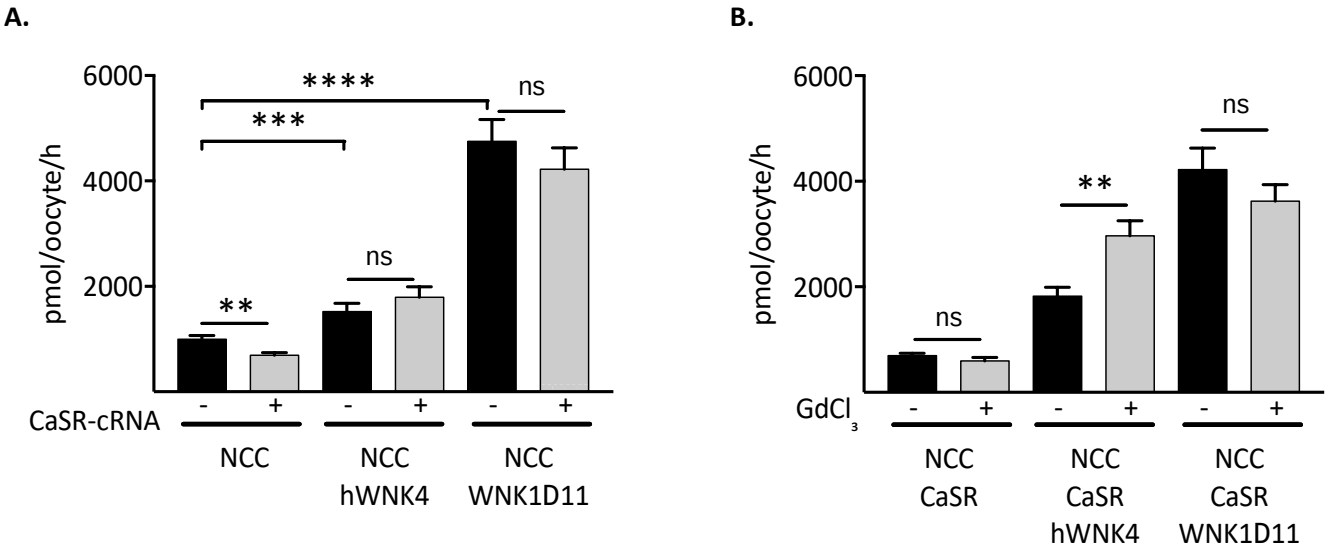
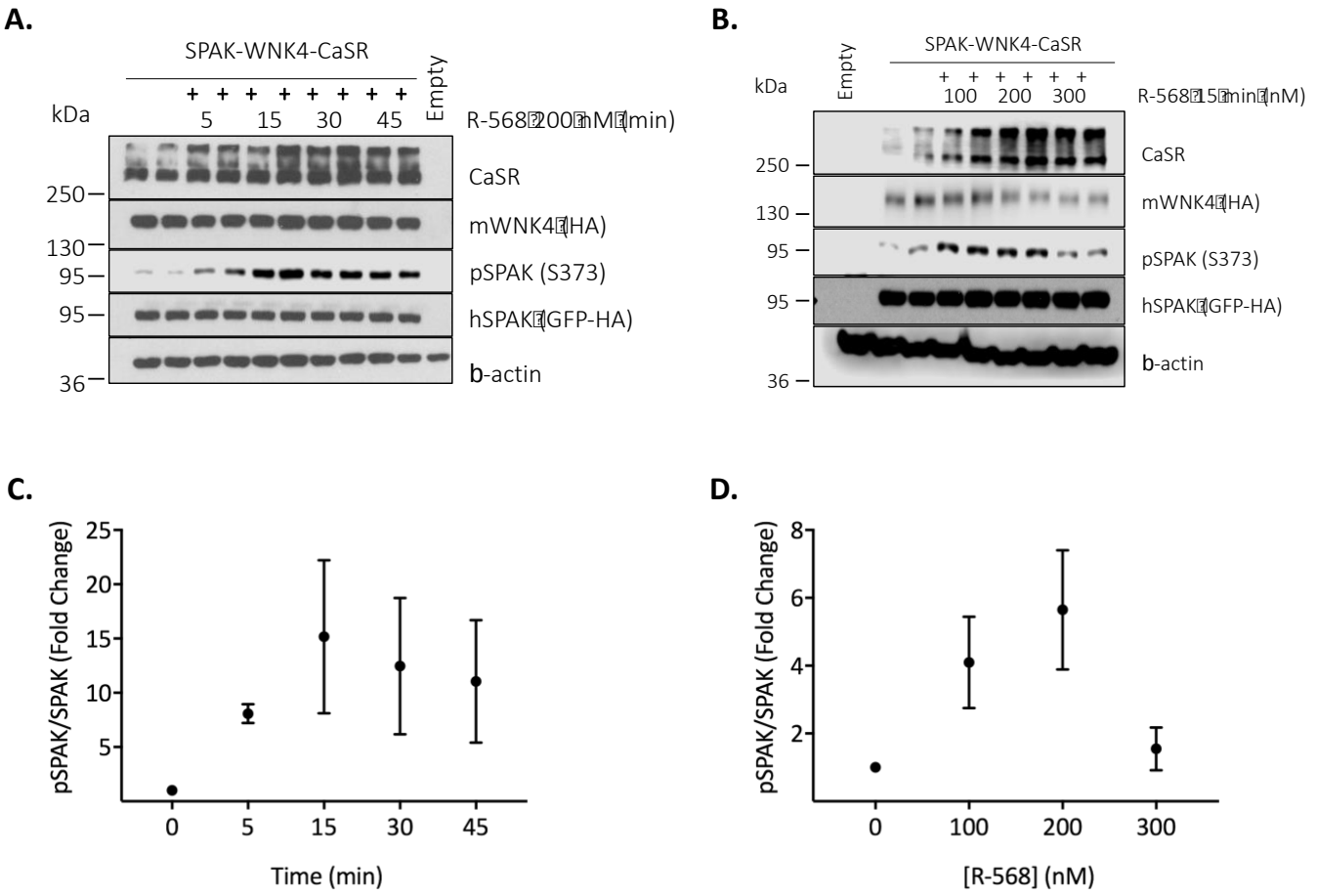
Supplemental Information

