Complete Methods

Animal Studies

Animal studies were performed in adherence to the NIH Guide for the Care and Use of Laboratory Animals and approved by The University of Maryland School of Medicine Institutional Animal Care and Use Committee. Mice were housed in groups of two to five per cage on a 12:12 h light/dark cycle; with lights on at 6 a.m. Tissues, blood and urine samples were collected between 8 and 10 am. Male mice were studied. Food and water were available ad libitum.

Creating Mice Carrying the Floxed CA-SPAK Allele

Two mutations, T243E and S383D, which render the kinase constitutively active,[23] were inserted into the full length N-terminal HA-epitope SPAK cDNA, and the mutant SPAK was incorporated into a construct that targets the mouse SPAK gene, STK39. The construct consisted of a 3.5 kb genomic DNA fragment as the 5' arm of recombination, followed by a loxP site, the neomycin resistance gene cassette, a second loxP site, the SPAK mutant, the mouse phosphoglycerate kinase-1 polyadenylation sequence (pA), and a 6.5 kb fragment as the 3' arm of recombination (Supplement Figure 1A). Arms of recombination were dropped into the construct vector using BAC clone bMQ-410m10 and recombineering. The loxP site is in frame with the remainder of the SPAK protein and encodes a short 12 amino acid peptide. Successful insertion of construct into the STK39 gene creates a SPAK KO, and allows CA-SPAK to expressed under control of the native SPAK promoter following recombination with Cre-recombinase.

129/SvEvTac-derived ES cells were electroporated with the targeting vector, and ES cells were grown on fibroblast feeder cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, gentamicin (50 μg/ml), LIF (1,000 U/ml), β-mercaptoethanol (90μM) and G418 (0.2 mg/ml). Two hundred and sixty neomycin-resistant colonies were picked and analyzed by Southern blot analysis. Recombination reduces the size of an XbaI fragment from 14 kb (wild-type allele) to 7 kb

(mutant allele) as an additional XbaI site is introduced upstream of the second loxP site (Supplemental Figure 1A, C). Three positive clones containing the floxed CA-SPAK allele were identified and injected into C57BL6 blastocysts, thereby generating 4 chimeric males of high chimeric percentage. Two of these males gave germline transmission of the floxed CA-SPAK allele. Male mice carrying the floxed CA-SPAK allele were backcrossed with C57BL6/J females

Creating DCT1 specific and Global CA-SPAK Mice

To drive early DCT (DCT1)-specific expression within the kidney, male mice homozygous for floxed CA-SPAK were bred with female mice that express Cre recombinase under the control of the parvalbumin promoter (The Jackson Laboratory, B6.129P2-Pvalb$^{tm1(Cre)Arbr/J}$). Within the kidney, parvalbumin is specifically expressed within the DCT1[24, 25]. The progeny of these crosses (PV-CRE + CA-SPAK, one copy each) were backcrossed for 10 generations with wild-type C57BL/6J mice, then interbred to produce mice homozygous for the floxed CA-SPAK alleles and Cre recombinase. During the course of our studies, we found animals heterozygous for Cre recombinase expressed CA-SPAK as efficiently as animals having two copies of Cre recombinase. Thus to avoid any off-target effects of Cre protein expression, all studies were performed on animals having a single copy of Cre recombinase. With Cre expression, transcription of the CA-SPAK targeting cassette occurs using the native SPAK gene promoter. However, in the absence of Cre, the CA-SPAK cassette prevents expression of the native gene with these animals being effectively SPAK-null (See Supplemental Figure 1). Neither genotype exhibited any obvious physical or behavioral abnormalities and both were produced in the expected Mendelian ratio and normal litter size (6-8 pups per litter).

To facilitate the molecular and physiological comparison of CA-SPAK to WT animals, several mice homozygous for floxed CA-SPAK and homozygous for Cre recombinase were crossed with WT C57Bl/6J (purchased from Jackson Laboratory) mice. The resulting progeny were further crossed to yield animals homozygous for either floxed CA-SPAK or WT SPAK, and heterozygous for Cre

recombinase. Neither genotype exhibited any obvious physical or behavioral abnormalities and both were produced in the expected Mendelian ratio and normal litter size (6-8 pups per litter).

For global CA-SPAK expression, male mice homozygous for CA-SPAK alleles were bred with female mice that express Cre recombinase under the control of the E2a promoter (The Jackson Laboratory, B6.FVB-Tg(Ella-cre)C5379Lmgd/J). Interestingly, these crosses never produced offspring that were homozygous for CA-SPAK, suggesting whole body expression of CA-SPAK is lethal (See Supplemental Table 1).

Genotyping

Genomic DNA was harvested from tail snips collected at weaning using Extract-N-Amp Tissue PCR Kit (Sigma). Quantitative PCR was then used to genotype CA-SPAK offspring by tracking the Cre recombinase gene (Fwd primer - ACCTGAAGATGTTCGCGATTATCT; Rev primer - ACCGTCAGTACGTGAGATATCTT), the 2LoxP allele of SPAK (Fwd primer - TACACTTCATTCTCAGTATTGTTTTGCC; Rev primer - TGATGATATCCAACATGGAACCTCC), and the WT allele of SPAK (Fwd primer - GTACGAGCTCCAGGAGGTTATCG; Rev primer - TTACTGGGTTCCAGCTCCGCC). At the end of each experimental protocol genomic DNA was collected from a piece of kidney to insure Cre recombinase had properly excised the one loxP site and enabling expression of the CA-SPAK coding cassette (1loxP allele; Fwd – AAAAGGCCCACAGCAGCAGAAC; Rev – ACCGAGATCTCTGAGTTCTCTTC). A Roche LightCycler 480 qPCR system and LightCycler 480 SYBER Green I Master reagents were used to quantify copy number. All reactions were performed in triplicate for each animal. Relative transcript abundance was calculated using the Pfaffl equation,[38] a derivation of the ΔΔCt method. Unlike the latter, the Pfaffl equation accounts for actual efficiency of doubling within the linear range of amplification, a value that can vary depending on template concentration. The values represented in the figures are normalized Pfaffl values relative to WT. Roche Cyber Green Master Mix was used to conduct PCR reaction.

