Supplemental Informations

1- Supplemental methods

Generation of the Tg$^{(B1\text{-}hPDS)}$ transgenics: The human SLC26A4 cDNA was ligated into the pBluescript cloning vector containing 6.9kbp of the human ATP6V1B1 promoter[1, 2]. The SV40 late region polyadenylation signal was cloned downstream of the cDNA. The transgene includes the 5'-flanking region of the *ATP6V1B1* gene extending to but excluding the endogenous translational start codon, the human *SLC26A4* cDNA, with its own translational start site, and the SV40 late region polyadenylation signal, and is referred as B1-hPDS. Transgene integrity was confirmed by restriction digest and bidirectional sequencing of ligation sites. To prepare for injection, the transgene was linearized from the vector by SalI and NotI digestion, followed by gel purification using an electroelution method and then concentrated using ElutipD columns (Whatman). The transgene was then further concentrated by ethanol precipitation and resuspended in low EDTA injection buffer (10mM tris 0.1mM EDTA). Transgene integrity was confirmed by restriction digest and bidirectional sequencing of ligation sites. Transgenic mice were created by the University of Utah transgenic mouse core facility using standard procedures as described[1, 2]. Genotyping demonstrated that 63 pups were positive for transgene integration. One founder transmitted the transgene in a Mendelian fashion and was subsequently used for all the experiments described. The mice that were used in the present study were F6. Mendelian transmission of the transgene and southern blot analyses (Suppl. Fig S1) were compatible with a single site of integration. The transgenic founder was crossed with wild-type C57BL/6 x CBA F1 mice and the resulting hemizygous offspring were analyzed. The control mice consisted of sex-matched wild type littermates.

Genotyping: Mouse genomic DNA was prepared from tail tissue by standard methods. To detect the B1-hPDS transgene, PCR primers were designed to amplify across the hPDS cDNA and SV40 Polyadenylation signal sequence to provide a PCR product of 345 bp (Forward primer: 5'-

AGAGGGTCAAGGTTCCATTTTAG-3'; Reverse primer: 5'-CAAACCACAACTAGAATGCAGTG-3'). PCR consisted in denaturation 95°C for 7min followed by 40 cycles of amplification (30 sec at 95°C, 30 sec at 57°C, 30 sec at 72°C) and 5 min at 72°C.

RNA extraction and reverse transcription: Animals were killed and kidneys were harvested and rapidly frozen in liquid nitrogen. Snap-frozen kidneys (six kidneys for each condition) were homogenized in RLT-Buffer (Qiagen, Basel, Switzerland) supplemented with ß-mercaptoethanol to a final concentration of 1%. Total RNA was extracted from 200 µl aliquots of each homogenized sample using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Quality and concentration of the isolated RNA preparations were analyzed by the ND-1000 spectrophotometer (NanoDrop Technologies). Total RNA samples were stored at –80°C. Each RNA sample was diluted to 100 ng/µl and 3 µl used as a template for reverse transcription using the Bioline cDNA synthesis kit (BioLine, Randolph, MA). For reverse transcription, 1 µg of RNA template were diluted in a 10-µl reaction mix that contained (final concentrations) RT buffer (1X), oligo $(dT)_{18}$ (2.5 µM), RNase inhibitor (0.5 U/µl), the Bioline reverse transcriptase enzyme (2.5 U/µl), dNTP mix (0.5 µM each), and RNase-free water.

Real-time quantitative PCR: Primers were designed using Primer Express software from Applied Biosystems for GAPDH (Forward 5'-GCACAGTCAAGGCCGAGAAT-3'; Reverse 5'-GCCTTCTCCATGGTGGTGAA-3'), human pendrin (Forward 5'-AAATCTCAAGAGGGTCAAGGTTC-3'; Reverse 5'-ACATCAAGTTCTTCTTCCGTCAG-3'), mouse Atp6v1b1 (Forward 5'-ATCAATGTGCTCCCATCCCTCT-3'; Reverse 5'-AATGCGCTTCAGCATCTCTTTC-3'). Primers were chosen to produce amplicons ≤150 bp that spanned intron-exon boundaries as to exclude amplifying genomic DNA. The specificity of all primers was first tested on mRNA derived from kidney. Real-time PCR was performed using a 5 ng of cDNA quantity on a LightCycler (Roche Diagnostics, Meylan, France) with LightCycler 480 SYBR Green I Master qPCR kit (Roche Diagnostics, Meylan,

