SPAK/OSR1 regulate NKCC1 and WNK activity: analysis of WNK isoform interactions and activation by T-loop trans-autophosphorylation

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Mutations in the WNK [with no lysine (K) kinase] family instigate hypertension and pain perception disorders. Of the four WNK isoforms, much of the focus has been on WNK1, which is activated in response to osmotic stress by phosphorylation of its T-loop residue (Ser[382]). WNK isoforms phosphorylate and activate the related SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) protein kinases. In the present study, we first describe the generation of double-knockin ES (embryonic stem) cells, where SPAK and OSR1 cannot be activated by WNK1. We establish that NKCC1 ($Na^+/K^+/2Cl^-$ co-transporter 1), a proposed target of the WNK pathway, is not phosphorylated or activated in a knockin that is deficient in SPAK/OSR1 activity. We also observe that activity of WNK1 and WNK3 are markedly elevated in the knockin cells, demonstrating that SPAK/OSR1 significantly influences WNK activity. Phosphorylation of another regulatory serine residue, Ser[1261], in WNK1 is unaffected in knockin cells, indicating that this is not phosphorylated by SPAK/OSR1. We show that WNK isoforms interact via a C-terminal CCD (coiled-coil domain) and identify point mutations of conserved residues within this domain that ablate the ability of WNK isoforms to interact. Employing these mutants, we demonstrate that interaction of WNK isoforms is not essential for their T-loop phosphorylation and activation, at least for overexpressed WNK isoforms. Moreover, we finally establish that full-length WNK1, WNK2 and WNK3, but not WNK4, are capable of directly phosphorylating Ser[382] of WNK1 in vitro. This supports the notion that T-loop phosphorylation of WNK isoforms is controlled by trans-autophosphorylation. These results provide novel insights into the WNK signal transduction pathway and provide genetic evidence confirming the essential role that SPAK/OSR1 play in controlling NKCC1 function. They also reveal a role in which the downstream SPAK/OSR1 enzymes markedly influence the activity of the upstream WNK activators. The knockin ES cells lacking SPAK/OSR1 activity will be useful in validating new targets of the WNK signalling pathway.

Key words: coiled-coil domain, Na^+/Cl^- co-transporter (NCC), $Na^+/K^+/2Cl^-$ co-transporter 1 (NKCC1), osmotic stress, protein kinase, signal transduction.

INTRODUCTION

Mutations in the genes encoding WNK1 [with no lysine (K) kinase-1] or WNK4 result in an inherited hypertension syndrome termed Gordon's syndrome or PHAII (pseudohypoaldosteronism type II) [1]. Mutations within a neuronal-specific splice variant of WNK1 have been shown to cause the hereditary sensory neuropathy type II pain perception disorder [2,3]. All isoforms of WNK possess an N-terminal kinase catalytic domain that is unusual in that the catalytic lysine residue normally found in subdomain II of most other kinases is replaced with another lysine residue in subdomain I [4,5]. Other than an N-terminal catalytic domain that is followed by an auto-inhibitory region, which suppresses basal kinase activity [6] and 1–3 CCDs (coiled-coil domains), the WNK isoforms contain no other obvious functional domains (see Figure 3A). Furthermore, outside these regions there is no significant sequence homology between WNK isoforms.

The best-characterized WNK substrates comprise the closely related and highly homologous STE20 kinase members SPAK (SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1), which are activated following phosphorylation [7–9]. WNK isoforms interact with SPAK as well as OSR1 and phosphorylate these enzymes at two conserved residues, namely the T-loop threonine residue (Thr[233] in human SPAK) and the serine residue in the S-motif (Ser[373] in human SPAK) (reviewed in [10]). T-loop phosphorylation triggers activation of SPAK and OSR1, whereas the role of S-motif phosphorylation is unclear, as the mutation of this residue does not affect SPAK or OSR1 activation [7,11]. SPAK and OSR1 kinases phosphorylate and activate members of the electroneutral cation-coupled chloride co-transporters (SLC12), including NCC (Na^+/Cl^- co-transporter), NKCC1 ($Na^+/K^+/2Cl^-$ co-transporter 1) and NKCC2, which are targets for the blood-pressure-lowering thiazide diuretic and loop diuretic drugs [9,12–16]. Knockin mice expressing a form of SPAK in which the T-loop residue is changed to alanine to prevent activation by WNK isoforms have low blood pressure and reduced phosphorylation of NCC in the kidney [17]. SPAK knockout mice display a similar phenotype [18].