Animals found to be homozygous for the 2loxP alleles and heterozygous for Cre were considered CA-SPAK, while animals found to be homozygous for the 2loxP alleles but lacking Cre were considered SPAK KO. Animals lacking the 2loxP alleles but found to be heterozygous for Cre were considered WT.

Dietary Manipulation

At approximately 6-7 weeks of age, all mice being studies were switched from the house diet to our control diet containing 1% potassium, 0.32% sodium, 0.9% chloride (TD.88238 from Harlan Teklad). The control diet is matched in composition to the various experimental diets except for salt content. The animals were acclimated to the control diet for at least 10 days prior to the beginning of the study. At 8–10 weeks of age the animals were assigned to a dietary regimen that consisted of either: 1) the control diet, 2) high-salt diet (1% potassium, 1.6% sodium, 2.9% chlorine; TD.10432), or 3) high-potassium diet (5.2% potassium, 0.3% sodium, 0.9% chlorine; TD.10432). All diets were purchased from Harlan Teklad and designed with assistance of a Teklad certified dietician.

Metabolic Cage Studies

Renal function was evaluated in metabolic cage clearance studies. After acclimation (3 days) in metabolic cages (Nalgene (Thermo Scientific), # NALGE650-0322), food and water consumption was assessed and urine samples were collected. Urine samples were collected several times a day to prevent contamination from food, water, and fecal matter. Urine samples were collected in tubes containing mineral oil to prevent evaporation. Kidney electrolyte handling was assessed in 24 hour measurements of Fractional Excretion (FE), which are calculated as the rate of urinary excretion of a solute (UxV, where Ux is the urinary concentration of substance, x, and V is the urinary flow rate) relative to the filtered load (FEx = UxV/eGFR*Px, where eGFR is the estimated glomerular filtration rate calculate by creatinine clearance (see below), and Px is the plasma concentration). Trans-Tubular

Potassium Gradient (TTKG), an index of distal potassium secretion, was calculated as follows; TTKG = (UK/PK)/(U_{Osm}/P_{Osm}), where UK and PK are the urinary and plasma [K^+] and U_{Osm} and P_{Osm} are the osmolarity of urine and plasma respectively.

Blood Pressure Measurements

Blood pressure measurements were made in conscious mice using a DSI (Data Sciences International) telemetry based system. The catheters from PA-C10 telemeters were surgically inserted into the internal carotid artery following the manufacturer's instruction. Following the surgery, the animals were allowed to recover for 4 days before beginning measurements. Dataquest ART 4.2 software was used to create a sampling program that measured the blood pressure of each animal for 20 s every 10 min.

Sample Collection, Preparation, and Analysis

Animals were anesthetized by intraperitoneal injection with ketamine/xylazine (100 mg/kg of ketamine, 10 mg/kg of xylazine). Once an animal was unconscious, a kidney was removed; cortex and medulla were separated by free-hand dissection and flash frozen in liquid nitrogen. Blood samples were collected from the carotid artery. Blood chemistry and gases (Na^+, K^+, Cl^-, HCO_3^-, pH, hematocrit, and BUN) were measured using from a 100ul aliquot of whole blood using an i-STAT EC8+ cartridge and an i-STAT1 Handheld Analyzer (Axaxis). The remaining fraction of blood was immediately spun-down to separate formed elements and plasma, the latter was subsequently isolated and frozen for later analysis of renin activity, aldosterone, and creatinine levels. Urine sodium, potassium and chloride analysis was performed in our lab using an Easylyte Analyzer (Medica Corporation). Plasma and urine creatinine levels were measured using the QuantiChrom Creatinine Assay Kit (BioAssay Systems) following the manufacturer's protocol. Plasma aldosterone levels were assayed using the Aldosterone EIA Kit manufactured by Cayman Chemical. Plasma renin activity was determined using a Renin Assay

Kit from Sigma-Aldrich (MAK157) following the manufacture's protocol.

Sample Preparation for Western Blotting

Mouse kidney tissue (cortex or medulla) was sonicated in HEENG buffer (20mM Hepes (pH 7.6), 125mM NaCl, 1mM EDTA, 1mM EGTA, 10% glycerol) containing 1% Triton and 0.5% SDS with protein and phosphatase inhibitor. Samples were taken from the freezer and immediately placed into HEENG buffer and then cut into small pieces using small scissors. The tissue was sonicated 2 times on ice at 8-second pulses (20 seconds between pulses) and allowed to sit at room temperature for 15 minutes before being rotated at 4°C for 1 hour followed by high-speed centrifugation (15,000 rpm) for 10 min to pellet insoluble material. The supernatant was collected and quantified for protein yield using a bicinchoninic acid protein assay reagent kit (Pierce). After incubating in Laemmli buffer supplemented with 2-mercaptoethanol (room temperature for 30 min), 20 μg of kidney protein per sample/well was resolved on precast TGX SDS-PAGE gels (4-20% gradient) purchased from BioRad, and transferred using BioRad TurboBlot system. The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk for 1 h at room temperature. Membranes were then incubated in 5% nonfat dry milk containing primary antibody (4°C, overnight), washed in TBS-T 10 minutes (3 times), incubated in 5% nonfat dry milk containing HRP-conjugated secondary antibody, and then washed for 10 minutes (3 times) in TBS-T. Bound antibodies were then revealed using enhanced chemiluminescence reagent (Pierce) and fluorography.

