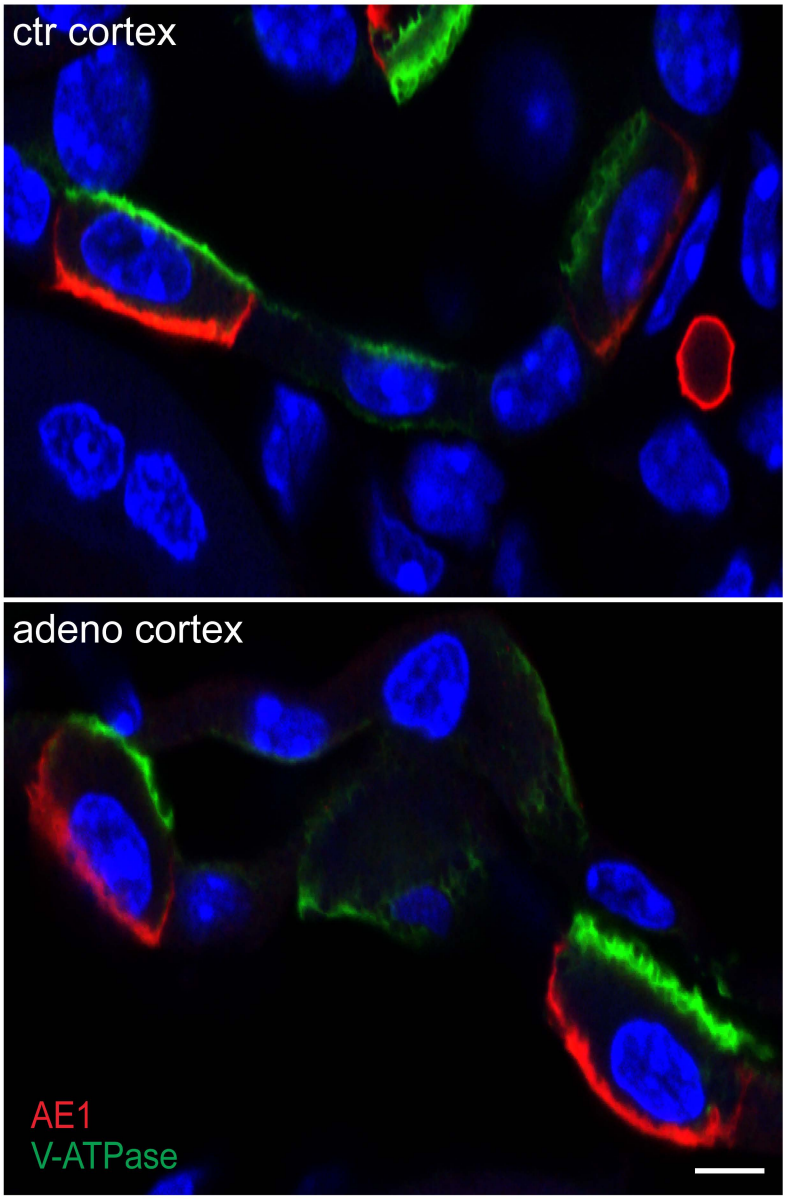
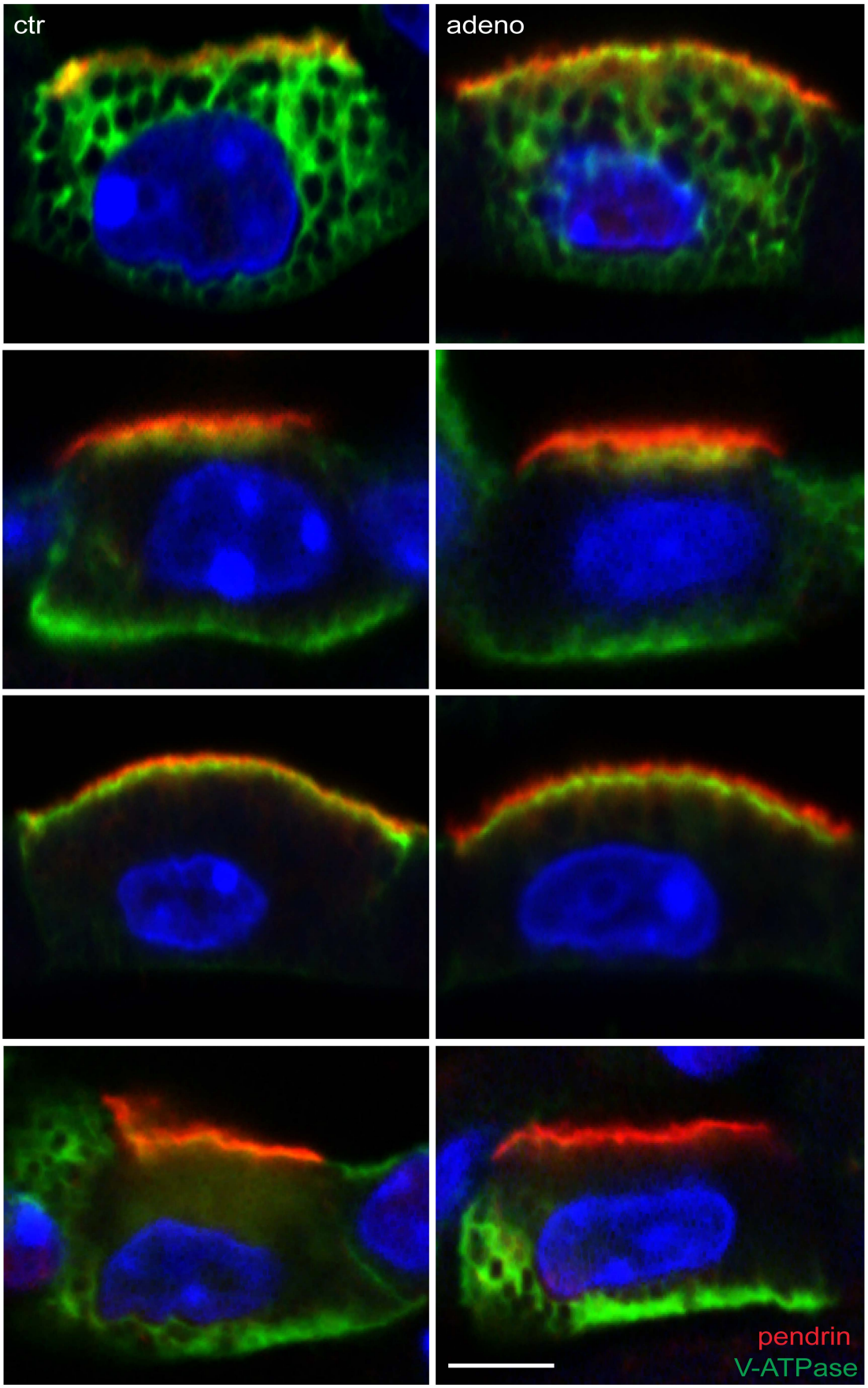
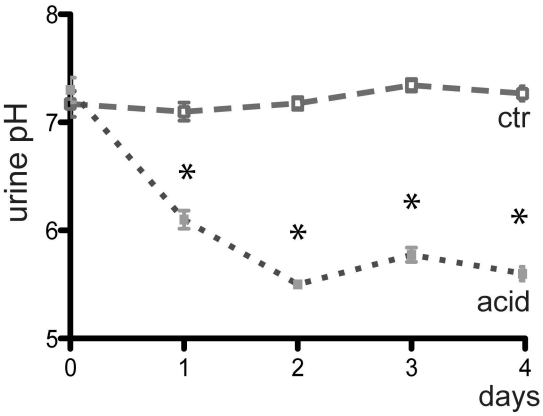


