Title: Renal tubular SGK1 deficiency causes impaired K+ excretion via loss of regulation of NEDD4-2/WNK1 and ENaC.

Authors: Al-Qusairi L, Basquin D, Roy A, Stifanelli M, Rajaram RD, Debonneville A, Nita I, Maillard M, Loffing J, Subramanya AR, Staub O

Journal: American journal of physiology. Renal physiology

Year: 2016 Aug 1

Issue: 311

Volume: 2

Pages: F330-42

DOI: 10.1152/ajprenal.00002.2016

Renal-tubular SGK1 deficiency causes impaired K^+ excretion via the loss of regulation of NEDD4-2/WNK1 and ENaC

Lama Al-Qusairi [1,5], Denis Basquin[1,5], Ankita Roy[2], Matteo Stifanelli[1], Renuga Devi Rajaram[1], Anne Debonneville[1], Izabela Nita[1] , Marc Maillard[3], Johannes Loffing[4,5], Arohan R. Subramanya[2], and Olivier Staub[1,5]

[1] Department of Pharmacology & Toxicology, University of Lausanne, Lausanne, Switzerland

[2] Department of Medicine, University of Pittsburgh School of Medicine and VA Pittsburgh Healthcare System, Pittsburgh, Pennsylvania, USA

[3] Service of Nephrology, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland

[4] Institute of Anatomy, University of Zurich, Zurich, Switzerland

[5]National Centre of Competence in Research "Kidney.ch", Switzerland

Corresponding author:
Prof. Olivier Staub
Department of Pharmacology and Toxicology
University of Lausanne
Rue de Bugnon 27
CH-1005 Lausanne, Switzerland
Tel: +41-21 692 54 07
FAX: +41-21-692-53 55
Email: olivier.staub@unil.ch

Running Title: The role of SGK1/NEDD4-2/WNK1 pathway in K^+ clearance

The stimulation of postprandial K^+ clearance involves aldosterone-independent and -dependent mechanisms. In this context, SGK1, an ubiquitously expressed kinase, is one of the primary aldosterone induced proteins in the aldosterone sensitive distal nephron (ASDN). Germline inactivation of *SGK1* suggests that this kinase is fundamental for K^+ excretion under conditions of K^+ load, but the specific role of renal SGK1 remains elusive. To avoid compensatory mechanisms that may occur during nephrogenesis, we employed inducible, nephron-specific $Sgk1^{Pax8/LC1}$ mice to assess the role of renal-tubular SGK1 in K^+ regulation. Under standard diet, these animals exhibited normal K^+ handling. When challenged by a high K^+ diet (HKD), they developed severe hyperkalemia, accompanied by a defect in K^+ excretion. Molecular analysis revealed reduced NEDD4-2 phosphorylation and total expression. γENaC expression and α/γENaC proteolytic processing were also decreased in the mutant mice. Moreover, WNK1, which displayed in control mice punctuate staining in the DCT and diffuse distribution in the CNT/CCD, was diffused in the DCT and less expressed in the CNT/CD of $Sgk^{Pax8/LC1}$ mice. Moreover, SPAK phosphorylation, and NCC phosphorylation/apical localization were reduced in the mutant. Consistent with the altered WNK1 expression, increased ROMK apical localization was observed. In conclusion, our data suggest that renal-tubular SGK1 is important in the regulation of K^+ excretion via the control of NEDD4-2, WNK1 and ENaC.

INTRODUCTION

Maintenance of plasma potassium (K^+) level within normal physiological range (3.5 to 5.0 mM) is crucial for proper function of excitable cells (e.g., neurons, skeletal and cardiac myocytes) and represents a significant homeostatic challenge that is mediated by the tight coordination of K^+ excretion via the kidney (and to a lesser extent colon), and K^+ storage occurring mainly in skeletal muscle (67). It is well accepted that control of K^+ balance by the kidney involves both aldosterone-dependent and -independent mechanisms (55, 56, 64). Renal K^+ secretion takes place essentially in the so-called aldosterone sensitive distal nephron (ASDN) as defined by Loffing *et al.* (35). One of the most prominent proteins stimulated by aldosterone in the ASDN is the otherwise ubiquitously expressed serum- and glucocorticoid-induced kinase 1 (SGK1) (28, 34). Studies performed in cellular and animal models suggest that SGK1 controls renal Na^+ reabsorption through regulation of the epithelial Na^+ channel ENaC and Na^+,Cl^--cotransporter NCC (3, 10, 17-19, 37, 65). This involves phosphorylation of S222, S246 and S328 residues of the ubiquitin-protein ligase NEDD4-2 (5, 17, 52), known to ubiquitylate and negatively regulate ENaC and NCC (1, 3, 22, 26, 45, 51). On the other hand, it has also been suggested that SGK1 acts via phosphorylation of WNK kinases (12, 44, 48).

Several *in vitro* and *in vivo* reports confer to SGK1 a positive role in the regulation of K^+ secretion. In heterologous expression systems SGK1 is able to stimulate the K^+-channel ROMK (KCNJ1) cell surface expression (38, 66). Huang *et al.* (23) reported that mice harboring constitutive gene inactivation of *Sgk1* (*Sgk1*$^{-/-}$) exhibited a defect in acute K^+ excretion after 30-60 min of intravenous K^+ load. At 6 days of high K^+ diet (HKD), *Sgk1*$^{-/-}$ mice developed hyperkalemia but excreted K^+ similarly to control mice (23). Surprisingly, ROMK membrane expression was enhanced in *Sgk1* $^{-/-}$ mice (23). The ubiquitous expression of SGK1 together with the involvement of the colon, the skeletal muscle and the liver in K^+ homeostasis,

leave the possibility that the impaired ability of *Sgk1*$^{-/-}$ mice to handle K^+ load was due to renal and extra-renal SGK1 (6, 29).

To decipher the specific role of renal-tubular SGK1 in K^+ homeostasis, we took advantage of the previously described inducible, nephron specific *Sgk1*$^{Pax8/LC1}$ model (18). These mice showed a Na^+ losing phenotype when Na^+ supply was restricted, which correlated with decreased NCC and ENaC protein levels, and reduced NEDD4-2 phosphorylation (18). Here, we show that even though SGK1 is expressed in several organs involved in K^+ homeostasis, the action of renal-tubular SGK1 is crucial for proper renal K^+ excretion and is mediated mainly by ENaC. Our data suggest that NEDD4-2 and WNK1 are key players in SGK1-mediated K^+ homeostasis.

MATERIAL AND METHODS

Induction of renal-tubule specific SGK1 mutant mice and the verification of the renal tubular specificity. Inducible, renal-tubule specific *SGK1$^{flox/flox}$/Pax8-rTA/LC1* KO (*SGK1$^{Pax8/LC1}$*) or homozygous littermate controls *Sgk1^{Pax8}* or *Sgk1^{LC1}* mice were generated as previously described (18). Mice were housed in a temperature-controlled facility (19–22°C) with a 12:12-h light-dark cycle. To induce gene deletion, 21 to 24 day old mice were treated with doxycycline (2 mg/ml in 2% sucrose in drinking water) for 12 days. Male mice were used in the study after 1 to 3 weeks following doxycycline treatment. Genotype was identified by PCR performed on ear biopsies as described previously (18). Experimental protocols were designed with respect to the Swiss Animal Welfare Act and approved by the veterinary administration of the Canton de Vaud, Switzerland, authorization number is 2590.

Dietary manipulation. Control *(Sgk1^{Pax8}* or *Sgk1^{LC1}*) and *Sgk1$^{Pax8/LC1}$* doxycycline treated mice were fed a standard (0.3% K^+; Sniff) or high-K^+ diet (HKD) (5% K^+, K^+_3-citrate was used as K^+ supplement; Sniff) for the periods indicated in the related results.

Plasma and organs collection. Mice were anesthetized by Ketamine/Xylazine intraperitoneal injection; blood was collected by exsanguination from the retro-orbital plexus in SARSTEDT heparin contained micro tubes and plasma was separated according to manufacturer's instructions. Mice were then humanely euthanized by cervical dislocation.

Metabolic cages, urine and plasma analysis. After 2 days of adaptation in metabolic cages (Indulab, Cat. #3600M021), data related to bodyweight, food and water intake were registered and 24h urine samples were collected as described previously (18). Urine analysis (Na^+, K^+, Ca^{2+}, Mg^{2+}, PO_4^{3-}, Creatinine, Urea) was performed by the Laboratory of Clinical

Chemistry at the Lausanne Hospital (CHUV) using a Modular Analytics System (Roche Diagnostics). Plasma Na^+ and K^+ levels were measured with a flame photometer (Cole-Palmer Instrument). Plasma aldosterone measurement was performed at the Service of Nephrology of the CHUV using the radioimmunoassay kit ALDOSTERONE-RIACT according to the manufacturer's instructions.

Protein lysates preparation and immunoblotting. Frozen tissues were homogenized using buffer containing TrisHCl 50mM, pH7.5, EDTA 1mM, EGTA 1mM, Sucrose 0.27M, NaF 50mM, Na-pyrophosphate 5mM in addition to protease inhibitors purchased from ROCHE (Complete Cat. #11836145001). Protein homogenates were then centrifuged at 10'000g for 10 min at 4°C, supernatant was collected and protein concentration was measured using the BradFord method (Uptima, Cat #UPF86420). For ROMK detection, an additional ultracentrifugation step at 100'000g for 1h was performed for membrane enrichment. Immunoblots were carried out and proteins quantified as previously described (45).

Antibodies. Anti-NCC antibodies (43, 53). pT233SPAK (43); Total SPAK (Millipore #072271); Anti αENaC (53); anti β and γ (63); anti NEDD4-2 (26), anti pS222NEDD4-2 (18); anti-S328NEDD4-2 (20); anti pS256SGK1 (Santa Cruz, # SC-16744); anti GAPDH (Millipore, #MAB374); anti actin (Sigma-Aldrich, #A-5316); anti WNK1 (Exon 12; (47)); anti p382WNK1/WNK4 (Pan-pWNK (55)).

Generation and characterization of ROMK antibody. Anti-ROMK antibodies are directed against a sequence of 49 residues (342-391) of rat ROMK protein (NFGKTVEVETPHCAMCLYNEKDARARMKRGYDNPNFVLSEVDETDDTQM). Guinea pigs were immunized by GST fusion proteins containing the ROMK peptide. Antibodies were

generated at Cocalico Biologicals. Inc. (Reamstown, PA, USA). Sera of the immunized animals were collected and affinity-purified using the Maltose Binding Fusion Protein (MBP) system. The specificity of the antibodies was validated by Western blotting of lysates of HEK293 cell transfected with a plasmid encoding ROMK and kidneys from WT and ROMK knockout mice (*Kcnj1* KO) (36) kept under HKD for 5 days to enhance ROMK protein expression (Fig. 5).

