# Regulator of G-protein signaling 14 protects the liver from ischemia reperfusion injury by suppressing TAK1 activation

## Supplementary Methods and Materials

### *Animals*

Male mice (8-10 weeks of age, 25±2 g) were housed in polycarbonate cages within a specific pathogen-free, temperature-controlled (23±2°C) facility, with a 12-h light/dark cycle. Food and water were provided ad libitum to the animals. Animal protocols were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University and were conducted in accordance with the guidelines outlined by the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication 86-23, revised 1985). C57BL/6 mice were used to produce *RGS14* knockout (*RGS14*-KO) and hepatocyte-specific RGS14 transgene (RGS14-TG) strains. The CRISPR/Cas9 system was used to generate the *RGS*14-KO mouse line. First, the single-guide RNA (sgRNA) for the target gene was predicted using an online design tool (http://chopchop.cbu.uib.no/). Then, pUC57-sgRNA (Addgene 51132) was used as the backbone vector to construct the RGS14-sgRNA expression vector. After acquiring the sgRNA and pST1374-Cas9 mRNA transcription products (Addgene 44758), the mixture was injected into C57BL/6 mouse single-cell fertilized eggs using the FemtoJet 5247 microinjection system. The fertilized eggs were transplanted into a surrogate female mouse, and after 19-21 d, F0 generation mice were obtained. Genomic DNA samples were extracted from the ear tissues of immature mice and the expression of

RGS14 was quantified. The *RGS14*-KO mice were identified by performing PCR using primers P1: 5'-CTGTGTGGACACTCCCATCC-3' and P2: 5'-ACCACAGAGAGAAGCAGCAC-3'.

To generate RGS14-TG mice, the CDS region of the mouse RGS14 was amplified and inserted into the Alb promoter expression vector to construct the Alb-RGS14 transgene vector. The PvuI restriction endonuclease were used to conduct plasmid linearization, and then the vector was purified and injected into mice to obtain the F0 generation. After successful transformants were identified, the Alb-RGS14-positive mouse was subjected to crossing with a wild-type mouse. Finally, a stable hepatocyte-specific RGS14 transgenic mouse was obtained. The RGS14-TG mice were identified by performing PCR using the primers TG-check F1: 5'-GAACCAATGAAATGCGAGGT-3' and TG-check R1 5'-AAGATCTGAAGCTGCTGTGC-3'.

***Hepatic IRI model***

We used an established partial (70%) liver warm ischemia model as previously described methods (1). Mice were anesthetized by intraperitoneal injection (sodium pentobarbital 80 mg/kg, Sigma-Aldrich, P3761), and after the anesthesia was completed, laparotomy was performed along the midline of the abdomen to expose the liver. The blood supply of the left lateral/median lobes of the liver was interrupted by non-invasive clamps. After 1 h of ischemia, the clamp was removed for reperfusion. The mice were anesthetized to collect serum and liver samples for further analysis.

Sham-operated mice were subjected to the same procedure without vascular occlusion.

***Liver damage assessment***

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using the ADVIA 2400 Chemistry System (Siemens, Tarrytown, NY, USA) according to the manufacturer's instructions. Histopathological analysis was performed as previously described methods(1). Liver samples were fixed in 10% formalin, dehydrated, embedded in paraffin and sliced into 5-μm-thick continuous paraffin sections. Next, the samples were stained with hematoxylin and eosin (HE, [cat. no. G1004; Wuhan Servicebio Biotechnology Co., Ltd.]; eosin [cat. no. BA-4024; Zhuhai Basso Biotechnology Co., Ltd.]). Liver tissue necrosis was observed under a microscope.

***Immunofluorescence staining***

Paraffin-embedded liver sections were stained with CD11b and TUNEL after conduction of deparaffinization and rehydration.

CD11b: The sections were blocked using 10% BSA before being incubated with primary antibodies against mouse CD11b (Boster, BM3925, 1:800 dilution) overnight at 4°C. After performing washing steps using PBS, the secondary antibodies (Alexa Fluor 568 goat anti-rabbit IgG (H+L), Invitrogen A11036, Massachusetts, USA) were added to the samples, which were then incubated for 1 h at 37°C to detect the presence of inflammatory cells. The nuclei of the cells were labeled with DAPI. The TUNEL

staining kit (Roche, 11684817910) was performed to detect cell apoptosis following the manufacturer's instructions.

