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Dominant functional role of the novel phosphorylation site S811 in the human renal NaCl cotransporter

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ABSTRACT: The NaCl cotransporter (NCC) is essential for electrolyte homeostasis and control of blood pressure. The human *SLC12A3* gene, which encodes NCC, gives rise to 3 isoforms, of which only the shortest isoform [NaCl cotransporter isoform 3 (NCC₃)] has been studied extensively. All NCC isoforms share key phosphorylation sites at T55 and T60 that are essential mediators of NCC function. Recently, a novel phosphorylation site at S811 was identified in isoforms 1 and 2 [NaCl cotransporter splice variant (NCC_{SV})], which are only present in humans and higher primates. The aim of the current study, therefore, is to investigate the role of S811 phosphorylation in the regulation of NCC by a combination of biochemical and fluorescent microscopy analyses. We demonstrate that hypotonic low-chloride buffer increases S811 phosphorylation, whereas phosphorylation-deficient S811A mutant hinders phosphorylation at T55 and T60 in NCC_{SV} and NCC₃. NCC_{SV} S811A impairs NCC₃ activity in a dominant-negative fashion, although it does not affect plasma membrane abundance. This effect may be explained by the heterodimerization of NCC_{SV} with NCC₃. Taken together, our study highlights the dominant-negative effect of NCC_{SV} on T55 and T60 phosphorylation and NCC activity. Here, we reveal a new function of NCC_{SV} in humans that broadens the understanding on NCC regulation in blood pressure control.—Tutakhel, O. A. Z., Bianchi, F., Smits, D. A., Bindels, R. J. M., Hoenderop, J. G. J., van der Wijst, J. Dominant functional role of the novel phosphorylation site S811 in the human renal NaCl cotransporter. *FASEB J.* 32, 4482–4493 (2018). www.fasebj.org

KEY WORDS: hypertension • kidney • NCC • splice variant

Hypertension (high blood pressure) affects more than 1.3 billion people worldwide and can lead to severe cardiovascular complications, including cerebrovascular disease, heart failure, ischemic heart disease, and renal failure (1–6). This makes hypertension the greatest contributing risk factor to morbidity and mortality worldwide. The cause of hypertension is identified in only ~10% of cases (secondary hypertension) (7, 8). It is of critical importance to increase our understanding of the pathophysiology of hypertension. Kidneys play a

key role in the control of blood pressure by regulating sodium (Na⁺) excretion (9–13). Fine-tuning (5–10%) of Na⁺ excretion primarily takes place in the distal convoluted tubule (DCT) by Na⁺ reabsorption *via* the NaCl cotransporter (NCC) (14–16).

Since the cloning of NCC, 3 splice isoforms of NCC have been identified that are the result of alternative splicing of the *SLC12A3* gene (14). Thus far, most research has focused on the shortest NCC isoform, NaCl cotransporter isoform 3 (NCC₃), as the other 2 isoforms are not present in rodents (17). NCC isoforms 1 and 2, which result from the alternative splicing of exon 20, are 9 aa longer than NCC₃ (17, 18). Subsequent studies have confirmed the presence of both long and short forms of the exon 20—20a and 20b, respectively—which possibly indicates a splicing donor site (19). Compared with NCC isoform 1, isoform 2 lacks 1 aa at glutamine residue 95 within the amino (N)-terminal domain; therefore, NCC isoforms 1 and 2 are practically indistinguishable and, in this study, are collectively referred to as NCC splice variant (NCC_{SV}). Of interest, NCC_{SV} is only present in humans and higher primates. In the human kidney, NCC_{SV} mRNA comprises ~44% of the total NCC content (14).

The importance of NCC in the regulation of blood pressure is illustrated by such diseases as Gitelman syndrome (OMIM: 263800) (20–22) and familial hyperkalemic

ABBREVIATIONS: DCT, distal convoluted tubule; eGFP, enhanced green fluorescent protein; ENaC, epithelial Na⁺ channel; FHHt, familial hyperkalemic hypertension; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonance energy transfer; HA, hemagglutinin; HCTZ, hydrochlorothiazide; HEK293, human embryonic kidney 293; ICS, image cytometry standard; NCC₃, NaCl cotransporter isoform 3; NCC, NaCl cotransporter; NCC_{SV}, NaCl cotransporter isoform splice variant; SPAK, ste20-related proline-alanine-rich kinase; TBS-T, Tris-buffered saline and Tween-20; tNCC, total NaCl cotransporter; WNK, with no lysine; YFP, yellow fluorescent protein

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hypertension (FHHt; OMIM: 145260) (23–26). Gitelman syndrome is caused by loss-of-function mutations in the *SLC12A3* gene that encodes NCC (20–22), which results in renal Na⁺ wasting and hypotension (18, 27). In contrast, FHHt is a form of hypertension that results from the gain of function of NCC as a consequence of mutations in genes that code for key signaling molecules (23, 28). In FHHt, mutations have been identified in *WNK1* and *WNK4*, which encode the with no lysine (WNK) kinases (23), or in *KLHL3* and *CUL3*, which code for Kelch-like 3 and Cullin 3, respectively (28). WNK kinases phosphorylate and activate Ste20-related proline-alanine-rich kinase (SPAK) and oxidative stress response 1, which, in turn, phosphorylate T55 and T60 in NCC (14, 29, 30). Phosphorylation of these sites is essential for NCC activity (16, 29, 31, 32) and plasma membrane abundance (33, 34).

Of interest, Gonzales *et al.* (17) have described a novel phosphorylation site on S811, which is only present in NCC_{SV}. Our group has previously shown that NCC_{SV} plays a role in physiologic (14) and pathophysiologic (35) processes; however, the importance of NCC_{SV} S811 phosphorylation in NCC_{SV} and NCC₃ function remains unknown. Therefore, the aim of the current study was to investigate the role of NCC_{SV} S811 in the phosphorylation and activity of NCC_{SV} and NCC₃. We used a combinational approach of immunoblotting with phospho-specific Abs, cell-surface biotinylation, and fluorescence microscopy to examine the influence of S811 on the phosphorylation, membrane abundance, and activity of NCC. In addition, a phospho-specific Ab against S811 was used to assess S811 phosphorylation under hypotonic low-chloride (Cl[−]) condition. Finally, the interaction of NCC_{SV} with NCC₃ was assessed by fluorescence lifetime imaging microscopy (FLIM).

MATERIALS AND METHODS

Mutagenesis and constructs

NCC isoform 1 (accession no. NP_000330.2) was used in all experiments to represent NCC_{SV}. For coimmunoprecipitation, enhanced green fluorescent protein (eGFP) C-terminal-coupled human NCC₃-eGFP, NCC_{SV}-eGFP, and the phospho-mutants of NCC_{SV}-eGFP—constitutively phosphorylated S811D and non-phosphorylated S811A—in pCMV-SPORT6 were generated as described in Tutakhel *et al.* (14) and Dimke *et al.* (16). In addition, human NCC₃, NCC_{SV}, and NCC_{SV} phospho-mutants were cloned into the hemagglutinin (HA) pCINeo-IRES-mCherry vector by using standard PCR and restriction cloning. For FLIM and coimmunoprecipitation experiments, NCC_{SV} and NCC₃ were cloned into the pCINeo vector, and human codon optimized mCitrine and mCherry tags were fused to the C-terminal end separated by a 15-residue-long linker region (3× GGGGS) by using PCR and uracil excision-based cloning (36). For fluorescence microscopy measurements, NCC₃, NCC_{SV}, NCC_{SV} phospho-mutants (S811D and S811A), and NCC₃ phospho-mutant (T55A/T60A) were cloned into the pCINeo-IRES-eCFP vector by using restriction cloning. The vector that carries halide-sensitive yellow fluorescent protein (YFP) and the mKate gene was described by Vijftigschild *et al.* (37) and kindly provided by Dr. Jeffrey M. Beekman (University Medical Center, Utrecht, The Netherlands).

Cell culture and transfection

Cell culture and transfection was performed as described in Tutakhel *et al.* (14). In brief, human embryonic kidney 293 (HEK293) cells were grown in DMEM medium that was supplemented with 10% v/v fetal bovine serum, 100 μM nonessential amino acids, and 2 mM L-glutamine. Cells were transiently transfected by using polyethylenimine (1:6, DNA: polyethylenimine; Polysciences, Warrington, PA, USA). Cells that were transfected with empty vectors were used as control (mock). For cotransfection, cells were transfected in a DNA ratio of 1 to 1.