Immunohistochemistry. Mice were anesthetized by Ketamine/Xylazine i.p. injection. Cardiac perfusions with PBS followed by PFA 4% were performed. Before freezing, fixed kidneys were kept in 30% sucrose in PBS solution at 4°C overnight. Immunostaining was performed on 5µ cryosections using the primary antibodies listed in the antibody section below. Alexa Fluor-488 conjugated goat anti-rabbit (Life-Technologies, Cat #A-11008,), Alexa Fluor-546 conjugated to goat anti-guinea pig (Thermo Fisher Scientific, Cat #A11074) or Alexa Fluor-546 conjugated to donkey anti-sheep (Thermo Fisher Scientific; Cat #A21098) were used. Images in the Figures are representatives of data obtained from 3 control and 2 $Sgk1^{Pax8/LC1}$ mice.

RNA extraction and TaqMan® Gene Expression Assays. Total RNA was purified from total kidneys using TRIzol (Ambion) and precipitated with isopropanol. cDNA was synthetized from 2–5 µg of total RNA using SuperScript II reverse transcriptase (Life-Technologies) and random hexamers (Promega). TaqMan Gene Expression Assays (Life Technologies) were used to analyze gene expression. Primers/probes were: *Nedd4l* (NEDD4-2; ThermoFisher Scientific Mm00459584_m1), *Sgk1* (Mm00441380_m1), *Scann1a* (αENaC; Mm00803386_m1), *Scann1b* (βENaC; Mm00441215_m1), *Scann1c* (γENaC; Mm00441228_m1), *Renin-1* (**Mm02342889_g1);** *Gapdh* (Mm99999915_g1).

Statistical analysis. The significance of the metabolic parameters of the experimental groups under different diets were analyzed using Two-way ANOVA followed by Bonferroni multiple comparisons tests. Values were considered significant when $\alpha < 0.05$. When involving only one factor, the data were statistically analyzed using an unpaired Student's t test. Values were considered significant when $p \leq 0.05$. Data are represented as means ± standard error of the mean (sem).

RESULTS

Sgk1$^{Pax8/LC1}$ mice display suppression of SGK1 in the kidney, but not in the liver.

The present model, *Sgk1$^{Pax8/LC1}$*, was described previously; recombination of genomic DNA was not only reported for the kidney, but also the liver (18), consistent with *Pax8* promotor activity as reported previously (57). We therefore analyzed *Sgk1* mRNA and SGK1 protein expression in the liver and compared it to the kidney (Fig. 1). Whereas there was recombination as seen previously in both organs (Fig. 1A), mRNA and protein levels of SGK1 (using an anti pT256-SGK1 antibody) were not affected in the liver, whereas they were strongly reduced in the kidney (Fig. 1B-D). We therefore conclude that SGK1 is primarily deleted in the nephron; we cannot exclude, however, that there is some suppression in a minor subpopulation of liver cells.

Severe hyperkalemia correlating with diminished K^+ excretion in Sgk1$^{Pax8/LC1}$ mice under challenging conditions.

To understand the involvement of renal-tubular SGK1 in K^+ homeostasis, we assessed metabolic parameters of control and inducible *Sgk1$^{Pax8/LC1}$* mice under normal (ND; containing 0.3 % K^+) and high K^+ diet (HKD, 5% of K^+; K^+_3-citrate) after 48 hours. We sacrificed the animals at the end of the daylight period, in order to have maximal aldosterone levels and assure highest difference between *Sgk1$^{Pax8/LC1}$* and controls. No significant differences in urine and plasma electrolytes were observed between the genotypes under ND (Fig. 2 and Table 1). After 2 days of HKD, *Sgk1$^{Pax8/LC1}$* mice developed severe hyperkalemia and mild hyponatremia (Fig. 2A, B), accompanied with elevated aldosterone levels (Fig. 2C), but no change of mRNA levels of renin 1 in the kidney (Fig. 2F). K^+ excretion was enhanced upon the consumption of HKD in both genotypes, but this increase was less important in *Sgk1$^{Pax8/LC1}$*mutants (Fig. 2D). No

significant difference in Na^+ excretion was observed between the two groups (Fig. 2E). After 5 days of HKD consumption, the mutant mice were capable to increase their urinary K^+ secretion to levels similar to the one observed in control mice, as no significant difference in K^+ excretion was observed between control (1664.2 +/- 71 mmol/day) and mutant (1319.5 +/- 124 mmol/day) mice after 5 days of HKD intake, suggesting that they come back into balance.

Taken together, these data suggest that basal levels of K^+ excretion do not require the renal-tubular SGK1, whereas the kinase appears to be crucial for the adjustment of K^+ elimination after a high K^+ load for 48 hours. Moreover, the finding that *Sgk1*$^{Pax8/LC1}$ show higher circulating aldosterone but no change in kidney renin 1 mRNA levels under basal and HKD conditions, suggests that the increased aldosterone levels are primarily a compensation for disturbed K^+ regulation.

Aberrant ENaC cleavage, expression and membrane localization in Sgk1$^{Pax8/LC1}$ *mice under HKD*

It was previously described that *Sgk1*$^{-/-}$ mice develop hyperkalemia when subjected to HKD (23). Electrophysiological measurements in isolated perfused cortical collecting ducts (CCDs) from $Sgk1^{-/-}$ animals showed lower absolute and amiloride-sensitive transepithelial potential differences, suggesting a defect in Na^+,K^+-ATPase and/or ENaC (23). We therefore assessed ENaC expression and localization including full-length and proteolytically processed forms, representing ENaC maturation and activation (24, 46). Under ND, ENaC showed similar protein expression and patterns in *Sgk1*$^{Pax8/LC1}$ versus control mice (data now shown). However, after 2 days of HKD, the cleavage product of αENaC, and both full-length and cleaved γENaC were decreased with no change in βENaC (Fig. 3A,B); this difference was accompanied by a decrease in αENaC apical localization and a reduction in intracellular and apical γENaC

expression (Fig. 3C). mRNA levels of the ENaC subunits did not change (not shown). These observations suggest that under HKD, the deficiency of renal epithelial SGK1 causes a defect in ENaC regulation.

Sgk1$^{Pax8/LC1}$ mice display alterations in NEDD4-2/WNK1pathway under HKD

As outlined above, it is well documented that SGK1 phosphorylates NEDD4-2, thereby interfering with NEDD4-2 dependent inhibition of ENaC and NCC (3, 5, 8, 17, 52). Because membrane localization of α- and γENaC was altered in *Sgk1$^{Pax8/LC1}$* mice under HKD, we evaluated the phosphorylation status of NEDD4-2 and observed a reduction in S222 and S328 phosphorylation in *Sgk1$^{Pax8/LC1}$* mice (Fig. 4A,B). Interestingly, total NEDD4-2 expression was also decreased (Fig. 4A-C) without any change in mRNA levels (not shown). No alteration in NEDD4-2 phosphorylation or protein levels was observed in the *Sgk1$^{Pax8/LC1}$* mice under normal diet (not shown). We have recently demonstrated that WNK1 is ubiquitylated and negatively controlled by NEDD4-2. Proteins levels of the "long" kinase active isoform (L-WNK1) protein levels were increased in *Neddl$^{Pax8/LC1}$* animals kept under high Na^+ diet and decreased in *Sgk1$^{Pax8/LC1}$* mice under low Na^+ diet (47). Under the present conditions (48h of HKD), Western blot analysis did not reveal a significant decrease in WNK1 in the *Sgk1$^{Pax8/LC1}$* mice (Fig. 4A,B), but we observed important changes by immunofluorescence analysis. The stainings in control mice showed that in the DCT (co-localization with NCC), WNK1 exhibited a punctate pattern (Fig. 4D). Such localization profile had been previously described for WNK4 (55). On the other hand, in the CNT/CD (co-localization with AQP2) of these mice, WNK1 was homogenously diffused within the cells (Fig. 4E). This segment-specific pattern of WNK1 may be related to differences in the signaling pathway associated to WNK1 in each segment. In contrast, in the *Sgk1$^{Pax8/LC1}$* mice, WNK1 in the DCT displayed a diffuse localization (Fig. 4D). In addition, its

expression was reduced in the CNT/CD compared to control littermates (Fig. 4E). These deregulations were related to a decrease in WNK activity in both DCT and CNT/CD of mutant compared to control mice as evidenced by the reduced staining with a p382WNK1/WNK4 (Pan-pWNK) antibody, an indicator of WNK1/WNK4 activity (Fig. 5). These data suggest that SGK1 controls NEDD4-2 phosphorylation also under HK conditions (as was the case with a low Na^+ diet (18)), and influences the subcellular localization/expression and activity of WNK1.

Sgk1$^{Pax8/LC1}$ KO mice show increased ROMK expression

SGK1 had been suggested to be a positive regulator of ROMK (32, 38, 44, 66, 68, 69). Surprisingly, however, in the hyperkalemic *Sgk1$^{-/-}$* KO mice, apical localization of ROMK was increased (23). One possible explanation for this apparent contradiction might be compensatory mechanisms taking place during nephrogenesis. We generated a novel anti-ROMK antibody in guinea pigs as described in Material and Methods, and validated the antibody by Western blotting (Fig. 6A,B). The antibody recognized transfected ROMK in HEK293 cells and in total kidney membrane extracts, whereas the corresponding bands were not visible in non-transfected cells or in kidneys from ROMK KO mice (*Kcnj1* KO mice). We then analyzed ROMK in *Sgk1$^{Pax8/LC1}$* kidneys from mice kept under HKD and found also in this model a slight increase in the fully-glycosylated form of ROMK in *Sgk1$^{Pax8/LC1}$* animals (Fig. 6C, D). Immunofluorescence analysis further revealed that ROMK protein abundance and membrane expression were enhanced both in the DCT and CNT/CD of *Sgk1$^{Pax8/LC1}$* animals as indicate the co-localization images of ROMK with either NCC or αENaC (Fig. 6E, F). This suggests that the hyperkalemia triggered by renal SGK1 deletion is independent of ROMK.

NCC and SPAK phosphorylation is altered in Sgk1$^{Pax8/LC1}$ mice

NCC dephosphorylation under K^+ load has been described in several reports (42, 53-55, 58). To analyze the response of NCC to the observed hyperkalemia in *Sgk1$^{Pax8/LC1}$* mice, we analyzed NCC phosphorylation and expression both under ND and after 2 days of HKD. We found that under ND, NCC phosphorylation and expression were similar in *Sgk1$^{Pax8/LC1}$* and control mice (not shown). Under HKD, NCC phosphorylation levels were much weaker in the mutant mice and no difference in total NCC expression was observed (Fig. 7A-C). In addition, immunofluorescence analysis of kidney sections revealed that NCC was less localized to the apical membrane in the KO mice (Fig. 7C). We then asked if this weak NCC phosphorylation correlated with changes in SPAK phosphorylation, a kinase known to phosphorylate and activate NCC (43). SPAK is phosphorylated by WNK kinases on threonine (T233) in the T-loop of the kinase domain that is crucial for SPAK activation (2, 61). Our IF analyses revealed that similarly to WNK1, total SPAK exhibited a punctuate pattern in the distal convoluted tubule (DCT) of control animals, which was less pronounced in *Sgk1$^{Pax8/LC1}$* mice (Fig. 7D). Phosphorylation at T233 was reduced in the DCT as compared to control animals consistent with the reduced NCC phosphorylation (Fig. 7E). Together, these data show that under HKD, NCC phosphorylation and membrane localization are both diminished in the *Sgk1$^{Pax8/LC1}$* mice, coinciding with a reduction in SPAK activation.

