Supplemental Information for Terker et al.

Potassium modulates electrolyte balance and blood pressure through effects on distal cell voltage and chloride

Supplemental Methods

Antibodies

The following antibodies were employed an are specific to the following proteins: NCC (Bostanjoglo et al., 1998), pNCC-T53 (McCormick et al., 2011b), WNK1 (Yang et al., 2007), WNK3 (Hoorn et al., 2011), N-terminal WNK4 (Yang et al., 2007), and NKCC2 (Schmitt et al., 2003). Commercial antibodies were used against myc (Sigma), pSPAK-S373/pOxSR1-S325 (Millipore), β-actin (Abcam), pSPAK-T233/pOxSR1-T185, pWNK1-S382, and C-terminal WNK4 (Division of Signal Transduction Therapy, University of Dundee), and Kir4.1 (Alomone Labs). Other antibodies were generous gifts from other investigators, including pNCC-T58 (Jan Loffing, Zurich), SPAK and OxSR1 (Eric Delpire, Vanderbilt), and CLC-K2 and Barttin (Thomas Jentsch, Berlin).

Mice

NCC (Schultheis et al., 1998 Oct 30), SPAK (Geng et al., 2009; McCormick et al., 2011a), KS OxSR1 (Terker et al., 2014), and AT_{1a} (Ito et al., 1995) knockout animals have all been reported previously. Wild-type controls for the KS $OxSR1^{-/-}$ mice were those littermates that had two floxed OxSR1 alleles, but lacked either the LC1 transgene, the Pax8 transgene, or both. Prior to use in reported studies, KS

OxSR1$^{-/-}$ mice were treated with doxycyline (2 mg/ml in 5% sucrose in drinking water) for 14 days to induce Cre-mediated recombination. Controls received only sucrose water.

Blood Pressure

For non invasive measuremens, mice were acclimated to the CODA-16 machine for five consecutive days before recording. In the dietary manipulation studies, mice were maintained on the baseline diet (high NaCl (6%)/normal K^+ (1%)) for one week before measurements were taken. After blood pressures were recorded for four days on the baseline diet, diets were changed to high NaCl/low K^+ (0%). Animals were allowed to acclimate to the new diet for at least four days and data were recorded after this period.

PCR Genotyping

Standard PCR on tail snips was used for animal genotyping. For NCC$^{-/-}$ (Schultheis et al., 1998 Oct 30) and $AT_{1a}^{-/-}$ (Terker et al., 2014), previously reported primers were used to distinguish knockouts from wild-type mice. To distinguish mice carrying at least one modified SPAK allele from wild-type mice, a previously reported approach was used (McCormick et al., 2011a). Western blots on protein extracts from cultured tail cells were then used to further distinguish SPAK$^{+/-}$ mice from SPAK$^{-/-}$ mice. For KS OxSR1$^{-/-}$ mice, previously published primers were used to identify animals that were homozygous for floxed exon 2 within the OxSR1 gene and

also carried at least one copy each of the Cre and Pax8 rtTA transgenes (Terker et al., 2014).

Immunofluorescence

For immunofluorescence, sections were brought to room temperature, washed in 1× PBS, incubated in 1× PBS with 0.5%Triton X for 30 min, washed in PBS, and blocked in 5% milk in PBS for 30 min. Primary antibody in 5% milk in 1× PBS was added for at least 1 hr or up to an overnight incubation, followed by a wash in PBS. Sections were incubated for 1 hr with secondary antibody in block, then washed. Images were acquired on a high-resolution wide field Core DV system (Applied Precision™). The system was an Olympus IX71 inverted microscope with a proprietary XYZ stage enclosed in a controlled environment chamber; differential interference contrast (DIC) transmitted light and a solid-state module for fluorescence. The camera used was a Nikon Coolsnap ES2 HQ. Representative images are shown.

Cell Culture

In siRNA knockdown experiments, 40nM siRNA (Invitrogen) was used. Cells were cultured in DMEM with high glucose, sodium pyruvate, and L-Glutamine (HyClone) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (p/s) for all experiments unless K^+-free medium was used. In K^+-free experiments, DMEM was prepared in our lab. DMEM prepared in our lab

contained the following: $CaCl_2$ (0.2 g/L), $Fe(NO_3)_3 \bullet 9H_20$) (0.0001 g/L), $MgSO_4$ (0.09767 g/L), $NaHCO_3$ (3.7 g/L), NaCl (6.4 g/L), NaH_2PO_4 (0.109 g/L), MEM Vitamins solution 100x (Sigma), MEM Amino Acids (50x) (GIBCO) supplemented with Glycine (0.03 g/L) and L-Glutamine (0.584 g/L), D-glucose (4.5 g/L), Phenol red (0.0159 g/L), and Sodium Pyruvate (0.11 g/L). For experiments using DMEM prepared in our lab, control groups were supplemented with K^+ gluconate (1.257 g/L), RbCl (0.65 g/L), or Choline Cl (0.75 g/L) followed by filtration and supplementation with 10% FBS and p/s. Final K^+ concentrations after FBS addition was measured to be 0.9 mM for the low K^+ medium and 4.4 mM for the normal K^+ medium.

