CCAAT/enhancer-binding protein homologous protein promotes ROS-mediated liver ischemia and reperfusion injury by inhibiting mitophagy in hepatocytes

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Supplementary Materials and Methods

Chimeric mice generation

The chimeric mice were generated as previously described [1]. Bone marrow cells were obtained from the femurs and tibias of 8-week-old male WT mice. 1×10^7 bone marrow cells were intravenously injected into 5-week-old WT or *CHOP* KO mice which had received lethally irradiated to generate chimeric mice. The mice were housed under

specific pathogen-free conditions for four weeks after the bone marrow transplantation and then were subjected to a warm hepatic IR model. The properties of the chimeric mice are as follows: KO mice that received WT bone marrow (WT→KO) represents the mice expressing CHOP only in myeloid cells, while the liver parenchymal cells including hepatocytes are *CHOP* deficiency; WT mice that received WT bone marrow (WT→WT) represents a chimeric control mice.

Liver IR injury model

A mouse model of segmental (70%) warm hepatic IR model was performed as previously described [2]. Briefly, mice were anesthetized by 2.5% isoflurane. The arterial and portal venous blood supply to the cephalad liver lobes was interrupted with an atraumatic clip for 90min. Mice were sacrificed at 6h of reperfusion. Sham controls (n=6) underwent the same procedure, but without vascular occlusion. In some experiments, mice were administered intraperitoneally with 3-Methyladenine (3-MA, 30 mg/kg, Sigma, St. Luis, MO) and Mito TEMPO (MT, 3 mg/kg, Sigma, St. Luis, MO), separately or together, 3 h prior to liver IR onset.

Serum biochemical analysis

Serum alanine aminotransferase (sALT) levels were measured by an automated chemical analyzer (Olympus Automated Chemistry Analyzer, Tokyo, Japan). The serum Myeloperoxidase (MPO) levels of the patients were measured by ELISA Kit according to the manufacture's protocols (Cusabio Technology, US).

Histopathology

Liver tissues were incubated in 4% paraformaldehyde for at least 24h, then embedded in paraffin. The specimens were sectioned at 4μm and stained with hematoxylin and eosin (H&E). The severity of IR injury was scored using Suzuki's criteria on a scale from 0-4. No necrosis, congestion/ centrilobular ballooning is given a score of 0, while severe congestion and >60% lobular necrosis is giver a score of 4.

TUNEL staining

Paraffin section of liver tissues were treated in toluene and then dehydrated by a graded series of ethanol solutions. TUNEL staining of liver tissues was performed using a fluorescent detection kit according to the manufacturer's instructions.

Primary human and murine hepatocytes isolation

Primary human hepatocytes were isolated and cultured according to previously described methods [3]. Primary murine hepatocytes were isolated from 8 weeks old WT and *CHOP* KO mouse using a two-stage collagenase perfusion method as described [4]. The hepatocytes were seeded in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 u/ml penicillin and 100 μg/ml streptomycin.

Cell treatment

Hypoxia/reoxygenation treatment performed as previously described [5]. For hypoxia,

the medium was replaced with serum- and glucose-free DMEM/F12 that was equilibrated with 5% CO_2, 94% N_2, and 5% O_2. After 60min of hypoxia, cells were incubated under normoxic conditions (air/5% CO_2) for 6 h. Primary human hepatocytes were treated with 5 mM 4-phenylbutyric acid (4-PBA) or PBS as control for 2 h prior to H/R onset. In some experiments, primary murine hepatocytes were pretreated with 3-MA (10 mM, Sigma, Saint Louis, MO, USA) and MT (10 μM, Sigma, Saint Louis, MO, USA) 2 h prior to H/R onset, respectively or together. For Drp1 inhibition, the cells were treated with mitochondrial division inhibitor-1 (Mdivi-1, 50 μM, Sigma, Saint Louis, MO, USA) 2 h prior to H/R onset. Viability of hepatocytes was quantified by the Cell Counting Kit-8 assay (Dojindo, Kumamoto, Japan) and the cytotoxicity was measured by the lactate dehydrogenase (LDH) activity (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Extraction of mitochondrial fraction

