT, alkylated with iodoacetamide, and proteolytically digested with the protease Lys-C (1:100, w/w) in 8M urea for 4h at 37°C. Samples were then diluted to 2M urea with 50 mM ammonium bicarbonate and further digested with trypsin (1:75, w/w) overnight at 37°C[3, 4]. The resulting peptide digests are desalted and dimethyl-labelled "on column"[5]. Control (WT) mice derived peptides are labelled "light", whereas *Jnk*$^{\Delta hepa}$ mice derived peptides were labelled "heavy". The labelled peptide digests are mixed in a 1:1 ratio, dried down and resuspended in 1 M glycolic acid in 80% ACN, 5% TFA). Phosphopeptide enrichment was performed using MagReSyn® Ti-IMAC beads (ReSyn Biosciences, Gauteng, South Africa) according to the manufacturer's instructions. Samples were then analysed on a nanoLC-MS/MS System (LC: Ultimate 3000 (Dionex, Dreieich, Germany); MS: Orbitrap Elite

(Thermo)). The raw data obtained from the mass spectrometer were processed and quantified with MaxQuant (version 1.4.1.2). The relevant MS and MS/MS spectra were searched against a forward-decoy Swissprot Homo sapiens database (version 09/2014).

Transplantation of bone marrow-derived cells

We transferred bone marrow from *Jnk*$^{\Delta hepa}$ and control (WT) mice into 6 week-old *Jnk*$^{\Delta hepa}$ and control isogeneic recipients (n=6-7 mice per group) after ablative γ-irradiation, as described previously[6]. Two months after BMT mice were subjected to either 4 weeks treatment with CCl_4 (0.6 mL/kg body weight, diluted in corn oil, injected intraperitoneally every 3 days) or BDL. At this time, mice were sacrificed and samples collected.

Serum parameters

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and alkaline phosphatase (AP) activity (UV test at 37°C) were measured in the Central Laboratory Facility at University Hospital RWTH Aachen according to standard procedures.

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table I. Origin and clinical features of liver samples from patients with DILI. (f: female; m: male; Bili: total bilirrubin; GLDH: glutamate dehydrogenase; TPT: prothrombin time; INR: international normalized ratio; αPTT: activated partial thromboplastin time; NSAID: non-steroidal anti-inflammatory drug).

Supplementary Figure 1. (A) Activation of pJNK in liver samples of patient samples of normal liver human tissue (C3, C4). **(B+C)** PCR blots of tail DNA from *Wt* and *Jnk1*$^{\Delta hepa}$ (upper panel) and control (*Wt*), *Jnk2*$^{-/-}$, and *Jnk*$^{\Delta hepa}$ (lower panel) mice confirmed the respective phenotype of interest.

Supplementary Figure 2. (A+B) A basal study of untreated female (♀) and male (♂) 8-week-old control (*Wt)* or *Jnk*$^{\Delta hepa}$ mice was performed. Representative macroscopic view of livers (scale bars: 10mm) and H&E staining of liver sections (scale bars 100μm) from each mice group is shown. **(C+D)** Serum AST and ALT was represented in a graph. **(E+F)** Liver weight (LW) and body weight of these mice is shown, and the liver *versus* body weight ratio (%LW/BW) calculated.

Supplementary Figure 3. (A) Control (*Wt*), *Jnk1*$^{\Delta hepa}$ and *Jnk*$^{\Delta hepa}$ mice were injected with APAP for 8h and sacrificed. The liver *versus* body weight ratio (%LW/BW) was then calculated. **(B)** Isoprostanes, indicators of lipid peroxides were measured in livers of 8h APAP-treated control (*Wt*) and *Jnk*$^{\Delta hepa}$ mice, and represented as pg/ml. **(C)** Densitometry analysis of the protein expression levels of P38 was performed in these mice and represented as arbitrary units of fluorescence (AUF).

Supplementary Figure 4. (A) Control (Wt) or *Jnk*$^{\Delta hepa}$ mice were injected with GalN and 30 min later with LPS. 8h thereafter mice were sacrificed. **(B)** Survival curve of

control (*Wt*) or *Jnk*$^{\Delta hepa}$ mice treated with GalN+LPS and sacrificed 8h later. **(C)** Serum levels of AST, ALT and GLDH were determined after GalN+LPS-treated control (*Wt*) or *Jnk*$^{\Delta hepa}$ mice. **(D)** Macroscopic (upper panel; Scale bars: 10mm) and microscopic (lower panel; Scale bars: 100μm) appearance of control (*Wt*) or *Jnk*$^{\Delta hepa}$ mice after 8h GalN+LPS treatment **(E)** The percentage of liver *vs* body weight ratio was calculated and represented in a graph.

Supplementary Figure 5. (A) Representative macroscopic view of control (*Wt)*, *Jnk2*$^{-/-}$ and *Jnk*$^{\Delta hepa}$ livers, 28 days after repeated injections of CCl_4. Corn-oil injections were used as controls. **(B)** Representative Sirius red staining of paraffin sections from the same livers. **(C+D)** Quantification of Sirius red and CollagenIA1 stainings were performed using Image J© Software and represented as percentage of area fraction. **(E)** Densitometry analysis of the protein expression levels of αSMA was performed in these mice and represented as arbitrary units of fluorescence (AUF). (**F)** mRNA levels of *Timp1* and *Mmp2* were determined by qRT-PCR in liver tissues of (n=5-10). Data are expressed as mean±SEM (*p<0.05; **p<0.01; ***p<0.001).

Supplementary Figure 6. Control (*Wt*), *Jnk2*$^{-/-}$ and *Jnk*$^{\Delta hepa}$ livers were subjected for 4 weeks of repeated CCl_4 injections. **(A)** mRNA levels of *Pcna* were determined by qRT-PCR in liver tissues (n=6-8; **p<0.01). **(B)** Densitometry analysis of the protein expression levels of PCNA and CyclinD was performed in these mice and represented as arbitrary units of fluorescence (AUF).

Supplementary Figure 7. The inflammatory profile is increased in absence of JNK in hepatocytes. **(A)** Representative CD11b staining performed on frozen liver sections of control (*Wt*), *Jnk2*$^{-/-}$ and *Jnk*$^{\Delta hepa}$ livers after 4 weeks of repeated CCl_4 injections.

Scale bars: 100μm. Quantification of positive cells was performed in 7-10 view-fields. **(B)** Representative F4/80 staining performed on frozen liver sections of the same livers. Scale bars: 100μm. Quantification of F4/80 positive cells per view field is shown. mRNA levels of *IL1α* **(C)**, *IL1β* **(D)**, *Mcp1* **(E)** and *Tnfα* **(F)** were determined by qRT-PCR in liver tissue (n=6-8). Data are expressed as mean±SEM (*p<0.05; **p<0.01; ***p<0.001).

Supplementary Figure 8. Venny diagram shows comparative gene expression overlap in untreated 8 week-old control (*Wt*) and $Jnk^{\Delta hepa}$ livers and primary hepatocytes. **(A)** Up-regulated genes. **(B)** Down-regulated genes. **(C+D)** Validation of gene array analysis by mRNA expression was performed in liver tissue and primary hepatocytes of 8 week-old untreated control (*Wt*), $Jnk1^{\Delta hepa}$ and $Jnk^{\Delta hepa}$ mice. The mRNA expression levels *Bad* and *Jnk2* are shown (*p<0.05; **p<0.01;***p<0.001).

