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Supplementary Information for

Adhesion-GPCR Gpr116 (ADGRF5) Expression Inhibits Renal Acid Secretion.

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Full Description of Methods

Cell Culture and Intracellular Ca^{2+} Measurements

HEK-293 human embryonic kidney cells were grown at 37°C in a humidified CO_2 atmosphere in DMEM containing 10% FBS, 1% P/S and 1% L-glutamine. For $[Ca^{2+}]_i$ measurements, cells were plated on ploy-L-lysine-coated x-well Tissue Culture Chamber Glass (Sarstedt). The cells were loaded with Fura-2-AM (1 µg/mL) in low-Ca^{2+} HBSS for 30 min at RT, then washed and incubated for 10 min at RT in the same low-Ca^{2+} HBSS. Cells were then mounted onto the stage of a Zeiss Axio Observer A1 inverted microscope equipped for fluorescence imaging and visualized through a 20x EC Plan-NEOFLUAR objective. Excitation (340nm/380nm), image acquisition and data analyses were performed using Image-1 MetaMorph software as well as AxoScope 10.7. $[Ca^{2+}]_i$ was measured as the ratio of fluorescence emitted at 510 nm when the cells were alternately excited at 340 nm and 380 nm ([F340/F380]). Synthetic agonist peptide (p16) corresponding to amino acids 1051-1066 of Gpr116 was synthesized as the following(1): NH_2-TSFSILMSPDSPDPGS-NH_2. p16 peptide was dissolved in DMSO at 100mM and diluted in HBSS immediately prior to experiments. Control peptide (F3A) was synthesized as the following: NH_2-TS<u>*A*</u>SILMSPDSPDPGS-NH_2.

Split-open Isolated Tubules

Mice were euthanized between 10:00 and 14:00, then kidneys were cut into thin slices and placed in ice-cold bath solution containing (in mM) 150 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 glucose, and 10 HEPES (pH 7.35). Collecting ducts (CDs) were individually isolated from the cortex of each animal by morphological identification (coarse surface, bifurcations) and adhered to poly-L-lysine-coated coverglasses. Adhered collecting ducts were placed in a perfusion chamber mounted on an inverted Nikon Eclipse Ti Microscope and split open with two sharpened micropipettes controlled with micromanipulators. For intracellular calcium measurements, CDs were loaded with Fura-2-AM (2 µM) for 30 min, washed, then placed back in the perfusion chamber for intracellular Ca^{2+} measurements. Three CDs each were collected from six mice for intracellular Ca^{2+} measurements. Data analysis was performed using Nikon NIS-Elements software version 4.3.

For intracellular pH measurements, split-opened CDs were loaded with 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein (BCECF) by incubation with 15 µM BCECF-AM acetoxymethyl ester in the bath solution for 40 min at room temperature followed by a washout with the bath solution for additional 10 min. Gpr116 synthetic agonist peptide (p16, 100 µM) was added during incubation with BCECF, as necessary. CDs were placed in an open-top imaging study chamber (RC-26GLP; Warner Instruments, Hamden, CT, USA) with a bottom coverslip viewing window and the chamber attached to the microscope stage of a Nikon Ti-S Wide-Field Fluorescence Imaging System (Nikon Instruments, Melville, NY, USA) integrated with Lambda XL light source (Sutter Instrument, Novato, CA, USA) and QIClick 1.4 megapixel monochrome CCD camera (QImaging, Surrey, BC, Canada) via NIS Elements 4.3 Imaging Software (Nikon Instruments, Melville,

NY, USA). Cells were imaged with a 40X Nikon Super Fluor objective and regions of interest (ROIs) were drawn for individual cells. The BCECF fluorescence intensity ratio was determined by excitation at 495 nm and 440 nm and calculating the ratio of the emission intensities at 520 nm every 5 seconds. Experiments were performed under permanent perfusion of a solution containing (in mM): 150 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 glucose and 10 HEPES at 1.5 ml/min rate. No significant BCECF bleaching was detected during the timeline of experiments. The changes in the ratio were converted into changes in pH_i by performing a calibration in high K^+ solutions (145 mM KCl) with predefined pH (6.0, 7.0 and 8.0, adjusted by HCl and KOH, respectively) in the presence of 15 μM nigericin, as was shown previously (2). Four individual CDs from four different mice were used for each experimental set. For intracellular acidification, a solution containing (in mM) 40 NH_4Cl 110 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 glucose and 10 HEPES was applied to the recording chamber for 2 min. The rate of proton extrusion from ICs was calculated as a linear slope of the initial pH_i recovery rate from the lowest pH_i values for each individual cell after removal of the acidification pulse, as was similarly done previously (3).

Following pH_i measurements, split-opened CDs were fixed with 10% neutral buffer formalin for 15 min at room temperature. After fixation, the samples were permeabilized by addition of 0.1% Triton in PBS for 5 min and washed in PBS 3 times for 5 min. Nonspecific staining was blocked with 10% normal goat serum (Jackson Immunoresearch, USA) in PBS for 30 min at room temperature. After washing with PBS (3 times for 5 min) the samples were incubated for 1.5 hours at room temperature in the dark with anti-aquaporin 2 labeled with ATTO-550 (1:100 dilution; Alomone labs) in 1% serum + 0.1% Triton in PBS. After washing with PBS (3 times for 5 min) the samples were stained with 4',6-diamidino-2-phenylindole (DAPI) (300 nM concentration, Calbiochem, San Diego, CA, USA) to visualize nuclei. The samples were dehydrated and mounted with a permanent mounting media (Thermo Scientific, Pittsburg, PA, USA). Labeled tubules were examined with an inverted Nikon Eclipse Ti fluorescent microscope using a 40X Plan-Fluor oil-immersion (1.3 NA) objective. Samples were excited with 405 and 561 nm laser diodes and emission captured with a 16-bit Cool SNAP HQ^2 camera (Photometrics) interfaced to a PC running NIS elements software. Cells without AQP2 expression were defined as ICs.

