Supplements

The protease aPC ameliorates renal I/R-injury by restricting YB-1 ubiquitination

Running title: aPC regulates YB-1 in renal IRI

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Supplementary materials and methods

Materials

The following antibodies were used in the current study: rabbit polyclonal antibody to β-actin, rabbit polyclonal antibody to YB-1, rabbit polyclonal antibody to OTUB1, mouse monoclonal antibody to ubiquitin, rabbit polyclonal antibody to K48-linkage specific polyubiquitin and rabbit isotype IgG (Cell Signaling Technology, Frankfurt, Germany); rat monoclonal antibody to EPCR (Sigma-Aldrich, Taufkirchen, Germany); rabbit polyclonal antibody to megalin, rabbit polyclonal antibody to HIF-1α, mouse monoclonal antibody to PAR1, mouse monoclonal antibody to PAR2, rabbit polyclonal antibody to PAR3 and goat polyclonal antibody to PAR4 (Santacruz, Heidelberg, Germany); rabbit polyclonal antibody to KIM1, (Abcam, Cambridge, UK). The following HRP conjugated secondary antibodies were used: goat anti-rabbit IgG-HRP, rabbit anti-mouse IgG-HRP, rabbit anti-rat IgG-HRP, rabbit anti-goat IgG-HRP (Abcam, Cambridge, UK).

Other reagents used in the current study were: human plasma thrombin, MG132, DMEM, benzamidine and Bradford reagent, ANTI-FLAG® M2 Affinity Gel, antibiotic/antimycotic solution (10,000 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin B; A5955), APO transferrin, hydrocortisone, amphotericin B (Sigma-Aldrich, Taufkirchen, Germany); trypsin-EDTA, fetal bovine serum, heat inactivated horse serum, insulin/transferrin/selenium, DMEM/F12, PBS, HBSS, TRIZOL, Superscript III Reverse Transcriptase Kit, soybean trypsin inhibitor (Life Technologies, Darmstadt, Germany); interferon γ (Cell Sciences, Canton, MA); protease inhibitor cocktail (Roche diagnostics GmbH, Mannheim, Germany); BCA reagent, shRNA vectors for YB-1 and OTUB1, transfection reagent Turbofect (Thermo Fisher Scientific, Waltham, MA, USA); OTUB1 ORF overexpression construct (OriGene, Rockville, MD, USA); ZipTip C18, PVDF membrane and immobilion enhanced chemiluminescence reagent (Millipore GmbH, Germany); cycloheximide (New England Biolabs, Frankfurt, Germany); ketamine (Pfizer, Karlsruhe, Germany); xylazine (Bayer, Leverkusen, Germany); mouse PAR agonists and control peptides (Bachem, Weil am Rhein, Germany); mouse PAR1 agonist peptide (P1 AP2) (GenScript, Aachen, Germany); DAB substrate Kit for peroxidase (Vector Laboratories, CA, USA); Trypsin, GoTag PCR kit (Promega, Mannheim, Germany); protein A/G-agarose beads (Santacruz, Heidelberg, Germany); human protein C (CEPROTIN®) and Prothromplex NF600 (Baxter, Vienna, Austria); SPECTROZYME® PCa (LOXO, Heidelberg, Germany); collagenase (Worthington Biochemical Corp., Lakewood, N.J.); recombinant human epidermal growth factor (R&D Systems, Minneapolis, MN).

Renal ischemia reperfusion injury model

All mice were anesthetized with sodium ketamine (100 mg/kg body weight, i.p.) and xylazine (10 mg/kg body weight, i.p.). In preliminary experiments we determined that equal dosing was required and sufficient in the different genotypes. Mice were routinely observed during the post-operative recovery phase and no differences in the recovery time between the different genotypes were noticed.

Body temperature was maintained by placing the mice on a 37°C thermostatically controlled operating platform. Post-surgery mice were kept in a heated environment during the recovery phase.

The surgery procedure did not differ among the groups. Only age-matched mice were used. Body fluid was maintained in all mice by subcutaneous administration of 300 µL 0.9% normal saline pre-operatively. A midline abdominal incision was made and both kidneys were exposed. The main renal arteries and veins were identified using a stereotactic microscope (Olympus, Germany), and great care was taken to identify all vascular branches. All renal arteries and veins were then bilaterally occluded for 30 min with nontraumatic microaneurysm clamps (F.S.T Instruments, Germany). To help maintain thermoregulation during surgery, the intestine was relocated and the abdomen was temporarily closed with few stitches. After 30 min of renal ischemia the abdomen was reopened and the clamps were removed. The kidneys were inspected for at least 1 minute to ensure restoration of blood flow (as indicated by a pink color) and 0.5 ml of pre-warmed (37°C) normal saline was instilled into the abdominal cavity. The abdomen was closed with continuous 4-0 polypropylene sutures. All animals received subcutaneous analgesic (buprenorphine 0.1 mg/kg) at the end of surgery. Mice were placed in a temperature controlled (~35°C) environment during the recovery phase and regularly inspected. After full recovery animals were returned to their cages with free access to food and water. Sham surgery consisted of an identical procedure without application of the microaneurysm clamps. Animals were sacrificed 24h after renal ischemia reperfusion injury or sham surgery to obtain blood and tissue samples.