For high Cl- experiments, either mannitol (41.5 g/L) or choline Cl^- (15.9 g/L) was added to the normal and low K^+ medium. Cells were transfected (or induced with 1 ug/ml tetracycline) on day 0. Medium was then changed (or 4,4'-Diisothiocyano2,2'-stilbenedisulfonic acid (DIDS) (100uM) or $BaCl_2$ (10 mM) was added) on day 1, followed by cell lysis on day 2 after which total protein quantification and Western blots were performed.

Measurement of Intracellular Chloride

To measure intracellular Cl^-, cells were loaded for 1 hr with the chloride-sensitive fluorescent dye, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) (14 mM), in either normal K^+ or low K^+ HBBS. Standard curves were produced using the Stern-Volmer equation from readings of cells that were loaded with MQAE in high K^+ HBBS supplemented with nigericin (7uM) and

tributyltin (10 uM) with stepwise increases in buffer [Cl^-] as explained previously (Verkman, 1990). After loading, cells were washed 3x and fluorescence was measured on a FlexStation plate reader (Molecular Devices) using 350nm excitation and 460nm emission.

Measurement of K^+ Reversal Potentials

Kcnj10 DNA (0.5 mg) was diluted with 200 ml serum free DMEM and further mixed with 4 ml Turbofect transfection reagent for the transfection of cells cultured in 35 mm Petri dish. Cells transfected with the vector alone were used as a control and their background currents were subtracted from that of the experimental groups. After 15 min incubation at room temperature, the mixture of the transfection agents was applied to the cells followed by additional 24 hr incubation before use.

For measuring the K^+ reversal potential using perforated whole-cell patch, the cells were superfused with HEPES-buffered NaCl solution containing 5 mM K and 140 mM Na^+. The tip of the pipette was filled with a pipette solution containing 140 mM KCl, 2 mM $MgCl_2$, 1mM EGTA, and 5 mM HEPES (pH 7.4). The pipette was then back-filled with amphotericin B (20 μ g/0.1 ml) containing the pipette solution. After forming a high resistance seal (>2 GΩ), the membrane capacitance was monitored until the whole-cell patch configuration was formed. The currents were low-pass filtered at 1 KHz, digitized by an Axon interface (Digidata 1322) and data were analyzed using the pClamp software system 9 (Axon).

DCT and Distal Nephron Model

The model is based on that generated by (Weinstein, 2005). In that work, NCC flux was represented by a kinetic model (equation 10) in which net flux through the transporter, J_{Na}, was a function of luminal and cytosolic Na^+ and Cl^- concentrations, and proportional to a transporter density, x_T. In the present work, Cl^--dependent transporter density is represented by the equation

$$\frac{x_T}{x_{T0}} = -4.0\left[\frac{[Cl^-]_i}{18.6} - 1.0\right]$$

The calculations for **Figure 7** utilize the epithelial model, with specified luminal and peritubular solute concentrations. Luminal solute concentrations are identical to those published (table 3 in (Weinstein, 2005)), with the exception that luminal NaCl has been reduced by 25 mM ($[Na^+]$ = 40.0 and $[Cl^-]$=30.9 mM). Peritubular concentrations are unchanged, with the exception of variable KCl, ranging from 2.0 to 8.0 mM, by KCl addition (rather than K^+-for-Na^+ substitution).

Human Studies

The order of diets was randomized. The amounts of Na^+ and K^+, as well as caloric content, were recorded for all foods consumed. On the morning of the fifth day of each diet, urine was collected and centrifuged (van der Lubbe et al., 2012). Samples from the first diet were frozen at -80°C until the volunteers finished the second diet, at which point both samples were loaded on a 3-8% (wt/vol) Tris-acetate gel (Invitrogen) and Western blots were performed. Loaded sample was

normalized to urine creatinine concentration measured by modified Jaffe method (Pointe Scientific).

References

Bostanjoglo, M., Reeves, W.B., Reilly, R.F., Velazquez, H., Robertson, N., Litwack, G., Morsing, P., Dorup, J., Bachmann, S., and Ellison, D.H. (1998). 11Beta-hydroxysteroid dehydrogenase, mineralocorticoid receptor, and thiazide-sensitive Na-Cl cotransporter expression by distal tubules. Journal of the American Society of Nephrology : JASN *9*, 1347-1358.

