Supplementary Material

Complete methods

Animal experiments

All animal experiments were conducted according to the German law governing the welfare of animals, and were approved by the Committee on Research Animal Care, Regierungspräsidium Freiburg (G-11/99). Animal procedures were also approved by the Departmental Director of 'Services Vétérinaires de la Préfecture de Police de Paris' and by the ethical committee of the Paris Descartes University. Mice were housed under a 12 hours dark/light cycle condition and controlled temperature of 23°C, fed ad libitum with normal mice chow. Only male mice were used for the study. Mice were 4-6 months old unless otherwise specified.

Breeding and genotyping were performed using standard protocols. Female *ATP6AP2-floxed* mice ($PRR^{flox/flox}$, generously provided by Michael Bader and Genevieve Nguyen) were crossed with male *Pax8.rtTA;tetO.Cre* mice to generate *ATP6AP2* tubule-specific male KO mice ($PRR^{fx/Y}$; *Pax8.rtTA;tetO.Cre*). $TetOcre^{-}$ littermates served as controls. All mice were crossed to C57/Bl6 background. For the induction of ATP6AP2 deletion pregnant female mice received doxycycline hydrochloride (Fagron, Barsbüttel, Germany) via the drinking water (2mg/ml with 5% sucrose, protected from light) at E16.5 up to birth (P0). At P0, doxycycline was replaced by normal drinking water. To assess the expression pattern of Pax8 following our inducible strategy, we crossed the *Pax8.rtTA;tetO.Cre* mice with the *reporter line* ($Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo/J}$) to generate mice expressing eGFP fluorescence in the Pax8 positive cells and Td-Tomato fluorescence in the negative ones. For urine collection individual mice were studied in metabolic cages (Techniplast, Marcoussis, France). After 3 days of adjustment 24h urine output was collected under paraffin oil. Retro-orbital blood was sampled in isoflurane-anaesthetized mice.

dDAVP challenge: After adjustment and collection of baseline parameters in metabolic cages, mice were injected *i.p.* with dDAVP (1µg/Kg of body weight (BW)) (n=5 control vs. 4 *cKO*). Urine collection was performed after an additional 5 h in metabolic cages. Placing the mice on a cold plate stimulated complete emptying of the bladder.

Acid load challenge: Mice were fed with HCl 0.3M enriched standard chow for 6 days (n=4 control vs. 5 *cKO*). Urine collection was performed daily, while blood gas analysis carried out at day 3 and 6 by retro-orbital puncture through an ABL 77 pH/blood-gas analyzer (Radiometer).

Salt Restriction: Mice were switched from 0.3% sodium containing diet (INRA) to a NaCl free diet for 7 days (n=6 control vs. 6 *cKO*). Urine collection for determining sodium excretion was performed after 0, 8, 24, 48, 72, 96, 120, 144, 168 hours from the switch.

Furosemide challenge: Mice were studied in metabolic cages (n=5 control vs. 6 *cKO*). After adjustment, urine was collected after 3h after *i.p.* injection of either vehicle either furosemide (2mg/Kg BW). Placing the mice on a cold plate stimulated complete emtpying of the bladder.

Telemetry BP recording and Angiotensin II infusion: mice (n=4 control vs. 4 *cKO*) were anesthetized with isofluorane and a BP telemeter (modelTA11PA-C10; Data Sciences International, St. Paul, MN) was implanted in the left femoral artery. A single dose of amoxicillin (20 mg/kg ip, clamoxyl; SmithKlineBeecham, Nanterre, France) and ketoprofen (5

mg/kg *i.p.*, Profenid; Aventis, Paris, France) was administered after the surgery. Mice were adapted for 1 week, and BP values from the last three days of the adaptation period were averaged to define the basal BP. The morning of day 1, angiotensin II filled osmotic mini-pumps (Alzet 1004) were implanted subcutaneously. Angiotensin II (Sigma-Aldrich A9525) was administered for 15 days at a rate of 1µg/kg of BW/min.

Biochemical and hormonal analyses

Urinary pH was measured using pH-meter (Mettler Toledo, Viroflay, France). Urine osmolality was determined with a freezing point osmometer (Axel Löser Meßtechnik, Berlin, Germany). Urinary creatinine was analysed by an enzymatic colorimetric creatinine kit (Labor+Technik Lehmann, Berlin, Germany). Urinary ammonium and titrable acidity were measured with a DL 55 titrator (Mettler Toledo). Urinary sodium was measured via flame photometry (Instruments Laboratory, Bedford, MA, USA).

Plasma renin was measured by radioimmunoassay as previously described[1]. 24 h urinary aldosterone excretion was measured via RIA (DPC Dade Behring, Paris, France). Urinary PGE2 was measured by Prostaglandin E2 EIA kit (Cayman Chemical, Ann Arbor, Michigan, USA). Urinary ATP was measured with the FLAA kit (Sigma-Aldrich).

Tissue Angiotensin II measurement was performed as previously reported[2]. CTX/OSOM and ISOM/IM were rapidly dissected on an ice-cold plate. Both kidneys were homogenized in ice-cold methanol by a Fast-Prep24 5G homogenizer. The homogenate was then centrifuged at 12000 g for 10 min at 4°C. The supernatant was transferred into a pre-weighed tube and dried out by overnight vacuum centrifugation before weighting back the tube for dry-tissue weight evaluation. Samples were then re-suspended in EIA buffer from the kit (Peninsula Lab International, San Carlos (CA), USA). Data are expressed as concentration/gram of tissue dry-weight. At baseline, n=5 control vs. 6 *cKO,* and under Ang II infusion, n=5 control vs. 6 *cKO* mice per group were used.

Tubule dissection and quantitative PCR

Left kidneys were perfused with incubation solution (140 mM NaCl, 0.4 mM KH2PO4, 1.6 mM K2HPO4, 1 mM MgCl2, 10 mM Na-acetate, 1 mM a-ketoglutarate, 1.3 mM Ca-gluconate, 3.75 mg/ml glycine, 0.48 mg/ml trypsin inhibitor, 0.25 mg/ml DNase I, pH 7.4) containing 1 mg/ml collagenase II through the aorta. Harvested kidneys were finely minced in 1 mm thick slides. The tissue was digested in the incubation buffer for 10 minutes at 37°C while shaking. Five serial washing of 5 min were performed. Before of each wash the supernatant was collected and used for manual tubular dissection based on their morphological and topological characteristics.

