Supplementary Materials for

αKlotho Mitigates Progression of Acute Kidney Injury to Chronic Kidney Disease via Activation of Autophagy

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Running title: αKlotho and autophagy in AKI and CKD

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MATERIALS AND METHODS

Antibodies and chemicals: The following antibodies were used for immunoblotting and/or immunohistochemistry: Rat monoclonal anti-αKlotho antibody (KM2076), mouse monoclonal anti-Flag M2 (Sigma-Aldrich, St. Louis, MO), mouse monoclonal antibody against β-actin (Sigma Aldrich, St. Louis, MO), mouse monoclonal antibody against α-smooth muscle actin (α-SMA) (Sigma Aldrich, St. Louis, MO), rabbit anti-LC3 (Novus Biologicals, Littleton, CO), guinea-pig anti-p62 (ProgenBiotechnik GmbH, Heidelberg, Germany), Lotus tetragonolobus lectin (LTL) (Vector labs, Burlingame, CA), goat polyclonal antibody against Tamm-Horsfall protein (THP) (Santa Cruz Biotech, Santa Cruz, CA), rabbit polyclonal antibody against Na^+-Cl^- cotransporter (NCC) (EMD Millipore, Billerica, MA), mouse monoclonal antibody against Calbindin D-28 (CB28) (Santa Cruz Biotech, Santa Cruz, CA), mouse monoclonal antibody against CTGF and rabbit polyclonal antibody against Collagen I (Abcam Inc., Cambridge, MA). Secondary Abs coupled to horseradish peroxidase for immunoblotting, or to FITC, Alexa Fluor, or Cy5, and Syto-61 fluorescent nuclear acid stain for IHC were purchased from Molecular Probes/Invitrogen (Molecular Probes Inc., Eugene, OR). Bafilomycin A1 (Baf), LiCl, 3-methyladenine (3-MA) and 30% hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO); Rapamycin and Wortmannin from LC laboratories (Woburn, MA). Baf and Rapamycin were dissolved in DMSO in working concentrations of 200 nM and 0.5 μM respectively for cell culture. The final volume of DMSO in cell culture media never exceeded 1/100. The working concentration of LiCl was 10 mM, 3-MA 10 mM, and Wortmannin 10 μM. Lactate dehydrogenase (LDH) release kit was purchase from Clontech laboratories, Inc. (Mountain View, CA).[1,2]

Plasmids: GFP–LC3 fusion plasmid was kindly provided by Dr. Noboru Mizushima. Full length cDNA rat LC3 was inserted into pEGFP-C1, a GFP fusion protein expression vector (Clontech Laboratories Inc., Mountain View, CA).[3] GFP-human Collagen I 1α2 plasmid driven by CMV promoter was purchased from OriGene Technologies (Rockville, MD). All plasmids were confirmed by sequencing analysis at Core Laboratory in UT Southwestern Medical Center.

Animal models and experiments: All animal work was conducted strictly following the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Animals were housed in a temperature-controlled room with a 12:12 hour light-dark cycle and were given *ad libitum* access to tap water and standard rodent chow prior to assignment of experiment. Special rodent diets are mentioned below.

Genetic αKlotho-deficient mice were generated by transgenic disruption of the *αKlotho* locus resulting in hypomorphic expression.[4] Transgenic mice overexpressing mouse αKlotho (*Tg-Kl*) was described previously.[4,5] All mice were *129 S1/SVlmJ* (*129 SV*) background. The homozygous (*kl*/*kl*; extremely low to zero αKlotho levels); heterozygous (*kl*/+; low αKlotho levels); wild-type (*WT;* normal αKlotho levels); and transgenic overexpressor (*Tg-Kl;* high αKlotho levels) mice were genotype-confirmed by genomic PCR.[4,5]

Transgenic mouse with GFP-LC3 reporter was a kind gift from Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). Genotyping protocol was shown in the literature.[6,7] Second transgenic mouse with RFP;GFP-LC3 reporter was a kind gift from Dr. Joseph Hill (UT Southwestern Medical Center, Dallas, USA). Genotyping protocol was published.[8] These mouse lines were cross-mated with *WT* mice*129 S1/SVlmJ* (*129 SV*) for ~ 5 generation and was subjected to surgery. The *RFP;GFP-LC3* reporter mouse line was also

cross-mated with *kl/+*, *WT* and *Tg-Kl* mouse lines respectively to generate three new mouse lines: *kl/+;RFP;GFP-LC3*, *WT;RFP;GFP-LC3*, and *Tg-Kl;RFP;GFP-LC3*.