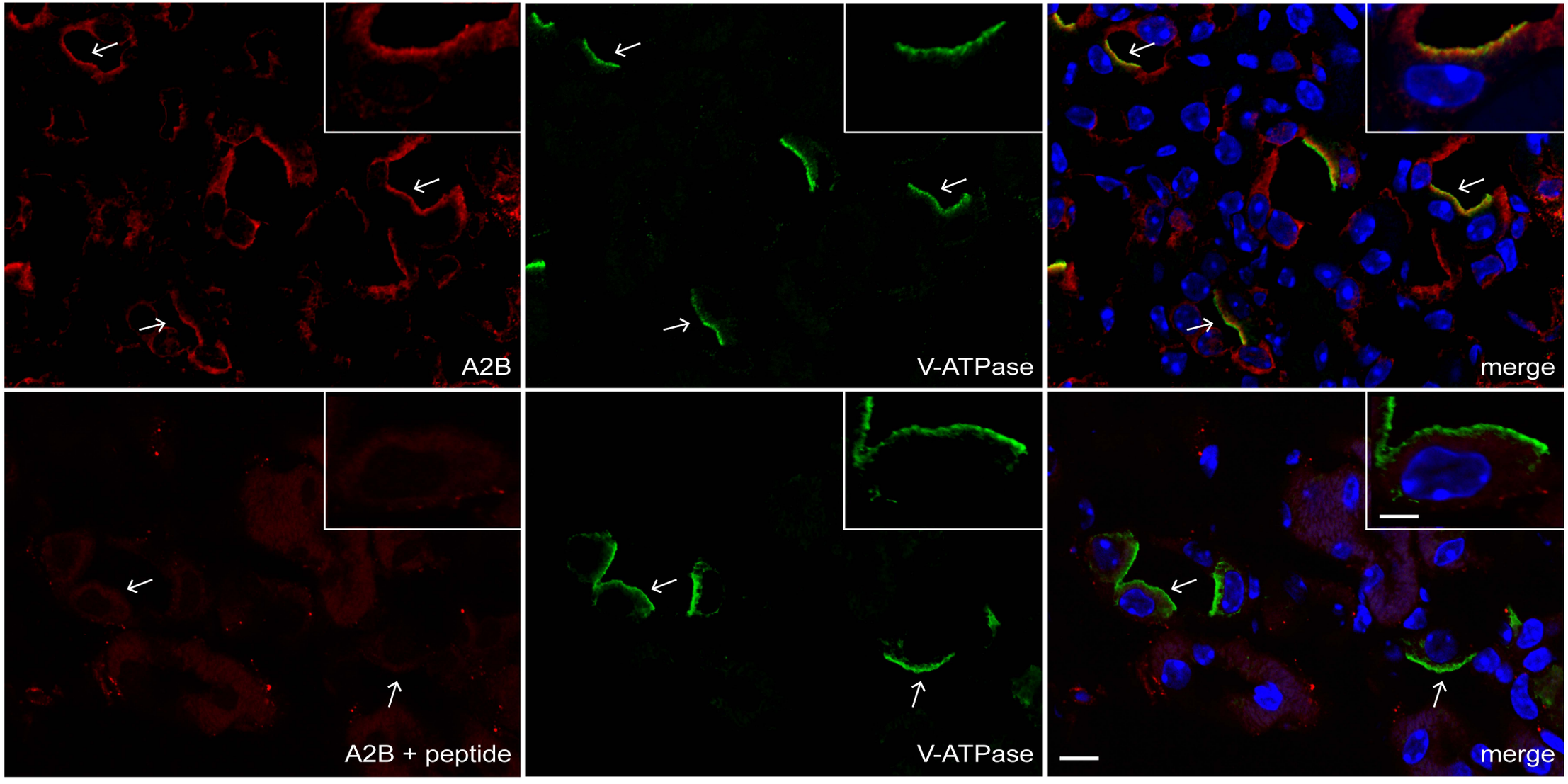
Battistone MA, et. al. Suppl. Figure 1.



