MUTATIONS AFFECTING THE CONSERVED ACIDIC WNK1 MOTIF CAUSE INHERITED HYPERKALEMIC HYPERCHLOREMIC ACIDOSIS

SUPPLEMENTAL INFORMATIONS OF METHODS

IN VITRO FUNCTIONAL CHARACTERIZATION

Vectors

The pGH19-L- and KS-WNK1-Δ11 plasmids carrying the A634T, D635E, Q636E, Q636R or D635N mutations were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and checked by direct sequencing. The primers used are referenced in Supplemental Table 7B. We subcloned the smaller fragment of the SnaB1-BamH1 digestion from pGH19-L-WNK1-Δ11 and pGH19-KS-WNK1-Δ11 plasmids carrying the mutation into pCDNA3-L and KS-WNK1-Δ11 plasmids linearised by SnaB1-BamH1. The LigaFast Rapid DNA Ligation System (Promega) was used according to the manufacturer's instructions. A pCMV6-mKLHL3 (Origene) plasmid encoded Myc- and Flag-tagged mouse KLHL3. We deleted the Flag tag by subcloning the HindIII-EcoRV digestion fragment of pCMV6-mKLHL3 into the same plasmid digested with HindIII-PmeI. pcDNA-hCUL3 encodes HA-tagged human CUL3 is a gift from C. Rochette-Egly (Department of Functional Genomics and Cancer, University of Strasbourg, Illkirch, France).

Flag-hKLHL3 cDNA was purchased from the MRC-PPU facility of the University of Dundee and was subcloned into pCDNA5/FRT/(His)6-Protein C vector (derived from pCDNA5/FRT/V5-His (Invitrogen) as described by Derivery and Gautreau (1). All tags are fused at the N-terminus of KLHL3 cDNA. The Ubiquitin-HA expressing vector was a gift from Gervaise Loirand (INSERM UMR1087, Nantes, France)

Mutant proteins in HEK293T cells

Cell culture conditions

The stable and inducible cell lines derived were grown in DMEM medium (Gibco) supplemented with 10% (v/v) Fetal Calf Serum (Biological Industries), Penicillin/Streptomycin (0.1 mg/ml each) (Gibco), Hygromycin B 200 µg/ml (Invivogen), Blasticidin 7,5 µg/ml (Invivogen). (His)6-Protein C-Flag-hKLHL3 was induced with 10 µg/ml tetracycline (Sigma).

Immunoprecipitation

For Lysis/immunoprecipitation in denaturing conditions cell pellets were lysed at 4°C in 1% SDS + Buffer A (150 mM NaCl, 10 mM Tris-HCl pH 8) containing Complete Protease Inhibitors (Roche) and deubiquitinase inhibitors (1,25 mg/ml N-Ethylmaleimide and 2 mM 1,10-Phenanthroline, Sigma)) and immediately boiled. Samples were diluted by adding 4x volume of solution containing 1% Triton-X and 150 mM NaCl, 10mM Tris-HCl (pH 8), 1mM EDTA, Complete Protease Inhibitors (Roche) and deubiquitinase inhibitors (1,25 mg/ml N-Ethylmaleimide and 2 mM 1,10-Phenanthroline). The

resultant lysates were immunoprecipitated with anti-myc antibody (9B11, Cell Signalling) at 4°C. Immunoprecipitates were washed in 1% IGEPAL CA-630 (Sigma), 300 mM NaCl, 10 mM Tris-HCl (pH 8), 1 mM EDTA, 1,25 mg/ml N-Ethylmaleimide and 2 mM 1,10-Phenanthroline and eluted in Laemmli sample buffer. For lysis/immunoprecipitation in native conditions cell pellets were lysed at 4°C in 10 mM Tris-HCl (pH 7,8), 150 mM NaCl, 1mM EDTA, 1% IGEPAL CA-630 containing Complete protease inhibitors (Roche). The resultant lysates were immunoprecipitated with anti-myc antibody (9B11, Cell Signalling) at 4°C, washed in lysis buffer and eluted in Laemmli sample buffer.

MOUSE EXPERIMENTAL STUDIES

Radio-Telemetry Monitoring of Blood Pressure and Heart Rate

Briefly, the monitoring system consists of a transmitter (PA-C10, Data Sciences International), receiver panel, consolidation matrix and personal computer with accompanying software. Mice were anesthetized with Ketamine/xylasine. A small incision was made in the middle of neck for insertion of the telemetry transmitter and a flexible catheter of the transmitter was surgically placed in the isolate left carotid artery and advanced down to the aortic arch. The body of the transmitter was placed subcutaneously in the right ventral flank of the animal. Mice were allowed recovering for 2 weeks in individual cages.

Baseline values for blood pressure and heart rate parameters were recorded for 6 consecutive days, every 30 minutes for 30 seconds. After baseline recording, animals were fed either with a specific diet or with a specific drug (see below). Under challenge, systolic, diastolic blood pressure (BP) and heart rate were continuously monitored to visualize effects of each diet and drug.

Mouse kidney immunoblotting

For *ENaC, NCC, SPAK/OSR and NKCC2,* renal cortex samples were dissected and homogenized in a cold extraction buffer containing 0.25M sucrose, 20mM tris-Hepes pH 7.4, proteases and phosphatase inhibitors (Complete and PhosSTOP tablets; Roche Diagnostics). The homogenates were then subjected to a first centrifugation (4,000g for 15 min) to obtain post-nuclear fractions. The supernatant was centrifuged at 17,000g for 20 min: the resulting pellet corresponds to the plasma membranes-enriched fraction.