Protein quantification was performed by scanning autofluorograms and measuring the integrated density of protein bands using ImageJ software. Bands were measured in the linear range of the fluorographic signal. Duplicate gels were processed and developed in parallel. After detection of the target, membranes were stripped and reprobed for tubulin as a loading control. Unless otherwise stated, each protein signal was divided by its own tubulin signal to yield a tubulin-normalized signal. These data are presented and analyzed as the relative abundance, denoting the tubulin-normalized test

signal relative to the average of the wild-type normalized signal.

Immunolocalization

Anesthetized mice were fixed by perfusion with 2% paraformaldehyde in PBS via the left ventricle for 5 min at room temperature. The kidneys were then removed and fixed for additional 24 h at 4 °C, rinsed in PBS, and embedded in paraffin. Cross-sections 3-µm thick, cut at the level of the papilla, were picked up on chrome-alum gelatin-coated glass coverslips and dried on a warming plate. The sections were then deparaffinized in two xylene baths and two absolute ethanol baths, 5 min each, and rehydrated in a graded ethanol series to distilled water.

For epitope retrieval, the coverslips were placed in a pH 8 aqueous solution containing Tris (1 mM), EDTA (0.5 mM), and SDS (0.02%). The retrieval solution was heated to boiling in a microwave oven, transferred to a conventional boiling water bath for 15 min, and then allowed to cool to room temperature before the sections were thoroughly washed in distilled water to remove the SDS.

Sections were preincubated for 30 min with Image-iT blocking solution (Invitrogen), rinsed in PBS, and then preincubated an additional 30 min in a solution of 2% BSA, 0.2% fish gelatin, 5% normal donkey serum, and 0.2% sodium azide in PBS. Tissues were thoroughly rinsed with Tris-buffered saline (TBS) to remove PBS. Incubations with specific antibodies (as described above), diluted in TBS containing 1% BSA, 0.2% fish gelatin, 0.1% Tween 20, 10 mM $CaCl_2$, and 0.2% sodium azide, took place overnight in a humid chamber at 4 °C. After thorough washing in high salt wash (incubation medium plus added sodium chloride at 0.5 M), various Alexa Fluor 405-, 488-, 568-, and 649-conjugated donkey anti-mouse, anti-rabbit, anti-chicken, and anti-guinea pig IgG antibodies (Jackson Laboratories) were used to visualize specific target protein.

Quantitative analysis of confocal images (apical fluorescence intensity and co-localization) was performed using Improvision Volocity 5 by a trained investigator who was blind as to identity of the sample groups.

Quantification of ROMK intracellular localization in kidney sections.

ROMK intracellular localization was determined by measuring the pixel intensity from the tubule lumen toward the intracellular space at 0.4 μm–increments using Volocity 5 3D Image Analysis Software (PerkinElmer). A plot profile line was drawn exactly perpendicular to the cell apical membrane at the point to be measured, and the density profile was plotted. The peak intensity value was taken along with the pixel intensity three pixels from the peak in the direction of the cytoplasm. This later value provided a measure of background label and ROMK label not associated with the apical membrane and was subtracted from the peak intensity and taken as the apical membrane signal. A total of fifty cells from random selection of 5 WT and 5 *CA-SPAK KI* mice ($n \geq 250$ cells per group) were measured and compared.

Morphometric Analysis of DCT and ASDN in Kidney Sections

Nephron segments (TAL, DCT1, DCT2, CNT1, CNT, and CCD) were identified in coronal kidney sections by confocal microscopy (Zeiss LSM 510, ×10 objective lens) and segment specific antibody labeling (NKCC2, TAL; NCC, total DCT; parvalbumin, DCT1; calbindin + NCC, DCT2; calbindin along CNT1; calbindin + AQP2, CNT; AQP2 alone, CCD). Images were tiled, and the entire cortical area was analyzed for each section. Nephron segment length were determined with a curvilinear stereometric system developed by Merz,[53] and used previously by our group.[38, 39] For these measurements, a curve linear test grid composed of evenly spaced curved lines was superimposed over the images of the cortex. Tubule length, L, was determined by $L = I \times D$, where *L* is the linear boundary length being measured, *I* is the number of intersections between the curvilinear test grid and the boundary, and *D* is the diameter of the grid semicircles (21 μm). The boundary in this case was one outer edge of the tubule being measured, but limited by the axial extent of the apical membrane. The length of each identifiable DCT1, DCT2, CNT1, CNT, and CCD was measured and normalized to the area of the

cortex in the image ($\mu m/mm^2$). The curve linear grid also contained a small dot at the midpoint of each semicircle. The dots were used in point counting to measure the total tubular area of each nephron segment. The fractional-area of each tubule type was calculated by dividing the number of dots falling on each tubule type by the total number of dots falling on the cortex. The dots on the grid were 21 µm apart, and thus each dot represented 421 μm^2 of area. Using this conversion, the fractional-area could be expressed as $\mu m^2/mm^2$. Sections from 6 animals of each genotype were evaluated in this manner, and the figures represent the average of each genotype ($n = 6$).

