Complete Methods

Animal study approval.

All animal use and welfare adhered to the NIH Guide for the Care and Use of Laboratory Animals following protocol reviews and approval by the Institutional Laboratory Animal Care and Use Committee of the University of Utah Health Sciences Center.

Generation of inducible whole nephron-specific adenylyl cyclase 3 knockout mice.

Mice transgenic for Pax8-rtTA and LC-1 transgenes were bred with mice containing loxP-flanked (floxed) exons 4-6 of the *Adcy3* gene[1] to yield inducible nephron-specific AC3 knockout mice that were homozygous for the floxed *Adcy3* gene and hemizygous for the Pax8-rtTA and LC-1 transgenes. As previously described[2, 3], the Pax8-rtTA transgene contains 4.3 kb of the 5' untranslated region along with exon 1, intron 1, exon 2 and part of intron 2 of the *Pax8* gene driving expression of the reverse tetracycline transactivator (rtTA). The LC-1 transgene encodes tetracycline-inducible bicistronic Cre recombinase and luciferase[2, 3]. To obtain mice lacking *Adcy3* gene in the nephron ($AC3^{-/-}$), 1-month old male mice that were homozygous for the floxed *Adcy3* gene and hemizygous for Pax8-rtTA and for LC-1 transgenes were given doxycycline (2 mg/ml) in 2% sucrose drinking water for 14 days, followed by at least 21 days without doxycycline before conducting experiments. A similar protocol was also used in control littermates that were homozygous for the floxed *Adcy3* gene and hemizygous for LC-1, but lacked Pax8-rtTA.

Mice which were heterozygous for Pax8-rtTA and LC-1 transgenes and homozygous for the floxed *Adcy3* gene were born at the expected frequency and showed no developmental abnormalities. These mice lived up to at least 1 year of age and had no apparent histologic renal abnormalities.

Genotyping

Tail DNA from AC3$^{-/-}$ and control littermates was PCR amplified with the following primers: AC3 - F 5'-ctgctttgtcattacaatttcc-3' and AC3 - R 5'-tgaggactgcctttctagag-3', which yield a 275 bp product from the floxed *Adcy3* gene and a 241 bp product from the wild-type allele[3]. Transgenes were identified using the following primers: Pax8-rtTA - F 5′-ccatgtctagactggacaaga-3′ and R 5′-catcaatgtatcttatcatgtctgg -3′ which yields a 600 bp product; and LC-1 - F 5′-tcgctgcattaccggtcgatgc-3′ and R 5′-ccatgagtgaacgaacctggtcg-3′ which yields a 480 bp product[2].

Analysis of mRNA.

RNA from renal cortex and papilla from nephron AC3 KO and control mice was extracted using guanidinium isothiocyanate and acid phenol, reverse transcribed and cDNA levels TRPM6 and GAPDH determined using real-time PCR (StepOne Plus, Applied Biosystems, Foster City, CA). PCR was performed according to instructions provided by the manufacturer using the *Taq*man Gene Expression Assay (Applied Biosystems, Carlsbad, CA) with Adcy3 (Cat.# Mm00460371_m1), TRPM6 (Cat.# Mm00463112_m1) and GAPDH (Cat.# Mm99999915_g1) primers.

Metabolic cage studies and electrolyte measurement

Nephron AC3 KO and control mice were placed in metabolic cages and given 9 ml of a gelled diet made from 248 g of PMI rodent powdered diet (LD101, LabDiet, Richmond, Indiana), 7 g gelatin and 110 ml water with free access to drinking water for 3 days. 24-hr urine collection was done each day and urine for day 3 of a normal diet was analyzed for volume, Na^+, K^+, Mg^{2+} and Ca^{2+}. Mice fed a low Mg^{2+} diet (negligible Mg^{2+} concentration, TD.93106, Harlan Laboratories, Madison, Wisconsin) were placed in metabolic cages for 7 days. Blood was collected at day 0 and 7 and analyzed for Mg^{2+} content. Urine collected at days 0, 2, 3 and 7 were similarly analyzed for Mg^{2+} content. Blood was taken from the heart and was used to analyze

plasma Mg^{2+} concentration. Electrolyte concentrations were determined in the clinical laboratory at Associated Regional University Pathologists.

Cell culture and transfection

HEK293 cells were grown at 37 °C in DMEM (Biowhittaker Europe, Vervier, Belgium) supplemented with 10% (v/v) FCS (PAA Laboratories, Linz, Austria), nonessential amino acids, and 2 mM L-glutamine in a humidified 5% (v/v) CO_2 atmosphere. Cells were seeded in 12-well plates and subsequently transfected with 1 μg (per well in a 12 wells plate) of HA-tagged human TRPM6 in the pCINeo-IRES-GFP mammalian expression vector[4] or of a human TRPM6-GFP fusion construct[5] using Lipofectamine 2000 (Invitrogen, Breda, the Netherlands). A similar protocol was followed for a murine TRPM7 construct, a kind gift from Dr. David E. Clapham. Mutations were introduced in the wild type HA-tagged TRPM6 construct using the QuikChange site-directed mutagenesis kit (Agilent, Amstelveen, the Netherlands). Approximately 36 hours after transfection, cells for electrophysiology and fluorescence imaging were seeded on glass coverslips coated with 50 μg/ml fibronectin (Roche, Mannheim, Germany). Experiments were started 2 hours after seeding the cells.