France). Briefly, 2µl (2.5 ng/µl) cDNA, 0.5 µl of each primer (25 µM), 2 µl RNAse free water, 5 µl SYBR Green Master mix (Roche Applied Biosystems, Meylan, France); for a final volume of 10 µl. Reaction conditions were as follows: 95oC for 10 minutes followed by 45 cycles of 95oC for 20 seconds, 62oC for 20 seconds, and 72°C for 20 seconds. All reactions were run in duplicate. A negative control was amplified in the absence of the reverse transcriptase. Cycle thresholds were recorded at within the linear range of fluorescence intensity, which was set at 0.06. Gene expression was normalized to that glyceraldehyde 3-phosphate dehydrogenease (GAPDH). Relative expression ratios were calculated as R=2(Ct(GAPDH)-Ct(test gene)), where Ct represents the cycle number at the threshold 0.06.

Southern Blot analyses of Genomic DNA: A 894 bp fragment of the hPDS coding sequence was excised by EcoRI and served as a probe template. The probe was radiolabelled with 32P-dCTP (Hartmann Analytik, Braunschweig, Germany) and purified with Illustra ProbeQuant G-50 columns (GE Healthcare, Waukesha, WI, USA). Phenol-Chloroform purified genomic DNA (5 µg) from wild-type and transgenic B1-hPDS mice was digested with either BamHI or EcoRI (Thermo, Waltham, MA, USA), respectively, and separated on 0.8% agarose gels. Digested genomic DNA was blotted on Hybond-XL membranes (GE Healthcare, Waukesha, WI, USA) and hybridized with radiolabelled probes following standard protocols.

Generation and characterization of a anti human pendrin antibody: The synthetic peptide (APGGRSEPPQLPEYS) corresponding to amino-acids 3 to 18 of the N-terminal end of human pendrin protein was synthesized by the DB-BioRun Laboratory (Nantes, France), coupled to keyhole limpet hemocyanin with the Imject Maleimide Activated Immunogen Conjugation Kit (Pierce), and used to immunize rabbits to generate polyclonal antiserum (DB-BioRun Laboratory, Nantes, France). Supplemental Figure 2 shows that analyses of mouse and human kidney sections labeled with this new antibody revealed exactly the same pattern of labeling in both species that was consistent with known pendrin expression. Pre-immune serum did not yield

any labeling, and all staining was abolished when the antiserum was pre-incubated with the immunizing peptide. Moreover, when the antiserum was used to stain kidney sections obtained from mice with pendrin disruption[3] no signal was detected which confirmed that this antiserum is specific for pendrin. (Supplemental Figure 1).

Biochemical and hormonal measurements: Urine creatinine (enzymatic method) was measured with a Konelab 20i auto-analyzer (Thermo Electron Corporation, Eragny Parc, France). Urinary chloride was measured with a DL 55 titrator (Mettler Toledo, Viroflay, France). Urinary Na^+ and K^+ were measured by flame photometry (IL943, Instruments Laboratory, Lexington, MA). Urine aldosterone was measured by RIA (DPC Dade Behring, La Défense, France). Blood was collected by tail incision on mice anesthetized by peritoneal injection of a mixture (0.1ml/g body weight) of ketamine (Imalgene®, Rhône Mérieux, Lyon, France; 10%) and xylazine (Rompun®, Bayer AG, Leverkusen, Germany; 5%) and [Na^+], [K^+], hematocrit and [Cl^-] were measured with an ABL 77 pH/blood-gas analyzer (Radiometer, Copenhagen, Denmark). Blood gases analyses were performed by retro-orbitary punction on awake animals and pH, PCO_2, and PO_2 were measured with an ABL 77 pH/blood-gas analyzer (Radiometer, Copenhagen, Denmark). Blood bicarbonate concentration was calculated by the autoanalyzer from the measured values using the Henderson-Hasselbach equation.

Measurements of blood pressure by radiotelemetry :

Animals were anaesthetized with pentobarbital (50 mg/kg) by intraperitoneal (IP) injection. Telemetric devices were implanted in 6 wild-type and 6 $Tg^{B1-hPDS}$ according to the standard protocols. Briefly, catheters were inserted into the right carotid artery and attached to a radiotransmitter (Physio Tel HD-X11; Data Sciences International) located on the back. Mice were allowed to recover for two to three days at which time they were placed in metabolic cages (Hatteras Instruments, Cary, NC) and permitted to acclimate to the cages and the gel diet for an additional five days. Blood pressure was continuously recorded using a telemetry receiver (Physio Tel DSI receiver; Data Sciences International).