For BKC and ROMK 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 inhibitors, 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 kidney extracts were then submitted to SDS/PAGE electrophoresis, and immunoblotting was performed as described. The following antibodies were used: NCC and WNK4 (gifts from D. Ellison),

ENaC (α- and γ-subunits; gifts from J. Loffing), NCC phospho-Thr55 (S908B), SPAK (S637B), OSR1 (S149C) and SPAK/OSR1 phospho-S motif (S383/325) (S670B) and NKCC2 (S669D). The last five antibodies were obtained from the Division of Signal Transduction Therapy of the University of Dundee. Phospho-NKCC2 antibody is a gift of K. Mutig. Rabbit anti-ROMK antibodies produced by James Wade and Paul Welling at the University of Maryland School of Medicine were used at 1:4000 as previously described (2). Rabbit anti-BK alpha antibodies were purchased from Alomone Labs (APC-021) diluted 1:1000 (3). Rabbit anti-tubulin was purchased from Cell Signaling Technology (#2144, 1:3000).

For ENaC, NCC, SPAK/OSR and NKCC2, control gels were run prior to western blotting and were stained with Coomassie. Several representative bands were then quantified by densitometry to assure equality of loading between samples (4). Quantification of the band(s) corresponding to the protein of interest was performed by densitometry using the Image J software. Densitometric values were normalized to the mean of the control group that was defined as 100%, and results were expressed as mean ± s.e.m (5).

For ROMK and BK channel, 20μg of protein extract was used after quantification using a bicinchoninic acid protein assay reagent kit (Pierce). 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

WNK1 and ROMK1 Immunolocalization in the cortical nephron

WNK1 immunofluorescence

Kidneys were fixed by perfusion in the aorta with 10% formol - pH 7.0 (Microm-Microtech, France). Dissected kidneys were washed in cold PBS and frozen in isopentane cooled with liquid nitrogen or paraffin-embedded. 4 μm thick sections were blocked with PBS-0.02% BSA and then incubated overnight at 4 °C with the primary antibodies, after an antigen-retrieval step for paraffin sections in Target Retrieval Solution, Citrate pH 6.1 (Agilent Dako). The following primary antibodies were used: rabbit anti-WNK1 (1/250, A301-515A**,** Bethyl Laboratories), goat anti-AQP2 (1/500, Santa Cruz; sc515770), sheep anti-NCC (S964B) and anti-NKCC2 (1/250, S669D) both from Division of Signal Transduction Therapy of the University of Dundee. After three 10-min washes with PBS, the sections were incubated with the two fluorophore-conjugated secondary antibodies [AlexaFluor™ 488 Donkey-anti Sheep, AlexaFluor™ 546 Goat-anti Rabbit (Invitrogen™, ThermoFisher Scientific)] for two hours at room temperature and then washed with PBS. Sections were mounted with the Mountant Permafluor medium (ThermoFisher Scientific). Representative images were acquired with an inverted Olympus IX83 microscope.

ROMK1 Immunofluorescence

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 (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 solution (1 mM Tris, 0.5 mM EDTA, and 0.02% SDS). The retrieval solution and sections were heated to boiling in a microwave oven, transferred to a conventional boiling water bath (15 min), and then cooled to room temperature before the sections were thoroughly washed in distilled water to remove the SDS.

Sections were preincubated for 30 min with 2% BSA, 0.2% fish gelatin, and 0.2% sodium azide in PBS. Incubations with specific antibodies (listed above), diluted in PBS containing 1% BSA, 0.2% fish gelatin, 0.1% Tween 20, 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), the anti-ROMK was detected with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Rockland) and enhanced with Alexa Fluor 488-conjugated donkey anti-goat IgG (Jackson Laboratories). Anti-guinea pig sodium chloride cotransporter was detected with Alexa Fluor 568-conjugated donkey anti-guinea pig IgG (Jackson Laboratories), while mouse anti-calbindin D28 was detected with Alexa Fluor 633-conjugated donkey anti-mouse IgG (Invitrogen). Unconjugated secondary antibodies from Jackson Laboratories and Rockland were coupled to the respective fluorophores using kits from Invitrogen. Quantitative analysis of images was performed as described in supplemental methods.

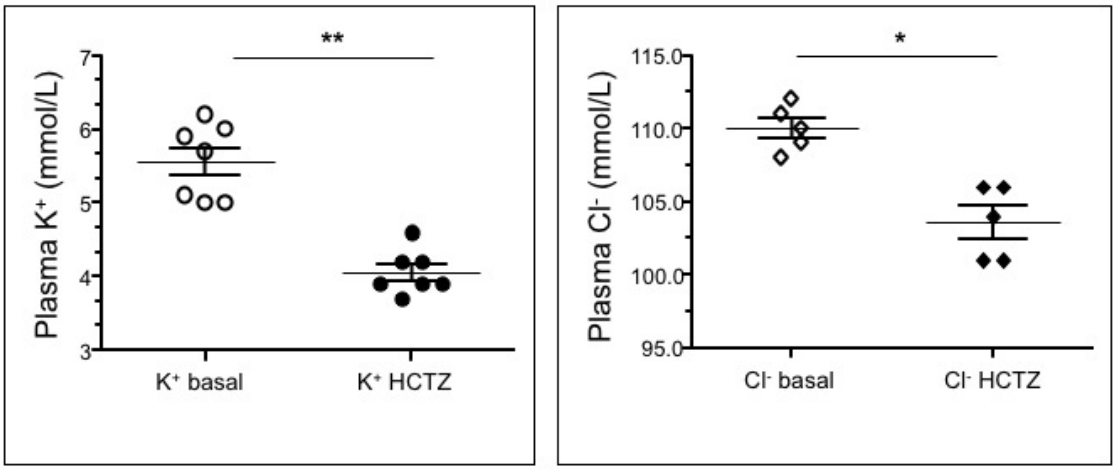
Quantitative analysis of images of ROMK immunofluorescence.