Both total liver lysates and primary hepatocyte lysates were extracted with ice-cold RIPA buffer (Beyotime, Shanghai, China) containing protease and phosphatase inhibitors (Beyotime, Shanghai, China). Mitochondrial proteins of the liver tissues were extracted using the Mitochondria Extraction Kit for Tissues (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, fresh liver tissues were homogenized in an ice-cold isolation buffer using a Dounce homogenizer. Homogenates were centrifuged at 700 × g for 5 min at 4℃, and the supernatants were further centrifuged at 10000 × g for 10 min at 4℃. The pellet mainly containing the

mitochondrial fraction was washed and resuspended in storage buffer.

Mitochondrial proteins of the primary hepatocytes were extracted using the Mitochondria Extraction Kit for Cells (Beyotime, Shanghai, China) according to the manufacturer's instructions. The primary hepatocytes were suspended in isolation buffer and were kept at 4℃ for 15 min. Then, the cells were homogenized in an ice-cold isolation buffer using a Dounce homogenizer. Homogenates were centrifuged at 600 × g for 10 min at 4℃, and the supernatants were further centrifuged at 10000 × g for 10 min at 4℃. The pellet was resuspended in storage buffer as mitochondrial fraction.

ATP production assay

ATP production of the mice livers was detected with an ATP Bioluminescent Assay Kit (Sigma, Saint Louis, MO, USA) according to the manufacturer's protocol.

Western blot

Protein extracted from liver tissues and primary hepatocytes was subjected to 4-12% SDS-PAGE and transferred to polyvinylidene difluoride nitrocellulose membrane. Monoclonal anti-CHOP, LC3BI-II, p62, p-AMPK, ATG3, ATG5, Beclin-1, Drp1, phospho-Drp1 Ser616, β-actin antibodies (Cell Signaling Technology, MA, USA) and monoclonal anti-VDAC and Cox1 (Abcam, Cambridge, MA, USA) were used.

Beclin-1 knockdown

In vitro studies, hepatocytes were cultured and transiently transfected with Beclin-1 specific siRNA (Beclin-1-siRNA, Thermo Fish Scientific, Carlsbad, CA) or non-specific siRNA (NS-siRNA, Thermo Fish Scientific, Carlsbad, CA) as control using lipofectamine 3000 reagent (Thermo Fish Scientific, Carlsbad, CA). In vivo studies, Beclin-1-siRNA and NS-siRNA was complexed with Invivofectamine 3.0 Reagent (Thermo Fish Scientific, Carlsbad, CA) according to the manufacturer's instruction. The complex (100 μl, 1.5 mg/kg) was administered by tail vein injection daily for 3 days prior to the onset of liver ischemia.

Reactive oxygen species (ROS) detection

The ROS production in mice liver was detected using a luminal-based chemiluminescent probe (L-012, R&D Systems, Minneapolis, MN, USA) according to the manufacture's instruction. Liver tissues were immediately homogenized and 2 mg of tissue lysate was loaded in a 96-well plate in 250 μl PBS, 50 mM L-012 was added and detected for luminescence activity in FLx800TM Multi-Detection Microplate Reader (Biotek, Winooski, Vermont, USA). ROS production in primary hepatocytes was measured using DCFDA Cellular ROS Assay Kit (Abcam, Cambridge, MA, USA) according to the manufacture's instruction. Cell fluorescence was detected by a fluorescent plate reader with excitation wavelength at 485 nm and emission wavelength at 535 nm. Hepatic ROS levels were detected using DHE staining. Frozen liver tissues were stained with DHE (10 μM) (Sigma, St Louis, MO, USA) at 37 ℃ for 30 m. The sections were observed under a fluorescence microscope.