Immunoblotting and cell surface biotinylation

Lysis buffer for cell lysis contained: 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 3% (w/v) CHAPS, 1 mM Na-orthovanadate, 10 mM Na-2-glycerophosphate, 50 mM NaF, 270 mM sucrose, 0.1% (v/v) 2-mercaptoethanol, pepstatin (1 μg/ml), PMSF (1 mM), leupeptin 5 μg/ml and aproptin 5 μg/ml. TBST (TBS containing Tween 20) consisted of Tris/HCl (pH 7.5), 0.15 M NaCl and 0.2% (v/v) Tween 20. Cells were lysed in 0.1 ml of ice-cold lysis buffer. The lysates were clarified by centrifugation (13,000 rpm, 4°C, 15 min) and the supernatants frozen in liquid nitrogen and stored at −20°C. Cell lysates were subjected to SDS-

PAGE then transferred on a PDVF membrane (Millipore). The membranes were incubated for 1 hour with TBST containing 5% (w/v) non-fat dried skimmed milk powder. The membranes were then incubated overnight at 4°C with either mouse anti-HA-tag (6E2) antibody (1:5,000, Bioké, Leiden, The Netherlands) or mouse anti-β-actin antibody (1:5,000, Sigma-Aldrich), diluted in 5% (w/v) non-fat dried skimmed milk in TBST. The next day, membranes were washed with TBST and incubated with a mouse peroxidase-coupled secondary antibody for 1 hour at room temperature (1:10,000, Jackson ImmunoReasearch, Suffolk, UK). After subsequent washes with TBST, the proteins were revealed using the SuperSignal West Pico and Femto chemiluminescence kits (Thermo Scientific).

For cell surface biotinylation experiments, HEK293 cells were transfected and cell surfaces were biotinylated for 30 min at 4˚C 48 hours later by adding sulfo-NHS-LC-LC-biotin (Pierce, Rockford IL, USA). Subsequently, protein lysates were incubated overnight with neutravidin-agarose beads (Pierce) to isolate cell surface biotinylated proteins.

Electrophysiology

All experiments were performed at room temperature. All experiments were undertaken and analyzed using an EPC-9 amplifier and the Patchmaster software (HEKA electronics, Lambrecht, Germany). The sampling interval was set to 200 μs for whole-cell recordings and to 20 μs for single channel cell-attached and outside-out recordings. Whole-cell recordings were acquired with a low-pass filter set at 2.9 kHz, while single channel recordings had a low-pass filter set at 5 kHz. The electrical noise was further reduced using the Humbug 50/60 Hz noise eliminator (Quest Scientific, Vancouver, Canada). Whole-cell patch clamp pipettes were pulled from thin wall borosilicate glass (Harvard Apparatus, March-Hugstetten, Germany) and had resistance between 1 and 3 MΩ when filled with the pipette solution. Series resistance compensation was set

to 75-95% in all whole-cell experiments. Current densities were obtained by normalizing the current amplitude (obtained at +80 mV 200 s after break-in) to the cell capacitance.

Single channel patch clamp pipette were made of thick wall borosilicate glass (Harvard Apparatus) and had resistance between 8 and 11 MΩ when filled with the pipette solution. Single channel recordings were analyzed using the Qub software package[6].

Solutions and compound application

The extracellular solution contained (in mM): 150 NaCl, 1 $CaCl_2$, 10 HEPES and pH adjusted to 7.4 using NaOH[4]. The pipette solution was made of (in mM): 150 NaCl, 10 Na_2EDTA, 10 HEPES and pH adjusted to 7.2 using NaOH[4]. Unless otherwise mentioned, cells were pre-incubated 15 minutes at 37°C in bath solution containing the compound of interest or vehicle (0.1% v/v DMSO and/or 0.1% v/v EtOH).

Total Internal Reflection Fluorescence (TIRF) microscopy

A microscope with 63×1.45 oil immersion objective (Olympus) was used to image GFP-labeled TRPM6 channels. Baseline fluorescence intensity from the cells was established for 15 minutes before switching to a solution-containing vehicle (0.1% v/v EtOH) or FSK (10 μM). 20 frames at 0.2 s interval were acquired every 2 or 3 min. To quantify the fluorescence, areas between 5 and 10% of the footprint of the cell were averaged and normalized according to the following equation: relative change = $(F(t)-F(0))/F(0)$, where F(0) is the intensity at the beginning of the time series and F(t) is intensity at a given time t. Images were background-subtracted.