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 4 $Wnk^{+/+}$ and 5 $Wnk^{+/delE631}$ mice ($n \geq 250$ cells per group) were measured and compared. Nephron segments (DCT2 and CNT) were identified in coronal kidney sections by confocal microscopy (Zeiss LSM 510, ×10 objective lens) and segment specific antibody labeling (DCT2; calbindin alone, CNT; calbindin + AQP2). The same ROMK antibody used for immunoblotting was used for IF labeling (diluted 1:80). Mouse anti-calbindin D-28kDa was purchased from Swant (PV27, 1:600) and a chicken anti-aquaporin 2 antibody produced by James Wade at the University of Maryland School of Medicine (1:100, Ref ID 25893600).

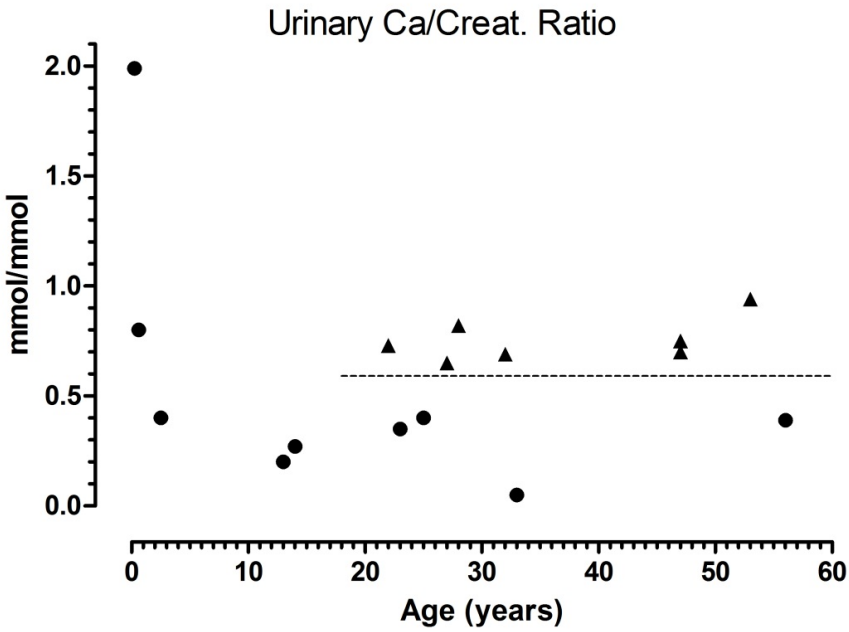
References

1. Derivery E, and Gautreau A. Assaying WAVE and WASH complex constitutive activities toward the Arp2/3 complex. *Methods Enzymol.* 2010;484:677-95.
2. Wade JB, Fang L, Coleman RA, Liu J, Grimm PR, Wang T, et al. Differential regulation of ROMK (Kir1.1) in distal nephron segments by dietary potassium. *Am J Physiol Renal Physiol.* 2011;300(6):F1385-93.
3. Zemen BG, Lai MH, Whitt JP, Khan Z, Zhao G, and Meredith AL. Generation of Kcnma1fl-tdTomato, a conditional deletion of the BK channel alpha subunit in mouse. *Physiol Rep.* 2015;3(11).
4. Picard N, Eladari D, El Moghrabi S, Planes C, Bourgeois S, Houillier P, et al. Defective ENaC processing and function in tissue kallikrein-deficient mice. *J Biol Chem.* 2008;283(8):4602-11.
5. Terris J, Ecelbarger CA, Nielsen S, and Knepper MA. Long-term regulation of four renal aquaporins in rats. *Am J Physiol.* 1996;271(2 Pt 2):F414-22.

