Supplementary data for:

Steap3 deficiency in hepatocytes protects the liver against ischemia/reperfusion injury by suppressing TAK1

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Supplementary Materials and Methods

Animals

Male mice (8-10 weeks of age, 25±2 g) were housed in a specific pathogen-free and temperature-controlled (23 ± 2°C) environment with a 12h light/dark cycle. Food and water were available ad libitum. Humane care was given to the animals in adherence with the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH; publication 86-23 revised in 1985). All the animal procedures were approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University. *Steap3*-KO mice were purchased from the Texas A&M Institute for Genomic Medicine (IST13594C11, TIGM，Germany). Identification primer sequences of *Steap3*-KO mice, F：5'-CTTGCAAAATGGCGTTACTTAAGC-3' and R：5'-CACGCTATAACACCGCCCT-3'. *Steap3*-HTG mice were generated using the following method: the full-length consensus CDS of the mouse *Steap3* (CCDS48343.1) gene was cloned downstream of the Alb promoter, and an Alb-*Steap3* transgene vector was obtained. The vector was linearized and microinjected into pronuclear stage embryos. Two-cell-stage embryos were transplanted into the oviducts of pseudo-pregnant foster mothers. Genomic DNA was extracted from The toes or tail tissue of newborn mice for screening by PCR. The following primers were used: F: 5’-GAGCGAGTCTTTCTGCACAC-3’ and R: 5’-AACACGGCCACAAAGATGAC-3’.

Warm hepatic ischemia/reperfusion (I/R) injury mouse model

A model of warm I/R injury of the liver (70%) was established as previously

described(1). Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Sigma-Aldrich, P3761). Then, midline laparotomy was performed to expose the liver. Next, the portal vein, hepatic artery and bile duct above the branching to the right lateral lobe were clamped with microvascular clips to interrupt the blood supply to the left lateral/median lobes of the liver. Successful ischemia surgery was identified by bleaching of the ischemic liver lobes. During ischemia, the abdomen was covered, rectal temperature was monitored, and body temperature was maintained at 37±0.5°C with a thermostatic blanket. After 60 min of ischemia, the blood vessel clamp was removed for reperfusion. The liver color recovered after reperfusion. The abdomen was closed by continuous abdominal sutures. The absence of ischemic color changes and the lack of a response to reperfusion were criteria for immediate sacrifice and exclusion from further analysis. At each indicated time point after reperfusion, the mice were anesthetized to collect liver samples and serum for further analysis. Sham control mice underwent the same procedure without clamping the vasculature.

Examination of liver function and serum concentrations of inflammatory factors

The extent of liver damage in animals was assessed by measuring the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with the ADVIA 2400 Chemistry System (Siemens, Tarrytown, NY, USA) according to the manufacturer's protocols. The inflammatory state was assessed by measuring serum cytokines and chemokines using commercial enzyme-linked immunosorbent assay (ELISA) kits (Murine Tnf-α Standard ABTS ELISA Development Kit, PeproTech, 900-T54, Rocky Hill, NJ, USA; Mouse/Rat Ccl2/JE/MCP-1 Quantikine ELISA Kit, R&D Systems, MJE00, Minneapolis, MN, USA) according to the manufacturer's protocol.

Histological and immunohistochemical staining

Hematoxylin and eosin (H&E) and immunohistochemical staining were performed with paraffin-embedded liver sections(2). Paraffin-embedded tissue samples were sectioned serially at 4- to 5-μm thickness. After deparaffinization and rehydration, the sections were stained with H&E to visualize the pattern in necrotic areas of the liver. The results are expressed as the average percentage of necrotic area relative to the whole visual field area. The Steap3 expression profile in mice was determined by incubating liver sections with a rabbit anti-mouse Steap3 (AVIVA, OAAN00117) primary antibody. After an overnight incubation with the primary antibody at 4°C, the sections were incubated with HRP-conjugated secondary antibodies (A21020, 1:500 dilution; Abbkine, Wuhan, China), and the Steap3 immunocomplexes were visualized using DAB (ZLI-9018; Zhongshan Biotech, Beijing, China). Histological images

were observed and captured under a light microscope (Olympus, Tokyo, Japan).