Most of animal experiments were performed in 3 month-old mice unless specifically stated. Three AKI models in mice were generated using established methods from our laboratory:[2,9,10] (1) Bi-lateral ischemic reperfusion injury (Bi-IRI):[10] Under anesthesia, both renal arteries were carefully dissected from the vascular bundle and clamped for 30 minutes with arterial clips to induce ischemia. (2) Uninephrectomy plus contralateral ischemia reperfusion injury (Npx-IRI):[9] Under anesthesia, the right kidney was removed and left renal artery was clamped with arterial clips for ischemia ranging from 20 to 45 minutes (period of ischemia) as described above. For the AK-transition-to-CKD study, we selected 30 minutes of ischemia. After clips were removed, the kidneys were observed for 5 minutes to ensure reperfusion. Sham animals underwent laparotomy of the same duration and manual manipulation of the kidney without arterial clamping.

At predetermined times after acute insult; 24-hour urine was collected. Under anesthesia, blood was drawn and kidneys were harvested, instantly snap-frozen in liquid nitrogen, and stored at -80°C for further processing. Plasma and urine chemistry of animals were analyzed using a Vitros Chemistry Analyzer (Ortho-Clinical Diagnosis, Rochester, NY).

Dietary treatment: To explore whether high phosphate diet affects transition of AKI to CKD, we started to treat animals 2 weeks after the acute insult with high Pi rodent chow 2.0% Pi (Harlan Teklad TD.08020) vs. normal rodent chows containing 0.7% Pi (Harlan Teklad 2016) for 18 weeks.

Blood, urine, and kidney samples collection: At designated time points after AKI induction, mice were placed individually in metabolic cages (Hatteras Instruments Inc., Cary, NC) for 24-

h urine collections. Urine samples were centrifuged (1,000 g x 10 min at 20°C) and the supernatants were stored at −20°C until analysis. Mice were anesthetized with Isoflurane and blood samples were collected in heparinized tubes, centrifuged (3,000 g x 5 min at 4°C), and the plasma was separated and stored at −80°C until analysis. At termination, mice were euthanized under anesthesia, and the kidneys were isolated and sliced. One slice were fixed with 4% paraformaldehyde for histological and immunohistochemical studies; the remaining parts of kidneys were snap-frozen in liquid N_2 and stored at −80°C until RNA or protein extraction and analysis.

Kidney histology and histopathology: Tissues were fixed in 4% paraformaldehyde (PFA) (16 hours at 4ºC), and 4 μm sections of paraffin embedded kidney tissues were stained with Hematoxylin and Eosin (H&E), Periodic acid Schiff (PAS) and Trichrome, and then examined and photographed by a renal pathologist blinded to the experimental protocol using an Axioplan 2 Imaging system (Carl Zeiss Micro-Imaging, Inc. Thornwood, NY). A semi-quantitative pathological scoring system was used as described.[9] A renal pathologist blinded to the experimental conditions evaluated the images.

To evaluate renal fibrosis, 4 μm paraffin embedded kidney sections were stained with Trichrome stain, and then examined and photographed by a renal pathologist blinded to the experimental protocol using an Axioplan 2 Imaging system (Carl Zeiss Micro-Imaging, Inc. Thornwood, NY). The fibrotic area and fibrosis intensity were quantified with Image J program with published methods.[11]

Transmission electron microscopy: Kidney slices were fixed overnight with 2.5% glutaraldehydeand 2% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.4) and samples were prepared as described in literatures.[12,13] The ultrathin sections were cut on an

ultracryomicrotome (Ultramicrotome Reichert Ultracut E; Leica Microsystems, Wetzlar, Germany) and were visualized with Jeol 1200 EX transmission electron microscope (Jeol Ltd., Akishima, Japan) in a blind manner.

Immunohistochemistry and immunoblotting in the kidney: Immunohistochemistry protocols and immunobloting protocols were performed as described in literatures.[2,9,10]

Real time RT-PCR: Total RNA was extracted from mouse kidneys using RNAeasy kit (Qiagen, Germantown, MD). Complimentary DNA was generated with oligo-dT primers using SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). Primers used for qPCR were: mouse *αKlotho* forward- AAC CAG CCC CTT GAA GGG AC, and *αKlotho* reverse- TGC ACA TCC CAC AGA TAG AC; and *cyclophilin* forward- TGC TCT TTT CGC CGC TTG CT, and *cyclophilin* reverse- TCT GCT GTC TTT GGA ACT TTG TCT G with conditions previously described.[14] The reaction was performed in triplicate for each sample. Data were expressed at amplification number of $2^{-\Delta\Delta Ct}$ normalized to *cyclophilin* and compared to controls.

Albumin and creatinine measurements: Using previously published methods,[11] plasma and urine creatinine concentrations were measured using a P/ACE MDQ Capillary Electrophoresis System and photodiode detector (Beckman-Coulter, Fullerton, CA).[15] Specific urine albumin concentrations were determined by the Exocell mouse albumin ELISA kit (Exocell, Philadelphia, PA).