Bioinformatic tools

Prediction of putative phosphorylation sites was obtained by submitting the human TRPM6 amino acid sequence to the NetPhosK1 and NetPhosK2 web servers (*http://www.cbs.dtu.dk/services/NetPhosK/)*. Alignment of protein sequences was performed using ClustalW2 web server (*http://www.ebi.ac.uk/Tools/msa/clustalw2/*).

Statistical analysis

All results are depicted as mean ± standard error of the mean (SEM). Direct comparisons of means between conditions were performed using student t-test. When multiple groups were compared, one-way ANOVA and Fisher's post-hoc tests were used to assess statistical significance. Difference with P values < 0.05 were considered statistically significant and indicated by a (*).

References

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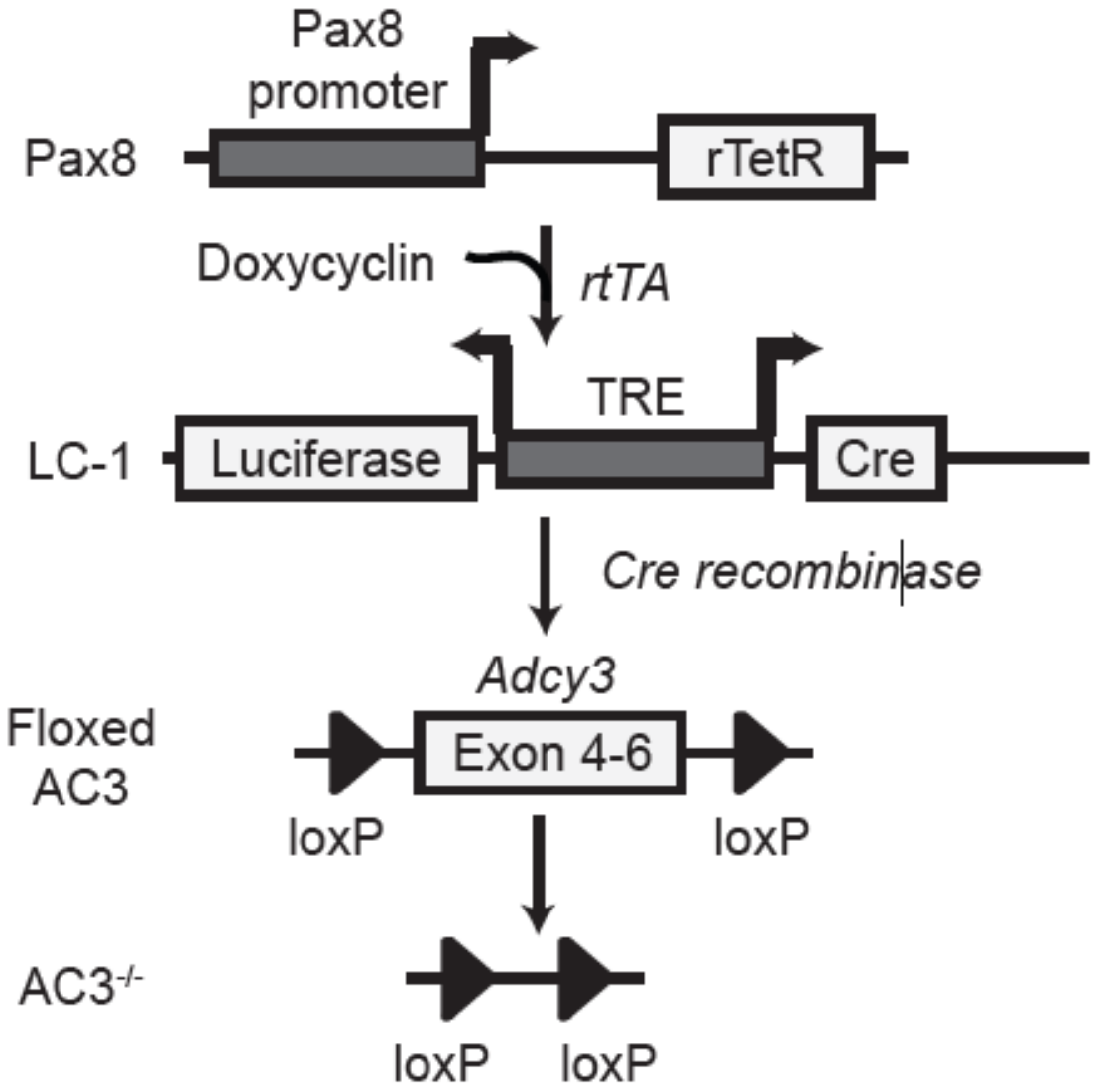

FIGURE S1 Mice containing the Pax8-rtTA and LC-1 transgenes were bred with mice containing loxP-flanked exons 4-6 of the *Adcy3* gene (AC3). Mice homozygous for floxed *Adcy3* and heterozygous for Pax-8 and LC-1 transgenes received daily doxycycline injections (2 mg/ml) for 14 days. Mice were allowed to recover for at least 21 days.