Immunofluorescence and TUNEL staining

Immunofluorescence staining and TUNEL staining were performed as described previously(3). In brief, liver sections were prepared through fixation in 4% neutral paraformaldehyde for more than 48 h, followed by embedding in paraffin. Then, the liver tissue samples were cut into 5-μm sections. For the analysis of the infiltration of inflammatory cells, mouse liver sections were first blocked with 10% goat serum and then incubated with a rabbit anti-mouse CD11b (Abcam, ab75476, Cambridge, UK) primary antibody at 4°C overnight. Then, the sections were washed with phosphate-buffered saline (PBS) and incubated with the appropriate secondary antibody for 1 h at 37°C. The secondary antibody used was a donkey anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (Thermo Fisher Scientific, A-10042, Massachusetts, USA). Apoptosis in paraffin-embedded liver sections was detected to evaluate liver injury by the TUNEL method (Roche, 11684817910) according to the manufacturer's protocol described previously. Nuclei were labeled with DAPI. Images were acquired under a fluorescence microscope (OLYMPUS BX51TRF, Tokyo, Japan) and with DP2-BSW software (version 2.2, Tokyo, Japan). The images were analyzed with Image-Pro Plus (version 6.0).

Quantitative real-time polymerase chain reaction (RT-PCR)

Quantitative RT-PCR was performed as described previously(4). Briefly, total mRNA was extracted from liver tissue and cultured cells using TRIZOL reagent (Invitrogen) and checked for quality using a Nanodrop 2000 (Thermo Fisher Scientific, Madison, WI, USA) and gel electrophoresis. Two micrograms of RNA was reverse transcribed into DNA with the Transcriptor First-Strand cDNA Synthesis Kit (no. 04896866001, Roche, Munich, Germany) according to the manufacturer′s instructions. Quantitative RT-PCR was performed with SYBR Green PCR Master Mix (Cat# 04887352001, Roche) to determine the expression levels of our genes of interest. The results were normalized against β-actin expression. The primer sequences of the target genes for RT-PCR are provided in Supplementary Table 1.

Western blot analysis

Western blot analyses were performed as previously described(5). Briefly, tissue or cell samples were lysed in RIPA lysis buffer (65 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing dissolved protease inhibitor cocktail tablets (04693132001; Roche) and phosphatase inhibitor tablets (4906837001; Roche). Protein concentrations were

quantified using BCA protein assay kit (23225; Thermo Fisher Scientific). Then, total protein samples were separated on 10% SDS-PAGE gel and transferred to PVDF membrane (IPVH00010; Millipore). Membranes were blocked with 5% skim milk at room temperature for 1 h and incubated overnight with the indicated primary antibodies at 4°C. The membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. Signals were visualized with enhanced chemiluminescence (ECL) reagents (170-5061; Bio-Rad, Hercules, CA, USA) and captured by the ChemiDoc XRS+ System (Bio-Rad). Protein expression was quantified by ImageJ software, and GAPDH was used as a control.

Isolation of primary hepatocytes and a hepatocyte hypoxia/reoxygenation (H/R) model

Primary hepatocytes were isolated from the liver of male mice aged 6-8 weeks using the collagenase perfusion method as previously described, and cells with more than 80% viability were used for further experiments(2). Briefly, after anesthesia by an intraperitoneal injection of sodium pentobarbital (50 mg/kg, SIGMA-ALDRICH, P3761), the liver of mice was fully digested by administering a solution containing 0.5% collagenase type IV (catalog no. 17104-019; Thermo Fisher Scientific) through the portal vein. The liver was then excised, minced, filtered through a 70-μm cell strainer (catalog no. 352350; Falcon, BD Biosciences) and centrifuged at 50 g for 2 min to obtain primary hepatocytes, which were further purified with a 50% Percoll solution (no. 17-0891-01, GE Healthcare Life Sciences, Buckinghamshire, England). The isolated cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2, water-saturated incubator at 37°C overnight. For H/R experiments, the medium was changed to sugar-free, serum-free DMEM. Cell hypoxia conditions (1% O_2, 5% CO_2, and 94% N_2) were created in a modular incubator chamber (Biospherix, Lacona, NY, USA). After 6h of hypoxia, the cells were returned to normal air conditions (95% air, 5% CO_2) and complete medium at the indicated time point to simulate liver I/R injury in vitro. The cells and related culture medium were collected for further experiments.

Plasmid construction

The entire homo Steap3 cDNA was cloned into pcDNA5-HA, pcDNA5-Flag and pHAGE-3×flag plasmids to express HA-tagged Steap3 and Flag-tagged Steap3 recombinant proteins. pcDNA5-HA-TAK1, pcDNA5-Flag-TAK1, and Flag-tagged TAK1 truncations were constructed using the same methods. GST-tagged Steap3 and GST-TAK1 were obtained by cloning Steap3 or TAK1 cDNA into the pcDNA5-GST-HA vector. Expression plasmids encoding truncated Steap3 (1-258,

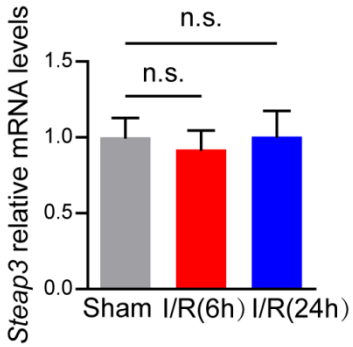
259-488) or TAK1 (1-300, 1-390, 301-579 and 391-579) were amplified using PCR and cloned into pcDNA5-flag and pcDNA5-HA, respectively, using standard molecular biology techniques. The primers used in this study are listed in Supplementary Table 3.