***Confocal microscopy***

L02 cells were co-transfected with HA-RGS14 and Flag-TAK1 plasmids for 24 h, then washed with PBS and fixed with 4% formaldehyde for 30 minutes. The cells were rinsed again with PBS and treated with 0.1% Triton X-100, and then incubated with mouse anti-Flag tag and rabbit anti-HA tag antibodies overnight at 4 °C. After washed with PBS and labeled with fluorophore-conjugated secondary antibodies for 1h at room temperature under dark conditions. And DAPI (S36939; Invitrogen; Carlsbad, CA, USA) were used for nuclei staining. The images were captured with the confocal laser scanning microscope (TCS SP8; Leica; Wetzler, Germany). The antibodies used were listed in Supplementary Table 2.

***Immunohistochemistry staining***

The paraffin-embedded liver sections were subjected to immunostaining with Cleaved-caspase3(C-Caspase3) and RGS14 after deparaffinization and rehydration. The sections were incubated in EDTA at 100℃ for 20 min and then blocked in BSA. Next, the primary antibodies against mouse C-caspase3 (CST, 9664, 1:150 dilution) and mouse RGS14 (Boster, BA3100-2, 1:100 dilution) were added to the samples and incubated overnight at 4°C. After the sections were rewarmed for 30 min at 37°C, they were subjected to washing steps with PBS and incubated with secondary antibodies

(ZSGB-bio, PV-9001). Finally, 3'-diaminobenzidine (DAB) (ZSGB-bio, ZLI--9018) was used to visualize the sections. Hematoxylin was used to label the nuclei.

**Cell culture and hypoxia-reoxygenation (HR) experiment**

Hepatocyte L02 cells were purchased from the ATCC and cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. To inhibit TAK1 activation, hepatocytes were treated with the TAK1 inhibitor (5Z)-7-oxozeaenol (O9890-1 MG, Sigma, St. Louis, MO, USA) at a concentration of 2 μM. HR experiments were performed in which hepatocytes in the rapid growth phase were treated with hypoxia (1% $O_2$, 5% $CO_2$) in sugar-free, serum-free DMEM for 6 h. Next, the cells were cultured in normal air conditions (95% air, 5% $CO_2$) and 10% fetal bovine serum medium for 6 h.

***Real-time quantitative polymerase chain reaction (RT-qPCR)***

Total RNA was extracted from animal tissues and cells using TRIzol reagent (Invitrogen). RNA concentrations and purity were detected using a NanoDrop 2000 (Thermo Fisher Scientific, Madison, WI, USA), and RNA integrity was detected using gel electrophoresis. cDNA was synthesized with 2 μg RNA using HiScript III RT SuperMix for qPCR (+gDNA wiper; cat. no. R312; Vazyme, Nanjing, China). RT-qPCR analyses were performed using ChamQTM SYBR qPCR Master Mix (cat. no. Q311-02; Vazyme) to quantify mRNA expression. The primer sequences used in this study are listed in Supplementary Table 1.

***Western-blotting***

In this study, liver or cell samples were lysed in RIPA lysis buffer and then boiled at 95°C for 15 min. Protein concentrations were measured using a BCA protein assay kit (cat. no. 23225; Thermo Fisher Scientific). Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes. The membranes were incubated at 4°C overnight with primary antibodies, followed by incubation with corresponding secondary antibodies. A ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA, USA) was used to detect protein signals. ImageJ software was used to quantify protein levels. All antibodies used for western blotting are listed in Supplementary Table 2.