***In vitro* microperfusion of mouse CCDs:** Kidneys were removed and cut into 1 to 2 mm coronal slices that were transferred into a chilled dissection medium containing (mM): 118 NaCl, 25 $NaHCO_3$, 2.0 K_2HPO_4, 1.2 $MgSO_4$, 2.0 calcium lactate, 1.0 sodium citrate, 5.5 glucose, and 12 creatinine, pH 7.4, and gassed with 95% O_2-5% CO_2. CCD segments were isolated from cortico-medullary rays under a dissecting microscope with a sharpened forceps. Because CCDs are highly heterogeneous, relatively short segments (0.45-0.6 mm) were dissected to maximize the reproducibility of the isolation procedure. *In vitro* microperfusion was performed as described by Burg et al. [4]. Briefly, isolated CCDs were rapidly transferred to a 1.2 ml temperature- and environmentally-controlled chamber, mounted on an inverted microscope, and perfused and bathed initially at room temperature with dissection solution. The specimen chamber was continuously suffused with 95% O_2-5% CO_2 to maintain pH at 7.4. Once secure, the inner perfusion pipette was advanced and the tubule was opened with a slight positive pressure. The opposite end of the tubule was then pulled into a holding collection pipette. In the holding collection pipette, 2 to 3 cm of water-saturated mineral oil contributed to maintain the tubule open at a low flow rate of perfusion. The perfusing and collecting end of the segment was sealed into a guard pipette using Dow-Corning 200 dielectric fluid (Dow Corning Corp., Midland, MI). The tubules were then warmed to 37°C and equilibrated for 20 minutes while the collection rate was adjusted to a rate of 1-4 nl/min. The length of each segment was measured using an eyepiece micrometer. Because CCDs from mice are frequently unstable and collapse, measurements were conducted during the first 90 minutes of perfusion. Usually, collections from 4 periods of 15 minutes were performed in which 25 to 30 nanoliters of fluid were collected. The volume of the collections was determined under water-saturated mineral oil with calibrated volumetric pipettes. 20 nanoliters were required for $[Na^+]$, $[K^+]$ and [creatinine] measurements. Transepithelial voltage (V_{te}) was measured continuously using Ag-AgCl electrodes connected to 0.15 M NaCl-agar bridges inserted in the perfusion pipette and bathing solutions. The initial value after rewarming of the tubule was noted. Values of each period were averaged.

Measurements of Na^+, K^+ and creatinine concentrations with high-pressure liquid chromatography*:* Cation concentrations were measured as previously described[5]. The Dionex-500 system (Dionex DX-500, Dionex Corp., Voisins le Bretonneux, France) consisted of an AS50 autosampler, a GP50 gradient pump, an ED40 electrochemical detector (Na^+ and K^+), and an AD20 UV absorbance detector 220 nm (creatinine). The signal-to-noise ratio of the conductivity measurement was enhanced by employing a cation self-regenerating suppressor (CSRS-ultra, 4mm) that was set in the autosuppression recycle mode. The HPLC column consisted of a Dionex IonPac CS12 column (4 x 250 mm), equipped with a guard column CG12A, (4 x 50 mm). The mobile phase consisted of 18mM methanesulfonic acid. Tubular fluid, perfusion solution, and standard solutions were drawn under mineral oil with a calibrated pipette (about 20 nl) and transferred to a vial containing 39 µl to the mobile phase of the HPLC with $LiNO_3$ as an internal standard. Peaks of each measured analyte (Na^+, K^+, Creatinine) were adjusted with the value of the Li^+ internal standard to limit the variations due to automatic injection. In each run of experiments, perfusion and bath solutions were tested in 4 or 5 replicates and the reproducibility of the measure was evaluated: Coefficient of variation (CV) < 0.10 for K^+ determination, and CV <0.05 for creatinine determination. In addition, a calibration curve for each analyte was tested with correlation coefficients >0.98.

Measurement of fluid absorption: Creatinine was used as the volume marker, and therefore was added to the perfusion solutions (both perfusate and bath) at a concentration of 12 mM. The rate of fluid absorption (*J*v) was calculated as *J*v = (Vperf – Vcoll)/L, with Vperf = Crcoll/Crperf x Vcoll.

