Supplementary data for:

Tripartite motif-containing 27 attenuates liver ischemia/reperfusion injury by suppressing TAK1 via TAB2/3 degradation

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Supplementary Materials and Methods

Animals

Trim27-KO mice were created using the CRISPR/Cas9 system (1). The guide sequence-guide RNA (sgRNA) of the target DNA region was predicted by the online CRISPR design tool (http://chopchop.cbu.uib.no/), and Trim27-sgRNA expression vector was constructed using pUC57-sgRNA (Addgene, 51132) as the backbone vector. After mixing the purified products of Cas9 mRNA and sgRNA, the mixture was injected into the C57BL/6 mouse single-cell fertilized egg through the FemtoJet 5247 microinjection system, and the injected fertilized egg was transplanted into a surrogate female mouse. F0 generation mice obtained after about 19-21 days of pregnancy. Two weeks after birth, the ear tissues of the mice were taken out to extract the genomic DNA. The *Trim27*-KO mice were genetically identified using the following primers, F: 5'-CTTCGTGGAGCCTATGATGC-3'; R: 5-GACAGGGAGGAAATGACGGC-3'.

To generate Trim27-HTG mice, the full-length consensus CDS of the mouse *Trim27* (CCDS36607.1) gene was cloned downstream of the Alb promoter, and an Alb-*Trim27* transgene vector was obtained. This construct was then microinjected into pronuclear stage embryos. Two-cell embryos were transplanted into the oviducts of pseudo-pregnant foster mothers. Genomic DNA was extracted from the ear tissue of newborn mice for screening by PCR. The following primers were used, F: 5'-GAACCAATGAAATGCGAGGT-3'; R: 5'-CGCCGTCTCTTCTTCAAGTC-3'.

Mouse liver I/R injury model

We used an established 70% warm hepatic I/R injury model as previously described (2). In general, after the mice were anesthetized by an intraperitoneal injection of

sodium pentobarbital (50 mg/kg, Sigma-Aldrich, P3761), midline laparotomy was performed to expose the liver. Next, the portal vein, hepatic artery and bile duct above the branching to the left lateral lobe were clamped with microvascular clips to interrupt the blood supply to the left lateral/median lobes of the liver. By confirming the ischemic liver lobes were bleached to identified that the ischemic operation was successful. During ischemia, cover the abdomen with a thermostatic blanket, monitor rectal temperature, and maintain body temperature at 37 ± 0.5 ° C. No ischemic color change and no response to reperfusion are criteria for exclusion for further analysis
After ischemia for 1 h, the clamp was released for reperfusion. At each indicated time point after reperfusion, the mice were anesthetized to collect liver samples and serum for further analysis. Mice that underwent the same surgical procedure without vasculature occlusion served as sham controls.

Cell culture and treatment

The hepatocyte L02 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin. For treatment, DMSO (D2650-100ML, Sigma), MG132 (SML1135-5MG, Sigma), and Chloroquine (CQ) (C6628-50G, sigma) were used. For the hypoxia-reoxygenation (H/R) experiment, hepatocytes were challenged with hypoxia conditions (1% O2, 5% CO2, and 94% N2) in a modular incubator chamber (Biospherix, Lacona, NY, USA) with sugar-free, serum-free DMEM. For reoxygenation, the cells were returned to normal air conditions (95% air, 5% CO2) and medium. To generate TRIM27 overexpressed or knock down cell lines, lentiviral vectors were packaged in HEK293T cells using the plasmids containing Flag-TRIM27 or shRNA and packaging plasmids. The lentivirus mediated delivery of Flag-TTIM7 or shRNA into L02 cells in the same manner as previously described (3).