Total RNA was extracted with the RNAqueous-Micro Total RNA Isolation Kit (Life Technologies) and eluted in 12µl. Reverse transcription was performed using random primers and the transcriptor first strand DNA kit (Roche). The synthetized cDNA was used to assess Atp6ap2 and Rpl26 gene expression by quantitative PCR. Following primers were used: Atp6ap2: Forward 5'-TCATCTCCGCTTCAGCAACC-3', Reverse 5'-AAAACAACAGACCCTGGCGA-3' ; Rpl26: Forward 5'-GCTAATGGCACAACCGTC-3', Reverse 5'-TCTCGATCGTTTCTTCCTTGTAT-3'. FastStart DNA Master SYBR Green 1 kit was used to perform the qPCR on a Lightcycler 480 (Roche).

Immunoblotting

Animals were sacrificed by cervical dislocation. Kidneys were harvested and placed in ice-cold NaCl 0.9%. Cortex/outer stripe of outer medulla (CTX/OSOM) and inner medulla/inner

stripe of outer medulla (IM/ISOM) were excised under a stereo microscope and placed into ice-cold isolation buffer (250 mM sucrose, 20 mM Tris-Hepes, pH 7.4) containing protease inhibitors. Cortical and medullary minced tissues were homogenized in 1 ml and 0.5 ml homogenization buffer, respectively, by using an ultraturrax homogenizer at maximum speed (24000 rpm) for 30 sec. The homogenate was centrifuged at 4000 g for 15 min, and the supernatant was centrifuged at 17,000 g for 30 min at 4°C. The pellet was resuspended in isolation buffer. Protein contents were determined using the Bradford protein assay (Bio Rad Laboratories, Hercules, CA).

Membrane proteins were solubilized in SDS-loading buffer (62.5 mM Tris HCl, pH 6.8, 2% SDS, 100 mM dithiotreitol, 10% glycerol and bromophenol blue), and incubated at room temperature for 30 min. Electrophoresis was initially performed for all samples on 7.5% polyacrylamide minigels, which were stained with Coomassie blue to provide quantitative assessment of loading, as previously described[3]. For immunoblotting, proteins were transferred electrophoretically (XCell II Blot Module, Invitrogen Life Technologies) for 2 h at 4°C from unstained gels to nitrocellulose membranes (Amersham) and then stained with 0.5% Ponceau S in acetic acid to check uniformity of protein transfer onto the nitrocellulose membrane. Membranes were first incubated in 5% non fat dry milk in phosphate-buffered saline, pH 7.4 (PBS) for 1 h at room temperature to block nonspecific binding of antibody, followed by overnight at 4°C with the primary antibody (anti-NHE3 1:400; anti-NKCC2 1:2,000; anti-NCC 1:10,000; anti-ATP6V1B1 1:1,000; anti-pendrin 1:1,000; anti-α-ENaC 1:2,000 ; anti-γ-ENaC 1:10,000; anti-AQP2 1:250) in PBS containing 1% non fat dry milk. After four 5 min washes in PBS containing 0.1% Tween-20, membranes were incubated with 1:10000 dilution of goat anti-rabbit IgG (Bio-Rad) or horse anti-goat IgG (Vector Laboratories, Burlingame, CA) conjugated to horseradish peroxidase in PBS containing 5% nonfat dry milk for 2 hours at room temperature. Blots were washed as above, and luminol-enhanced chemiluminescence (ECL, Perkin Elmer Life Science Products) was used to visualize bound antibodies. Detection of chemiluminescence was performed using the mini-LAS imaging system (Fuji). Quantification of each band was performed by densitometry using Image J software. Densitometric values were normalized to the mean for the control group that was defined as 100% and results were expressed as mean ± S.E.

Immunohistochemistry

Kidneys were fixed *in situ* by retrograde perfusion of the aorta with a solution of PFA 4% in phosphate buffer. Harvested kidneys were washed in ice-cold phosphate buffer for 30 minutes before freezing in cold isopentane. Transversal cut cryosections (6 µm thick) were used for Masson Trichrome staining and immunofluorescence, longitudinal cut sections for quantification of the length of renal papilla.

For immunohistochemistry, cryosections were pre-incubated 1h with goat or donkey serum according to the host species of the applied secondary antibody. Then, sections were incubated overnight with primary antibody at 4°C (guinea pig anti-AE1 1:1,000; goat anti-Atp6ap2 1:100, rabbit anti-AQP2 1:2,000, guinea pig anti-p62 1:500, rat anti-Lamp2 1:500, rabbit anti-NKCC2 1:1,000, guinea pig anti-pendrin 1:10,000, rabbit anti B1 subunit of H^+ATPase 1:1000[4], anti-renin 1:30,000[5]). After washing with PBS, 1-hour incubation with fluorophore conjugated secondary antibody was performed. Additional washing in PBS preceded the mounting of the coverslips with fluorescence mounting medium (DAKO). Each experiment included three mice per group. Representative pictures were acquired with a Leica Confocal SP8 microscope.

Antibody sources

The sources of the antibodies used in immunohistochemistry and immunoblotting were as follows. Antibodies against alpha and gamma subunits of ENaC, and NKCC2 were a kind gift from J. Loffing (University of Zürich, Zürich, Switzerland). Anti-NCC antibody was a gift from D. Ellison (Oregon Health and Science University, Portland, OR). Antibody against pendrin was a gift from P. Aronson (Yale University, New Haven, CN). Guinea pig anti-AE1 and anti-pendrin antibodies were kindly provided by C. Wagner (University of Zürich). Anti-rabbit AQP2 antibody was kindly provided by S. Frische (Department of Biomedicine, Aarhus University, Denmark). Antibody against NHE3 was kindly provided by M.A. Knepper (NIH, NHLBI, Bethesda, MD, USA). Antibody against goat AQP2 was from Santa Cruz (sc9882). Anti-goat Atp6ap2 antibody was from Abcam (ab5959). Anti-guinea pig p62 was obtained from PROGEN (Heidelberg, Germany, cat. no. gp62-c), and anti-LC3 antibody was from Cell Signaling (D3U4C). Anti-rat LAMP2 was kindly provided by the DSHB Hybridoma Bank.