DISCUSSION

This study shows that mice with inducible deletion of renal-tubular SGK1 exhibit reduced K^+ excretion as compared to control animals, leading to severe hyperkalemia when subjected to HKD. The reduced K^+ excretion is correlated to altered NEDD4-2 and WNK1 in addition to reduced apical membrane expression of ENaC. Our observations confirm partly the previous report by Huang *et al.* demonstrating that total *Sgk1*$^{-/-}$ mice display normal K^+ handling when fed standard diet, but develop hyperkalemia, although without a difference in K^+ excretion, when challenged by HKD (23). We also observed elevated aldosterone levels (but no difference in renin 1 mRNA expression in the kidney) in *Sgk1*$^{Pax8/LC1}$ mice both under standard and K^+ rich diets similarly to *Sgk1*$^{-/-}$ mice (23). Given the similar K^+ content in the HKD used in both studies (5% K^+), the lower K^+ excretion observed under chronic HKD in *Sgk1*$^{Pax8/LC1}$ but not *Sgk1*$^{-/-}$ mice suggests that renal-tubular SGK1 plays a critical role in the regulation of K^+ excretion under HKD and its role may be attenuated by compensatory mechanisms triggered during nephrogenesis in *Sgk1*$^{-/-}$ mice. Extra-renal SGK1 had been shown previously to play a role in K^+ homeostasis as suggested by experiments performed in *Sgk1*$^{-/-}$ animals demonstrating that hepatic SGK1 regulates kalemia via its role in insulin-stimulated K^+ uptake (6). In our mice, we found no evidence for suppression of SGK1 in the liver; although we cannot exclude suppression of SGK1 in a subpopulation of liver cells, we consider it as unlikely that hepatic SGK1 plays a major role in this model. Finally, we cannot exclude that strain background differences may contribute to the alterations as well.

Our molecular analyses suggest that the effect of SGK1 on K^+ balance is mediated at least in part by the positive regulation of ENaC, implicating phosphorylation and consequent interference with NEDD4-2 action (5, 17-19, 21, 25, 31, 52). After 2 days of HKD, NEDD-2 phosphorylation and total expression were reduced in *Sgk1*$^{Pax8/LC1}$ with no alteration of its

mRNA level. Similar observations were made in *Sgk1*$^{Pax8/LC1}$ animals under low salt diet showing reduced NEDD4-2 phosphorylation on both S222 and S328 sites (18). However, the decrease in NEDD4-2 total expression found here might be due to the hyperkalemia and/or to increased aldosterone levels. Indeed, van der Lubbe *et al.* had shown that NEDD4-2 expression was decreased in rats fed with HKD (59). Moreover, Loffing-Cueni *et al.* had described that low Na^+ diet caused reduction in NEDD4-2 expression, and provided evidence that it was aldosterone dependent inhibition of NEDD4-2 levels (33). Taken together, this suggests that aldosterone inhibits NEDD4-2 action via 1) SGK1-dependent phosphorylation (likely a rapid mechanism of regulation) and 2) lowering its expression by an uncharacterized post-transcriptional mechanism (slow or late mode). NEDD4-2 phosphorylation at S328 interferes with regulation of ENaC (17, 52). Consistent with this, we observed under HKD lower expression of γENaC, as well as decreased cleavage of α- and γENaC, features that had been associated with increased ubiquitylation of ENaC (24, 27, 49, 50). Our findings are in line with previous reports showing the decrease in γENaC cleavage in *Sgk1*$^{-/-}$ mice treated by aldosterone for 7 days (19). These *Sgk1*$^{-/-}$ mice exhibited a Na^+ losing phenotype under low salt diet, but intriguingly an increase in amiloride-sensitive Na^+ currents, as measured by whole-cell patch-clamping in the CCD. In contrast, low amiloride-sensitive transepithelial potential differences (a marker for ENaC activity) in isolated CCDs from *Sgk1*$^{-/-}$ mice under HKD were reported by Huang *et al.* (23). Similarly to the *Sgk1*$^{-/-}$ mice, *Sgk1*$^{Pax8/LC1}$ did not exhibit Na^+ loss compared to control mice when fed HKD despite the observed decrease in α and γENaC inactivation and NCC. One possible explanation is an increase in Na^+ reabsorption in the proximal tubules as previously reported in Sgk1$^{-/-}$ under low salt diet (65). Interestingly, transgenic mice with specific deletion of αENaC in renal-tubules (39) or only in the CNT (13, 41) exhibit hyperkalemia and decreased K^+ excretion. In view of what precedes, we consider it as very likely that the low ENaC cleavage and membrane localization in the *Sgk1*$^{Pax8/LC1}$ mice account

at least in part for the observed decrease in K^+ excretion, by limiting the electrogenic driving force for K^+ secretion.

We also observed that *Sgk1*$^{Pax8/LC1}$ mice exhibited decreased phosphorylation and apical localization of NCC after 2 days of HKD. Vallon *et al.*, had shown that *Sgk1*$^{-/-}$ mice fed with HKD (5% K^+) for 7 days exhibit decreased NCC phosphorylation and total protein expression (58). The longer exposure to HKD may explain this more severe NCC alteration observed by these authors. Our data suggest that the decrease in NCC apical localization in *Sgk1*$^{Pax8/LC1}$ mice is likely an effect of reduced NEDD4-2 phosphorylation leading to increased activity towards NCC (3). The deregulation of NCC in our model was accompanied by reduced phosphorylation of WNK1/4 S382 and SPAK T233. Recently, Roy *et al.* have reported that L-WNK1 kinase, known to stimulate NCC via SPAK/OSR1 kinase, is a target of NEDD4-2 (47) and the expression of L-WNK1 was reduced in *Sgk*$^{Pax8/LC1}$ mice fed with a low Na^+ diet. Though we did not detect a statistically significant reduction in WNK1 protein expression in *Sgk*$^{Pax8/LC1}$ mice under our conditions, we did observe a striking alteration in the WNK1 localization pattern in both the DCT and the CNT/CD. Taken together with the observation that WNK1 phosphorylation was reduced under HKD conditions, our data suggest that SGK1 regulates NCC phosphorylation via WNK kinases, and the NEDD4-2/WNK1/SPAK pathway participates in this process. Another mechanism for NCC regulation was proposed by Rozansky & collaborators, who suggested that phosphorylation of WNK4 by SGK1 on S1196 inhibits WNK4 dependent downregulation of NCC (48). However, regulation of NCC by WNK4 is complex, and can be both inhibitory and stimulatory, depending on the physiological context (9). To our knowledge, no antibodies against this WNK4 phosphorylation site are currently available (32, 44), hence we were not able to test this possibility in our model. It was also reported that intracellular Cl^- is able to inhibit WNK kinases (4, 40) and evidence was presented

that low plasma K^+ leads to lower intracellular [Cl^-] and stimulation of WNK kinase activity (i.e. WNK1, WNK3 and WNK4 (7, 54, 55)). Consequently, hyperkalemia may cause an increase in intracellular [Cl^-] and inhibition of the WNK kinases. Taken together, the deregulation in NCC apical localization and phosphorylation in *Sgk1*$^{Pax8/LC1}$ mice under HKD may be due to decreased NEDD4-2 phosphorylation and reduced L-WNK1 expression and activity, exacerbated by hyperkalemia. Moreover, it is important to note that the reduced phosphorylation levels of NCC is likely a secondary effect of the hyperkalemia and not the cause of it.

The regulation of ROMK by SGK1 is still under debate. Several *in vitro* reports suggest a positive regulation of the channel by SGK1 (11, 38, 66). However, *in vivo* studies do not confirm this hypothesis (23, 56). Our data show that ROMK protein levels and membrane localization are increased in *Sgk*1$^{Pax8/LC1}$ mice under HKD, indicating that a deregulation of ROMK cannot account for the decrease in K^+ excretion in *Sgk1*$^{Pax8/LC1}$ animals. This is in agreement with the observation made in *Sgk*1$^{-/-}$ and aldosterone synthase-KO mice, which exhibit hyperkalemia together with increased ROMK membrane expression when fed with HKD (23, 56). Importantly, *in vitro* and *in vivo* evidences suggest that L-WNK1 inhibits ROMK by stimulating its endocytosis (14, 30, 60, 62). Consequently, the increased ROMK levels are compatible with the observed decrease of WNK1 expression and activity in our mouse model. We note that the findings regarding ROMK regulation in our mice indicate that the mechanisms of K^+ excretion, affected by SGK1, may also involve other channels/transporters such as the BK (15), Kir4.1 (Kcnj10) (70) channels or the H^+,K^+-ATPase (16). This will be addressed by future studies.

Taken together, the renal-tubular SGK1 plays a crucial role in K^+ homeostasis via the regulation of NEDD4-2 mediated ENaC inhibition. *Sgk1*$^{Pax8/LC1}$ mice exhibit an alteration of WNK1 puncta in the DCT and a decrease of WNK1 staining in the CNT/CD. This is correlated with decreased SPAK/NCC phosphorylation and increased ROMK expression/apical localization in the DCT/CNT/CD.

ACKNOWLEDGMENTS

We thank Drs. Bernard Rossier, Laurent Schild, and the members of the Staub lab for comments and critically reading the manuscript. We also thank Andrée Tedjani, Sebastien Desarzens, and Jérémy Vidal for technical help, and Alessia Spirli for help with the statistical analysis. This work was supported by the Swiss National Science Foundation Grant # 310030_159735 (to OS) and 310030_143929/1 (to JL) the National Centre of Competence in Research “Swiss Kidney.ch” (to OS and JL), networking support by the COST Action ADMIRE BM1301 (to OS and JL), Novartis Foundation for medical-biological research (to OS), NIH grant R01DK098145 (to ARS). LAQ was supported by a fellowship of the Marie Curie Co-funding IKPP (International Fellowship Program on Integrative Kidney Physiology and Pathophysiology).