Buffers

The following buffers were used: isotonic control buffer, which contained (mM) 135 NaCl, 5 KCl, 0.5 CaCl₂, 0.5 MgCl₂, 0.5 Na₂HPO₄, 0.5 Na₂SO₄, 15 HEPES/Tris, pH 7.4; hypotonic low Cl[−] buffer, which contained (mM) 67.5 Na-gluconate, 2.5 K-gluconate, 0.25 CaCl₂, 0.25 MgCl₂, 0.5 Na₂HPO₄, 0.5 Na₂SO₄, 7.5 HEPES/Tris, pH 7.4; dissection buffer, which contained (mM) 300 sucrose, 25 imidazole, 1 EDTA, HCl, pH 7.2, and freshly added protease inhibitors (μM) 50 PMSF, 20 aprotinin, 20 leupeptin, 10 pepstatin A; PBS supplemented with (mM) 1 MgCl₂, 0.5 CaCl₂, NaOH, pH 8.0; lysis buffer, which contained (mM) 50 Tris/HCl, pH 7.5, 1 EGTA, 1 EDTA, 10 Na⁺ β-glycerophosphate, 1 Na-orthovanadate, 50 Na-fluoride, 10 Na-pyrophosphate, 270 sucrose, 150 NaCl, 1% (v/v) Triton X-100, and freshly added protease inhibitors (μM) 50 PMSF, 20 aprotinin, 20 leupeptin, 10 pepstatin A; buffer A, which contained (mM) 50 Tris/HCl, pH 7.5, 100 EGTA; 1× Laemmli sample buffer, which contained (mM) 40 DTT, 12 Tris/HCl, pH 6.8, 0.4% (w/v) SDS, 2% (v/v) glycerol, 0.002% (w/v) bromophenol blue; and Tris-buffered saline and Tween-20 (TBS-T), which contained (mM) 140 NaCl, 20 Tris/HCl, pH 7.5, 0.1% (v/v) Tween-20. NaI solution contained (mM) 140 NaI, 4.2 KCl, 1.4 CaCl₂, 1 MgCl₂, 5.5 D-glucose, 10 HEPES/Tris, pH 7.4, 300 mOsmol/kg H₂O.

Cell treatment

At 48 h post-transfection, HEK293 cells were washed once with PBS. Cells were then incubated for 30 min at 37°C with isotonic control buffer or hypotonic low Cl[−] buffer. Cells were subsequently used to isolate total cell membrane fractions, cell-surface biotinylation, or for coimmunoprecipitation.

Isolation of total cell membrane fractions

Ten volumes of ice-cold dissection buffer were added to HEK293 cells and cells were homogenized by 20 passages through a 21-gauge needle (Eclipse; Becton Dickinson, Franklin Lakes, NJ, USA) that was fitted to a 1-ml syringe (Becton Dickinson). Homogenized cells were centrifuged at 4000 g for 8 min at 4°C, after which the supernatant was centrifuged at 16,000 g for 40 min at 4°C. The pellet of the 16,000 g centrifugation was resuspended in ice-cold dissection buffer and protein concentration was determined by using the Bradford method (MilliporeSigma, Burlington, MA, USA). Proteins were eluted in 1× Laemmli sample buffer. Samples were incubated for 15 min at 65°C before immunoblotting. For immunoblotting of total cell membrane fractions, 20 μg protein was loaded onto gels.

Cell-surface biotinylation

We performed cell-surface biotinylation as described in De Groot *et al.* (38). HEK293 cells were subsequently lysed in ice-cold lysis

buffer and centrifuged at 16,000 g at 4°C. Protein concentrations were determined by using the Bradford method (MilliporeSigma). Equal amounts of protein were incubated with neutravidin beads (Pierce, Rockford, IL, USA) for 2 h at 4°C. Beads were washed 3 times with lysis buffer and eluted in 1× Laemmli sample buffer. Samples were incubated for 15 min at 65°C before immunoblotting.

FLIM

FLIM images were generated from HEK293 cells at 16 h after transfection with NCC₃ and NCC_{SV} that contained mCitrine and/or mCherry C-terminal fusion plasmids. The total amount of DNA per transfection was 2 µg (1:1 donor acceptor ratio) in WillCo-dish (diameter, 35 mm; WillCo Wells, Amsterdam, The Netherlands) with a cell density of 1.2×10^6 . Imaging and measurements were performed as described in Verboogen *et al.* (39). In brief, samples were washed and imaged in live cell imaging solution (HEPES-buffered saline solution; Thermo Fisher Scientific, Waltham, MA, USA). Photon traces in PicoQuant Photon Trace (PT3; PicoQuant, Berlin Germany) format were used to construct FLIM images in image cytometry standard (ICS). Conversion of PT3 files to ICS was performed by using PT32ICS conversion software (39). For FLIM statistics of single cells, photons were pooled for each individual cell and fitted with exponential decay functions that were convoluted with the instrument response function using OriginPro2016 (OriginLab, Northampton, MA, USA). Single pixel-fitted FLIM images were generated for individual cells—with at least 1,000,000 photons per cell—using TRI2 software (v.2.8.6.2; Gray Institute, Oxford, United Kingdom) (40, 41). Settings used for TRI2 were as follows: 7×7-pixel circular binning and thresholding at 15–100% intensity. Lifetime imaging pictures were generated by using Fiji ImageJ (National Institutes of Health, Bethesda, MD, USA; <https://imagej.net/Fiji/>) (42).

Coimmunoprecipitation

Transfected HEK293 cells were lysed in ice-cold lysis buffer for 1 h at 4°C. Cell lysate was subsequently collected and centrifuged at 16,000 g at 4°C. Protein concentrations were determined by using the Bradford method. Anti-green fluorescent protein (GFP) or anti-HA Abs were coupled to protein A/G plus agarose beads (Santa Cruz Biotechnology, Dallas, TX, USA) by gentle agitation for 2 h at 4°C. Equal amounts of protein were subsequently incubated with GFP- or HA-conjugated beads for 2 h at 4°C. GFP-conjugated beads were used for coimmunoprecipitation of mCitrine-tagged NCC to demonstrate binding to mCherry-tagged NCC. Coimmunoprecipitation of HA-tagged NCC was performed with HA-conjugated beads to detect interaction with eGFP-tagged NCC. Beads were washed 3 times with ice-cold lysis buffer, and proteins were eluted in 1× Laemmli sample buffer. Samples were incubated for 15 min at 65°C before immunoblotting. Coimmunoprecipitation was analyzed by anti-GFP, -HA, and -mCherry Abs.

Immunoprecipitation

Transfected HEK293 cells were lysed in ice-cold lysis buffer for 1 h at 4°C. Cell lysate was subsequently collected and centrifuged at 16,000 g for 10 min at 4°C. Protein concentrations were determined by using the Bradford method (MilliporeSigma). Anti-phosphorylated S811 (pS811) NCC_{SV} Ab was covalently coupled 0.5 µg:1 µl (Ab:bead) to protein A/G plus agarose beads (Santa Cruz Biotechnology) by gentle agitation for 2 h at 4°C. Beads were washed once with ice-cold PBS and 3 times with 100 mM sodium

borate (pH 9.3). For covalent cross-linking, beads were incubated twice with 20 mM dimethyl pimelimidate (MilliporeSigma) for 30 min at room temperature under gentle agitation. Beads were washed 3 times with 50 mM glycine (pH 2.5) and once with 200 mM Tris/HCl, pH 8.0. For neutralization, beads were incubated in 200 mM Tris/HCl, pH 8.0, for 2 h at room temperature. Equal amounts of protein were subsequently incubated with NCC_{SV} pS811-conjugated agarose beads for 2 h at 4°C under gentle agitation. Immunoprecipitates were washed 3 times with ice-cold lysis buffer and twice with buffer A. Proteins were eluted in 1× Laemmli sample buffer. Samples were incubated for 15 min at 65°C before immunoblotting. Input and immunoprecipitated fractions were analyzed by anti-total NCC (tNCC) Ab.

Immunoblotting

Samples in Laemmli buffer were loaded on an 8% w/v SDS-PAGE gel, transferred to PVDF membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA) that were subsequently blocked for 30 min with TBS-T that contained 5% w/v skimmed dried milk powder. Membranes were subsequently incubated with primary Abs that were dissolved in 5% w/v in nonfat dry milk in TBS-T overnight at 4°C. All PVDF membranes were probed once with indicated primary Abs. Membranes were then incubated with horseradish peroxidase-conjugated secondary Abs that were diluted in 1% w/v nonfat dry milk in TBS-T for 1 h at room temperature and visualized with the imaging system (ChemiDoc XRS; Bio-Rad, Hercules, CA, USA) using ECL (Thermo Fisher Scientific). Immunoreactive bands were quantified by using Image Studio Lite software (Li-Cor Biosciences, Lincoln, NE, USA).