Cell culture: The opossum kidney cell (OK) cells were maintained in high glucose DMEM medium described in literature.[15] OK cells were treated with H_2O_2, and/or autophagy modulators (inducers or suppressors) in the presence or absence of αKlotho protein for 16 hours. Cell lysates were made[1,2] and subjected to immunoblot for LC3 protein. Supernatants

from cell culture media were harvest for measurement of LDH release with LDH cytotoxicity detection kit as we described previously.[1,2] GFP-LC3 was transiently transfected into OK cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were treated with H_2O_2, and/or autophagy modulators (inducers or suppressors) for 16 hours. After fixation with 4% paraformaldehyde, cells were stained with anti-flag antibody for αKlotho and with rhodamine-phalloidin for counterstain. Fluorescent microscopy analysis was performed as we described previously.[2,16]

Qualitative and quantitative analysis of collagen in OK cells: To qualitatively evaluate collagen deposition *in vitro,* GFP-collagen Iα2 plasmid driven by CMV promoter (CMV-GFP-Col1) was transiently transfected into OK cells. Twenty-four hours after transient transfection, OK cells were treated with αKlotho protein or modulators of autophagic flux for 24 hours. OK cells were stained with anti-αKlotho antibody to detect αKlotho, counterstained with rhodamine-phalloidin; and underwent laser confocal microscopy. Cells were stained with Sirius Red/Fast Green collagen staining kit (Chondrex, Inc., Redmond, WA) for quantitative analysis following kit's instruction. Briefly OK cells grown in glass coverslips were stained simultaneously for total protein (Fast Green) and collagen (Picrosirius Red). The dye was subsequently eluted from the section, and the optical density (OD) of the eluate was read at 540 and 605 nm using a spectrophotometer. The amount of collagen in the section was normalized by total protein with following equation: collage I/protein = [(OD540-(OD605*0.291))/0.0378]/(OD605/0.00204) according to the manufacturer's instruction.

Statistical analyses: Data are expressed as Means ± SD. Statistical analysis was performed using unpaired *Student t*-test, or one-way analysis of variance (ANOVA) followed by post hoc

Student-Newman-Keuls test when applicable. P value ≤ 0.05 was considered statistically significant.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Kidney pathology in CKD mice. *WT* mice at 3 months old were subjected to bilateral ischemia reperfusion injury (Bi-IRI), uninephrectomy and contralateral ischemia reperfusion injury (Npx-IRI), or laparotomy for Sham, and fed normal rodent chow or high Pi diet (2.0% Pi) respectively for 18 weeks starting 2 weeks after acute kidney insults. Representative micrographs of Periodic acid–Schiff (PAS) staining in the kidney sections from 4 animals of each group, scale bar = 100 μm. Upper panel: CKD mice were fed with normal phosphate rodent chow (NP); Bottom panel: CKD mice were fed with high phosphate rodent chow (HP).

Supplementary Figure 2. Autophagy flux in the kidney was up-regulated by bilateral ischemia reperfusion injury (Bi-IRI). Three months old *WT* mice were subjected to AKI induced by Bi-IRI and mice were sacrificed at Day 2. (**A**) Protein expression of LC3-I, LC3-II and p62 in the kidney of AKI mice. Upper panel: representative immunoblots for LC3, p62, and β-actin protein in the kidney. Bottom panel: summary of immunoblots from three independent experiments. (**B**) Immunofluorescence images for LC3 in the kidneys of transgenic GFP-LC3 mice at Day 2. Upper panel: representative LC3 (green) and Syto 61 (blue for nuclear stain). Scale bar = 50 μm. Bottom panel: the number of GFP-LC3 puncta per renal tubule (50 tubules analyzed per sample). Results are means ± SD from 5 independent experiments. Statistical significance was assessed by unpaired *Student-t* test, and accepted when *: $P<0.05$; **: $P<0.01$ between two groups for **A** and **B**. (**C**) Protein expression of αKlotho, LC3-I and LC3-II, and p62 in the kidney of mice with different genetic αKlotho levels at 6 weeks old without renal insult. Left panel: representative immunoblots for αKlotho, LC3-II/LC3-I, p62, and β-actin protein in the kidney. Right panel: summary of immunoblots from 4 independent experiments.

Results are means ± SD from 4 independent experiments. Statistical significance was assessed by one-way ANOVA followed by Student-Newman-Keuls test, and accepted when *: P<0.05; **: P<0.01 between two groups.

Supplementary Figure 3. Localization of LC3 punctas in renal tubular segments in AKI mice. RFP;GFP-LC3 reporter mice underwent 30-minute of ischemia followed by 24 hours reperfusion. The kidney sections were subject to immunofluorescent staining and photographed at 63x original amplification. (**A**) Lotus tetragonolobus lectin (LTL) staining in proximal convoluted tubules (PT). (**B**) Tamm-Horsfall protein (THP) staining in thick ascending limbs (TAL). (**C**) Na^+-Cl^- cotransporter (NCC) staining in distal convoluted tubules (DT). (**D**) Calbindin D-28 (CB28) staining in later distal convoluted tubules. Scale bar = 50 mm. CB28: Calbindin D-28; DT: distal renal tubules; NCC: Na^+-Cl^- cotransporter; PT: proximal tubules; TAL: thick ascending limbs; THP: Tamm-Horsfall protein.