Preparation of activated protein C

Activated protein C was generated as previously described with slight modifications.[1,2] Briefly, prothrombin complex (Prothromplex NF600), containing all vitamin K dependent coagulation factors, was reconstituted with sterile water and supplemented with $CaCl_2$ at a final concentration of 20 mM. The column for purification of protein C was equilibrated at room temperature with 1 liter of washing buffer (0.1 M NaCl, 20 mM Tris, pH 7.5, 5 mM benzamidine HCl, 2 mM Ca^{2+}, 0.02% sodium azide). The reconstituted prothombin complex was gravity eluted on a column filled with Affigel-10 resin covalently linked to a calcium-

dependent monoclonal antibody to PC (HPC4). The column was washed first with two column volumes of washing buffer and then two column volumes with a wash buffer rich in salt (0.5 M NaCl, 20 mM Tris, pH 7.5, 5 mM benzamidine HCl, 2 mM Ca^{2+}, 0.02% sodium azide). Then the benzamidine was washed off the column with a buffer of 0.1 M NaCl, 20 mM Tris, pH 7.5, 2 mM Ca^{2+}, 0.02% sodium azide. To elute PC the column was gravity eluted with elution buffer (0.1 M NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA, 0.02% sodium azide, pH 7.5) and 3 ml fractions were collected. The peak fractions were identified by measuring absorbance at 280 nm. The peak fractions were pooled. The recovered PC was activated with human plasma thrombin (5% w/w) and incubated for 3 h at 37°C. To isolate activated protein C (aPC) ion exchange chromatography with FPLC (ÄKTAFPLC®, GE Healthcare Life Sciences) was used. First, thrombin was removed with a cation exchange column MonoS (GE Healthcare Life Sciences). Then a MonoQ anion exchange column (GE Healthcare Life Sciences) was equilibrated with 10% of a 20 mM Tris, pH 7.5, 1 M NaCl buffer. After applying the solution that contains aPC a 10-100% gradient of a 20 mM Tris, pH 7.5, 1 M NaCl buffer was run through the column to elute aPC at a flow of 1-2 ml/min under continuous monitoring of OD and conductivity. APC eluted at ~36 mS/cm by conductivity or at 40% of the buffer. Fractions of 0.5 ml were collected during the peak and pooled. Proteolytic activity of purified aPC was ascertained with the chromogenic substrate SPECTROZYME® PCa.

***In vivo* aPC capture assay**

Mice were anesthetized (sodium ketamine, 100 mg/kg body weight, i.p., and xylazine 10 mg/kg body weight, i.p.) and injected *via* the tail vein with human PC (20 µg in a final volume of 100 µl 1xPBS) or 1xPBS (100 µl) per mouse. After 10 min blood samples were collected from the vena cava into 0.38% sodium citrate and 50 mM benzamidine HCl (final concentrations). Human aPC was captured from these plasma samples using an antibody highly specific for human aPC (HAPC 1555), and the activity of the captured human protein C was determined using the chromogenic substrate SPECTROZYME® PCa as previously described.[3]

Determination of serum BUN and creatinine

Mice were anesthetized 24h after reperfusion with sodium ketamine (100 mg/kg body weight, i.p.) and xylazine (10 mg/kg body weight, i.p.) and sacrificed. Blood samples were obtained from the abdominal vena cava and collected into tubes pre-filled with sodium citrate (final concentration 0.38%). Plasma was obtained by centrifugation at 2000g for 10 min. Renal dysfunction was evaluated by measuring serum levels of blood urea nitrogen (BUN) and creatinine according to the manufacturer's instructions. Serum BUN was measured using a

kinetic test kit with urease (Roche Diagnostics, Cobas c501 module) and creatinine was determined by an enzymatic based kit (Roche Diagnostics, Cobas c501 module) in the Institute of Clinical Chemistry and Pathobiochemistry, medical faculty, Otto-von-Guericke University, Magdeburg, Germany. [4]

Histology and immunohistochemistry

Sacrificed mice were perfused with ice-cold PBS and then with 4% buffered paraformaldehyde. Tissues were further fixed in 4% buffered paraformaldehyde for 2 days at 4°C, embedded in paraffin and processed for sectioning. Kidney injury was evaluated using hematoxylin and eosin stained histological sections. Images of the outer third of the kidney sections were randomly chosen and captured using an Olympus Bx43 Microscope (Olympus, Hamburg, Germany). All tubuli within an image were individually scored on a scale of 0-4 based on the cellular damage as indicated by morphological signs of cell-swelling and tubular dilatation.[5] The following scores were assigned: 0 – no cellular or tubular damage visible; 1 – damage visible, but less than 25% of the tubuli affected; 2 – 25% to 50% tubular damage; 3 – 50% to 75% tubular damage; and 4 – more than 75% damage. At least 5 random images per mouse and at least 5 mice per group were included into each group.

Immunohistochemical analyses of YB-1 and OTUB1 was conducted essentially as previously described.[3] Paraffin embedded tissue sections were deparaffinised and rehydrated, incubated with a specific primary antibody (1 h, at room temperature), washed 3 times with PBS, and incubated with an appropriate, horseradish peroxidase-conjugated secondary antibody. Peroxidase activity was detected using a DAB substrate (3,3'-diaminobenzidine) and slides were counterstained with hematoxylin.[3] Control images were obtained following incubation with a non-specific primary antibody and were used for background correction. All histological analyses were done by two independent blinded investigators. Images were obtained using an Olympus Bx43 Microscope (Olympus, Hamburg, Gemany) at 20x or 40x magnification.

Analyses of human tissue samples

Paraffin sections of human kidney biopsies obtained from patients with acute renal failure were scored for the severity of tubular injury (H.J.G.). YB-1 staining was done by a blinded investigator (K.S.) and staining intensity was scored by two independent and blinded investigators (W.D. and H.W). Control sections were obtained from patients undergoing tumor nephrectomy, but without any other renal disease. All patients and controls were Caucasian. The study complied with the Declaration of Helsinki. Tissue samples were collected according to the guidelines of the local ethics committees after giving written informed consent (Ethic-Committee-No: 068/1999). Immunohistochemical staining was

essentially performed as outlined above using a rabbit anti-YB1 antibody (primary antibody, abcam, ab12148) and a TRITC labelled polyclonal anti-rabbit antibody (secondary antibody, Dako R0156). Sections were counterstained with DAPI.