Abs

The following Abs were used: anti-tNCC (1:2000; EMD Millipore); anti-NCC pT55 and anti-NCC pT60 (equivalent mouse NCC pT53 and pT58; 1:2000) as described in Pedersen *et al.* (43) (PhosphoSolutions, Aurora, CO, USA); anti-HA (1:5000; Cell Signaling Technology, Danvers, MA, USA); anti-GFP (generated in house, detects eGFP and mCitrine; 1:5000); anti-mCherry (1:1000; Abcam, Cambridge, United Kingdom); and anti-β-actin (1:20,000; MilliporeSigma). Anti-NCC_{SV} pS811 Ab [residue 811 of human NCC phosphorylated at S811, RGARP(pS)VSGALD] was raised in sheep and was affinity purified on the appropriate antigen (MRC-PPU Reagents, Dundee, United Kingdom). Secondary Abs used were as follows: horseradish peroxidase-conjugated goat anti-rabbit (1:10,000; MilliporeSigma) and goat anti-mouse (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). All Abs have been validated for their linearity in detection (Supplemental Fig. 1).

NCC activity measurements using YFP-mKate

NCC activity was performed as described in Valdez-Flores *et al.* (22). NCC is able to transport NaI-like NaCl in a hydrochlorothiazide (HCTZ)-sensitive manner (22). In brief, HEK293 cells were cotransfected with the halide-sensitive YFP-mKate construct (37) and the appropriate human NCC pCINeo-IRES-eCFP construct or pCINeo-IRES-eCFP mock. The total amount of DNA per transfection was 2 µg (0.2 µg YFP-mKate + 1.8 µg NCC) in a WillCo-dish with a cell density of 1.2×10^6 . Nineteen hours after transfection and before measurements, cells were incubated with hypotonic low Cl[−] buffer for 10 min at 37°C. Cells were then placed in an incubation chamber that contained hypotonic low Cl[−] buffer (500 µl) and attached to the stage of an inverted microscope (Axiovert 200M; Carl Zeiss, Jena, Germany). NCC-expressing HEK293 cells were identified by detecting eCFP

fluorescence using a monochromator at 430 nm excitation, and the emitted light was subsequently directed by a 480AF30 dichroic mirror (Omega Optical, Brattleboro, VT, USA). After 30 s, an equal volume of NaI solution was added to the incubation chamber (final concentration of Na^+ and I^- : 105 and 70 mM, respectively). Changes in intracellular halide concentrations were monitored by simultaneous measurements of halide-sensitive YFP fluorescence and halide-insensitive fluorescence of mKate (37). Halide-sensitive YFP was excited at 488 nm by using a monochromator. Fluorescence-emitted light was directed by a 505DRLPXR dichroic mirror (Omega Optical) through a 535af26 emission filter. mKate was excited at 543 nm, and fluorescence-emitted light was directed by a 560DRLP dichroic mirror (Omega Optical) through a 565ALP emission filter onto a Cool-Snap HQ monochrome charge-couple device camera (Photometrics, Tucson, AZ, USA). The integration time of the charge-couple device camera was set at 200 ms for both YFP and mKate with a sampling interval of 3 s. We performed all measurements at 37°C. Quantitative image analysis was performed with Metamorph 6.0 (Molecular Devices, Sunnyvale, CA, USA). For each wavelength, mean fluorescence intensity was monitored in an intracellular region and, for the purpose of background correction, an extracellular region of identical size. After background correction, the fluorescence emission ratio of the green and red fluorescence of YFP and mKate, respectively, was used to determine changes in intracellular halide content as a measure of NCC activity. Signal specificity was assessed by adding 100 μM HCTZ or an equivalent amount of its solvent, DMSO, together with the hypotonic low Cl^- buffer incubation for 10 min at 37°C. We determined the thiazide-sensitive uptake taking by the difference of the measurements with and without HCTZ.

Statistics

Immunoblot data were analyzed by comparing integrated optical densities of bands by 1-way ANOVA with Tukey's multiple comparisons *post hoc* test if multiple groups were compared. In FLIM and NCC activity measurements, all depicted data are

averages of at least 3 independent experiments, and all statistical analyses were performed by using a 1-way ANOVA with Dunnett's multiple comparison *post hoc* test that compared control condition with all other conditions. Depicted values are presented as means $\pm$ SEM of at least 3 independent experiments, and values of $P < 0.05$ were considered statistically significant. All data were analyzed by using Prism 6 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Effect of S811 on NCC_{SV} phosphorylation at T55 and T60

To examine the role of phosphorylation at S811 in NCC_{SV} , we generated NCC_{SV} S811D and S811A plasmids that represent the constitutively phosphorylated and non-phosphorylated forms of S811, respectively. HEK293 cells that were transfected with the respective plasmids were subjected to hypotonic low Cl^- buffer, which is a known stimulus of NCC phosphorylation at T55 and T60 (Fig. 1A) (29, 44). Of interest, NCC_{SV} S811A did not demonstrate a significant stimulation of T55 and T60 phosphorylation upon hypotonic low Cl^- buffer compared with isotonic control buffer (Fig. 1 and Supplemental Fig. 2A). Of note, tNCC density does not differ significantly (Supplemental Fig. 2A). NCC_3 , NCC_{SV} , and NCC_{SV} S811D demonstrated significantly higher phosphorylation at T55 and T60 upon hypotonic low Cl^- buffer treatment compared with isotonic control buffer (Fig. 1 and Supplemental Fig. 2A). No difference in T55 and T60 phosphorylation was observed between NCC_{SV} and NCC_3 after treatment with hypotonic low Cl^- buffer. Of note, isotonic control buffer did not result in T55 and T60 phosphorylation in any of the conditions

Figure 1. S811A mutant of NCC_{SV} , but not S811D, prevents phosphorylation at T55 and T60. A) Representative immunoblot of total cell membrane fractions isolated from HEK293 cells that were transfected with mock, indicated NCC isoforms, and NCC_{SV} mutants treated with isotonic control (Co) or hypotonic low Cl^- (Hy) buffer. Immunoblots were probed with anti-tNCC, anti-phosphorylated T55 (pT55), and T60 (pT60) NCC Abs and depict the immunoreactive band of ~130 kDa. β -Actin was used as sample loading control. B, C) Densitometry analysis of immunoreactive bands of pT55 and pT60-to-tNCC ratio. We determined the abundance of tNCC and pT55 and pT60 upon treatment with hypotonic low Cl^- buffer relative to treatment with isotonic control buffer. We subsequently compared the relative abundance in hypotonic low Cl^- condition of NCC_{SV} , NCC_{SV} S811D, and NCC_{SV} S811A with the relative abundance of NCC_3 . For each condition, 20 μg protein was loaded the gels. OD, optical density. Values are expressed as means $\pm$ SEM of at least 3 independent experiments. *Significance of hypotonic low Cl^- buffer compared with its own control; #significance compared with NCC_{SV} ; $P < 0.05$.

tested (Fig. 1B, C and Supplemental Fig. 2A, black bars). Immunoreactive bands were not observed in cells that were transfected with empty vector (mock) (Fig. 1A).

Effect of S811 phosphorylation on phosphorylation of NCC₃ at T55 and T60

As NCC_{SV} and NCC₃ are coexpressed at the apical membrane of epithelial cells that line the DCT (14), we hypothesized that S811 phosphorylation could affect phosphorylation at T55 and T60 of NCC₃. Coexpression of NCC_{SV} S811A and NCC₃ resulted in significantly less phosphorylation at T55 and T60 compared with coexpression of NCC_{SV} and NCC₃ when treated with hypotonic low Cl⁻ buffer. Of interest, this decrease was >50% for both T55 and T60 (Fig. 2B, C and Supplemental Fig. 2B, white bars). Coexpression of NCC₃ with NCC_{SV} or NCC_{SV} S811D demonstrated significantly higher phosphorylation at T55 and T60 when treated with hypotonic low Cl⁻ buffer compared with isotonic control buffer (Fig. 2 and Supplemental Fig. 2B). Similar to that shown in Fig. 1, NCC_{SV} S811A alone did not demonstrate significant differences in T55 and T60 phosphorylation between isotonic control and hypotonic low Cl⁻ buffer treatment (Fig. 2 and Supplemental Fig. 2B). In isotonic control buffer, there were no difference in the levels of T55 and T60 phosphorylation in any of the conditions tested (Fig. 2B, C and Supplemental

Fig. 2B, black bars). Cells that were transfected with mock vector did not display immunoreactive bands (Fig. 2A).