Coimmunoprecipitation (Co-IP) and Glutathione S-transferase pulldown assays

Co-IP assays were performed as previously described to identify interactions of Steap3 with other factors(6). For co-IP experiments, HEK293T cells were cotransfected with the indicated plasmids, and the cells were lysed with ice-cold IP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40) containing a protease inhibitor cocktail (04693132001, Roche). The obtained cell lysates were incubated with protein G-agarose beads (AA104307, Bestchrom) and the indicated antibody for 3 h at 4℃. The immunoprecipitated proteins were washed and analyzed by Western blot analysis. For pulldown assays, GST-Steap3 and GST-TAK1 fusion proteins were purified with glutathione-sepharose 4B beads (AA009305, Bestchrom) and then incubated with purified Flag-tagged proteins for an additional 4 h. Proteins that interacted were eluted and subjected to Western blot analysis.

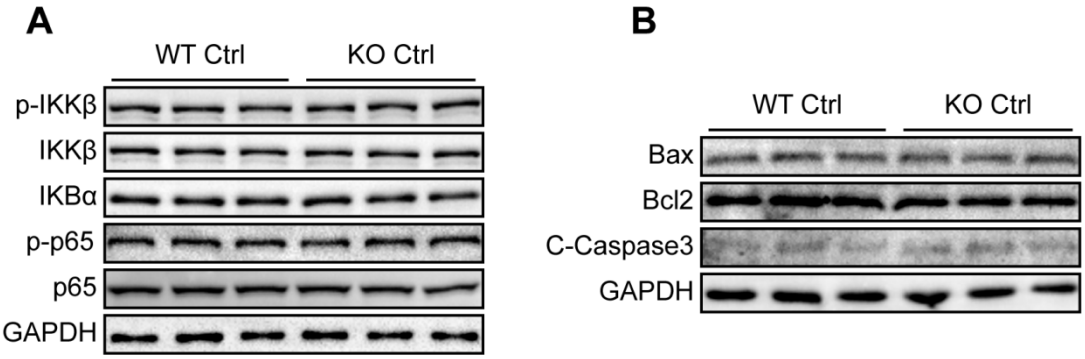
Supplementary Figures

Supplementary Fig. 1



Supplementary Fig1. The mRNA of *Steap3* was not affected in the liver upon I/R operation. The mRNA levels of *Steap3* in the liver of mice at 6 h and 24 h after hepatic I/R operation.

Supplementary Fig. 2



Supplementary Fig 2. Steap3 deficiency didn't affect inflammatory and apoptosis during hepatic I/R injury. (A) Western blot analysis of the Bax, Bcl2 and cleaved caspase-3 levels in cultured primary hepatocytes isolated from WT and *Steap3*-KO mice that without H/R stimulation. GAPDH served as the loading control. (B) The protein levels of NF-κB signaling pathway molecules in cultured primary hepatocytes isolated from WT and *Steap3*-KO mice that without H/R stimulation. GAPDH served as the loading control.

Supplementary Tables

Supplementary Table 1. Primers for real-time PCR detection.

Gene	Specie		Sequence5'---3'
Steap3	mice	F	AGTTCAGCTTCGTGCAGTCC
		R	AGCAGCGTGAGTGTGAATGT
Il6	mice	F	TAGTCCTTCCTACCCCAATTTCC
		R	TTGGTCCTTAGCCACTCCTTC
Il1b	mice	F	CCGTGGACCTTCCAGGATGA
		R	GGGAACGTCACACACCAGCA
Ccl2	mice	F	TACAAGAGGATCACCAGCAGC
		R	ACCTTAGGGCAGATGCAGTT
Cxcl10	mice	F	ATGACGGGCCAGTGAGAATG
		R	ATGATCTCAACACGTGGGCA
Bcl2	mice	F	TGGTGGACAACATCGCCCTGTG
		R	GGTCGCATGCTGGGGCCATATA
Bax	mice	F	TGAGCGAGTGTCTCCGGCGAAT
		R	GCACTTTAGTGCACAGGGCCTTG
Bad	mice	F	CCAGAGTTTGAGCCGAGTGAGCA
		R	ATAGCCCCTGCGCCTCCATGAT
β-actin	mice	F	GTGACGTTGACATCCGTAAAGA
		R	GCCGGACTCATCGTACTCC
Il6	human	F	GAGTAGTGAGGAACAAGCCAGA
		R	AAGCTGCGCAGAATGAGATGA
Il1b	human	F	TCGCCAGTGAAATGATGGCT
		R	TGGAAGGAGCACTTCATCTGTT
Ccl2	human	F	ATAGCAGCCACCTTCATTCCC
		R	CAGCTTCTTTGGGACACTTGC
Bcl2	human	F	GGTGGGGTCATGTGTGTGG
		R	CGGTTCAGGTACTCAGTCATCC
Bax	human	F	CCCGAGAGGTCTTTTTCCGAG
		R	CCAGCCCATGATGGTTCTGAT