***In situ* hybridization**

In situ hybridization on thin sections was performed as previously reported[6]. Briefly, sense and anti-sense riboprobes for Atp6ap2 were prepared by reverse transcription from the Atp6ap2 exon 9 cDNA cloned into the vector Topo pCRII using the digoxigenin RNA labeling kit from Roche. Dissected kidneys were fixed with paraformaldehyde (4% PFA in PBS, overnight at 4°C) and incubated in a sucrose solution (30% sucrose in PBS, overnight at 4°C). Equilibrated samples were embedded in Tissue-Tek, frozen on dry ice and cut into 12 µm sections. Cryosections were fixed with PFA (4% in PBS, 20 min at RT) and digested with proteinase K (10 µg/ml, 10 min at RT). After refixation (4% PFA, 20 min), sections were acetylated (0.25% acetic anhydride, 0.1 M triethanolamine, pH 8.0, 10 min). Acetylated sections were prehybridized in hybridization buffer (50% formamide, 4× SSC, 2× Denhardt's solution, 5% dextran sulfate, 100 µg/ml yeast tRNA, 5 h at RT) and then hybridized with the Atp6ap2 probe (overnight at 60°C with 1 µg/ml of the digoxigenin-labeled riboprobe). After a series of washing steps (wash 1: 0.2× SSC, 30 min at 60°C; wash 2: 50% formamide, 2× SSC, 30 min at RT; wash 3: 0.2× SSC, 10 min at RT; wash 4: 0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween-20, pH 7.4, 15 min at RT) the sections were blocked with BSA (3% in Tris buffered saline, 2 h at RT) and incubated with anti-digoxigenin antibodies (alkaline phosphatase-conjugated Fab fragments (Roche), diluted 1:2,000, overnight at 4°C). The slides were rinsed and equilibrated with detection buffer (0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). Finally, the hybridization signal was developed with BM Purple (Roche) and the slides were photographed under an Axio Imager microscope (Zeiss).

Electron Microscopy

After *in situ*-perfusion with 4% glutaraldehyde in 0.1M Na cacodylate buffer (pH 7.4), kidney samples were rinsed in cacodylate buffer and post-fixed in 1% osmium tetroxide (EMS) in 0.1 M cacodylate buffer for 1 h at room temperature. After further washing in double distilled water, the samples were stained *en bloc* in aqueous 2% uranyl acetate for 1 h. The samples were dehydrated through a graded ethanol series to 100% ethanol, then infiltrated with a solution of 1:1 EPON (Ted Pella, Inc, Redding, CA): 100% ethanol overnight. They were then placed in fresh EPON for several hours and embedded in EPON at 60°C overnight. Ultrathin sections were cut on a Leica EMUC7 ultramicrotome, collected on formvar-coated grids, stained with lead citrate and uranyl acetate, and examined in a JEOL 1011 transmission electron microscope at 80 kV. Images were acquired using an AMT (Advanced Microscopy Techniques, Danvers, MA) digital imaging system.

For immunogold staining, other kidney samples fixed in 4% paraformaldehyde were embedded in LRWhite resin as previously described[7]. Ultrathin sections were incubated for two hours with rabbit anti-AQP2 antibodies raised against the AQP2 C-terminus[8], rinsed and incubated for 45 min with goat anti-rabbit IgG conjugated with 10 nm gold particles (Ted Pella Inc., Redding CT). Sections were rinsed again in PBS and counterstained with lead citrate and uranyl acetate before imaging as above in the JEOL 1011 TEM.

***Ex vivo* microperfusion and intracellular pH measurements**

Intracellular pH (pH_i) measurements were performed as shown previously[9]. Cortical CD (CCD) segments were isolated from corticomedullary rays under a dissecting microscope and placed on a microperfusion stage equipped with an inverted fluorescence microscope (Axiovision A1; Zeiss). pH_i was measured with imaging-based dual excitation-wavelength fluorescence microscopy: intracellular dye was excited alternatively at 500 and 440 nm with a light-emitting diode (Optoled, Cairn Research, Faversham, UK). Emitted light was collected through a dichroic mirror, passed through a 530 nm filter and focused onto an EM-CCD camera (iXon, Andor Technology, Belfast, Ireland) connected to a computer. The measured light intensities were digitalized with 14-bit precision (16384 gray level scale) for further analysis.

Tubules were loaded for 20 min with 5×10^{-6} mol/L of the pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF; Thermo Fisher) in a sodium containing solution (140 mM NaCl, 1 mM CaCl2, 1.2 mM MgSO4, 2 mM K2HPO4, 10 mM Hepes, 5.5 mM glucose, 5 mM alanine at pH 7.4). Luminal and peri-tubular sodium was removed by perfusing tubules with Na^+-free, ammonium-free solution where N-methyl-D-Glutamine$^+$ ($NMDG^+$) replaced Na^+. Then, tubules were exposed for 18 min to the same sodium free solution, before inducing intracellular alkalinisation with a 3 min challenge of 20mM NH4Cl-enriched sodium free bath. Recovery of the pH_i was stimulated by applying a sodium free bath for additional 6 minutes. Variations in pH_i were recorded by changes in fluorescence of BCECF. ICs were identified by fluorescein-conjugated peanut lectin (Vector Labs). Three control and four *Atp6ap2 cKO* tubules (number of cells: 11 *vs.* 21) from 3 mice each were studied. pH_i was calibrated at the end of each experiment using the high-$[K^+]$/nigericin technique (HEPES-buffered, 95-mM K^+ solution containing 10 µM of the K^+/H^+ exchanger nigericin) by using four different calibration solutions (pH: 6.5, 6.9, 7.2 and 7.5). Andor IQ software (Andor Technology) was used for data analysis.