Crcoll and Crperf are the concentrations of creatinine in the collected fluid and perfusate, respectively. Vcoll is the collection rate at the end of the tubule. L is the length of the tubule.

Calculation of the rate of absorption of Na and K: For each collection, Na^+ flux (JNa) and K^+ flux (*J*K) where calculated and reported to the length of the

tubule:

JNa =[([Na]perf x Vperf)– ([Na]coll x Vcoll)] /L

JK =[([K]perf x Vperf)– ([K]coll x Vcoll)] /L

Therefore, positive values indicate net absorption, whereas negative values indicate net secretion of the ion. For each tubule, the mean of the 4 collection periods was used.

Intracellular pH measurements on isolated microperfused tubules

During intracellular pH measurement experiments, the average tubule length exposed to bath fluid was limited to 300 – 350 µm in order to prevent motion of the tubule.

Two solutions were used, differing in their content in chloride. The composition of the solutions were as follows ; chloride-containing solution was composed of (in mM) 119 NMDG-Cl, 23 NMDG-HCO_3, 2 K_2HPO_4, 1.5 $CaCl_2$, 1.2 $MgSO_4$, 10 HEPES, and 5.5 D-glucose ; chloride-free solution was composed of (in mM) 119 NMDG-gluconate, 23 NMDG-HCO_3, 2 K_2HPO_4, 7.5 Ca-gluconate, 1.2 $MgSO_4$, 10 HEPES, and 5.5 D-glucose. All solutions was adjusted to pH 7.40 and continuously bubbled with 95% O_2/5% CO_2.

At the beginning of all experiments, tubules were bathed and perfused with the Cl-containing solution.

To identify principal and intercalated cells, we labeled the apical membrane of intercalated cells by adding fluorescent peanut lectin (PNA,Vector Labs) to the luminal perfusate for 5 minutes and observed which cells were fluorescent. Intracellular pH in CCD cells was assessed with imaging-based, dual excitation-wavelength fluorescence microscopy with use of the fluorescent probe 2',7'-Bis- *(2-Carboxyethyl)*-5- *(And-6)*- carboxyfluorescein (BCECF, Molecular probes). Tubules were loaded for ~20 min at room temperature with $5x10^{-6}$mol/L of the acetoxymethyl ester of BCECF added to the peritubular fluid. Loading was continued until the fluorescence intensity at 440 nm excitation wavelength was at least one order of magnitude higher than background fluorescence. The loading solution was then washed out by initiation of bath flow and the tubule was equilibrated with dye-free solution for 5-10 minutes. Bath solution was delivered at a rate of 20 ml/min and warmed

to 37±0.5°C by water jacket immediately upstream to the chamber. During the fluorescence recording, the Cl-containing solution was delivered to the perfusion pipette via a chamber under an inert gas (N_2 pressure around 1 bar) connected through a manual 6-way valve. With this system, opening of the valve instantaneously activated flow of one solution. The majority of the fluid delivery to the pipette exited the rear of the pipette system through a drain port at a rate of 4 ml/min. This method resulted in a smooth and complete exchange of the luminal solution in less than 3 to 4 s as measured by the time necessary for appearance of colored dye at the tip of the perfusion pipette. After 3-minutes recording, luminal fluid was instantaneously (at the rate of 4 ml/min in the draining) replaced by the corresponding Cl-free solution for 3 minutes. Finally, the luminal solution was changed again for the Cl-containing solution.

Intracellular dye was excited alternatively every 2 seconds at 440 and 500 nm with a light-emitting diode (Optoled, Cairn Research, Faversham, UK). Emitted light was collected through a dichroïc mirror, passed through a 530 nm filter and focused onto a EM-CCD camera (iXon, Andor Technology, Belfast, Ireland) connected to a computer. The measured light intensities were digitized with 14-bit precision (16384 grey level scale) for further analysis. For each tubule, 3-4 intercalated cells were analyzed and the mean grey level was measured with the Andor IQ software (Andor Technology, Belfast, Ireland). Background fluorescence was subtracted from fluorescence intensity to obtain intensity of intracellular fluorescence.

Intracellular dye was calibrated at the end of each experiment using the high $[K^+]$-nigericin technique. Tubules were perfused and bathed with a HEPES-buffered, 95-mM K^+ solution containing 10 µM of the K^+/H^+ exchanger nigericin. Four different calibration solutions, titrated to pH 6.9, 7.3, 7.5 or 7.8 were used.