Battistone MA, et. al. Suppl. Figure 2.



Battistone MA, et. al. Suppl. Figure 3.



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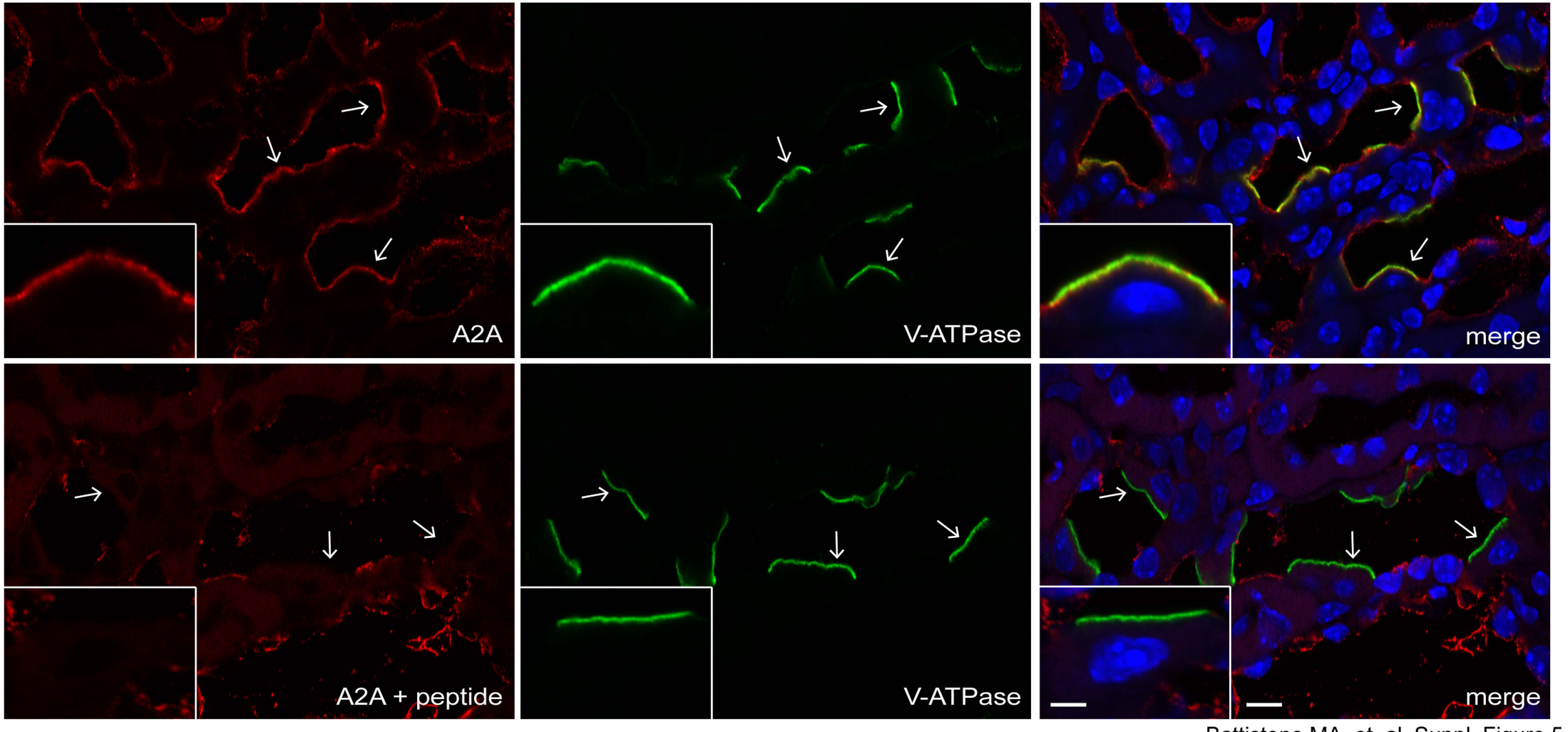
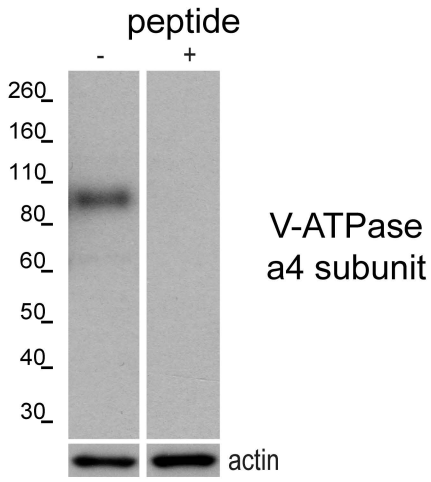


Battistone MA, et. al. Suppl. Figure 5.



Battistone MA, et. al. Suppl. Figure 6.

Supplementary Figure legends

Supplementary Figure 1: Adenosine does not affect cortical A-ICs. Confocal microscopy images showing double-labeling of the kidney cortex for the V-ATPase A subunit (green) and AE1 (red) to identify A-ICs. No effect in V-ATPase apical membrane was detected in cortical A-ICs from adenosine-treated mice (**B**) versus saline-treated mice (**A**). Bar = 5μm.

Supplementary Figure 2: Adenosine does not affect cortical pendrin-positive ICs. Confocal microscopy images showing double-labeling of the kidney cortex for the V-ATPase A subunit (green) and pendrin (red). A variety of V-ATPase staining patterns were observed, including cytosolic, bipolar, apical and basolateral, while pendrin was always apical, as previously shown. No detectable effect in the distribution of V-ATPase was observed in adenosine-treated mice (**right panels**) versus saline-treated mice (**left panels**). Bar = 2.5μm.

Supplementary Figure 3: Urine pH in acidotic treatment. A decrease in urine pH was observed during the first two days in mice that were given NH_4Cl in their drinking water, followed by a stable more acidic urine pH compared to control. Between 4 and 8 mice per group were examined each day. * $P<0.001$.

Supplementary Figure 4: Characterization of the rabbit anti-A2B antibody. Double-labeling for the A2B receptor (red, using the Alomone antibody) and the V-ATPase A subunit (green) of kidney medulla visualized by confocal microscopy. The A2B receptor is enriched in the apical membrane of A-ICs (arrows, upper panels), identified by their positive apical labeling for the V-ATPase. A2B labeling was also

observed in the apical membrane of principal cells (negative for the V-ATPase) and in thick ascending limbs. Staining was abolished when the antibody was pre-incubated with the immunizing peptide (lower panels). Bar = 5 μm. Inset Bar = 2.5 μm.

