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Supplemental Methods

Experimental animals

All animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, in compliance with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*. All mice were on the C57BL/6J background. B6N;129-*Ilk*^{tm1Star}/J mice carrying loxP sites between exons 4 and 5 and after exon 12 were generated by Dr. René St-Arnaud from McGill University (Montréal, Canada) and are available from Jackson laboratory (Stock No: 023310). Transgenic mice harboring aquaporin 2 (*Aqp2*) Cre recombinase (*Aqp2*-Cre+) were generated by Dr. Günther Schütz's group from the German Cancer Research Center (Heidelberg, Germany) and obtained from Dr. Wenzheng Zhang of Albany Medical Center (Albany, NY).

To generate PC specific *Ilk* knockout mice, homozygous floxed *Ilk* male mice (*Ilk* $^{fl/fl}$) were crossed with female *Aqp2*-Cre+ mice to generate *Ilk* $^{fl/+}$; *Aqp2*-Cre+ mice. *Ilk* $^{fl/+}$; *Aqp2*-Cre+ mice and *Ilk* $^{fl/fl}$ mice were then crossed to generate homozygous knockout (*Ilk* $^{fl/fl}$; *Aqp2*-Cre+) mice. The littermates without *Aqp2*-Cre (*Ilk* $^{fl/fl}$; *Aqp2*-Cre−) were used as control.

For the necrostatin-1 (Nec-1, N9037, Sigma-Aldrich, St. Louis, MO) treatment experiment, Nec-1 was first dissolved in DMSO and then diluted in phosphate-buffered saline (PBS). 2 weeks old wildtype and *Ilk* $^{fl/fl}$; *Aqp2*-Cre+ mice were given 1.65 mg/kg Nec-1 or PBS containing 1% DMSO through intraperitoneal injection every two days for 2 weeks. Mice were sacrificed at the age of 4 weeks. Blood and urine were collected at that time.

Kidney tissue preparation

Kidney tissues were collected as following. Briefly, mice were anesthetized using isoflurane (3% inhalant). The right kidney was snap frozen in liquid nitrogen and stored at -80°C for protein and RNA analysis. The left kidney was fixed by perfusion through the left ventricle with PBS (0.137 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) followed by paraformaldehyde lysine-periodate (PLP) fixative (4% paraformaldehyde, 75 mM lysine-HCl, 10 mM sodium periodate and 0.15 M sucrose in 37.5 mM sodium phosphate). Freshly perfusion-fixed kidneys were cut into 5 mm slices and incubated in PLP fixative overnight at 4°C. After washing with PBS for three times, kidney slices were kept in PBS containing 0.01% sodium azide at 4°C until use.

For cryosections, kidney slices were immersed in 30% sucrose in PBS overnight at 4°C, and then embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA). 4 μm cryosections were cut using a Leica CM3050S cryostat (Leica Biosystems, Buffalo Grove, IL), mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at −20°C until use.

For paraffin embedding, kidney slices were fixed in formalin overnight at 4°C followed by three washes with PBS. Slices were dehydrated in an ethanol gradient of 50%, 70%, 95% and 100%, then xylene, and finally immersed in paraffin through an automatic Leica TP1020 tissue processor (Leica Biosystems) over 24 hours. After embedded in paraffin, 3 μm sections were cut using a Leica RM 2025 rotary microtome (Leica Biosystems) and stored at room temperature until use.

Measurement of serum creatinine and blood urea nitrogen

Blood samples were collected from mice at the time of tissue harvesting. Serum was stored in a −80°C freezer until use. Serum creatinine concentration was measured by modified Jaffe method using the QuantiChrom Creatinine Assay Kit (Cat. No. DICT-500, BioAssay Systems, Hayward, CA). Briefly, 30 µl serum, water (as blank) or standard (2 mg/dL) was added to each well of a 96-well plate in triplicates. Then 200 µl working reagent (mixed by 100 µl reagent A and 100 µl reagent B) was added to each well. Optical density was measured at 0 min (OD_0) and then at 5 min (OD_5) using 510 nm of wave length. Serum creatinine concentrations were calculated using the formula provided by the manufacturer.