Caspase-3 activity assay

Caspase-3 activity was measured by a Caspase-3 Activity Assay Kit (Calbiochem, La Jolla, CA, USA) as previously described [2]. Cells were lysed in cold cell lysis buffer provided in each colorimetric assay kit. Cytosolic extracts (30 μg protein in 50 μl cell lysis buffer) and 5 μl chromophore substrate were incubated at 37℃ for 2h. Caspase-3 activity was assayed in terms of the absorbance of 405 nm with a plate reader.

Transmission electron microscopy (TEM)

TEM was performed at the Bioscience Electron Microscopy Centre of Nanjing Medical University with standard protocols. Fresh mouse liver fragments were collected in fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, PH 7.4) and chopped into 1 mm cubes. Images were captured using a bright field FEI Tecnai Biotwin G2 transmission electron microscope operated at an accelerating voltage of 80 kV and equipped with an AMT 2k XR40 CCD camera.

Immunofluorescence staining

For immunofluorescence of liver tissues, 8 μm cryostat sections were thawed at room temperature for 30 min, fixed with ice-cold PFA at 4℃ for 30 min, and permeabilized with 0.2% Triton X 100 in PBS. The sections were blocked with 1X PBS/0.1% Tricon X 100/10% fetal bovine serum/1% BSA for 1 h, and overnight incubated with primary CHOP mouse mAb (Cell Signaling Technology, MA, USA) and Albumin Rabbit mAb

(Abcam, Cambridge, MA, USA) at 4℃. The next day, samples were incubated with the goat anti-rabbit and goat anti-mouse secondary antibodies (Abcam, Cambridge, MA, USA). Then, the slides were incubated with DAPI and mounted with one drop of VECTASHIELD medium. The expression of PDH and Lamp1 in primary hepatocytes were detected by immunofluorescence staining using primary mouse PDH mAb and rabbit Lamp1 mAb (Abcam, Cambridge, MA, USA). The secondary, biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA, USA) was incubated with immunoperoxidase (ABC Kit, Vector) according to the manufacture's instruction. After incubation with secondary goat anti-mouse Texas Red-conjugated IgG (Sigma, Saint Louis, MO, USA), the hepatocytes were pre-mounted with VECTASHIELD medium with DAPI. Images were captured and observed with a confocal microscope (Carl Zeiss).

Patients' study

The pericarcinomatous tissues were obtained from six hepatocellular carcinoma patients undergoing hepatectomy with portal triad clamping at 15 min and 30 min after ischemia. Another eight successive patients who were preparing for partial hepatectomy with portal triad clamping were randomly divided into NAC group or control (CON) group (four patients in each group) according to the presence or absence of N-acetylcysteine (NAC, Hangzhou Minsheng Pharmaceuticals, China) administration. Patients in the NAC group were injected with NAC at 150 mg/kg/day for 3 days prior to the operation, while the patients in the CON group were injected with saline. The serum ALT levels were analyzed at 1 day and 3 days after resection. In addition, the

liver specimens from another 3 patients undergoing liver resection for hemangioma without portal occlusion were used to isolate primary hepatocytes for in vitro studies.