1. Supplemental Figures



Supplementary Fig. 1. **CaSR activates NCC in a WNK4-dependent manner in *X. laevis* oocytes. A.** The presence of none-activated CaSR has no effect on WNK4 or WNK1-induced activation of NCC. Functional expression assay shows the thiazide-sensitive Na^+ uptake (pmol/oocyte/h) in groups of oocytes injected with NCC, NCC + hWNK4, NCC + WNK1D11 cRNA (black bars) or together with CaSR cRNA (grey bars), as stated. **p<0.005 vs. NCC. ***p=0.0005 vs. NCC. ****p<0.0001 vs. NCC. **B.** Activation of CaSR with $GdCl_3$ increased the activity of NCC only in the presence of WNK4. Uptake was performed in control conditions (black bars) or after stimulation with $GdCl_3$ 80 µM for 15 min (gray bars). **p<0.005 vs. its own control (NCC CaSR hWNK4 with no stimulation).



Supplementary Fig. 2. CaSR phosphorylates SPAK in a time- and dose-dependent manner in HEK-293 cells. **A and B.** Representative immunoblots of time- and dose- curves of HEK-293 cells transfected with SPAK-GFP-HA, mWNK4-HA and hCaSR. The day before the experiment, cells were serum-starved in a calcium free isotonic medium and left overnight. On the day of the experiment, cells were switched to an isotonic solution containing 0.75 mM $CaCl_2$ + the indicated concentrations of R-568 for the indicated times. **C. and D.** Densitometric analyses are shown in the graphs below the representative immunoblot. Each point in the graphs depicts the mean ± S.E.M of at least 3 independent experiments.

2. Complete Methods

2.1. NCC functional experiments in *X. laevis* oocytes extended

The use of *Xenopus laevis* frogs was approved by the Animal Care and Use committee of our institution. Oocytes were surgically extracted from Tricaine (0.17%) anesthetized adult female *Xenopus laevis* frogs and incubated in Ca^{2+}-free ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4) together with type B collagenase for 1.5 hours. After four washes with ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4) oocytes were incubated overnight at 16°C in ND96 medium, supplemented with 5 mg/100 ml gentamicin. The next day, oocytes were microinjected with 50 nl of H_2O alone or containing 0.2-0.4 µg/µl of NCC, hCaSR, hWNK4, or hWNK1 cRNA in various combinations. Oocytes were then maintained at 16 °C with daily changes of ND96 medium with gentamicin until the day of the experiment. cRNA for injection was transcribed *in vitro* from cDNA linearized at the 3' end using T7 RNA polymerase mMESSAGE kit (Ambion). All experimental data is based on a minimum of 3 independent experiments.