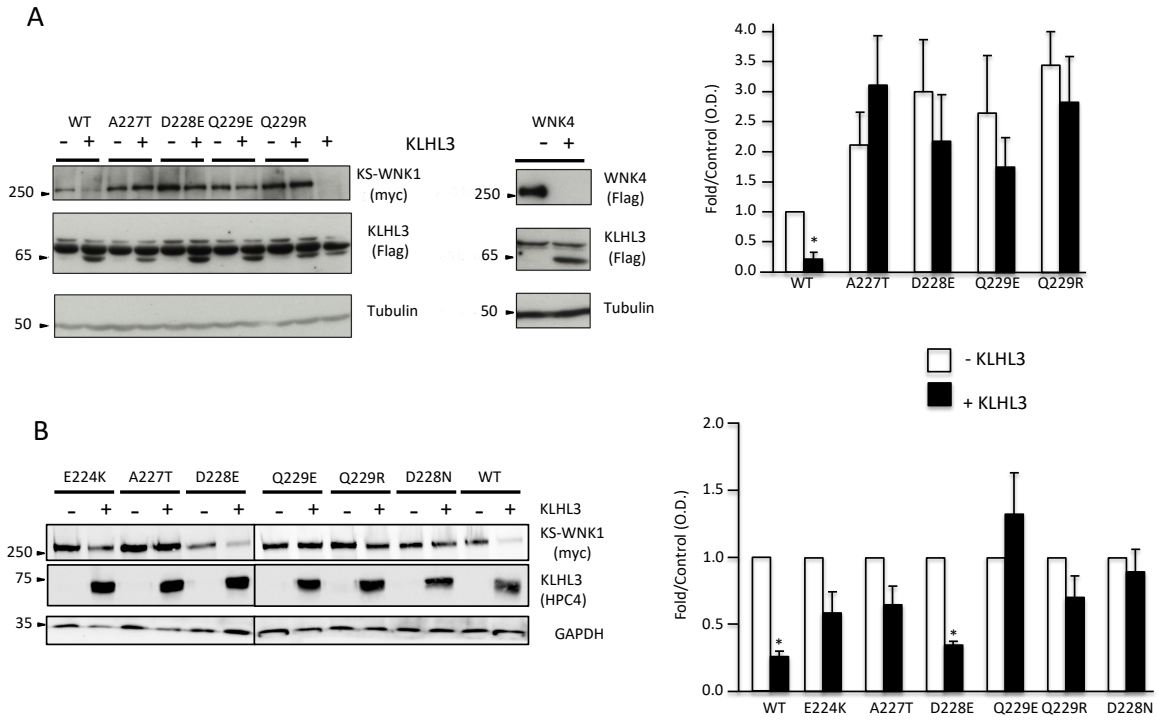
SUPPLEMENTAL FIGURES



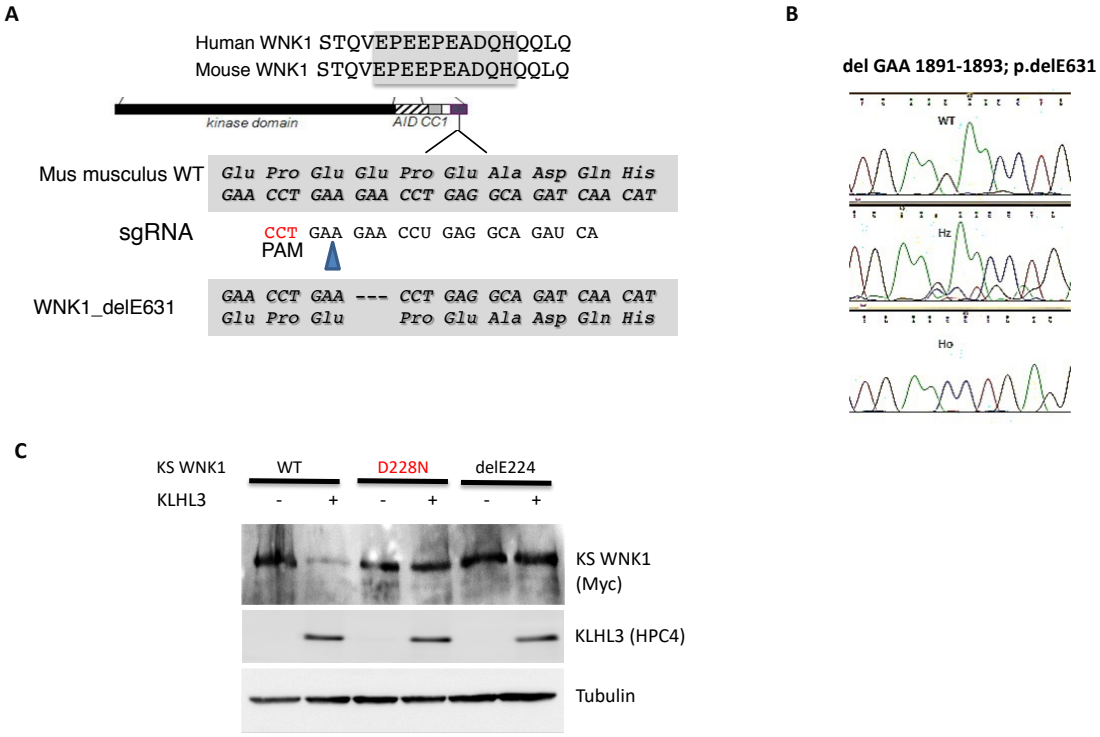
Supplemental Figure 1 : Sensitivity to hydrochlorothiazide in patients with *WNK1* ex7 mutations.
Correction of hyperkalemia (left panel) and hyperchloremia (right panel) with hydrochlorothiazide (6.25 to 25 mg/day) in five to seven subjects with WNK1 ex7 mutations.



Supplemental Figure 2: Random urinary Ca/Creat ratio in patients with *WNK1* ex7 mutations.
Patients with high ratio for age. Patients with normal ratio for age. Random urinary Ca/Creat reference values : <6months: 0.10-2.6 mmol/mmol; 6-12months: 0.09-2.2 mmol/mmol; 1-2 years:0.07-1.5 mmol/mmol; 2-3 years: 0.06-1.4 mmol/mmol; 3-5 years 0.05-1.1 mmol/mmol; 5-7 years: 0.04-08 mmol/mmol and > 0.6 mmol/mmol in adults **(15).**



Supplemental Figure 3: Effects of KLHL3 expression on KS-WNK1 wild-type and several KS-WNK1 ex7 mutants
A. *Xenopus laevis* oocytes were injected with cRNA encoding wild-type or mutant KS-WNK1, or WNK4, in the presence or absence of cRNA encoding KLHL3. 48 hours after injection proteins were extracted from oocytes and analyzed by immunoblotting using the indicated antibodies (anti-c-myc for KS-WNK1, anti-Flag for WNK4 and KLHL3, and anti-tubulin for loading control). The numbering on KS-WNK1 corresponds to the numbering on L-WNK1 minus 407 residues. Shown is a representative blot of three independent experiments (left panel).. Right panel shows the quantification of immunoblots for c-myc (KS-WNK1) normalized to total proteins and expressed relative to control (KS-WNK1 without KLHL3). Data were analyzed by one-way Anova analysis followed by Sidak's multiple comparisons test. * $p<0.05$. n = 3, except Q229R (n=2).
B. *HEK293T cells:* Flp-In T-Rex 293 cells stably and inducibly expressing (His)6-Protein C-Flag-hKLHL3 were transfected with myc-tagged KS-WNK1 (WT or mutants), as indicated. 34 hours post-transfection cells were induced with tetracycline. 14 hours later (48h post-transfection) cells were harvested and lysed in denaturing conditions. Cell lysates were subjected to immunoblot analysis with the indicated antibodies. Densitometric analysis was performed using the FUJI FILM Multi Gauge software. Results are shown as mean ±SEM. *$p<0.05$ compared to normalized controls. Shown is a representative blot of three independent experiments (left panel). Right panel shows the quantification of immunoblots.