Supplementary Figure 5: Characterization of the rabbit anti-A2A antibody. Double-labeling for the A2A receptor (red, using the Alomone antibody) and the V-ATPase A subunit (green) of kidney medulla visualized by confocal microscopy. A2A is enriched in the apical membrane of A-ICs (arrows, upper panels), identified by their positive apical labeling for the V-ATPase. Expression of A2A was also observed in the apical membrane of principal cells (negative for the V-ATPase) and in thick ascending limbs. Staining was abolished when the antibody was pre-incubated with the immunizing peptide (lower panels). Bar = 5 μm. Inset Bar = 2.5 μm.

Supplementary Figure 6: Characterization of the rabbit anti-V-ATPase a4 subunit antibody. The specificity of the rabbit V-ATPase a4 subunit antibody was tested by WB using a mouse kidney medulla membrane fraction. A single band at approximately 100-kDa was detected using the antibody (-), and was abolished when the antibody was pre-incubated with the immunizing peptide (+). Actin was used a loading control.

Supplementary Materials and Methods

Animals

Adult C57BL/CBAF1 wild type male mice were purchased from Jackson Laboratories. In addition, transgenic mice that express EGFP under the control of the ATP6V1B1 gene promoter were used. The generation and characterization of these mice have been previously described[1], and they are further referred to as B1-EGFP transgenic mice. All procedures described were reviewed and approved by the Massachusetts General Hospital (MGH) Subcommittee on Research Animal Care and were performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. For tail vein injections, animals were anesthetized (pentobarbital; 60 mg/kg i.p.) and treated with 40 μL of saline solution or a saline solution containing adenosine, ADORA2A or ADORA2B agonists (1.5 μmoles/kg of body weight), or DMSO (0.1%). For acid loading *in vivo* experiments, 1.5% NH_4Cl (0.28 M) and 1% sucrose were added to the drinking water for 4 days, a well-established protocol to induce acid/base disturbances in mice[2-4].

Chemicals and reagents

Membrane permeable cAMP analog 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (cpt-cAMP), V-ATPase inhibitor concanamycin A (ConA), 3-isobutyl-1-methylxanthine (IBMX) and adenosine were obtained from Sigma-Aldrich (St. Louis, MO, USA). The ADORA2B receptor agonist (BAY 60-6583) and antagonist (PSB1115), the ADORA2A agonist (PSB0777 ammonium salt) and antagonist (ANR94) were obtained from TOCRIS (Avonmouth, Bristol, UK). mPKI (14-22) amide (myristoylated) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Seminaphthorhodafluor-5F-5-(and 6)-carboxylic acid, acetoxymethyl ester acetate

(SNARF-4F 5-(and-6)-Carboxylic Acid) (SNARF-5F-AM), Nigericin and Valinomycin were obtained from Thermo Scientific Tecnology (Waltham, MA, USA).

Immunofluorescence labeling

Mice were perfused via the left cardiac ventricle with phosphate buffered saline (PBS) followed by paraformaldehyde-lysine-periodate fixative (PLP) as previously described[5]. PLP-fixed kidney slices were cryoprotected in PBS containing 0.9M sucrose, embedded in Tissue-Tek OCT compound 4583 (Sakura Finetek, Torrance, CA) and frozen at -20°C. Sections were cut at 5-10 μm and picked up onto Fisherbrand Superfrost Plus microscope slides (Fisher Scientific, Pittsburg, PA) and stored at 4°C. Kidney sections were hydrated in PBS solution, and treated with sodium dodecyl sulfate (SDS) for 4 min[6]. ADORA2A and ADORA2B antibodies required additional antigen retrieval, and sections were microwaved for 2 min in EDTA-TRIS pH 9. Slides were blocked in 1% bovine serum albumin (BSA), and incubated with different primary antibodies at 4°C overnight, and with the secondary antibodies for 1 h at room temperature (RT). The primary antibodies used were chicken polyclonal antibody against the V-ATPase A subunit (1:400) and rabbit polyclonal antibody against the V-ATPase A subunit (1:400) made and purified in our laboratory[7, 8], two rabbit polyclonal antibodies against ADORA2A (1:50, Abcam, Cambridge, MA, USA; and 1:20, Alomone, Jerusalem BioPark, Israel), two rabbit polyclonal antibodies against ADORA2B (1:50, Millipore, Billerica, MA, USA; and 1:20, Alomone), anti rabbit polyclonal antibody against Slc26a4 (pendrin; 1:100, Thermo Fisher Scientific), anti rabbit polyclonal against Slc4a1 (AE1; 1:400; generously provided by Dr. Seth Alper group in Beth Israel Hospital, Boston, MA, USA). A mouse monoclonal antibody against the V-ATPase B1 subunit (1:100, Abcam) was used for the *in situ* PLA studies. In addition, we generated