Image Processing

As described in the morphometric analysis section above, four different markers were used to identify the DCT and ASDN segments. Quantitative analysis revealed changes in DCT1 and CNT abundance but these are not entirely obvious by eye when all markers are included in the image. For this reason, Figure 6 only shows DCT1 and CNT segments in a two-color image, against a bright field image. For imaging processing, imageJ64 software (NIH) was used. To identify and visualize DCT1, overlapping signals from the Parvalbumin and NCC channels were selected, and merged to single color (green). To identify and visualize the CNT overlapping signals from AQP2 and Calbindin signals were selected and merged to another color (red). Selected DCT1, CNT, and bright field images were then merged.

Statistical Analysis

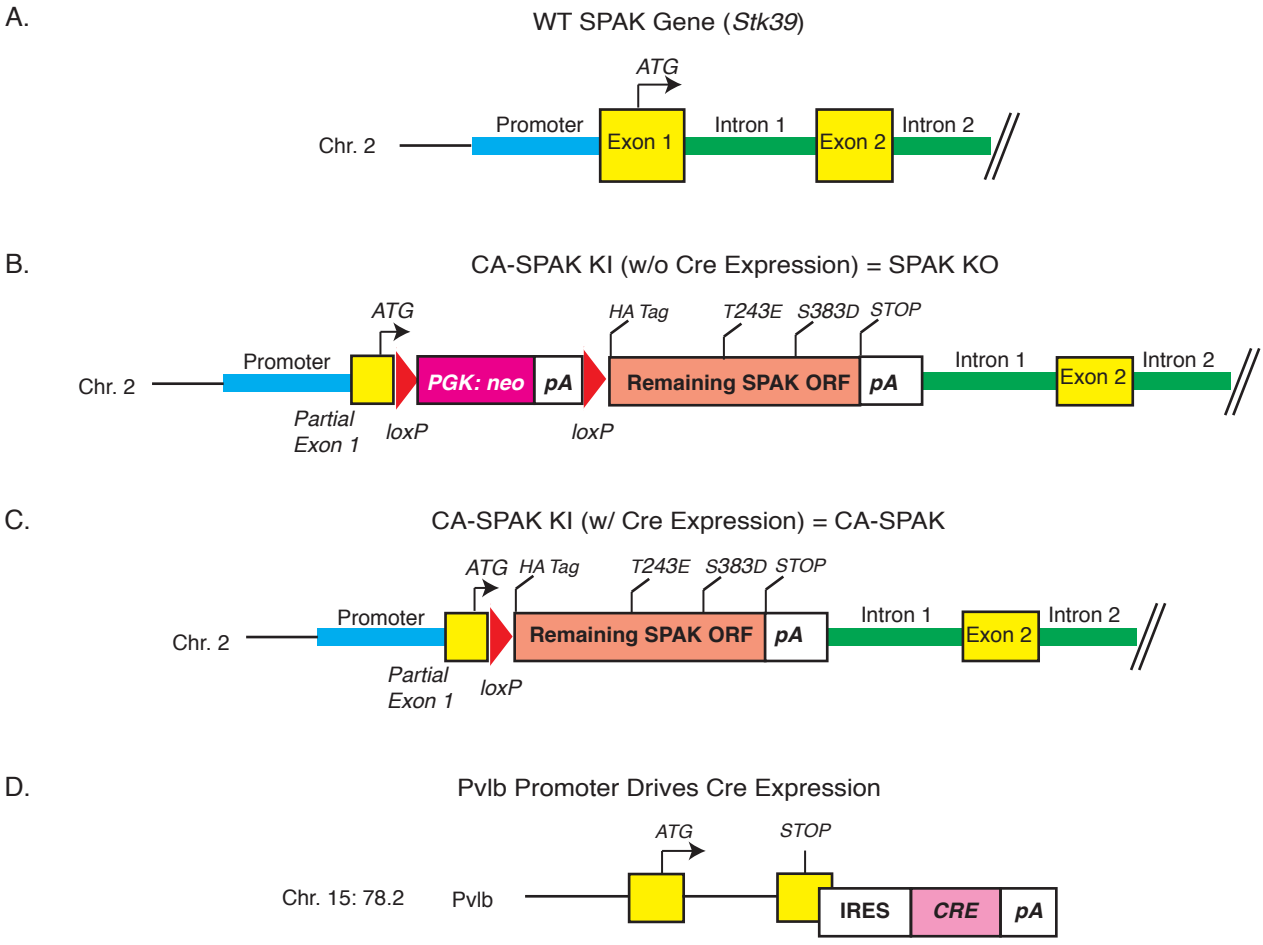
Data are presented as means ± SEM. Statistical analysis was performed using GraphPad PRISM version 6. Statistical significance was determined using One-way ANOVA when comparing a single dependent variable in all three genotypes. Two-way ANOVA was used in HCTZ and dietary manipulation studies to determine if there is an interaction between two variables (e.g., diet and genotype or drug and genotype) and the dependent variable (e.g., blood pressure, plasma potassium concentration, Fractional excretion). For both One-way and Two-way ANOVA analyses, a multiple

comparisons analysis was performed post hoc using Tukey's Multiple Comparison test. $P < 0.05$ was considered significant.