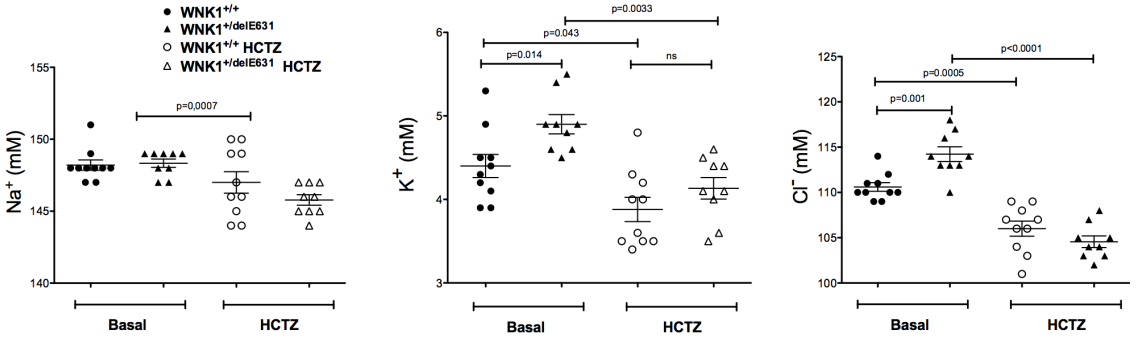


Supplemental Figure 4: Generation of the *WNK1*$^{+/delE631}$ mouse model using the CRISPR-Cas9 technology
A. The acidic motif of human and mouse WNK1 are compared. A sgRNA was designed for this motif and introduced in mouse zygotes together with Cas9 protein and a 160 nucleotide ssODN in order to obtain a double strand cleavage 3 nucleotides upstream of the protospacer adjacent motif (PAM) site (5'NGG3') and homologous recombination replacing the Asp635 by a Glu. No homologous recombination event was observed but 7 animals presented a deletion of the E631 codon.
B. DNA sequences of the wild type (upper panel) heterozygous (middle panel) and homozygous (lower panel) mice for the *WNK1* E631 deletion
C. Flp-In T-Rex 293 cells stably and inducibly expressing (His)6-Protein C-Flag-hKLHL3 were transfected with myc-tagged KS- WNK1 (WT, D228N and E224del mutant). Cell lysates were subjected to immunoblot analysis with the indicated antibodies.

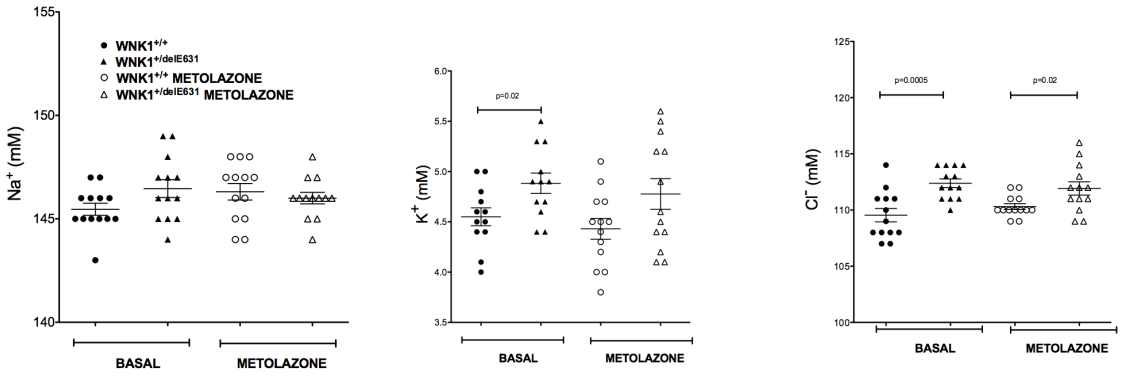
A

	SBP			DBP		
	Basal	Metolazone	p	Basal	Metolazone	p
Wnk1$^{+/+}$ (n=4)	117.4 ±4.8	123.5 ±5.7	ns	86.1 ±5.1	81.2 ±11.7	ns
Wnk1$^{+/delE631}$ (n=3)	125.6 ±5.3	114.4 ±13.6	ns	81.4 ±4.2	74.9 ±11.4	ns
	Basal	HCTZ	p	Basal	HCTZ	p
Wnk1$^{+/+}$ (n=6)	125±5.4	121.8±4.1	ns	94.8±3.7	91.6±3.2	0.0026
Wnk1$^{+/delE631}$ (n=7)	131±4.5	122.7±3.9	0.010	98.8±2.3	92.8±2	0.0043

B



C



Supplemental Figure 5 :

A. Blood Pressure Effects of HCTZ and Metolazone In Wnk1$^{+/delE631}$ male mice
Systolic (SBP) and diastolic (DBP) blood pressures measured by telemetry in Wnk1$^{+/+}$ and Wnk1$^{+/delE631}$ mice during 6 nights (basal) and at least 4 nights for HCTZ and Metolazone treatments.

B. Biological effects of HCTZ in Wnk1$^{+/delE631}$ male mice
Plasma electrolytes were measured using an i-STAT® system (Abbott) and EC-8+ cartridges test on Wnk1$^{+/+}$ (n=13) and Wnk1$^{+/delE631}$ (n=8) mice before and at the end of the treatment. Mutant mice had significantly higher plasma potassium (4.4±0.1 vs 4.9±0.1 mmol/L, p=0.01) and chloride levels (110.6±0.5 vs 114.2±0.8 mmol/L, p=0.001) compared to wild type littermates in basal conditions, but these differences which were corrected by HCTZ (3.9±0.1 vs 4.1±0.1 mmol/L, ns and 106±0.8 vs 104.6±0.6 mmol/L, ns respectively).

C. Biological effects of metolazone in Wnk1$^{+/delE631}$ male mice
Plasma electrolytes were measured in the same conditions. In basal conditions and compared to wild-type littermates (n=13), mutant mice had significantly higher plasma potassium (4.55±0.09 vs 4.9±0.1 mmol/L, p=0.02) and chloride levels (109.5±0.6 vs 112.4±0.4 mmol/L, p=0.0005), differences which were not corrected by metolazone (4.4±0.1 vs 4.8±0.15 mmol/L, p=0.037 and 110.3±0.3 vs 111.9.6±0.6 mmol/L, p=0.02 respectively).