References

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Supplemental Figures

Figure S1

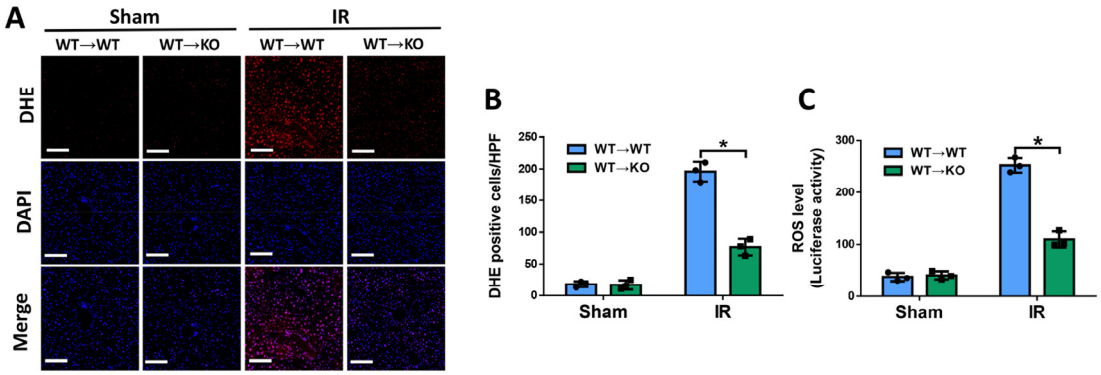

Figure S1. *CHOP* deficiency reduces IR-induced ROS production in hepatocytes

WT→WT and WT→KO mice were subjected to 90 min of partial warm liver ischemia, followed by 6 h of reperfusion. (A) Immunofluorescence staining of dihydroethidium (DHE, red) and DAPI (blue) in liver tissues. (scale=100μm) (B) Quantification of DHE+ cells per high power field. (C) ROS activity in liver tissues. n = 6 mice/group. All results were representative of at least two independent experiments. *p <0.05. Values were expressed as mean ± SD.

Figure S2

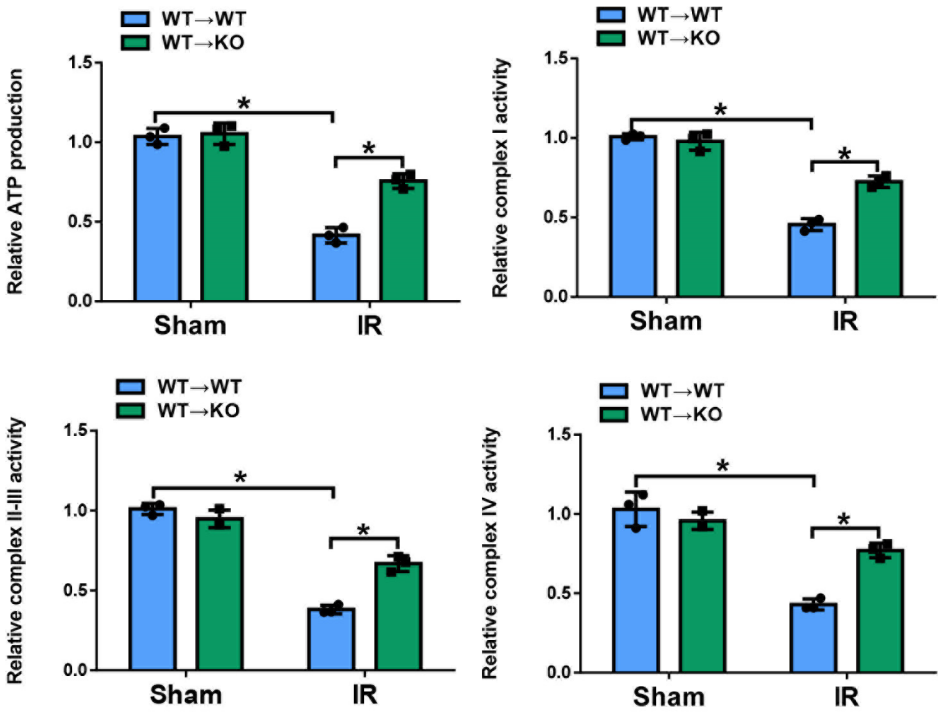

Figure S2. *CHOP* deficiency prevents IR-induced mitochondria damage in hepatocytes

WT→WT and WT→KO mice were subjected to 90 min of partial warm liver ischemia, followed by 6 h of reperfusion. Relative mitochondrial ATP production, respiratory chain complex I activity, respiratory chain complex II+III activity and respiratory chain complex IV activity of the livers.