References

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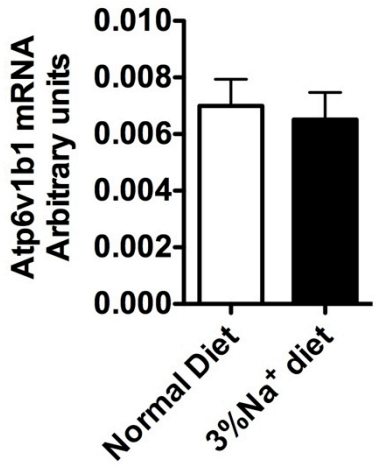
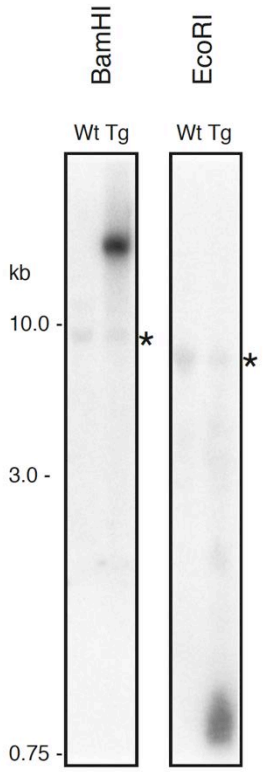
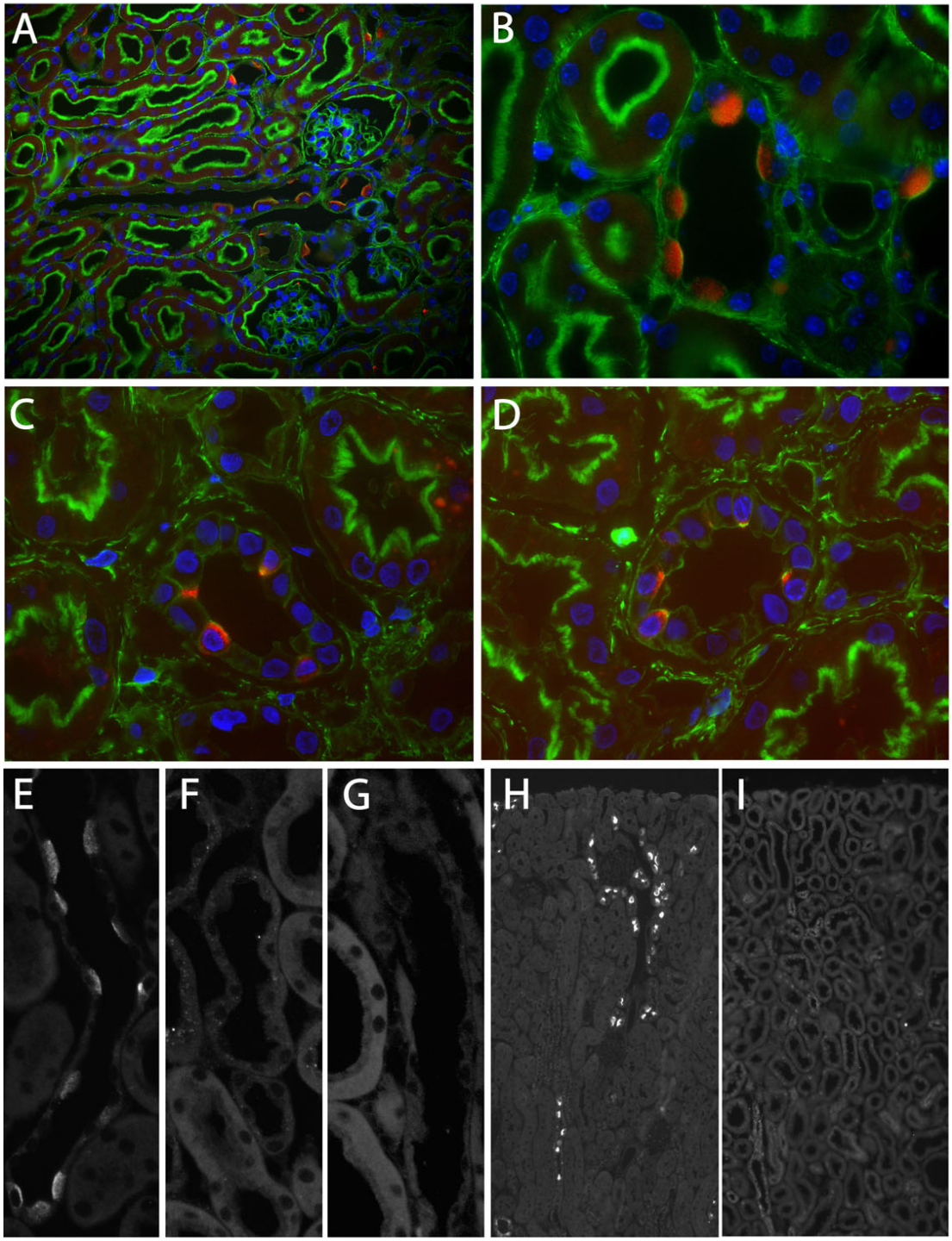
2- Supplemental figures