Supplementary Figure 9. (A) Primary hepatocytes were isolated from control (*Wt*), $Jnk2^{-/-}$ and $Jnk^{\Delta hepa}$ mice. A total number of 500.000 cells were seeded in 6-well plates and cultivated for up to 48h. Visible light microphotographs were taken in untreated hepatocytes. Scale bars: 100μm. **(B)** Representative TUNEL stainings of primary hepatocytes. Dead cells are stained green; total cells were counter-stained with DAPI (blue). Scale bars: 100μm. **(C)** Staining for BrdU was performed in coverslips and counterstained to DAPI (blue). Scale bars: 100μm. **(D)** BrdU-positive cells were counted in untreated control (*Wt*) and $Jnk^{\Delta hepa}$ hepatocytes, and displayed in a graph.

Supplementary Figure 10. Primary hepatocytes were isolated from control (*Wt*) and $Jnk^{\Delta hepa}$ mice. A total number of 500.000 cells were seeded in 6-well plates and cultivated for up to 12h. **(A)** Reactive oxygen species (ROS) were measured in

presence or absence of APAP and/or SP600125, an inhibitor of the JNK pathway. **(B)** Annexin V (green)/Ethidium Homodimer III (red)/DAPI (blue) was performed and microphotographs were taken. **(C)** Quantification of the double Annexin V/Ethidium Homodimer III/ DAPI-positive cells was graphed. Values are mean±SEM from at least 6 mice per group (*p<0.05; **p<0.01).

Supplementary Figure 11. (A) Hepatocytic protein extracts from 8h-APAP-treated control (*Wt*) and $Jnk^{\Delta hepa}$ primary hepatocytes were labelled with beads, digested with trypsin, and the phosphopeptides were enriched, and analyzed using mass spectrometry. **(B)** The expression of phosphoMAPK/CDK substrates was assessed in untreated and 8h-APAP-treated control (WT) and $Jnk^{\Delta hepa}$ primary hepatocytes. Potential target proteins of JNK with matching molecular weights are displayed on the right. Values are mean±SEM from at least 6 mice per group (**p<0.01 and ***p<0.001).

SUPPLEMENTARY REFERENCES

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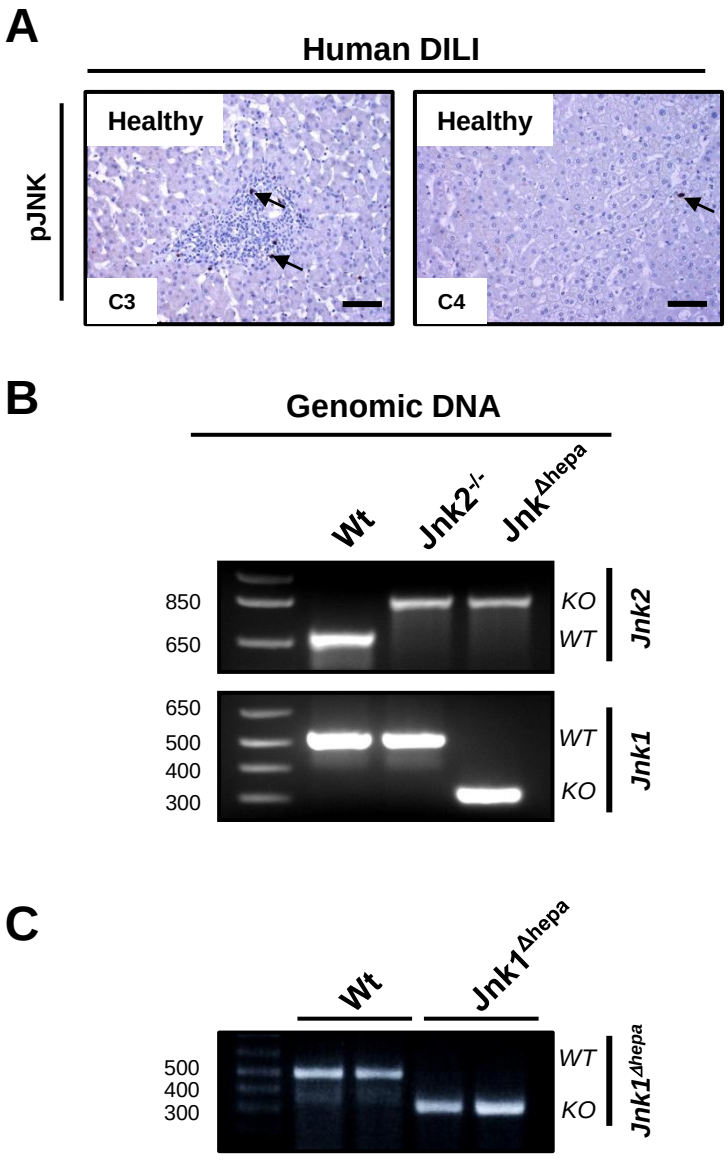
Author names in bold designate shared co-first authors