The cellular activity of WNK1 is stimulated by hyperosmotic or hypotonic low chloride conditions [11,19,20]. Phosphorylation of a conserved serine residue present in all WNK isoforms (Ser[382] in WNK1) is required for activation of WNK1 [11]. The isolated kinase domain of WNK1, when expressed in bacteria, is phosphorylated at Ser[382], suggesting that WNK isoforms are capable of auto-phosphorylating this residue [6,11]. However, it has not been demonstrated experimentally that WNK isoforms can directly phosphorylate Ser[382] in the T-loop of WNK1 *in vitro*. Furthermore, kinase-inactive WNK1 is still phosphorylated at its T-loop *in vivo*, raising the possibility that a distinct upstream kinase mediates phosphorylation [11]. It has also been reported that WNK isoforms interact with one another [20–23], but whether this is required for WNK activation and/or SPAK/OSR1 phosphorylation is not known. Moreover, there is also controversy regarding which regions mediate association of WNK isoforms, with two reports suggesting that is mediated via the N-terminal catalytic domain [20,21], whereas other studies have implicated the C-terminal non-catalytic region [22,23].

In the present study we initially generated double knockin ES (embryonic stem) cells expressing forms of SPAK and OSR1 that cannot be activated by WNK isoforms and employed these to establish that SPAK/OSR1 regulate the phosphorylation and activation of NKCC1 *in vivo*. We also found that the activity of WNK1 and WNK3 is markedly elevated and not activated further in response to osmotic stress in cells lacking SPAK/OSR1 activity, suggesting that SPAK/OSR1 can significantly influence WNK isoform activity. We also provide evidence that the ability of full-length WNK isoforms to interact is mediated via a CCT (conserved C-terminal) coiled-coiled domain and identify mutations that inhibit such interactions. In addition, we demonstrate that full-length WNK1, WNK2 and WNK3, but not WNK4, can directly phosphorylate the T-loop of WNK1 *in vitro*, providing further evidence that WNK activity is regulated by a trans-autophosphorylation reaction. Overall, these results provide further information on the mechanism by which activity of WNK isoforms is controlled.

MATERIALS AND METHODS

Materials

Sequencing-grade trypsin was from Promega. Complete™ protease inhibitor cocktail tablets were from Roche. Protein G–Sepharose, glutathione–Sepharose and [γ-^{32}P]ATP were from Amersham Biosciences. Tween 20, Triton X-100 and dimethyl pimelimidate were from Sigma-Aldrich. Colloidal Blue staining kit and Precast SDS polyacrylamide Bis-Tris gels were from Invitrogen. Phosphocellulose P81 paper was from Whatman. All peptides were synthesized by Dr Graham Bloomberg (Molecular Recognition Centre, University of Bristol School of Medical Sciences, Bristol, U.K.).

General methods

Restriction enzyme digests, DNA ligations, generation of knockin mouse ES cells, other recombinant DNA procedures, electrophoresis, cell culture and transfections were performed using standard protocols. All mutagenesis was performed using the QuikChange site-directed mutagenesis method (Stratagene) with KOD polymerase (Novagen). DNA constructs used for transfection were purified from *Escherichia coli* DH5α using QIAGEN kits according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing, which was performed by the Sequencing Service (School of Life Sciences,

University of Dundee, Dundee, U.K.), using DYEnamic ET terminator chemistry (GE Healthcare) on Applied Biosystems automated DNA sequencers.

