**Supplementary Methods**

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“Vasopressin-Dependent b-Catenin Phosphorylation at Ser552 and Branching Structure of Mouse Collecting Duct System”

**Detailed Methods**

**Mice.** All animal experimental procedures were carried out in accordance with National Heart, Lung, and Blood Institute [NHLBI] animal protoco**l H-0047R6**, approved by the NHLBI Animal Care and Use Committee. Pathogen-free, male, 6-to 8-week-old C57BL/6 mice (Taconic) were used. In order to generate the Ctnnb1 T551A/S552A knock-in mutant mice, CHOPCHOP (https://chopchop.cbu.uib.no) was employed to obtain all the possible sgRNAs, and one sgRNA (CAACGGCGCACCTCCATGGG) was selected and purchased. A single-strand donor oligonucleotide containing the desired mutation was synthesized (IDT, https://www.idtdna.com/pages). The sgRNA (5 ng/ul) and donor oligonucleotides (100 ng/ul) were co-microinjected with Cas9 mRNA (20 ng/ul) into the pronuclei of zygotes collected from C57BL/6 mice. Donor nucleotide sequences are given in Supplemental Figure 1 (<https://esbl.nhlbi.nih.gov/Databases/Catenin-RNA-seq/Data/>). Injected embryos were cultured in M16 medium overnight in a 37 oC incubator with 6% CO2. In the next morning, embryos at 2-cell stage of development were implanted into the oviducts of pseudo-pregnant surrogate mothers. Offspring born to foster mothers were genotyped by PCR and Sanger Sequencing.

**Urinary osmolality**. Spot urines were collected by holding the mice over a piece of parafilm. Osmolality was determined by vapor pressure osmometry (Wescor).

**Immunoblotting.** Immunoblotting was carried out in either kidney tissue or cultured mpkCCD cells using standard procedures in our laboratory.1, 2 Antibodies are identified in figure legends.

**Microdissection of CCDs and branched connecting tubule (CNT)/** **initial collecting tubule (iCT) structures.** Mice were euthanized via cervical dislocation. The kidneys were perfused via the left ventricle with ice-cold Dulbecco's PBS (DPBS; Thermo Scientific), followed by reperfusion with the dissection buffer (5 mM HEPES, 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2.5 mM disodium phosphate, 1.2 mM magnesium sulfate, 5.5 mM glucose, 5 mM Na acetate, pH 7.4) with 1.5 mg/ml collagenase B (Roche). The collagenase-perfused kidneys were harvested and cut into thin slices along the cortical-medullary axis with a sharp razor blade. The digestion was continued in vitro in the same collagenase-containing dissection buffer. The digestion was monitored until optimal for single tubule microdissection, typically ~30 minutes. The microdissection of CCDs or branched CNT/iCT structures were carried out under a Wild M8 dissection stereomicroscope equipped with on-stage cooling. The collecting ducts were washed by transferring them to dishes containing ice-cold DPBS and manually ‘swimming’ them into debris-free fluid using a pipet or sharpened tungsten needle.

**Immunocytochemistry of microdissected CCDs or branched CNT/iCT structures.** After the branched CNT/iCT structures or CCDs were isolated, tubules were transferred to a glass bottom culture dish (MatTek, #P35G-0-20-C) coated with Cell-Tak (Corning, # CLS354241-1EA). The tubules were then fixed for 20 minutes with 4% paraformaldehyde in PBS and permeabilized for 20 minutes with permeabilization buffer (PBS with 0.3% Triton X-100 and 0.1% BSA). After permeabilization, tubules were blocked for 30 minutes with blocking buffer (PBS with 1% BSA and 0.2% gelatin). Primary and secondary antibodies are indicated in the figure legends. The anti-Na+/K+ ATPase antibody was produced in our laboratory by immunizing rabbits or chickens with a synthetic peptide corresponding to the entire COOH-terminal tail of mouse ATP1A1 protein (sequence C-DEVRKLIIRRRPGGWVEKETYY) conjugated to keyhole limpet hemocyanin (KLH).3

**Morphometry of microdissected CCDs**. Determination of the numbers of cells per unit length in microdissected CCDs from the mice was carried out using DAPI to label nuclei followed by counting of the DAPI-labeled structures. Confocal fluorescence images were recorded with a Zeiss LSM980 confocal microscope using a 20X objective lens by Z-stack scanning. 3D images are reconstructed using Z-stack files, and cell counting was performed on three-dimensional reconstructed tubule images using IMARIS Scientific Image Processing & Analysis software (v10.2.0, Bitplane, Zurich, Switzerland). Counting was automated using IMARIS “spot analysis” for nuclei. DAPI fluorescence was quantified to assess relative DNA abundances in individual nuclei, allowing construction of histograms of DAPI fluorescence intensity to assess relative DNA amounts in each nucleus. The DAPI-labeled microdissected CCDs were also labeled with anti-AQP2 (rabbit, 1:500, Knepper Lab, #K5007) followed by a secondary antibody (Alexa Fluor 568 goat anti-rabbit). IMARIS was used to quantify the diameter of the microdissected tubules.