Cell culture

BUMPT cells were routinely cultured in DMEM containing 10% FBS and maintained at 33°C in the presence of interferon γ (10 U/ml) to enhance expression of a thermosensitive T antigen.[6] Under these conditions cells remain undifferentiated and proliferate. To induce differentiation, BUMPT cells were grown for 2 days at 37°C in the same medium without interferon γ. Differentiation was ascertained by expression of Na^+/glucose transporter and megalin, both specific markers of proximal tubular cells. Only differentiated cells were used for hypoxia and reoxygenation experiments.

Mouse primary renal proximal tubular epithelial cells (rTEC) were isolated by using a modification of previously described methods[7,8]. Mice were scarified by cervical dislocation. Kidneys were immediately removed and placed in cold (4 °C) Hanks Balanced Salt Solution (HBSS) (Life Technologies, Germany) with 1% antibiotic/antimycotic additive (Sigma-Aldrich, Germany). After removal of renal capsules kidneys were bisected and the renal medulla was discarded. The remaining cortical tissue was minced and transferred to 10 ml HBSS containing collagenase (200 units/ml; Worthington Biochemical Corp., Lakewood, N.J.) and Soybean Trypsin Inhibitor (0.5mg/ml, Life Technologies, Germany). The tubuli containing suspension was incubated (37 °C, 70 rpm) for 15 minutes, re-suspended with a 10 ml pipette, and incubated again for 15 minutes. Following digestion the suspension from each kidney was re-suspended again and then distributed into two 15 ml conical tubes (two tubes with ~5 ml each per kidney). Density sedimentation with horse serum was used to inactivate enzymes and enrich for rTECs. To accomplish this, 5 ml of sterile, heat inactivated horse serum (Life Technologies, Germany) was added to each tube and the tube was vortexed for 30 seconds. After sedimentation of tissue remnants for 1 minute the supernatant containing the rTECs was transferred to another tube and centrifuged (7 minutes, 200xg). The cell pellet was washed once with 10 ml of HBSS and centrifuged (200xg, 7 minutes). The supernatant was discarded and the rTECs isolated from one kidney were re-suspended and pooled in 60 ml of DMEM/F-12 culture media (Life Technologies, Germany) containing insulin/ transferrin/ selenium (5 µg/ml, 2.75 µg/ml, and 3.35 ng/ml, respectively, Life Technologies, Germany), APO transferrin (2.0 µg/ml), hydrocortisone (40 ng/ml, Sigma-Aldrich, Germany), recombinant human epidermal growth factor (rhEGF, 0.01 µg/ml, R&D Systems, Minneapolis, MN), and 1% antibiotic/antimycotic solution (10,000 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 µg/ml amphotericin B, Sigma-Aldrich Germany). rTECs were incubated at 37 °C with 5% CO_2. Culture media was replaced initially after 24 hours and

subsequently every 48–72 hours using DMEM/F-12 without rhEGF. By staining cells for megalin a purity of at least 95% was confirmed. For routine passage cells were rinsed with a calcium- and magnesium-free PBS and exposed to trypsin (0.05%)-EDTA (Life Technologies, Germany) for 1 min. rTEC cells were grown in 12-well or P-100 dishes and experiments conducted at confluence.

***In vitro* hypoxia and reoxygenation model**

Hypoxia and reoxygenation of BUMPT cells and rTECs was conducted as previously described.[9] Differentiated and 80% confluent BUMPT cells or rTECs were serum deprived overnight (0.5% FBS). For hypoxia injury cells were kept in HBSS (Life Technologies, Darmstadt, Germany) in a hypoxic atmosphere containing 1% O_2, 94% N_2, 5% CO_2. A hypoxia chamber (Stemcell, Grenoble, France) was used to maintain cells under hypoxic conditions. After 6 h hypoxia cells were returned to complete medium and 21% O_2 ("reoxygenation" period). As controls we used cells which were likewise serum-starved and maintained in HBSS for 6 h, but these cells were continuously maintained at 21% O_2. Cells were harvested for RNA and protein isolation at various time-points after reoxygenation. In a subset of experiments cells were pre-treated (30 min before hypoxia) with aPC (20 nM), the protein biosynthesis inhibitor cycloheximide (10 µg/ml), the proteasome inhibitor MG132 (10 µM), PAR1, PAR2, PAR3, or PAR4 agonist peptide or control peptide (each 10 µM, see Supplementary Table S1), or blocking antibodies towards PAR1, PAR2, PAR3, PAR4, or EPCR (each 20 µg/ml). Two different PAR-1 activating peptides were used, corresponding to the thrombin-specific tethered ligand (P1 AP2, TFLLR) or the aPC-specific tethered ligand (P1 AP1, NPNDKYEPFWEDEEKNESGL). In all cases cells were pre-treated for 30 min. All concentrations are final concentrations in the cell-culture medium.

Proteomics

We transfected BUMPT cells with a flag-tagged YB-1 overexpression construct or an appropriate control construct. Cells were lysed 24h after transfection in RIPA buffer and protein complexes were pulled down with an ANTI-FLAG® M2 Affinity Gel. Immunoprecipitated samples were diluted with 50mM ABC (ammonium bicarbonate) and then reduced with 25 mM DTT (dithiothreitol) in 20mM ABC for 60 min at 60°C. This was followed by alkylation using 100 mM of IA (2-Iodoacetamide) in 20 mM ABC for 30 min at 37°C in dark. These modified proteins were digested using trypsin 20 ng/µl for 15-16 h by incubating at 37°C. The digestion was stopped using 1% acetic acid and samples were purified using ZipTip C18 (Millipore Corp., Billerica, MA, USA). This mixture was subjected to LC-MS/MS analysis. The digested peptides were first enriched on a nanoAcquity UPLC 2G-V/Mtrap Symmetry C18 pre-column (2 cm length, 180 µm inner diameter and 5 µm particle