Blood urea nitrogen (BUN) content was analyzed by Stanbio Urea Nitrogen Kit (Stanbio Laboratory, Boerne, TX) according to manufacturer's instruction. Briefly, 20 µl serum, water or standard solution was added to each tube in triplicates. Then 1 ml BUN color reagent and 2 ml BUN acid reagent were added to each tube. After incubating in boiling water bath for 10 min, tubes were immediately immersed in cold water for 3 min. Optical density was measured at 520 nm. Serum BUN concentrations were calculated according to manufacturer's instruction.

Immunofluorescence staining and immunoblotting

Paraffin embedded kidney sections were deparaffinized in xylene and rehydrated with a graded series of ethanol (100% and 95%, 80%, and 70% ethanol in distilled water) and water. After treatment with Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) at 95°C for 20 min, kidney sections were subjected to immunostaining. For immunofluorescence staining of cryosections, slides were rehydrated in PBS and incubated with 1% SDS in PBS for 4 min followed by washing with PBS for three times. Both deparaffinized sections and cryosections were blocked with 1% (w/v) bovine serum albumin in PBS, and incubated with primary antibodies of various dilutions from 1:100 to 1:3000 (Supplemental Table 1). Meanwhile, corresponding normal rabbit IgG (A6154, Sigma-Aldrich) and mouse IgGκ (sc-516176, Santa Cruz, CA) were used to serve as negative control. After washing with PBS for three times, slides were incubated with corresponding fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 1 hour. After three more washes with PBS, slides were mounted and viewed under a Nikon Eclipse 90i epifluorescence (Nikon Instruments, Melville, NY) or a Zeiss LSM800 confocal microscope with Airyscan super-resolution capabilities (Carl Zeiss Microscopy, Thornwood, NY). Analysis of fluorescence intensity was performed using ImageJ software (NIH; https://imagej.nih.gov/). Immunofluorescence staining of kidney with MLKL, CD68 and Ly6G was performed on cryosections, while immunostaining with other primary antibodies was performed on paraffin embedded sections. Quantification of the number of F4/80 or Ly6G positive cells was performed manually in 10 randomly selected fields (magnification: 400 x) for each specimen. Data presented as the number of F4/80 or Ly6G positive cells per high power field (HPF) with a magnification of 400 x.

Immunofluorescence staining of cultured cells was performed similarly as kidney sections with

minor modification. Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed with PBS for three times. After permeabilization with 0.01% Triton X-100 in PBS for 4 min, they were subjected to routine immunostaining for MLKL or phospho-MLKL as described above.

Immunoblotting of kidney tissue lysate or cell lysate was performed as follows. Briefly, kidney or cell lysates were homogenized in lysis buffer (16 mM NaF, 8 mM Na_3VO_4, 0.5% NP-40, 0.1% Triton-X-100, 0.1% SDS in PBS) containing protease inhibitor cocktail (Bimake, Houston, TX). Lysates were obtained by centrifugation at 12,000 g for 15 min at 4°C. Total protein was measured using the BCA assay following manufacturer's instruction (Pierce, Thermo Scientific, Waltham, MA). Protein lysates were separated by SDS-polyacrylamide gel electrophoresis, then electrophoretically transferred to polyvinylidene difluoride membranes (MilliporeSigma, Burlington, MA) using a semi-dry transfer cell (Bio-Rad, Hercules, CA). After blocking with 5% non-fat milk in Tris-buffered saline-0.1% Tween (TBS-T), membranes were incubated with primary antibodies of various concentration (Supplemental Table 1) at 4°C overnight. After washing with TBS-T, the horseradish peroxidase (HRP) conjugated secondary antibodies were applied to membranes (Santa Cruz). Finally, membranes were developed using the ECL Pro Kit (PerkinElmer, Waltham, MA) and exposed to autoradiographic film (Santa Cruz). Relative intensity of protein expression was quantified by optical density using the Quantity One software (Bio-Rad).

To detect urinary NGAL by immunoblotting, 3 μl spot urine collected from each mouse was mixed with 5 μl SDS-PAGE sample loading buffer, then subjected to routine SDS-PAGE and immunoblotting. 2 μg of purified recombinant NGAL protein (230-3002s, Raybiotech, Peachtree Corners, GA) was loaded as a standard for quantification.