Supplemental Table 1. Genotyping Results for Global CA-SPAK (G-CA-SPAK) Expression							
Litters	Total Number of Pups Delivered						Pups/Litter
9	59						6.6
	Male			Female			
	G-CA-SPAK-/-	G-CA-SPAK+/-	G-CA-SPAK+/+	G-CA-SPAK-/-	G-CA-SPAK+/-	G-CA-SPAK+/+	
	10	22	0	8	19	0	

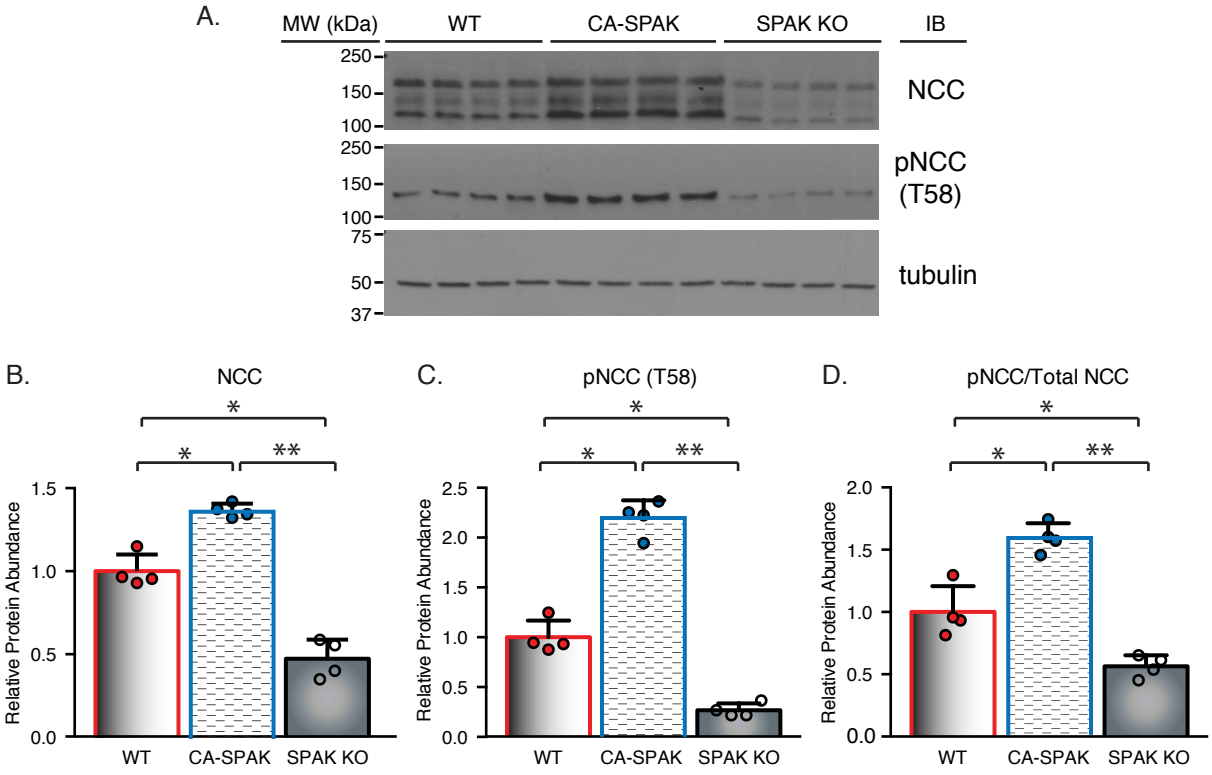
Supplemental Table 2. List of Antibodies Used During Study				
Antibody (species-antigen)	Use	Dilution	Source or Company w/ product ID	Reference (PMID)
ms = mouse sh = sheep rb = rabbit ch = chicken gp = guinea-pig	WB = western blot IF = immunolocalization			
ch – Aquaporin2	IF, WB	1:100, 1:1000	James Wade, U. of Maryland S.O.M.	25893600
rb – ROMK	IF, WB	1:80, 1:3000	James Wade, U. of Maryland S.O.M.	21454252
gp – NCC	IF	1:200	James Wade, U. of Maryland S.O.M.	22977235
rb – NCC	WB	1:500	James Wade, U. of Maryland S.O.M.	22977235
ch – pNCC (T58)	IF, WB	1:50, 1:100	James Wade, U. of Maryland S.O.M.	22977235
ch – NKCC2	IF	1:100	James Wade, U. of Maryland S.O.M.	12145305
rb – NKCC2	WB	1:6000	Mark Knepper - NIH	11053048
rb – pNKCC2 (R5)	WB	1:1000	Biff Forbush – Yale University	12145305
rb – SPAK	IF, WB	1:250, 1:700	Eric Delpire – Vanderbilt S.O.M	12386165
rb - parvalbumin	IF	1:500	Swant - #PV27	22977235
ms – calbindin D-28kDa	IF	1:600	Sigma – C8666	25893600
rb - tubulin	WB	1:3000	Cell Signaling - #2144	25893600
rb - HA	WB	1:1000	Cell Signaling - #3724	
rb- WNK1	WB	1:1000	Arohan Subramanya – U. of Pittsburgh	26241057
Sh - pWNK1 (S382)	WB	1:500	MRC-PPU Reagents – Dundee Scotland	25565204
rb - WNK4	WB	1:1000	Novus Biologicals – NB600-284	19401467
ms - ENaC (alpha)	WB	1:1000	Stress Marq Biosciences – 14E10	20966128
rb – EnaC (beta)	WB	1:500	Larry Palmer – Cornell University	16554417
rb - ENaC (gamma)	WB	1:1000	Larry Palmer – Cornell University	16554417
rb - BK-alpha	WB	1:1000	Alamone – APC-021	26537348
rb - BK-B1	WB	1:750	Alamone – APC-036	19458125
rb - BK-B4	WB	1:500	Alamone – APC-061	20299355