size) from Water Corporation and separated using NanoAcquity BEH130 C18 column (10 cm length, 100 µM inner diameter and 1.7 µm particle size from Water Corporation) on a nanoAcquity UPLC. The separation was achieved by the formation of a linear gradient over 92 min using buffer A (2% acetonitrile, 2% DMSO in water with 0.1% acetic acid) and buffer B (5% DMSO in acetonitrile with 0.1% acetic acid; gradient: 1-5% buffer B in 2 min, 5-25% B in 63 min, 25-60% B in 25 min, 60-99% B in 2 min). The peptides were eluted at a flow rate of 400 nL/ min and were analyzed using LTQ-Orbitrap Velos mass spectrometer (Thermo Electron Corporation, Germany) equipped with a nano-ESI source installed with a Picotip Emmitter (New Objective, USA).

The MS was operated in positive mode and in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Survey full scan MS spectra (from *m*/*z* 300 to 1700) were acquired in the Orbitrap with resolution, *R*=30 000 with a target value of 1 x E6. The method allowed sequential isolation of the twenty most intense ions depending on signal intensity and were subjected for CID fragmentation with an isolation width of 2 Da and a target value of 3 x E4 or with a maximum ion time of 100 ms. Target ions already selected for MS/MS were dynamically excluded for 60 s. General MS conditions were electrospray voltage, 1.7 kV; no sheath and auxiliary gas flow, capillary temperature of 300°C. Ion selection threshold was 2000 counts for MS/MS, activation time of 10 ms, and activation energy of 35% normalized were also applied for MS/MS. Only doubly and triply charged ions were triggered for tandem MS analysis.

The raw data acquired on the MS instrument was further analysed for protein identifications using Proteome Discoverer 1.4.1.14 (Thermo Scientific, USA). The MS spectral data was searched against human FASTA formatted Uniprot/SwissProt database using SequestHT algorithm. Database searches were performed with carbamidomethylation on cysteine as fixed modification and oxidation on methionine as variable modification. Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 10 ppm peptide ion and 0.8 Da MS/MS tolerances.[10] Peptides with a false discovery rate (FDR) of less than 1% were accepted and estimated by Percolator. Gene ontology (GO) classification and location, biological process, and molecular function was performed using ProteinCenter software (Thermo Scientific).

Immunoblotting and Immunoprecipitation

Following deep anaesthesia and before perfusion of animals the renal artery and vein of one kidney was ligated, then removed and flash frozen in liquid nitrogen for protein and RNA isolation. Proteins were isolated using RIPA buffer and the concentration was determined using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA).

Kidney and cell extracts were separated by SDS/PAGE and transferred to PVDF membranes. Membranes were blocked in Tris-buffered saline with 0.1% Tween 20 with 5% non-fat dry milk or bovine serum albumin. Membranes were incubated with appropriate primary antibodies overnight at 4°C. After washing 5 times with 1 x TBS-T membranes were incubated with an appropriate secondary peroxidase-conjugated antibody, and immunoreactive proteins were visualized using an enhanced chemiluminescence system (Millipore, Darmstadt, Germany). For Immunoprecipitation, protein lysates were precleared with protein A/G-agarose beads to reduce non-specific binding. Cleared protein lysates were incubated with anti-YB-1 or irrelevant IgG antibodies. Antigen-antibody complexes were precipitated following incubation with protein A/G-agarose beads at 4°C overnight by centrifugation, and washed in cold lysis buffer (RIPA-buffer). The precipitates were boiled for 5 min in SDS loading buffer and subjected to immunoblotting.[11]

RT-PCR

Kidney tissue was thawed on ice and transferred into TRIZOL (Life Technologies, Darmstadt, Germany) for isolation of total RNA following the manufacturer's protocol. Quality of total RNA was ensured on an agarose gel and by analyses of the A260/280 ratio. The reverse transcription reaction was conducted with 1 µg of total RNA using the Super Script reagents and oligo(dT) primers (Life Technologies, Darmstadt, Germany). cDNA was amplified using the primers listed in the Supplementary Table S2.

Supplementary Tables

Name	Abbreviation	Peptide sequence
PAR1 agonist peptide (aPC)	P1 AP1	NPNDKYEPFWEDEEKNESGL
PAR1 agonist peptide (aPC) control	CON P1	GDENENEKPNWYELKEPDSF
PAR1 agonist peptide (thrombin)	P1 AP2	TFLLR
PAR1 agonist peptide (thrombin) control	CON P1	RLLFT
PAR2 agonist peptide	P2 AP	SLIGRL
PAR2 agonist peptide control	CON P2	LSIGRL
PAR3 agonist peptide	P3 AP	SFNGGP
PAR3 agonist peptide control	CON P3	FSNGGP
PAR4 agonist peptide	P4 AP	AYPGKF
PAR4 agonist peptide control	CON P4	YAPGKF

Supplementary Table S1: PAR agonist peptides and control peptides sequences

Gene	Forward	Reverse
mPAR1	5’ CCGGCACTGATTGGCAGTT 3’	5’ GACTGGATCGGATACACCACC 3’
mPAR2	5’ CACCACCTGCCACGATGT 3’	5’ CGATTCACAGTGCGGACAC 3’
mPAR3	5’ ATGGGCATCAACCGCTAC 3’	5’ GCTGTCGGTATTGTGGTAG 3’
mPAR4	5’ AACGCCTCACTACTGGACTCT 3’	5’ GAGCCAGCTAATCGGAAGGTC 3’
mEPCR	5’ AATGCCTACAACCGGACTCG 3’	5’ ACCAGTGATGTGTAAGAGCGA 3’
mβ-actin	5’CCGTAAAGACCTCTATGCCAACA 3’	5’ CGGACTCATCGTACTCCTGCT 3’

Supplementary Table S2: Sequences of mouse primers used within the current study.