Geng, Y., Hoke, A., and Delpire, E. (2009). The Ste20 kinases Ste20-related proline-alanine-rich kinase and oxidative-stress response 1 regulate NKCC1 function in sensory neurons. The Journal of biological chemistry *284*, 14020-14028.

Hoorn, E.J., Walsh, S.B., McCormick, J.A., Fürstenberg, A., Yang, C.-L., Roeschel, T., Paliege, A., Howie, A.J., Conley, J., Bachmann, S., et al. (2011). The calcineurin inhibitor tacrolimus activates the renal sodium chloride cotransporter to cause hypertension. Nature Medicine *17*, 1304-1309.

Ito, M., Oliverio, M.I., Mannon, P.J., Best, C.F., Maeda, N., Smithies, O., and Coffman, T.M. (1995). Regulation of blood pressure by the type 1A angiotensin II receptor gene. Proceedings of the National Academy of Sciences of the United States of America *92*, 3521-3525.

McCormick, J.A., Mutig, K., Nelson, J.H., Saritas, T., Hoorn, E.J., Yang, C.-L., Rogers, S., Curry, J., Delpire, E., Bachmann, S., et al. (2011a). A SPAK isoform switch modulates renal salt transport and blood pressure. Cell Metab *14*, 352-364.

McCormick, J.A., Nelson, J.H., Yang, C.L., Curry, J.N., and Ellison, D.H. (2011b). Overexpression of the Sodium Chloride Cotransporter Is Not Sufficient to Cause Familial Hyperkalemic Hypertension. Hypertension *58*, 888-894.

Schmitt, R., Klussmann, E., Kahl, T., Ellison, D.H., and Bachmann, S. (2003). Renal expression of sodium transporters and aquaporin-2 in hypothyroid rats. Am. J. Physiol. Renal Physiol. *284*, F1097-1104.

Schultheis, P.J., Lorenz, J.N., Meneton, P., Nieman, M.L., Riddle, T.M., Flagella, M., Duffy, J.J., Doetschman, T., Miller, M.L., and Shull, G.E. (1998 Oct 30). Phenotype resembling Gitelman's syndrome in mice lacking the apical Na+- Cl- cotransporter of the distal convoluted tubule. J.Biol.Chem. *273*, 29150-29155.

Terker, A.S., Yang, C.-L., McCormick, J.A., Meermeier, N.P., Rogers, S.L., Grossman, S., Trompf, K., Delpire, E., Loffing, J., and Ellison, D.H. (2014). Sympathetic stimulation of thiazide-sensitive sodium chloride cotransport in the generation of salt-sensitive hypertension. Hypertension *64*, 178-184.

van der Lubbe, N., Jansen, P.M., Salih, M., Fenton, R.A., van den Meiracker, A.H., Danser, A.H., Zietse, R., and Hoorn, E.J. (2012). The phosphorylated sodium chloride cotransporter in urinary exosomes is superior to prostasin as a marker for aldosteronism. Hypertension *60*, 741-748.

Verkman, A.S. (1990). Development and biological applications of chloride-sensitive fluorescent indicators. The American journal of physiology *259*, C375-388.

Weinstein, A.M. (2005). A mathematical model of rat distal convoluted tubule. I. Cotransporter function in early DCT. American journal of physiology. Renal physiology *289*, F699-720.

Yang, C.L., Liu, X., Paliege, A., Zhu, X., Bachmann, S., Dawson, D.C., and Ellison, D.H. (2007). WNK1 and WNK4 modulate CFTR activity. Biochemical and biophysical research communications *353*, 535-540.