and purified a novel rabbit polyclonal antibody against the last 10 amino acids (KHQKSQLQSFTIHEDAVEGDHC) of the C terminal tail of the V-ATPase a4 subunit (1:100) (Suppl. Fig. 6). The secondary antibodies used were: donkey Alexa 488-conjugated anti-chicken IgY, donkey alexa 488-conjugated anti-rabbit IgG, and donkey cy3-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories). All antibodies were diluted in DAKO medium (Dako, Carpinteria, CA). Slides were mounted with Vectashield mounting medium containing the DNA marker DAPI (Vector Laboratories, Burlingame, CA), and were examined using a Bio-Rad Radiance 2000 confocal microscope (version 4.1, Zeiss Laboratories), a Zeiss LSM800 confocal microscope equipped with Airyscan super resolution capabilities (Zeiss Laboratories) and Nikon Eclipse 90i epifluorescence microscope (Nikon Instruments, Melville, NY).

Immunogold electron microscopy

Small pieces (1mm^3) of tissues were excised from the inner stripe of the outer medulla of PLP-fixed kidneys and dehydrated through a graded series of ethanol solutions up to 100%. Tissue pieces were infiltrated with 100% LR White resin (EMS, Hatfield, PA) on a rotator and embedded in fresh LR White in gelatin capsules and polymerized 24-48 h at 50°C. Ultrathin (70nm) sections were cut using a Reichert Ultracut E ultramicrotome and collected onto formvar-coated grids (EMS, Hatfield, PA). Grids were incubated for one hour at RT on drops of primary antibody (rabbit anti V-ATPase A subunit, 1:100) followed by incubation in a secondary gold conjugate for 1h (goat anti-rabbit 15nm). Grids were rinsed with deionized water and contrast-stained using 2.0% aqueous uranyl acetate. Grids were examined at 80 kV in a JEOL1011 transmission electron microscope (Peabody, MA) equipped with an AMT digital camera and proprietary image capture software (Advanced Microscopy Techniques, Danvers, MA). For the

quantification of apical V-ATPases in electron micrographs of A-ICs, gold particles located on the apical plasma membrane and microvilli, were counted for each cell using the software FIJI (National Institutes of Health, Bethesda, MD). The length and the width of each cell were also measured using the software FIJI.

RNA extraction, reverse transcription and PCR

RNA isolation, cDNA synthesis and PCR were performed as described previously[9, 10]. RNA from the kidney medulla was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA), and RNA from EGFP^{+}-ICs was isolated by PicoRNA KIT (Thermo Fisher Scientific). Genomic DNA contamination was degraded with RNase-free DNase set (Qiagen). PCR products were resolved on 1% agarose gels. The sequences of: the ADORA1 primers were CCAAGCTTACCACCCTGAGT (Forward) and TGGTGTGTGAGTGTGTGTTCA (Reverse); the ADORA2A primers were TGGGCTGGAAGACAGAAACT (Forward) and CACAGGGCAGCTCTGAACTA (Reverse); the ADORA2B primers were AAACTGGAGAGACGAAGTCCT (Forward) and TTTTCACACTTTAGGTCCCAAT (Reverse); the ADORA3 primers were CTCTAGTGGGGCTGGACGTA (Forward) and CCCACACAGGTGGATTTAGC (Reverse). Sequences for GAPDH were used as controls[11].

Cell fractionation and Western blotting (WB)

Whole kidney medulla, and EGFP^{+}-ICs isolated by FACS from the medulla were homogenized in ice-cold buffer containing 300 mM sucrose, 1 mM EDTA, 20 mM Tris-HCl pH 7.4 and complete protease inhibitors and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN). Cell fractionation was performed using a well-characterized sequential centrifugation procedure (modified from[12-14]). Briefly, samples

were spun at 4,000g at 4°C for 10 min, and supernatants were centrifuged at 17,000g at 4°C for 30 min. The 17,000g pellets are enriched in plasma membrane. Supernatants were centrifuged for the third time at 100,000g at 4°C for 1 h. The 100,000g pellets are enriched in intracellular vesicles. For electrophoresis, the pellets were incubated at 70°C for 10 min in a reducing sample buffer containing lithium dodecyl sulfate (LDS) and DTT (NuPAGE, Invitrogen, Carlsbad, CA, USA). Samples were run on a NuPage 4% 12% Bis-Tris Gel (Invitrogen) and transferred onto Sequi-Blot PVDF membranes (Bio-Rad, Hercules, CA). The membranes were incubated in 5% nonfat milk, were incubated overnight at 4°C with primary antibody (rabbit anti V-ATPase a4 subunit antibody (1:1,000)) and followed by HRP-conjugated goat anti-rabbit secondary antibody (1:5,000, 1h, RT). Proteins were detected using Western Lighting Blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences).