NCC_{SV} and NCC₃ form hetero- and homodimers

To assess whether the effect of NCC_{SV} S811 phosphorylation on NCC₃ T55 and T60 phosphorylation could be exerted *via* dimerization of NCC_{SV} with NCC₃, we used a Förster resonance energy transfer (FRET) –based microscopy approach with C-terminal mCitrine and mCherry fusions of NCC_{SV} and NCC₃, respectively. FRET was measured by using FLIM microscopy, which has the advantage that the lifetime (τ) is not dependent on excitation power and probe concentration and is therefore less technically challenging than ratiometric FRET analysis (45). The amount of FRET, which represents the interaction between the donor and acceptor pair, is measured as the lifetime of the mCitrine fluorescent signal. This signal shortens as a result of the energy transfer to the mCherry probe in the case of protein dimerization (39, 46). Coexpression of NCC_{SV}-mCitrine with either NCC_{SV}-mCherry or NCC₃-mCherry in HEK293 cells resulted in a significant decrease in lifetime as demonstrated by phasor plots (Fig. 3B) and single-cell analysis (Fig. 3A), which indicated the formation of both hetero- and homodimers of NCC_{SV} with NCC_{SV} and NCC₃, respectively. We further assessed the

Figure 2. NCC_{SV} S811A inhibits phosphorylation at T55 and T60 of NCC₃. A) Representative immunoblot of total cell membrane fractions isolated from HEK293 cells that were cotransfected with NCC₃ and NCC_{SV} or NCC_{SV} mutants in a 1-to-1 ratio and treated with isotonic control (Co) or hypotonic low Cl⁻ (Hy) buffer. Immunoblots were probed with anti-tNCC, anti-phosphorylated T55 (pT55), and T60 (pT60) NCC Ab that depict the immunoreactive band of ~130 kDa. β-Actin was used as sample loading control. B, C) Densitometry analysis of the immunoreactive bands of pT55 and pT60 divided by immunoreactive bands of tNCC. We determined the abundance of tNCC and pT55 and T60 upon treatment with hypotonic low Cl⁻ buffer relative to treatment with isotonic control buffer. We compared the relative abundance in hypotonic low Cl⁻ condition of each combination with the relative abundance of NCC₃ cotransfected with NCC_{SV}. For each combination, 20 μg protein was loaded on gels. OD, optical density. Values are expressed as means ± SEM of at least 3 independent experiments. *Significance of hypotonic low Cl⁻ buffer compared with its own control; #significant compared with NCC₃ + NCC_{SV}; P < 0.05.

dimerization of NCC_{SV} and NCC₃ by performing a competition experiment in which NCC_{SV}-mCitrine was coexpressed with unlabeled NCC₃ as a competitor together with NCC₃-mCherry. Competition with unlabeled NCC₃ resulted in an increase of the donor lifetime compared with HEK293 cells that coexpressed NCC_{SV}-mCitrine and NCC_{SV}-mCherry (Fig. 3B). In addition, we used SLC41A1 as negative control; this is an Mg²⁺ transporter that localizes to the apical membrane of the DCT (47, 48), but does not interact with NCC_{SV}. Therefore, NCC_{SV}-mCitrine and SLC41A1-mCherry were coexpressed in HEK293 cells, which did not colocalize (Supplemental Fig. 3A) and demonstrated no significant lifetime decrease—in line with 2 proteins that do not interact (Supplemental Fig. 3A, B). Dimerization, as observed by FRET/FLIM, was further established by coimmunoprecipitation experiments (Fig. 3C and Supplemental Fig. 3C): NCC₃ demonstrated an interaction between NCC₃ and NCC_{SV}, NCC_{SV} S811D, or NCC_{SV} S811A.

Effect of S811 phosphorylation on NCC function

As NCC activity is regulated by its phosphorylation (29), we used a functional assay to study the effect of S811 phosphorylation on the transport activity of NCC_{SV} and NCC₃. The respective plasmids were cotransfected in HEK293 cells with halide-sensitive YFP that was coupled to the halide-insensitive fluorescent red protein, mKate. Before measurements, transfected HEK293 cells were incubated in hypotonic low Cl[−] buffer to maximally increase NCC phosphorylation and activity (22). After 30 s, NaI was added to the medium, which induced a significant reduction in the YFP-to-mKate ratio in NCC₃-expressing cells, which was indicative of NCC transport activity (Fig. 4A, B). Pretreatment with HCTZ (40 min, 100 μM), a well-known inhibitor of NCC, completely abrogated this response (Fig. 4B). As described in Valdez-Flores *et al.* (22), NCC activity is measured as the difference in the YFP-to-mKate ratio

Figure 3. NCC_{SV} and NCC₃ form hetero- and homodimers. *A*) NCC_{SV}-mCitrine was transiently cotransfected with NCC₃-mCherry or NCC_{SV}-mCherry in HEK293 cells. FRET between labels was monitored by fluorescence lifetime imaging. Representative confocal microscopy (left) and convoluted FLIM (right) images of NCC_{SV}-mCitrine (green) with NCC₃-mCherry (red) or NCC_{SV}-mCherry (red). Scale bar, 10 μm. *B*) Whole-cell apparent fluorescence lifetimes demonstrating hetero- and homodimerization of NCC_{SV} and NCC₃ proteins in live cells. *C*) Coimmunoprecipitation with mCitrine-labeled NCC_{SV} and mCherry-labeled mock, NCC_{SV}, and NCC₃. GFP-coupled beads represent the immunoprecipitated NCC_{SV}-mCitrine fraction, and immunoblots were probed with anti-mCherry and anti-GFP Abs; depicting the immunoreactive NCC band of ~130 kDa. As the GFP Ab binds to mCitrine, it demonstrates that NCC_{SV}-mCitrine (bound to GFP-coupled beads) interacts with both NCC_{SV}- and NCC₃-mCherry. As a negative control, NCC_{SV}-mCitrine and mock were coexpressed in HEK293 cells and did not demonstrate an interaction between mCitrine and mCherry. Total cell lysate (input) was probed with anti-mCherry, anti-GFP, or anti-β-actin Abs. β-Actin was used as sample loading control. *N* ≥ 3; *n* > 20/condition. *Significant difference between NCC_{SV}-mCitrine + mock and other conditions; #significant differences from NCC_{SV}-mCitrine + NCC₃-mCherry + NCC₃; *P* < 0.05.

Figure 4. S811A exhibits significantly impaired NCC activity. **A)** Model of NCC activity assay in HEK293 cells that depicts the coexpression of an NCC isoform with a halide-sensitive YFP coupled to the red fluorescent protein, mKate. The latter is insensitive to changes in intracellular halide concentrations. Preincubation of these cells in hypotonic low Cl^- solution that contains 70 mM Na-gluconate (NaGluc) for 40 min and the subsequent addition of NaI (final Na^+ and I^- concentration: 105 and 70 mM, respectively) resulted in a change in YFP fluorescence, which is corrected for the red fluorescent iodide-insensitive (mKate) signal. This allows for the determination of changes in intracellular iodide concentrations by using the YFP-to-mKate ratio as a measure of NCC activity. **B)** NaI was added 30 s after the start ($t = 0$), and the YFP-to-mKate ratio was plotted over time for mock and NCC₃-expressing HEK293 cells that were treated with or without thiazide [HCTZ, 100 μM ; $N = 3$, mock (DMSO, ∇); $N = 3$, NCC₃ HCTZ ($\blacktriangle$); $N = 3$, NCC₃ DMSO ($\circ$)]. **C)** Activity of NCC₃, NCC_{SV}, NCC_{SV} S811D, NCC_{SV} S811A, NCC₃ T55A/T60A, NCC₃ + NCC_{SV}, NCC₃ + NCC_{SV} S811D, and NCC₃ + NCC_{SV} S811A is expressed as the difference in the YFP-to-mKate ratio ($\Delta \text{YFP/mKate}$ ratio, $t = 0 \text{ s} - t = 180 \text{ s}$). Thiazide-specific NCC activity was determined by the ratio difference of the measurements with and without HCTZ. **D)** Representative immunoblot of total lysate (input) and the biotinylated fractions of HEK293 cells that were transfected with NCC₃, NCC_{SV}, or the indicated mutants and probed with anti-HA and β -actin Abs. β -Actin was used as sample loading control. **E)** Densitometry analysis of cell-surface biotinylation. Optical densities of bead fractions were divided by the corresponding optical densities of the input samples ($N = 3$). Values are expressed as means $\pm$ SEM. $N = 3$; $n > 65/\text{condition}$. *Significant difference in activity between NCC₃ and all other constructs; #significant difference in activity between NCC₃ + NCC_{SV} and all other constructs; $P < 0.05$.

($\Delta \text{YFP/mKate}$ ratio) between $t = 0$ and $t = 180 \text{ s}$ after the addition of NaI. This is plotted as the HCTZ-specific NCC activity: $\Delta \text{YFP/mKate}$ ratio without HCTZ – $\Delta \text{YFP/mKate}$ ratio with HCTZ (Fig. 4C). Cells that expressed NCC_{SV} S811A alone or in combination with NCC₃ displayed significantly lower NCC activity compared with NCC₃ and NCC₃ + NCC_{SV} (Fig. 4C). The NCC₃ phosphodeficient T55A/T60A mutant (NCC₃ T55A/T60A) was included as negative control and demonstrated significantly lower NCC activity compared with wild-type NCC₃ and NCC₃ + NCC_{SV} (Fig. 4C). Of note, the basal YFP-to-mKate ratio at $t = 0 \text{ s}$ was not significantly different between the various conditions tested (Fig. 4B).