Supplemental Figure 1

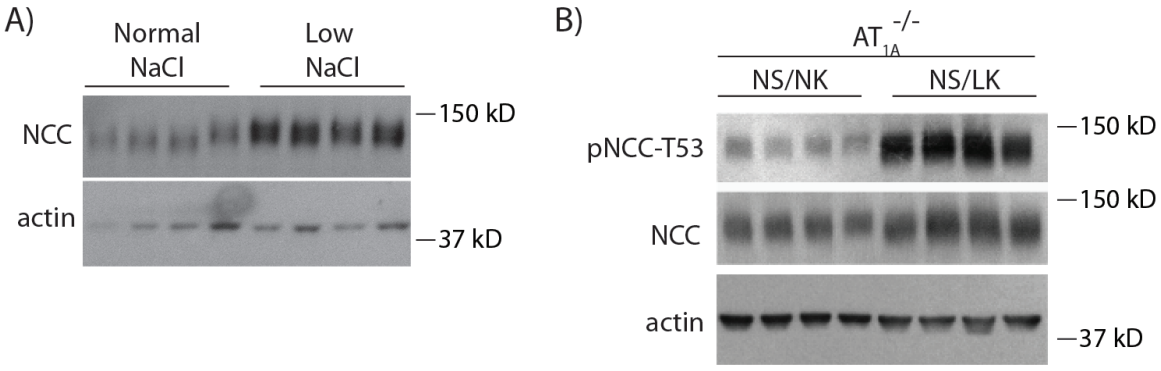

Figure S1, related to Figure 1: Effects of dietary manipulations on NCC abundance in wild type and $AT_{1a}^{-/-}$ mice. A) Western blot of kidney from wild type mice consuming normal NaCl and low NaCl diets. The low NaCl diet increased NCC abundance significantly (106 ± 30% increase). $p<0.05$ by unpaired t-test. B) Western blot of kidney from $AT_{1a}^{-/-}$ mice consuming normal NaCl /normal K^+ (NS/NK) and normal NaCl /low K^+ (NS/LK) diets. The NS/LK increased pNCC-T53 (278 ± 27% increase) and NCC (70 ± 9% increase) abundance. $p<0.001$ by unpaired t-test for both. Numbers indicate mean ± sem.