Sample Analysis

Animals were housed in metabolic cages (VWR, PA) for 24 hours before collection of data. Urine was collected into tubes containing mineral oil. Cages were cleaned daily following collection of urine. Urine was spun at 3000 rpm to remove debris. Urine pH measurements were performed using an Orion PerpHecT ROSS combination micro electrode (Thermo Scientific, MA). Urine osmolality was measured on a 3320 Micro Osmometer (Advanced Instruments, MA) after dilution in water. Urine electrolyte concentrations were measured on an EasyLyte Plus Analyzer (Medica, MA) containing a Na^+, K^+, and Cl^- electrode according to manufacturer's guidelines. Urine titratable acid was measured using previously published methods(4, 5). Briefly, urine was acidified by the addition of

equal volume of 0.1 M HCl, then boiled for 2 minutes and then cooled to 37°C. The volume of 0.4 M NaOH required to titrate the urine sample back to pH 7.4 was measured. Samples of DI water were analyzed in parallel and net titratable acidity was calculated as:

$$\frac{(V_{NaOH^{urine}} - V_{NaOH^{water}})}{V_{urine}} \times 0.4\ M\ NaOH$$

Urine ammonia was measured using an A7553 Ammonia Assay Kit (Pointe Scientific, MI). Urine creatinine was measured using the Mouse Creatinine Enzymatic Assay Kit (Crystal Chem, IL). Blood chemistry was measured using whole blood harvested between 10:00 and 14:00 from the facial vein with an i-STAT EC8+ cartridge and an i-STAT1 VetScan Handheld Analyzer (Abaxis, CA). For female mouse analysis in Figure S6, mice were ventilated and blood samples were taken from the left ventricle (arterial samples).

Immunofluorescence

Mice were euthanized between 10:00 and 14:00 and their kidneys were harvested and fixed in 10% buffered formalin. Kidneys were placed in 30% sucrose for cryopreservation before being embedded in Tissue-Tek O.C.T. Compound (VWR, PA). Frozen kidneys were sectioned at 8 µm on a cryostat. Slides were prepared for immunostaining by antigen retrieval in 10 mM sodium citrate, 0.5% Tween-20, pH 6.0 at 70°C for 30 min. Sections were permeabilized using 0.3% Triton-X and blocked in 3% BSA at RT for at least 1 h. Primary antibodies (Table 1) were diluted in blocking buffer and sections were incubated overnight at 4°C. Following incubation, sections were washed three times in PBS and incubated with secondary antibodies for 1 h at RT. For experiments using two primary antibodies from the same species, primary antibodies were added sequentially, and secondary antibodies used in excess before addition of the next primary antibody. Nuclei were counterstained with DAPI. Fluorescence images were captured on a Zeiss LSM 700 confocal microscope and processed in Zeiss Zen software.

Cell-type image analysis was performed on 20x stitched-images captured on a Keyence BZ-X700 fluorescence microscope. Images were processed with Image-J using a Shanbhag threshold and the particle counting function.

Immunogold electron microscopy

Kidney tissues were obtained between 10:00 and 14:00 following perfusion-fixation in 4% paraformaldehyde/75 mM lysine-HCL/10 mM sodium periodate (PLP) in 0.15 M sucrose in 37.5mM sodium phosphate buffer, and cut into transverse slices. Small (1 mm x 1mm) pieces of cortex and medulla (inner medulla/outer stripe) were excised from the slices and rinsed several times in PBS, dehydrated through a graded series of ethanol solutions to 100%, then allowed to pre-infiltrate in a 1:1 mix of LR-White embedding resin (EMS, Hatfield, PA):100% ethanol. Specimens were then placed into 100% LR White resin for an additional several hours of infiltration, then transferred into fresh LR White resin in gelatin

capsules and allowed to polymerize 24-48 h at 50°C. Ultrathin (70 nm) sections were cut using a Leica EMUC7 ultramicrotome and collected onto formvar-coated grids (EMS, Hatfield, PA). Sections were incubated for 1 h at room temperature in primary antibody (rabbit antivacuolar ATPase 'A' subunit) as previously described (diluted 1:200 in DAKO antibody diluent)(6). Specimens were rinsed several times in PBS, incubated in a secondary gold conjugate for 1 h at room temperature (goat anti-rabbit IgG diluted 1:200, 15 nm, Ted Pella #15727), then rinsed several times with distilled deionized water and contrast-stained using 2.0% aqueous uranyl acetate.

Grids were examined at 80 kV in a JEOL 1011 transmission electron microscope (Peabody, MA) equipped with an AMT digital camera and proprietary image capture software (Advanced Microscopy Techniques, Danvers, MA). For quantification of apical V-ATPases in electron micrographs, gold particles located on the apical plasma membrane and microvilli and within 75 nm from the apical membrane (unless associated with discernable subapical vesicles) were counted manually for each cell. ImageJ was used to measure cell width (as a straight line between tight junctions) and apical membrane length (as freehand line).