Hematoxylin and eosin (H&E) staining

H&E staining was conducted as follows. Briefly, paraffin-embedded kidney sections were deparaffinized, rehydrated and nuclei were stained with Weigert's iron hematoxylin (Sigma-Aldrich). Sections were then rinsed in running tap water and differentiated with 0.3% acid alcohol, followed by 2 min of incubation with Scott's tap water (238 mM sodium bicarbonate, 29 mM magnesium sulphate in distilled water). The sections were then stained with 1% eosin (Sigma-Aldrich) for cytosol staining. The slides were finally dehydrated and mounted with cytoseal 60 mounting media (Thermo). Nuclei were stained dark brown, while the cytoplasm and extracellular matrix were stained pink.

Tubular injury score was calculated based on the percentage of tubules in kidney sections that displayed tubular damage, including tubular dilation or atrophy, tubular necrosis, loss of the brush border, or cast formation. The degree of injury was graded semi quantitatively and blindly by two independent researchers from 10 randomly chosen fields of each kidney section. The score was given according to the extent of injury involved in each field as follows: 0, normal; 1, involvement less than 10%; 2, involvement up to 11 to 25%; 3, involvement up to 26 to 75%; 4, extensive damage involving more than 75% of the observed tubules.

Picrosirius red staining

Paraffin-embedded kidney sections were stained using the Picrosirius red stain kit following manufacturer's instruction (24901, Polysciences Inc, Warrington, PA). Briefly, after deparaffinization and rehydration, paraffin-embedded kidney sections were stained with Weigert's iron hematoxylin for 8 min, and incubated with solution A for 2 min at room temperature. Sections were then rinsed in water and incubated with solution B for 60 min, followed by 2 min of incubation with solution C. The slides were then immersed in 70 % ethanol for 45 sec, dehydrated and mounted using cytoseal 60 mounting media. The Picrosirius red stained kidney sections were examined by a Zeiss LSM800 confocal microscope (Carl Zeiss Microscopy) under normal and polarized light. The staining for type I collagen fibers stains yellow to orange, and type III collagen stains green under polarized light.

Masson's trichrome staining

Paraffin-embedded kidney sections were stained using the Trichrome stains kit (Masson, Sigma-Aldrich) according to manufacturer's instruction. Briefly, paraffin-embedded kidney sections were deparaffinized, rehydrated and incubated with preheated Bouin's solution at 56 °C for 15 min. The sections were then washed in tap water, followed by staining with Weigert's iron hematoxylin for 5 min. Sections were washed in tap water for 5 min, rinsed in deionized water and then stained in Biebrich Scarlet-Acid Fucshin for 5 min. After incubating with working phosphotungstic/phosphomolybdic acid solution for 5 min, sections were placed in Aniline blue solution for 5 min, followed by 2 min of incubation with 1% acetic acid. Finally, sections were dehydrated and mounted. The Masson's trichrome stained kidney sections were examined by the Zeiss LSM800 confocal microscope (Carl Zeiss Microscopy). In Masson's trichrome staining, collagen fibers stained blue, cytoplasm stained pink, and nuclei stained dark brown.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as follows. Briefly, PLP fixed kidney slices were post-fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C for 24 hours. The slices were incubated with 1% osmium tetroxide in cacodylate buffer at room temperature for 1 hour, followed by several rinses in 0.1 M sodium cacodylate buffer. After dehydration in a graded ethanol series from 50% to 100%, followed by brief dehydration in propylene oxide, kidney slices were infiltrated with Eponate resin (Ted Pella, Redding, CA) and embedded in fresh Eponate at 60°C. Ultra-thin sections were cut, stained with uranyl acetate and lead citrate, and examined under a JEOL JEM 1011 transmission electron microscope (JEOL, Peabody, MA). Images were taken using an AMT digital imaging system (Advanced Microscopy Techniques, Danvers, MA).

RNA isolation and Quantitative real time-PCR

Mouse kidney RNA was extracted using QIAshredder and RNeasy purification kits (Qiagen, Valencia, CA). The synthesis of cDNA from mRNA was performed following the protocol of the

High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was carried out using a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA) and the Power up SYBR Green PCR Master Mix (Life Technology, Carlsbad, CA). The level of specific mRNA was normalized to the level of housekeeping gene β-actin expression. The relative mRNA expression was determined by the $2^{-\Delta\Delta Ct}$ method. The sequences of primers that used for the study are summarized in Supplemental Table 2.