Antibodies

The antibodies against the following peptides were raised in sheep and affinity-purified on the appropriate antigen by the Division of Signal Transduction Therapy Unit at the University of Dundee: WNK1 (residues 2360–2482 of human WNK1, S062B), WNK2 (residues 1605–1871 of human WNK2, S140C), WNK3 (residues 1142–1461 of human WNK3, S156C), WNK4 (residues 1221–1243 of human WNK4, S064B), WNK2 mouse (residues 1650–1808 of mouse WNK2, S385C), WNK3 mouse (residues 1145–1508 of mouse WNK3, S346C). Validation of the specificity of the human and mouse WNK isoform antibodies is presented in Supplementary Figures S1 and S2 at http://www.BiochemJ.org/bj/441/bj4410325add.htm). Additional antibodies were against WNK1 pS382 [residues 375–389 of human WNK1 phosphorylated on Ser[382], KRASFAK(*P*)SVIGTPEF, S099B], WNK1 pS1261 [residues 1254–1268 of human WNK1 phosphorylated on Ser[1261], AGRRFIV(*P*)SPVPESRL, S854B], SPAK mouse (residues 439–453 of mouse SPAK, QSLSVHDSQAQPNAN, S150C), OSR1 mouse (residues 389–408 of mouse OSR1, SAHLPQPAGQMPTQPAQVSL, S149C), SPAK/OSR1 (T-loop) phospho-Thr[233]/Thr[185] [residues 226–238 of human SPAK or residues 178–190 of human OSR1, TRNKVRK(*P*)TFVGTP, S204C], SPAK/OSR1 (S-motif) phospho-Ser[373]/Ser[325] [residues 367–379 of human SPAK, RRVPGS(*P*)SGHLHKT, which is highly similar to residues 319–331 of human OSR1, in which the sequence is RRVPGS(*P*)SGRLHKT, S670B], NKCC1 phospho-Thr[203], Thr[207] and Thr[212] [residues 198–217 of human NKCC1, HYYYD(*P*)THTN(*P*)TYYLR(*P*)TFGHNT, S763B], NKCC1 total (residues 1–260 of shark NKCC1, S841B), moesin (full-length human moesin, S135C) and GST (glutathione transferase) (full-length bacterial GST, S902A). The anti-FLAG antibody (F1804) was purchased from Sigma–Aldrich. Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Pierce. Pre-immune IgG used in control immunoprecipitation experiments was affinity-purified from pre-immune serum using Protein G–Sepharose.

Buffers

Buffer A contained 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 1 mM DTT (dithiothreitol). Lysis buffer contained 50 mM Tris/HCl, pH7.5, 1 mM EGTA, 1 mM EDTA, 1 mM Na$_3$VO$_4$, 50 mM NaF, 5 mM Na$_4$P$_2$O$_7$, 0.27 M sucrose, 1 % (v/v) Triton X-100, 1 mM benzamidine, 0.1 mM PMSF and 1 mM DTT. TBS-T contained 2 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.2 % Tween-20. SDS sample buffer contained 50 mM Tris/HCl, pH 6.8, 1 % SDS, 1 % (v/v) 2-mercaptoethanol and 6.5 % glycerol. Basic buffer contained 15 mM Hepes, pH 7.5, 135 mM NaCl, 5 mM KCl, 0.5 mM CaCl$_2$, 0.5 mM MgCl$_2$, 0.5 mM Na$_2$HPO$_4$ and 0.5 mM Na$_2$SO$_4$. Hypotonic low chloride buffer contained 7.5 mM Hepes, pH 7.5, 67.5 mM sodium gluconate, 2.5 mM potassium gluconate, 0.25 mM CaCl$_2$, 0.25 mM MgCl$_2$, 0.5 mM Na$_2$HPO$_4$ and 0.5 mM Na$_2$SO$_4$.

Cell culture, transient transfections and stimulations

HEK-293 (human embryonic kidney 293) cells were cultured on 10- or 15-cm-diameter dishes in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Non-transfected Flp-In T-Rex-HEK-293 cells were

grown as normal HEK-293 cells; however, the medium was supplemented with 100 μg/ml Zeocin and 15 μg/ml blasticidin. Stable Flp-In T-Rex-HEK-293 cell lines were purchased from Invitrogen and grown as normal HEK-293 cells; however, the medium was supplemented with 15 μg/ml blasticidin and 100 μg/ml Hygromycin B. For HEK-293 transient transfection experiments, each dish of adherent HEK-293 cells was transfected with 20 μl of 1 mg/ml poly(ethylenimine) (Polysciences) and 5–10 μg of plasmid DNA as described previously [24]. Cells were stimulated with either basic or hypotonic low chloride or hypertonic conditions for 30 min. The cells were lysed in 0.3–0.75 ml ice-cold lysis buffer, the lysates were clarified by centrifugation at 4°C for 15 min at 26 000 g and supernatants were frozen in liquid nitrogen. Protein concentration was quantified using the Bradford method [24a].