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Supplementary Figure 5. 3-MA abolished αKlotho associated up-regulation of autophagy and protection of kidney cell against oxidative stress. OK cells were treated with H_2O_2 with or without 3-methyladenine (10 mM). In addition, recombinant αKlotho protein (0.4 nM) was added. (**A**) Lactate dehydrogenase (LDH) in supernatants released from OK cells was used for assessment of cell injury. (**B**) Protein expression of LC3-I, LC3-II, and p62 protein

in OK cells. Left panel: representative immunoblots for LC3-I and LC3-II, p62, and β-actin protein. Right panel: summary of immunoblots from three independent experiments. Data are means ± SD. (**C**) Representative immunocytochemistry for αKlotho (blue), LC3 (green) and phalloidin (red) in OK cells. OK cells were seeded on coverslips and transfected with GFP-LC3 plasmid. After 24 hours of transfection, cells were treated with H_2O_2 and/or 3-MA. Vehicle (upper panel) or recombinant αKlotho protein (bottom panel) was added to examine whether αKlotho suppresses cell injury induced by H_2O_2. Scale bar = 20 μm. (**D**) Quantification of the number of GFP-LC3 puncta per cell in OK cells (50 cells analyzed per sample). Results represent as means ± SD from three independent experiments. Statistical significance was assessed by one-way ANOVA followed by Student-Newman-Keuls test, and accepted when *: P<0.05; **: P<0.01 between two groups for **A**, **B** and **D**.

Supplementary Figure 6. Up-regulation of autophagy reduced collagen I accumulation in OK cells. OK cells were transfected with GFP-Col1 plasmid, and treated with autophagy inducers: LiCl (10 mM) or rapamyocin (0.5 μM), and inhibitors: Baf (200 nM) or wortmannin (10 μM), and αKlotho protein. NaCl (10 mM) or DMSO (1/1000 dilution) were used as vehicle for control. Upper panel: Representative immunocytochemistry for Col1 (green) and phalloidin (red). Scale bar = 100 μm. Bottom panel: Collagen amount in OK cells was stained and quantified with Sirius red/fast green kit. Baf: Bafilomycin A1