Supplement Figure 1



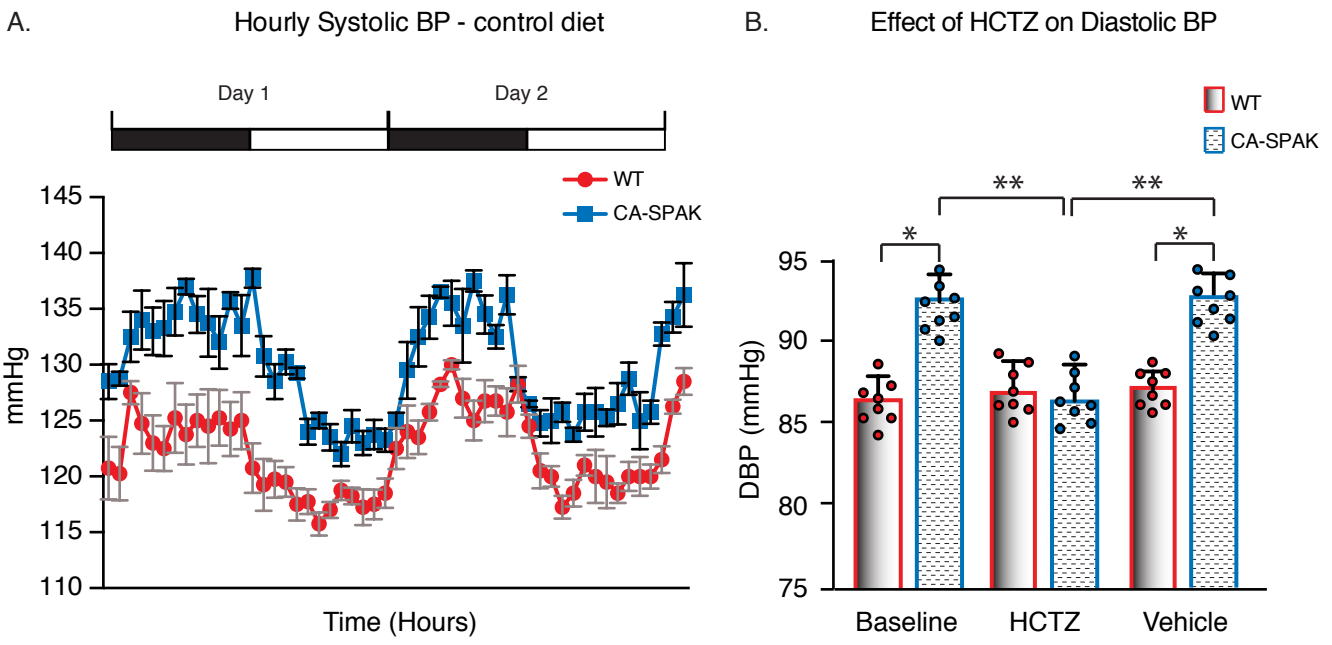
Supplemental Figure 1. **Targeted CA-SPAK Knockin Strategy. (A)** WT SPAK gene, showing promoter and first two exons. **(B)** Targeting knockin cassette, containing floxed Neo, followed by the SPAK reading frame with phospho-mimic mutations (T243E & S383D), an HA-tag and an in-frame stop codon, was inserted after the start codon of SPAK. In the absence of Cre-recombinase, the cassette renders the native gene inactive and thus the animals are functionally SPAK-null. **(C)** In the presence of Cre-recombinase, the Neo selection cassette is excised, allowing CA-SPAK transcription under the control of the native SPAK gene promoter. **(D)** DCT1 specific expression of CA-SPAK was driven by Cre-recombinase under the control of the parvalbumin promoter for these studies. In the kidney, parvalbumin is exclusively expressed in the early DCT. The individual elements of the above genes are not draw to scale.

Supplemental Figure 2



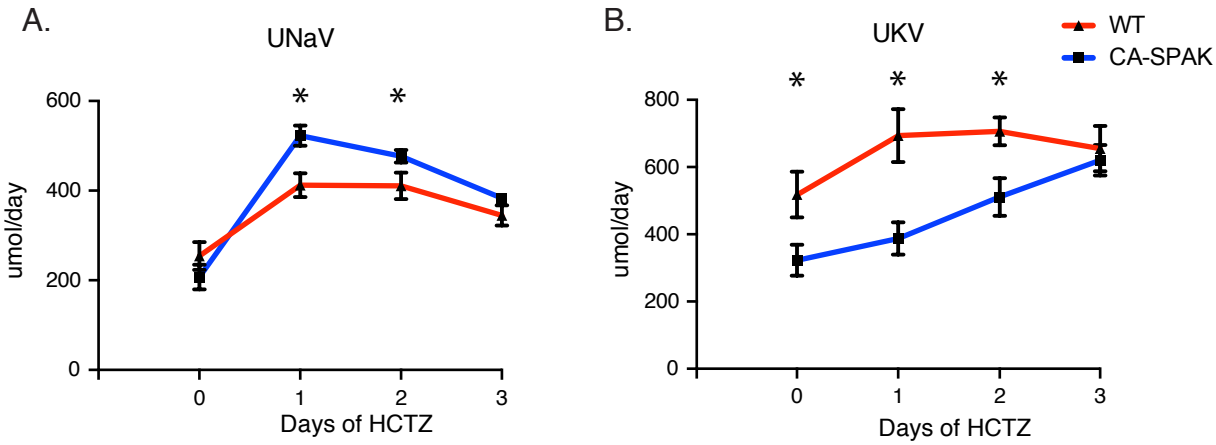
Supplemental Figure 2. NCC and pNCC (T58) abundance in WT, CA-SPAK and SPAK KO littermates. **(A)** Representative western blot and quantification of **(B)** total NCC, **(C)** pNCC and **(D)** the ratio of pNCC to total NCC in WT, CA-SPAK, and SPAK KO mice. Dots and wickers are mean + SEM (n=4, *P< 0.05 vs WT, **P< 0.05 CA-SPAK vs SPAK KO).