Measurement of tissue osmolality

The measurement of tissue osmolality was performed as reported previously [10]. Briefly, a longitudinal section of the kidney was cut in order to include the entire papilla. Then, CTX/OSOM, ISOM and IM were dissected and placed in a pre-weighted tube. The tissue was dried-out under vacuum for 2.5 h. A volume of 40, 30 and 20 µl of deionized water was added to the CTX/OSOM, ISOM and IM samples, respectively. Tubes were sealed to minimize evaporation of the water and warmed at 95°C and then placed overnight at 4°C. After gentle sonication in water bath for 15 min, the samples were spun down at 8,000 g for 1 min and the supernatant collected for measuring the osmolality.

Supplementary references

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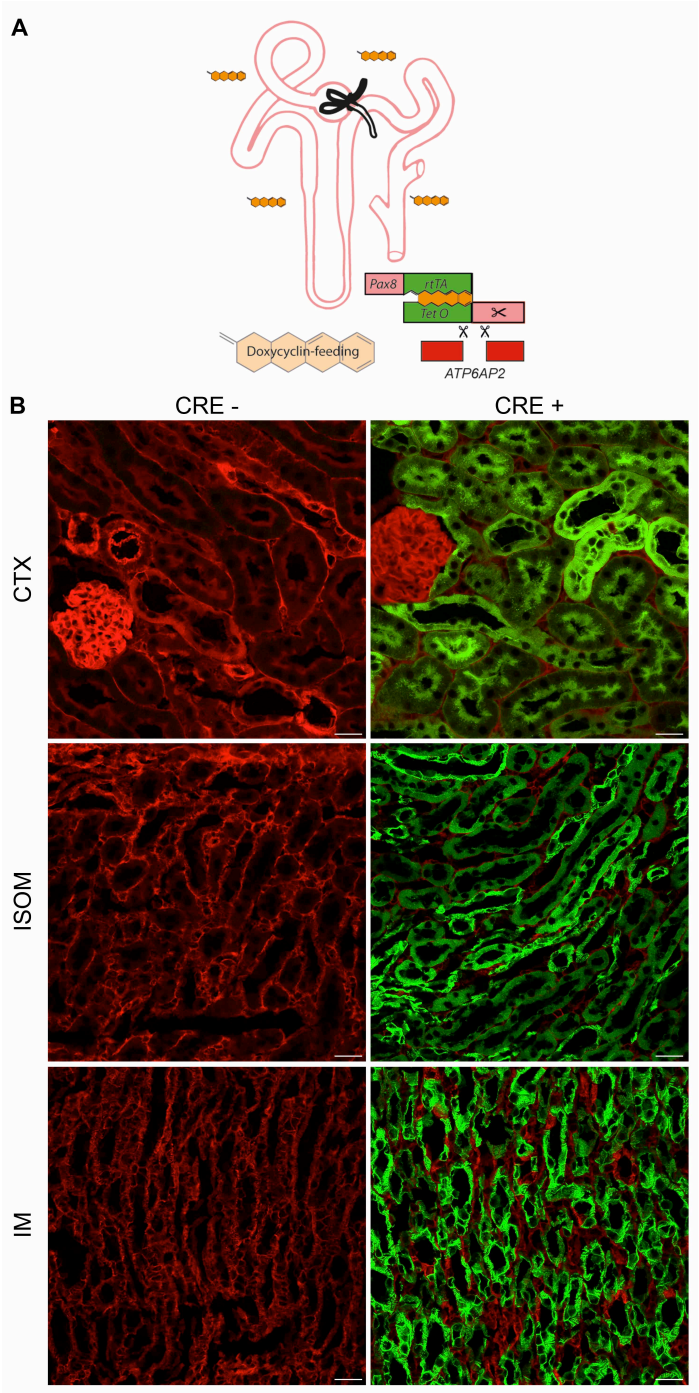

Figure S1: *Atp6ap2* depletion strategy

(A) Scheme of the strategy to generate *Atp6ap2 cKO*. Pax8rtTA expression occurs in the pink labelled cells. Cre-Lox recombination was induced at E16.5 by doxycycline administration. (B) Representative pictures from Pax8.rtTA;tetO.Cre -(Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo/J}$) mice and their CRE negative control (left). Doxycycline administration at E16.5 induces eGFP expression in all the epithelial cells of the nephron, but not in the glomerulus and the vascular cells. eGFP expression seemed to be lower in the proximal tubules (identified by the presence of a luminal brush-border), consistent with the absence of effective downregulation of ATP6AP2 in the proximal tubule. Importantly, the intensity of eGFP in the medulla was similar to the intensity observed in cortical nephron segments identified as "non-proximal tubules" (i.e, thick ascending limbs, distal convoluted tubules, connecting

tubules and cortical collecting ducts) due to the lack of a brush border. Cre negative control mice do not respond to doxycycline and do not express eGFP. Scale bar: 25 µm.