Effect of S811 on the abundance of NCC at the plasma membrane

To determine the effect of S811 phosphorylation on the localization of NCC at the plasma membrane, HEK293 cells

that were transfected with mock, NCC₃, NCC_{SV}, NCC_{SV} S811D, or S811A mutants were subjected to cell-surface protein biotinylation. NCC protein was demonstrated in total cell lysate (input) and the biotinylated fraction upon both isotonic control and hypotonic low Cl^- conditions (Fig. 4D). We corrected the optical density of the biotinylated fraction for the input fraction to assess NCC protein abundance at the plasma membrane. No significant difference was observed between the conditions tested, which indicated an equal amount of NCC protein abundance at the plasma membrane (Fig. 4E). Cells that were transfected with mock vector did not display immunoreactive bands of NCC in the biotinylated fractions or the input (Fig. 4D).

Generation of specific Abs against NCC_{SV} pS811

The NCC_{SV} pS811-specific epitope [RGARP(pS)VSGALD] was used to generate a phospho-specific NCC_{SV} pS811 Ab.

We performed immunoprecipitation of the cell lysate of HEK293 cells that were transfected with mock, NCC₃, or NCC_{SV} by using NCC_{SV} pS811-conjugated beads (Fig. 5A). We subsequently used a commercially available NCC Ab that detected both NCC₃ and NCC_{SV} to determine the total input as well as the specific phosphorylated fraction after immunoprecipitation. Of importance, this tNCC Ab only detected the immunoprecipitated fraction of NCC_{SV} under the hypotonic low Cl⁻ buffer condition. This indicates that the S811 residue is phosphorylated in hypotonic low Cl⁻ buffer. Immunoreactivity was not present in cells that were transfected with mock or NCC₃ (Fig. 5A).

DISCUSSION

The current study demonstrates that S811 acts as a dominant regulatory site for the phosphorylation of T55 and T60 in NCC_{SV} and NCC₃. Moreover, our findings demonstrate that the phosphorylation-deficient S811A mutant decreases the transport activity of both NCC_{SV} and NCC₃. The effect of S811 phosphorylation on NCC₃ activity likely occurs *via* the formation of heterodimers. Together, these

findings provide evidence that S811—present only in NCC_{SV}—is a crucial new residue involved in NCC regulation.

Our findings demonstrate that S811 has a dominant-negative effect on T55 and T60 phosphorylation. Richardson *et al.* (44) previously showed that mutation of T60 to alanine in NCC₃ markedly inhibited the phosphorylation of neighboring residues, such as T55, induced by hypotonic low Cl⁻ treatment. In addition, the T60A mutant strongly inhibited NCC activity, whereas this was less prominent for the T55A mutant (44). This implies that T60 can exert a dominant-negative effect over other phosphorylation sites in NCC₃ (44). Several other studies have demonstrated that T60 is a key phosphorylation site for NCC activity (29, 34, 44). This has been highlighted by a loss of function mutation at T60 in NCC that is linked to Gitelman syndrome (34, 49). It has also been previously shown that defective T60 phosphorylation corrects the phenotype of FHt in mice (34). Our study adds a new important site that is solely present in NCC_{SV} at S811. Of importance, this site could have a regulatory effect on NCC, as the S811A mutant prevented T55 and T60 phosphorylation and inhibited activity in both NCC_{SV}

Figure 5. Increased NCC_{SV} pS811 upon intracellular Cl⁻ depletion and schematic model for NCC_{SV} S811 regulation. **A**) Immunoprecipitation of pS811-conjugated beads with cell lysate from HEK293 cells that were transfected with mock, NCC₃, or NCC_{SV}. Indicated NCC isoforms were treated with isotonic control (Co) or hypotonic low Cl⁻ (Hy) buffer. pS811-coupled beads represent the immunoprecipitated NCC_{SV} pS811 fraction, and immunoblots were probed with anti-tNCC Ab. Representative immunoblot depicts the immunoreactive NCC_{SV} pS811 band of ~130 kDa. **B, C**) NCC_{SV} S811A mutant not only abolishes the hypotonic low Cl⁻-induced phosphorylation at T55 and T60 in NCC_{SV}, but also prevents phosphorylation at T55 and T60 in NCC₃. **B**) WNK kinases are autophosphorylated (activated) when the intracellular Cl⁻ concentration decreases. WNKs can phosphorylate and activate SPAK and oxidative stress response 1 (OSR1), which, in turn, phosphorylate T55 and T60 in NCC. Hypotonic low Cl⁻ buffer increases S811 phosphorylation, most likely *via* the WNK-SPAK/OSR1 signaling pathway. **C**) The dominant-negative effect

of NCC_{SV} S811A on the phosphorylation of T55 and T60 in NCC_{SV} and NCC₃ may occur *via* the formation of heterodimers between NCC_{SV} and NCC₃. IP, immunoprecipitation.

and NCC₃ (Fig. 5). The inhibitory effect of S811A on NCC function is in line with our previous data that show that *Xenopus laevis* oocytes that expressed NCC_{SV} S811A exhibited decreased ²²Na⁺ uptake compared with oocytes that expressed wild-type NCC_{SV} (14). Furthermore, it was shown that WNK4 inhibits both NCC₃ and NCC_{SV} function independent of S811, as the NCC_{SV} S811D mutant was not protected from the inhibitory effects of WNK4 (14). Of interest, our results now demonstrate that the S811 residue is phosphorylated upon intracellular Cl⁻ depletion (Fig. 5A). It is known that T55 and T60 are phosphorylated by the activation of the WNK-SPAK signaling pathway upon Cl⁻ depletion (29, 30); therefore, it is likely that S811 phosphorylation upon intracellular Cl⁻ depletion (Fig. 5A) is also mediated by the activation of the WNK-SPAK signaling pathway. However, prediction software suggested that the S811 phosphorylation site is highly similar to a PKA consensus site. Therefore, hormones that regulate the PKA pathway could potentially be involved in the regulation of NCC_{SV}-mediated Na⁺ transport in DCT. There is a growing body of evidence that demonstrates that NCC abundance and phosphorylation is affected by PKC or PKA downstream of angiotensin II or vasopressin signaling, respectively (43, 50–52).

It has previously been shown that NCC mRNA in the human kidney comprises 55% of NCC₃ and 45% of NCC_{SV} (14). To mimic this physiologic distribution, we performed a cotransfection experiment with NCC_{SV} S811A and NCC₃ that demonstrated significantly less phosphorylation at T55 and T60 compared with cells that were cotransfected with NCC_{SV} and NCC₃. We have demonstrated that NCC₃ and NCC_{SV} can dimerize, which results in a mixture of hetero- and homodimers of the 2 variants. Of interest, the observed decrease in the levels of phosphorylation was >50% for both T55 and T60, which suggests that NCC_{SV} S811A not only affected NCC_{SV}, but also blocked T55 and T60 phosphorylation in NCC₃ (Fig. 5). Furthermore, cells that coexpressed NCC_{SV} S811A and NCC₃ demonstrated significantly impaired NCC activity, which confirmed the dominant-negative effect of NCC_{SV} S811 on NCC₃ function. Observations that NCC_{SV} is significantly expressed in the kidney (14), together with the fact that S811 can affect T55 and T60 phosphorylation and function in NCC_{SV} and NCC₃, implies a potential key role for S811 in NCC-mediated renal salt handling.

Although the NCC_{SV}-to-NCC₃ mRNA ratio in the human kidney is 45%, on average, the ratio of NCC_{SV} to NCC₃ expression varied from 19 to 63% in 7 human kidneys (14). The large difference in the NCC_{SV}-to-NCC₃ ratio suggests that the splicing of NCC is highly regulated in humans (19). In humans, it has been shown that ~95% of genes are alternatively spliced (53). The production of alternatively spliced mRNA is diversely regulated by a variety of external stimuli or/and genetic makeup. Between human individuals, altered splicing can lead to differences in alternative spliced mRNAs that can impact the abundance or function of a

protein (54, 55). Mechanisms of alternative splicing machinery are highly variable and not well understood (54). In several well-documented examples, such natural variation of alternative splicing has indeed been demonstrated to contribute to the development of various diseases, such as cancer and diabetes (56–60). Moreover, altered splicing of genes is suggested to influence drug response (61); therefore, unraveling the mechanism behind the splicing ratio of NCC_{SV} to NCC₃ in the kidney might revise our understanding of salt transport and the response to diuretics in the distal part of the nephron.