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Figure S1

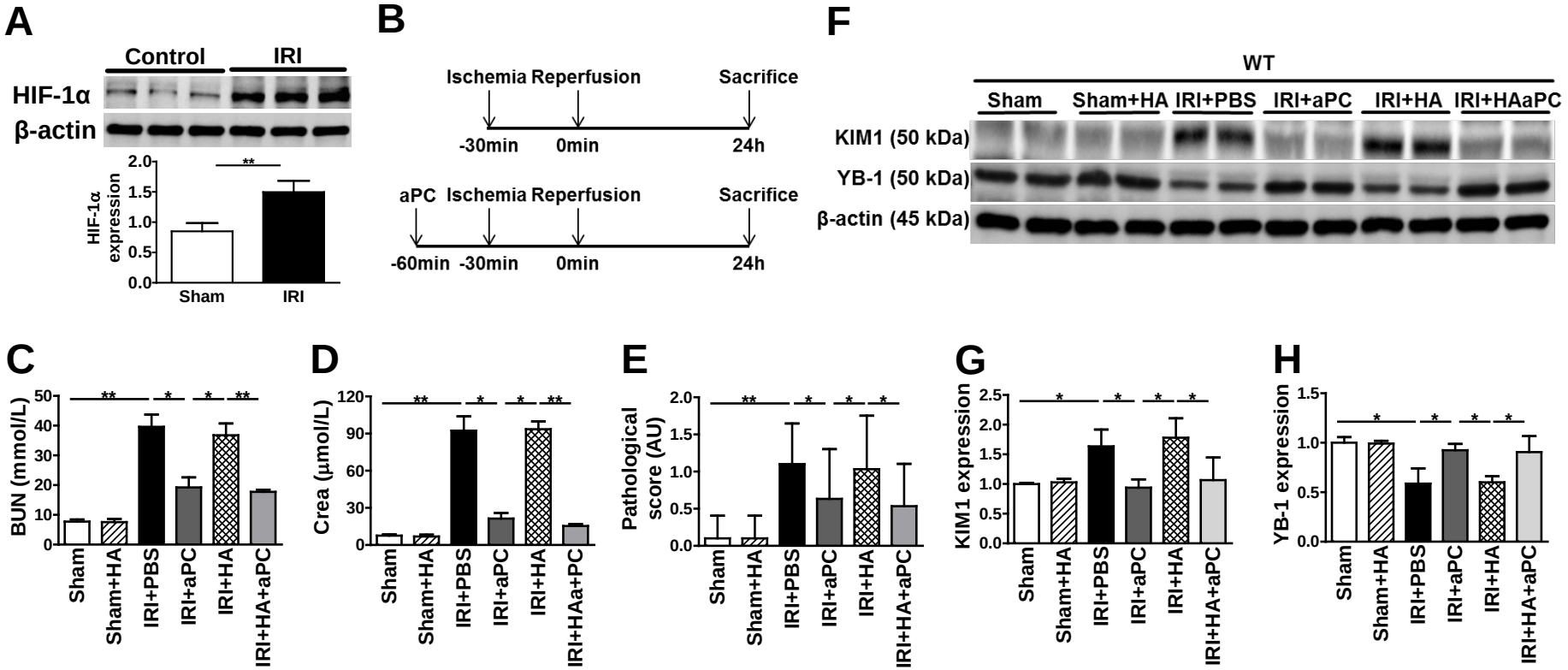

Figure S1: aPC's protective effect in renal IRI is independent of its anticoagulant function

A: Efficient hypoxic stress was ascertained by determining HIF-1α expression in renal cortex extracts. Exemplary immunoblot of HIF-1α (132 kDa) and β-actin (loading control, 45 kDa) and bar graph summarizing results.

B: Scheme of the murine renal IRI model. Renal pedicles were bilaterally occluded for 30 min (ischemia) and the mice were sacrificed 24h after reperfusion. In a subset of mice aPC (0.5 mg/kg body weight i.p.) was administered 30 min prior to renal ischemia (bottom).

C-E: Serum urea nitrogen (BUN, C), creatinine (Crea, D), and pathological score (E) in control (sham, open bars), experimental (ischemia reperfusion injury, IRI+PBS, black bars), and wild type IRI mice receiving either aPC (0.5 mg/kg aPC, dark grey, IRI+aPC) or aPC (0.5 mg/kg) pre-incubated with the HAPC1573 antibody (light grey bars, IRI+HA+aPC). Treatment with the antibody HAPC1573 alone has no impact in sham (Sham+HA, striated) or IRI (IRI+HA, chequered) wild-type mice on these parameters.

F-H: Treatment of wild-type IRI mice with aPC or aPC–HAPC1573 complex (0.5 mg/kg) efficiently diminished KIM1 expression while preserving YB-1 levels; representative immunoblots (F) of kidney lysates obtained from control (Sham, open bars), IRI (black), and IRI mice treated with aPC (dark grey) or the aPC–HAPC1573 (light grey) complex. The antibody HAPC1573 itself has not impact on KIM1 or YB-1 expression in wild-type sham (Sham+HA, striated) or IRI (IRI+HA, chequered) mice. Bar graphs (G, H) summarizing results.

Bar graphs representing mean value ± SD of at least 6 mice per group (B, C, D); ★: $P<0.05$, ★★: $P<0.01$ (ANOVA).