SUPPLEMENTAL TABLE 1 : CLINICAL AND GENETIC CHARACTERISTICS OF THE INITIAL FHHt PEDIGREE 29

A. Basic clinical and biological characteristics

Family Id	Sex (M/F)	Age (years)	BMI (Kg/m^2)	SBP/DBP (mmHg)	K^+ (mmol/L)	Cl^- (mmol/L)	CO2 (mmol/L)	Creatinine (µmol/l)
II-1	F	47	23.1	104/70	5.7	107	21.1	62
III-1	F	28	18.1	105/77	5.3	108	21.2	53
III-2	F	23	19.7	101/57	5.2	109	19	71
IV-1	M	7	17.6	91/43	6.2	112	17	48
IV-2	F	4	14.7	102/52	5.9	110	19	41
IV-3	F	0.4	17.3	93/59	5.7	112	17	41

B. Results of the linkage analysis using genome wide SNP array

Chr.	Position (bp, hg19)	LOD score	Size (Mb)	Protein coding genes (n)
1	90025620-101802189	1.8	11.8	114
6	462341-12431410	1.8	12.0	104
10	93485377-114335290	1.8	20.8	253
11	5544589-15156100	1.8	9.6	159
12	768966-10214318	1.8	9.4	181
13	103961473-106552546	1.8	2.6	2
16	6458443-8781022	1.8	2.8	12

C. Main results of the whole exome sequencing

Variants	Case II-1	Case IV-2	Control	Only affected
Coding (exons, exon boundaries, splicing)	10,484	10,384	10,304	6,563
Novel Nonsynonymous	681	640	644	71
Linkage location	8	8	5	4
Probably Damaging (Polyphen2, SIFT, MutPred Server)	6	6	4	SLC30A7 KIF11 TCTN3 WNK1

SUPPLEMENTAL TABLE 2: *IN SILICO* PATHOGENICITY PREDICTIONS FOR *WNK1* MISSENSE MUTATIONS AT EXON 7

Family affected	Mutation		Charge change	Grantham distance	PolyPhen-2	Sift	MutationTaster	MutPred
	Nucleotide change	Amino acid change						
K-88	c.1891G>A	E631K	acid to basic	56	Probably damaging (p=0.999)	Deleterious (score: 0)	Disease-causing (p-value: 1.000)	Gain of ubiquitination at E631 (P= 0) Gain of methylation at E631 (P= 0.0199) Loss of helix (P = 0.0558) Gain of glycosylation at E631 (P= 0.0571) Gain of loop (P= 0.2045)
K-58, K-75	c.1900G>A	A634T	hydrophobic to neutral	58	Possibly damaging (p=0.553)	Deleterious (score: 0)	Disease-causing (p-value: 0.776)	Gain of helix (P= 0.0143) Loss of loop (P = 0.0203) Gain of relative solvent accessibility (P = 0.0289) Gain of phosphorylation at A634 (P = 0.0298) Loss of sheet (P = 0.0483)
K-91	c.1903G>A	D635N	acid to neutral	23	Probably damaging (p=1.000)	Deleterious (score: 0)	Disease-causing (p-value: 1.000)	Gain of loop (P = 0.0097) Loss of helix (P = 0.0138) Gain of glycosylation at P632 (P = 0.1505) Loss of solvent accessibility (P = 0.2882) Loss of stability (P = 0.308)
K-29 K-30	c.1905T>A	D635E	acid to acid	45	Possibly damaging (p=0.867)	Deleterious (score: 0)	Disease-causing (p-value: 0.664)	Loss of loop (P = 0.0073) Gain of helix (P = 0.0078) Gain of glycosylation at P632 (P = 0.1112) Loss of stability (P = 0.6081) Gain of sheet (P = 0.6509)
K-73	c.1906C>G	Q636E	neutral to acid	29	Probably damaging (p=0.996)	Deleterious (score: 0)	Disease-causing (p-value: 0.607)	Gain of helix (P = 0.0078) Loss of loop (P = 0.0112) Gain of relative solvent accessibility (P = 0.2363) Gain of glycosylation at P632 (P = 0.2458) Gain of solvent accessibility (P = 0.3089)
K-03, K-76	c.1907A>G	Q636R	neutral to basic	43	Probably damaging (p=0.998)	Deleterious (score: 0)	Disease-causing (p-value: 0.77)	Loss of loop (P = 0.0073) Gain of helix (P = 0.0078) Gain of solvent accessibility (P = 0.1505) Loss of glycosylation at P632 (P = 0.1796) Gain of relative solvent accessibility (P = 0.2363)

For SIFT: the change is predicted to be deleterious with a score <0.05
For MutationTaster: the change is predicted to be disease causing with a score p >0.5,
For MutPred: the change is predicted to be deleterious with a general score g > 0.75; in addition this tool gives scores (*p*) for 5 structural and functional properties. Combinations of high values of general scores and low values of property scores are referred to as hypotheses : scores with *g*> 0.5 and *p*< 0.05 are referred to as actionable hypotheses; scores with *g*> 0.75 and *p*< 0.05 are referred to as confident hypotheses and scores with *g*> 0.75 and *p*< 0.01 are referred to as very confident hypotheses.

