Aldosterone regulates NCC indirectly, via plasma potassium

Supplemental Figures and Methods

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Full Methods

Animals

All studies were approved by Oregon Health and Science University's Animal Care and Usage Committee (Protocol IS3286). Kidney-specific MR knockout (KS MR$^{-/-}$) mice were generated through use of the Pax8-rtTA/LC1 system[1] as our lab has previously reported[2]. Double transgenic Pax8-rtTA/LC1 mice were bred with mice homozygous for the MR allele in which exon 3 is floxed[3] to obtain MR$^{fl/+}$/Pax8-rtTA/LC1. These mice were then interbred to obtain MR$^{fl/fl}$/Pax8-rtTA/LC1 animals. To induce Cre-mediated recombination of the MR gene, MR$^{fl/fl}$/Pax8-rtTA/LC1 mice were treated with 2mg/ml doxycycline hyclate in 5% sucrose drinking water for two weeks. Control animals were genetically identical, but treated with only sucrose water for the two-week period. For some experiments, animals lacking one of the transgenes were included treated with only sucrose water and used as controls. Following treatment, all animals were returned to regular drinking water for at least two weeks before use. All mice used for experiments were 12-24 weeks old.

PCR Genotyping

Genomic DNA extracts were prepared from tail snips by heating overnight at 55°C in 300µL digestion solution containing 5mM EDTA, 200mM NaCl, 100mM Tris (pH 8.0), 0.2% SDS and 0.4 mg/ml Proteinase K. The following day, supernatant was transferred to a new tube and 1mL 100% ethanol was added followed by centrifugation at 16,000xg for 30 min at room temperature. Supernatant was poured off and 1mL 70% ethanol was added followed by centrifugation at 16,000xg

for 20 min at room temperature. Supernatant was poured off and DNA pellet was immediately resuspended in 300μL Tris-EDTA buffer (pH 7.5). Residual ethanol was evaporated at 55°C for 2hr before DNA was used for PCR. DNA was stored at -20°C.

Primers used to detect presence of Pax8-rtTA and LC1 transgenes were reported previously[4]. Primers used to detect presence of floxed or wild-type MR allele were: 5'-CTGGAGATCTGAACTCCAGGCT-3', 5'-TAGAAACACTTC GTAAAGTAGAGCT-3', and 5'-CCTAGAGTTCCTGAGCTGCTGA-3'.

Animal diets and measurement of food consumption

For Na^+ deprivation studies, a Na^+-deficient diet (0.01-0.02% Na^+, 0.8% K^+, Harlan Laboratories TD. 90228) was used. For custom diets, a gel diet was prepared from 38.4% powder diet, 61.4% water, and 0.15% agar. The Na^+-deficient diet was supplemented with NaCl (0.49% NaCl) to prepare the normal salt control diet. For K^+ loading studies, a K^+-deficient diet (15-30 ppm K^+, Harlan Laboratories TD.88239) was supplemented with KCl to prepare normal (1% K^+) and high K^+ (5% K^+) diets. For studies using the diet deficient in both Na^+ and K^+ (Harlan Laboratories TD.08168), control groups were fed the Na^+-deficient diet. Food consumption was determined by tracking the dry weight of food present in cages of individually housed mice.

Blood pressure measurement

Blood pressure was measured by radiotelemetry using PA-C10 transmitters (Data Sciences Inc.) implanted in the left carotid arteries of study mice as previously reported[4]. Animals were allowed at least 7 days to recover from surgery before

measurements were recorded every 10 minutes. Two-hour averages were used to calculate mean arterial pressures (MAP).

Metabolic cage studies

Animals were acclimated to metabolic cages (Hatteras Instruments) for two days prior to urine collection. Animals were fed a gel diet with indicated mineral content (Harlan Laboratories, see Animal diets and measurement of food consumption section) and urine was collected under water-saturated light mineral oil every 24 hours. Urine Na^+ and K^+ content were determined by flame photometry.