Proximity Ligation Assay

In situ interactions were detected by using the proximity ligation assay (PLA) kit Duolink II (Olink Bioscience, Uppsala, Sweden). The PLA probe anti-rabbit minus binds to the V-ATPase a4 subunit antibody, whereas the PLA probe anti-mouse plus binds to the V-ATPase B1 subunit antibody. The Duolink PLA only generates a signal when the two PLA probes have bound to each other, which only takes place if both proteins are closer than 40 nm, indicating their interaction[15]. Briefly, PLP-fixed kidney sections were incubated, as previously described, with antigen retrieval (1% SDS, for 4 min) and with blocking agent (1% BSA in PBS, for 15 min). Primary antibodies (1:50) were, then, applied at 4 °C overnight. Slides were washed three times in PBS for 10 min. PLA probes detecting rabbit and mouse antibodies were diluted in DAKO medium in a concentration of 1:5 and applied to the slides for 1h in a humidity chamber pre-

heated at 37 °C. Unbound PLA probes were removed by washing two times in a buffer (containing 0.01 M Tris, 0.15 M NaCl and 0.05% Tween 20) for 5 min. The samples were incubated with the ligation solution consisting of Duolink II Ligation stock (1:5) and Duolink Ligase (1:40) diluted in water for 30 min at 37 °C. After ligation, slides were washed again as described above. The Duolink Amplification and Detection stock was diluted 1:5 with addition of polymerase (1:80) and was applied to the slides for 100 min. After final washing steps with a buffer (containing 0.2 M Tris and 0.1 M NaCl), slides were mounted using Duolink In Situ Mounting Medium containing DAPI. Negative controls were performed with omission of primary antibodies.

Urine collection, adenosine assay and determination of urine pH

Mouse urine was collected directly from the bladder with a fine-gauge needle. Urine samples were centrifuged at 1,000g for 5 min at 4°C to remove any particulates, and then, pre-treated with adenosine convertor and urine clarifier (provided in the kit) and with a 50 % suspension of Catalase Beads (BioVision, Milpitas, CA, USA) at RT for 15 min. The pre-treated urine samples were centrifuged at 1,000g for 1 min and the supernatants were used for the assay. Adenosine was measured using adenosine deaminase followed by a multi-step enzymatic approach resulting in the generation of an intermediate that reacts with the adenosine probe with the formation of a fluorescent product (BioVision). Fluorescence was measured at Ex/Em = 535/587 nm using a DTX880 Multimode Plate Reader (Beckman Coulter, Inc.). Adenosine concentrations were extrapolated from the linear adenosine standard curves provided by the company. Urine osmolality were measured using an osmometer (Wescor, Logan, UT).

For pH measurement, urine samples were collected, before and 15 min after tail vein injection with adenosine. Urination was triggered by gentle massage of the abdomen

and the pH was measured immediately using pH micro-fine paper strips (range pH 5.5 to pH 8.0, 0.2 increment, Hydrion). pH assessment was conducted in a "blind manner" in de-identified samples.

Isolation of EGFP$^+$-ICs from B1-EGFP mice

Isolation of EGFP$^+$-ICs from the inner stripe of the outer medulla and the inner medulla of B1-EGFP mice was performed, as we previously described[16]. EGFP-negative cells, which correspond to all other cell types in the medullary kidney, were also collected. Briefly, kidney medulla were dissected, minced, and incubated in isolation buffer containing 1 mg/ml collagenase I (Invitrogen), 1 mg/ml collagenase II (Sigma), and 10 mM HEPES (pH 7.5) in RPMI 1640 medium (Invitrogen) at 37°C with gentle shaking (1,400 rpm) every 1 min (for 10s each time) for a duration of 45 min. Isolated cells were passed through a 70-μm filter; washed once in PBS containing 2% fetal bovine serum and 2 mM EDTA; and centrifuged at 500g for 5 min 4°C. Cells were either suspended in complete RPMI medium to be used for live cell imaging, or in PBS containing 2% fetal bovine serum and 2 mM EDTA for FACS. Cells were further passed through a 35-μm filter for FACS isolation performed at the MGH Flow Cytometry Core facility (Boston, MA). The validity and assessment of contamination of our FACS isolation technique have been characterized previously[9, 17].