Supplemental Figure 2

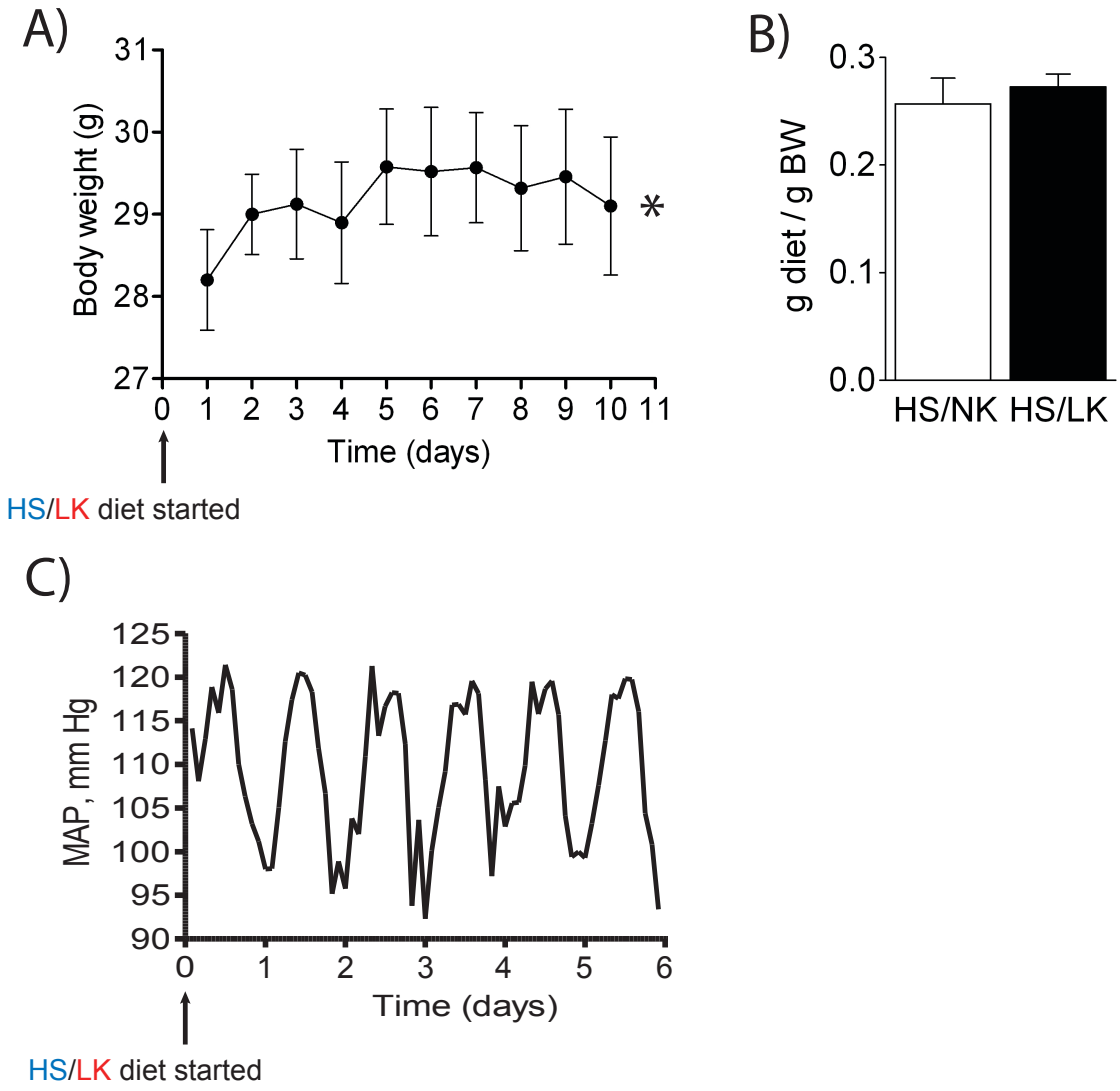

Figure S2, related to Figure 2: Effects of dietary potassium intake on body weight and food consumption. A) Body weight measurements for wild type mice maintained on HS/LK diet for 10 days. Animals were fed a HS/NK diet for 7 days before being switched to HS/LK on day 0. The HS/LK diet slightly increased body weight. * $p<0.05$ by one-way ANOVA with repeated measures. Data points represent mean ± sem. B) Food consumption of wild type mice consuming HS/NK and HS/LK diets. Bars represent mean ± sem for the amount of food consumed on day 7 of the indicated diet. There was no difference in food consumption between the two diets. C) 24-hour mean arterial pressure (MAP) recording from Slc12a3$^{-/-}$ mice on a HS/LK diet. Trace represents averaged 2-hour averages of 2 animals. Mice were maintained on a HS/NK diet for one week until time 0, when they were switched to HS/LK.

Supplemental Figure 3

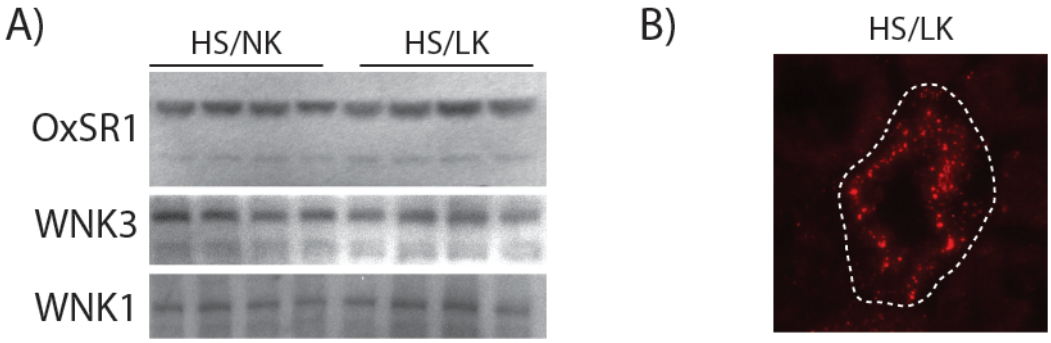

Figure S3, related to Figure 3: A) Western blot of kidney from mice consuming HS/NK and HS/LK diets. The HS/LK diet did not change the total kidney abundance of OxSR1, WNK1, or WNK3. Actin loading control is same as **Fig 1A**. B) Immunofluorescence imaging of WNK4 in the DCT with a C-terminal WNK4 antibody. WNK4 localized to cytoplasmic puncta on HS/LK diet similar to data obtained with an N-terminal WNK4 antibody (see **Fig 3G**). Dotted line outlines DCT.