To explore the molecular mechanism by which NCC_{SV} S811 exerts a dominant-negative effect on NCC₃, we conducted FLIM and coimmunoprecipitation experiments. Here, we have demonstrated that both the constitutively phosphorylated and non-phosphorylated NCC_{SV} S811 mutants form heterodimers with NCC₃, which indicates that the effect of S811 on the phosphorylation of NCC₃ can occur *via* interaction between the 2 isoforms. Phosphorylation status of S811 did not affect the interaction between NCC_{SV} and NCC₃. Of importance, our findings show, for the first time to our knowledge, that NCC_{SV} and NCC₃ form hetero- and homodimers in living cells and that NCC_{SV} competes with NCC₃ on the functional level. Homodimerization of 2 NCC₃ has been proposed in previous studies that used immunoblotting (22, 62–64), and it has also been found that NCC is present at the plasma membrane in a dimerized form (63, 64). Whereas it has been demonstrated for other proteins that dimerization occurs at the surface of the plasma membrane (65), our data now suggest that the dimerization of NCC already occurs in the cytosol, as a decreased lifetime of NCC₃ was observed in organellar membranes. Dimerization of transmembrane proteins is a common mechanism that precedes advanced functional regulation of a protein. For example, in the tyrosine kinase pathway, Erb2/Erb3 cross-phosphorylate each other after dimerization (66, 67). From its dimeric nature, NCC resembles the NaKCl cotransporter (64, 68), which suggests that other members of the family of electroneutral cation-chloride cotransporters form similar oligomeric structures. Of interest, it has recently been demonstrated that NCC can interact with the epithelial Na⁺ channel (ENaC) (69). Moreover, inhibition of NCC also affected the function of ENaC, likely *via* the direct interaction of both proteins (70). As NCC and ENaC are coexpressed along the later part of the DCT, it has been suggested that this interaction can regulate Na⁺ reabsorption in the DCT (70, 71). Heterodimerization of NCC_{SV} with NCC₃ suggests that NCC_{SV} phosphorylation adds an additional regulatory mechanism by affecting the phosphorylation of N-terminal T55 and T60 of NCC₃ *via* dimerization.

NCC abundance at the plasma membrane is a powerful mechanism with which Na⁺ reabsorption in the kidney is modulated. In the current study, cell-surface biotinylation of NCC-transfected cells revealed that a change in S811 phosphorylation of NCC_{SV} does not

affect the abundance of NCC_{SV} at the plasma membrane, where NCC is functionally active. Moreover, there is no change in the plasma membrane abundance of NCC in any of the NCC isoforms or mutants when cells are exposed to hypotonic low Cl⁻ stress. Our findings indicate that, upon hypotonic low Cl⁻ stress, translocation of NCC to the plasma membrane is not affected. Rather, intrinsic T55 and T60 phosphorylation of NCC at the plasma membrane is increased. Our findings are in agreement with previous studies that have demonstrated that NCC surface expression is not affected by the phosphorylation status of T60 or S811 in *X. laevis* oocytes (14, 29). Similarly, the triple mutation of T55, T60, and Ser73 to alanine did not significantly change NCC abundance at the plasma membrane (33). Previously, a relationship between T60 phosphorylation and the membrane stability of NCC has been suggested (34). However, several studies have demonstrated a decreased abundance of NCC₃ T60A in both the biotinylated fractions and input fraction, which suggests an unaltered abundance at the plasma membrane (33, 34).

Taken together, our results demonstrate that S811 of NCC_{SV} plays a crucial role in NCC function and could therefore be a key factor in renal salt handling. Additional experiments are needed to find the kinases that regulate S811 phosphorylation, which will provide more insight into the influence of S811 on NCC activity and might be crucial in elucidating the pathogenesis of essential hypertension. FJ

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AUTHOR CONTRIBUTIONS

O. A. Z. Tutakhel, F. Bianchi, R. J. M. Bindels, J. G. J. Hoenderop, and J. van der Wijst conceived and designed the research; O. A. Z. Tutakhel, F. Bianchi, D. A. Smits, and J. van der Wijst performed the research; O. A. Z. Tutakhel, F. Bianchi, and D. A. Smits analyzed the data; O. A. Z. Tutakhel, F. Bianchi, D. A. Smits, and J. van der Wijst drafted the manuscript; and all authors read and approved the final manuscript.

REFERENCES

- Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., Blaha, M. J., Dai, S., Ford, E. S., Fox, C. S., Franco, S., Fullerton, H. J., Gillespie, C., Hailpern, S. M., Heit, J. A., Howard, V. J., Huffman, M. D., Judd, S. E., Kissela, B. M., Kittner, S. J., Lackland, D. T., Lichtman, J. H., Lisabeth, L. D., Mackey, R. H., Magid, D. J., Marcus, G. M., Marelli, A., Matchar, D. B., McGuire, D. K., Mohler E. R. III, Moy, C. S., Mussolino, M. E., Neumar, R. W., Nichol, G., Pandey, D. K., Paynter, N. P., Reeves, M. J., Sorlie, P. D., Stein, J., Towfighi, A., Turan, T. N., Virani, S. S., Wong, N. D., Woo, D., and Turner, M. B.; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. (2014) Heart disease and stroke statistics—2014 update: a report from the American Heart Association. *Circulation* **129**, e28–e292
- Narayan, K. M., Ali, M. K., and Koplan, J. P. (2010) Global noncommunicable diseases—where worlds meet. *N. Engl. J. Med.* **363**, 1196–1198
- Murray, C. J., and Lopez, A. D. (2013) Measuring the global burden of disease. *N. Engl. J. Med.* **369**, 448–457
- Mills, K. T., Bundy, J. D., Kelly, T. N., Reed, J. E., Kearney, P. M., Reynolds, K., Chen, J., and He, J. (2016) Global disparities of hypertension prevalence and control: a systematic analysis of population-based studies from 90 countries. *Circulation* **134**, 441–450
- Cameron, A. C., Lang, N. N., and Touyz, R. M. (2016) Drug treatment of hypertension: focus on vascular health. *Drugs* **76**, 1529–1550
- Bloch, M. J. (2016) Worldwide prevalence of hypertension exceeds 1.3 billion. *J. Am. Soc. Hypertens.* **10**, 753–754
- Carretero, O. A., and Oparil, S. (2000) Essential hypertension. Part I: definition and etiology. *Circulation* **101**, 329–335
- Messerli, F. H., Williams, B., and Ritz, E. (2007) Essential hypertension. *Lancet* **370**, 591–603
- Mullins, L. J., Bailey, M. A., and Mullins, J. J. (2006) Hypertension, kidney, and transgenics: a fresh perspective. *Physiol. Rev.* **86**, 709–746
- Dahl, L. K., and Heine, M. (1975) Primary role of renal homografts in setting chronic blood pressure levels in rats. *Circ. Res.* **36**, 692–696
- Bianchi, G., Fox, U., Di Francesco, G. F., Bardi, U., and Radice, M. (1973) The hypertensive role of the kidney in spontaneously hypertensive rats. *Clin. Sci. Mol. Med. Suppl.* **45**(Suppl 1), 135s–139s
- Coffman, T. M., and Crowley, S. D. (2008) Kidney in hypertension: guyton redux. *Hypertension* **51**, 811–816
- Boudoulas, K. D., Triposkiadis, F., Parissis, J., Butler, J., and Boudoulas, H. (2017) The cardio-renal interrelationship. *Prog. Cardiovasc. Dis.* **59**, 636–648
- Tutakhel, O. A., Jeleń, S., Valdez-Flores, M., Dimke, H., Piersma, S. R., Jimenez, C. R., Deinum, J., Lenders, J. W., Hoenderop, J. G., and Bindels, R. J. (2016) Alternative splice variant of the thiazide-sensitive NaCl cotransporter: a novel player in renal salt handling. *Am. J. Physiol. Renal Physiol.* **310**, F204–F216
- Ellison, D. H., Velázquez, H., and Wright, F. S. (1987) Thiazide-sensitive sodium chloride cotransport in early distal tubule. *Am. J. Physiol.* **253**, F546–F554
- Dimke, H., San-Cristobal, P., de Graaf, M., Lenders, J. W., Deinum, J., Hoenderop, J. G., and Bindels, R. J. (2011) γ-Adducin stimulates the thiazide-sensitive NaCl cotransporter. *J. Am. Soc. Nephrol.* **22**, 508–517
- Gonzales, P. A., Pisitkun, T., Hoffert, J. D., Tchapyjnikov, D., Star, R. A., Kleta, R., Wang, N. S., and Knepper, M. A. (2009) Large-scale proteomics and phosphoproteomics of urinary exosomes. *J. Am. Soc. Nephrol.* **20**, 363–379
- Simon, D. B., Nelson-Williams, C., Bia, M. J., Ellison, D., Karet, F. E., Molina, A. M., Vaara, I., Iwata, F., Cushner, H. M., Koolen, M., Gainza, F. J., Gitleman, H. J., and Lifton, R. P. (1996) Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat. Genet.* **12**, 24–30
- Mastroianni, N., Bettinelli, A., Bianchetti, M., Colussi, G., De Fusco, M., Sereni, F., Ballabio, A., and Casari, G. (1996) Novel molecular variants of the Na-Cl cotransporter gene are responsible for Gitelman syndrome. *Am. J. Hum. Genet.* **59**, 1019–1026
- Urbanová, M., Reiterová, J., Stěrková, J., Lněnička, P., and Ryšavá, R. (2011) DNA analysis of renal electrolyte transporter genes among patients suffering from Bartter and Gitelman syndromes: summary of mutation screening. *Folia Biol. (Praha)* **57**, 65–73
- Vargas-Poussou, R., Dahan, K., Kahila, D., Venisse, A., Riveira-Munoz, E., Debaix, H., Grisart, B., Bridoux, F., Unwin, R., Moulin, B., Haymann, J. P., Vantyghem, M. C., Rigothier, C., Dussol, B., Godin, M., Nivet, H., Dubourg, L., Tack, I., Gimenez-Roqueplo, A. P., Houillier, P., Blanchard, A., Devuyst, O., and Jeunemaitre, X. (2011) Spectrum of mutations in Gitelman syndrome. *J. Am. Soc. Nephrol.* **22**, 693–703