REFERENCES

1. **Abriel H, Loffing J, Rebhun JF, Pratt JH, Schild L, Horisberger JD, Rotin D, and Staub O**. Defective regulation of the epithelial Na+ channel by Nedd4 in Liddle's syndrome. *J Clin Invest* 103: 667-673, 1999.
2. **Alessi DR, Zhang J, Khanna A, Hochdorfer T, Shang Y, and Kahle KT**. The WNK-SPAK/OSR1 pathway: Master regulator of cation-chloride cotransporters. *Sci Signal* 7: re3, 2014.
3. **Arroyo JP, Lagnaz D, Ronzaud C, Vazquez N, Ko BS, Moddes L, Ruffieux-Daidie D, Hausel P, Koesters R, Yang B, Stokes JB, Hoover RS, Gamba G, and Staub O**. Nedd4-2 Modulates Renal Na+-Cl- Cotransporter via the Aldosterone-SGK1-Nedd4-2 Pathway. *J Am Soc Nephrol* 22: 1707-1719, 2011.
4. **Bazua-Valenti S, Chavez-Canales M, Rojas-Vega L, Gonzalez-Rodriguez X, Vazquez N, Rodriguez-Gama A, Argaiz ER, Melo Z, Plata C, Ellison DH, Garcia-Valdes J, Hadchouel J, and Gamba G**. The Effect of WNK4 on the Na+-Cl- Cotransporter Is Modulated by Intracellular Chloride. *J Am Soc Nephrol* 26: 1781-1786, 2015.
5. **Bhalla V, Daidie D, Li H, Pao AC, LaGrange LP, Wang J, Vandewalle A, Stockand JD, Staub O, and Pearce D**. Serum- and glucocorticoid-regulated kinase 1 regulates ubiquitin ligase neural precursor cell-expressed, developmentally down-regulated protein 4-2 by inducing interaction with 14-3-3. *Mol Endocrinol* 19: 3073-3084, 2005.
6. **Boini KM, Graf D, Kuhl D, Haussinger D, and Lang F**. SGK1 dependence of insulin induced hypokalemia. *Pflugers Arch* 457: 955-961, 2009.
7. **Castaneda-Bueno M, Cervantes-Perez LG, Rojas-Vega L, Arroyo-Garza I, Vazquez N, Moreno E, and Gamba G**. Modulation of NCC activity by low and high K(+) intake: insights into the signaling pathways involved. *Am J Physiol Renal Physiol* 306: F1507-1519, 2014.
8. **Chandran S, Li H, Dong W, Krasinska K, Adams C, Alexandrova L, Chien A, Hallows KR, and Bhalla V**. Neural precursor cell-expressed developmentally down-regulated protein 4-2 (Nedd4-2) regulation by 14-3-3 protein binding at canonical serum and glucocorticoid kinase 1 (SGK1) phosphorylation sites. *J Biol Chem* 286: 37830-37840, 2011.
9. **Chavez-Canales M, Zhang C, Soukaseum C, Moreno E, Pacheco-Alvarez D, Vidal-Petiot E, Castaneda-Bueno M, Vazquez N, Rojas-Vega L, Meermeier NP, Rogers S, Jeunemaitre X, Yang CL, Ellison DH, Gamba G, and Hadchouel J**. WNK-SPAK-NCC cascade revisited: WNK1 stimulates the activity of the Na-Cl cotransporter via SPAK, an effect antagonized by WNK4. *Hypertension* 64: 1047-1053, 2014.
10. **Chen S, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, and Pearce D**. Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc Natl Acad Sci U S A* 96: 2514-2519, 1999.
11. **Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, and Pearce D**. Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc Natl Acad Sci U S A* 96: 2514-2519, 1999.
12. **Cheng CJ, and Huang CL**. Activation of PI3-kinase stimulates endocytosis of ROMK via Akt1/SGK1-dependent phosphorylation of WNK1. *J Am Soc Nephrol* 22: 460-471, 2011.
13. **Christensen BM, Perrier R, Wang Q, Zuber AM, Maillard M, Mordasini D, Malsure S, Ronzaud C, Stehle JC, Rossier BC, and Hummler E**. Sodium and potassium balance depends on alphaENaC expression in connecting tubule. *J Am Soc Nephrol* 21: 1942-1951, 2010.
14. **Cope G, Murthy M, Golbang AP, Hamad A, Liu CH, Cuthbert AW, and O'Shaughnessy KM**. WNK1 affects surface expression of the ROMK potassium channel independent of WNK4. *J Am Soc Nephrol* 17: 1867-1874, 2006.
15. **Cornelius RJ, Wen D, Li H, Yuan Y, Wang-France J, Warner PC, and Sansom SC**. Low Na, high K diet and the role of aldosterone in BK-mediated K excretion. *PLoS One* 10: e0115515, 2015.
16. **Crambert G**. H-K-ATPase type 2: relevance for renal physiology and beyond. *Am J Physiol Renal Physiol* 306: F693-700, 2014.
17. **Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraibi A, Pratt JH, Horisberger JD, Pearce D, Loffing J, and Staub O**. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. *EMBO J* 20: 7052-7059, 2001.

18. **Faresse N, Lagnaz D, Debonneville A, Ismailji A, Maillard M, Fejes-Toth G, Naray-Fejes-Toth A, and Staub O**. Inducible kidney-specific Sgk1 knockout mice show a salt-losing phenotype. *Am J Physiol Renal Physiol* 302: F977-985, 2012.
19. **Fejes-Toth G, Frindt G, Naray-Fejes-Toth A, and Palmer LG**. Epithelial Na+ channel activation and processing in mice lacking SGK1. *Am J Physiol Renal Physiol* 294: F1298-1305, 2008.
20. **Flores SY, Loffing-Cueni D, Kamynina E, Daidie D, Gerbex C, Chabanel S, Dudler J, Loffing J, and Staub O**. Aldosterone-Induced Serum and Glucocorticoid-Induced Kinase 1 Expression Is Accompanied by Nedd4-2 Phosphorylation and Increased Na+ Transport in Cortical Collecting Duct Cells. *J Am Soc Nephrol* 16: 2279-2287, 2005.
21. **Gleason CE, Frindt G, Cheng CJ, Ng M, Kidwai A, Rashmi P, Lang F, Baum M, Palmer LG, and Pearce D**. mTORC2 regulates renal tubule sodium uptake by promoting ENaC activity. *J Clin Invest* 125: 117-128, 2015.
22. **Harvey KF, Dinudom A, Cook DI, and Kumar S**. The Nedd4-like Protein KIAA0439 Is a Potential Regulator of the Epithelial Sodium Channel. *J Biol Chem* 276: 8597-8601, 2001.
23. **Huang DY, Wulff P, Volkl H, Loffing J, Richter K, Kuhl D, Lang F, and Vallon V**. Impaired regulation of renal K+ elimination in the sgk1-knockout mouse. *J Am Soc Nephrol* 15: 885-891, 2004.
24. **Hughey RP, Mueller GM, Bruns JB, Kinlough CL, Poland PA, Harkleroad KL, Carattino MD, and Kleyman TR**. Maturation of the epithelial Na+ channel involves proteolytic processing of the alpha- and gamma-subunits. *J Biol Chem* 278: 37073-37082, 2003.
25. **Ichimura T, Yamamura H, Sasamoto K, Tominaga Y, Taoka M, Kakiuchi K, Shinkawa T, Takahashi N, Shimada S, and Isobe T**. 14-3-3 proteins modulate the expression of epithelial Na+ channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. *J Biol Chem* 280: 13187-13194, 2005.
26. **Kamynina E, Debonneville C, Bens M, Vandewalle A, and Staub O**. A novel mouse Nedd4 protein suppresses the activity of the epithelial Na^+ channel. *FASEB J* 15: 204-214, 2001.
27. **Knight KK, Olson DR, Zhou R, and Snyder PM**. Liddle's syndrome mutations increase Na+ transport through dual effects on epithelial Na+ channel surface expression and proteolytic cleavage. *Proc Natl Acad Sci U S A* 103: 2805-2808, 2006.
28. **Lang F, Stournaras C, and Alesutan I**. Regulation of transport across cell membranes by the serum- and glucocorticoid-inducible kinase SGK1. *Mol Membr Biol* 31: 29-36, 2014.
29. **Lang F, and Vallon V**. Serum- and glucocorticoid-inducible kinase 1 in the regulation of renal and extrarenal potassium transport. *Clin Exp Nephrol* 16: 73-80, 2012.
30. **Lazrak A, Liu Z, and Huang CL**. Antagonistic regulation of ROMK by long and kidney-specific WNK1 isoforms. *Proc Natl Acad Sci U S A* 103: 1615-1620, 2006.
31. **Liang X, Peters KW, Butterworth MB, and Frizzell RA**. 14-3-3 isoforms are induced by aldosterone and participate in its regulation of epithelial sodium channels. *J Biol Chem* 281: 16323-16332, 2006.
32. **Lin DH, Yue P, Rinehart J, Sun P, Wang Z, Lifton R, and Wang WH**. Protein phosphatase 1 modulates the inhibitory effect of With-no-Lysine kinase 4 on ROMK channels. *Am J Physiol Renal Physiol* 303: F110-119, 2012.
33. **Loffing-Cueni D, Flores SY, Sauter D, Daidie D, Siegrist N, Meneton P, Staub O, and Loffing J**. Dietary sodium intake regulates the ubiquitin-protein ligase nedd4-2 in the renal collecting system. *J Am Soc Nephrol* 17: 1264-1274, 2006.
34. **Loffing J, Flores SY, and Staub O**. Sgk kinases and their role in epithelial transport. *Annu Rev Physiol* 68: 461-490, 2006.
35. **Loffing J, Zecevic M, Feraille E, B. K, Asher C, Rossier BC, Firestone GL, Pearce D, and Verrey F**. Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. *Am J Physiol Renal Physiol* 280: F675-F682, 2001.
36. **Lu M, Wang T, Yan Q, Yang X, Dong K, Knepper MA, Wang W, Giebisch G, Shull GE, and Hebert SC**. Absence of small conductance K+ channel (SK) activity in apical membranes of thick ascending limb and cortical collecting duct in ROMK (Bartter's) knockout mice. *J Biol Chem* 277: 37881-37887, 2002.
37. **Naray-Fejes-Toth A, Canessa CM, Cleaveland ES, Aldrich G, and Fejes-Toth G**. sgk is an aldosterone-induced kinase in the renal collecting duct. *J Biol Chem* 274: 16973-16978, 1999.