Western Blot

Mice were anesthetized between 10:00 and 14:00 and exsanguinated by perfusion of ice-cold PBS in to the left ventricle. Organs were harvested and disrupted using a dounce homogenizer in RIPA buffer containing Halt protease inhibitors (Thermo Scientific) and then sonicated using a CV188 Ultrasonic Processor (Cole Palmer, IL). Tissues were spun at 16,000 x *g* at 4°C and the supernatant protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific). 25 µg of reduced protein was loaded into Bolt 4-12% Bis-Tris Plus gels (Invitrogen) according to manufacturer's guidelines and run in Bolt MOPS SDS running buffer. Gels were transferred onto nitrocellulose membranes using an iBlot 2 (Invitrogen) with NC regular stacks. Membranes were blocked with SuperBlock (PBS) blocking buffer (Thermo Scientific) for 1 h at RT. Primary antibodies (Table 1) were diluted in blocking buffer and left overnight at 4°C on an orbital shaker. Membranes were washed with 0.1% Tween-20, incubated with secondary antibody and then developed with SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific). Membranes were imaged on a Amersham 680 Imager (GE, PA).

Quantitative RT-PCR

RNA was isolated using a modified Trizol method combined with the RNeasy Mini Kit (Qiagen, DE). cDNA was produced with the QuantiTect reverse transcription kit with gDNA Wipeout (Qiagen). TaqMan real-time PCR assays (Thermo Scientific) were used according to manufacturer's guidelines (Table 2). Quantitative PCR amplification was performed on an Applied Biosystems QuantStudio 6 PCR system (Thermo Scientific). Baseline and threshold values were set according to manufacturer's instructions.

Bronchoalveolar Lavage (BAL)

Mice were anesthetized between 10:00 and 14:00 by sodium pentobarbital and BAL fluid was collected by intratracheal intubation followed by serial lavaging with 0.9% saline (2 x 0.7 mL). Pooled fluid was centrifuged at 1000 x *g* for 10 min at 4°C to remove cells. Protein was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific).

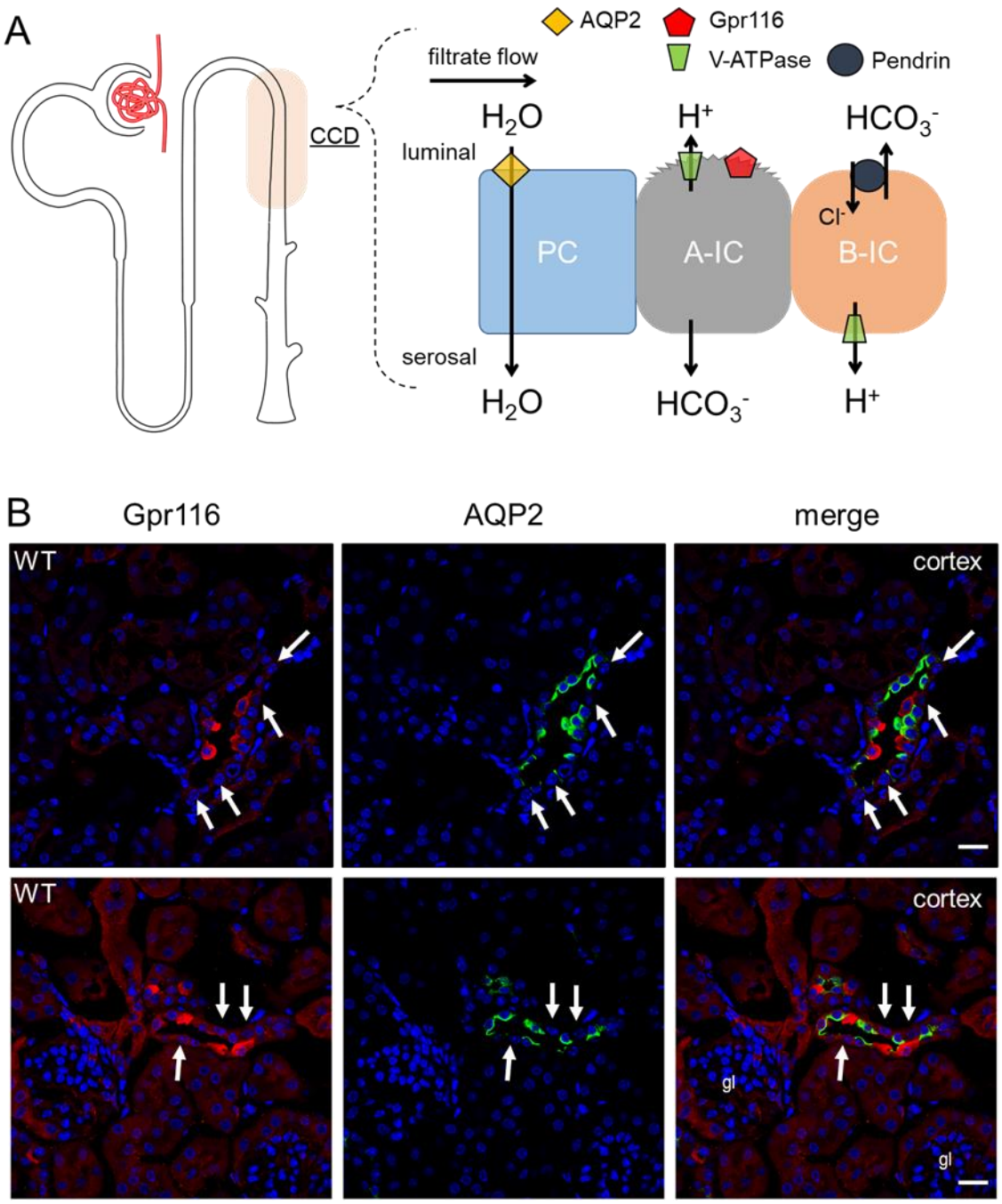

Fig. S1. Gpr116 expression in the cortex. (A) Diagram of the murine cortical collecting duct (CCD) showing water-transporting principal cells (PC), acid-secreting type-A intercalated cells (A-IC), and bicarbonate-secreting type-B intercalated cells (B-IC). Bicarbonate exchange is mediated by pendrin on the apical membrane of B-ICs. (B) Immunofluorescence images demonstrate Gpr116-positive cells amongst AQP2-positive principal cells. However, not every AQP2-negative cell is Gpr116-positive in cortical collecting ducts (arrows). Glomeruli are labeled (gl). Scale bar is 20μm.

