Supplemental Information (Jiang M et al.)

Detailed Methods

Reagents and antibodies: Cisplatin, chloroquine and rapamycin were purchased from Sigma (St. Louis, MO). Carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) and 7-amino-4-trifluoromethyl coumarin (AFC) were from Enzyme Systems Products (Livermore, CA). Unless indicated, all other reagents were from Sigma. The following primary antibodies were used: anti-LC3 from Abcam (Cambridge, MA) and Novus Biologicals (Littleton, CO); anti-Atg5, anti-p62 and anti-cyclophilin B from Abcam; anti-Atg7, anti-p53, anti-phospho-p53 (ser15), anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-p38, and anti-phospho-p38 from Cell Signaling (Danvers, MA); anti-p21 from Santa Cruz (Santa Cruz, CA); anti-β-actin from Sigma. All secondary antibodies for immunoblot analysis were from Thermo Scientific (Rockford, IL).

Animals: C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). $Atg7^{flox/flox}$ mouse breeders were described previously.[1] PEPCK-Cre transgenic mice were originally obtained from Dr. Volker Haase (University of Pennsylvania, Philadelphia, PA).[2] $Atg7^{flox/flox}$ mice were bred with PEPCK-Cre mice to generate $Atg7^{flox/flox}$; PEPCK-Cre mice (see Figure 3A for the breeding protocol). For the purpose of comparison, PT-Atg7-KO and wild-type littermates were examined in the same experiments. Mice were housed in a pathogen-free animal facility of Charlie Norwood VA Medical Center under a 12/12-hour light/dark pattern with free access to food and water. All animal experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Charlie Norwood VA Medical Center.

Genotyping: Genomic DNA was extracted from mouse tail biopsy for PCR analysis according to a modified protocol from Dr. Zhenyu Yue (Mount Sinai School of Medicine, New York, NY). Two sets of PCR were performed to detect the floxed and wild-type alleles of Atg7 gene. In the first reaction, the floxed allele produced a 500bp fragment whereas wild-type allele generated a 1.5kb product by the primers Atg7s (TGGCTGCTACTTCTGCAATGATGT) and Intron 13as (GAATATTCTAATTCAACCAGACCTAGGT). The second reaction using the primers Intron 13s (GCTGGTTAAAGACTGTCTAATAAAGAGCA) and Mid-intron 13as (CTGCCGCTGAGCCCTGAGAGAGGCCT) amplified a 600bp product for wild-type allele and was negative for the floxed allele. Additionally, as described in our recent work,[3] a 370bp fragment was amplified by the primers ACCTGAAGATGTTCGCGATTATCT and ACCGTCAGTACGTGAGATATCTT to confirm the presence of PEPCK-Cre gene. All the primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Experimental models of acute kidney injury: Male mice of 8-10 weeks old were used for experiments. For cisplatin injury, mice were intraperitoneally injected with a single dose of cisplatin at 25mg/kg or 30mg/kg; control animals were injected with a comparable volume of saline. To test the effect of chloroquine, 60mg/kg chloroquine was injected (i.p.) 1 hour prior to cisplatin administration and then daily after cisplatin treatment. To test the effect of rapamycin, 1 mg/kg rapamycin was injected (i.p.) 1 hour prior to and 1 day after cisplatin administration. Renal ischemia-reperfusion was induced in mice as described in our recent work.[4, 5] Briefly, after anesthetized with pentobarbital (50mg/kg, i.p.), mice

were kept on a Homeothermic Blanket Control Unit (Harvard Apparatus Ltd, U.K.) to monitor and maintain body temperature at ~36.5ºC. Renal pedicles were exposed by flank incisions for bilateral clamping to induce 25 minutes of ischemia. The clamps were then released for reperfusion. Color change of kidneys was observed to indicate sufficient ischemia and reperfusion. Sham control mice underwent the same operation without renal pedicle clamping.

Renal function: Renal function was determined by blood urea nitrogen (BUN) and serum creatinine using commercial kits from Stanbio Laboratory (Boerne, TX). In brief, blood samples were collected for coagulation and centrifugation at room temperature to collect serum. For BUN, the reaction was conducted at 100°C for 10-12 minutes and the absorbance at 520nm was recorded by the end of reaction. For serum creatinine, samples were added to a pre-warmed (37°C) reaction mixture and the absorbance at 510nm was monitored kinetically at 20 and 80 seconds of reaction. BUN and creatinine levels (mg/dl) were then calculated based on standard curves.

Histological examination and TUNEL assay: Kidney tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at 4μM. Hematoxylin-eosin staining was performed using standard procedures and renal tubules with the following histopathological changes were considered injured: loss of brush border, tubular dilation and disruption, cast formation and cell lysis. Tissue damage was examined in a blind manner and scored by the percentage of damaged tubules: 0, no damage; 1, <25%; 2, 25-50%; 3, 50-75%; 4, >75%. Apoptotic cells in renal tissue were identified by TdT-mediated

dUTP nick-end labeling (TUNEL) assay using an in situ cell death detection kit (Roche Applied Science, Indianapolis, IN) as described recently.[4, 5] Briefly, tissue sections were deparaffinized and permeabilized with 0.1M sodium citrate, PH 6.0 at 65°C for 30 minutes. The sections were then incubated with a TUNEL reaction mixture for 1 hour at 37ºC in a humidified/dark chamber. Positive nuclear staining was detected by fluorescence microscopy. For quantification, 10-20 fields were randomly selected from each tissue section and the amount of TUNEL-positive cells per mm^2 was evaluated.