Supplemental Figure 3



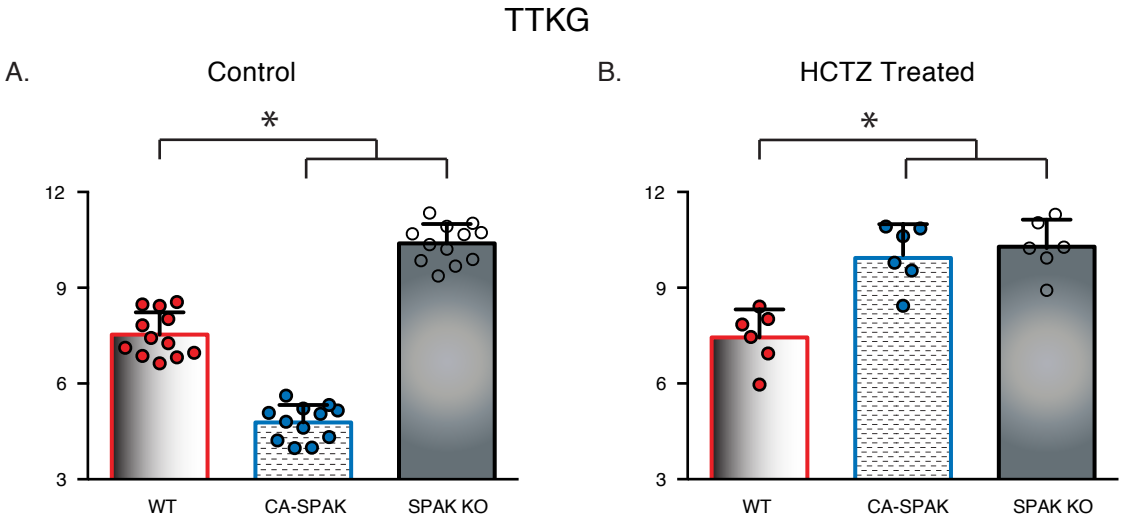
Supplemental Figure 3. Characterization of WT and CA-SPAK blood pressure. (A) Hourly telemetric measurements of blood pressure (dark bar, awake period at night; open bar, day). CA-SPAK animals retain the typical diurnal variation in systolic blood pressure, but at all time points CA-SPAK mice are hypertensive relative to WT mice. Each data point and wicker is the average hourly SBP + SEM (n=8). **(B)** Diastolic blood pressure is also elevated in CA-SPAK and is normalized with HCTZ treatment. (n=8, *P< 0.05 WT vs CA-SPAK for the same treatment, **P< 0.05 different treatments within the same genotype).

Supplemental Figure 4



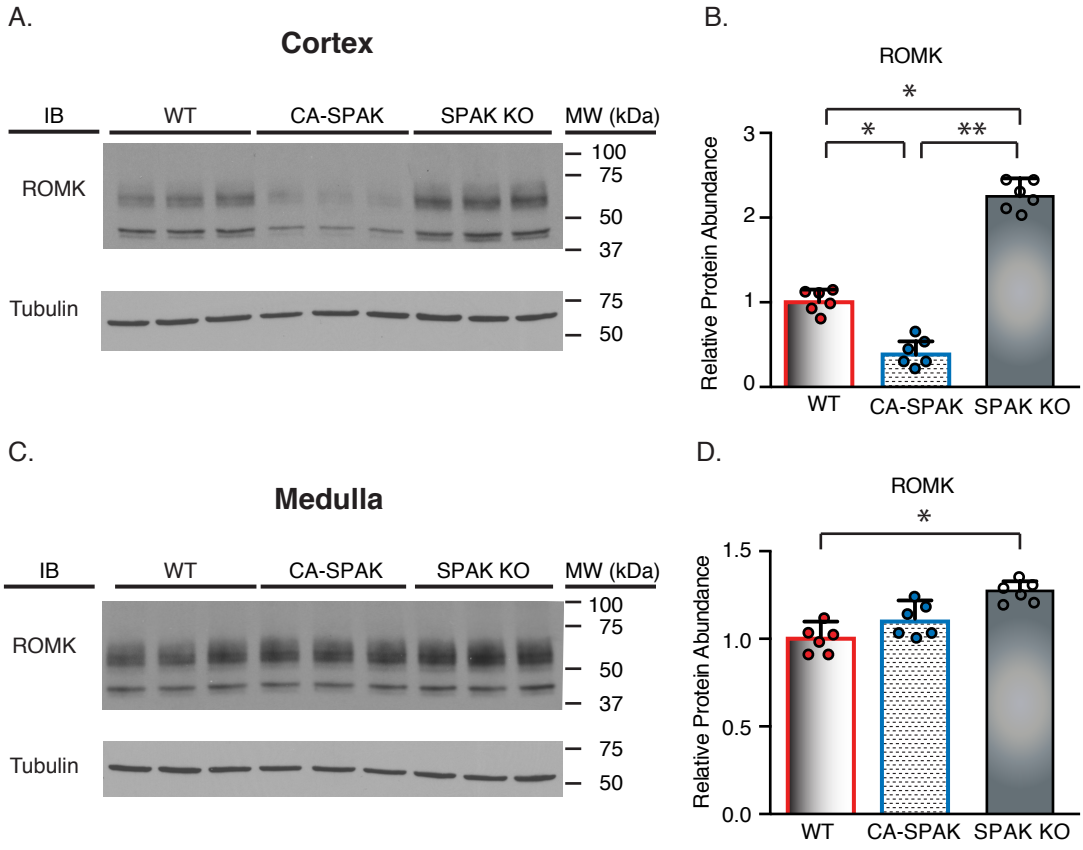
Supplemental Figure 4. **Delayed Restoration of Urinary Potassium Excretion in CA-SPAK mice.** The temporal response of urianry sodium **(A)** and potassium excretion **(B)** in WT and CA-SPAK mice follwing HCTZ treatement. Dots and wickers are mean ± SEM (n=6, *P < 0.05 WT vs CA-SPAK undergoig same treatment) .