Supplemental Figure S1. Effects of high salt diet on *Atp6v1b1* mRNA.
Wild type C57BL/6 mice were fed either a normal salt (0.3% Na^+ as NaCl salt) or high salt diet (3% Na^+) for two weeks. RT-PCR analysis were performed using the primers shown in suppl. table S1. Results were normalized to GAPDH as described above in the method section. Results are the mean ± S.E., n = 6 in both groups. The absence of statistical difference was tested by unpaired Student's t test.

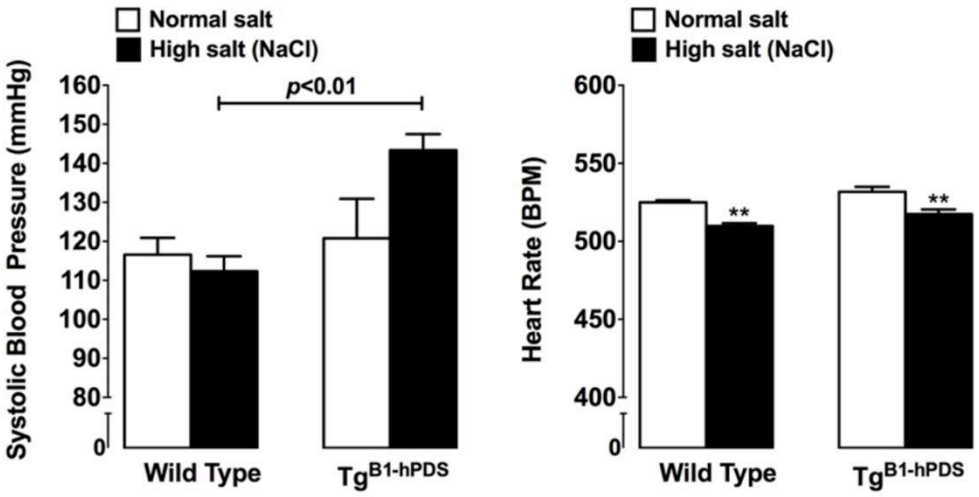


Supplemental Figure S2. Southern Blot analyses of Genomic DNA from WT or TgB1-hPDS mice. Genomic DNA from wildtype (Wt) and transgenic mice (Tg) was digested with either BamHI or EcoRI as indicated above. Digestion with BamHI which does not cut within the transgenic construct, revealed a single band of strong intensity supportive of a single integration site. An additional genomic digest with EcoRI, which also excises the probe template, resulted in a single band corresponding to the size of the probe. Binding of the probe to fragments which include the endogenous murine Pds sequence are marked by asterisks.



Supplemental Figure S3. Characterization of a new anti human pendrin antibody. Low magnification (A) and high magnification (B) of mouse kidney sections, and high magnification (C, D) of human kidney sections labeled with the rabbit anti human pendrin antibody (*red labeling*), plasmic membranes were counterstained with FITC-conjugated phalloidin, and nuclei were counterstained with DAPI. The pattern of labeling was identical in human and mouse kidney, and was consistent with the known localization of pendrin in β-ICs. Staining of mouse kidney sections (E), was completely abolished when the antibody was pre-incubated with the immunizing peptide in excess (F),

and was absent when pre-immune serum was used instead of the immune serum (G). Staining was also absent in sections obtained from mice with pendrin disruption (Pds-/-) (I) compared to WT mice (H) and stained using same procedure than in panel A and B demonstrating the specificity of the signal.