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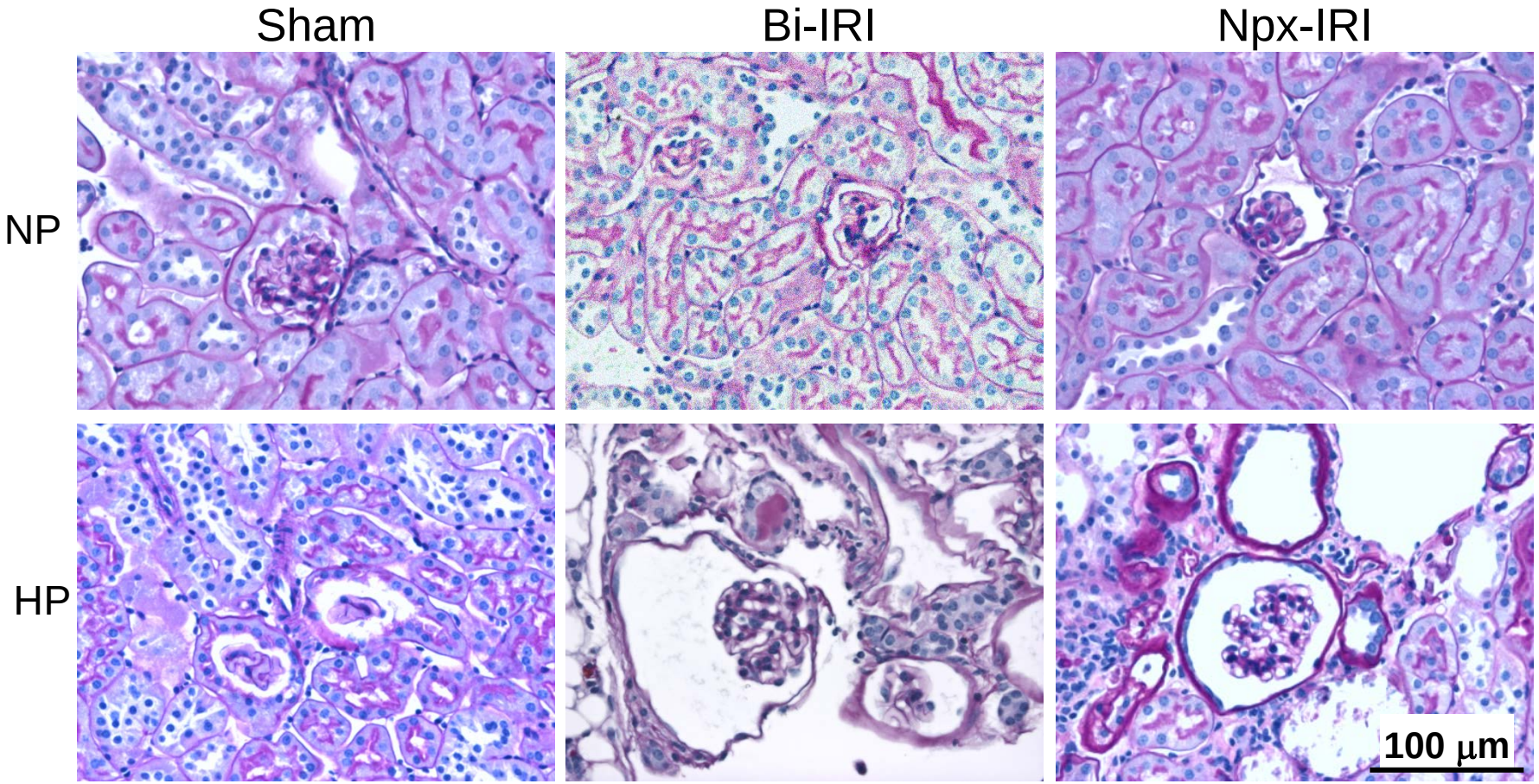
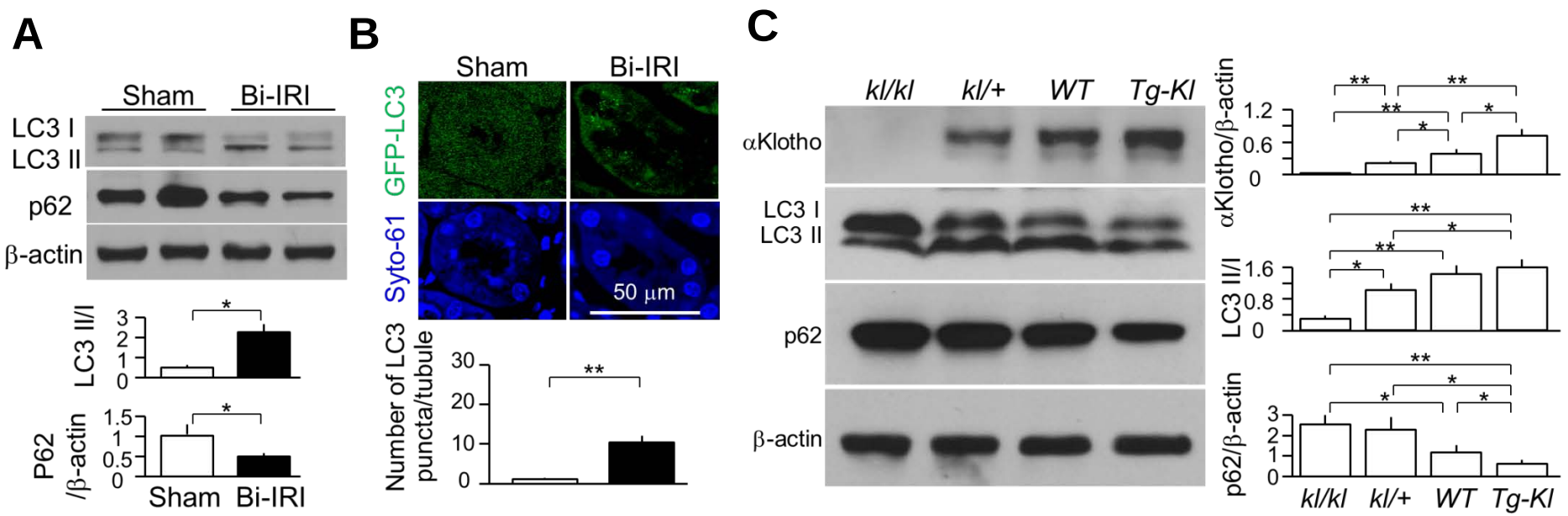


Supplementary Figure 1. Kidney pathology in CKD mice. *WT* mice at 3 months old were subjected to bilateral ischemia reperfusion injury (Bi-IRI), uninephrectomy and contralateral ischemia reperfusion injury (Npx-IRI), or laparotomy for Sham, and fed normal rodent chow or high Pi diet (2.0% Pi) respectively for 18 weeks starting 2 weeks after acute kidney insults. Representative micrographs of Periodic acid–Schiff (PAS) staining in the kidney sections from 4 animals of each group, scale bar = 100 mm. Upper panel: CKD mice were fed with normal phosphate rodent chow (NP); Bottom panel: CKD mice were fed with high phosphate rodent chow (HP).