Supplemental Figure 4

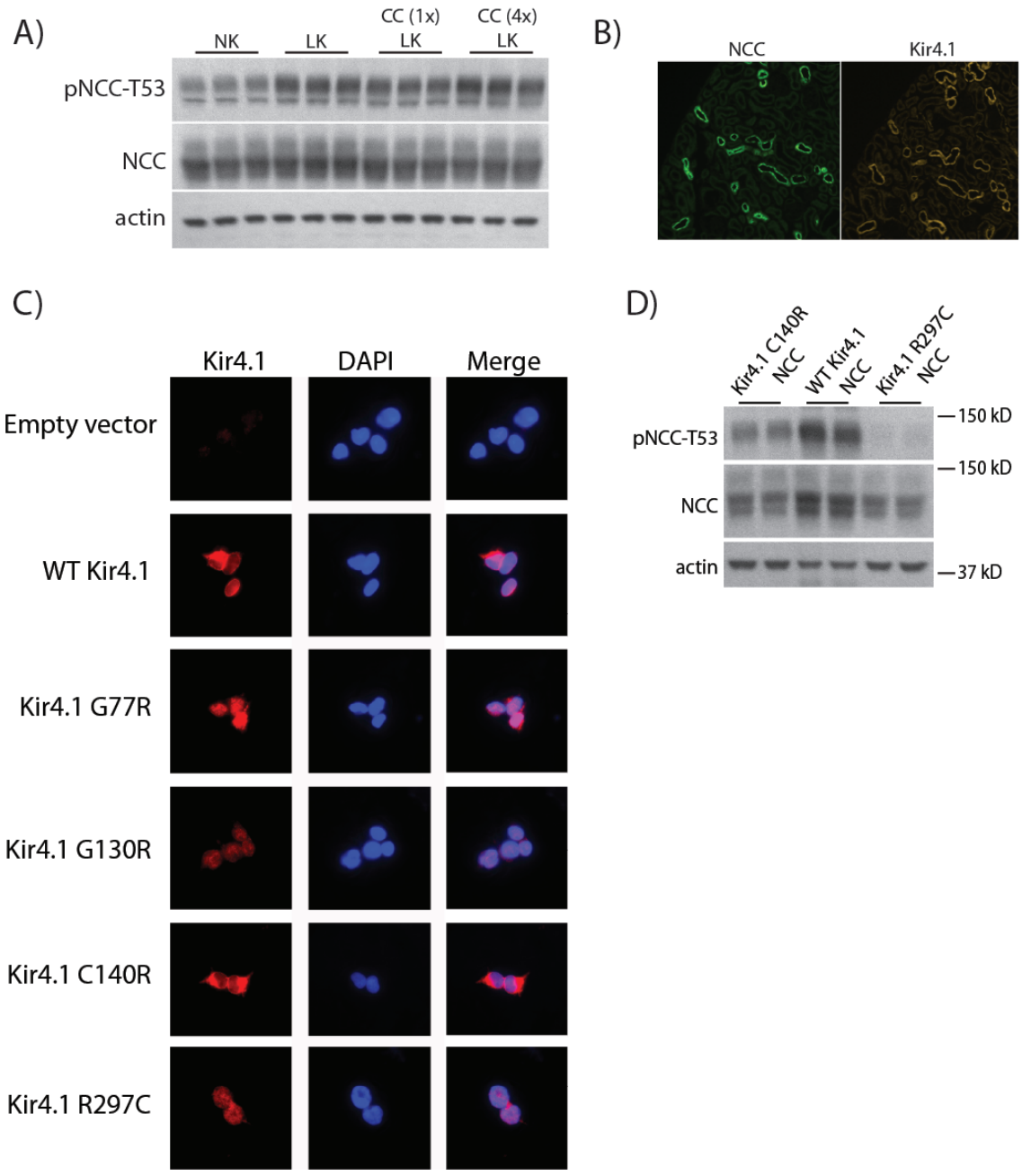

Figure S4, related to Figure 5: A) Western blot of HEK cells cultured in NK, LK, LK medium supplemented with choline Cl^- equimolar to the RbCl added in **Fig 4E** (CC 1x), and LK medium with four times as much choline Cl^- (CC 4x). LK increased pNCC-T53 as before (48 ± 2.2% increase) ($p<0.01$ by unpaired t-test corrected for multiple comparisons) whereas adding choline Cl^- did not prevent this effect. B) Immunofluorescence staining for NCC and Kir4.1 on consecutive kidney sections. Kir4.1 is highly expressed in cells that also express NCC. C) Cell immunofluorescence staining for WT and mutant Kir4.1 expressed in HEK cells. Cells have no endogenous Kir4.1 expression. D) Original image used for first two panels of **Fig 5B**.

Supplemental Figure 5

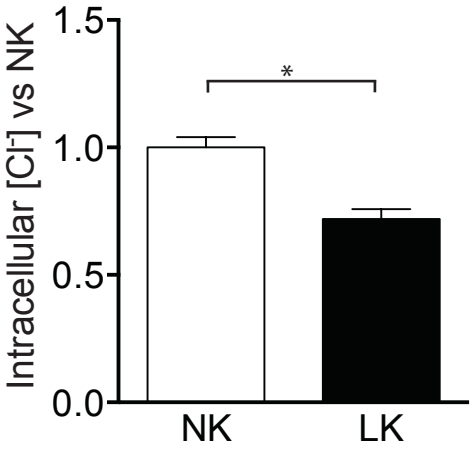

Figure S5, related to Figure 6: Effects of LK medium on relative intracellular [Cl^-] of cultured mouse DCT cells. Incubation conditions are as in Figure 6. Bars represent mean ± sem. * indicates $P<0.05$.