Generation of stable T-REx cell lines

Non-transfected Flp-In T-RExHEK-293 cells were co-transfected with 0.5 μg of pcDNA5-FRT vector encoding FLAG-tagged WNK isoforms and 4.5 μg of pOG44 Flp recombinase vector with 20 μl of 1 mg/ml polyethylenimine (Polysciences) as described previously [24]. At 36 h post-transfection, the cells were put under selection in medium containing 15 μg/ml blasticidin and 100 μg/ml Hygromycin B. Expression of the FLAG-tagged WNK isoforms in the stable cell lines was induced by addition of 1 μg/ml final concentration of doxycycline 12–24 h prior to harvesting.

Generation and genotyping of SPAK and OSR1 knockin ES cells

Mice were maintained under specific pathogen-free conditions in accordance with the regulations set by the University of Dundee and the U.K. Home Office. Female SPAK$^{+/T243A}$ OSR1$^{+/T185A}$ mice were induced to superovulate by the injection of PMSG (pregnant mare serum gonadotropin). This was followed 48 h later by the injection of HCG (human chorionic gonadotropin). These mice were then mated with male SPAK$^{+/T243A}$ OSR1$^{+/T185A}$ mice. Blastocysts were removed at 2.5 days post-coitus and cultured on 24-well plates on a feeder layer of mitotically inactivated primary mouse embryonic fibroblasts for 1–2 weeks to allow the ES cells to grow. Wells were trypsinized and 80 % of the aliquot was frozen into two batches, whereas the remaining 20 % was used to grow cells for DNA preparation. Cells were genotyped by PCR for SPAK and OSR1 [17].

Acquisition and genotyping of gene-trapped WNK3 knockout ES cells

Mouse ES cells that were gene-trapped for WNK3 (gene trap RRJ530) were purchased from the Gene Trap Consortium (http://www.genetrap.org). As the *WNK3* gene is located on the X chromosome and ES cells obtained from the Gene Trap Consortium are male XY cells, the prediction was that the targeted WNK3 cells would lack expression of WNK3. WNK3 knockout cells were genotyped by PCR using the WNK3 forward primer TGACATCAGGAACCTTAAAGACG and reverse primer CCACCCTCAGTCCAGTATCC (Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410325add.htm). These primers detect the inserted fragment (500 bp) that was used to disrupt expression of WNK3.

Immunoblotting

Cell lysates (20–40 μg), purified proteins or immunoprecipitates in SDS sample buffer were subjected to electrophoresis on a polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated for 5 min with TBS-T containing 10 % (w/v) non-fat dried skimmed milk powder. The membranes were then immunoblotted in 5 % (w/v) non-fat dried skimmed milk powder in TBS-T with the indicated primary antibodies for 2 h at room temperature. Sheep antibodies were used at a concentration of 1–2 μg/ml, whereas commercial antibodies were diluted 1000-fold. The incubation with phospho-specific sheep antibodies was performed with the addition of 10 μg/ml of the dephosphopeptide antigen used to raise the antibody. The blots were then washed six times with TBS-T and incubated for 45 min at room temperature with secondary horseradish peroxidase-conjugated antibodies diluted 5000-fold in 5 % (w/v) non-fat dried skimmed milk powder in TBS-T. After repeating the washing steps, the signal was detected with the enhanced chemiluminescence reagent. Immunoblots were developed using an automatic film processor (SRX-101; Konica Minolta Medical) and films were scanned with a 300 dpi resolution on a scanner (PowerLook 1000; UMAX). Figures were generated using Photoshop/Illustrator (Adobe).

Expression and purification of WNK1 (1–667, D368A) and full-length OSR1 in *E. coli*

All pGEX-6P-1 constructs were transformed into BL21 *E. coli* cells, and 1 litre cultures were grown at 37°C in Luria–Bertani broth containing 100 μg/ml ampicillin until the attenuance at 600 nm was 0.8. Isopropyl β-D-thiogalactopyranoside (30 μM) was then added and the cells were cultured for a further 16 h at 26°C. Cells were isolated by centrifugation at 26 000 g, resuspended in 40 ml of ice-cold lysis buffer and lysed in one round of freeze/thawing, followed by sonication (Branson Digital Sonifier; ten 15 s pulses with a setting of 45 % amplitude) to fragment DNA. Lysates were centrifuged at 4°C for 15 min at 26 000 g. The GST-fusion proteins were affinity-purified on 0.5 ml of glutathione–Sepharose and eluted in buffer A containing 0.27 M sucrose and 20 mM glutathione. Alternatively, to cleave GST, the resin was incubated overnight with GST-PreScission protease (50 μg/0.5 ml beads).