Blood collection and electrolyte measurements

Whole blood was collected via cardiac puncture under anesthesia. Blood was immediately transferred into heparinized tubes, 80µL of which was loaded into a Chem8+ cartridge for electrolyte measurement by i-STAT analyzer (Abbot Point of Care Inc). The remaining blood volume was centrifuged at 2,000xg for 5 min at room temperature. Plasma was removed and stored at -20°C.

Kidney Western blot

Kidneys were removed, snap frozen in liquid nitrogen, and stored at -80°C until homogenization. Using a Potter Homogenizer, they were homogenized in cold homogenization buffer containing protease and phosphatase inhibitors as previously reported[2]. Homogenate was centrifuged at 6,000xg for 15 min at 4°C and supernatant was transferred to a new tube. Following total protein quantification,

protein was separated on either a 4-12% Bis-tris or 3-8% Tris-acetate gel (Invitrogen) and transferred overnight to a PVDF membrane, which was blocked with 5% nonfatmilk in PBS-Tween for one hour followed by a one-hour incubation with primary antibody. Membranes were then washed, incubated with an HRP-coupled secondary antibody (1:5,000, Invitrogen), washed again, and imaged using a Western Lightning kit (Perkin Elmer). Densitometry was performed with ImageJ (http://rsbweb.nih.gov/ij/) and all data has been normalized to actin.

Perfusion fixation and immunofluorescence imaging

Kidneys were fixed in vivo by retrograde abdominal aortic perfusion with 3% PFA[5]. 5µm sections were washed in PBS, incubated in 0.5% Triton-X in PBS for 30 minutes, and blocked in 5% nonfat milk in PBS for 30 min. Sections were then incubated overnight in primary antibody at 4°C. The next day, sections were washed in PBS, incubated in Cy-3-coupled secondary antibody (Invitrogen), and embedded in elvanol mounting medium. Sections were imaged on an AXIO Imager M2 microscope (Zeiss).

Antibodies

The total NCC[6], pNCC-T53[7], α-, β-, and γ-ENaC antibodies have all been previously reported[8]. Commercial antibodies were used to detect SGK1 (Cell Signaling) and actin (Abcam).

Aldosterone infusion and plasma concentration measurement

Aldosterone was infused at a concentration of 240 ug/kg/d for 7 days via osmotic minipump (Alzet). Pumps were primed overnight in a 0.9% NaCl solution at 37°C prior to implantation. Plasma aldosterone concentration was measured by ELISA (IBL America). Sham group underwent surgery, but had no pump implanted.

Fludrocortisone and amiloride studies

Animals were treated with fludrocortisone (17 mg/L), amiloride (50 mg/L), or both via drinking water for 5-7 days before sacrifice.

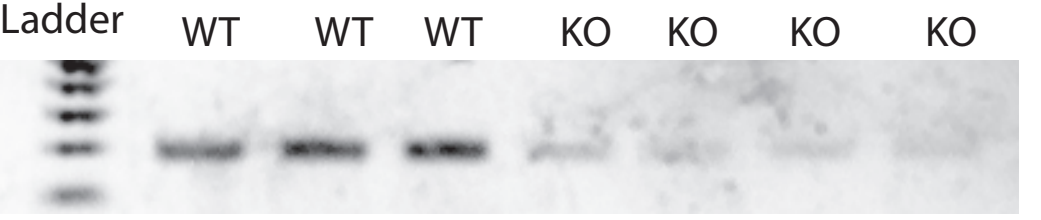
Statistics

The null hypothesis was tested using unpaired t tests, two-way ANOVA, two-way ANOVA for repeated measures, and appropriate post-hoc tests as indicated in the figure legends and text.