Cell culture

LLC-PK1 cells and mCCDC11 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Corning, Corning, NY) containing 10% fetal bovine serum in a 5% CO_2/95% air humidified atmosphere at 37°C. Cells were trypsinized in 0.25% trypsin-EDTA, and plated on standard tissue culture dishes for adherent cell (353002, Corning). Cells were allowed to grow for 24 hours, then an ILK inhibitor, cpd22 of 0.5, 1 and 2 μM was added and incubated with cells for 24 hours (407331, Calbiochem, San Diego, CA). After treatment for 24 hours, cells were harvested and lysed in lysis buffer, and prepared for electrophoresis (SDS-PAGE) and immunoblotting. All experiments were repeated at least three times.

Cell viability assay

Two assays were performed to assess cell viability, including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Affymetrix, Santa Clara, CA) assay and cellular DNA content quantification assay. The LLC-PK1 were seeded on 96 well plates and grown to approximately 80% confluence. Cells were then treated with 0.2% DMSO, ILK inhibitor cpd22 in the presence or absence of the necroptotic inhibitor necrostatin-1 (Nec-1, 50 μM). For MTT assay, 24 hours after treatment, MTT was added and incubated with cells at 37°C for 3 hours. After removal of MTT, DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 548 nm on a microplate reader (model DTX880, Beckman-Coulter, Fullerton, CA). Relative cell viability was presented as the percentage of treated group over control group, as $OD_{treat}/OD_{control}$ x100%.

Cell viability was also assessed by measuring cellular DNA content using the CyQUANT NF Assay Kit (C35006, Invitrogen). 24 hours after treatment as mentioned above, cells in a 96 well plate were incubated with the DNA binding dye at 37°C for 1 hour. Cellular DNA content was analyzed by measuring the fluorescence intensity by the SpectraMax M5 Multi-Mode microplate reader (Molecular Devices, San Jose, CA) with excitation at 485 nm and emission detection at 530 nm. Relative cell viability was presented as the percentage of treated group over control group, as $fluorescence_{treat}$ / $fluorescence_{control}$ x100%.

siRNA transfection

ILK siRNA (sense sequence: AAGGACACAUUCUGGAAGGGG, antisense sequence: CCUUCCAGAAUGUGUCCUUGG) was purchased from GE Dharmacon (Lafayette, CO).

mCCDC11 cells were seeded in 6-well plate and transfected with ILK siRNA (50 nM) or scrambled siRNA mixed with 5 μl Lipofectamine 2000 (Invitrogen) in 1 ml DMEM for 6 hours. 24 hours after transfection, some control cells (transfected with or without scrambled siRNA) were incubated with 2 μM cpd22, and 1 ng/ml recombinant TNF-α (300-01A, PeproTech) respectively. Cell viability assay and immunofluorescence staining were performed 48 hours after ILK siRNA transfection.

Statistical analyses

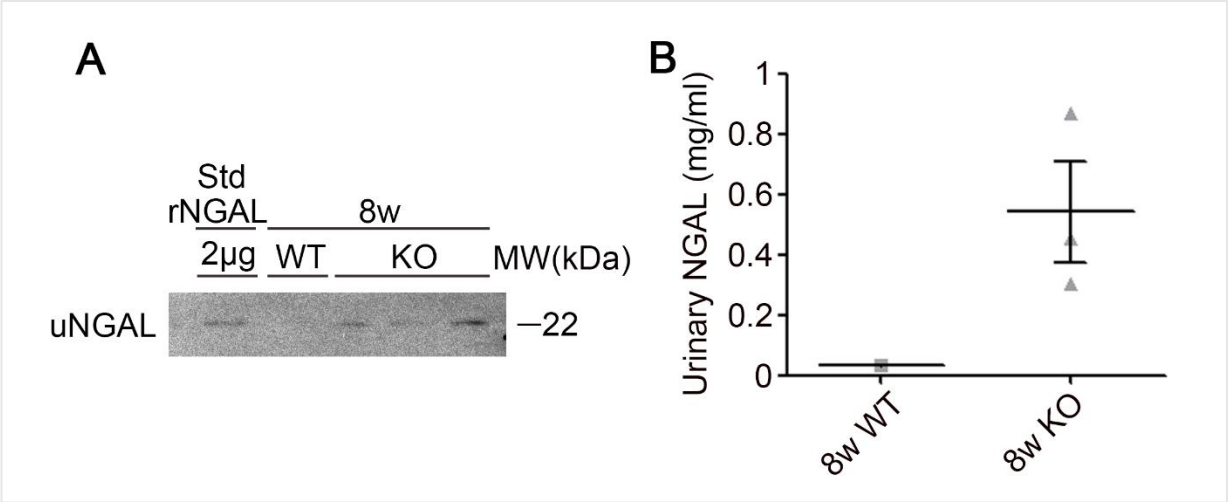
Data are shown as mean ± standard error, SE, of independent replicates ($n \geq 3$). Experimental data were analyzed with Student's *t*-test for two groups or with one-way ANOVA for multiple groups using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). A P value < 0.05 was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Individual P values are specified in figure legends.