38. **Palmada M, Embark HM, Yun C, Bohmer C, and Lang F**. Molecular requirements for the regulation of the renal outer medullary K(+) channel ROMK1 by the serum- and glucocorticoid-inducible kinase SGK1. *Biochem Biophys Res Commun* 311: 629-634, 2003.
39. **Perrier R, Boscardin E, Malsure S, Sergi C, Maillard MP, Loffing J, Loffing DC, Sorensen MV, Koesters R, Rossier BC, Frateschi S, and Hummler E**. Severe Salt-Losing Syndrome and Hyperkalemia Induced by Adult Nephron-Specific Knockout of the Epithelial Sodium Channel alpha-Subunit. *J Am Soc Nephrol* in press: 2016.
40. **Piala AT, Moon TM, Akella R, He H, Cobb MH, and Goldsmith EJ**. Chloride Sensing by WNK1 Involves Inhibition of Autophosphorylation. *Sci Signal* 7: ra41, 2014.
41. **Poulsen SB, Praetorius J, Damkier HH, Miller L, Nelson RD, Hummler E, and Christensen BM**. Reducing alphaENaC expression in the kidney connecting tubule induces pseudohypoaldosteronism type 1 symptoms during K+ loading. *Am J Physiol Renal Physiol* 310: F300-310, 2016.
42. **Rengarajan S, Lee DH, Oh YT, Delpire E, Youn JH, and McDonough AA**. Increasing plasma [K+] by intravenous potassium infusion reduces NCC phosphorylation and drives kaliuresis and natriuresis. *Am J Physiol Renal Physiol* 306: F1059-1068, 2014.
43. **Richardson C, Rafiqi FH, Karlsson HK, Moleleki N, Vandewalle A, Campbell DG, Morrice NA, and Alessi DR**. Activation of the thiazide-sensitive Na+-Cl- cotransporter by the WNK-regulated kinases SPAK and OSR1. *J Cell Sci* 121: 675-684, 2008.
44. **Ring AM, Leng Q, Rinehart J, Wilson FH, Kahle KT, Hebert SC, and Lifton RP**. An SGK1 site in WNK4 regulates Na+ channel and K+ channel activity and has implications for aldosterone signaling and K+ homeostasis. *Proc Natl Acad Sci U S A* 104: 4025-4029, 2007.
45. **Ronzaud C, Loffing-Cueni D, Hausel P, Debonneville A, Malsure SR, Fowler-Jaeger N, Boase NA, Perrier R, Maillard M, Yang B, Stokes JB, Koesters R, Kumar S, Hummler E, Loffing J, and Staub O**. Renal tubular NEDD4-2 deficiency causes NCC-mediated salt-dependent hypertension. *J Clin Invest* 123: 657-665, 2013.
46. **Rossier BC, and Stutts MJ**. Activation of the epithelial sodium channel (ENaC) by serine proteases. *Annu Rev Physiol* 71: 361-379, 2009.
47. **Roy A, Al-Qusairi L, Donnelly BF, Ronzaud C, Marciszyn AL, Gong F, Chang YP, Butterworth MB, Pastor-Soler NM, Hallows KR, Staub O, and Subramanya AR**. Alternatively spliced proline-rich cassettes link WNK1 to aldosterone action. *J Clin Invest* 125: 3433-3448, 2015.
48. **Rozansky DJ, Cornwall T, Subramanya AR, Rogers S, Yang YF, David LL, Zhu X, Yang CL, and Ellison DH**. Aldosterone mediates activation of the thiazide-sensitive Na-Cl cotransporter through an SGK1 and WNK4 signaling pathway. *J Clin Invest* 119: 2601-2612, 2009.
49. **Ruffieux-Daidie D, Poirot O, Boulkroun S, Verrey F, Kellenberger S, and Staub O**. Deubiquitylation regulates activation and proteolytic cleavage of ENaC. *J Am Soc Nephrol* 19: 2170-2180, 2008.
50. **Ruffieux-Daidie D, and Staub O**. Intracellular Ubiquitylation of the Epithelial Na+ Channel Controls Extracellular Proteolytic Channel Activation via Conformational Change. *J Biol Chem* 286: 2416-2424, 2011.
51. **Shi PP, Cao XR, Sweezer EM, Kinney TS, Williams NR, Husted RF, Nair R, Weiss RM, Williamson RA, Sigmund CD, Snyder PM, Staub O, Stokes JB, and Yang B**. Salt-sensitive hypertension and cardiac hypertrophy in mice deficient in the ubiquitin ligase Nedd4-2. *Am J Physiol Renal Physiol* 295: F462-470, 2008.
52. **Snyder PM, Olson DR, and Thomas BC**. Serum and Glucocorticoid-regulated Kinase Modulates Nedd4-2-mediated Inhibition of the Epithelial Na+ Channel. *J Biol Chem* 277: 5-8, 2002.
53. **Sorensen MV, Grossmann S, Roesinger M, Gresko N, Todkar AP, Barmettler G, Ziegler U, Odermatt A, Loffing-Cueni D, and Loffing J**. Rapid dephosphorylation of the renal sodium chloride cotransporter in response to oral potassium intake in mice. *Kidney Int* 83: 811-824, 2013.
54. **Terker AS, Zhang C, Erspamer KJ, Gamba G, Yang CL, and Ellison DH**. Unique chloride-sensing properties of WNK4 permit the distal nephron to modulate potassium homeostasis. *Kidney Int* 2015.
55. **Terker AS, Zhang C, McCormick JA, Lazelle RA, Meermeier NP, Siler DA, Park HJ, Fu Y, Cohen DM, Weinstein AM, Wang WH, Yang CL, and Ellison DH**. Potassium modulates

electrolyte balance and blood pressure through effects on distal cell voltage and chloride. *Cell Metab* 21: 39-50, 2015.
56. **Todkar A, Picard N, Loffing-Cueni D, Sorensen MV, Mihailova M, Nesterov V, Makhanova N, Korbmacher C, Wagner CA, and Loffing J**. Mechanisms of renal control of potassium homeostasis in complete aldosterone deficiency. *J Am Soc Nephrol* 26: 425-438, 2015.
57. **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, and Koesters R**. An efficient and versatile system for acute and chronic modulation of renal tubular function in transgenic mice. *Nat Med* 14: 979-984, 2008.
58. **Vallon V, Schroth J, Lang F, Kuhl D, and Uchida S**. Expression and phosphorylation of the Na+-Cl- cotransporter NCC in vivo is regulated by dietary salt, potassium, and SGK1. *Am J Physiol Renal Physiol* 297: F704-712, 2009.
59. **van der Lubbe N, Moes AD, Rosenbaek LL, Schoep S, Meima ME, Danser AH, Fenton RA, Zietse R, and Hoorn EJ**. K+-induced natriuresis is preserved during Na+ depletion and accompanied by inhibition of the Na+-Cl- cotransporter. *Am J Physiol Renal Physiol* 305: F1177-1188, 2013.
60. **Vidal-Petiot E, Elvira-Matelot E, Mutig K, Soukaseum C, Baudrie V, Wu S, Cheval L, Huc E, Cambillau M, Bachmann S, Doucet A, Jeunemaitre X, and Hadchouel J**. WNK1-related Familial Hyperkalemic Hypertension results from an increased expression of L-WNK1 specifically in the distal nephron. *Proc Natl Acad Sci U S A* 110: 14366-14371, 2013.
61. **Vitari AC, Deak M, Morrice NA, and Alessi DR**. The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. *Biochem J* 391: 17-24, 2005.
62. **Wade JB, Fang L, Liu J, Li D, Yang CL, Subramanya AR, Maouyo D, Mason A, Ellison DH, and Welling PA**. WNK1 kinase isoform switch regulates renal potassium excretion. *Proc Natl Acad Sci U S A* 103: 8558-8563, 2006.
63. **Wagner CA, Loffing-Cueni D, Yan Q, Schulz N, Fakitsas P, Carrel M, Wang T, Verrey F, Geibel JP, Giebisch G, Hebert SC, and Loffing J**. Mouse model of type II Bartter's syndrome. II. Altered expression of renal sodium- and water-transporting proteins. *Am J Physiol Renal Physiol* 294: F1373-1380, 2008.
64. **Welling PA**. Regulation of renal potassium secretion: molecular mechanisms. *Semin Nephrol* 33: 215-228, 2013.
65. **Wulff P, Vallon V, Huang DY, Volkl H, Yu F, Richter K, Jansen M, Schlunz M, Klingel K, Loffing J, Kauselmann G, Bosl MR, Lang F, and Kuhl D**. Impaired renal Na(+) retention in the sgk1-knockout mouse. *J Clin Invest* 110: 1263-1268, 2002.
66. **Yoo D, Kim BY, Campo C, Nance L, King A, Maouyo D, and Welling PA**. Cell surface expression of the ROMK (Kir 1.1) channel is regulated by the aldosterone-induced kinase, SGK-1, and protein kinase A. *J Biol Chem* 278: 23066-23075, 2003.
67. **Youn JH, and McDonough AA**. Recent advances in understanding integrative control of potassium homeostasis. *Annu Rev Physiol* 71: 381-401, 2009.
68. **Yue P, Lin DH, Pan CY, Leng Q, Giebisch G, Lifton RP, and Wang WH**. Src family protein tyrosine kinase (PTK) modulates the effect of SGK1 and WNK4 on ROMK channels. *Proc Natl Acad Sci U S A* 106: 15061-15066, 2009.
69. **Yun CC, Palmada M, Embark HM, Fedorenko O, Feng Y, Henke G, Setiawan I, Boehmer C, Weinman EJ, Sandrasagra S, Korbmacher C, Cohen P, Pearce D, and Lang F**. The Serum and Glucocorticoid-Inducible Kinase SGK1 and the Na(+)/H(+) Exchange Regulating Factor NHERF2 Synergize to Stimulate the Renal Outer Medullary K(+) Channel ROMK1. *J Am Soc Nephrol* 13: 2823-2830, 2002.
70. **Zhang C, Wang L, Zhang J, Su XT, Lin DH, Scholl UI, Giebisch G, Lifton RP, and Wang WH**. KCNJ10 determines the expression of the apical Na-Cl cotransporter (NCC) in the early distal convoluted tubule (DCT1). *Proc Natl Acad Sci U S A* 111: 11864-11869, 2014.

FIGURE LEGENDS

Fig. 1. *Sgk1* mRNA and SGK1 protein levels are decreased in the kidney, but not in the liver of *Sgk1*$^{Pax8/LC1}$ mutant mice. A: PCR on genomic DNA extracted from total kidney and liver showing the WT and mutant (KO) *Sgk1* bands after 12 days of doxycycline treatment. The intensity of the mutant band in the kidney is more pronounced than in the liver of the same animals. B: qRT-PCR (TaqMan) analysis of *Sgk1* mRNA in control and *Sgk1*$^{Pax8/LC1}$ KO. A significant decreased of *SGK1* mRNA level occurs in the kidney but not in the liver of the same animals, (n: 7 controls, 6 *Sgk1*$^{Pax8/LC1}$). C: Western blot (WB) analysis of phosphorylated SGK1 (p-T256) in protein lysates from total kidney (upper panel) and liver (lower panel) in control and *Sgk1*$^{Pax8/LC1}$ KO. Actin was used as a loading control. D: Protein quantification of C showing an almost complete loss of pSGK1 in the kidney of mutant mice compared to control with no alteration of pSGK1 levels in the liver, (n: 6 controls, 7 *Sgk1*$^{Pax8/LC1}$). *: p value < 0.05.

Fig. 2. K^+ excretion is defective in *Sgk1*$^{Pax8/LC1}$ mice under HKD but not ND. A, B: Plasma K^+ and Na^+ in control (white columns) and *Sgk1*$^{Pax8/LC1}$ (grey columns) mice showing significant hyperkalemia with a slight decrease in natremia in *Sgk1*$^{Pax8/LC1}$ under HKD (n: 5 controls, 7 *Sgk1*$^{Pax8/LC1}$) but not under ND (n: 12 controls, 11 *Sgk1*$^{Pax8/LC1}$). C: Plasma aldosterone is increased in *Sgk*1$^{Pax8/LC1}$ versus control mice under ND (n: 12 controls, 13 Sgk1$^{Pax8/LC1}$) and under HKD (n: 14 controls, 11 *Sgk1*$^{Pax8/LC1}$). D,E: 24h urinary K^+ and Na^+ excretion indicating reduced K^+ excretion in *Sgk*1$^{Pax8/LC1}$ mutant animals with no significant difference in Na^+ excretion (n: 12 controls, 13 *Sgk1*$^{Pax8/LC1}$); *: α value < 0.05, **: α value < 0.01. F: Quantitative qRT-PCR (Taqman) on RNA isolated from kidneys of control or *Sgk1*$^{Pax8/LC1}$ mice, kept under normal diet or 48h of HKD.