Bad	human	F	CCCAGAGTTTGAGCCGAGTG
		R	CCCATCCCTTCGTCGTCCT
β-actin	human	F	CATGTACGTTGCTATCCAGGC
		R	CTCCTTAATGTCACGCACGAT

Supplementary Table 2. Antibodies for immunoblot analyses.

Antibody	Manufacturer	Cat No.
Steap3	Abclonal	A0683
p-IKKα/β	Cell Signaling	2694
Ikkβ	Cell Signaling Technology	8943
IkBα	Cell Signaling Technology	4814
p-p65	Cell Signaling Technology	3033
p65	Cell Signaling Technology	8242
Bcl2	Cell Signaling Technology	3498
Bax	Cell Signaling Technology	2772
C-Caspase3	Cell Signaling Technology	9664
p-ERK	Cell Signaling Technology	4370
ERK	Cell Signaling Technology	4695
p-JNK	Cell Signaling Technology	4668
JNK	Cell Signaling Technology	9252
p-p38	Cell Signaling Technology	4511
P38	Cell Signaling Technology	8690
p-ASK1	Cell Signaling Technology	3765
ASK1	ABclonal	A6274
p-TAK1	Abcam	ab192443
TAK1	Cell Signaling Technology	5206
Flag	Medical &Biological Laboratories	M185-3LL
HA	Medical &Biological Laboratories	M180-3
GAPDH	Cell Signaling Technology	2118

Supplementary Table 3. Primers for plasmid construction.

Gene		Primer sequence(5’-3’)
Flag-*Steap3*	F	TCGGGTTTAAACGGATCCATGTCGCACCAGCCTGCTGTTG
	R	GGGCCCTCTAGACTCGAGTCATACGTGGCTCGTCTTCTCG

HA-*TAK1*	F	TCGGGTTTAAACGGATCCATGTCTACAGCCTCTGCCGCCT
	R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
GST-HA-*TAK1*	F	TCGGGTTTAAACGGATCCATGTCTACAGCCTCTGCCGCCT
	R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
GST-HA-*Steap3*	F	TCGGGTTTAAACGGATCCATGTCGCACCAGCCTGCTGTTG
	R	GGGCCCTCTAGACTCGAGTCATACGTGGCTCGTCTTCTCG
Flag-*TAK1*	F	TCGGGTTTAAACGGATCCATGTCTACAGCCTCTGCCGCCT
	R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
HA-*Steap3*	F	TCGGGTTTAAACGGATCCATGTCGCACCAGCCTGCTGTTG
	R	GGGCCCTCTAGACTCGAGTCATACGTGGCTCGTCTTCTCG
Flag-*TAK1* (1-300)	F	TCGGGTTTAAACGGATCCATGTCTACAGCCTCTGCCGCCT
	R	GGGCCCTCTAGACTCGAGTCAATACTGTAATGGCTCATCTGC
Flag-*TAK1* (301-579)	F	TCGGGTTTAAACGGATCCCCTTGTCAGTATTCAGATGAAGG
	R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
Flag-*TAK1* (1-390)	F	TCGGGTTTAAACGGATCCATGTCTACAGCCTCTGCCGCCT
	R	GGGCCCTCTAGACTCGAGTCAAGCACTCATCCTCTTGCCCTC
Flag-*TAK1* (391-579)	F	TCGGGTTTAAACGGATCCGACATGTCTGAAATAGAAGCTAGG
	R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
Flag-*Steap3* (1-258)	F	TCGGGTTTAAACGGATCCATGTCGCACCAGCCTGCTGTTG
	R	GGGCCCTCTAGACTCGAGTCAGAAGAACTTGTTCTGGCTTTCC
Flag-*Steap3* (259-488)	F	TCGGGTTTAAACGGATCCATGAAGCTGCCCGTGTCCGTGG
	R	GGGCCCTCTAGACTCGAGTCAGTCTGTGGGCAGCGTGAACTTG

Supplementary References

1. Sakai N, Van Sweringen HL, Schuster R, Blanchard J, Burns JM, Tevar AD, et al. Receptor activator of nuclear factor-κB ligand (RANKL) protects against hepatic ischemia/reperfusion injury in mice. Hepatology (Baltimore, Md.) 2012;55:888-897.
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