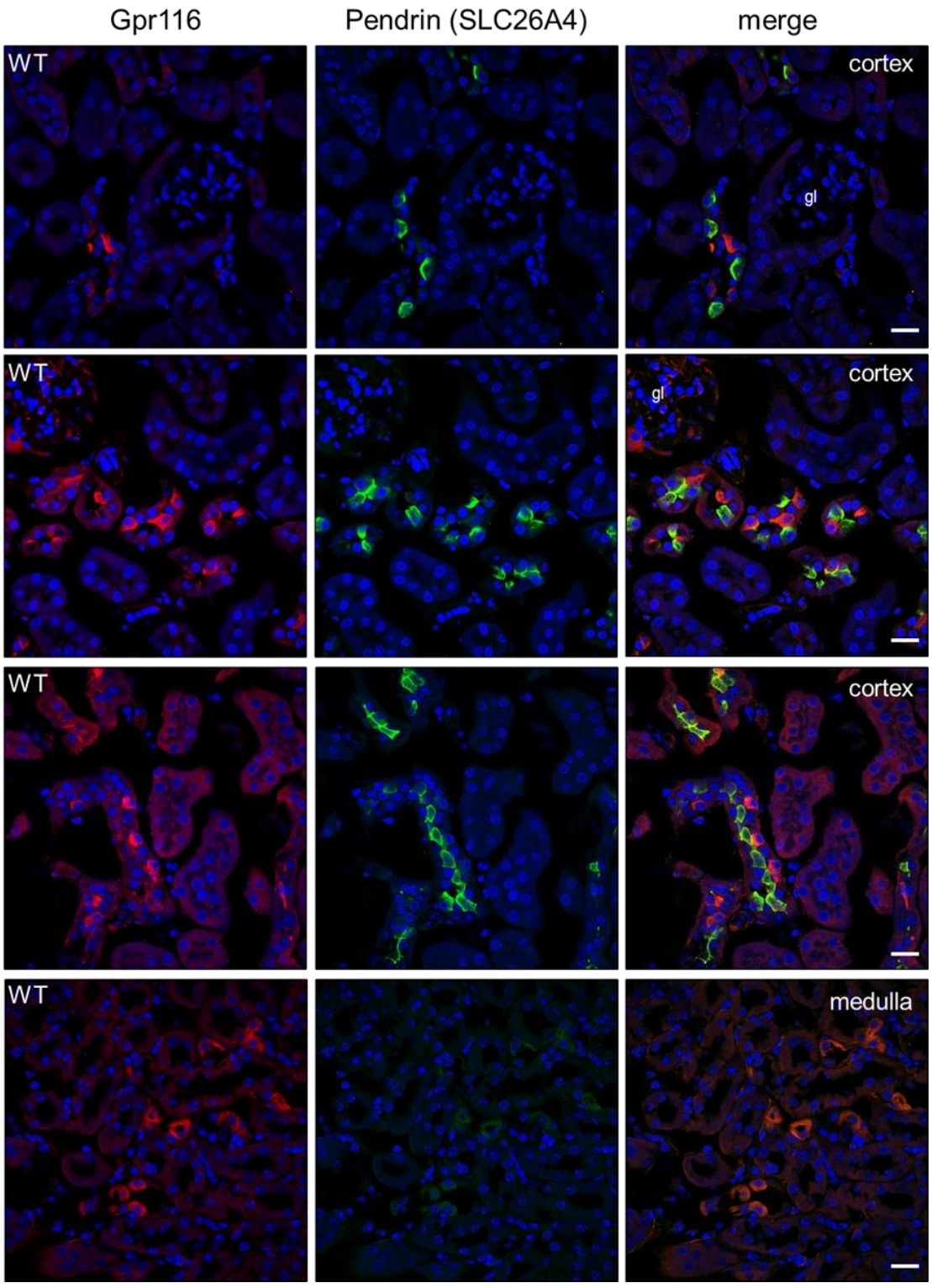

Fig. S2. B-type intercalated cells do not express Gpr116 (40x). Immunofluorescence images demonstrate Gpr116-positive cells do not express B-type intercalated cell marker pendrin (SLC26A4). Pendrin-positive cells are present in the cortex, but are not abundant in the medulla. Glomeruli are labeled (gl). Scale bar is 20μm.