Figure S2

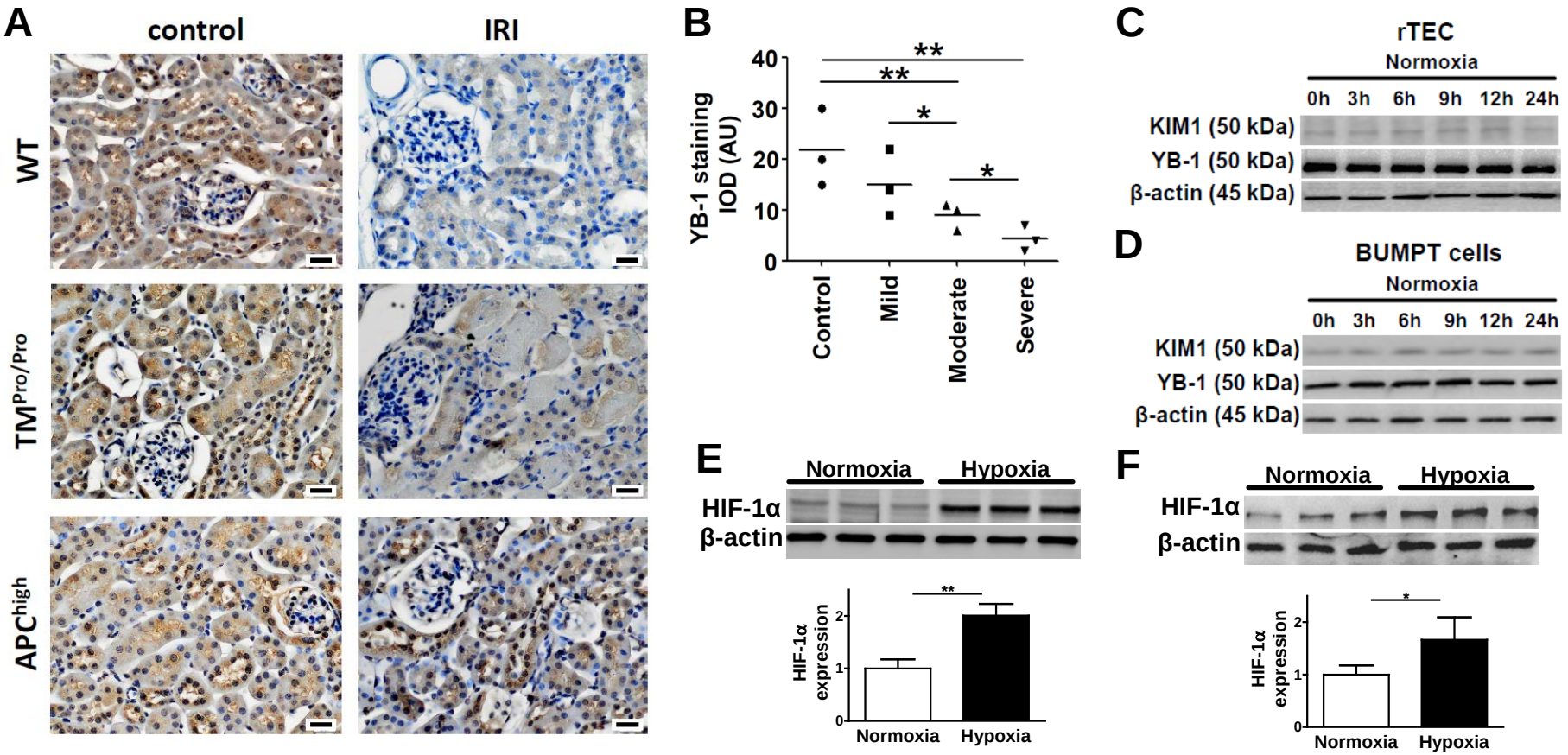

Figure S2: Loss of YB-1 expression following acute renal injury in mice and humans

A: Following IRI tubular YB-1 expression is diminished in WT and TM$^{Pro/Pro}$, but not in APC^{high} mice. Exemplary immunohistochemical images of renal paraffin embedded tissue sections obtained from control (sham-operated) or IRI mice. YB-1 antigen detected by HRP-DAB reaction (brown), hematoxylin counterstain (blue); scale bar: 20 µm.

B: Dot blot summarizing results of YB-1 expression in human renal biopsies of patients with acute renal injury graded as mild, moderate, or severe by an experienced pathologists. Expression of YB-1, determined by immunohistochemical analyses, declines with an increasing severity of acute renal injury. Dot blot corresponding to the exemplary immunohistochemical images shown in Figure 2B; ★: $P<0.05$, ★★: $P<0.01$ (Wilcoxon-Mann-Whitney-test).

C,D: Expression of KIM1 and YB-1 remains stable under normoxic conditions in primary rTEC (C) and BUMPT (D) cells. Representative immunoblots of three repeat experiments.

E,F: Efficient hypoxic stress was ascertained by determining HIF-1α expression in cell lysates of BUMPT cells (E) and rTECs (F). Exemplary immunoblot of HIF-1α (132 kDa) and β-actin (45 kDa, loading control) and bar graph summarizing results of three independent repeat experiments (each in triplicates).

Figure S3

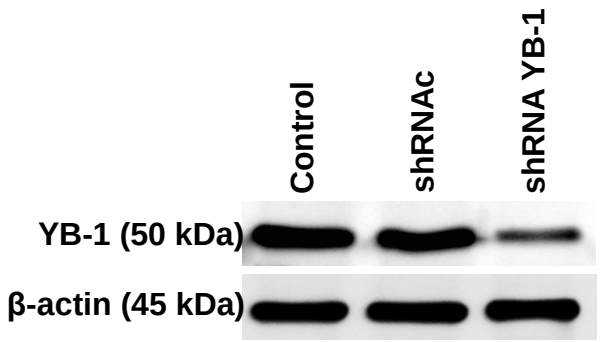

Figure S3: YB-1 knock down in tubular cells
Immunoblot of non-transfected tubular (BUMPT) cells and BUMPT cells stably transfected with non-specific control shRNA (shRNAc) or YB-1 specific RNA (shRNA YB-1). Exemplary immunoblot, showing reduced YB-1 expression in shRNA YB-1 cells; β-actin as loading control.