***Plasmid construction***

The full-length CDS sequences for RGS14 and TAK1 were amplified from homo cDNA, and then cloned into pcDNA5-Flag, pcDNA5-HA, and pcDNA5-Myc vectors, respectively, using the infusion method to construct the full-length overexpressed plasmids for RGS14 and TAK1 with FLAG, HA, and MYC tags. We designed primers to construct the truncated Flag-RGS14 (1-202, 203-489, 374-566) plasmid and the truncated plasmid of Flag-TAK1 (1-300, 1-480, 301-579, 481-579). Glutathione S-transferase (GST)-tagged RGS14 and GST-TAK1 were obtained by cloning RGS14 or TAK1 cDNA into the pcDNA5-GST-HA vector. The primers used in this study are listed in Supplementary Table 3

### *Construction of stable cell lines*

The primers used for *RGS14* knockdown and overexpression plasmid construction are listed in Supplementary Table 3. Lentiviral vectors were packaged in HEK293T cells using plasmids containing sh*RGS14* or shRNA and packaging plasmids. After 48 h, harvested virus and infected L02 cells. Finally, stable cell lines were obtained by treating the infected cells with puromycin. The stable cell lines were identified by western blotting and PCR.

### *Co-immunoprecipitation (Co-IP) and Glutathione S-transferase pulldown assays*

Co-IP assays were performed as described previously (1) to identify the interactions of RGS14 with other factors. For the Co-IP assay, HEK293T cells were co-transfected with the indicated plasmids, and polythyleneimine (PEI ;24765-1; Polysciences Inc., Warrington, PAOA, UK) was used as the transfection reagent according to the manufacturer's instructions. At 24 h after transfection, the cells were lysed with 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 (cat. no. 04693132001; Roche). Part of the lysate was used as input, and the remaining lysates were incubated with protein G-agarose beads (cat. no. AA104307; Bestchrom, Shanghai, China) and 1 μg of the indicated antibody overnight at 4℃. The immunoprecipitated proteins were washed six times with cold IP buffer and analyzed by western blot analysis. For the pull-down assay, GST-HA-RGS14 and GST-HA-TAK1 fusion proteins were purified with glutathione Sepharose 4B beads (cat. no. AA0072; Bestchrom) and then incubated with

purified FLAG-tagged proteins for an additional 4 h at 4℃. The proteins that interacted were eluted and subjected to western blot analysis.

***RNA-seq and data analysis***

A hierarchical nested clustering tree was constructed based on the similarity of different liver tissue from the *RGS14*-KO group and WT group after hepatic IRI. The unweighted pair-group method with arithmetic mean was adopted and then visualized using the hclust function. Sequencing was conducted using the MGISEQ-2000 with a read length of 50 bp. Reads were mapped to reference genome sequences (mm10/GRCm38) using the HISAT2 software (version 2.1.0). The files obtained were converted into binary BAM format using SamTools (Version 1.4). Fragments per kilobase of exon model per million reads mapped values were calculated using the StringTie software (version 1.3.3b) with default parameters. Differential gene expression was calculated using the DESeq2 software (version 1.2.10) based on the following two criteria: (1) fold change >1.5 and (2) adjusted P value <0.05. Finally, the fold change and P value were presented as a volcano plot using the ggplot2 package.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is an integrated database that contains genomic, chemical, and system functional information. The annotations of all genes in the selected genome were downloaded from the KEGG database. The Fisher's exact test was used to perform KEGG pathway enrichment analysis using our in-house R script and statistical significance was set at $P<0.05$. Visualization was conducted using the ggplot2 package.

Gene set enrichment analysis was performed using gene sets of GO terms to rank genes

based on their expression change and to analyze the whole-genome changes. The process was performed using the Java GSEA (version 3.0) platform. A P value <0.05 and a false discovery rate value <0.25 were considered significant.

Metascape (http://metascape.org/) is an online database that is updated regularly with data from GO, KEGG, Reactome and other databases (3). Enrichment analysis was performed according to the significantly changed genes identified in two groups. Then, the markedly enriched genes were selected and clustered to construct a network that was based on the similarities and relevance of the gene candidates. Different clusters are labeled with different colors.