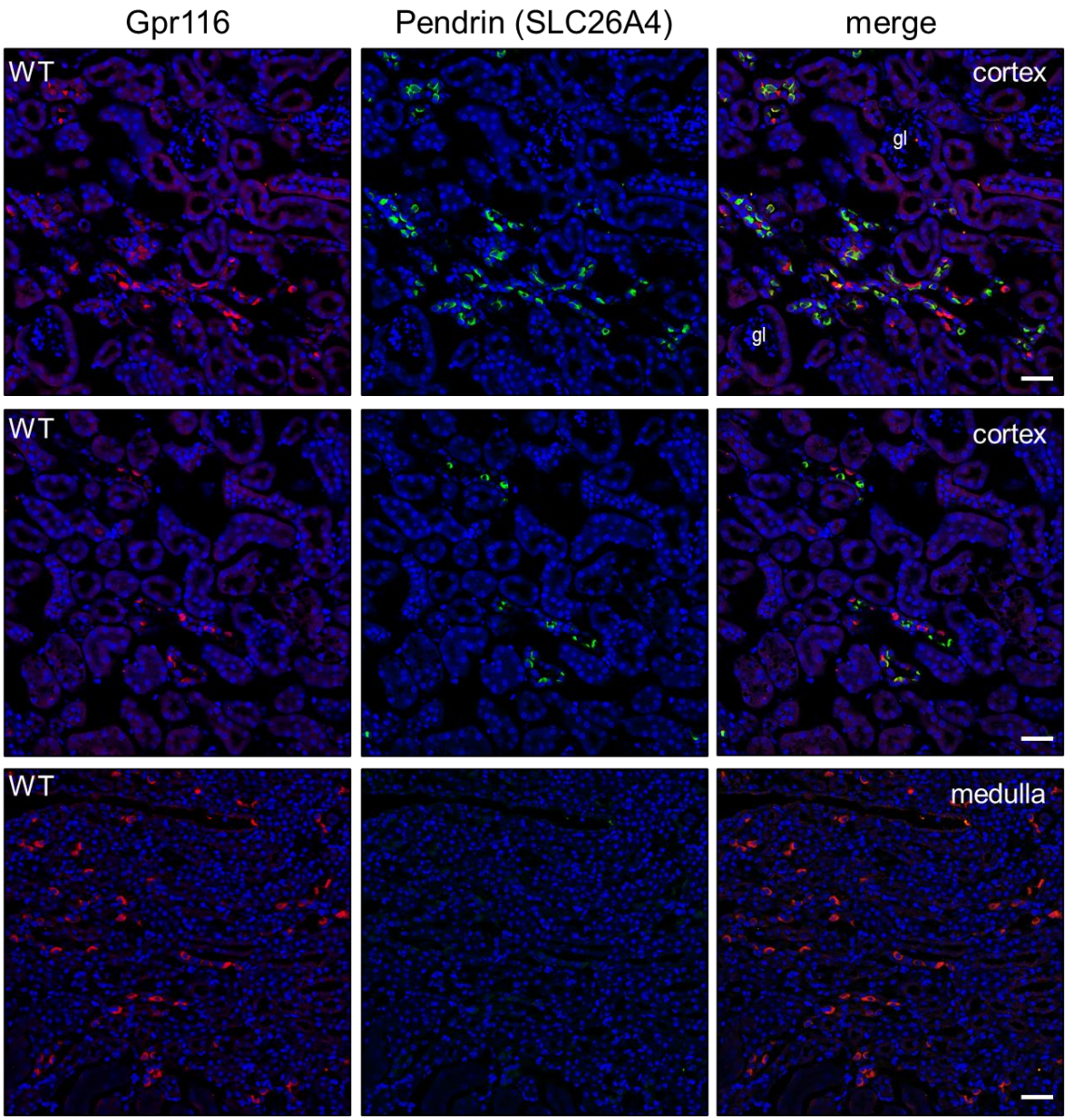

Fig. S3. B-type intercalated cells do not express Gpr116 (20x). Immunofluorescence images demonstrate Gpr116-positive cells do not express B-type intercalated cell marker pendrin (SLC26A4). Pendrin-positive cells are not abundant in the medulla. Glomeruli are labeled (gl). Scale bar is 40μm.