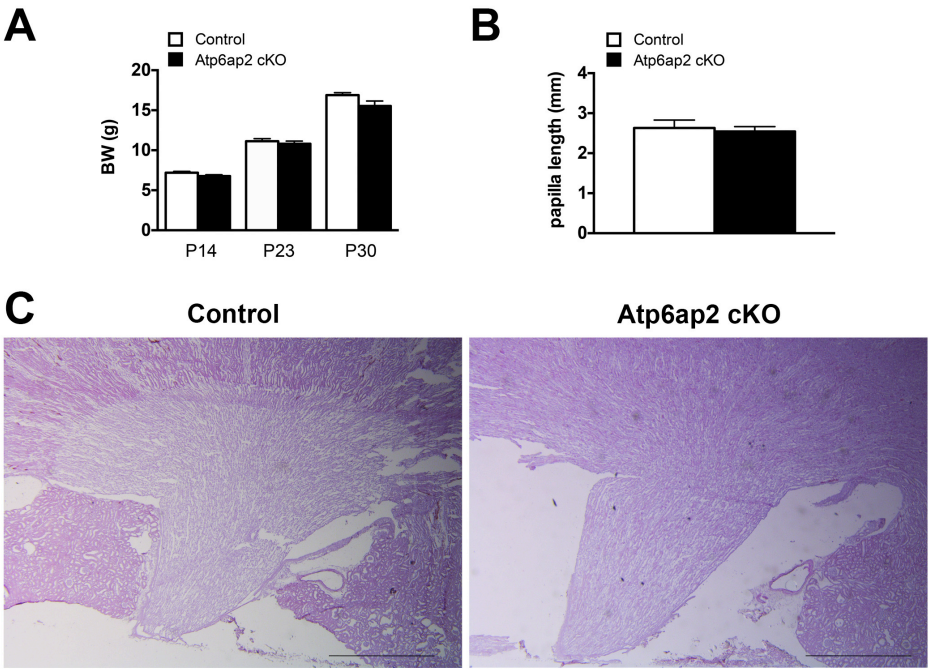

Figure S2: Body weight and renal morphology of *Atp6ap2 cKO* animals

(A) Body weight (BW) progression in control (white) and *Atp6ap2 cKO* mice (black) at 14 (n=19 control vs. 16 *cKO*), 23 (n=19 control vs. 16 *cKO*) and 30 days (n=5 control vs. 5 *cKO*) after birth. (B) Quantification of papilla length from control (white) and *Atp6ap2 cKO* (black) mice (n=4 control vs. 4 *cKO*). (C) Representative pictures of renal papilla from control and *Atp6ap2 cKO* mice stained with hematoxylin and eosin. Scale bar: 1 mm.

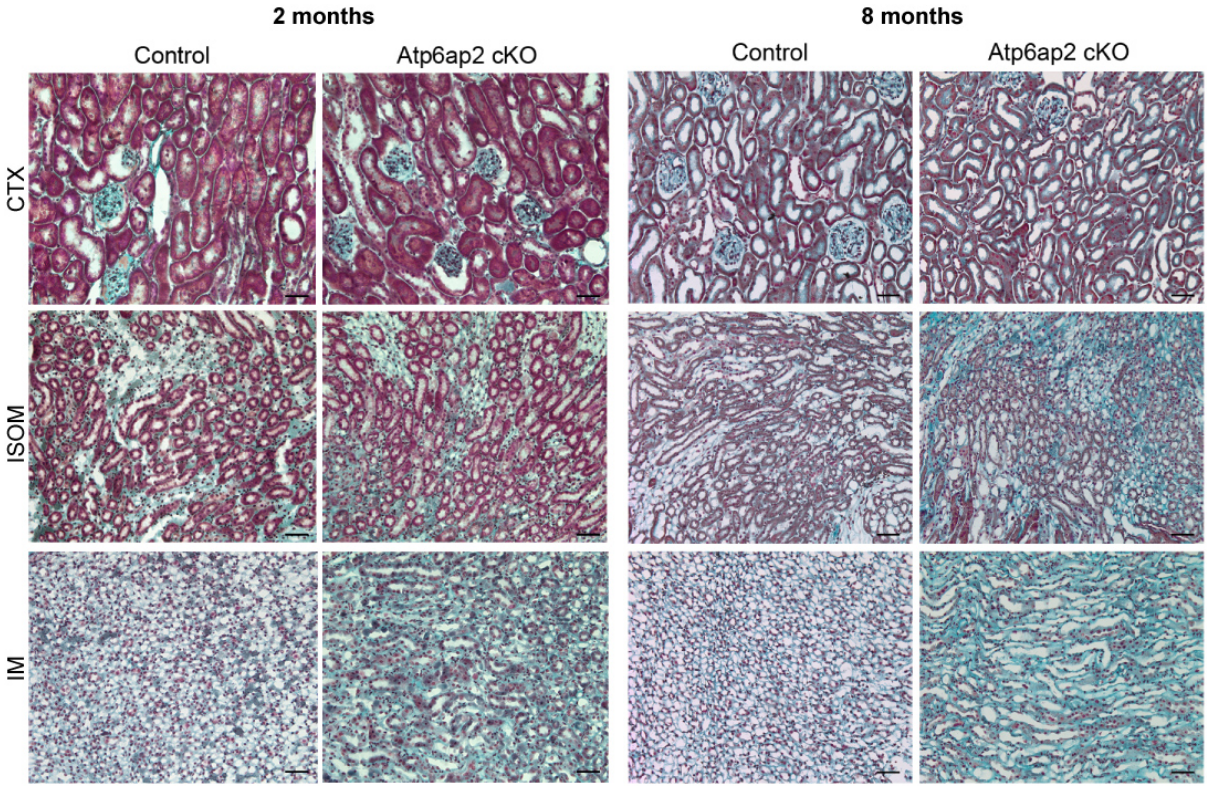

Figure S3: *Atp6ap2 cKO* mice develop medullary interstitial fibrosis

Representative pictures of renal CTX, ISOM and IM of control and *Atp6ap2 cKO* mice at two months of age (left panel) and 8 months of age (right panel) stained with Masson's trichrome. At both time points, fibrotic material is clearly increased in the mutant kidneys, especially in the IM. Scale bar: 50 µm.