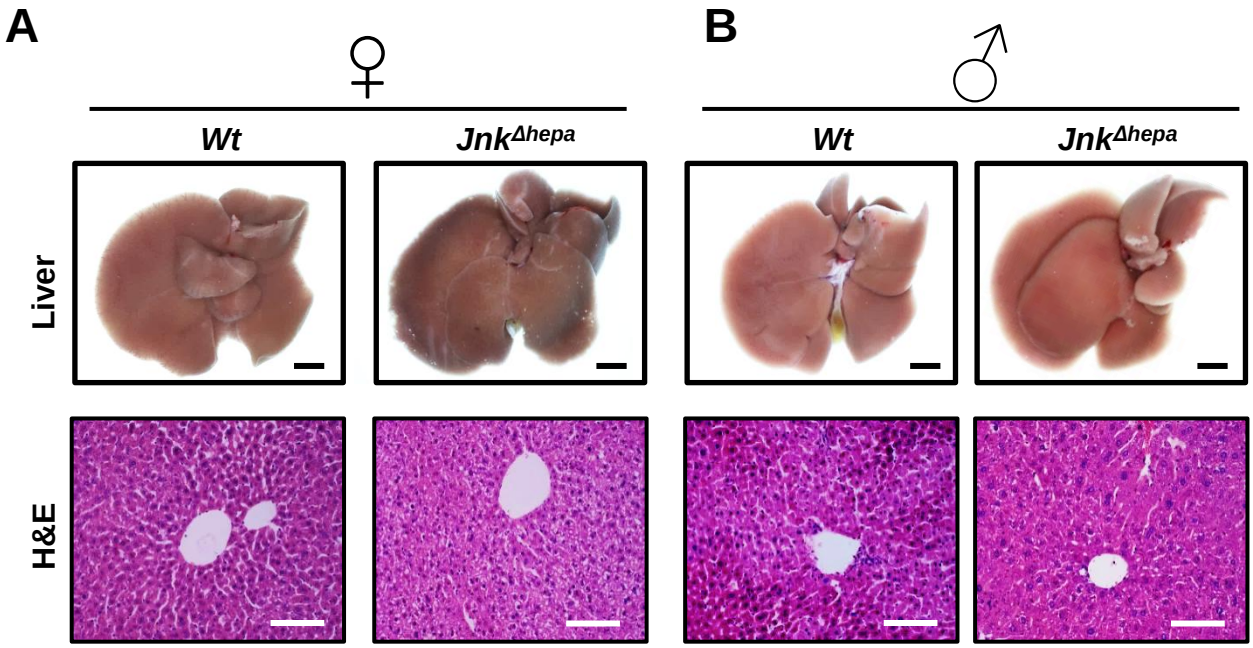
Suppl. Table I

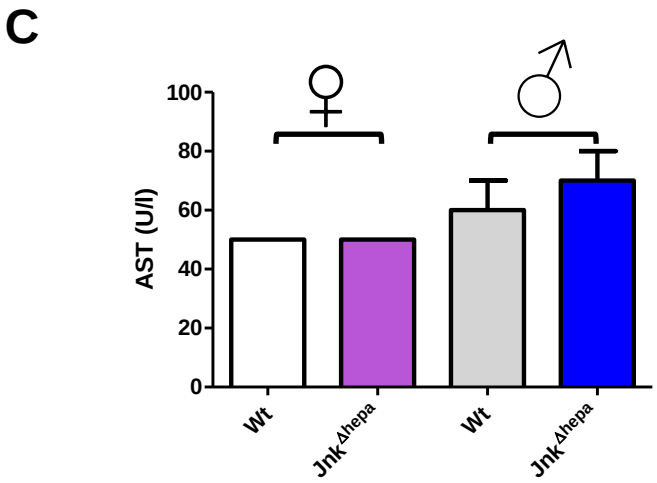
#	Sex	Age	Etiology	AST	ALT	Bili	GLDH	AP	GGT	TPT	INR	αPTT	Creatine	Phosphate
				U/l	*U/l*	*mg/dl*	*U/l*	*U/l*	*U/l*	*%*		*sec*	*mg/dl*	*mg/dl*
1	f	50	Paracetamol	16253	4320	3.90	622.60	86	447	17	3.39	56.50	1.01	-
2	f	23	Phenprocoumon	215	154	3.20	-	63	216	42	1.73	48.00	0.94	3.80
3	m	48	Autoimmune	1004	1068	28.50	18.50	141	148	48	1.44	31.70	0.56	2.50
4	f	63	NSAID	189	209	4.30	10.60	85	38	24	2.73	56.40	0.87	3.00

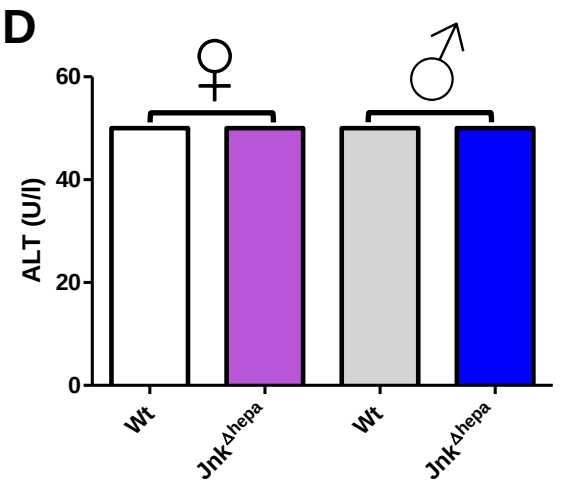
Origin and clinical features of liver samples from patients with DILI. (f: female; m: male; Bili: total bilirrubin; GLDH: glutamate dehydrogenase; TPT: prothrombin time; INR: international normalized ratio; αPTT: activated partial thromboplastin time; NSAID: non-steroidal anti-inflammatory drug).

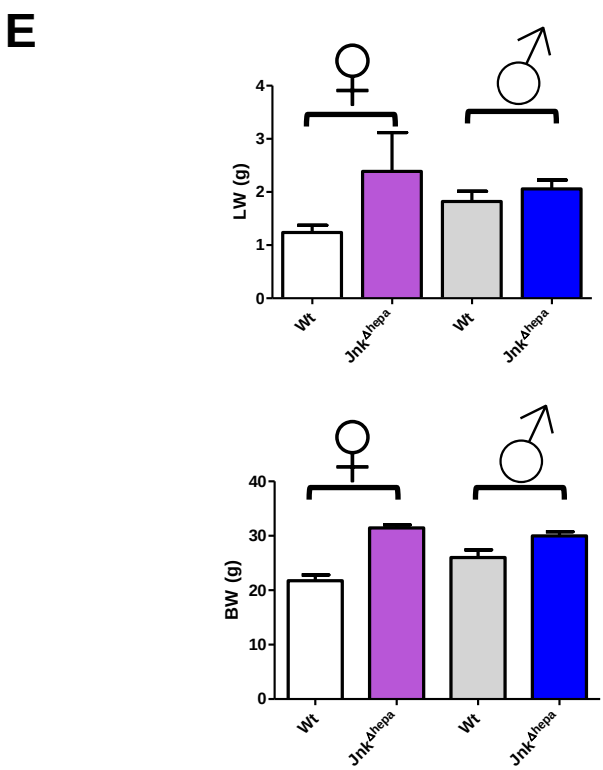

A
Human DILI
pJNK
Healthy
C3
Healthy
C4
B
Genomic DNA
Wt
Jnk2-/-
JnkΔhepa
850
650
KO
WT
Jnk2
650
500
400
300
WT
KO
Jnk1
C
Wt
Jnk1Δhepa
500
400
300
WT
KO
Jnk1Δhepa