SUPPL TABLE 3: CLINICAL SYMPTOMS AND MODALITY OF BLOOD PRESSURE MEASUREMENT OF THE 9 INDEX CASES

Id	Age at work-up (years)	Initial symptoms at discovery	Clinical symptoms	Hyperkalemia related symptoms	ECG anomalies	Co-morbidities	HBP	Modality of BP measurement
K3-1	25	HBP at 18 years, followed by HBP and hyperkalemia during first pregnancy	HBP	No	No	Obesity, active tobacco cons.	Yes	Multiple office BP measurements
K29-1	47	None : incidental findings during routine check-up	No	No	No	ovarian cancer	No	Multiple office BP measurements
K30-1	0.6	metabolic acidosis at birth, vomiting and poor weight gain	No	No	Yes	No	na	Multiple office BP measurements
K58-1	13	Paresthesia, cramps and family history of hyperkalemia	No	Paresthesia and cramps	Tall T waves	No	No	Multiple office BP measurements
K73-1	14	Chronic migraines and abdominal pain	No	No	na	Gilbert disease	No	Multiple office BP measurements
K75-1	32	Post-partum transient HBP follow-up	No	No	Normal	active tobacco cons.	No	Ambulatory BP measurement
K76-1	27	None : incidental findings during routine check-up	No	No	Tall T waves	Seminoma at 25 yo	No	Multiple office BP measurements
K88-1	24	na	HBP and short stature *	na	na	mild mental retardation	Yes	Multiple (Home BP: 135/90)
K91-1	2.5	na	na	na	na	na	No	na

* short stature present in the other members of the family

SUPPLEMENTAL TABLE 4: BASIC CLINICAL AND BIOCHEMICAL CHARACTERISTICS OF ALL CASES WITH *WNK1* EXON 7 MUTATIONS

	Clinical data					Plasma							Urine			
Id	Age (years)	Sex (F/M)	BMI (kg/m²)	SBP (mmHg)	DBP (mmHg)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)	CO_2 t (mmol/L)	Creatinine (µmol/L)	Protides (g/L)	Uric Acid (µmol/L)	Renin (mU/L)	Aldo (pmol/L)	UAG [§] (pmol/L)	Urinary Ca/creat
K3-1	25	F	32	148	116	140	5.5	108	20	77	82	181	< 5	227	54	0.4
K3-2	0.1	F	-	95	62	140	6.3	107	19	46	65	-	-	-	-	-
K29-1	47	F	23.1	104	70	136	5.7	107	21.1	62	65	194	2.4	53	50	0.7
K29-2	28	F	18.1	105	77	140	5.3	108	21.2	53	64	181	0.8	14	58	0.82
K29-3	23	F	19.7	101	57	139	5.2	109	19	71	64	190	2.3	23	43	0.35
K29-4	7	M	17.6	91	43	138	6.2	112	17	48	65	162	-	-	-	-
K29-5	4	F	14.7	102	52	136	5.9	110	19	41	64	126	-	-	-	-
K29-6	7	F	20.9	102	41	138	5.3	109	20	41	64	135	13.7	1001	41	0.97
K30-1	0.6	M	14.2	95	55	139	6.4	108	17	47	64	178	N.A.	N.A.	-	-
K58-1	13	F	19.9	105	55	140	6.0	111	21	46	-	167	3.9	132	-	0.2
K73-1	14	M	20.1	138	80	142	5.0	109	-	79	-	N.A.	<1	521	-	0.27
K75-1	32	F	18.6	117	85	141	5.1	110	21	58	64	193	3.5	75	136.5	0.69
K75-2	28	F	19.4	118	72	138	4.7	105	22	85	65		5.3	70	-	-
K76	27	M	20.9	134	81	144	6.4	112	24	85	67	N.A.	<0.1 ***	219	-	-
K88-1	22	F	19.2	148	100	140	7.1	107	19.5	54	74	150	<0.2 ***	1385	58.6	0.73
K88-2	56	M	22.5	125	90	140	5.2	105	19.4	77	73	160	0.2 ***	706	40	0.39
K88-3	47	F	18.3	140	100	139	6.1	106	18.6	63	69	160	0.28 ***	1061	45.4	0.75
K88-4	53	M	23.1	145	100	142	6.0	108	20.8	75	67	190	0.7 ***	958	45.5	0.94
K91	2.5	M	19.4	90	60	139	6.3	103	20	36	60		0.1 ***	226	15	0.4
Mean	23		20.1	116	73	140	5.8	109	20.0	60	67	169	3.3	351	53.4	0.59
SD	18		3.8	20	21	2	0.6	3	1.7	16	5	21	4.0	446	30.1	0.26

* Creatinine reference values in children: newborn: 21-75 µmol/L; 2months- 3 years: 15-37µmol/L; 3 – 7 years 27-52 37µmol/L

** Urinary Ca/creat reference values in children: <6months: 0.10-2.6 mmol/mol; 6-12months: 0.09-2.2 mmol/mol; 1-2years:0.07-1.5 mmol/mol and 2-3years: 0.06-1.4 mmol/mol

§ UAG (urinary anion gap) represents an indirect index of urinary ammonia excretion; in normal subjects UAG should be negative during acidosis.

£ Mean and SD values calculated on plasma renin values expressed in mU/L

*** unit in ng/ml/h

SUPPLEMENTAL TABLE 5 : BASAL PHENOTYPES OF HETEROZYGOUS AND HOMOZYGOUS WNK1^{delE631} MUTANT MICE

A

Blood Pressure Genotype	SBP mmHg basal	SBP mmHg High Na
Wt (n=5)	104.8±7.2	107.8±4.5
Htz $^{+/delE631}$ (n=6)	107.0±5.4 ^{ns}	110.8±3.9 ^{ns}
Hmz $^{delE631/del631}$ (n=8)	122.5±4.1 *,*	118.4±2.3 *,^{ns}

SBP represent the mean ±sem of 10 tail-cuff consecutive measurements either on normal diet (basal) or High NaCl (3%) diet
No statistical difference was observed between wild-type and heterozygous mutant mice either on basal or high Na diet
Statistical results are shown as first comparison wt vs Htz, 2nd comparison Htz and Hmz
* p<0.05