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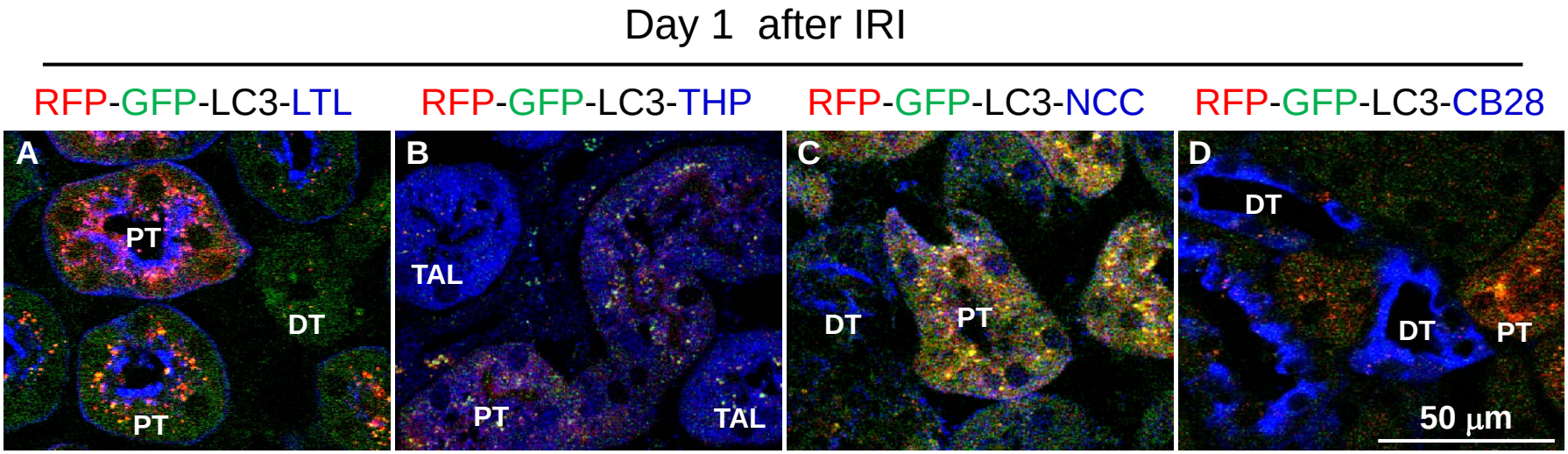

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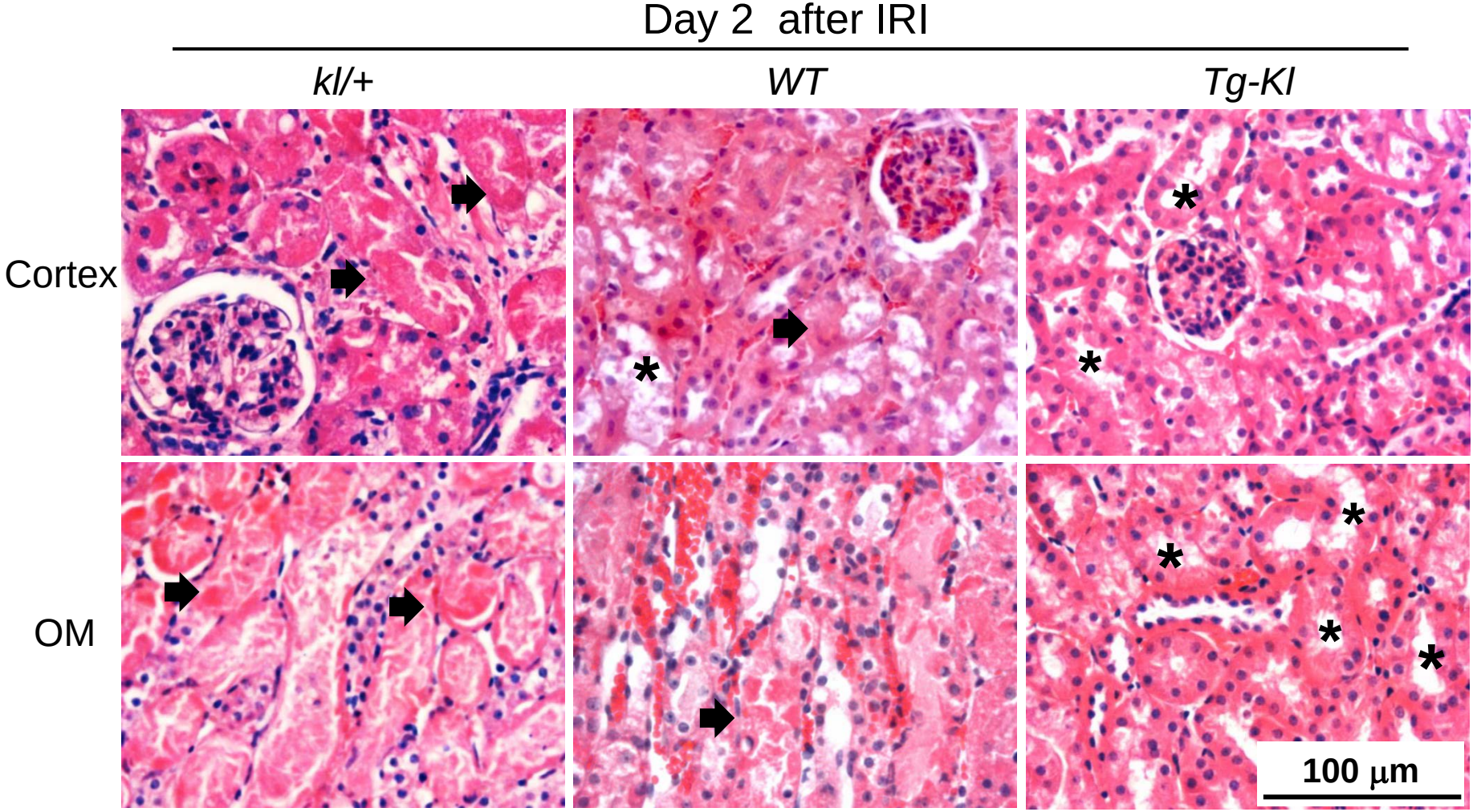