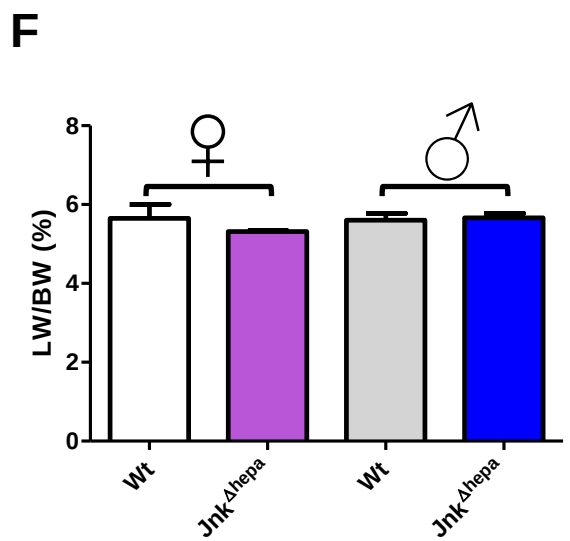
Suppl. Fig. 1


A
B
Wt
Jnk^Δhepa
Wt
Jnk^Δhepa
Liver
H&E


C
AST (U/l)
100
80
60
40
20
0
Wt
Jnk^Δhepa
Wt
Jnk^Δhepa

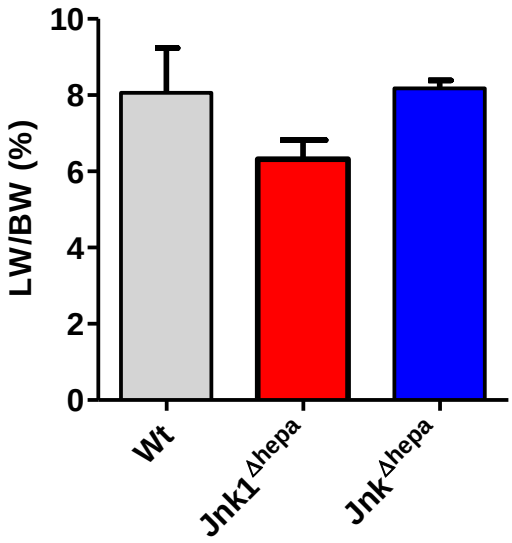

D
ALT (U/l)
60
40
20
0
Wt
Jnk^Δhepa
Wt
Jnk^Δhepa


E
LW (g)
4
3
2
1
0
BW (g)
40
30
20
10
0
Wt
Jnk^Δhepa
Wt
Jnk^Δhepa


F
LW/BW (%)
8
6
4
2
0
Wt
Jnk^Δhepa
Wt
Jnk^Δhepa

Suppl. Fig. 2

A



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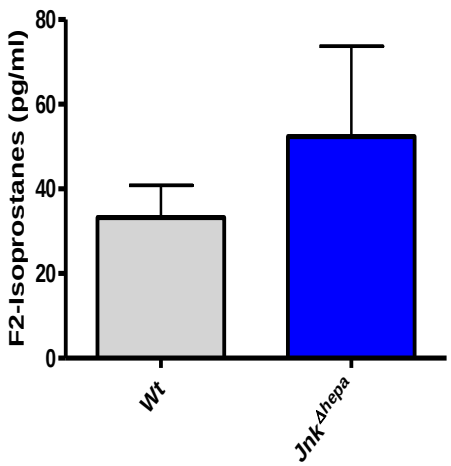
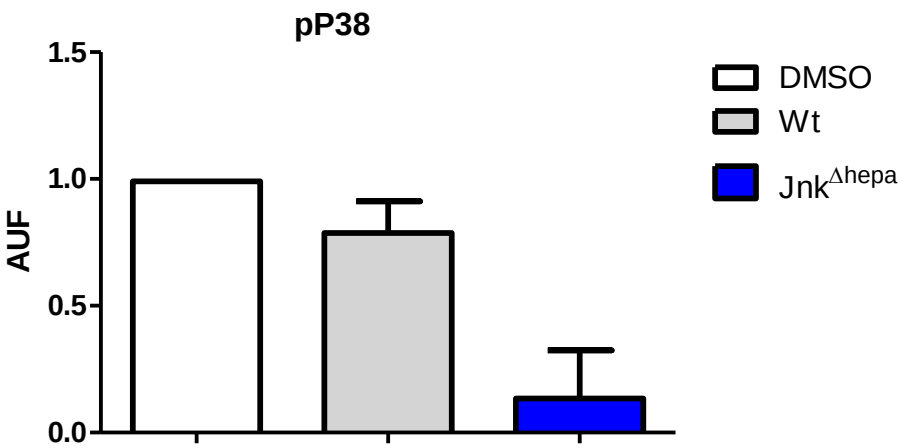