22. Valdez-Flores, M. A., Vargas-Poussou, R., Verkaar, S., Tutakhel, O. A. Z., Valdez-Ortiz, A., Blanchard, A., Treard, C., Hoenderop, J. G., Bindels, R. J., and Jeleń, S. (2016) Functionalomics of NCC mutations in Gitelman syndrome using a novel mammalian cell-based activity assay. *Am. J. Physiol. Renal Physiol.* **311**, F1159–F1167
23. Wilson, F. H., Disse-Nicodème, S., Choate, K. A., Ishikawa, K., Nelson-Williams, C., Desitter, I., Gunel, M., Milford, D. V., Lipkin, G. W., Achard, J. M., Feely, M. P., Dussol, B., Berland, Y., Unwin, R. J., Mayan, H., Simon, D. B., Farfel, Z., Jeunemaitre, X., and Lifton, R. P. (2001) Human hypertension caused by mutations in WNK kinases. *Science* **293**, 1107–1112
24. Lalioti, M. D., Zhang, J., Volkman, H. M., Kahle, K. T., Hoffmann, K. E., Toka, H. R., Nelson-Williams, C., Ellison, D. H., Flavell, R., Booth, C. J., Lu, Y., Geller, D. S., and Lifton, R. P. (2006) Wnk4 controls blood pressure and potassium homeostasis via regulation of mass and activity of the distal convoluted tubule. *Nat. Genet.* **38**, 1124–1132
25. Yang, C. L., Zhu, X., and Ellison, D. H. (2007) The thiazide-sensitive Na-Cl cotransporter is regulated by a WNK kinase signaling complex. *J. Clin. Invest.* **117**, 3403–3411
26. Rozansky, D. J., Cornwall, T., Subramanya, A. R., Rogers, S., Yang, Y. F., David, L. L., Zhu, X., Yang, C. L., and Ellison, D. H. (2009) Aldosterone mediates activation of the thiazide-sensitive Na-Cl cotransporter through an SGK1 and WNK4 signaling pathway. *J. Clin. Invest.* **119**, 2601–2612
27. Gitelman, H. J., Graham, J. B., and Welt, L. G. (1966) A new familial disorder characterized by hypokalemia and hypomagnesemia. *Trans. Assoc. Am. Physicians* **79**, 221–235
28. Boyden, L. M., Choi, M., Choate, K. A., Nelson-Williams, C. J., Farhi, A., Toka, H. R., Tikhonova, I. R., Bjornson, R., Mane, S. M., Colussi, G., Lebel, M., Gordon, R. D., Semmekrot, B. A., Poujol, A., Välimäki, M. J., De Ferrari, M. E., Sanjad, S. A., Gutkin, M., Karet, F. E., Tucci, J. R., Stockigt, J. R., Keppler-Noreuil, K. M., Porter, C. C., Anand, S. K., Whiteford, M. L., Davis, I. D., Dewar, S. B., Bettinelli, A., Fadrowski, J. J., Belsha, C. W., Hunley, T. E., Nelson, R. D., Trachtman, H., Cole, T. R., Pinsky, M., Bockenhauer, D., Shenoy, M., Vaidyanathan, P., Foreman, J. W., Rasoulpour, M., Thameem, F., Al-Shahrouri, H. Z., Radhakrishnan, J., Gharavi, A. G., Goilav, B., and Lifton, R. P. (2012) Mutations in Kelch-like 3 and Cullin 3 cause hypertension and electrolyte abnormalities. *Nature* **482**, 98–102
29. Pacheco-Alvarez, D., Cristóbal, P. S., Meade, P., Moreno, E., Vazquez, N., Muñoz, E., Díaz, A., Juárez, M. E., Giménez, I., and Gamba, G. (2006) The Na⁺:Cl⁻ cotransporter is activated and phosphorylated at the amino-terminal domain upon intracellular chloride depletion. *J. Biol. Chem.* **281**, 28755–28763
30. Alessi, D. R., Zhang, J., Khanna, A., Hochdörfer, T., Shang, Y., and Kahle, K. T. (2014) The WNK-SPAK/OSR1 pathway: master regulator of cation-chloride cotransporters. *Sci. Signal.* **7**, re3
31. Subramanya, A. R., Liu, J., Ellison, D. H., Wade, J. B., and Welling, P. A. (2009) WNK4 diverts the thiazide-sensitive NaCl cotransporter to the lysosome and stimulates AP-3 interaction. *J. Biol. Chem.* **284**, 18471–18480
32. Zhou, B., Zhuang, J., Gu, D., Wang, H., Cebotaru, L., Guggino, W. B., and Cai, H. (2010) WNK4 enhances the degradation of NCC through a sortilin-mediated lysosomal pathway. *J. Am. Soc. Nephrol.* **21**, 82–92
33. Hossain Khan, M. Z., Sohara, E., Ohta, A., Chiga, M., Inoue, Y., Isobe, K., Wakabayashi, M., Oi, K., Rai, T., Sasaki, S., and Uchida, S. (2012) Phosphorylation of Na-Cl cotransporter by OSR1 and SPAK kinases regulates its ubiquitination. *Biochem. Biophys. Res. Commun.* **425**, 456–461
34. Yang, S. S., Fang, Y. W., Tseng, M. H., Chu, P. Y., Yu, I. S., Wu, H. C., Lin, S. W., Chau, T., Uchida, S., Sasaki, S., Lin, Y. F., Sytwu, H. K., and Lin, S. H. (2013) Phosphorylation regulates NCC stability and transporter activity *in vivo*. *J. Am. Soc. Nephrol.* **24**, 1587–1597
35. Pathare, G., Tutakhel, O. A. Z., van der Wel, M. C., Shelton, L. M., Deinum, J., Lenders, J. W. M., Hoenderop, J. G. J., and Bindels, R. J. M. (2017) Hydrochlorothiazide treatment increases the abundance of the NaCl cotransporter in urinary extracellular vesicles of essential hypertensive patients. *Am. J. Physiol. Renal Physiol.* **312**, F1063–F1072
36. Nørholm, M. H. (2010) A mutant Pfu DNA polymerase designed for advanced uracil-excision DNA engineering. *BMC Biotechnol.* **10**, 21
37. Vijftigchild, L. A., van der Ent, C. K., and Beekman, J. M. (2013) A novel fluorescent sensor for measurement of CFTR function by flow cytometry. *Cytometry A* **83**, 576–584
38. De Groot, T., Lee, K., Langeslag, M., Xi, Q., Jalink, K., Bindels, R. J., and Hoenderop, J. G. (2009) Parathyroid hormone activates TRPV5 via PKA-dependent phosphorylation. *J. Am. Soc. Nephrol.* **20**, 1693–1704
39. Verboogen, D. R. J., González Mancha, N., Ter Beest, M., and van den Bogaart, G. (2017) Fluorescence lifetime imaging microscopy reveals rerouting of SNARE trafficking driving dendritic cell activation. *Elife* **6**, 3289
40. Barber, P. R., Ameer-Beg, S. M., Gilbey, J. D., Edens, R. J., Ezike, I., and Vojnovic, B. (2005) Global and pixel kinetic data analysis for FRET detection by multi-photon time-domain FLIM. *SPIE Proceedings* **5700**
41. Barber, P. R., Ameer-Beg, S. M., Gilbey, J., Carlin, L. M., Keppler, M., Ng, T. C., and Vojnovic, B. (2009) Multiphoton time-domain fluorescence lifetime imaging microscopy: practical application to protein–protein interactions using global analysis. *J. R. Soc. Interface* **6**(Suppl 1), S93–S105
42. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682
43. Pedersen, N. B., Hofmeister, M. V., Rosenbaek, L. L., Nielsen, J., and Fenton, R. A. (2010) Vasopressin induces phosphorylation of the thiazide-sensitive sodium chloride cotransporter in the distal convoluted tubule. *Kidney Int.* **78**, 160–169
44. Richardson, C., Rafiqi, F. H., Karlsson, H. K., Moleleki, N., Vandewalle, A., Campbell, D. G., Morrice, N. A., and Alessi, D. R. (2008) Activation of the thiazide-sensitive Na⁺:Cl⁻ cotransporter by the WNK-regulated kinases SPAK and OSR1. *J. Cell Sci.* **121**, 675–684
45. Wallrabe, H., and Periasamy, A. (2005) Imaging protein molecules using FRET and FLIM microscopy. *Curr. Opin. Biotechnol.* **16**, 19–27
46. Jares-Erijman, E. A., and Jovin, T. M. (2003) FRET imaging. *Nat. Biotechnol.* **21**, 1387–1395
47. Hurd, T. W., Otto, E. A., Mishima, E., Gee, H. Y., Inoue, H., Inazu, M., Yamada, H., Halbritter, J., Seki, G., Konishi, M., Zhou, W., Yamane, T., Murakami, S., Caridi, G., Ghiggeri, G., Abe, T., and Hildebrandt, F. (2013) Mutation of the Mg²⁺ transporter SLC41A1 results in a nephronophthisis-like phenotype. *J. Am. Soc. Nephrol.* **24**, 967–977
48. Goytain, A., and Quamme, G. A. (2005) Functional characterization of human SLC41A1, a Mg²⁺ transporter with similarity to prokaryotic MgtE Mg²⁺ transporters. *Physiol. Genomics* **21**, 337–342
49. Lin, S. H., Shiang, J. C., Huang, C. C., Yang, S. S., Hsu, Y. J., and Cheng, C. J. (2005) Phenotype and genotype analysis in Chinese patients with Gitelman's syndrome. *J. Clin. Endocrinol. Metab.* **90**, 2500–2507
50. Castañeda-Bueno, M., Arroyo, J. P., Zhang, J., Puthumana, J., Yarbrough O. III, Shibata, S., Rojas-Vega, L., Gamba, G., Rinehart, J., and Lifton, R. P. (2017) Phosphorylation by PKC and PKA regulate the kinase activity and downstream signaling of WNK4. *Proc. Natl. Acad. Sci. USA* **114**, E879–E886
51. Mutig, K., Saritas, T., Uchida, S., Kahl, T., Borowski, T., Paliege, A., Böhlick, A., Bleich, M., Shan, Q., and Bachmann, S. (2010) Short-term stimulation of the thiazide-sensitive Na⁺:Cl⁻ cotransporter by vasopressin involves phosphorylation and membrane translocation. *Am. J. Physiol. Renal Physiol.* **298**, F502–F509
52. Saritas, T., Borschewski, A., McCormick, J. A., Paliege, A., Dathe, C., Uchida, S., Terker, A., Himmerkus, N., Bleich, M., Demarets, S., Laghmani, K., Delpire, E., Ellison, D. H., Bachmann, S., and Mutig, K. (2013) SPAK differentially mediates vasopressin effects on sodium cotransporters. *J. Am. Soc. Nephrol.* **24**, 407–418
53. Pan, Q., Shai, O., Lee, L. J., Frey, B. J., and Blencowe, B. J. (2008) Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* **40**, 1413–1415
54. Lu, Z. X., Jiang, P., and Xing, Y. (2012) Genetic variation of pre-mRNA alternative splicing in human populations. *Wiley Interdiscip. Rev. RNA* **3**, 581–592
55. Pickrell, J. K., Marioni, J. C., Pai, A. A., Degner, J. F., Engelhardt, B. E., Nkadori, E., Veyrieras, J. B., Stephens, M., Gilad, Y., and Pritchard, J. K. (2010) Understanding mechanisms underlying human gene expression variation with RNA sequencing. *Nature* **464**, 768–772
56. Allagnat, F., Christulia, F., Ortis, F., Piro, P., Lortz, S., Lenzen, S., Eizirik, D. L., and Cardozo, A. K. (2010) Sustained production of spliced X-box binding protein 1 (XBP1) induces pancreatic beta cell dysfunction and apoptosis. *Diabetologia* **53**, 1120–1130
57. Skotheim, R. I., and Nees, M. (2007) Alternative splicing in cancer: noise, functional, or systematic? *Int. J. Biochem. Cell Biol.* **39**, 1432–1449