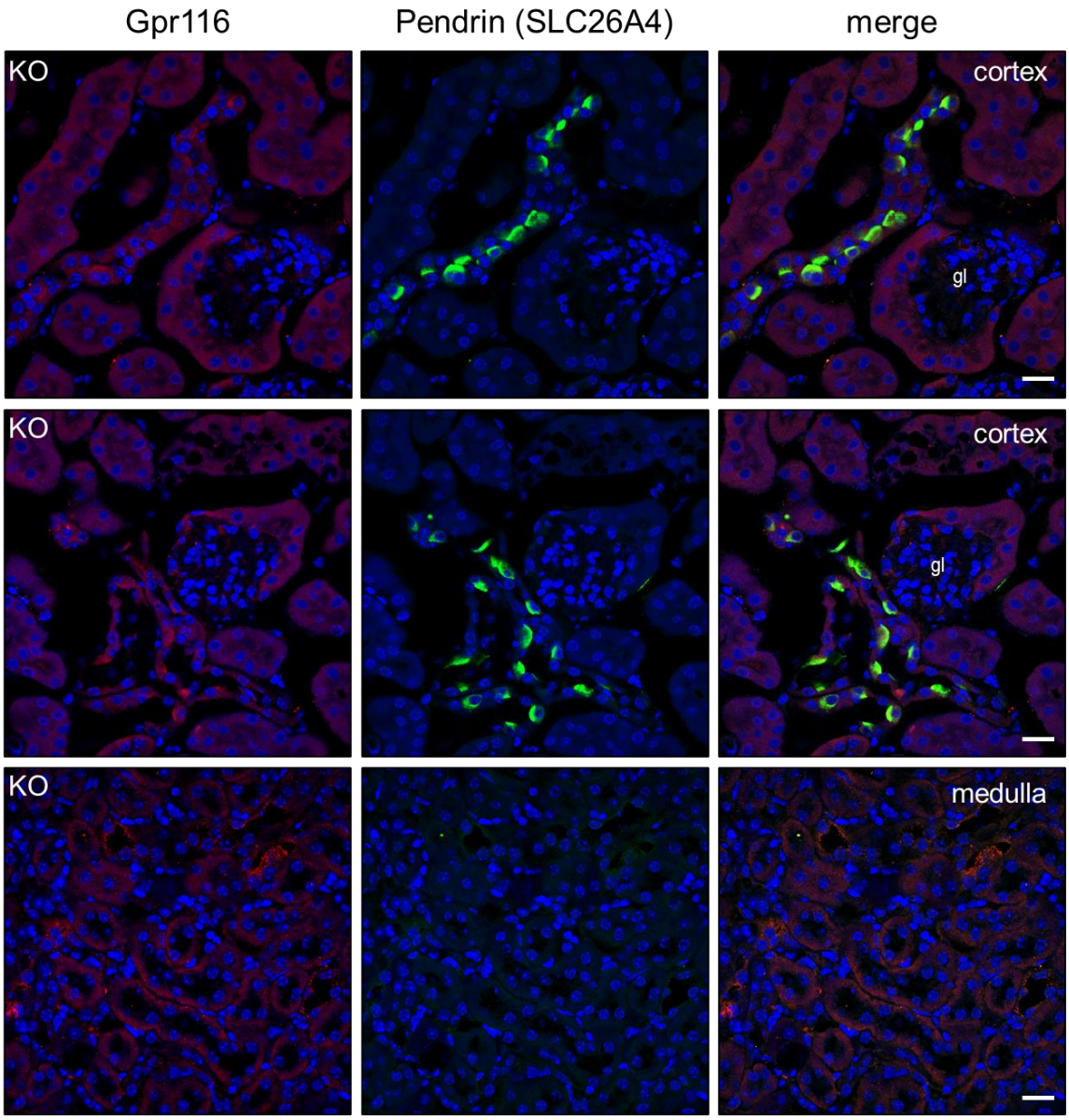

Fig. S4. Pendrin expression in Gpr116 KO kidney (40x). Pendrin-positive B-type intercalated cell expression in cortical collecting ducts. Glomeruli are labeled (gl). Scale bar is 20μm.

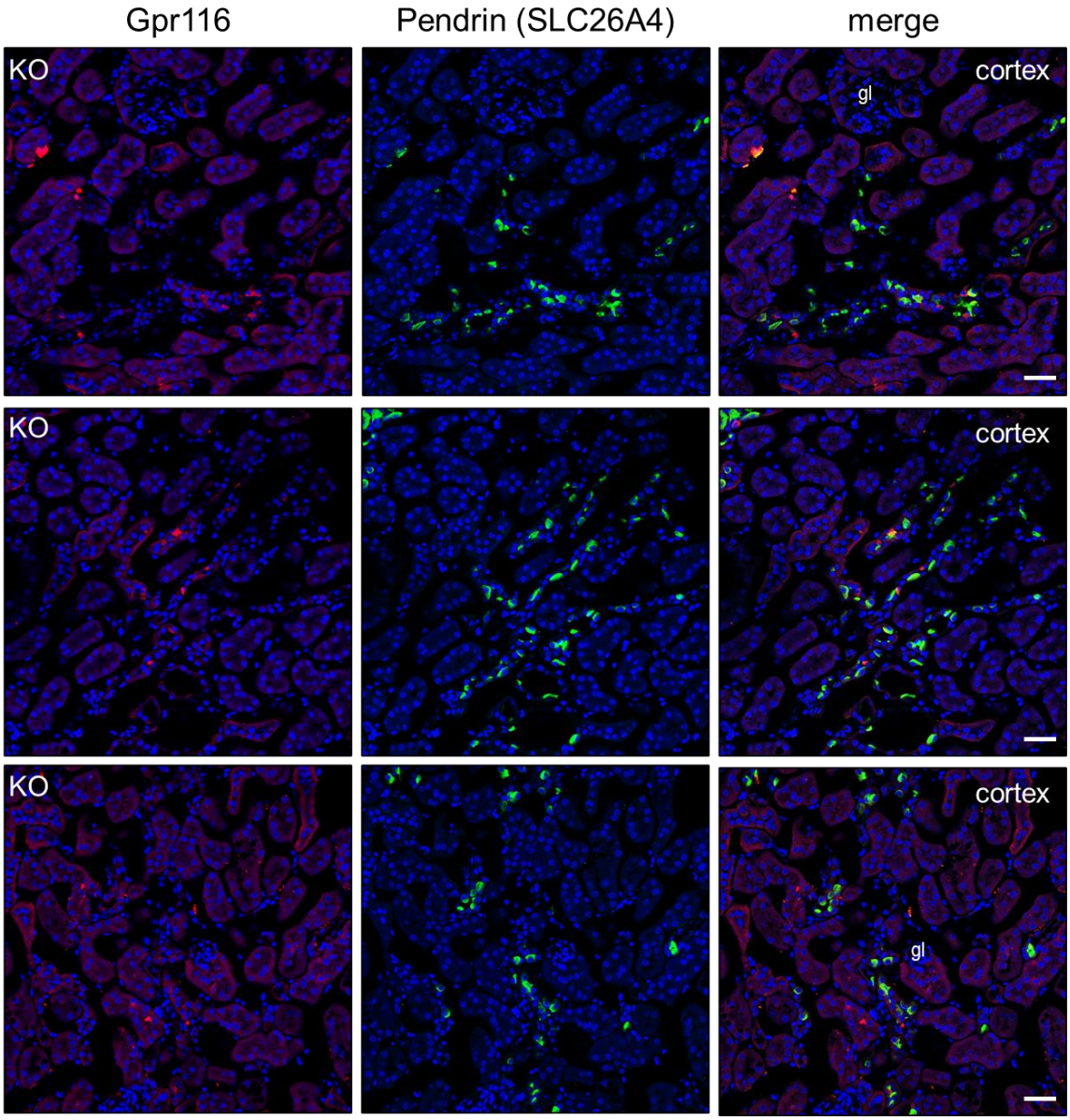

Fig. S5. Pendrin expression in Gpr116 KO kidney (20x). Pendrin-positive B-type intercalated cell expression in cortical collecting ducts. Glomeruli are labeled (gl). Scale bar is 40μm.