Plasmids

Cloning of human WNK1 and human WNK4 have been described previously [7]. In order to clone mouse WNK2 (NCBI accession number NP_083637; residues 1650–1808) and mouse WNK3 (NCBI accession number: XP_914679; residues 1145–1508), reverse transcription–PCR was carried out using mouse brain RNA (Stratagene) as a template and the SuperScript III 1 Step kit (Invitrogen). The resulting PCR products were digested with BamHI/NotI restriction enzymes and then ligated into pGex6 bacterial expression vector. The sequence was verified by DNA Sequencing & Services (MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Scotland, U.K.; http://www.dnaseq.co.uk) using Applied Biosystems Big-Dye Version 3.1. The GST-tagged protein was purified in the DSTT (Division of Signal Transduction Therapy, University of Dundee, Dundee, U.K.) by standard methods. Required amino acid mutations were introduced into the pCR2.1-TOPO clone using site-directed mutagenesis with QuikChange® (Stratagene) and sub-cloned into bacterial and mammalian expression vectors.

Purification of GST–WNK4 fragments

HEK-293 cells were transiently transfected with GST–WNK4 constructs and the cells were lysed in ice-cold lysis buffer 36 h post-transfection. The lysates were clarified by centrifugation at

$4\,^{\circ}$C for 15 min at $26\,000\,\boldsymbol{g}$. The clarified lysates (1 mg) were incubated with 5–$10\,\mu$l glutathione–Sepharose beads for 1 h at $4\,^{\circ}$C. The beads were washed three times with lysis buffer containing 0.5 M NaCl and 3 times with Buffer A. The purified proteins were eluted in $1\times$ SDS loading buffer and subjected to immunoblotting with the anti-GST and anti-WNK1 antibodies.

Purification of FLAG–WNK isoforms

Expression of the WNK isoforms was induced 12–24 h prior to harvest by addition of doxycycline to a final concentration of $1\,\mu$g/ml. The cells were stimulated with basic or hypotonic low chloride buffers for 30 min and lysed in 0.3–0.75 ml ice-cold lysis buffer. The lysates were clarified by centrifugation at $4\,^{\circ}$C for 15 min at $26\,000\,\boldsymbol{g}$. The FLAG-tagged WNK isoforms were affinity-purified by incubating 1 mg of clarified lysate with 5–$10\,\mu$l FLAG-agarose for 1 h at $4\,^{\circ}$C. The beads were washed three times with lysis buffer containing 0.5 M NaCl followed by two washes with Buffer A. The purification was subsequently either eluted in $1\times$ SDS loading buffer or used for activity measurements.

Gel filtration

HEK-293 cells or HEK-293 cells stably expressing wild-type or mutant WNK4 were lysed in a low volume of lysis buffer to obtain a protein concentration of >4 mg/ml and then centrifuged at $14\,000\,\boldsymbol{g}$ for 15 min to remove insoluble material. The supernatant was filtered through a $0.2\,\mu$m filter and subjected to desalting employing a GE Healthcare HiPrep™ 26/10 desalting column equilibrated with 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine and 1 mM DTT. The lysates were diluted to 6 mg/ml and 0.3 ml was injected on to a GE Healthcare Superose™ 6 10/300 GL column equilibrated with 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine and 1 mM DTT at a flow rate of 0.5 ml/min and 0.2 ml fractions were collected. A total of 0.2 ml of $2\times$ SDS sample buffer was added to each fraction and the fractions corresponding to molecular masses between the void volume and 44 kDa were electrophoresed on a polyacrylamide gel and immunoblotted with antibodies against WNK1, WNK3, OSR1, SPAK and FLAG.

Assay of WNK activity towards OSR1

FLAG-tagged WNK isoforms were purified as described above. The reaction mixture contained a final volume of $25\,\mu$l of Buffer A containing $2\,\mu$g of OSR1 (D164A), 0.1 mM $[\gamma\text{-}^{32}$P]ATP and 10 mM MgCl$_2$. The reaction was performed in a Thermomixer at $30\,^{\circ}$C for 30 min, and the reaction was terminated by addition of $5\,\mu$l of $5\times$ SDS loading buffer. The samples were electrophoresed on a polyacrylamide gel, which was stained with Colloidal Blue, dried and autoradiographed.