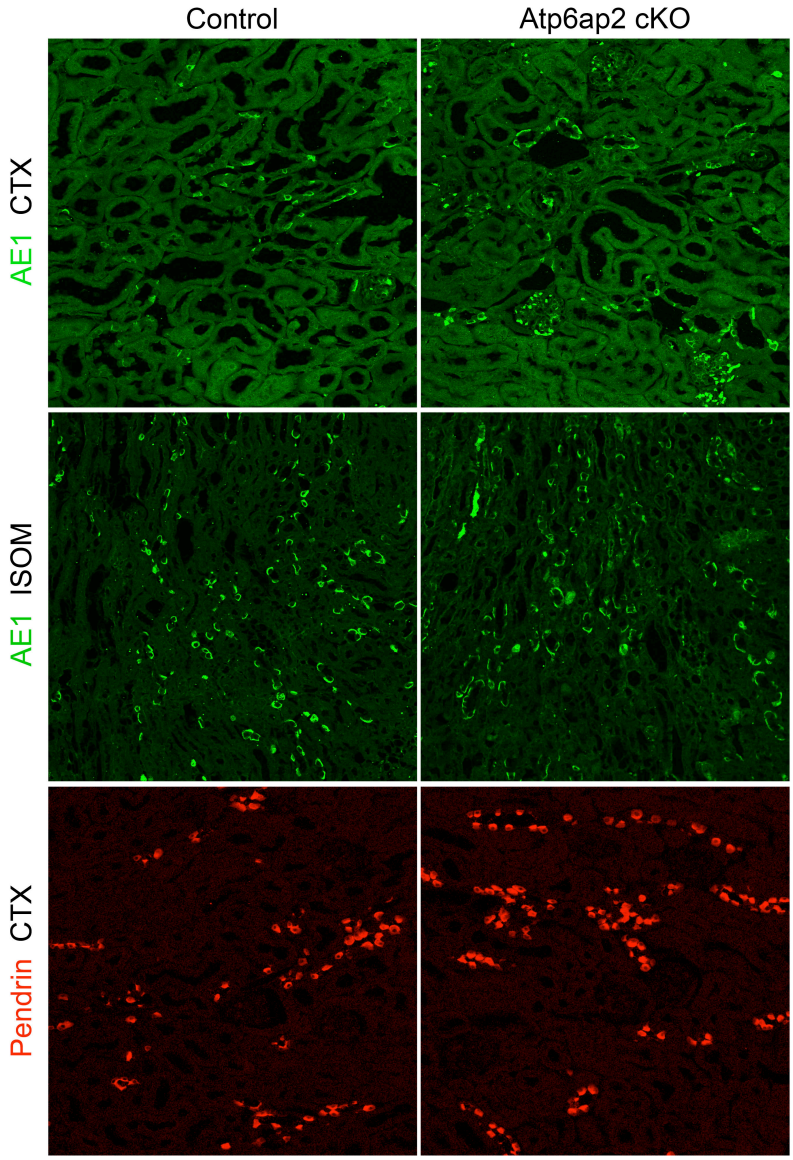

Figure S4: The distribution and number of α-IC and β-IC is not changed in *Atp6ap2 cKO* mice

Representative pictures of renal CTX and ISOM of control and *Atp6ap2 cKO* mice stained with anti-AE1 (green) and anti-pendrin (red) antibodies. Scale bar: 75 µm.

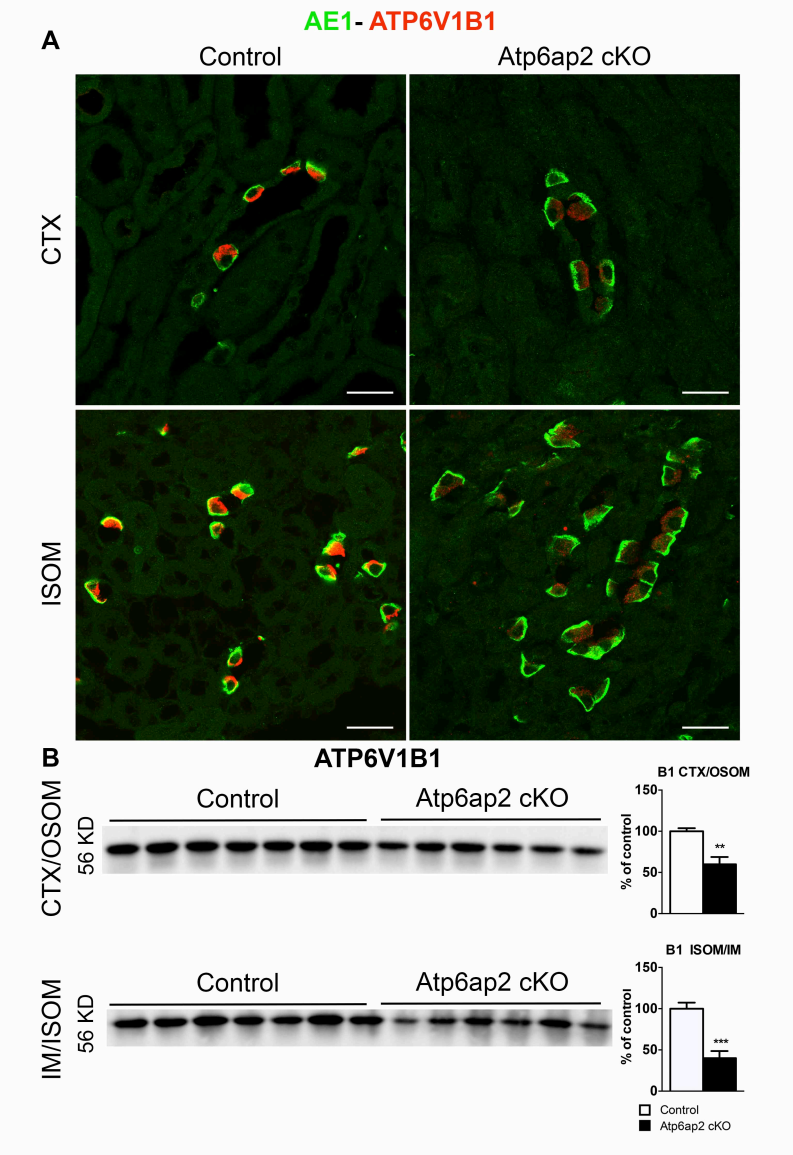

Figure S5: The B1 subunit of H^+ATPase is downregulated in *Atp6ap2 cKO* mice

(A) Representative pictures of renal CTX and ISOM of control and *Atp6ap2 cKO* mice co-labeled with anti-AE1 (green) and anti-ATP6V1B1 (red) antibodies. Scale bar: 25 µm. (B) immunoblotting of B1 subunit of ATP6V1B1 from membrane enriched samples of CTX/OSOM and IM/ISOM. ** is for p-value < 0.01; *** is for p-value < 0.001; Student's t-test.