References

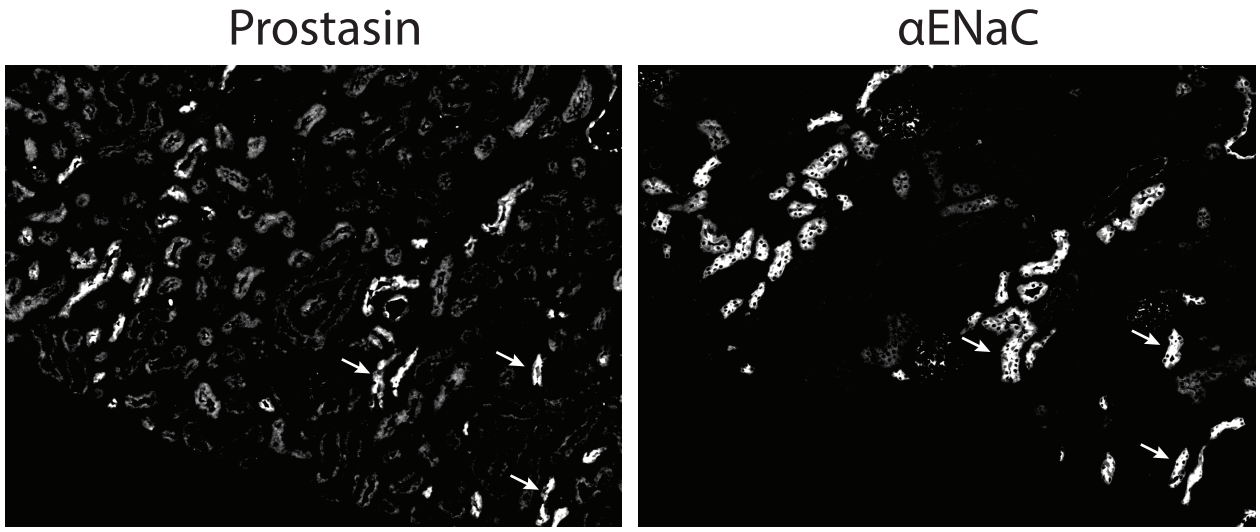
1. Traykova-Brauch, M, Schonig, K, Greiner, O, Miloud, T, Jauch, A, Bode, M, Felsher, DW, Glick, AB, Kwiatkowski, DJ, Bujard, H, Horst, J, von Knebel Doeberitz, M, Niggli, FK, Kriz, W, Grone, HJ, Koesters, R: An efficient and versatile system for acute and chronic modulation of renal tubular function in transgenic mice. *Nature medicine,* 14**:** 979-984, 2008.
2. McCormick, JA, Yang, CL, Zhang, C, Davidge, B, Blankenstein, KI, Terker, AS, Yarbrough, B, Meermeier, NP, Park, HJ, McCully, B, West, M, Borschewski, A, Himmerkus, N, Bleich, M, Bachmann, S, Mutig, K, Argaiz, ER, Gamba, G, Singer, JD, Ellison, DH: Hyperkalemic hypertension-associated cullin 3 promotes WNK signaling by degrading KLHL3. *J Clin Invest,* 124**:** 4723-4736, 2014.
3. Berger, S, Wolfer, DP, Selbach, O, Alter, H, Erdmann, G, Reichardt, HM, Chepkova, AN, Welzl, H, Haas, HL, Lipp, HP, Schutz, G: Loss of the limbic mineralocorticoid receptor impairs behavioral plasticity. *Proceedings of the National Academy of Sciences of the United States of America,* 103**:** 195-200, 2006.
4. Terker, AS, Zhang, C, McCormick, JA, Lazelle, RA, Zhang, C, Meermeier, NP, Siler, DA, Park, HJ, Fu, Y, Cohen, DM, Weinstein, AM, Wang, WH, Yang, CL, Ellison, DH: Potassium modulates electrolyte balance and blood pressure through effects on distal cell voltage and chloride. *Cell Metab,* 21**:** 39-50, 2015.
5. Saritas, T, Borschewski, A, McCormick, JA, Paliege, A, Dathe, C, Uchida, S, Terker, A, Himmerkus, N, Bleich, M, Demareetz, S, Laghmani, K, Delpire, E, Ellison, DH, Bachmann, S, Mutig, K: SPAK differentially mediates vascopressin effects on sodium cotransporters. *J Am Soc Nephrol,* 24**:** 407-418, 2013.
6. Bostanjoglo, M, Reeves, WB, Reilly, RF, Velazquez, H, Robertson, N, Litwack, G, Morsing, P, Dorup, J, Bachmann, S, Ellison, DH: 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, 1998.
7. McCormick, JA, Mutig, K, Nelson, JH, Saritas, T, Hoorn, EJ, Yang, C-L, Rogers, S, Curry, J, Delpire, E, Bachmann, S, Ellison, DH: A SPAK isoform switch modulates renal salt transport and blood pressure. *Cell Metab,* 14**:** 352-364, 2011.
8. Todkar, A, Picard, N, Loffing-Cueni, D, Sorensen, MV, Mihailova, M, Nesterov, V, Makhanova, N, Korbmacher, C, Wagner, CA, Loffing, J: Mechanisms of renal control of potassium homeostasis in complete aldosterone deficiency. *Journal of the American Society of Nephrology : JASN,* 26**:** 425-438, 2015.