Assay of WNK activity towards WNK1 Ser[382]

Immunoprecipitation of endogenous WNK1, WNK3 or transfected FLAG-tagged WNK isoforms were undertaken as described above. The reaction mixture contained a final volume of $25\,\mu$l of Buffer A containing $1\,\mu$g of recombinant GST–WNK1 (1–667, D368A) (expressed in *E. coli*), 0.1 mM ATP and 10 mM MgCl$_2$. The reaction was performed in a Thermomixer at $30\,^{\circ}$C for 20 min and the reaction was terminated by addition of $55\,\mu$l of $2\times$ SDS loading buffer. Fractions of the samples were electrophoresed on a polyacrylamide gel and immunoblotted with anti-FLAG and anti-WNK1 phospho-Ser[382] antibodies.

Immunoprecipitation and assay of OSR1

Clarified cell lysate (2 mg) was incubated with $5\,\mu$g of the OSR1 (mouse peptide) antibody conjugated to $5\,\mu$l of Protein G–Sepharose and incubated for 2 h at $4\,^{\circ}$C with gentle agitation. The immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl and twice with 1 ml of Buffer A. The OSR1 immunoprecipitates were assayed with the CATCHtide (cation chloride co-transporter peptide substrate) (RRHYYYDTHTNTYYLRTFGHNTRR) that encompasses the OSR1 phosphorylation sites on NKCC1 [15]. Assays were set up in a total volume of $50\,\mu$l in Buffer A containing 10 mM MgCl$_2$, 0.1 mM $[\gamma\text{-}^{32}$P]ATP and $300\,\mu$M CATCHtide (RRHYYYDTHTNTYYLRTFGHNTRR). After incubation for 30 min at $30\,^{\circ}$C, the reaction mixture was applied onto P81 phosphocellulose paper, the papers were washed in phosphoric acid, with the incorporation of ^{32}P-radioactivity in CATCHtide quantified by Cerenkov counting.

^{86}Rb uptake NKCC1 assay

ES cells were plated in 12-well plates (2.4 cm diameter/well) and the ^{86}Rb uptake assay was performed on cells that were 80 % confluent. Culture medium was removed from the wells and replaced with either basic/isotonic or hypotonic/hypertonic medium for 15 min at $37\,^{\circ}$C. Cell medium was removed by means of aspiration with a vacuum pump and replaced with stimulating medium plus inhibitors including 1 mM ouabain in the presence or absence of 0.1 mM bumetanide, an NKCC1 inhibitor, for a further 15 min. After this period, the medium was removed and the cells were washed with basic/isotonic medium to remove traces of hypo/hypertonic medium; next, they were re-incubated with only basic/isotonic medium plus inhibitors containing 2 μCi/ml ^{86}Rb for 5 min at $37\,^{\circ}$C. After this incubation period, cells were rapidly washed three times with the respective ice-cold non-radioactive medium. The cells were lysed in $300\,\mu$l of ice-cold lysis buffer and ^{86}Rb uptake tracer activity was quantified on a PerkinElmer liquid scintillation analyser.