**Supplementary Tables**

**Supplementary Table 1. Primers for real-time qPCR detection**

| **Gene** | | **Sequence5'---3'** |
|---|---|---|
| Mouse *RGS14* | F | GGCTTACTTCACTGAGTTCCTG |
| | R | ACTCGTGGTAGATGTTGTGGG |
| Mouse *Il6* | F | TAGTCCTTCCTACCCCAATTTCC |
| | R | TTGGTCCTTAGCCACTCCTTC |
| Mouse *Il1b* | F | CCGTGGACCTTCCAGGATGA |
| | R | GGGAACGTCACACACCAGCA |
| Mouse *Ccl2* | F | TACAAGAGGATCACCAGCAGC |
| | R | ACCTTAGGGCAGATGCAGTT |
| Mouse *Tnf* | F | CATCTTCTCAAAATTCGAGTGACAA |
| | R | TGGGAGTAGACAAGGTACAACCC |
| Mouse *Cxcl10* | F | ATGACGGGCCAGTGAGAATG |
| | R | ATGATCTCAACACGTGGGCA |
| Mouse *Bcl2* | F | TGGTGGACAACATCGCCCTGTG |
| | R | GGTCGCATGCTGGGGCCATATA |
| Mouse *Bax* | F | TGAGCGAGTGTCTCCGGCGAAT |
| | R | GCACTTTAGTGCACAGGGCCTTG |
| Mouse *Bad* | F | CCAGAGTTTGAGCCGAGTGAGCA |
| | R | ATAGCCCCTGCGCCTCCATGAT |
| Mouse *β-actin* | F | GTGACGTTGACATCCGTAAAGA |
| | R | GCCGGACTCATCGTACTCC |
| Human *RGS14* | F | CGCGGAAAACGTGACTTTCTG |

| | | |
|---|---|---|
| | R | CTGACGGTCGATGTTCACTGG |
| Human *Il6* | F | GAGTAGTGAGGAACAAGCCAGA |
| | R | AAGCTGCGCAGAATGAGATGA |
| Human *Il1b* | F | GCTGGAGAGTGTAGATCCCAAA |
| | R | TGCTTGAGAGGTGCTGATGT |
| Human *Ccl2* | F | ATAGCAGCCACCTTCATTCCC |
| | R | CAGCTTCTTTGGGACACTTGC |
| Human *Tnf* | F | TGGCGTGGAGCTGAGAGATA |
| | R | TGATGGCAGAGAGGAGGTTG |
| Human *Cxcl10* | F | GTGGCATTCAAGGAGTACCTC |
| | R | TGATGGCCTTCGATTCTGGATT |
| Human *Bcl2* | F | GGTGGGGTCATGTGTGTGG |
| | R | CGGTTCAGGTACTCAGTCATCC |
| Human *Bax* | F | CCCGAGAGGTCTTTTTCCGAG |
| | R | CCAGCCCATGATGGTTCTGAT |
| Human *Bad* | F | CCCAGAGTTTGAGCCGAGTG |
| | R | CCCATCCCTTCGTCGTCCT |
| Human *β-actin* | F | CATGTACGTTGCTATCCAGGC |
| | R | CTCCTTAATGTCACGCACGAT |

**Supplementary Table 2. Antibodies for immunoblot analyses**

| **Antibody** | **Cat No.** | **Manufacturer** |
|---|---|---|
| RGS14 | 16258-1-AP | Proteintech |
| p-IKKβ | 2694 | CST |
| IKKβ | A0714 | Abclonal |
| p-P65 | 3033 | CST |
| P65 | 8242 | CST |
| IκBα | 4814 | CST |
| Bcl2 | A19693 | Abclonal |
| C-Caspase3 | 9664 | CST |
| Bax | 2772 | CST |
| p-ERK | 4370 | CST |
| ERK | 4695 | CST |
| p-JNK | 4668 | CST |
| JNK | 9252 | CST |
| p-p38 | 4511 | CST |
| p38 | 8690 | CST |
| p-TAK1 | 4508 | CST |
| TAK1 | 5206 | CST |
| GAPDH | 2118 | CST |
| Actin | AC026 | Abclonal |
| Flag | 20543-AP | Proteintech |
| HA | 3724s | CST |
| Myc | AE009 | Abclonal |