Assessment of PKA-dependent protein phosphorylation

Sorted EGFP$^+$-ICs were incubated in RPMI in the absence or presence of DMSO (0.1%, ctr), cpt-cAMP (1 mM), adenosine (600 μM), IBMX (1.5 μM), ADORA2B agonist (BAY 60-6583, 600 μM) or ADORA2A agonist (PSB0777, 600 μM) for 15 min at 37°C. The cells were also incubated in the absence or presence of ADORA2A

antagonist (anr94, 10 μM), or ADORA2B antagonist (psb1115, 10 μM), or PKA inhibitor (mPKI, 10μM) for 15 min in RPMI at 37°C. At the end of the incubations, cells were washed with PBS at 500g for 5 min at 4°C. Total proteins from pellets were solubilized in RIPA buffer (containing complete protease and phosphatase inhibitors; Roche Applied Science) and the phosphorylated proteins were assessed by SDS–PAGE and WB. The monoclonal primary antibody used was an antibody that recognizes phosphorylated consensus motifs on PKA substrates (phospho-Ser/Thr residue with arginine at the -3 and -2 positions: RRXS*/T*; anti-pPKAs; 1:500; clone 100G7E, Cell Signaling, Danvers, MA, USA) and the corresponding secondary antibody was coupled to peroxidase. The blotted membranes were stripped (RT, 10 min, Thermo Scientific Technology) and the B1 V-ATPase antibody (1:1000) was used as a loading control.

Intracellular pH measurements

Isolated medullary kidney cells, from B1-EGFP mice, plated on plastic-bottomed μ-slides (Ibidi, Madison, Wisconsin, USA) were transferred to a thermostatically controlled stage equipped with a perfusion system maintained at 37°C on a Nikon A1 resonant scanning confocal inverted microscope (Nikon Instruments, Melville, NY) equipped with a 60X oil immersion objective and a Nikon Perfect Focus System. Only $EGFP^+$-ICs were visualized and selected for intracellular pH (pH_i) measurements. Cells were incubated with SNARF-4F 5-(and-6)-Carboxylic Acid (2.5 μM, Invitrogen), which is membrane permeant and is esterified in the cytoplasm within seconds to become membrane impermeant. SNARF-5F-AM was excited with 565nm laser and the emission was detected using a multi-anode photomultiplier tube spectral detector set up to collect the emitted light between 571- to 591 (581)-, and 621- to 681 (640)-nm wavelengths simultaneously[10]. After a 4-min incubation, cells were washed in control

solution (in mM: 125 NaCl, 5 KCl, 5 NMDG-Cl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 4 NaH_2PO_4, and 5 HEPES) to remove residual non-esterified SNARF-5F-AM in the solution before time-lapse imaging. Cells were then perfused in the absence of sodium for 3 min (Na-free solution containing in mM: 1 KCl, 134 NMDG-Cl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 4 KH_2PO_4, and 5 HEPES). An ammonium chloride (NH_4Cl) pulse was then applied in the absence of sodium (in mM: 1 KCl, 94 NMDG-Cl, 20 NH_4Cl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 4 KH_2PO_4, and 5 HEPES) for 1.5 min, followed by the Na-free solution for 3 min and finally, control solution again for 1 min. In separate groups, modulators (V-ATPase inhibitor concanamycin A (ConA; 1 μM), adenosine (600 μM) and cpt-cAMP (1 mM) were added 3 min before the NH_4Cl pulse and were present throughout the pH_i measurements. Images were taken every 4s and imported into Fiji software. The mean pixel intensity was recorded from an intracellular area and care was taken to avoid the nucleus and large intracellular vesicles. The 581- to 640-nm ratios were converted to pH using a calibration curve obtained by incubating cells with Nigericin and Valinomycin (10 μM) in the presence of a solution containing high-potassium at different pH values (pH:4 to pH:9). Proton secretion was determined from the initial rate of pH_i recovery ($\Delta pH_i/\Delta t$) following the NH_4Cl pulse. $\Delta pH_i/\Delta t$ was estimated from the linear portion of the pH_i recovery by using the linear regression function in Excel. The first pH_i value was chosen to be the lowest value within the first 8 s after the rapid drop in pH_i, and the last pH_i value was chosen to be the value after 0.47 min in zero sodium solution. These experiments were performed in the absence of nominal bicarbonate, thus minimizing the contribution of HCO_3^--transporting mechanisms in pH_i recovery.

Statistical analysis

The numeric data were analyzed using GraphPad Prism (Version 7; GraphPad Software, La Jolla, CA, USA). Data were analyzed using one-way or two-way ANOVA followed by a Tukey's post hoc test. A value of $P<0.05$ was considered significant. Data were expressed as the means ± SEM. For each set of data, at least four different animals were performed for each condition. Collection, analysis and interpretation of data were conducted in a "blind manner" in de-identified samples.

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