Figure S3

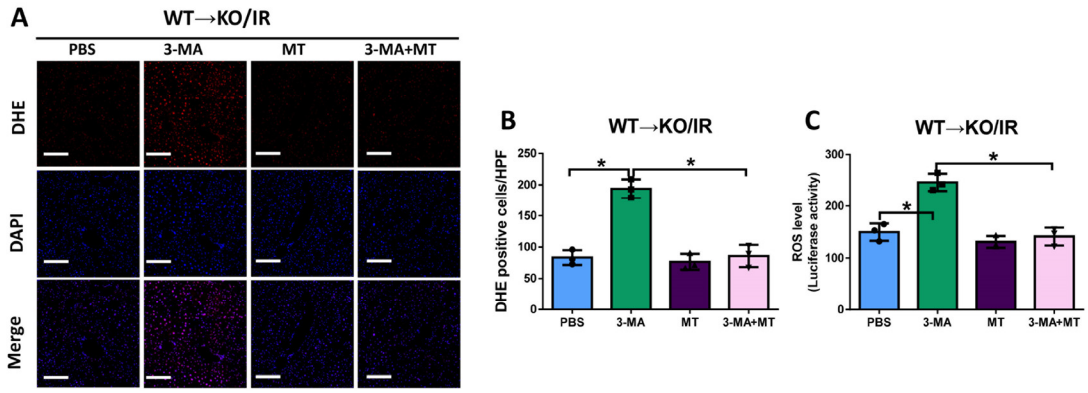

Figure S3. *CHOP* deficiency reduces IR-induced hepatocellular ROS production in a mitophagy dependent manner

WT→WT and WT→KO mice were administered intraperitoneally with 3-Methyladenine (3-MA, 30 mg/kg) and MT (3 mg/kg) separately or together, with PBS administration as control, 3 h prior to liver IR onset. (A) Immunofluorescence staining of dihydroethidium (DHE, red) and DAPI (blue) in liver tissues. (scale=100μm) (B) Quantification of DHE+ cells per high power field. (C) ROS activity in liver tissues. n = 6 mice/group. All results were representative of at least two independent experiments. *p <0.05. Values were expressed as mean ± SD.