C



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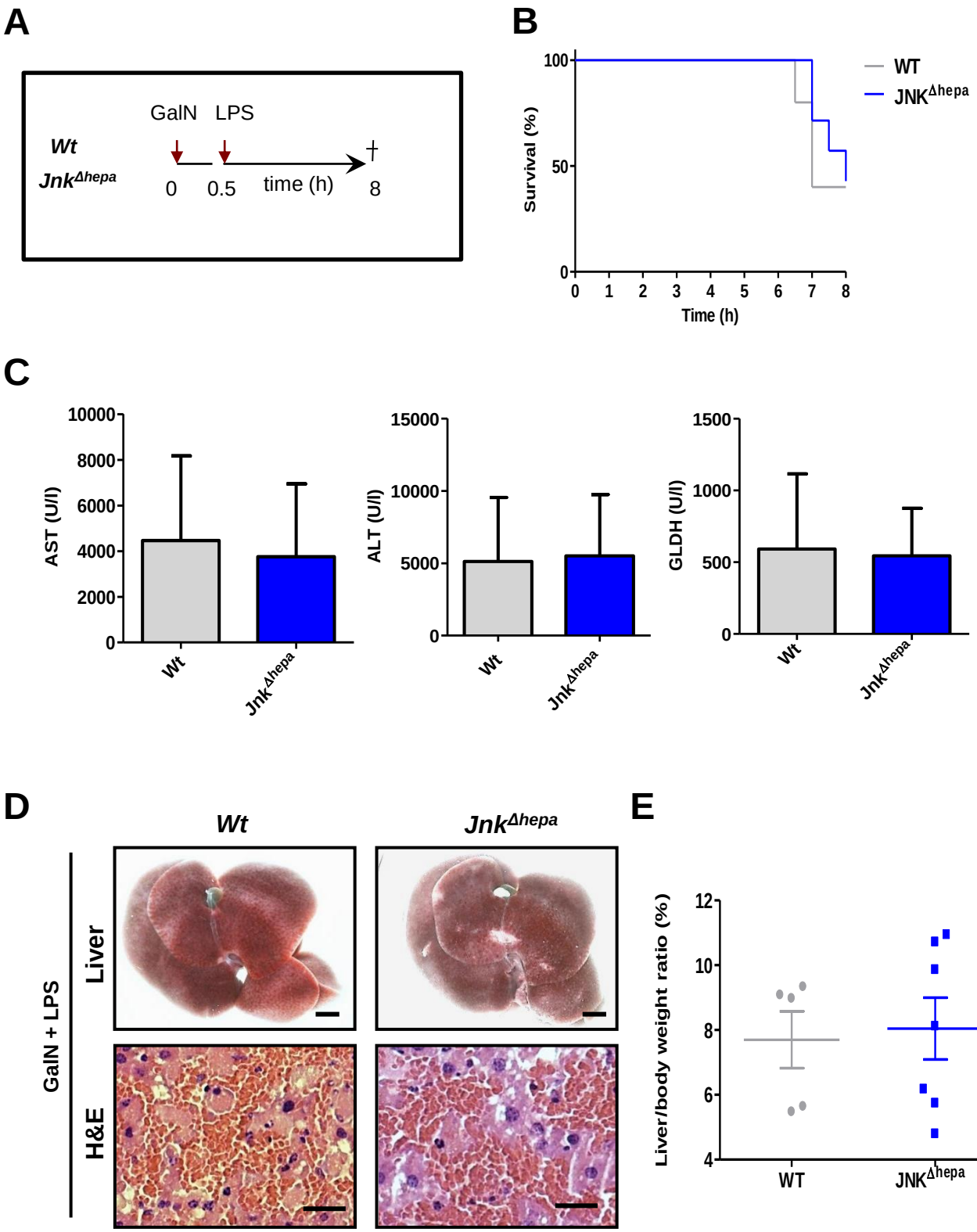
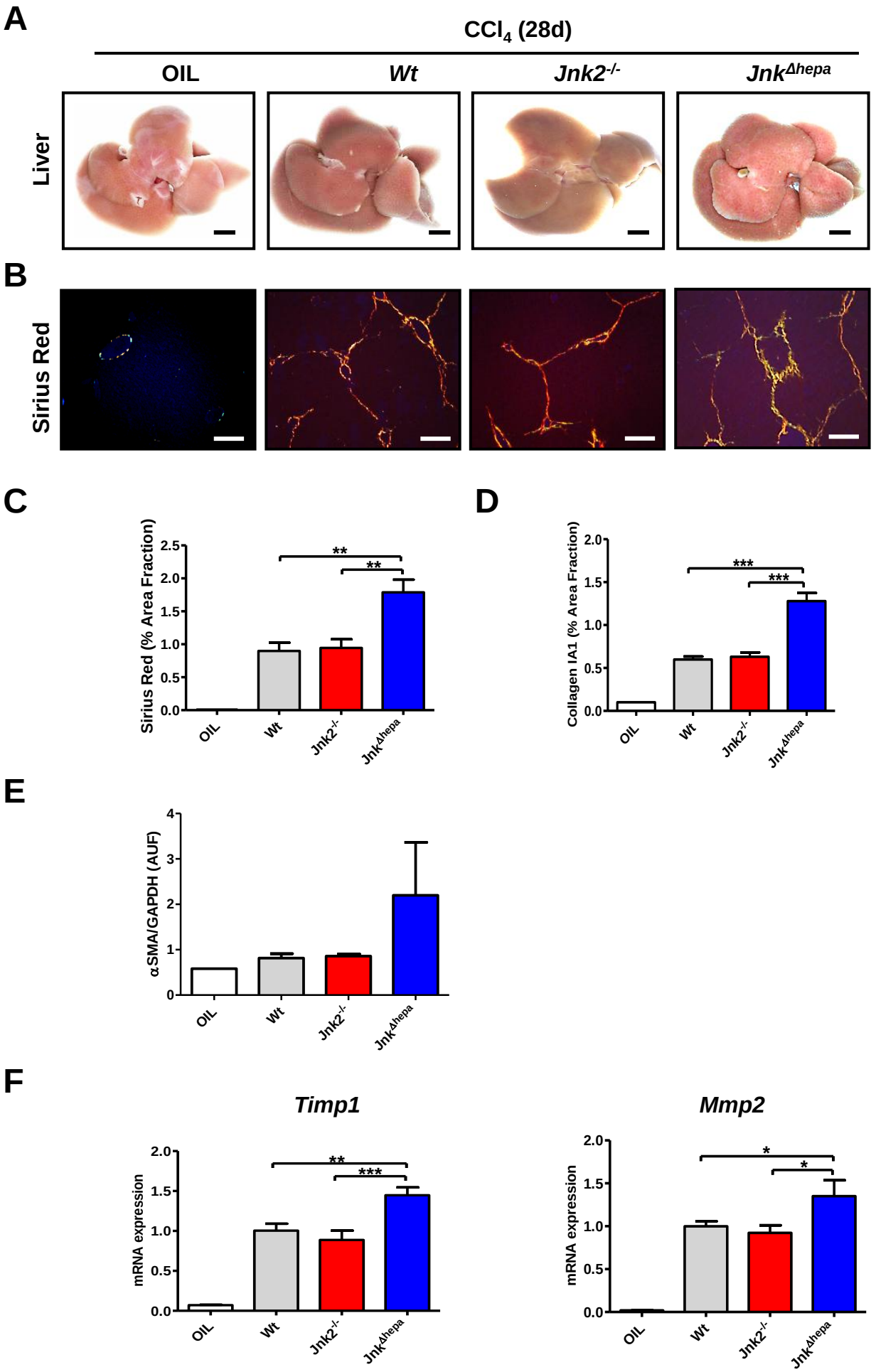
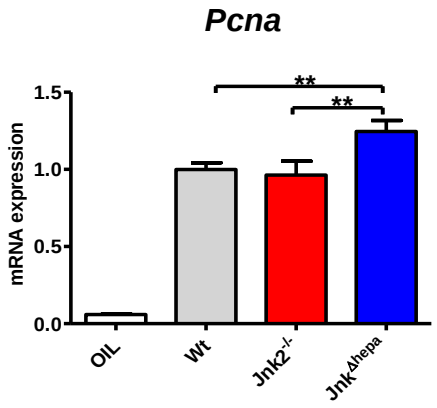

A
GalN
LPS
Wt
Jnk^Δhepa
0
0.5
time (h)
8
B
Survival (%)
Time (h)
WT
JNK^Δhepa
C
AST (U/l)
ALT (U/l)
GLDH (U/l)
Wt
Jnk^Δhepa
D
Wt
Jnk^Δhepa
GalN + LPS
Liver
H&E
E
Liver/body weight ratio (%)
WT
JNK^Δhepa

Suppl. Fig. 4


A
CCl4 (28d)
OIL
Wt
Jnk2-/-
JnkΔhepa
Liver
B
Sirius Red
C
Sirius Red (% Area Fraction)
D
Collagen IA1 (% Area Fraction)
E
αSMA/GAPDH (AUF)
F
Timp1
Mmp2
mRNA expression