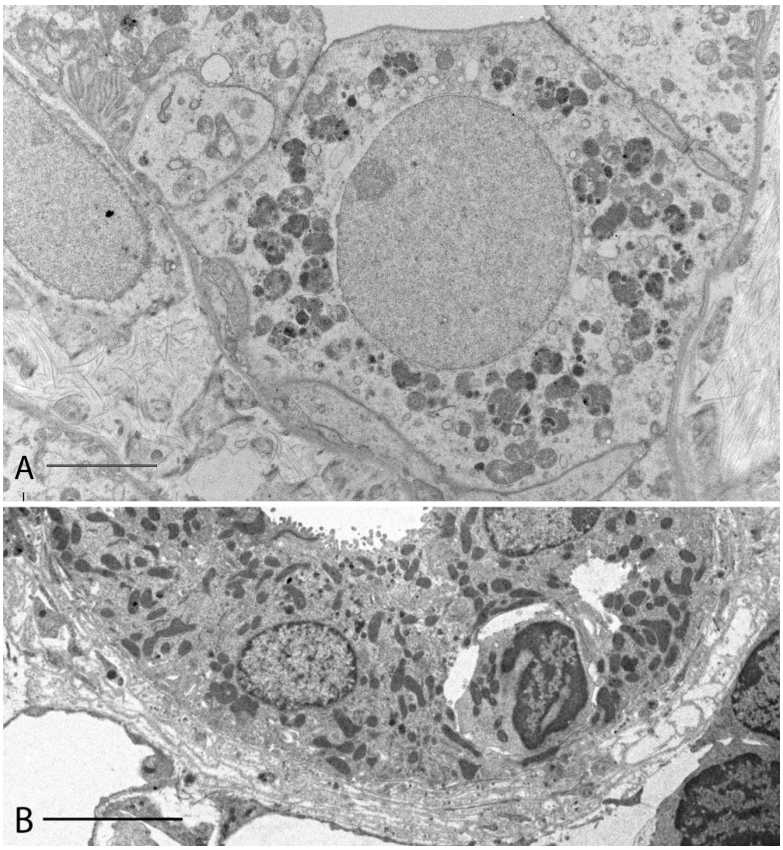

Figure S6: Ultrastructural analysis of young and old *Atp6ap2 cKO* mice

(A) Electron microscopy of *Atp6ap2 cKO* kidneys from a 2-month old mouse showing a medullary principal cell with accumulations of cytoplasmic organelles containing electron-dense material (see also Figure 4 and Figure S7). Basal to the basement membrane fibrillar material can be found, suggesting an early sign of fibrosis in agreement with the Masson's trichrome staining from Supplementary Figure 3. Scale bar: 2 µm. (B) shows another example of a representative thick ascending limb surrounded by fibrotic material in a 8-month old *Atp6ap2 cKO* mouse. A lymphocyte is also present within the epithelium. Scale bar: 5 µm.

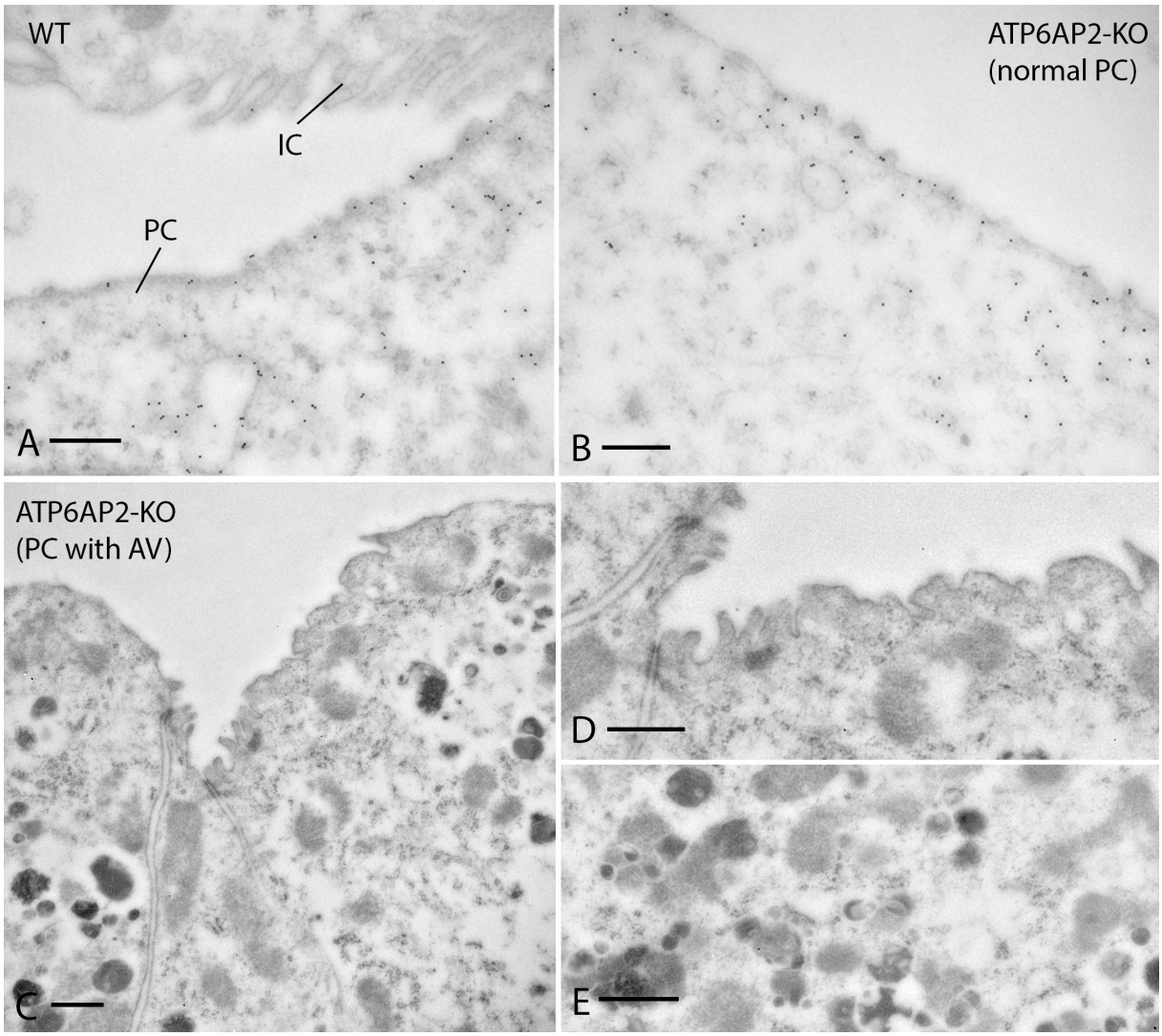

Figure S7: Immunogold localization of AQP2 in collecting duct principal cells of WT and *Atp6ap2 cKO* mice

(A) In WT mouse PCs, AQP2 is located in the apical plasma membrane and on cytoplasmic vesicles. Adjacent ICs show no specific labeling. (B) In *Atp6ap2 cKO* mice, some PCs appear unaffected by the *Atp6ap2 cKO* and have a normal pattern of AQP2 localization that resembles that seen in WT animals. (C) PCs, in which *Atp6ap2 cKO* results in the development of many autophagic vacuoles and dense bodies, have little or no detactable AQP2, either on the apical surface (D) or over the intracellular vesicles. Scale bars: 0.5 µm.