Immunoblot analysis: Kidney tissues from cortex and outer medulla were ground in liquid nitrogen and lysed in 2% SDS buffer containing protease inhibitor cocktail and nuclease. Protein concentration was determined with BCA reagent from Thermo Scientific. Equal amounts (50-100μg) of protein were loaded in each lane and separated on SDS-polyacrylamide electrophoresis gel. After transferred onto polyvinylidene difluoride membrane, blots were blocked with 5% milk and probed subsequently with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Antigens were visualized with an enhanced chemiluminescence kit (Thermo Scientific). Either β-actin or cyclophilin B was used to monitor protein loading and transferring.

Immunofluorescence staining of LC3: Mice were anesthetized and immediately fixed by perfusion with a modified Zamboni's fixative containing 4% paraformaldehyde and picric acid. Kidneys were harvested and further fixed with the same fixative overnight at 4°C. The tissues were then balanced with 30% sucrose in PBS at 4°C overnight and routinely embedded in paraffin. For antigen retrieval, deparaffinized tissue sections were incubated

with 10mM sodium citrate containing 0.05% Tween 20, PH 6.0 at 95-100°C for 1 hour. After blocked with 5% normal goat serum in PBS, the slides were subsequently incubated with 5μg/ml anti-LC3 at 4°C overnight and 1:500 Alexa Fluor 568 goat-anti-rabbit secondary antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Both of the primary and secondary antibodies were diluted with 2% BSA, 0.2% milk, 2% normal goat serum and 0.8% Triton X-100 in PBS. Negative controls were done by replacing the primary antibody with antibody diluent. To determine the localization of LC3 puncta in kidneys, the slides were further stained with fluorescein isothiocyanate-labeled lectins, phaseolus vulgaris agglutinin (proximal tubule marker) or peanut agglutinin (distal tubule marker) from Vector Laboratories (Burlingame, CA). Cell nuclei were counterstained with Hoechst33342. After mounted with an antifade reagent (Molecular Probes), the slides were examined by fluorescence microscopy. For quantification, 10-20 high magnification (×630) fields were randomly selected from each slide and the number of LC3 puncta per proximal tubule was evaluated.

Immunohistochemistry of p62: Kidneys were fixed in 4% paraformaldehyde overnight at 4°C and processed for routine embedding in paraffin. Tissue sections were deparaffinized and incubated with 0.1M sodium citrate, PH 6.0 at 65°C for antigen retrieval. After subsequently incubated with 0.1% phenylhydrazine and 0.03% H_2O_2 to block endogenous peroxidase activity and with a buffer containing 2% BSA, 0.2% milk, 2% normal donkey serum and 0.8% Triton X-100 to reduce non-specific binding, the slides were exposed to 1.5μg/ml anti-p62 at 4°C overnight. Negative controls were done by replacing the primary antibody with antibody diluent. Following blocking of endogenous avidin-biotin with a kit

from Vector Laboratories, the slides were further incubated with 1:500 biotinylated donkey-anti-mouse secondary antibody (Chemicon, Temecula, CA) for 1 hour at room temperature. Signals of the antigen-antibody complexes were amplified using Tyramide Signal Amplification (TSA™) Biotin System (PerkinElmer, Waltham, MA) and visualized with VECTASTAIN® ABC Standard kit and DAB Peroxidase Substrate Kit (Vector Laboratories) following protocols of the manufacturers.

Cisplatin treatment of primary proximal tubular cells and apoptosis determination: Primary proximal tubular cells were isolated from 5- to 6-week-old mice and cultured as described previously.[6] After 7 days growth, the cells were seeded in 35mm dishes at a density of $0.4x10^6$ cells per dish and reached 80 ~90% confluence by the next day. The cells were then exposed to 30μM cisplatin for 24 hours and apoptosis was determined by morphologic and biochemical methods as described before.[4-6] Morphologically, cells were stained with 10μg/ml Hoechst 33342. Cellular and nuclear morphology was examined by phase contrast and fluorescence microscopy, respectively. For each condition, four fields with ~200 cells per field were randomly selected and cells with typically apoptotic characterizations were counted to estimate apoptosis percentage. Biochemically, the enzymatic activity of caspases was measured. Cells were extracted with 1% Triton X-100 and 20μg protein were added to enzymatic reactions containing 50μM DEVD-AFC, a fluorogenic peptide substrate of caspases. After 1 hour incubation at 37ºC, fluorescence was measured at excitation 360nm/emission 530nm. Based on a standard curve constructed with free AFC, the fluorescence reading was converted into the nanomolar amount of liberated AFC per mg protein to indicate caspase activity.

Statistics: Qualitative data including immunoblots and cell images are representatives of at least three experiments. Quantitative Data were expressed as means ± SD. Statistical analysis was conducted using the GraphPad Prism software. Statistical differences in multiple groups were determined by multiple comparisons with ANOVA followed by Tukey's post-tests. Statistical differences between two groups were determined by 2-tailed unpaired or paired Student t-test. $P<0.05$ was considered significantly different.

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