Supplemental Figure 6

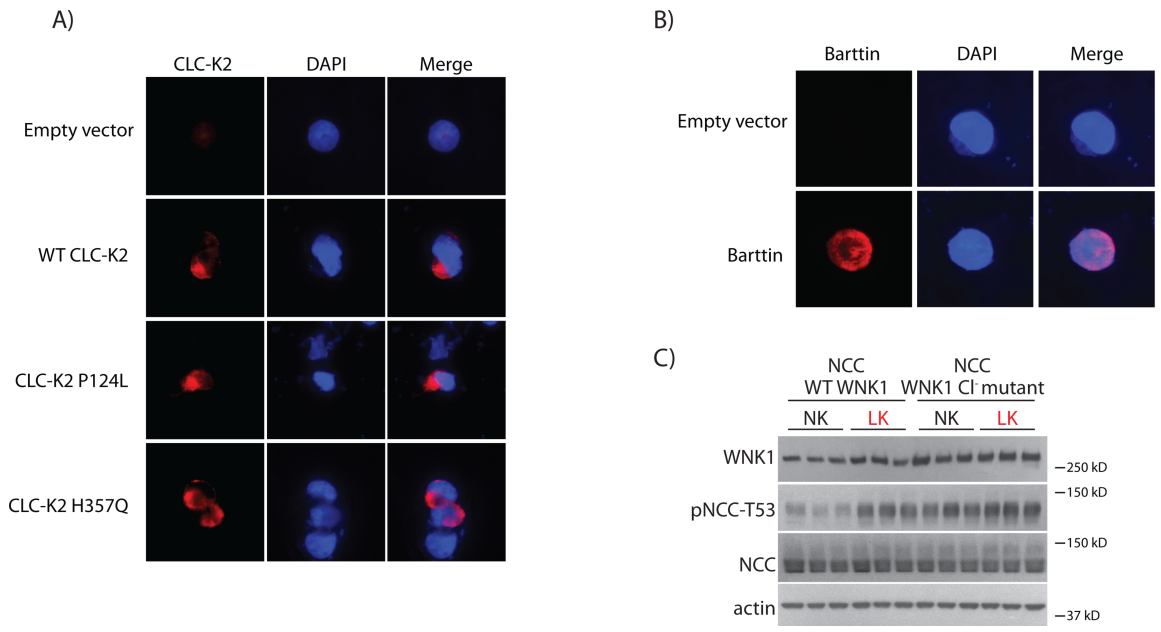

Figure S6, related to Figure 6: A) Cell immunofluorescence staining for WT and mutant CLC-K2 expressed in HEK cells. Cells have no endogenous CLC-K2 expression. B) Cell immunofluorescence staining for barttin subunit expressed in HEK cells. Cells have no endogenous barttin expression. C) Original image used for Figure 6G.

Table S1, related to Figure 1: Densitometry for Western blots from **Fig 1.**

Panel A		HS/NK	HS/LK
	pNCC-T53	1 ± 0.44	13.6 ± 0.95
	pNCC-T58	1 ± 0.74	7.62 ± 0.20
	NCC	1 ± 0.23	3.55 ± 0.09
	NKCC2	1 ± 0.096	1 ± 0.13

Panel C		HS/NK	HS/LK
	pNCC-T53	1 ± 0.20	10.3 ± 0.50
	NCC	1 ± 0.20	1.7 ± 0.23

Panel D		HS/NK	HS/LK
	pNCC-T53	1 ± 0.34	2.26 ± 0.07
	NCC	1 ± 0.047	1.12 ± .064

Panel E		HS/NK	HS/LK
	pNCC-T53	1 ± 0.008	4.35 ± 0.96

Table S2, related to Figure 3: Densitometry for Western blots from **Fig 3.**

Panel C		Vehicle	Amiloride	Amiloride + LK
	pNCC-T53	1 ± 0.12	0.16 ±0.092	1.38 ± 0.055
	NCC	1 ± 0.032	0.64 ± 0.11	0.83 ± 0.087
	NKCC2	1 ± 0.075	4.61 ± 1.06	1.38 ± 0.29

Panel D		HS/NK	HS/LK
	SPAK	1 ± 0.16	1.7 ± 0.16
	WNK4	1 ± 0.13	2.14 ± 0.27

Panel E		NS/NK	NS/LK
	WNK4	1 ± 0.16	2.17 ± 0.28

Panel H		HS/NK	HS/LK
	pNCC-T53	1 ± 0.29	11.5 ± 2.36
	NCC	1 ±.072	2.3 ± 0.14

Panel I		HS/NK	HS/LK
	pNCC-T53	1 ± 0.40	2.45 ± 0.14
	NCC	1 ± 0.17	1.67 ± 0.15

Panel J		SPAK-/-		SPAK-/- KS OxSR1-/-	
		HS/NK	HS/LK	HS/NK	HS/LK
	pNCC-T53	1 ± 0.25	6.78 ± 0.36	0.57 ± 0.13	3.72 ± 0.4
	NCC	1 ±0.11	1.80 ± 0.09	1.19 ± 0.029	1.33 ± 0.09
	OxSR1	1 ±0.054	0.95 ± 0.036	0.14 ± 0.014	0.29 ±0.034