Fig. 3. Deregulation of ENaC in *Sgk1*$^{Pax8/LC1}$ mice after 2 days of HKD. A: WB analysis of ENaC in control and *Sgk1*$^{Pax8/LC1}$ mice showing a decrease in cleaved (Cl.) αENaC and in full-length and cleaved γENaC, without alteration in βENaC. B: Protein quantification from A (n: 6 controls, 7 *Sgk1*$^{Pax8/LC1}$). C: Immunofluorescence (IF) images of ENaC in control and *Sgk1*$^{Pax8/LC1}$mutant mice showing a decrease in the apical localization of αENaC (upper panel) and in the total expression of γENaC (third panel). βENaC exhibits similar pattern in both genotypes (second panel). *: p value < 0.05, **: p value < 0.01

Fig. 4. Deregulation of the NEDD4-2/WNK1 pathway in *Sgk1*$^{Pax8/LC1}$ mice after 2 days of HKD. (A) WB analysis of NEDD4-2 (N4-2) and WNK1 revealing a significant decrease in total and phosphorylated NEDD4-2 levels in *Sgk1*$^{Pax8/LC1}$ mice. A slight (non-significant) alteration was observed in WNK1 total protein levels. (B) Protein quantification from (A), (n: 7 and 6 per group); *: p value < 0.05, **: p value < 0.01. (C) Immunofluorescence images of NEDD4-2 in control and *Sgk1*$^{Pax8/LC1}$ mutant mice showing a decrease of total NEDD4-2 expression in the KO animal. (D) Co-staining of NCC (red) (upper panel) and WNK1 (green) (middle panel), a punctate pattern of WNK1 observed in the DCT of control animal disappears in *Sgk1*$^{Pax8/LC1}$ mice where WNK1 exhibit a diffused pattern. (E) Co-staining of AQP2 (red) and WNK1 (green) showing the decrease in WNK1 expression in the CNT/CD of mutant compared to control animals.

Fig. 5. WNKs phosphorylation and likely activity is decreased in both DCT and CNT/CD of *Sgk1*$^{Pax8/LC1}$ under HKD. A: Co-staining of AQP2 (green) and Pan-pWNK (red) showing reduced WNK phosphorylation in the CNT/CD of mutant compared to control animals. B: Co-staining of NCC (green) and Pan-pWNK (red) showing decreased WNK phosphorylation in the DCT of *Sgk1*$^{Pax8/LC1}$ compared to control animals.

Fig. 6. ROMK localization altered in *Sgk1*$^{Pax8/LC1}$ mice under HKD. A,B: Validation of anti ROMK antibodies. A: Western blotting of ROMK from membrane enriched preparation of kidney extracts of control and *Kcnj1 (ROMK)* KO mice after 5 days of HKD. Fg: fully glycosylated, Pg partially glycosylated, Ng non glycosylated ROMK. B: Western blotting of ROMK of untransfected HEK293 cells (1) and cells transfected with ROMK (2). C: WB analysis of ROMK in control and *Sgk1*$^{Pax8/LC1}$ mice after 2 days of HKD, Ng: non glycosylated, Pg: partially glycosylated, Fg: Fully glycosylated. D: Protein quantification from A revealing a slight increase in the fully glycosylated form of ROMK. E: Co-staining of ROMK (red) and ENaC (green) in the CNT/CD (D) F: ROMK (red) and NCC (green) in the DCT. ROMK is more enhanced at the apical membrane in the *Sgk1*$^{Pax8/LC1}$ versus control mice in both ENaC and NCC expressing segments. Note that ROMK exhibits more pronounced apical localization in the DCT than in the CNT/CD.

Fig. 7. Alteration in SPAK and NCC phosphorylation and cellular localization after 2 days of HKD. A: WB analysis of NCC in control and *Sgk1*$^{Pax8/LC1}$ mice showing reduced NCC phosphorylation of several phosphorylation sites without alteration in NCC total expression in the *Sgk1*$^{Pax8/LC1}$ mice. B: Protein quantification of A (n: 7 for both genotypes); *: $p < 0.05$, **: $p < 0.01$). C: Immunofluorescence images of total and T53 phosphorylated NCC in control and *Sgk1*$^{Pax8/LC1}$ mice. The apical localization (upper panel) and the phosphorylation state of NCC (lower panel) are both altered in the *Sgk1*$^{Pax8/LC1}$ animals. D: IF images of total SPAK showing a punctate pattern of SPAK in the control mice which becomes less pronounced in the *Sgk1*$^{Pax8/LC1}$ animals. E: Co-staining of NCC (green) (upper panel) and T233 phosphorylated SPAK (red) (middle panel) showing decreased SPAK phosphorylation in the DCT of *Sgk1*$^{Pax8/LC1}$ mice.

	Normal diet (0.3% K)		high K diet (5% K)	
	WT (12)	KO (13)	WT (12)	KO (13)
Body weight (g)	25.4 +/- 0.5	24.8 +/- 0.5	23.7 +/- 0.5	22.4 +/- 0.4
Food intake 24h/ body weight (g 24h/g of BW)	0.14 +/- 0.01	0.15 +/- 0.004	0.151 +/- 0.007	0.125 +/- 0.007
Water intake 24h/ body weight (ml 24h/g of BW)	0.18 +/- 0.01	0.21 +/- 0.01	0.34 +/- 0.02 *	0.33 +/- 0.02 *
Urine volume (ml 24h)	1.62 +/- 0.22	1.74 +/- 0.26	2.79 +/- 0.27	2.61 +/- 0.32
PO4 24/Creat	46.8 +/- 3.3	52.7 +/- 1.9	13.30 +/- 0.5	13.27 +/- 0.7
Mg 24/Creat	0.75 +/- 0.07	0.85 +/- 0.07	0.78 +/- 0.10	1.09 +/- 0.23
Ca 24 /Creat	1.59 +/- 0.5	2.38 +/- 0.68	1.10 +/- 0.16	1.01 +/- 0.13
Urea 24/Creat	530 +/- 84	436 +/- 77	307 +/- 13	319 +/- 20
Creat 24h/body weight (μmol 24h/g of BW)	162 +/- 29	135 +/- 11	304 +/- 14	264 +/- 18

Table 1: Metabolic parameters of control and SGK1$^{Pax8/LC1}$ KO mice under normal diet and after 2 days of high K^+ diet. No significant differences were observed in these parameters between control and mutant mice. (n=12 WT and 13 *SGK1*$^{Pax8/LC1}$ KO mice in both diets).
Note that water intake 24h/body weight was significantly different between ND and HKD with no significant difference in urine volume between both diets which may result from the high variability due to the evaporation in metabolic cages. *= $P < 0.05$.

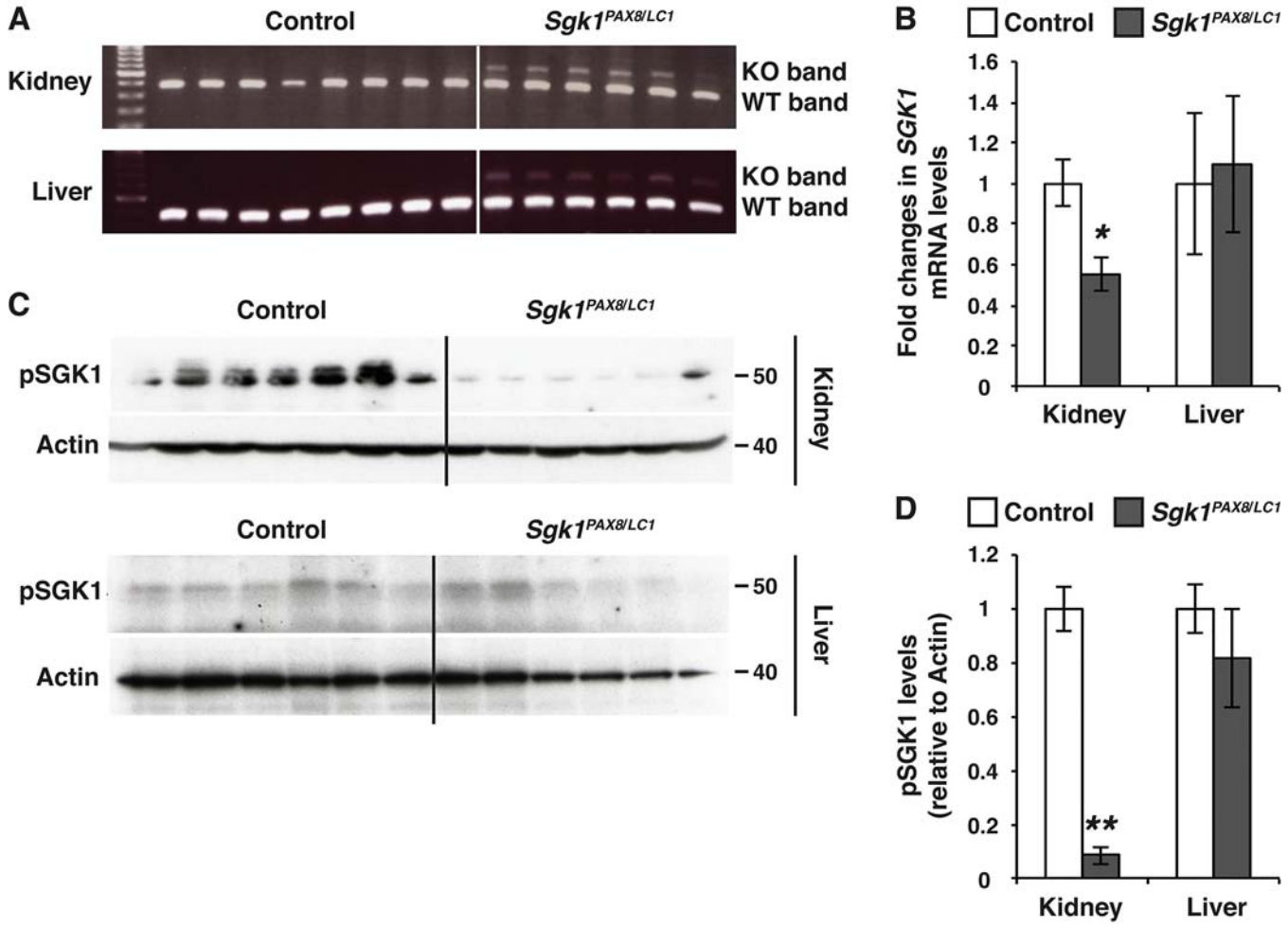
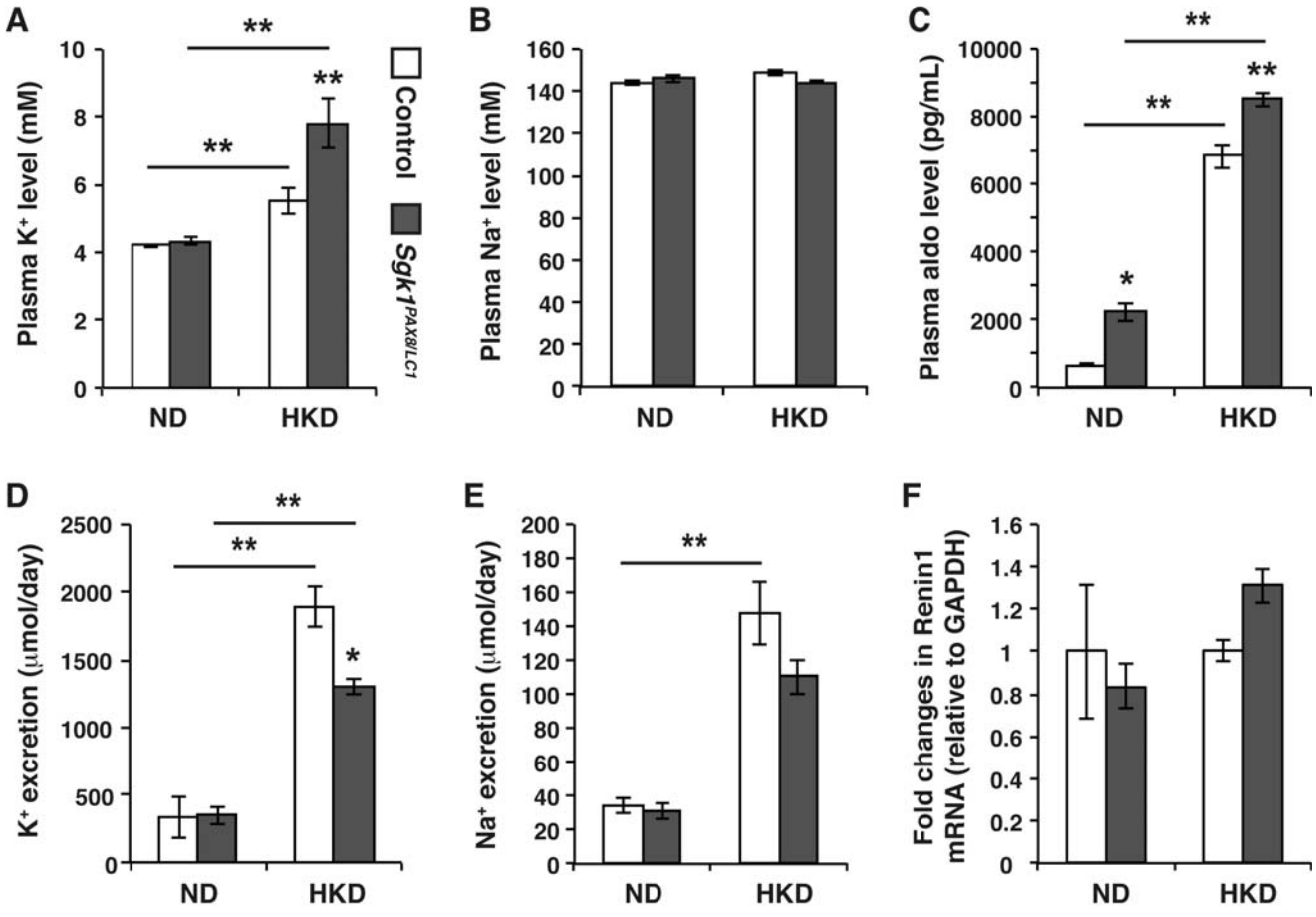