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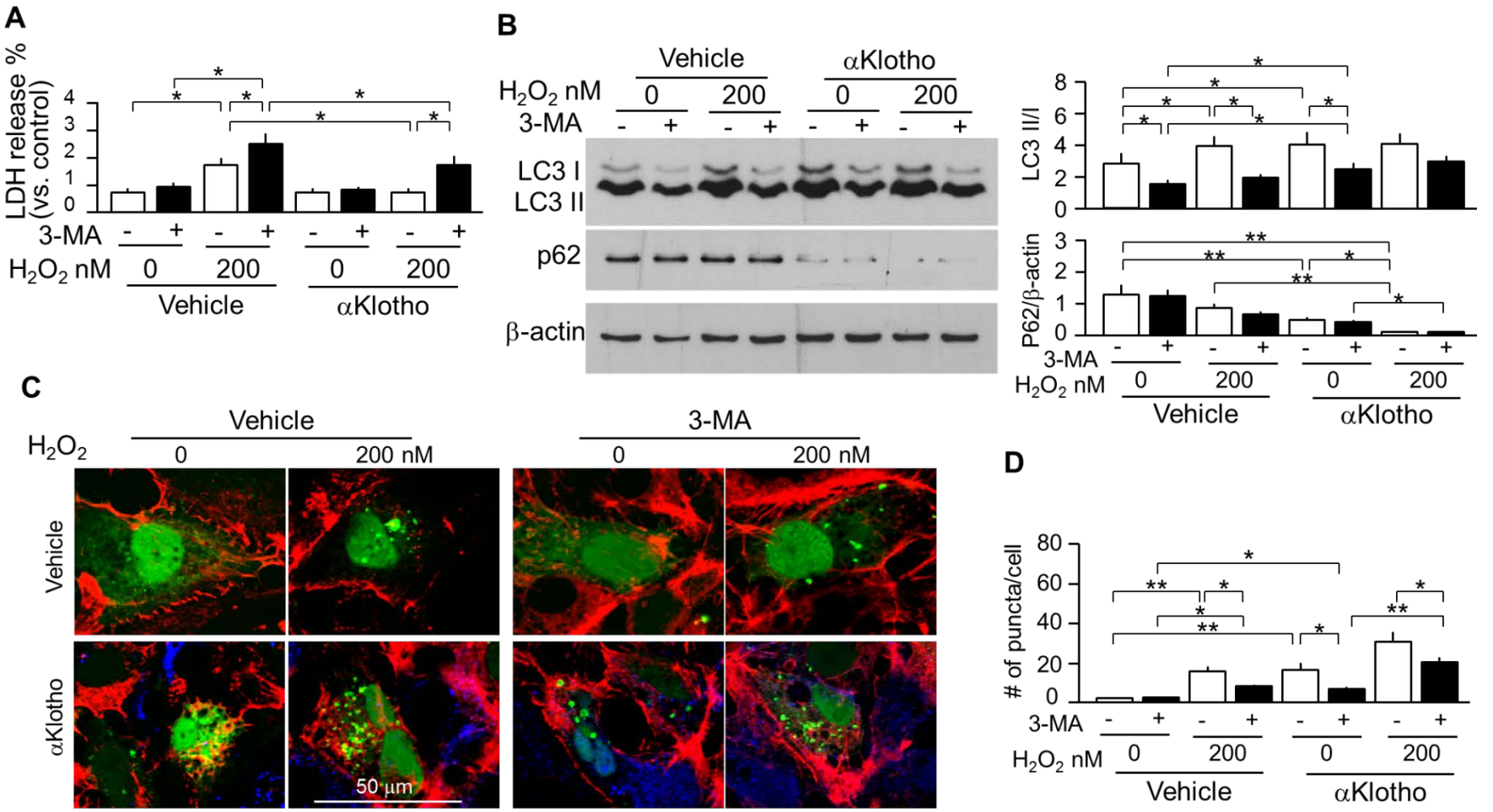