RESULTS

Generation of double knockin ES cells lacking SPAK and OSR1 activity

We previously described the generation of knockin mice in which the T-loop threonine residue in OSR1 (Thr[185]) and SPAK (Thr[243]) was mutated to alanine in order to prevent activation by WNK isoforms [17]. These mice were used to generate single OSR1[185A/185A], single SPAK[243A/243A] and double OSR1[185A/185A]/SPAK[243A/243A] knockin ES cell lines as described in the Materials and methods section. Similar levels of OSR1 protein expression were observed in wild-type and knockin ES cell lines. Immunoblot analysis revealed no obvious expression of SPAK protein in wild-type ES cells, suggesting that SPAK is either not expressed or only present at very low levels in these cells (Figures 1A and 1C). The wild-type and knockin ES cells were stimulated under hypotonic low chloride conditions (Figure 1A) or with sorbitol (Figure 1C) and OSR1 activity and T-loop phosphorylation was assessed. In wild-type cells, hypotonic low chloride (Figure 1A) and sorbitol (Figure 1C) induced significant activation of OSR1, which was accompanied by phosphorylation of Thr[185]. As expected, no detectable OSR1 activity or phosphorylation at Thr[185] was observed in the single OSR1[185A/185A] or double OSR1[185A/185A]/SPAK[243A/243A] knockin ES cell lines stimulated with hypotonic low chloride conditions (Figure 1A) or sorbitol (Figure 1C). We also assessed the phosphorylation
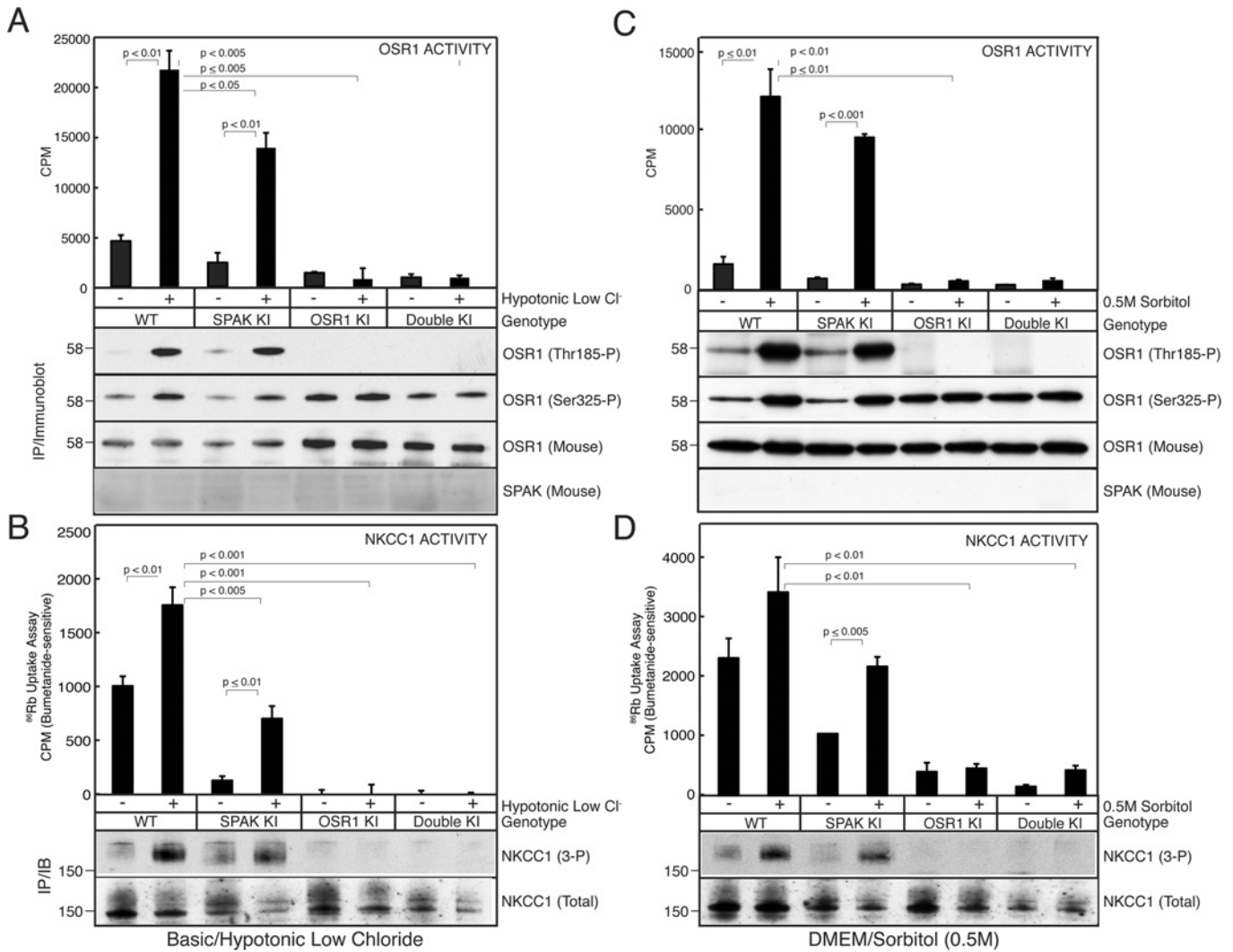
Figure 1   Evidence that SPAK/OSR1 regulate NKCC1 activity