58. Sveen, A., Kilpinen, S., Ruusulehto, A., Lothe, R. A., and Skotheim, R. I. (2016) Aberrant RNA splicing in cancer; expression changes and driver mutations of splicing factor genes. *Oncogene* **35**, 2413–2427
59. Zhang, W., Duan, S., Bleibel, W. K., Wisel, S. A., Huang, R. S., Wu, X., He, L., Clark, T. A., Chen, T. X., Schweitzer, A. C., Blume, J. E., Dolan, M. E., and Cox, N. J. (2009) Identification of common genetic variants that account for transcript isoform variation between human populations. *Hum. Genet.* **125**, 81–93
60. Hull, J., Campino, S., Rowlands, K., Chan, M. S., Copley, R. R., Taylor, M. S., Rockett, K., Elvidge, G., Keating, B., Knight, J., and Kwiatkowski, D. (2007) Identification of common genetic variation that modulates alternative splicing. *PLoS Genet.* **3**, e99
61. Heinzen, E. L., Yoon, W., Tate, S. K., Sen, A., Wood, N. W., Sisodiya, S. M., and Goldstein, D. B. (2007) Nova2 interacts with a *cis*-acting polymorphism to influence the proportions of drug-responsive splice variants of SCN1A. *Am. J. Hum. Genet.* **80**, 876–883
62. Rieg, T., Tang, T., Uchida, S., Hammond, H. K., Fenton, R. A., and Vallon, V. (2013) Adenylyl cyclase 6 enhances NKCC2 expression and mediates vasopressin-induced phosphorylation of NKCC2 and NCC. *Am. J. Pathol.* **182**, 96–106
63. De Jong, J. C., Willems, P. H., Mooren, F. J., van den Heuvel, L. P., Knoers, N. V., and Bindels, R. J. (2003) The structural unit of the thiazide-sensitive NaCl cotransporter is a homodimer. *J. Biol. Chem.* **278**, 24302–24307
64. Frindt, G., and Palmer, L. G. (2009) Surface expression of sodium channels and transporters in rat kidney: effects of dietary sodium. *Am. J. Physiol. Renal Physiol.* **297**, F1249–F1255
65. Lin, W. C., Iversen, L., Tu, H. L., Rhodes, C., Christensen, S. M., Iwig, J. S., Hansen, S. D., Huang, W. Y., and Groves, J. T. (2014) H-Ras forms dimers on membrane surfaces *via* a protein–protein interface. *Proc. Natl. Acad. Sci. USA* **111**, 2996–3001
66. Maruyama, I. N. (2014) Mechanisms of activation of receptor tyrosine kinases: monomers or dimers. *Cells* **3**, 304–330
67. Schlessinger, J. (2004) Common and distinct elements in cellular signaling *via* EGF and FGF receptors. *Science* **306**, 1506–1507
68. Moore-Hoon, M. L., and Turner, R. J. (2000) The structural unit of the secretory Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1) is a homodimer. *Biochemistry* **39**, 3718–3724
69. Schmitt, R., Ellison, D. H., Farman, N., Rossier, B. C., Reilly, R. F., Reeves, W. B., Oberbäumer, I., Tapp, R., and Bachmann, S. (1999) Developmental expression of sodium entry pathways in rat nephron. *Am. J. Physiol.* **276**, F367–F381
70. Mistry, A. C., Wynne, B. M., Yu, L., Tomilin, V., Yue, Q., Zhou, Y., Al-Khalili, O., Mallick, R., Cai, H., Alli, A. A., Ko, B., Mattheyses, A., Bao, H. F., Pochynyuk, O., Theilig, F., Eaton, D. C., and Hoover, R. S. (2016) The sodium chloride cotransporter (NCC) and epithelial sodium channel (ENaC) associate. *Biochem. J.* **473**, 3237–3252
71. Wynne, B. M., Mistry, A. C., Al-Khalili, O., Mallick, R., Theilig, F., Eaton, D. C., and Hoover, R. S. (2017) Aldosterone modulates the association between NCC and ENaC. *Sci. Rep.* **7**, 4149

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