Figure S4

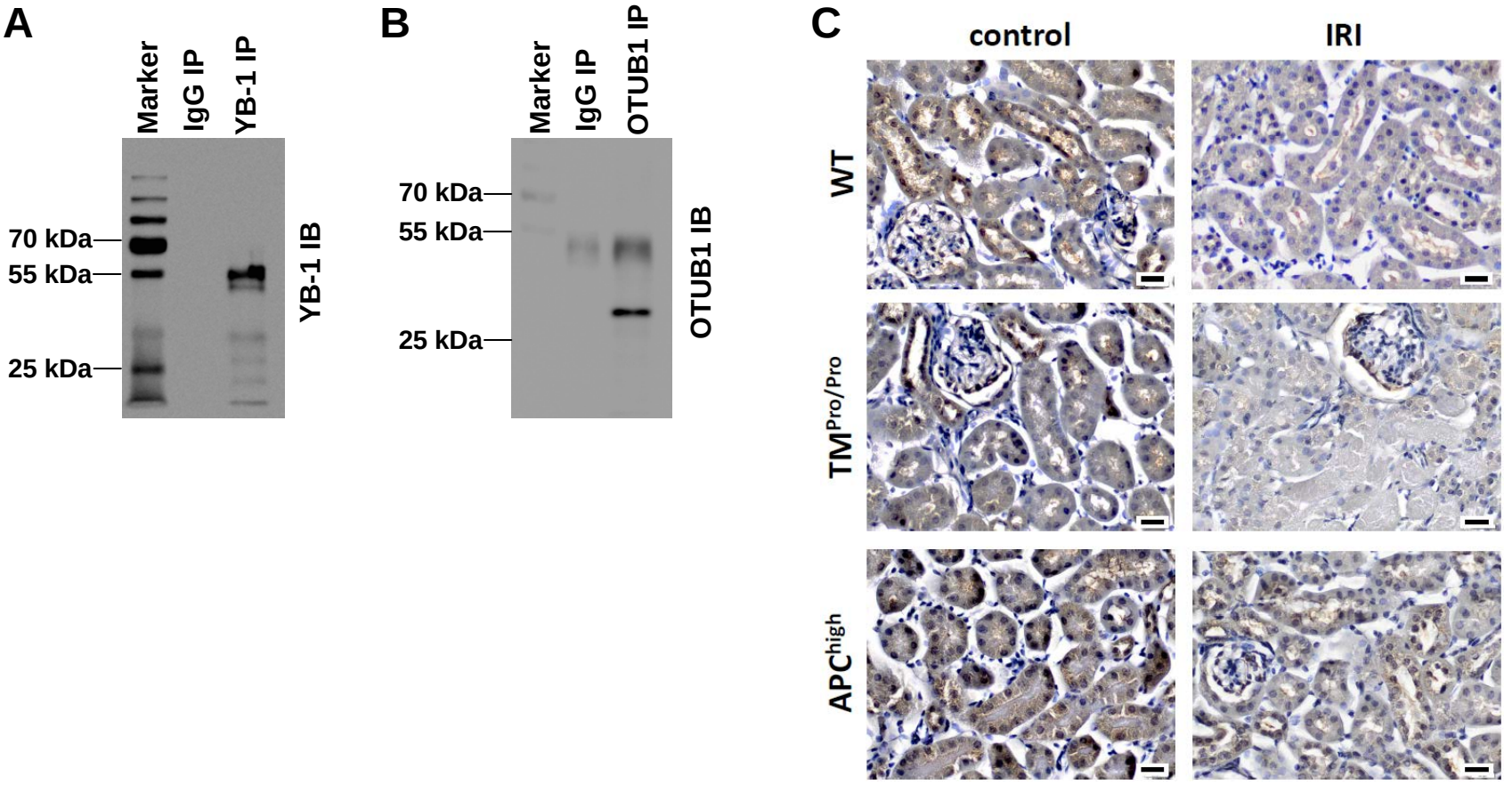

Figure S4: IgG controls for immunoprecipitation experiments and OTUB1 expression in renal tissue

A: Immunoblotting of YB-1 (YB-1 IB) following immunoprecipitation using antibodies against YB-1 or IgG control (IgG IP) from whole cell lysates of control mouse tubular cells.

B: Immunoblotting of OTUB1 (OTUB1 IB) following immunoprecipitation using antibodies against OTUB1 or IgG control (IgG IP) from whole cell lysates of control mouse tubular cells.

C: Following IRI tubular OTUB1 expression is diminished in WT and $TM^{Pro/Pro}$, but not in APC^{high} mice. Exemplary immunohistochemical images of renal paraffin embedded tissue sections obtained from control (sham-operated) or IRI mice. OTUB1 antigen detected by HRP-DAB reaction (brown); hematoxylin counterstain (blue); scale bar: 20 µm.