Fig. 1
Al-Qusairi et al.



Al-Qusairi et al.
Fig. 2

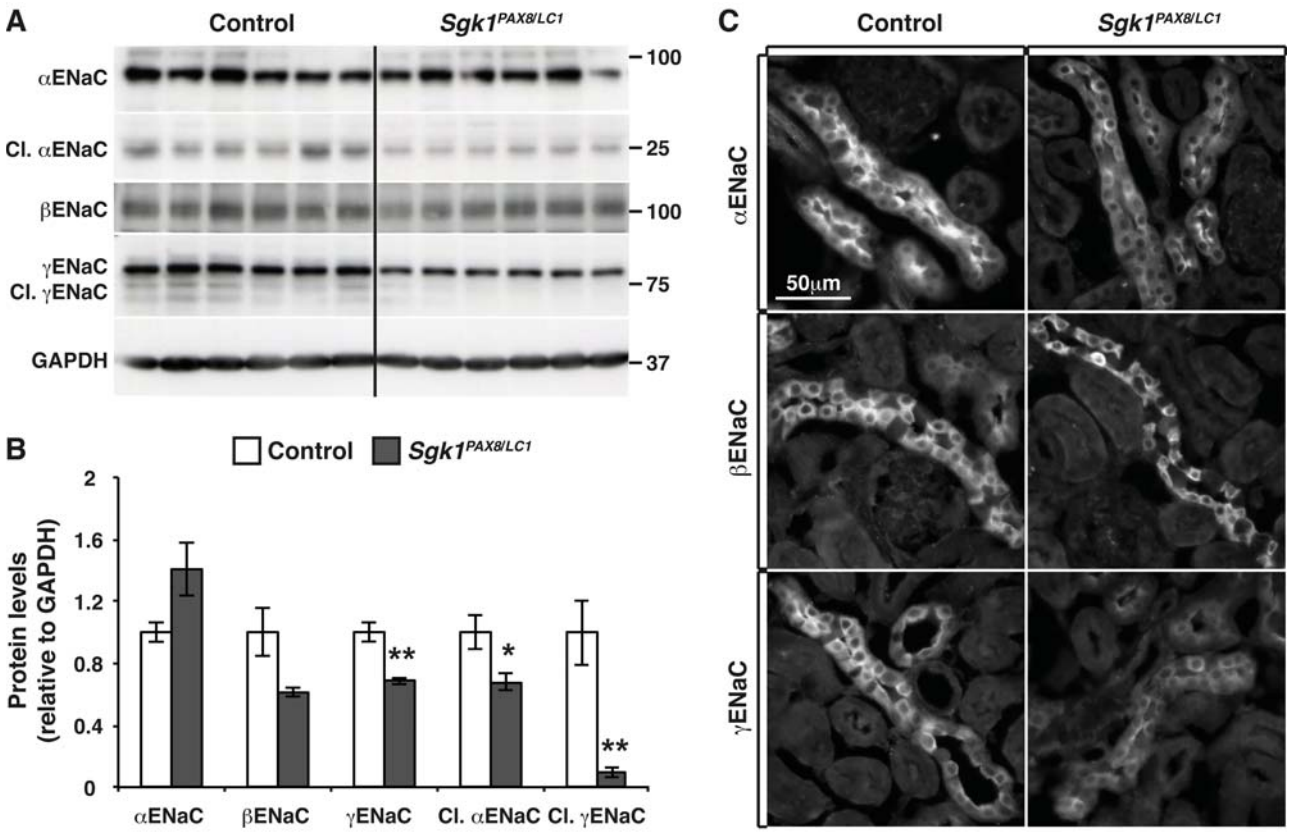


Fig.3
Al-Qusairi et al.

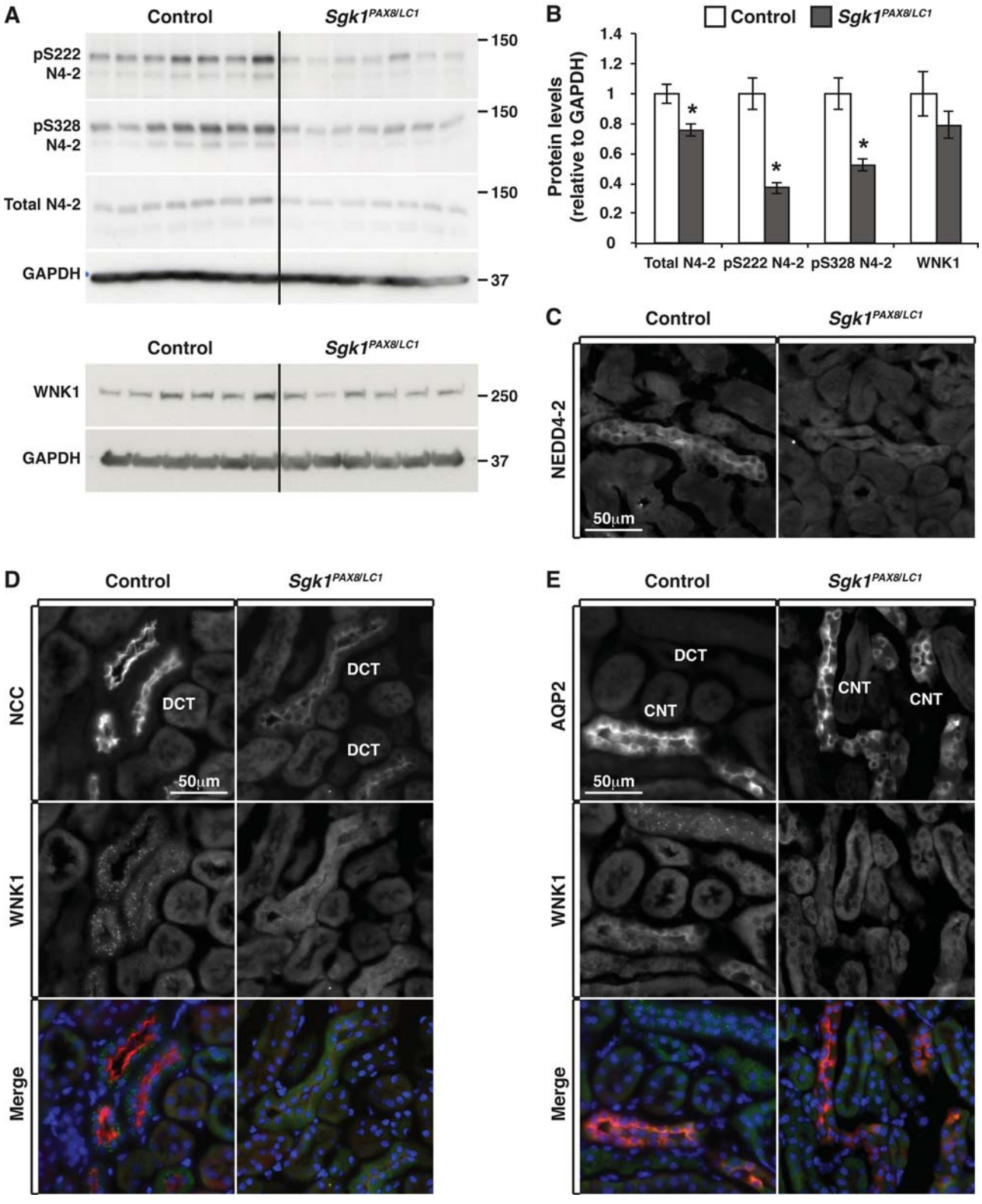


Fig.4
Al-Qusairi et al.