Suppl. Fig. 5

A



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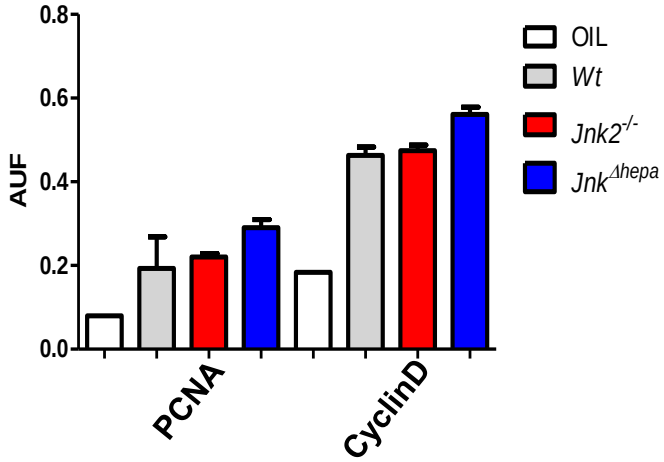
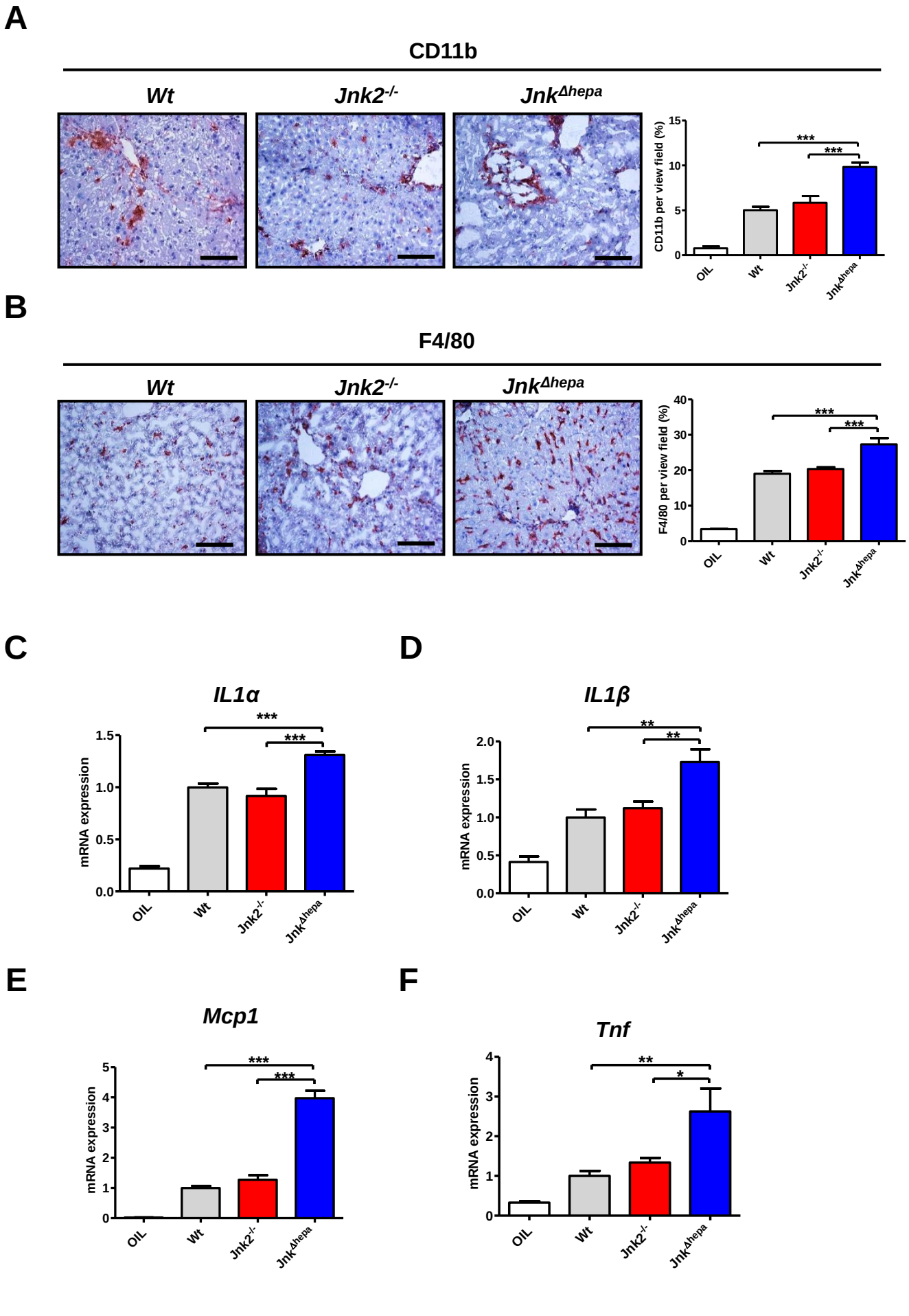
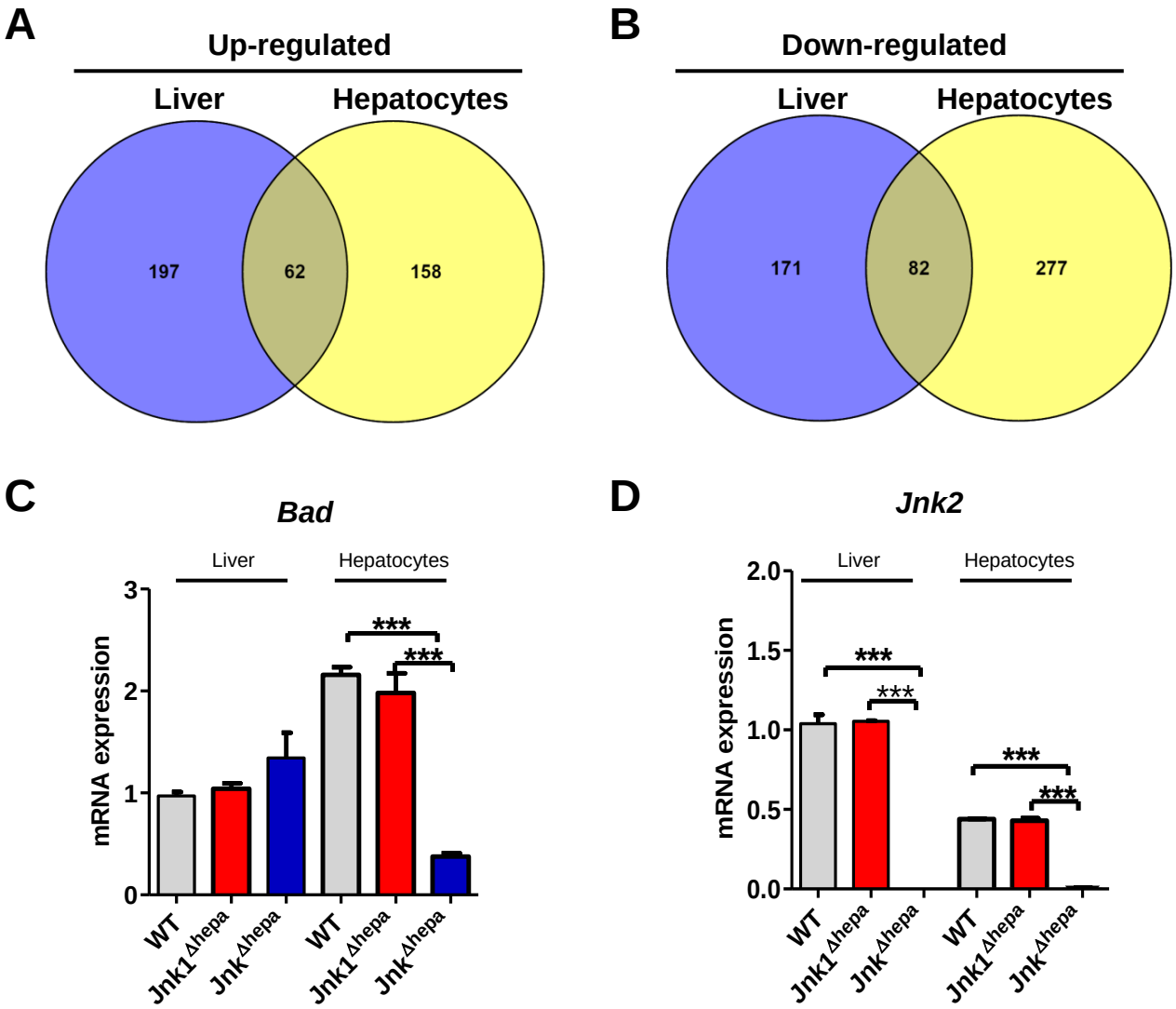