Figure S5

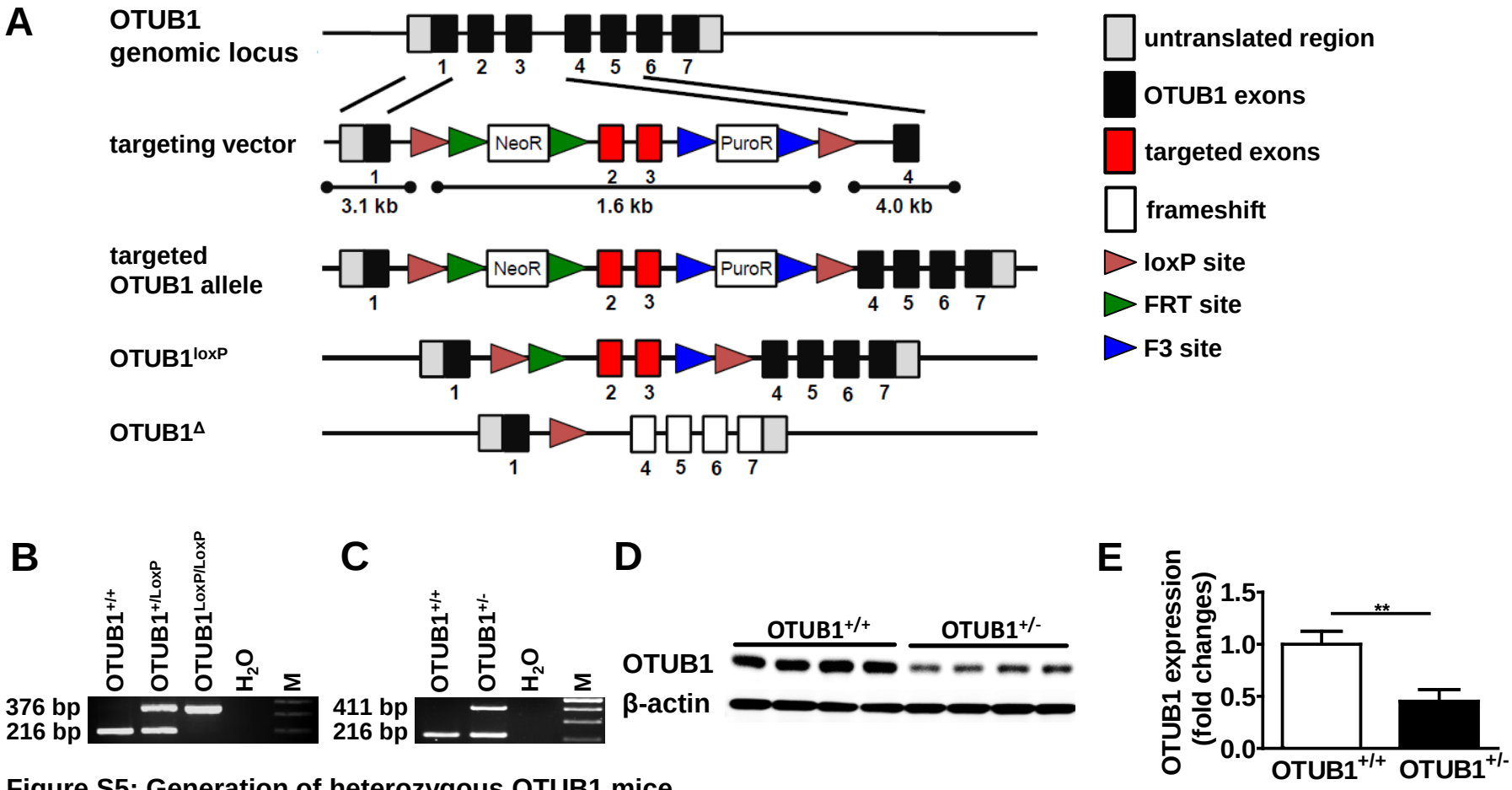

Figure S5: Generation of heterozygous OTUB1 mice

A: Schematic representation of the gene targeting strategy to obtain heterozygous OTUB1 mice. Initially, a targeting construct containing a NeoR and PuroR expression cassette flanking exon 2 and 3 of the OTUB1 gene and two loxP-sites was generated and used to target murine embryonic stem cells. The NeorR and PuroR cassettes were removed by flp-mediated recombination, yielding $OTUB1^{LoxP}$ mice. Subsequently, exon 2 and 3 were removed by breeding heterozygous $OTUB1^{LoxP}$ mice with C57BL/6 Rosa 26-$Cre^{+/-}$ mice, resulting in germline inactivation of one OTUB1 allel. **B:** PCR of tail DNA from the indicated mice showing a 216 bp band in case of $OTUB1^{+/+}$, a 216 and a 376 bp band in case of heterozygous $OTUB1^{+/LoxP}$, and a 376 bp band in case of $OTUB1^{LoxP\ /LoxP}$ mice. **C:** Standard PCR of tail DNA obtained from $OTUB1^{+/+}$ mice (216 bp) or $OTUB1^{+/-}$ (411 bp) mice. **D,E**: Exemplary immunoblot showing OTUB1 expression in renal cortex samples obtained from $OTUB1^{+/+}$ and $OTUB1^{+/-}$ mice (D) and bar graph summarizing results of 8 mice per group (E); mean ± SD; *t*-test, ✶✶: $P<0.01$.

Figure S6

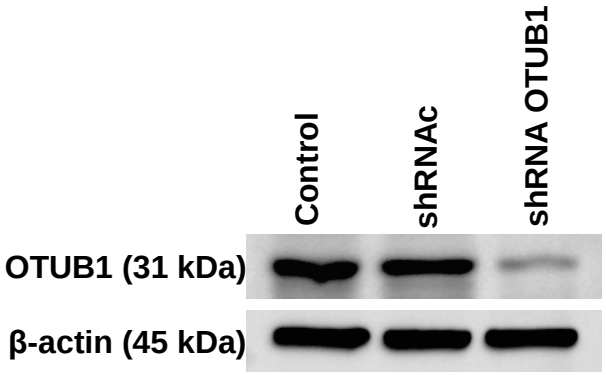

Figure S6: OTUB1 knock down in tubular cells
Immunoblot of non-transfected tubular (BUMPT) cells and BUMPT cells stably transfected with non-specific control shRNA (shRNAc) or OTUB1 specific RNA (shRNA OTUB1). Exemplary immunoblot, showing reduced OTUB1 expression in shRNA OTUB1 cells; β-actin as loading control.