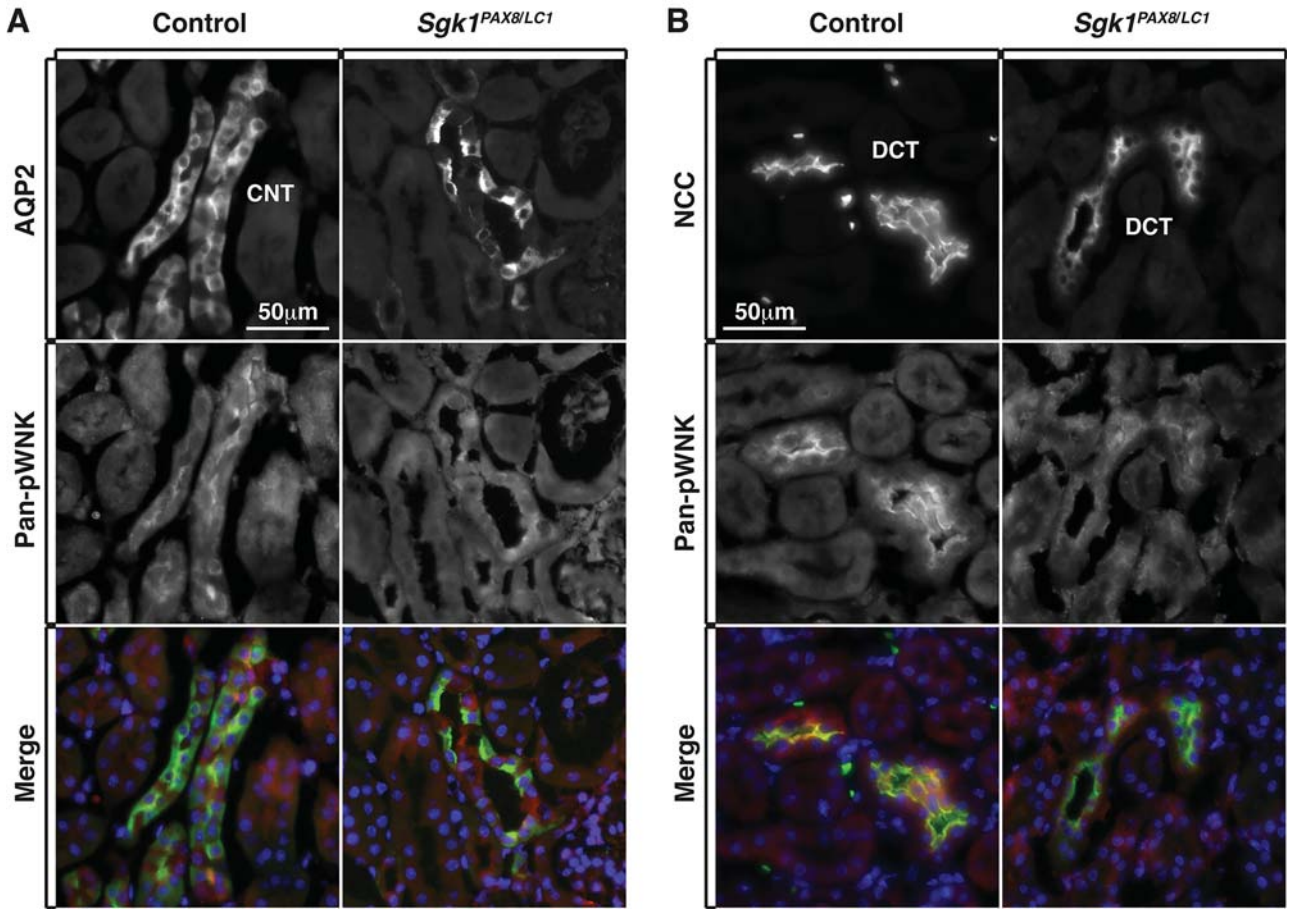
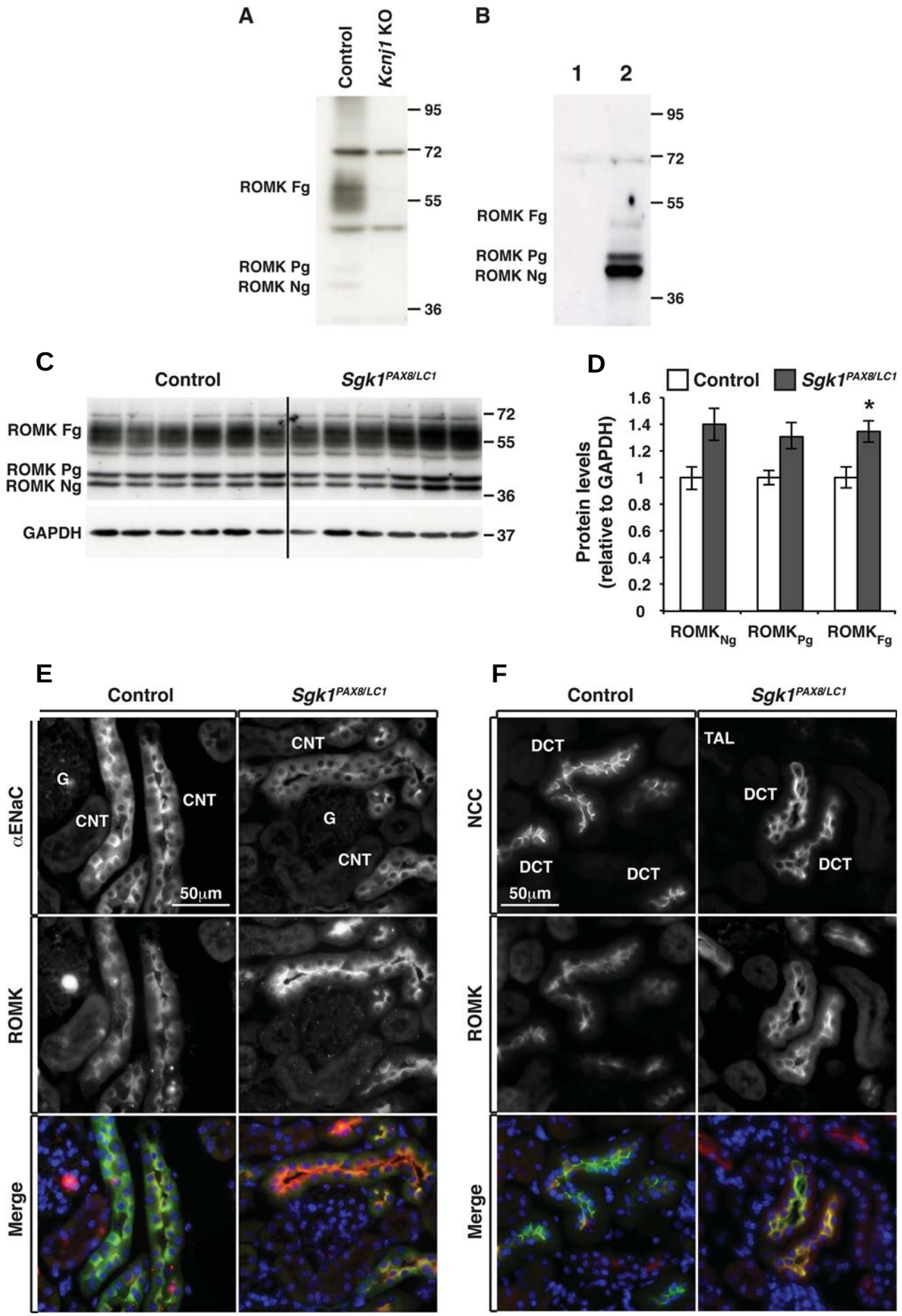
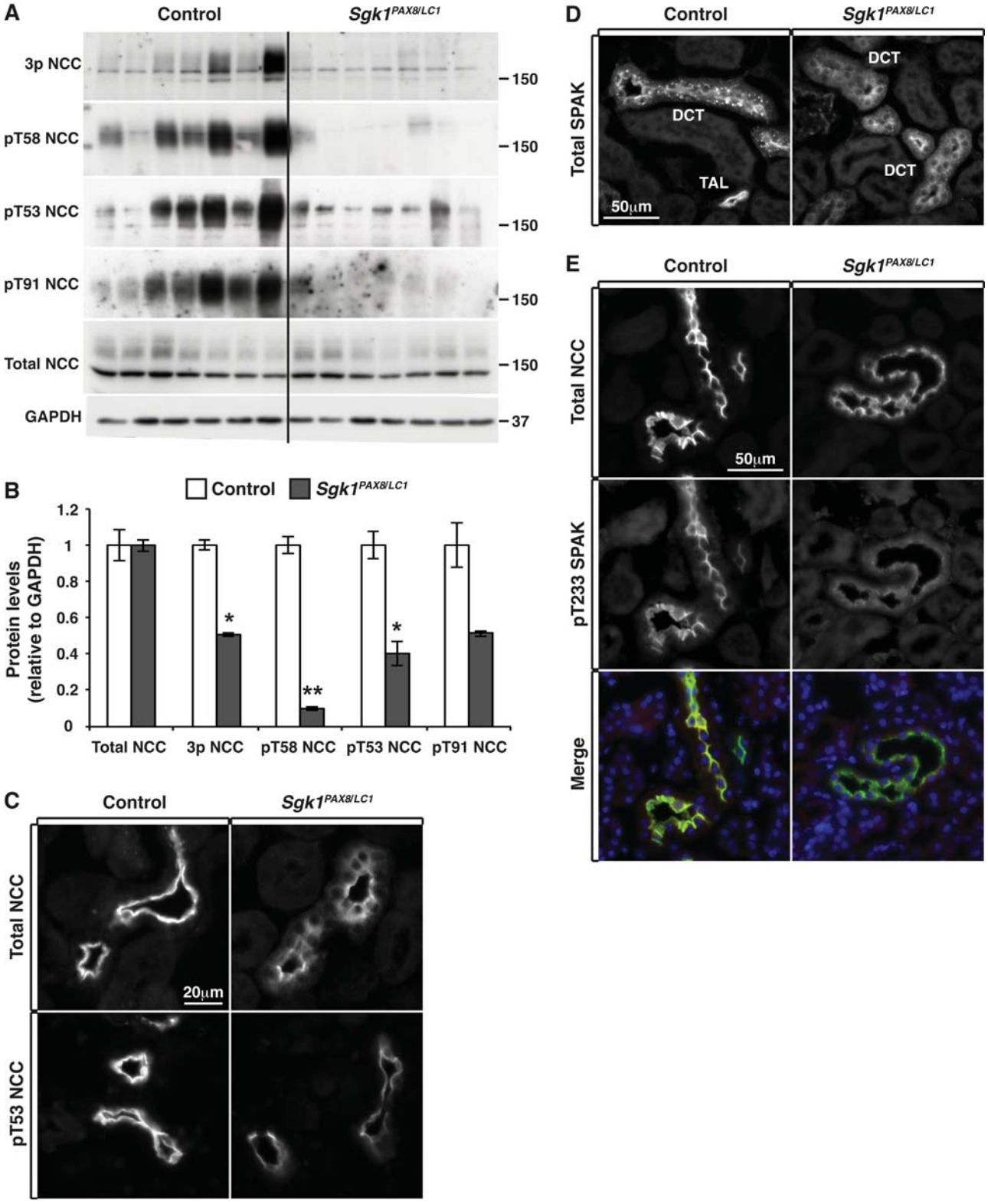


Fig. 5
Al-Qusairi et al.



Al-Qusairi et al.,
Fig. 6



Al-Qusairi et al.
Fig.7