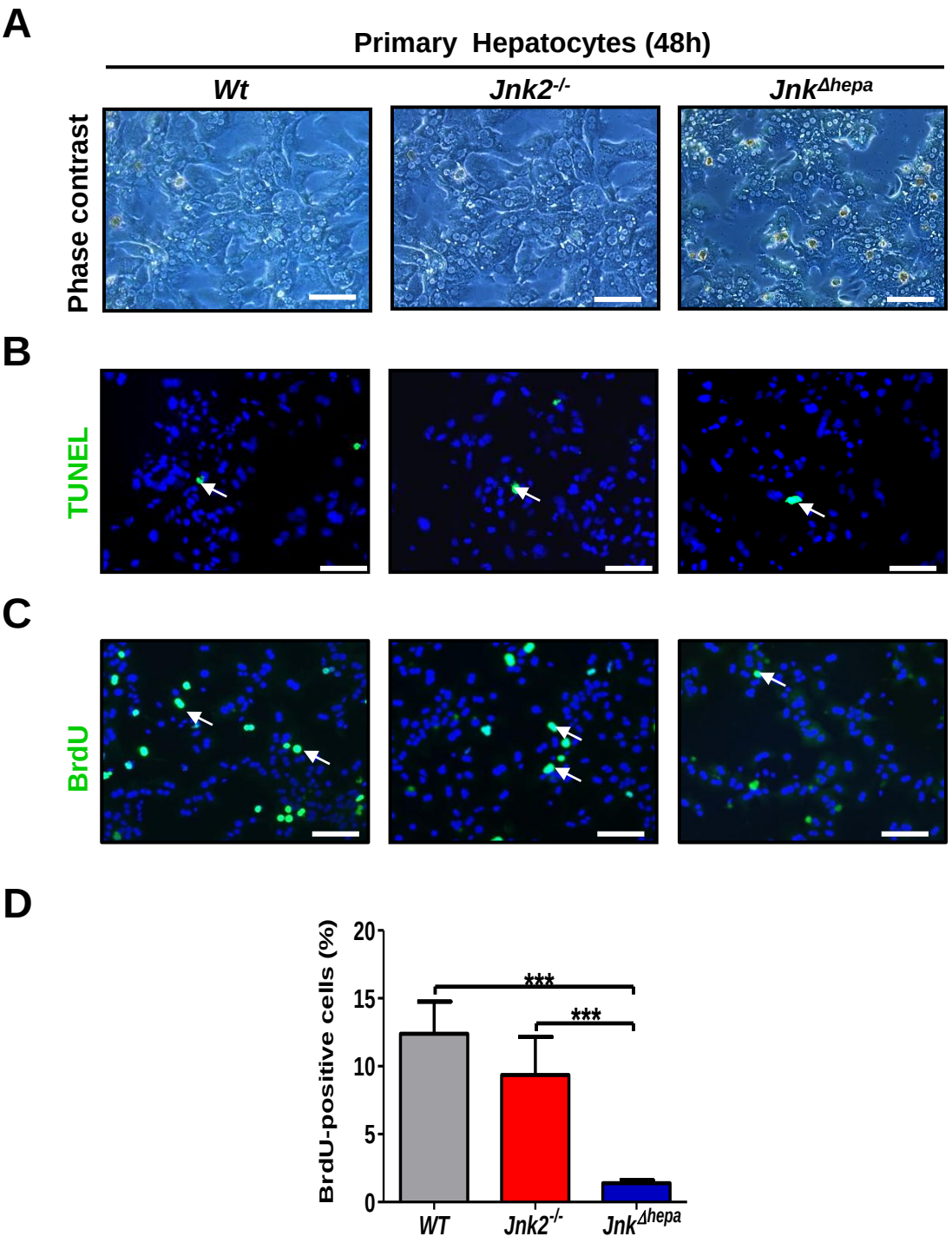
Suppl. Fig. 6


A
CD11b
Wt
Jnk2-/-
JnkΔhepa
CD11b per view field (%)
***
***
OIL
Wt
Jnk2-/-
JnkΔhepa
B
F4/80
Wt
Jnk2-/-
JnkΔhepa
F4/80 per view field (%)
***
***
OIL
Wt
Jnk2-/-
JnkΔhepa
C
IL1α
mRNA expression
***
***
OIL
Wt
Jnk2-/-
JnkΔhepa
D
IL1β
mRNA expression
**
**
OIL
Wt
Jnk2-/-
JnkΔhepa
E
Mcp1
mRNA expression
***
***
OIL
Wt
Jnk2-/-
JnkΔhepa
F
Tnf
mRNA expression
**
*
OIL
Wt
Jnk2-/-
JnkΔhepa