Immunohistochemical and Immunofluorescence staining

For immunohistochemical and immunofluorescence staining, liver tissues were dehydrated, paraffin embedded and sectioned (4-5 μm per section). CD11b and c-Caspase3 were detected to analysis immune cell accumulation and apoptosis. Primary antibodies against mouse CD11b (Boster, BM3925, Wuhan, China) and c-Caspase3 (9664S, Cell Signaling, USA) were used. The secondary antibodies used were goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (Thermo Fisher Scientific, A-10042, Massachusetts, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining were performed to detect cell apoptosis in liver according to the manufacturer’s protocol (Roche, 11684817910) as described previously (2). Immunohistochemical staining was

visualized using 3,3′-diaminobenzidine (DAB) (ZLI-9032, Zhongshan Biotech, Beijing, China). Immunofluorescence was visualized using a fluorescence microscope (OLYMPUS DX51, Tokyo, Japan) and quantified using Image-Pro Plus software.

Immunoprecipitation assays and Glutathione S-transferase pulldown assays

Immunoprecipitation assays were performed as previously described to identify the interactions of Trim27 with other factors (2). Briefly, cells were harvested with ice-cold IP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40). 10% cell lysate was applied as input and the remaining cell lysate were precleared and immunoprecipitated with Protein G agarose beads (AA104307, Bestchrom, Shanghai, China) and the indicated antibodies at 4°C overnight. The beads were then washed with high-concentration IP buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM EDTA, 1% Triton X-100) and low-concentration IP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) twice and boiled with 2×SDS loading buffer at 95°C for 10 minutes, followed by western blot analyses.

GST pull-down assays were performed to examine the direct interaction between TRIM27 and TAK1 as previously described (2). Cells were harvested with ice-cold IP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40). 10% cell lysate was applied as input and the remaining cell lysate were precleared and immunoprecipitated with with Glutathione Sepharose 4B beads (AA0071, Bestchrom, Shanghai, China) at 4 °C for 3 h. Subsequently， the purified GST-HA-TAK1 or GST-HA-TRIM27 beads were washed for five times and incubated with Flag-TRIM27 or Flag-TAK1 protein at 4 °C overnight. Then, the beads were washed three times and boiled with 2×SDS loading buffer, followed by western blot analyses. GST protein was used as the negative control.

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

Quantitative RT-PCR analyses were performed for quantification of mRNA expression as described previously (2). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The isolated RNA was quantified and checked for quality using a Nanodrop 2000 (Thermo Fisher Scientific, Madison, WI, USA) and gel electrophoresis. The cDNA was synthesized with 2 ug of RNA using the HiScript® III RT SuperMix for qPCR (+gDNA wiper) (Cat# R312, Vazme, Nanjing, China) according to the manufacturer′s instructions. Quantitative RT-PCR was performed with ChamQ^{TM} SYBR qPCR Master Mix (Cat# Q311-02, Vazme, Nanjing, China). The results were normalized against β-actin expression. The primer sequences of the target genes used in this study are listed in Supplementary Table 1.

Western blot

For western blot studies, 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).The protein concentration was measured using a BCA protein assay kit (23225; Thermo Fisher Scientific). Then, the protein samples were separated by sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel and transferred to a nitrocellulose PVDF membrane and blocked with 5% milk. Membranes were incubated at 4°C overnight with the indicated primary antibodies. After the blots were rinsed extensively with TBS-T buffer, we incubated them with the corresponding secondary antibodies for 1 h at room temperature. Chemiluminescence was performed with enhanced chemiluminescence (ECL) reagents (170-5061; Bio-Rad, Hercules, CA, USA) and captured by the ChemiDoc XRS+ System (Bio-Rad). GAPDH and β-actin were used as a loading control. Antibodies used in this study are listed in Supplementary Table 2.