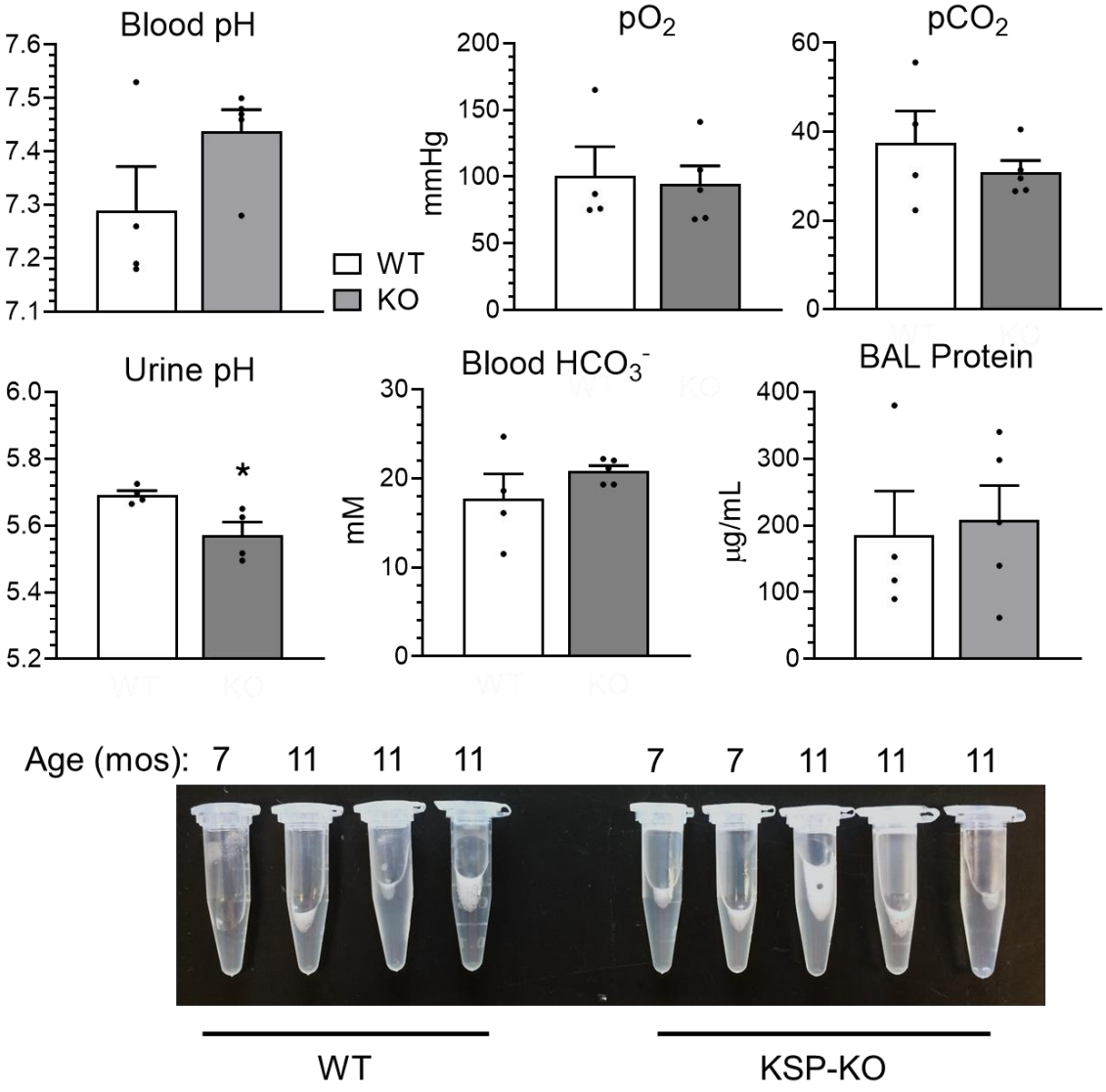

Fig. S6. Urine, blood and BAL analysis in female cohort of mice. Mice were ventilated and blood was collected from the left ventricle. Samples were immediately analyzed using an iSTAT handheld analyzer. BAL samples were collected immediately after blood collection. Samples were spun to remove cells and protein was quantified using a BCA protein assay kit. N=4 WT mice, 5 KO mice. * signifies $p < 0.05$.