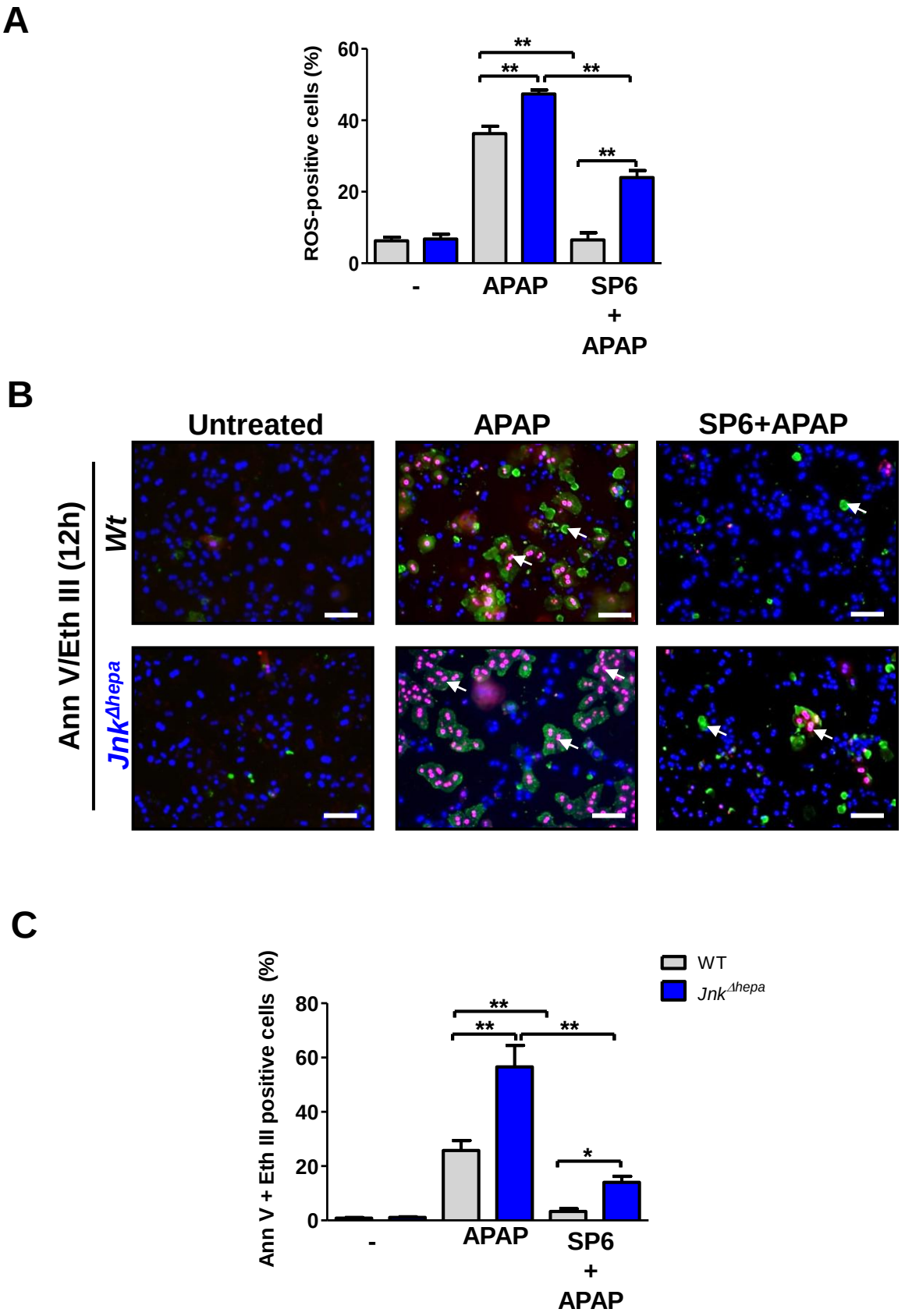
Suppl. Fig. 7


A
Up-regulated
Liver
Hepatocytes
197
62
158
B
Down-regulated
Liver
Hepatocytes
171
82
277
C
Bad
Liver
Hepatocytes
mRNA expression
3
2
1
0
***
***
WT
Jnk1 Δhepa
Jnk Δhepa
WT
Jnk1 Δhepa
Jnk Δhepa
D
Jnk2
Liver
Hepatocytes
mRNA expression
2.0
1.5
1.0
0.5
0.0
***
***
***
***
WT
Jnk1 Δhepa
Jnk Δhepa
WT
Jnk1 Δhepa
Jnk Δhepa

Suppl. Fig. 8

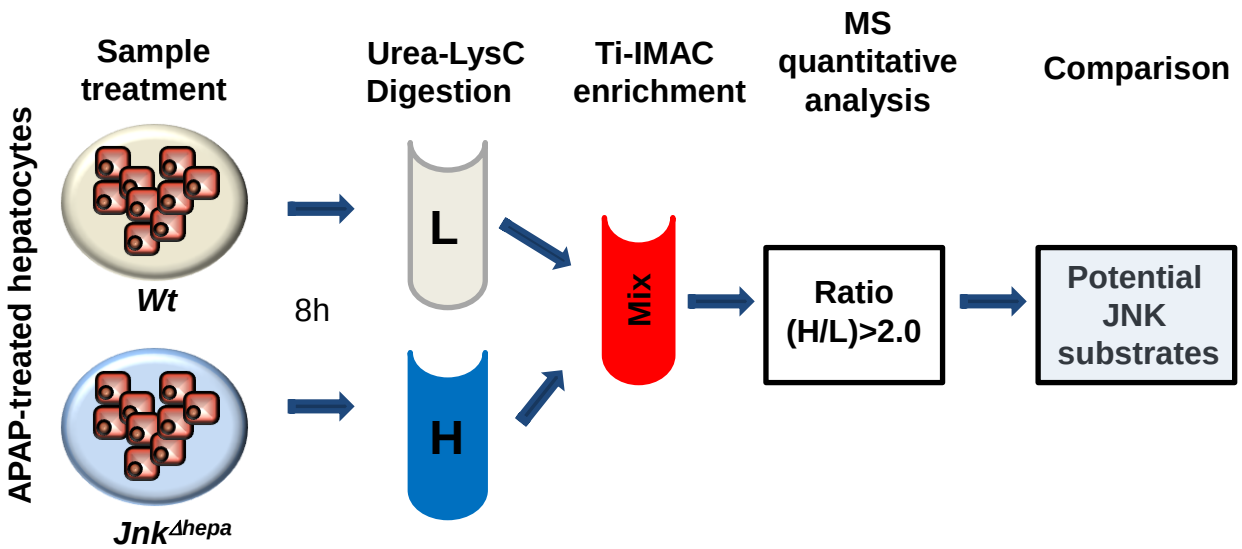

A
Primary Hepatocytes (48h)
Wt
Jnk2-/-
JnkΔhepa
Phase contrast
B
TUNEL
C
BrdU
D
BrdU-positive cells (%)
20
15
10
5
0
***
***
WT
Jnk2-/-
JnkΔhepa

Suppl. Fig. 9

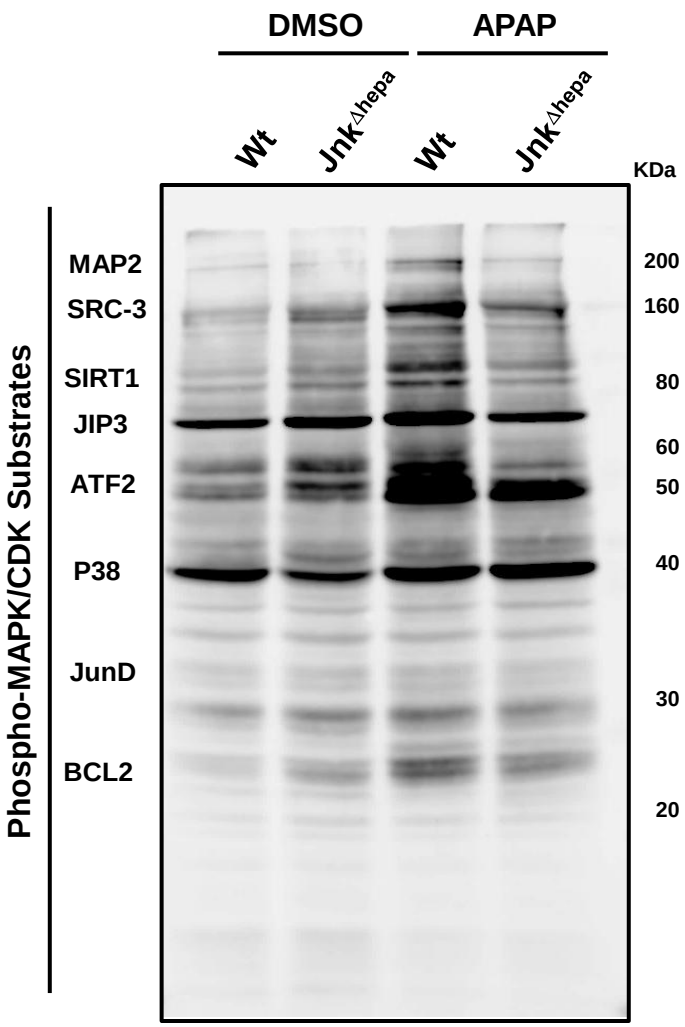

A
ROS-positive cells (%)
60
40
20
0
**
**
**
**
-
APAP
SP6
+
APAP
B
Untreated
APAP
SP6+APAP
Ann V/Eth III (12h)
Wt
Jnk Δhepa
C
Ann V + Eth III positive cells (%)
80
60
40
20
0
WT
Jnk Δhepa
**
**
**
*
-
APAP
SP6
+
APAP

Suppl. Fig. 10

A



B



Suppl. Fig. 11