Plasmid construction

The full-length homo *TRIM27* cDNA was cloned into pcDNA5-HA, pcDNA5-Myc and pHAGE-3×Flag plasmids to express HA-tagged TRIM27, Flag-tagged TRIM27 and Myc-tagged TRIM27 recombinant proteins. pcDNA5-HA-TAK1, pcDNA5-Flag-TAK1 and TAK1 truncations were constructed by the same methods. GST-tagged TRIM27 and GST-TAK1 were obtained by cloning TRIM27 or TAK1 cDNA into the pcDNA5-GST-HA vector. Expression plasmids encoding truncated TRIM27 (1-62aa, 1-132aa, 1-315aa, 63-132aa, 133-315aa and 316-513aa) or TAK1 (1-300aa, 1-480aa, 301-579aa and 481-579aa) were amplified using PCR and cloned into pcDNA5-GST-HA and pcDNA5-Flag plasmids, respectively, using standard molecular biology techniques. Several knockdown sequences for *TRIM27* were designed, and the knockdown sequences were constructed into the pLKO.1 vector. The primers used in this study are listed in Supplementary Table 3.

Supplementary Figures

Supplementary Fig. 1

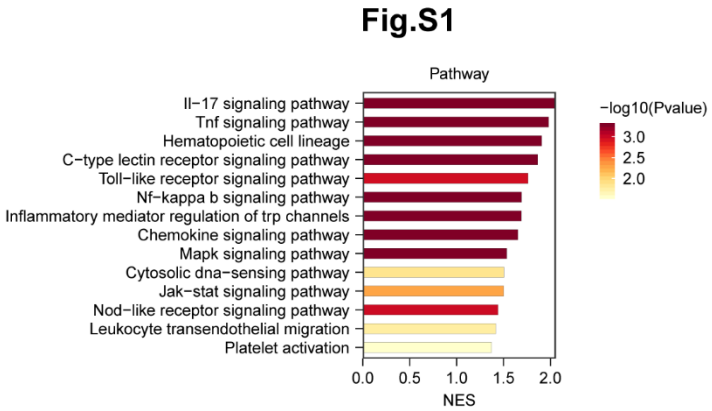

Fig. S1. GSEA showing the changes of inflammation-related pathways based on RNA-seq data set of WT and *Trim27*-KO groups.

Supplementary Tables

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

Gene	Specie		Sequence5'---3'
TRIM27	human	F	GGGCTTCAAGGAGCAAATCC
		R	AGGCTCAAGAGTTCAGCTCG
Tnfα	human	F	TGGCGTGGAGCTGAGAGATA
		R	TGATGGCAGAGAGGAGGTTG
Il6	human	F	GAGTAGTGAGGAACAAGCCAGA
		R	AAGCTGCGCAGAATGAGATGA
Il1b	human	F	GCTGGAGAGTGTAGATCCCAAA
		R	TGCTTGAGAGGTGCTGATGT
Bax	human	F	CCCGAGAGGTCTTTTTCCGAG
		R	CCAGCCCATGATGGTTCTGAT
Bcl2	human	F	GGTGGGGTCATGTGTGTGG
		R	CGGTTCAGGTACTCAGTCATCC
β-actin	human	F	CATGTACGTTGCTATCCAGGC
		R	CTCCTTAATGTCACGCACGAT
Trim27	mice	F	GGAGCAAATCCAGAACCGACT
		R	GCCCCGTTGATGCTGTTATAG
Tnfα	mice	F	CATCTTCTCAAAATTCGAGTGACAA
		R	TGGGAGTAGACAAGGTACAACCC
Il6	mice	F	TAGTCCTTCCTACCCCAATTTCC
		R	TTGGTCCTTAGCCACTCCTTC
Il1b	mice	F	CCGTGGACCTTCCAGGATGA
		R	GGGAACGTCACACACCAGCA

Ccl2	mice	F	TACAAGAGGATCACCAGCAGC
		R	ACCTTAGGGCAGATGCAGTT
Cxcl2	mice	F	GCGCCCAGACAGAAGTCATA
		R	CAGTTAGCCTTGCCTTTGTTCA
Bad	mice	F	CCAGAGTTTGAGCCGAGTGAGCA
		R	ATAGCCCCTGCGCCTCCATGAT
Bax	mice	F	TGAGCGAGTGTCTCCGGCGAAT
		R	GCACTTTAGTGCACAGGGCCTTG
Bcl2	mice	F	TGGTGGACAACATCGCCCTGTG
		R	GGTCGCATGCTGGGGCCATATA
β-actin	mice	F	GTGACGTTGACATCCGTAAAGA
		R	GCCGGACTCATCGTACTCC

Supplementary Table 2. Antibodies for immunoblot analyses.