Supplemental Figure 1



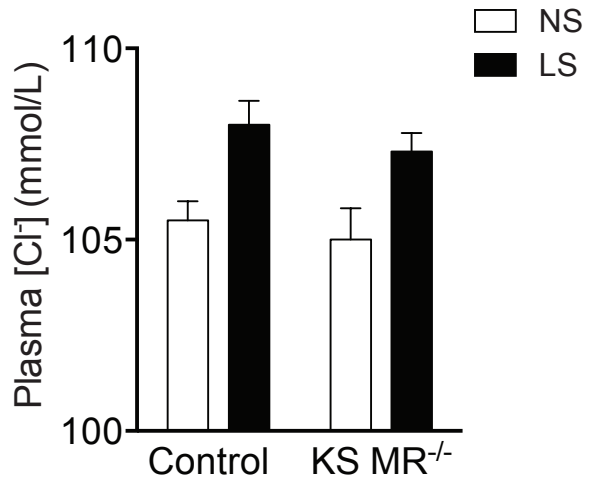
Semi quantitative RT PCR of mineralocorticoid receptor from kidney of mice following doxycycline treatment.

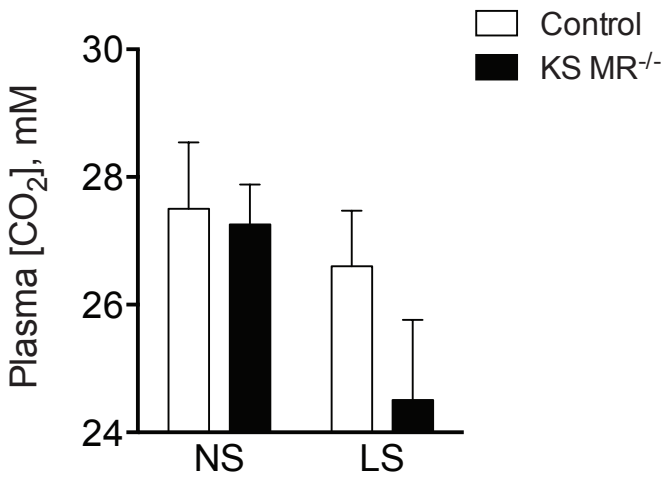
Supplemental Figure 2

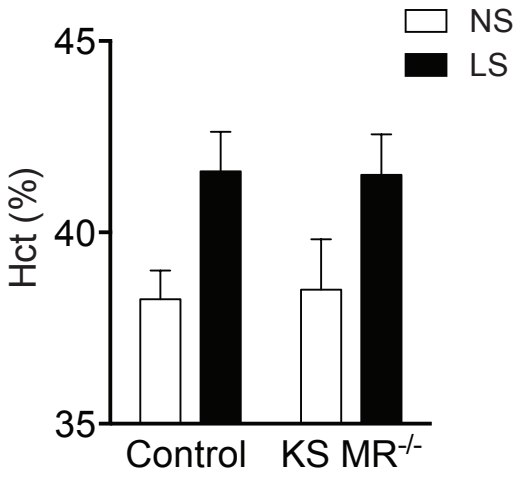


Serial cryostat kidney sections illustrating the striking co-localization between prostasin (left panel) and αENaC (right panel). Arrows show some typical tubule profiles that appear to express both proteins. There is some additional staining for prostasin in other nephron segments (identified by asterisks).