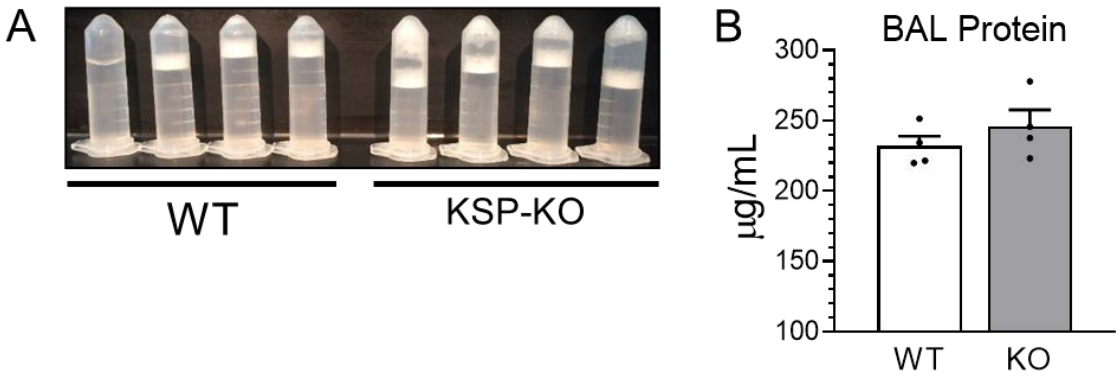

Fig. S7. Kidney-specific Gpr116 KO does not cause greater turbidity in BAL fluid. (A) Bronchoalveolar lavage fluid collected from WT and kidney-specific KO mice. (B) Quantification of BAL protein as measured by BCA protein assay. Bars are mean ± SEM. N=4 mice.

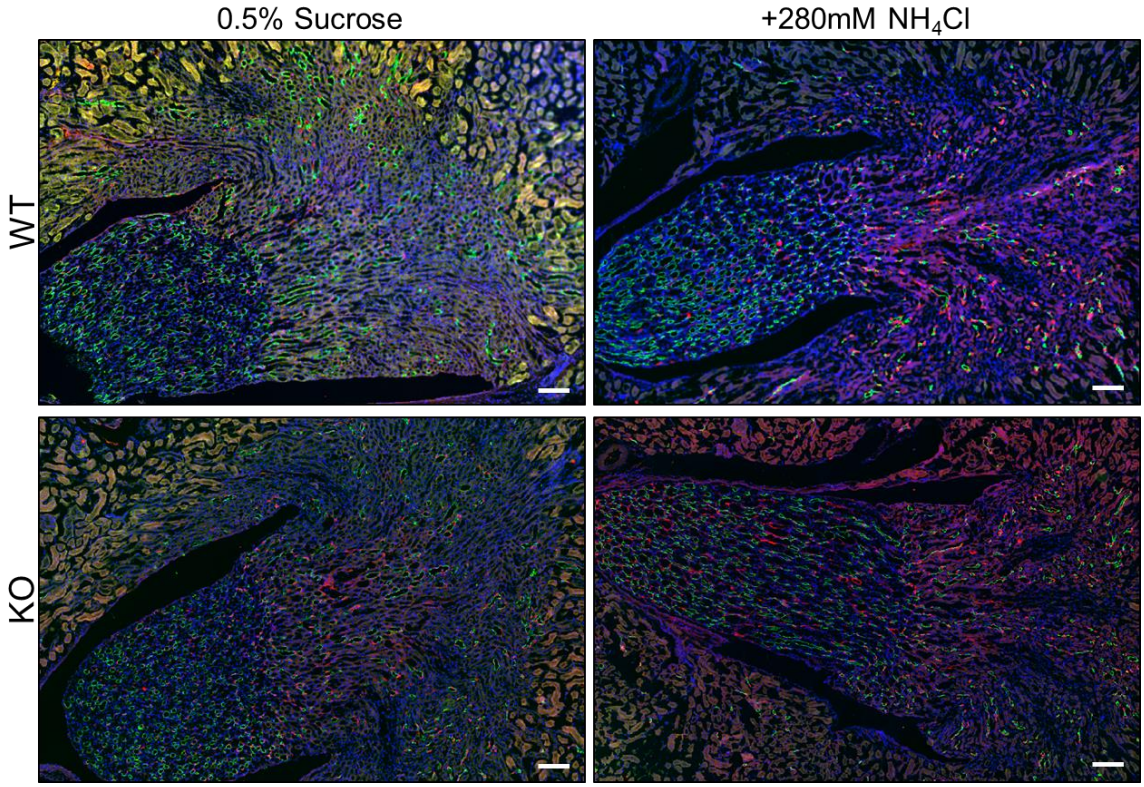

Fig. S8. Representative stitched images used to quantify AE1$^+$ and AQP2$^+$ cells in mouse medulla. Representative images demonstrating AQP2 (green) labeling of principal cells and AE1 (red) labeling of intercalated cells from mice drinking control water and water with NH_4Cl. Scale bars are 200µm.

SI Appendix References

1. K. Brown *et al.*, Epithelial Gpr116 regulates pulmonary alveolar homeostasis via Gq/11 signaling. *JCI Insight* **2** (2017).
2. J. A. Thomas, R. N. Buchsbaum, A. Zimniak, E. Racker, Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* **18**, 2210-2218 (1979).
3. V. Tomilin, M. Mamenko, O. Zaika, C. S. Wingo, O. Pochynyuk, TRPV4 deletion protects against hypokalemia during systemic K(+) deficiency. *American journal of physiology. Renal physiology* **316**, F948-F956 (2019).
4. J. C. Chan, The rapid determination of urinary titratable acid and ammonium and evaluation of freezing as a method of preservation. *Clin Biochem* **5**, 94-98 (1972).
5. P. Rajkumar *et al.*, Identifying the localization and exploring a functional role for Gprc5c in the kidney. *FASEB J* **32**, 2046-2059 (2018).
6. T. G. Paunescu *et al.*, cAMP stimulates apical V-ATPase accumulation, microvillar elongation, and proton extrusion in kidney collecting duct A-intercalated cells. *Am J Physiol Renal Physiol* **298**, F643-654 (2010).