48 hours after microinjection, NCC transport assay was conducted following our protocol[1-3] utilizing the radioactive tracer $^{22}Na^+$ (Perkin Elmer Life Sciences). Briefly, 10-15 oocytes per well were pre-incubated for 15 minutes in a Cl^--free ND96 medium (96 mM Na^+ isethionate, 2mM K^+ gluconate, 1.8 mM Ca^{2+}

gluconate, 1 mM Mg^{2+} gluconate and 5 mM HEPES, pH 7.4) containing 1mM ouabain, 100 µM amiloride, 100 µM bumetanide and in the presence or absence of 100 µM metolazone. Then, oocytes were transferred for uptake to a K^+-free uptake medium (40 mM NaCl, 56 mM Na^+ gluconate, 4 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4) containing ouabain, amiloride, bumetanide, with or without metolazone and with 1 µCi of $^{22}Na^+$ for 60 minutes at 32 °C. At the end of the uptake period, oocytes were transferred to ice-cold radioactive-free uptake medium and washed five times to remove any excess of radioactive tracer from oocytes membrane. Oocytes were finally placed individually in plastic tubes containing SDS 1% to be lysed and for radioactive tracer determination with β–cintillation counting. For treatment with $GdCl_3$, oocytes were pre-incubated for 15 minutes in the Cl^--free ND96 medium, then transferred to wells with or without 80 µM of $GdCl_3$ in the Cl^--free ND96 medium for 15 minutes, then washed one time in the uptake medium and then transferred for uptake.

2.2. Cell Culture and Transient Transfection Experiments

HEK-293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C under 95% air and 5% CO_2 in DMEM (11965-092) (Dulbecco's Modified Eagle's medium, Life Technologies) containing 1.8 mM Ca^{2+} and supplemented with 10% FBS (Invitrogen) and 100 U/ml penicillin and streptomycin (Invitrogen). These cells were transiently transfected with hCaSR, mWNK4-HA-FLAG, mWNK45A-HA[5] and hSPAK-GFP-HA DNA. hCaSR-E228K and hCaSR185Q were generated in our own laboratories. Cells were transfected with lipofectamine 2000 (Life Technologies) following

manufacturer's instructions. For treatment with NPS R-568 (Tocris Biosciences), cells were serum-depleted overnight and then the drug was added before to the culture medium and incubated at 37°C for 30 min. For treatment with PKC inhibitor bisindolylmaleimide I (BIM) (Cell Signaling Technology), the drug was added to the culture medium and left overnight. For treatment with WNK463, the drug was added to the culture medium to a final concentration of 4 µM for 2 h prior to the administration of NPS R-568.

For the time- and dose-response curves in Supplementary Fig 1A and B, cells were fasted overnight in a Ca^{2+}-free isotonic solution (135 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM Na_2SO_4, 0.5 mM Na_2HPO_4, 15 mM HEPES, 25 mM glucose, pH 7.4). On the next day, cells were switched to an isotonic solution phosphate- and sulphate-free with calcium (135 mM NaCl, 5 mM KCl, 0.75 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM HEPES, 25 mM glucose, pH 7.4) with or without NPS R-568 (Tocris Biosciences) for the time and dose indicated before lysis.

2.3. Western Blot and Immunoprecipitation

48 hours after transfection, cells were lysed with a lysis buffer (50 mM Tris-HCl (pH 7.5), 1mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% (wt/vol) Nonidet P-40 and 0.27 M sucrose) containing protease inhibitors (Complete, Roche). Cells were then pelleted by centrifugation at 20,000 g for 12 minutes at 4°C and supernatants were recovered. Protein concentration was quantified by the BCA protein assay. 20 µg of cell lysate was prepared with Laemmli buffer 5X and separated by electrophoresis on 7.5% SDS-PAGE. Cell lysates (0.3-0.5 mg of protein) that were used for immunoprecipation were incubated with 10 µl of