Supplemental Figure S4: Systolic blood pressure and heart rate measured in WT and $Tg^{B1\text{-}hPDS}$ mice by radio-telemetry. Mice were fed either normal (0.3% Na^+ as NaCl) or high salt (3% Na^+ as NaCl) for two weeks. Data are means ± S.E. of data from 5 independent mice in each group. Data were analyzed by ANOVA followed by bonferroni post hoc-test when appropriate. ** indicate p<0.01 vs. Normal salt diet.

3. Supplemental Tables

Supplemental Table 1 : Primers used in this study

	Forward primers	Reverse Primers
Mouse Gapdh	5’-GCACAGTCAAGGCCGAGAAT-3’	5’-GCCTTCTCCATGGTGGTGAA-3’
primers B1-hPds (genotyping)	5’-AGAGGGTCAAGGTTCCATTTTAG-3’	5’-CAAACCACAACTAGAATGCAGTG-3’
human PDS	5’-AAATCTCAAGAGGGTCAAGGTTC-3’	5’-ACATCAAGTTCTTCTTCCGTCAG-3’
Mouse Atp6v1b1	5’-ATCAATGTGCTCCCATCCCTCT-3’	5’-AATGCGCTTCAGCATCTCTTT-3’

Supplemental Table 2 : Physiological data from $Tg^{B1\text{-}hPDS}$ and WT mice fed either a normal salt or a high salt diet for two weeks

		0.8% NaCl (0.3% Na^+)		8%NaCl (3% Na^+)	
		$Tg^{B1\text{-}hPDS}$	WT	$Tg^{B1\text{-}hPDS}$	WT
Plasma	**[Na^+], mM**	148 ± 1	149 ± 1	154 ± 0.6	154 ± 1.4
	[K^+], mM	4.15 ± 0.19	4.03 ± 0.11	3.60 ± 0.25	3.76 ± 0.15
	[Cl^-], mM	117 ± 1.3	116 ± 0.9	119 ± 0.4	118 ± 0.4
	Ht, %	41 ± 1.02	42 ± 0.77	42 ± 0.6	44 ± 1.2
blood	**pH**	7.24 ± 0.01	7.23 ± 0.02	7.25 ± 0.01	7.28 ± 0.01
	[HCO_3^-], mM	21.3 ± 0.8	21.5 ± 0.8	22.0 ± 0.5	22.3 ± 0.3
	pCO_2, mmHg	51 ± 1.0	54 ± 1.8	52 ± 1.5	50 ± 0.9
urine	**pH**	5.76 ± 0.07	5.83 ± 0.06	6.22 ± 0.05	6.13 ± 0.03

Data are means ± S.E. of data obtained from 8 $Tg^{B1\text{-}hPDS}$ and 8 WT mice. Both diet conditions are presented in same table but are from two independent series and statistical significance was only assessed vs. WT fed same diet, using a two-tailed unpaired Student's *t*-test.

Supplemental Table 3 : Effect of a high salt diet on different renal transporters protein abundance. Densitometric analyzes of immunoblots of membrane fractions isolated from the renal cortex of $Tg^{B1\text{-}hPDS}$ mice or WT mice. All animals were pair-fed a high salt diet (3%Na+ as NaCl) for one week.

	WT	**KO**	***p* value**
NHE3	100 ± 9.9	75.5 ± 12	0.19
NKCC2	100 ± 5.4	109.5 ± 6.7	0.29
NCC	100 ± 8.1	58.0 ± 8.2	0.003*
pNCC	100 ± 16.5	58.0 ± 8.0	0.041*
αENaC 90kDa	100 ± 6.5	75.0 ± 5.3	0.012*
αENaC 30kDa	100 ± 9.1	55.3 ± 5.7	0.001*
γENaC	100 ± 12.8	64.3 ± 7.3	0.032*
mPds	100 ± 7.0	86.7 ± 11.9	0.33
B1-H-ATPase	100 ± 6.2	93.1 ± 11.9	0.45
Ndcbe	100 ± 3.9	61.4 ± 7.2	0.0005*

Data are mean ± S.E and are expressed as a percentage of control values.
Data obtained from 7 $Tg^{B1\text{-}hPDS}$ mice and 7 WT mice.
statistical significance was determined by a two-tails unpaired Student's t-test, a *p* value < 0.05 is considered as significant and labeled with *.