Table S3, related to Figure 4: Densitometry for Western blots from **Fig 4.**

Panel A		NK	LK
	pNCC-T53	1 ± 0.024	1.90 ± 0.18
	pNCC-T58	1 ±0.042	1.39 ± 0.025
	NCC	1 ± 0.014	1.03 ±0.087

Panel B		HS/NK	HS/LK
	pSPAK/pOxSR1	1 ± 0.053	2.31 ± 0.022

Panel C		Neg siRNA		WNK1 siRNA	
		NK	LK	NK	LK
	pNCC-T53	1 ± 0.085	5.91 ±0.027	1.10 ± 0.052	3.74 ± 0.098
	NCC	1 ± 0.048	1.01 ± 0.021	0.96 ± 0.038	0.98 ± 0.065
	WNK1	1 ± 0.24	1.57 ± 0.020	0.31 ± 0.038	0.60 ± 0.062
	pSPAK/pOxSR1	1 ± 0.06	2.43 ± 0.11	1.35 ± 0.037	1.84 ± 0.13

Panel D		Neg siRNA		WNK3 siRNA	
		NK	LK	NK	LK
	pNCC-T53	1 ± 0.31	2.03 ±0.076	1.01 ± 0.027	1.11 ± 0.048
	NCC	1 ± 0.083	1.09 ± 0.047	1.02 ± 0.015	0.97 ± 0.016
	WNK3	1 ± 0.042	0.97 ± 0.038	0.43 ± 0.022	0.035 ±0.030
	pSPAK/pOxSR1	1 ± 0.038	1.67 ± 0.021	0.90 ± 0.017	1.15 ± 0.022

Panel E		NK	LK	LK+Rb
	pNCC-T53	1 ± 0.18	3.67 ± 0.23	1.17 ± 0.23
	NCC	1 ± 0.011	0.95 ± 0.030	1.02 ± 0.083
	pSPAK/pOxSR1	1 ± 0.054	1.34 ± 0.12	0.63 ± 0.11

Panel F		Mannitol	BaCl2
	pNCC-T53	1 ± 0.030	0.32 ± 0.073
	NCC	1 ± 0.076	1.34 ± 0.028
	pSPAK/pOxSR1	1 ± 0.070	0.44 ± 0.026

Table S4, related to Figure 5: Densitometry for Western blots from **Fig 5.**

Panel B		WT	C140R	R297C	G77R
	pNCC-T53	1 ± 0.040	0.49 ± 0.051	0.38 ± 0.086	0.58 ± 0.095
	NCC	1 ± 0.024	0.63 ± 0.085	0.73 ± 0.067	0.95 ± 0.17

Panel C		WT	G130R
	pNCC-T53	1 ± 0.051	0.45 ± 0.045
	NCC	1 ± 0.028	0.92 ±0.011

Table S5, related to Figure 6: Densitometry for Western blots from **Fig 6.**

Panel C		Normal Cl-		High Cl-	
		NK	LK	NK	LK
	pNCC-T53	1 ± 0.16	1.68 ± 0.058	0.90 ±0.0069	1.02 ± 0.031
	NCC	1 ± 0.039	1.25 ± 0.012	1.30 ± 0.046	1.07 ± 0.027

Panel D		DMSO		DIDS	
		NK	LK	NK	LK
	pNCC-T53	1 ± 0.17	2.04 ± 0.11	1.14 ± 0.027	1.12 ± 0.13
	NCC	1 ± 0.015	1.10 ± 0.013	0.98 ± 0.076	0.90 ± 0.065

Panel E		WT CLC-K2		CLC-K2 P124L	
		NK	LK	NK	LK
	pNCC-T53	1 ± 0.11	1.6 ± 0.036	0.65 ± 0.055	0.57 ± 0.11
	NCC	1 ± 0.11	1.02 ± 0.034	3.01 ± 0.22	2.21 ± 0.24

Panel F		WT CLC-K2		CLC-K2 H357Q	
		NK	LK	NK	LK
	pNCC-T53	1 ± 0.052	1.44 ± 0.11	0.99 ± 0.088	0.97 ± 0.062
	NCC	1 ± 0.067	0.96 ± 0.028	0.81 ± 0.0096	0.73 ± 0.042

Panel G		WT WNK1		WNK1 Cl- mutant	
		LK	NK	LK	NK
	pNCC-T53	2.00 ± 0.13	1 ± 0.16	2.17 ± 0.063	2.02 ± 0.17
	NCC	1.02 ± 0.061	1 ± 0.08	0.96 ± 0.025	1.01 ± 0.029

Panel H		WT WNK1		WNK1 Cl- mutant
		NK	LK	NK
	pWNK1-S382	1 ± 0.42	3.34 ± 0.43	7.60 ± 0.12
	total WNK1	1 ± 0.11	1.15 ± 0.063	1.24 ± 0.14