agarose beads coated with anti-FLAG antibody (Sigma-Aldrich) at 4°C for 2 hours. Beads were collected by centrifugation and washed two times with 500 µl of IP buffer and one time with ice-cold PBS buffer. IP proteins were eluted with 20 µl of Laemmli buffer 2.5X at 95°C for 8 minutes and separated by electrophoresis on 7.5% SDS-PAGE. Proteins were electrotransferred onto PVDF membranes and immunoblotting was performed. Membranes were blocked for 1.5 hours in 10% (wt/vol) nonfat milk (BioRad) dissolved in Tris-buffered saline (TBS)-Tween20. Incubation with primary selective antibodies was conducted overnight at 4°C and with secondary antibodies for 1 hour at room temperature. The following primary commercial HRP-conjugated antibodies were used: anti-Flag 1:5000 (Sigma-Aldrich), anti-HA 1:2500 (Sigma-Aldrich), anti-beta-actin 1:2500 (Santa Cruz Biotechnology). Other commercial antibodies not HRP-conjugated were: monoclonal anti-CaSR 0.8 µl/ml (Sigma), pERK 1:4000 (Cell Signaling Technology) and pRRXS 1:2000 (Cell Signaling Technology). Polyclonal antibodies raised in sheep against NCC (S965B), phosphorylated NCC at threonines 45,50 and 55 (S995B), SPAK (S150C) and phosphorylated SPAK at serine 373 (S670BC). The concentrations used were 1-3 µg/ml. These antibodies were produced at the Medical Research Council phosphorylation unit at Dundee University. The specificity of these antibodies has been extensively proven. The secondary antibodies were HRP-conjugated: anti-mouse 1:2000 (GE), anti-Rabbit 1:10000 (Sigma) and anti-Sheep 1:10000 (Jackson). All antibodies were diluted in 5% (wt/vol) nonfat milk (BioRad) dissolved in TBS-Tween20. The immune reaction signal was detected by chemiluminescence using the Luminata Forte Western HRP substrate (Millipore).

2.4. Animal studies

Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington DC, 1996) and were approved by the Animal Care and Use committee of our institution.

2.4.1 Mice Studies. The strains used were C57BL/6 for the WT mice and SPAK$^{234A/243A}$ for the SPAK knock-in mice. For every study performed, only adult male mice were used. Mice (12-16-week-old), were kept in cages with free access to food and water. For the R-568 experiment, on the day of the experiment, mice were randomly assigned to vehicle or drug-administered groups. The calcimimetic NPS R-568 (Tocris Biosciences) was dissolved in 10% cyclodextrin and was administered (3 µg/g of weight) by oral gavage. 3 hours later, kidneys were extracted under deep anesthesia and frozen in liquid nitrogen. Kidneys were homogenized using a lysis buffer (250 mM sucrose, 10 mM triethanolamine, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate) supplemented with Complete tablets (Roche). Lysates were centrifuged 10,000 g for 10 minutes and supernatants with kidney protein extracts were recovered, quantified with a BCA assay and prepared for immunoblot.

For the furosemide (Sigma) experiment, an acute IP dose of 15 mg/kg was administered to C57BL/6 mice. 3 hours later, kidneys were extracted under deep anesthesia, frozen in liquid nitrogen and prepared for Western blot analysis.

2.4.2 Rat Kidney perfusion. The right kidney of male Wistar rats was mounted in the Langendorff system as previously described[6,7] and perfused with vehicle

(physiological saline with 10% cyclodextrin) or the calcimimetic NPS R-568 (Tocris Biosciences) at a rate of 0, 0.60 and 1.8 µg/ml/min[9] , which had no effect on the perfusion pressure. After 30 minutes of perfusion, the kidney was manually separated into the cortex and medulla and the cortex was frozen in liquid nitrogen. Kidneys were homogenized using a lysis buffer (see Western Blot and Immunoprecipitation above). Lysates were centrifuged 10,000 g for 10 minutes and supernatants with kidney protein extracts were recovered, quantified and prepared for immunoblot.

2.4.3 Immunofluorescence. Mice were anesthetized with pentobarbital (60 mg/kg). One kidney was extracted for Western blot analysis, and the other kidney was perfused with approximately 5 ml of 4 % (wt/vol) paraformaldehyde in PBS. Kidney was then extracted, incubated in 4% (wt/vol) paraformaldehyde in PBS for at least 3 h, and then incubated in 30% (wt/vol) sucrose in PBS overnight at room temperature. The kidneys were mounted in OCT (Tissue-Tek) and 5 µm sections were prepared. The slides were kept at -80°C until immunostaining. For immunostaining, tissues were hydrated in TBS-tween 0.1% and then incubated in blocking buffer for 1 h (3% (wt/vol) BSA in TBS-tween 1%). Then, slides were incubated with primary and secondary antibodies dissolved in 1% BSA in TBS-tween 0.1%. The following antibodies were used: anti-NCC[8] (1:100) and Alexa Fluor 488 (1:400) from Life Technologies.

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