Antibody	Cat No.	Manufacturer
TRIM27	15099	CST
GAPDH	60004-1-Ig	proteintech
P-Ikkb	2694	CST
Ikkb	0714	CST
IkBa	66418-1-lg	proteintech
p-p65	3033	CST
P65	8242	CST
β-actin	AC026	ABCLONAL
Bad	9292	CST
Bax	50599-2-lg	proteintech
Bcl2	3498	CST
C-Caspase3	9664	CST

Flag	M185-3LL	MBL
p-ERK	4370	CST
ERK	4695	CST
p-JNK	4668	CST
JNK	9252	CST
p-p38	4511	CST
P38	9212	CST
p-ASK1	3765	CST
ASK1	A6274	ABCLONAL
p-TAK1	4531	CST
TAK1	ab109526	ABCAM
Flag	20543-I-AP	Proteintech
HA	M180-3	MBL
Myc	M047-3	MBL

Supplementary Table 3. Primers for plasmid construction.

Gene	**Specie**		**Sequence5'---3'**
HA-TAK1	human	F	TCGGGTTTAAACGGATCCATGTCTACAGCCTCTGCCGCCT
		R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
Flag-TRIM27	human	F	TCGGGTTTAAACGGATCCATGGCCTCCGGGAGTGTGGC
		R	GGGCCCTCTAGACTCGAGTCAAGGGGAGGTCTCCATGG
GST-HA-TAK1	human	F	TCGGGTTTAAACGGATCCATGTCTACAGCCTCTGCCGCCT
		R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
GST-HA-TRIM27	human	F	TCGGGTTTAAACGGATCCATGGCCTCCGGGAGTGTGGC
		R	GGGCCCTCTAGACTCGAGTCAAGGGGAGGTCTCCATGG
Flag-TAK1	human	F	TCGGGTTTAAACGGATCCATGTCTACAGCCTCTGCCGCCT
		R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
Flag-TAK1(1-30	human	F	AGAGCCCGGGCGGATCCATGTCTACAGCCTCTGCCGC

0aa)		R	TCGACGAATTGCTCGAGTCAATACTGTAATGGCTCATCTGCTCC
Flag-TAK1(1-480aa)	human	F	AGAGCCCGGGCGGATCCATGTCTACAGCCTCTGCCGC
		R	TCGACGAATTGCTCGAGTCATAGTTGGTGATCCAGTGTAAGATAAGC
Flag-TAK1(301-579aa)	human	F	AGAGCCCGGGCGGATCCCCTTGTCAGTATTCAGATGAAGGAC
		R	TCGACGAATTGCTCGAGTCATGAAGTGCCTTGTCGTTTCT
Flag-TAK1(481-579aa)	human	F	TCGGGTTTAAACGGATCCATGCAGCCTCTAGCACCGTGCC
		R	GGGCCCTCTAGACTCGAGTCATGAAGTGCCTTGTCGTTTCT
GST-HA-TRIM27（1-62aa）	human	F	TCGGGTTTAAACGGATCCATGGCCTCCGGGAGTGTGGC
		R	GGGCCCTCTAGACTCGAGTCACTGCGGGAAGGTCTCCCG
GST-HA-TRIM27（1-132aa）	human	F	TCGGGTTTAAACGGATCCATGGCCTCCGGGAGTGTGGC
		R	GGGCCCTCTAGACTCGAGTCAGAGCGGCAGCACGCTGTGG
GST-HA-TRIM27（1-315aa）	human	F	TCGGGTTTAAACGGATCCATGGCCTCCGGGAGTGTGGC
		R	GGGCCCTCTAGACTCGAGTCATGAGTATAACTGAGCCTCTCTTAATTC
GST-HA-TRIM27（63-132aa）	human	F	TCGGGTTTAAACGGATCCAGGCACATGCGGCCCAACC
		R	GGGCCCTCTAGACTCGAGTCAGAGCGGCAGCACGCTGTGG
GST-HA-TRIM27（133-315aa）	human	F	TCGGGTTTAAACGGATCCGAGGAGGCGGTGGAGGGC
		R	GGGCCCTCTAGACTCGAGTCATGAGTATAACTGAGCCTCTCTTAATTC
GST-HA-TRIM27（316-513aa）	human	F	TCGGGTTTAAACGGATCCGTGGACGTGACTCTGGACCC
		R	GGGCCCTCTAGACTCGAGTCAAGGGGAGGTCTCCATGG
HA- TRIM27	human	F	TCGGGTTTAAACGGATCCATGGCCTCCGGGAGTGTGGC
		R	GGGCCCTCTAGACTCGAGTCAAGGGGAGGTCTCCATGG
Myc-Ub	human	F	TCGGGTTTAAACGGATCCATGCAGATTTTCGTGAAAACCCT
		R	GGGCCCTCTAGACTCGAGTTAACCACCACGAAGTCTCAACACA