Supplemental Table 1. List of primary antibodies used for immunoblotting (IB) and immunofluorescence staining (IF).

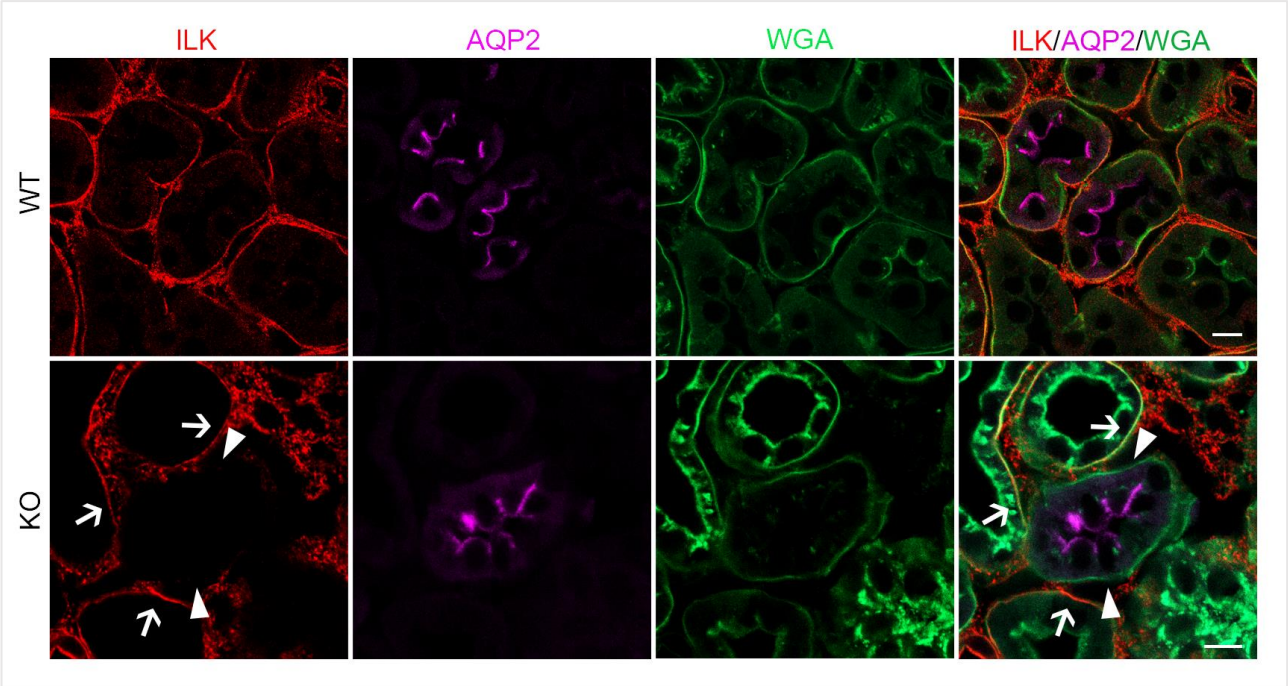
Antigen	Manufacturer	Catalog	Species	Dilution(IB)	Dilution(IF)
AQP2	Santa Cruz	sc-9882	goat	-	1:1800
ILK	Cell Signaling	3862s	rabbit	1:2000	1:1000
NGAL	R&D Systems	AF-1857	goat	1:1000	-
α-SMA	Sigma	A5228	mouse	1:1500	1:3000
collagen I	Sigma	C2456	mouse	-	1:1000
fibronectin	Sigma	F3648	rabbit	1:5000	1:2000
p-Smad3(Ser423/425)	Abcam	ab51451	rabbit	1:1000	-
Smad2/3	Santa Cruz	sc-133098	mouse	1:2000	-
GAPDH	Cell Signaling	2118s	rabbit	1:10000	-
NF-κB p65	Cell Signaling	8242	rabbit	1:1000	-
p-NF-κB p65(Ser536)	Cell Signaling	3033s	rabbit	1:1000	-
F4/80	Invitrogen	14-4801-85	rat	-	1:200
Ly6G	Biolegend	127601	rat	-	1:100
MLKL	EMD Millipore	MABC604	rat	1:1000	-
MLKL	ABGENT	AP14272b-ev	rabbit	-	1:500
p-MLKL (Ser345)	Abcam	ab196436	rabbit	-	1:1000
RIPK3	Santa Cruz	sc-374639	mouse	1:1000	-
p-RIPK3 (Thr231/Ser232)	Cell Signaling	57220s	rabbit	-	1:200
p-RIPK1 (Ser166)	Cell Signaling	31122s	rabbit	-	1:400
CD68	Novus	NB100-683	mouse	-	1:400
V-ATPase	Provided by Dr. Dennis Brown	-	chicken	-	1:800
Akt	Cell Signaling	4691s	rabbit	1:3000	-
p-Akt (Ser 473)	Cell Signaling	4058s	rabbit	1:4000	-
β-actin	Cell Signaling	4967s	rabbit	1:10000	-