**Glomerular counts in wild-type (WT) and Ser552Ala mice**. Whole kidney glomerular counts were done using the acid maceration method of Damidian *et al*.4 as modified by Peterson *et al*.5 Briefly, a mouse kidney was chopped into small pieces and then reduced to a suspension consisting of glomeruli and small renal tubule fragments by incubation in 6M HCl at 37 °C for 90 minutes. Glomeruli (identified by their round shape) were counted in triplicate in three aliquots of the total suspension under a Wild M8 dissection stereomicroscope and total glomerular number was calculated by multiplying by the ratio of the total volume to the aliquot-volume.

**Preparation of nuclear fractions.** Manually dissected inner medullas were transferred to a BeadBug™ tube prefilled with 3 mm Zicronium beads (Millipore Sigma, # Z763802-50EA) and homogenized (BeadBug™ 6, Benchmark Scientific, 3000rpm, 30 sec, 3 cycles) with 150-180 µL of lysis buffer (250 mM sucrose, 10 nM triethanolamine, pH = 7.6). Nuclear fractions were extracted by NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, # 78833), following the manufacturer’s instructions. Immunoblot analysis was used to assess the adequacy of nuclear purification by measuring Lamin A/C (rabbit, 1:1000, Cell signaling # 2032, a nuclear protein).

**Immunocytochemistry of kidney sections.** Mice underwent cervical dislocation and were immediately perfused via the left ventricle of the heart with ice-cold DPBS followed by 4% paraformaldehyde in DPBS. The kidneys were post-fixed in 4% paraformaldehyde solution, further processed with alcohol and xylene, and then embedded in paraffin blocks. 5 μm sections were mounted on glass slides for immunofluorescence staining. For dewaxing, the tissue sections were immersed in xylene, then gradually rehydrated by using decreasing concentrations of ethanol (100%, 100%, 95%, and 70%). After performing antigen retrieval using the antigen retrieval buffer (Elabscience, #E-IR-R220A), the tissue sections were permeabilized for 20 minutes with permeabilization buffer (PBS with 0.3% Triton X-100 and 0.1% BSA). Tissue sections were then blocked for 30 minutes with blocking buffer (PBS with 1% BSA and 0.2% gelatin). Primary antibodies were applied overnight at 4°C. The secondary antibody incubation was carried out for 1 hour at room temperature. Immunofluorescent labeling was analyzed using a Zeiss LSM980 confocal microscope using ZENBlue software (Zeiss). The primary and secondary antibodies are listed in the legends.

**Determination of** **nephron:CCD ratio in mice.** In mice and other mammals, multiple nephrons converge to form a single CCD in the cortical labyrinth via branching of CNTs and iCTs.6 The ratio of medullary thick ascending limb (MTAL) to OMCD provides a measure of cortical branching ratio (nephrons:CCD) because all structures between the MTAL and OMCD other than the CNT and iCT are unbranched (MTAL, cortical thick ascending limb, CCD in the medullary rays and OMCD).7

To measure the MTAL/OMCD ratio, kidneys from WT and Ser552Ala mice underwent perfusion fixation and embedding as described in the previous paragraph. Tissue sectioning was performed to obtain cross-sections of the inner stripe of the outer medulla perpendicular to the corticomedullary axis. The sections underwent immunofluorescence labeling for the Na-K-2Cl cotransporter NKCC2 (a marker for the MTAL) and for AQP2 (a marker for OMCD). After immunofluorescence staining, MTALs and OMCDs were counted in demarcated square fields.

**Small-sample RNA-seq in microdissected CCDs.** For RNA-seq analyses, four to eight CCDs were collected for each sample for a total length of 1.5-3.0 mm. mRNA collection and purification were performed by using a Direct-Zol RNA MicroPrep kit (Zymo Research, Irvine, CA). cDNA was generated using the SMART-Seq V4 Ultra Low RNA Kit (Takara Bio, Mountain View, CA). After 14 cycles of amplification, 1 ng of cDNA was “tagmented” and bar coded using a Nextera XT DNA Sample Preparation Kit (Illumina, #FC-131-1024). The final libraries were purified using AmPure XP magnetic beads (Beckman Coulter, Indianapolis, IN) and quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Sample quantity and quality were assayed on an Agilent 2100 bioanalyzer. cDNA library concentrations were normalized, and samples were pooled and sequenced on an Illumina Novaseq 6000 platform using a 50 bp paired-end modality. FastQC was used to evaluate sequence quality (software version 0.11.9). Adapter contamination was not significant, so read trimming was not performed. RNA-seq reads were indexed using STAR (2.7.6a) and aligned to the mouse reference genome from *Ensembl* (release 104) with the matching genome annotation file (release 104).8 Transcripts per million (TPM) and expected read counts were generated using RSEM (1.3.3).9 Unless otherwise specified, the computational analyses were performed on the NIH Biowulf High-Performance Computing platform.

**References**

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