**Supplementary Table 3. Primers for plasmid construction**

| Specie | Gene | | Sequence5'---3' |
|---|---|---|---|
| Human | Flag-RGS14 | F | TCGGGTTTAAACGGATCCATGCCAGGGAAG CCCAAGCACC |
| | | R | GGGCCCTCTAGACTCGAGTCAGAGGGCTGA GTCGGTGGTG |
| Human | HA-TAK1 | F | TCGGGTTTAAACGGATCCATGTCTACAGCCT CTGCCGCCT |
| | | R | GGGCCCTCTAGACTCGAGTCATGAAGTGCCT TGTCGTTTCT |
| Human | Flag-TAK1 | F | TCGGGTTTAAACGGATCCATGTCTACAGCCT CTGCCGCCT |
| | | R | GGGCCCTCTAGACTCGAGTCATGAAGTGCCT TGTCGTTTCT |
| Human | GST-HA | F | TCGGGTTTAAACGGATCCATGTCCCCTATAC TAGGTTATTGG |
| | | R | GGGCCCTCTAGACTCGAGAGCGTAATCTGGA ACATCGTATGGGTATTTTGG |
| Human | GST-HA-RGS14 | F | TCGGGTTTAAACGGATCCATGCCAGGGAAG CCCAAGCACC |
| | | R | GGGCCCTCTAGACTCGAGTCAGAGGGCTGA GTCGGTGGTG |
| Human | GST-HA-TAK1 | F | TCGGGTTTAAACGGATCCATGTCTACAGCCT CTGCCGCCT |
| | | R | GGGCCCTCTAGACTCGAGTCATGAAGTGCCT TGTCGTTTCT |
| Human | Myc-RGS14 | F | TCGGGTTTAAACGGATCCATGCCAGGGAAG CCCAAGCACC |
| | | R | GGGCCCTCTAGACTCGAGTCAGAGGGCTGA GTCGGTGGTG |
| Human | Flag-TAK1 (1-300) | F | TCGGGTTTAAACGGATCCATGTCTACAGCCT CTGCCGCCT |
| | | R | GGGCCCTCTAGACTCGAGTCATGAAGTGCCT TGTCGTTTCT |
| Human | Flag-TAK1 (301-579) | F | TTACAGTATAGCCTTTCTACTTACTACCAGC AATG |
| | | R | AGAAAGGCTATACTGTAATGGCTCATCTGCT CC |
| Human | Flag-TAK1 (1-480) | F | TCGGGTTTAAACGGATCCTGGACCCCTGATG ATTCCACAG |
| | | R | GGGCCCTCTAGACTCGAGTCATGAAGTGCCT TGTCGTTTCT |

| | | | |
|---|---|---|---|
| Human | Flag-TAK1 (481-579) | F | AGTCATCCAGCCTTTCTACTTACTACCAGCAATG |
| | | R | TAGAAAGGCTGGATGACTTCGAGTTGGCTTTTC |
| Human | Flag-RGS14 (1-202) | F | TCGGGTTTAAACGGATCCAGCCCTGACGCCACGAGG |
| | | R | GGGCCCTCTAGACTCGAGTCAGCCGAGGCGCGAGGAGC |
| Human | Flag-RGS14 (203-489) | F | CGCCTCGGCATCGAAGGCCTGGTGGAG |
| | | R | GCCTTCGATGCCGAGGCGCGAGGAGCC |
| Human | Flag-RGS14 (374-566) | F | TCGGGTTTAAACGGATCCATGCCAGGGAAGCCCAAGC |
| | | R | GGGCCCTCTAGACTCGAGTCAGAGCCTGTTTTCCAGCCGCAC |
| Human | RGS14-S | | TCGGGTTTAAACGGATCCATGCCAGGGAAGCCCAAGCACC |
| | RGS14-AS | | GGGCCCTCTAGACTCGAGTCAGAGGGCTGAGTCGGTGGTG |
| Human | RGS14-shRNA1 | F | CCGGCACGGAGGGTGAAAGTGAAAGCTCGAGCTTTCACTTTCACCCTCCGTG TTTTTG |
| | | R | AATTCAAAAACACGGAGGGTGAAAGTGAAAGCTCGAG CTTTCACTTTCACCCTCCGTG |
| Human | RGS14-shRNA2 | F | CCGGCCCACCTAGAACTCAGGATAACTCGAGTTATCCTGAGTTCTAGGTGGG TTTTTG |
| | | R | AATTCAAAAACCCACCTAGAACTCAGGATAACTCGAGTTATCCTGAGTTCTAGGTGGG |

**Supplementary References**

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3. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al.Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun. 2019; 10: 1523.