Supplemental Table 2. List of primers used for quantitative real-time PCR.

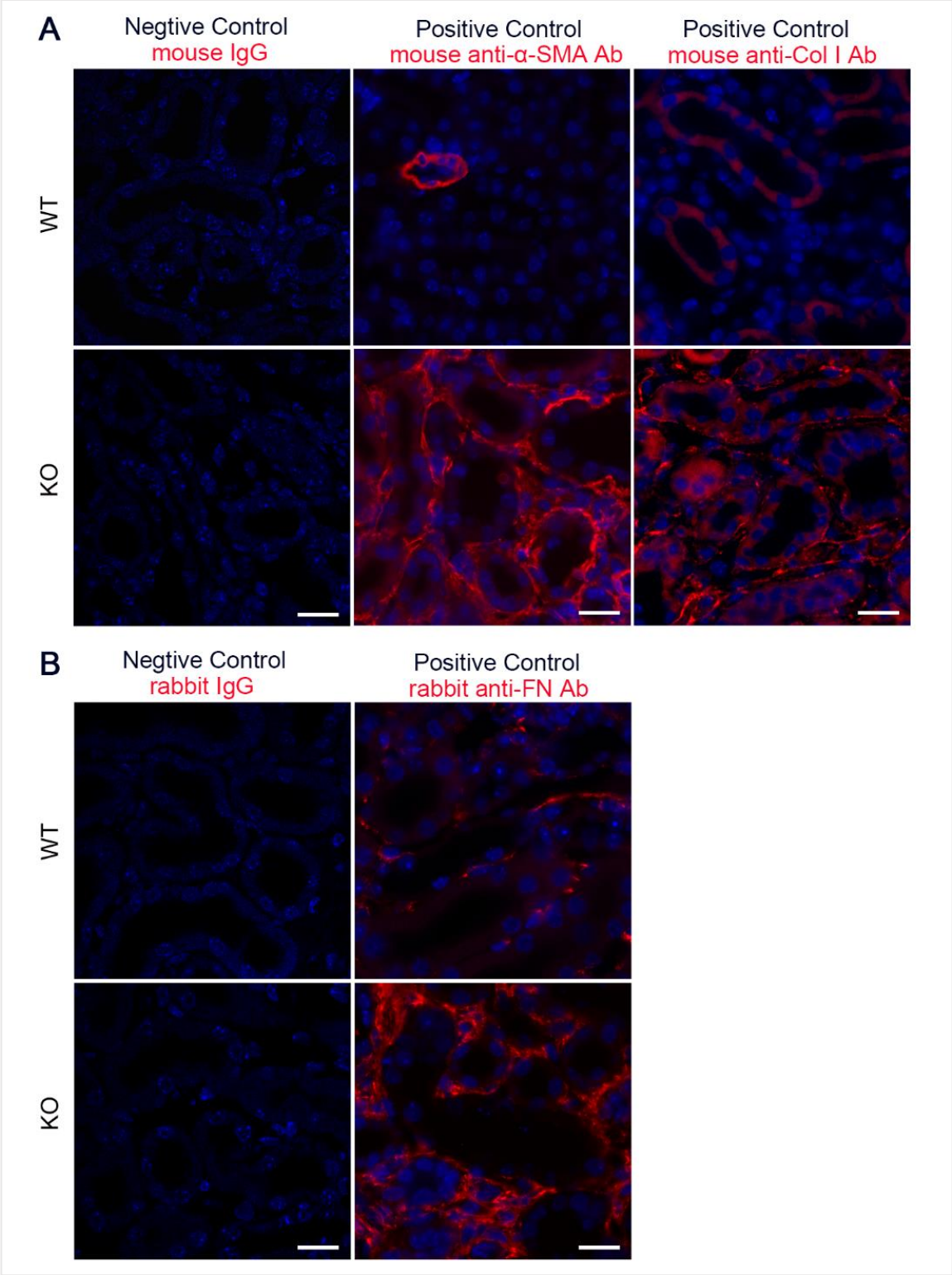
Gene	Forward	Reverse
Fibronectin	5’-CTG GAG TCA AGC CAG ACA CA-3’	5’-CGA GGT GAC AGA GAC CAC AA-3’
Collagen IA1	5’-GCT CCT CTT AGG GGC CAC T-3’	5’-CCA CGT CTC ACC ATT GGG G-3’
TGF-βi	5’-CCT CAC CTC CAT GTA CCA GAA-3’	5’-TGG AAA TGA CCTTGT CAATGA GAG-3’
TNF-α	5’- CCC TCA CAC TCA GAT CAT CTT CT -3’	5’- GCT ACG ACG TGG GCT ACA G -3’
IL-6	5’- CCA AGA GGT GAG TGC TTC CC -3’	5’- CTG TTG TTC AGA CTC TCT CC CT -3’
IL-1β	5’- GCA ACT GTT CCT GAA CTC AAC T -3’	5’- ATC TTT TGG GGT CCG TCA ACT -3’
MLKL	5’-ACA ATG AGT GTG CGC AGC CTC-3’	5’-CTA CGA GGA AAC TGG AGC TGC TG-3’
RIPK3	5’-ACA GGC CAT CCT TCC AGG AC-3’	5’- GCT TGG CTC TCT GGC AGA CAA G -3’
IL-33	5′-TCC TTG CTT GGC AGT ATC CA-3′	5′-TGC TCA ATG TGT CAA CAG ACG-3′
CXCL1	5′-CTG GGA TTC ACC TCA AGA ACA TC-3′	5′-CAG GGT CAA GGC AAG CCT C -3′