B

Biology Genotype	Na+ mmol/L	K+ mmol/L	Cl- mmol/L	HCO3- mmol/L
Wt (n=5/5)	144.4±0.4	4.3±0.03	110.8±0.8	23.5±1.0
Htz $^{+/delE631}$ (n=5)	145.2±0.8, ^{ns}	4.7±0.08 **	115.0±0.9, **	22.0±0.9 ^{ns}
Hmz $^{delE631/del631}$ (n=4)	146.0±0.7 ^{ns,ns}	4.9±0.14 **,^{ns}	113.8±1.1 *,^{ns}	22.1±1.5 ^{ns,ns}

No statistical difference was observed between wild-type and heterozygous or homozygous mutant mice for plasma Na+ and HCO3-
Statistical results are shown as first comparison wt vs Htz, 2nd comparison Htz and Hmz
* p<0.05 , ** p<0.01

SUPPLEMENTAL TABLE 6 : EFFECT OF A 1-WEEK HIGH NA DIET IN WNK1 WILD-TYPE AND HETEROZYGOUS MUTANT MICE (WNK1 DELE631+/-)

	Basal		High Na		Significance			
	WT n=4	delE631$^{+/-}$ n=4	WT n=4	delE631$^{+/-}$ n=4	p1 - *genotype - basal* wt-m	p2 *HNa* wt-m	p3 *- regimen - basal* wt-wt	p4 *vs HNa* m-m
*Blood Pressure (mmHg)**								
Night (activity)								
SBP mean	124.5	125.2	120.6	128.1	0.96	0.62	0.79	0.83
SEM	10.4	8.4	10.1	10.1				
DBP mean	94.8	92.6	90.5	94.2	0.80	0.68	0.59	0.87
SEM	5.6	5.8	4.7	7.2				
Day (rest)								
SBP mean	112.5	107.5	106.9	104.9	0.69	0.85	0.67	0.79
SEM	10.1	6.6	7.6	6.8				
DBP mean	85.1	80.7	84.0	81.5	0.55	0.72	0.87	0.91
SEM	5.6	4.3	3.8	5.4				
Urines								
Na/Creat (mmol/µmol)	15.9	11.1	216.1	158.3	ns	ns	0.09	0.003
SEM	1.3	4.1	108.2	37.5				
K/Creat (mmol/µmol)	28.5	36.4	31.8	28.9	ns	ns	0.85	0.17
SEM	0.8	2.3	22.5	4.8				
Aldosterone (nmol/L)£	3.2	5.5	0.57	1.24	0.08	0.03	0.003	0.001
SEM	0.4	0.8	0.07	0.18				
Aldo/creat	0.7	1.2	-	-	0.10	-	-	-
SEM	0.1	0.2						

SBP and DBP : average of 6 days on normal diet, average of 6 days on high Na diet
£ : On a high Na diet, urinary aldosterone went below the level of detection (<1.8 nmol/L)

SUPPLEMENTAL TABLE 7 : PRIMER SEQUENCES

A. Direct sequencing of Wnk1 exons 7 and 25 in patients

WNK1 exon	Forward	Reverse
7	TTCCCAGACAGGAATCAAGC	TTCCTTACCACCCCATCCTT
25	CCAGCAAGGTGAGTTGACAAA	GGAGAGCCAAATTCCTTTGA

B. Mutagenesis in PGH19-L and KS-WNK1, pCDNA-L and KS-WNK1 Plasmids

Mutation L-WNK1	Mutation KS-WNK1	Location	Forward
A634T	A227T	c.1900G>A	GAACCTGAAGAACCTGAGACAGATCAACATCAACAAC
D635E	D228E	c.1905T>A	TGAAGAACCTGAGGCAGAACAACATCAACAACTACAG
D635N	D228N	c.1903G>A	CCTGAAGAACCTGAGGCAAATCAACATCAACAACTAC
Q636E	Q229E	c.1906C>G	CCTGAAGAACCTGAGGCAGATGAACATCAACAAC
Q636R	Q229R	c.1907A>G	GAAGAACCTGAGGCAGATCGACATCAACAACTACAGTAC

Mutation L-WNK1	Mutation KS-WNK1	Location	Reverse
A634T	A227T	c.1900G>A	GTTGTTGATGTTGATCTGTCTCAGGTTCTTCAGGTTC
D635E	D228E	c.1905T>A	CTGTAGTTGTTGATGTTGTTCTGCCTCAGGTTCTTCA
D635N	D228N	c.1903G>A	TCTGCCTCAGGTTTTTCAGGTTCTACTTGTGTAGAAAC
Q636E	Q229E	c.1906C>G	GTTGTTGATGTTCATCTGCCTCAGGTTCTTCAGG
Q636R	Q229R	c.1907A>G	GTACTGTAGTTGTTGATGTCGATCTGCCTCAGGTTCTTC

C. Construction and analysis of the CRISPR-Cas9 mouse model

Sg-RNAs
Sg-RNA1 5'-CCUGAAGAACCUGAGGCAGAUCA-3'
Sg-RNA2 5'-CUCUGCUUCAGUUUCUACACAGG-3'
Sg-RNA3 5'-CCUGAGGCAGAUCAACAUCAACA-3

PCR and sequencing primers

WNK1 exon 7	Forward	Reverse
PCR	CTTTCGAGACCACCCCACTC	GCACATTTGTTTACTTCCCCAG
sequencing	TGCTGCTAGCACTGGCATAC	GCACATTTGTTTACTTCCCCAG

ssODN
5'ACCTCTGCTTCAGTTTCTACACAGGTAGAACCTGAAGAACCTGAGGCAGATCA
ACATCAACAGCTGCTGCAATATCAGCAACCTAGTATATCTGTGTTGT3'

D. RT-qPCR of renin

	Forward	Reverse
Renin	ATGAAGGGGGTGTCTGTGGGG	ATGTCGGGGAGGGTGGGCACCTG
mUbiquitin C	AGCCCAGTGTTACCACCAAG	ACCCAAGAACAAGCACAAGG