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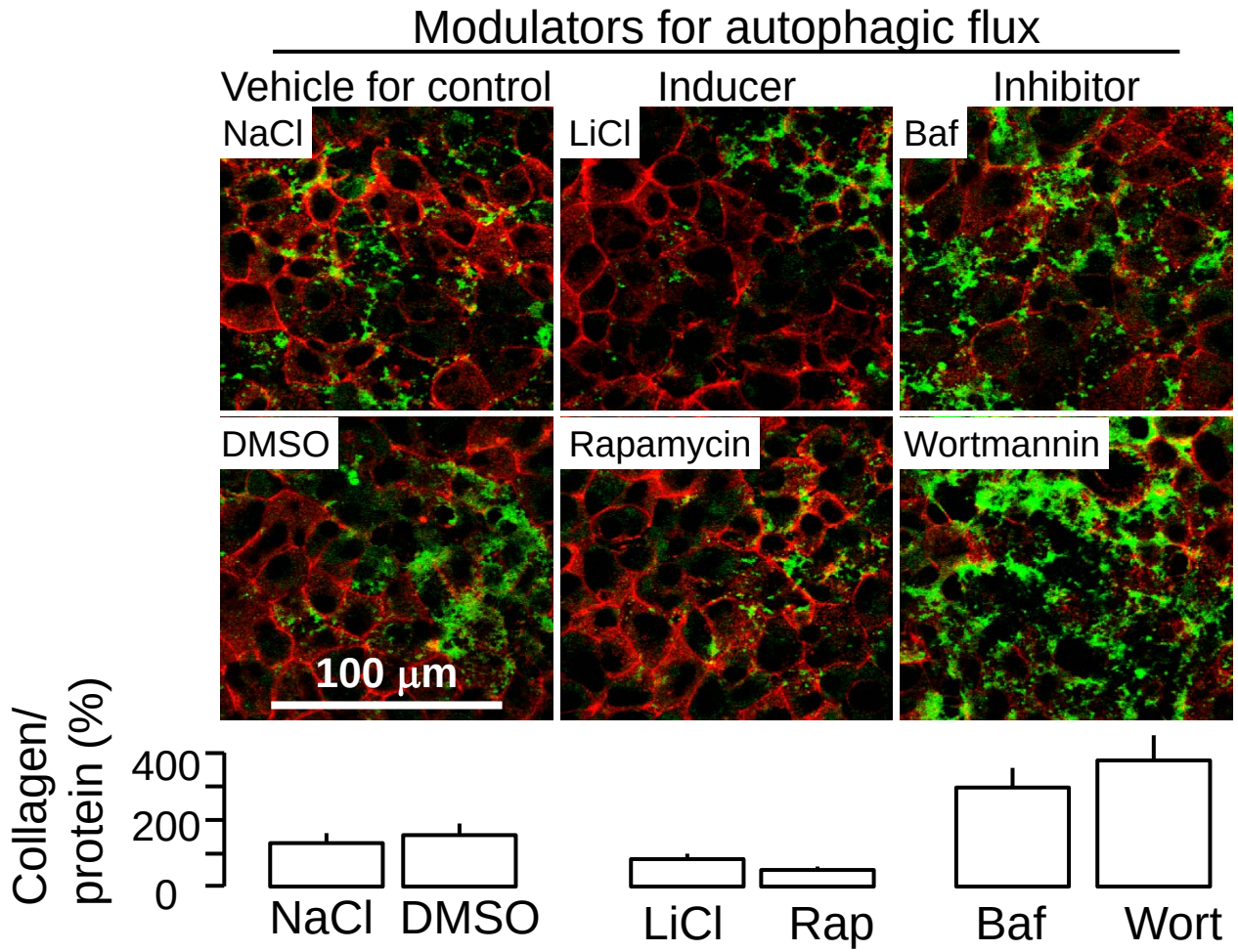

Supplementary Figure 6. Up-regulation of autophagy reduced collagen I accumulation in OK cells. OK cells were transfected with GFP-Col 1 plasmid, and treated with autophagy inducers: LiCl (10 mM) or rapamyocin (0.5 mM), and inhibitors: Baf (200 nM) or wortmannin (10 mM), and αKlotho protein. NaCl (10 mM) or DMSO (1/1000 dilution) were used as vehicle for control. Upper panel: Representative immunocytochemistry for Col 1 (green) and phalloidin (red). Scale bar = 100 mm. Bottom panel: Collagen amount in OK cells was stained and quantified with Sirius red/fast green kit. Baf: Bafilomycin A1, Rap: rapamycin; Wort: wortmannin