Supplemental Figure 3

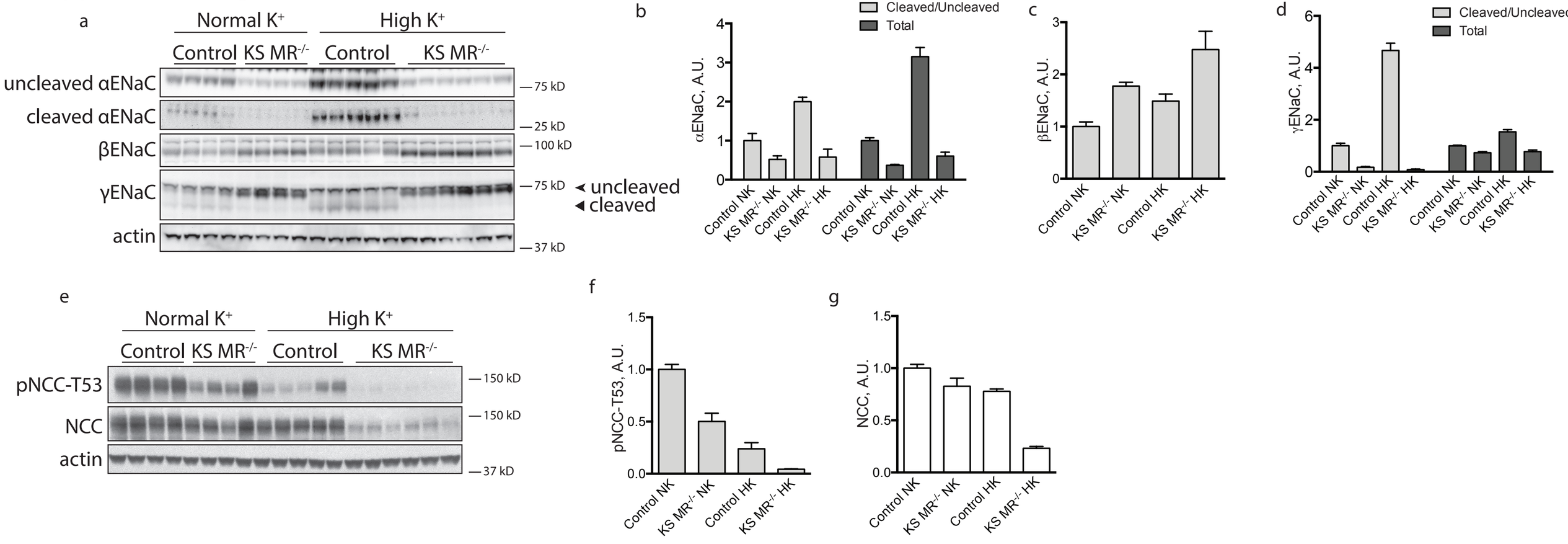






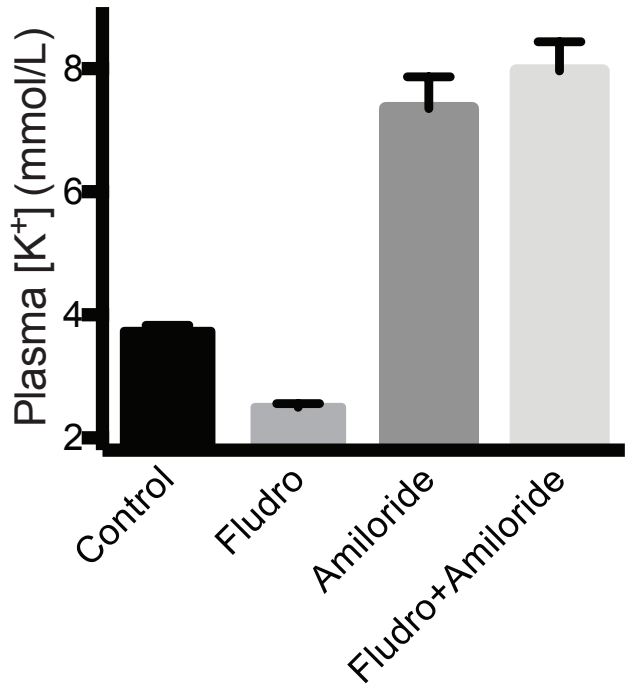
Comparison of plasma chloride and carbon dioxide, and hematocrit in control and MR knockout mice, when consuming a normal salt (NS) and low salt (LS) diet.

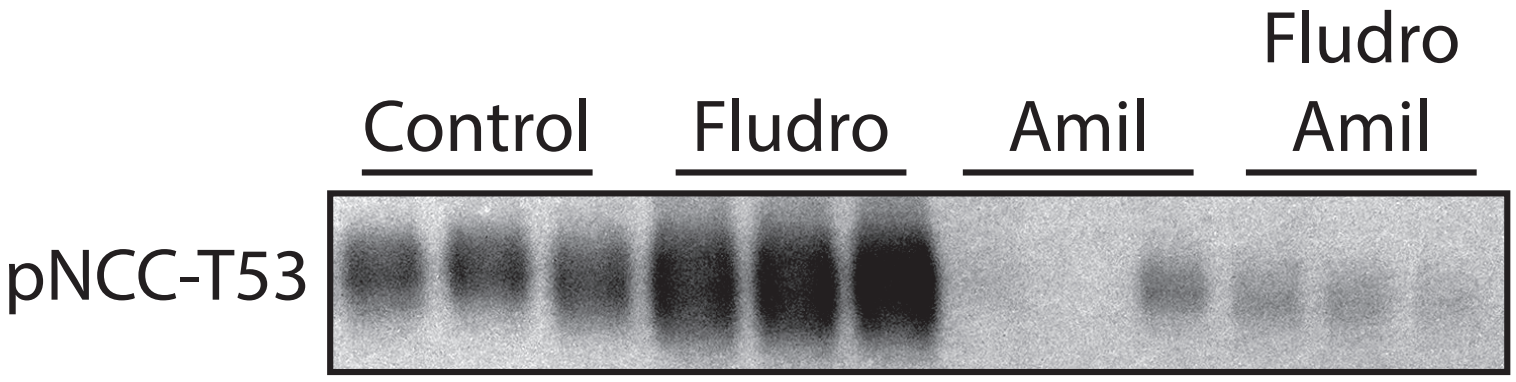
Supplemental Figure 4



Effects of high potassium intake in control and MR knockout mice. Panel a shows western blot of ENaC. Panels b-d quantify the results in panel a. Panel e shows western blot of NCC. The results are quantified in panels f and g. High K^+ intake increased the total abundance and the cleaved/uncleaved ratio of both α and γENaC in control animals, but had no effect on these parameters in knockouts. The interaction of diet and genotype was significant. The high K^+ diet did not affect βENaC to a statistically significant degree in either genotype. High K^+ intake reduced total NCC and pNCC abundance in both genotypes and the interaction of diet and genotype was significant for both. Two-way ANOVA was used with Tukey's post-hoc test. $p<0.05$ was judged to be significant. n=4 (control NK), 4 (knockout NK), 5 (control HK), and 6 (knockout HK).

Supplemental Figure 5





Results of treatment with fludrocortisone and amiloride, to prevent the change in plasma potassium concentration. Plasma K concentration is shown in the top panel. Western blot of phosphorylated NCC is shown below.