Figure S4

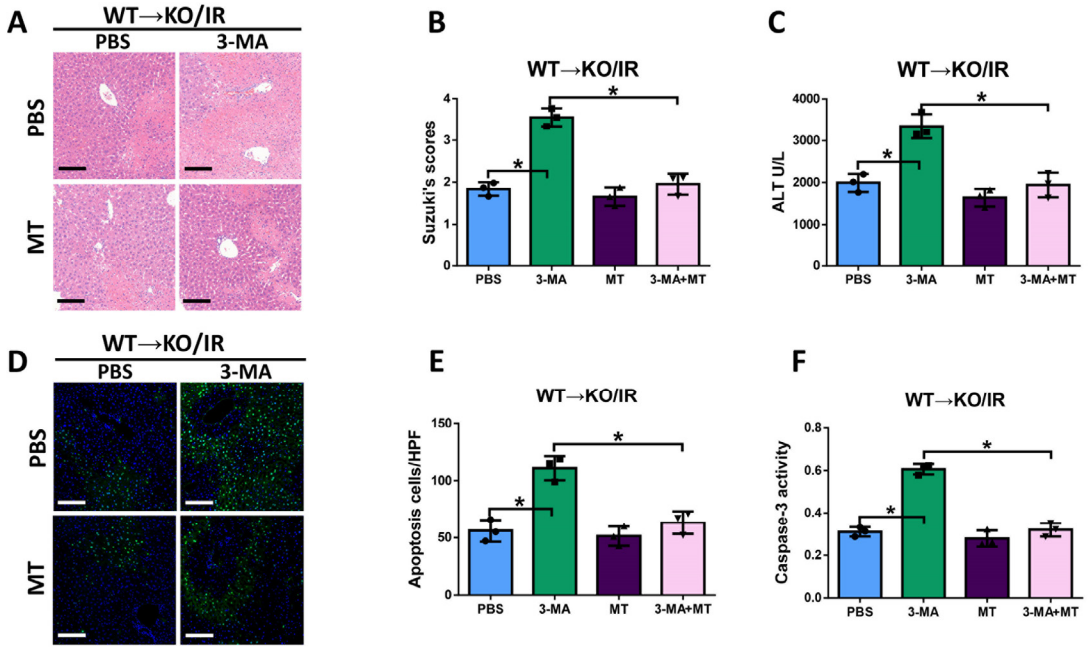

Figure S4. *CHOP* deficiency prevents liver IR injury in a mitophagy dependent manner

WT→WT and WT→KO mice were administered intraperitoneally with 3-Methyladenine (3-MA, 30 mg/kg) and MT (3 mg/kg) separately or together, with PBS administration as control, 3 h prior to liver IR onset. (A) Representative histological staining (H&E) of ischemic livers. (scale=100μm) (B) Liver damage was evaluated by Suzuki's histological score. (C) Liver function was evaluated by serum ALT levels. (D) Immunofluorescence staining of TUNEL+ cells in ischemic livers. (scale=100μm) (E) Quantification of TUNEL+ cells per high power field. (F) Liver Caspase-3 activity. n = 6 mice/group. All results were representative of at least two independent experiments. *p <0.05. Values were expressed as mean ± SD.

Figure S5

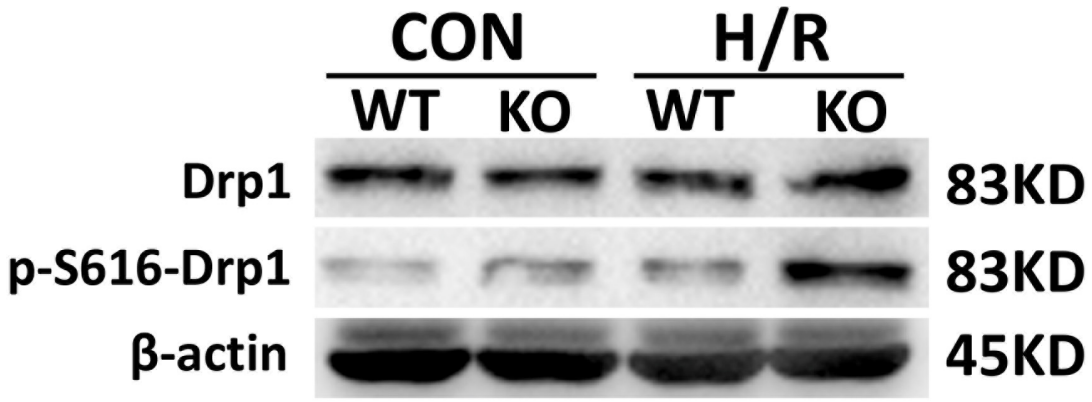

Figure S5. H/R increases the protein levels of p-S616-Drp1 in *CHOP* KO hepatocytes

Primary hepatocytes isolated from WT and *CHOP* KO mice were subjected to H/R. Total proteins of the primary hepatocytes were prepared for further studies. Western blot analysis of Drp1, p-S616-Drp1 and β-actin expression in the hepatocytes. Results were representative of at least two independent experiments.

Table S1. Demographics

Total numbers of patients	17
Study 1: Different duration of ischemia	6
Study 2: Isolation of primary hepatocytes	3
Study 3: NAC VS CON	8
Male/female	8/9
Mean age	56.72±7.86
Diagnosis	
Hepatocellular carcinoma	14
Hemangioma	3
Comorbidities	
Hepatitis B	12
Pre-operative treatment	
Entecavir	12