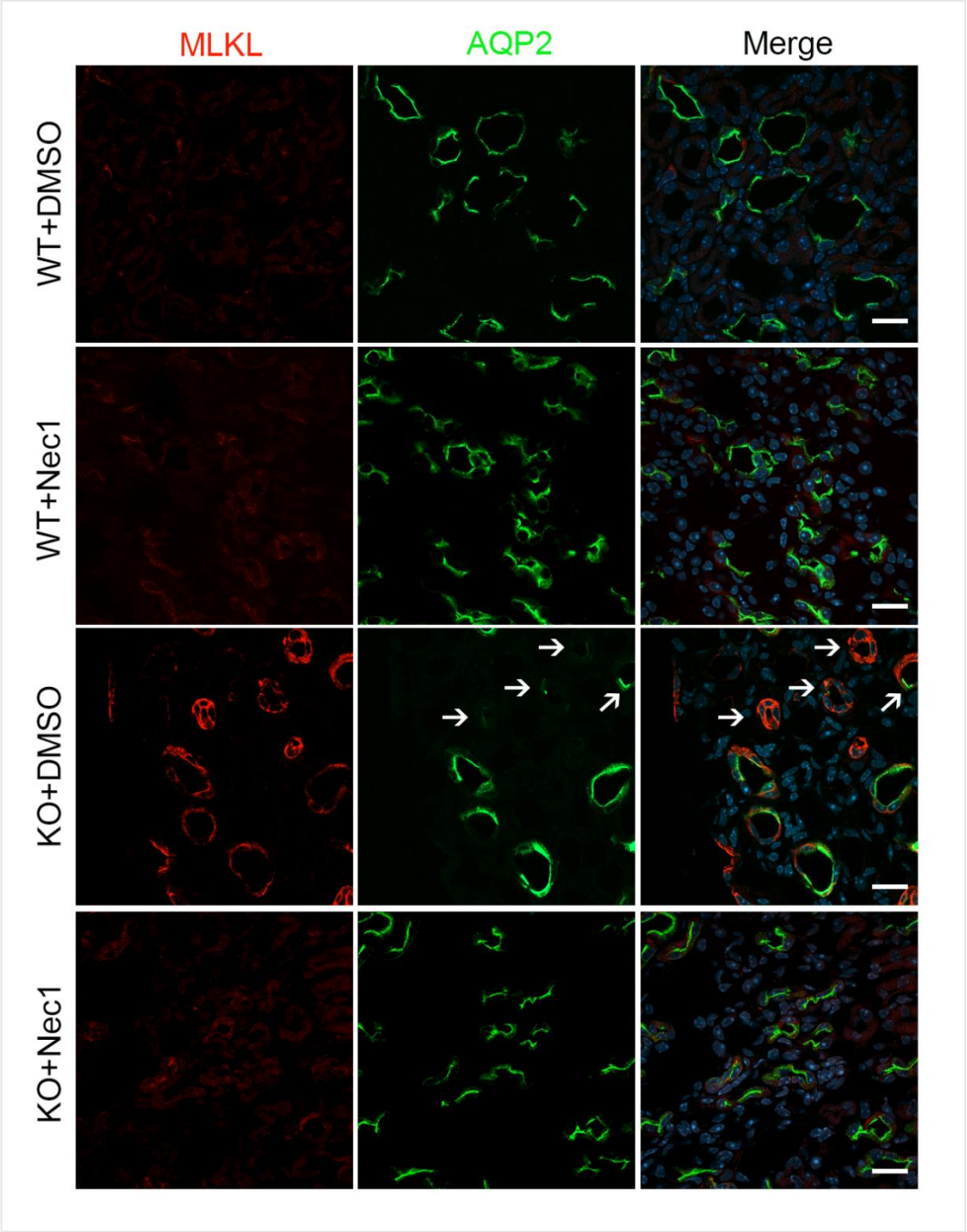
Supplemental Figure 1. (A) Increased urinary NGAL secretion in 8 weeks old KO mice was revealed by immunoblotting. 2 μg of purified recombinant NGAL (Std rNAGL) was loaded as a standard for calculation. (B) Urinary secretion of NGAL in 8 weeks wild type and KO mice was quantified by densitometry and normalized to the expression of Std rNAGL.



Supplemental Figure 2. ILK expression (red) in wild type (WT) and PC *Ilk* KO kidney was revealed by immunofluorescence staining under low magnification. AQP2 stained purple in PCs, and membrane stained green by WGA-FITC. Arrowheads indicate loss of basal ILK signal in AQP2 positive PCs. Arrows indicate intact ILK staining in non-CD tubules in *Ilk* KO kidney. Scale bar=10 μm.



Supplemental Figure 3. Immunofluorescence staining using isotype-matched immunoglobulin G (IgG) revealed minimal, non-specific background staining in the wild type and fibrotic *Ilk* KO kidney. Immunostaining of α-SMA, collagen I (A) and fibronectin (B) signals (red) was significantly increased in interstitium in *Ilk* KO kidney compared with wild type kidney. Immunostaining was performed using serial sections of the same kidney. Scale bar=20 μm.



Supplemental Figure 4. Necrostatin-1 (Nec-1) treatment blocked membrane accumulation of MLKL in *Ilk* KO PCs by immunofluorescence staining viewed under low magnification. AQP2 positive PCs stained green, and MLKL stained red. Arrows highlighted collecting ducts (CDs) that were stained positive for AQP2 in *Ilk* KO kidney. Scale bar=20 μm.