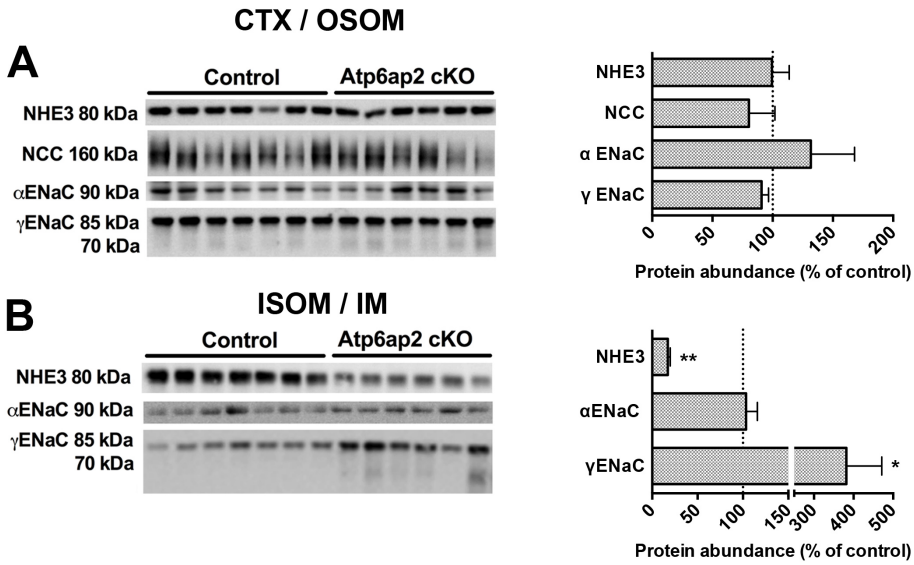

Figure S8: Expression of sodium transporters in *Atp6ap2 cKO* kidneys

Immunoblotting of the main sodium transporters along the nephron and their relative quantification (% of control) in CTX/OSOM (A) and ISOM/IM (B). Reduction of NHE3 in ISOM/IM samples most likely reflects TAL damage in the medulla. ** is for p-value < 0.01; * is for p-value < 0.001. Student's t-test.

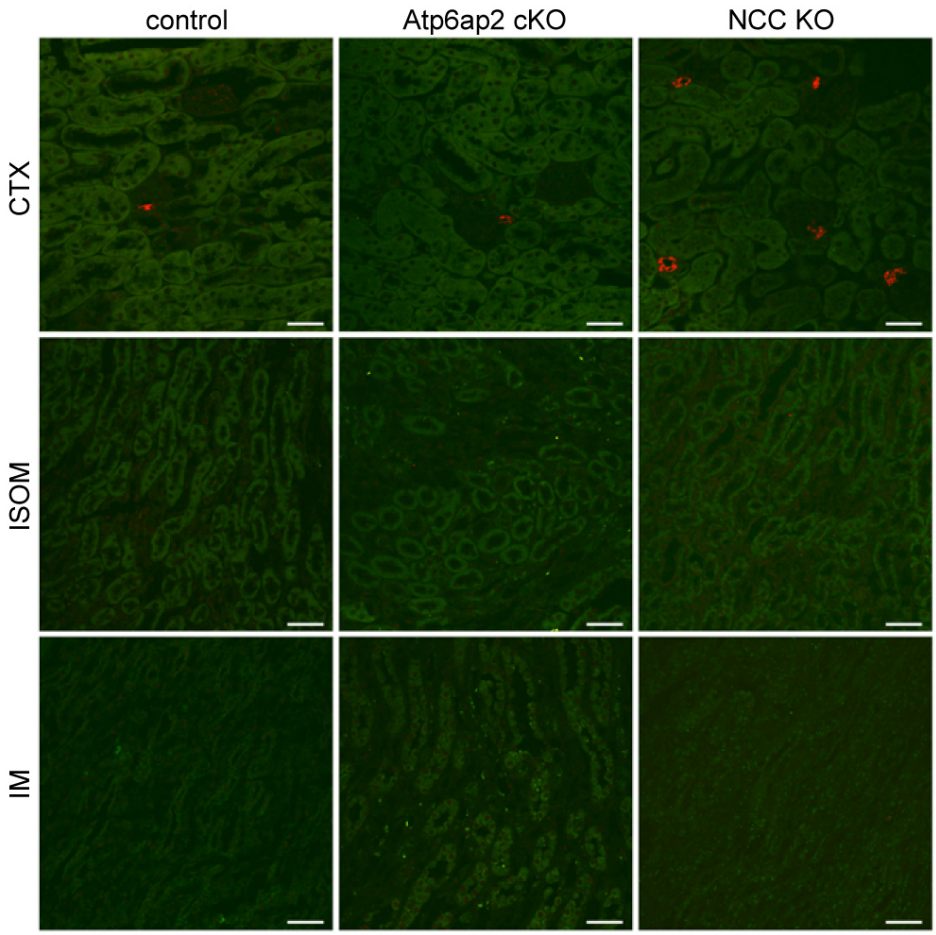

Figure S9: Renin expression in *Atp6ap2 cKO* kidneys

Immunostaining for renin (red) shows expression in the cortical juxtaglomerular apparatus in control (left) and *Atp6ap2 cKO* (middle) kidneys, but not in other parts of the kidney. In NCC KO kidneys there is prominent renin expression in hyperplastic juxtaglomerular cells. Also here, there is no expression in other kidney regions. Scale bar: 50 µm.