HA-TAB2	human	F	TCGGGTTTAAACGGATCCATGGCCCAAGGAAGCCACC
		R	GGGCCCTCTAGACTCGAGTCAGAAATGCCTTGGCATCTC
Flag-TAB3	human	F	TCGGGTTTAAACGGATCCATGGCGCAAAGCAGCCCACA
		R	GGGCCCTCTAGACTCGAGTCAGGTGTACCGTGGCATCTCG
Myc-TRIM27	human	F	TCGGGTTTAAACGGATCCATGGCCTCCGGGAGTGTGGC
		R	GGGCCCTCTAGACTCGAGTCAAGGGGAGGTCTCCATGG
shTRIM27-1	human	F	CCGGCAGGGCTGAAAGAATCAGGATCTCGAGATCCTGATTCTTTCAGCCCTG T TTTTG
		R	AATTCAAAAACAGGGCTGAAAGAATCAGGATCTCGAGATCCTGATTCTTTCA GCCCTG
shTRIM27-2	human	F	CCGGGCCCTACTTCAGTCTGAGTTACTCGAGTAACTCAGACTGAAGTAGGGC TTTTTG
		R	AATTCAAAAAGCCCTACTTCAGTCTGAGTTACTCGAGTAACTCAGACTGAAG TAGGGC
shTRIM27-3	human	F	CCGGGAGAAGATTGTTTGGGAGTTTCTCGAGAAACTCCCAAACAATCTTCTC TTTTTG
		R	AATTCAAAAAGAGAAGATTGTTTGGGAGTTTCTCGAGAAACTCCCAAACAA TCTTCTC

Supplementary References

1. **Chen L, Huang J, Ji YX,** Mei F, Wang PX, Deng KQ, et al. Tripartite Motif 8 Contributes to Pathological Cardiac Hypertrophy Through Enhancing Transforming Growth Factor β-Activated Kinase 1-Dependent Signaling Pathways. Hypertension (Dallas, Tex. : 1979) 2017;69:249-258.

2. **Guo WZ, Fang HB, Cao SL,** Chen SY, Li J, Shi JH, et al. Six-Transmembrane Epithelial Antigen of the Prostate 3 Deficiency in Hepatocytes Protects the Liver Against Ischemia-Reperfusion Injury by Suppressing Transforming Growth Factor-β-Activated Kinase 1. Hepatology (Baltimore, Md.) 2020;71:1037-1054.

3. **Yan ZZ, Huang YP, Wang X,** Wang HP, Ren F, Tian RF, et al. Integrated Omics Reveals Tollip as an Regulator and Therapeutic Target for Hepatic Ischemia-Reperfusion Injury in Mice. Hepatology (Baltimore, Md.) 2019;70:1750-1769.