Supplemental Figure 5



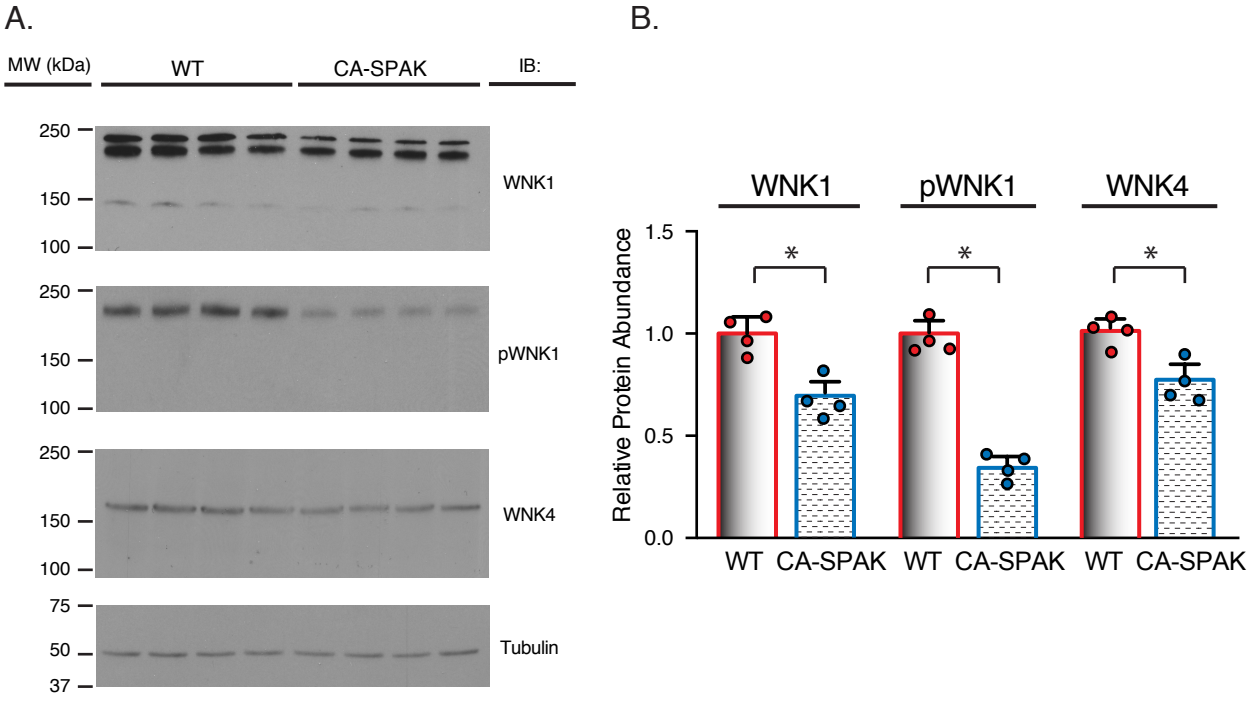
Supplemental Figure 5. Distal Nephron Potassium Secretion as Assessed by Trans-Tubular Potassium Gradient (TTKG). (A) Relative to WT, CA-SPAK mice display a decreased TTKG indicative of impaired potassium secretion from the ASDN (n=12 per genotype). **(B)** Chronic HCTZ treatment (3 days) restores TTKG of CA-SPAK to elevated level of SPAK KO mice. (n=6, *P< 0.05). WT (red), CA-SPAK (blue), and SPAK KO (gray) bars and wickers are mean + SEM, circles are individual data points from each mouse.

Supplemental Figure 6



Supplemental Figure 6. ROMK Abundance is Reduced in Kidney Cortex of CA-SPAK. (A) Western blots and **(B)** quantification of ROMK in CA-SPAK renal cortex, compared to WT and SPAK-KO littermates, and **(C & D)** Loop of Henle-enriched renal medulla (n=6 for each genotype, *P < 0.05 vs WT, **P < 0.05 CA-SPAK vs SPAK KO). WT (red), CA-SPAK (blue), and SPAK KO (gray) bars and wickers are mean + SEM, circles are individual data points from each mouse.

Supplemental Figure 7



Supplemental Figure 7. WNK expression is decrease in CA-SPAK mice. (A) Western blots and **(B)** quantification of WNK1. pWNK1, and WNK4 in CA-SPAK renal cortex, compared to WT littermates, (n=4 for each genotype, *P < 0.05 vs WT, bars and wickers are mean + SEM, circles are individual data points from each mouse.