(**A**) SPAK/OSR1 kinase-inactive mouse ES cells were stimulated with basic ($-$) or hypotonic low chloride ($+$) buffers and lysed. Wild-type (WT) (SPAK$^{+/+}$/OSR1$^{+/+}$), single SPAK knockin (KI) (SPAK$^{T243A/T243A}$/OSR1$^{+/+}$), single OSR1 knockin mice (SPAK$^{+/+}$/OSR1$^{T185A/T185A}$) and double knockin (SPAK$^{T243A/T243A}$/OSR1$^{T185A/T185A}$) cells were used. OSR1 was immunoprecipitated (IP) from the clarified lysates using the anti-OSR1 mouse peptide antibody. The immunoprecipitates were subjected to activity measurements using the CATCHtide [15]. A fraction of the immunoprecipitates was also subjected to immunoblot assay with the indicated antibodies. (**B**) Confluent ES cells were incubated with either basic ($-$) or hypotonic low chloride ($+$) buffers for 30 min in the presence of 1 mM ouabain (a known Na$^+$/K$^+$-ATPase inhibitor) and/or 0.1 mM bumetanide (a known NKCC1 inhibitor). ^{86}Rb uptake proceeded for 5 min and was then quantified by scintillation counting. Bumetanide-sensitive ^{86}Rb uptake (i.e. bumetanide-insensitive counts subtracted) is plotted for both basic and hypotonic low chloride conditions. The anti-NKCC1 antibody was used to immunoprecipitate NKCC1 from the ES cell extracts. The immunoprecipitates were subjected to immunoblot analysis using the indicated antibodies. (**C**) Same as (**A**) except that cells were stimulated with isotonic [($-$), DMEM] and hyperosmotic [($+$), 0.5 M sorbitol] medium. (**D**) Same as (**B**), except that cells were stimulated with isotonic [($-$), DMEM] and hyperosmotic [($+$), 0.5 M sorbitol] medium. The activity assay results are all presented as c.p.m. $\pm$ S.E.M. for triplicate samples. Statistical analyses were performed using the Student's t test. P values listed above each bar set refer to the comparison between non-stimulated and stimulated conditions for each ES cell type as well as a comparison of the wild-type stimulated with the stimulated single SPAK, OSR1 and double knockin ES cells. Statistical significance was determined at $P < 0.05$. Molecular masses are indicated in kDa on the left-hand side of the Western blots. IB, immunoblot.

of NKCC1 at residues phosphorylated by SPAK/OSR1 (Thr^{197}, Thr^{201} and Thr^{206}; numbering based on mouse sequence). This revealed that in wild-type as well as single SPAK$^{243A/243A}$ knockin ES cells, hypotonic low chloride conditions (Figure 1B) or sorbitol (Figure 1D) induced marked phosphorylation of NKCC1, accompanied by an increased ability to uptake ^{86}Rb$^+$ in a bumetanide (NKCC1 inhibitor)-sensitive manner. Strikingly, no detectable NKCC1 phosphorylation or ^{86}Rb$^+$ bumetanide-sensitive uptake was observed in the single OSR1$^{185A/185A}$ or double OSR1$^{185A/185A}$/SPAK$^{243A/243A}$ knockin ES cell lines (Figures 1B and 1D). The finding that NKCC1 phosphorylation and activity is abolished in the single OSR1$^{185A/185A}$ ES cells also supports the notion that SPAK is not significantly expressed in ES cells.

Increased WNK1 and WNK3 activity in OSR1$^{185A/185A}$/SPAK$^{243A/243A}$ knockin ES cells

We observed that the basal phosphorylation of OSR1 at the S-motif residue (Ser^{325}), which is phosphorylated by WNK isoforms, was markedly elevated in the single OSR1$^{185A/185A}$ or double OSR1$^{185A/185A}$/SPAK$^{243A/243A}$ knockin ES cells and not further increased by hypotonic low chloride (Figure 1A) or sorbitol (Figure 1C). The degree of phosphorylation of OSR1 at Ser^{325} in knockin cells lacking OSR1 activity in control medium was similar to phosphorylation observed in wild-type cells challenged with osmotic stress (Figures 1A and 1C). These findings suggested that WNK activity was markedly elevated in knockin ES cells
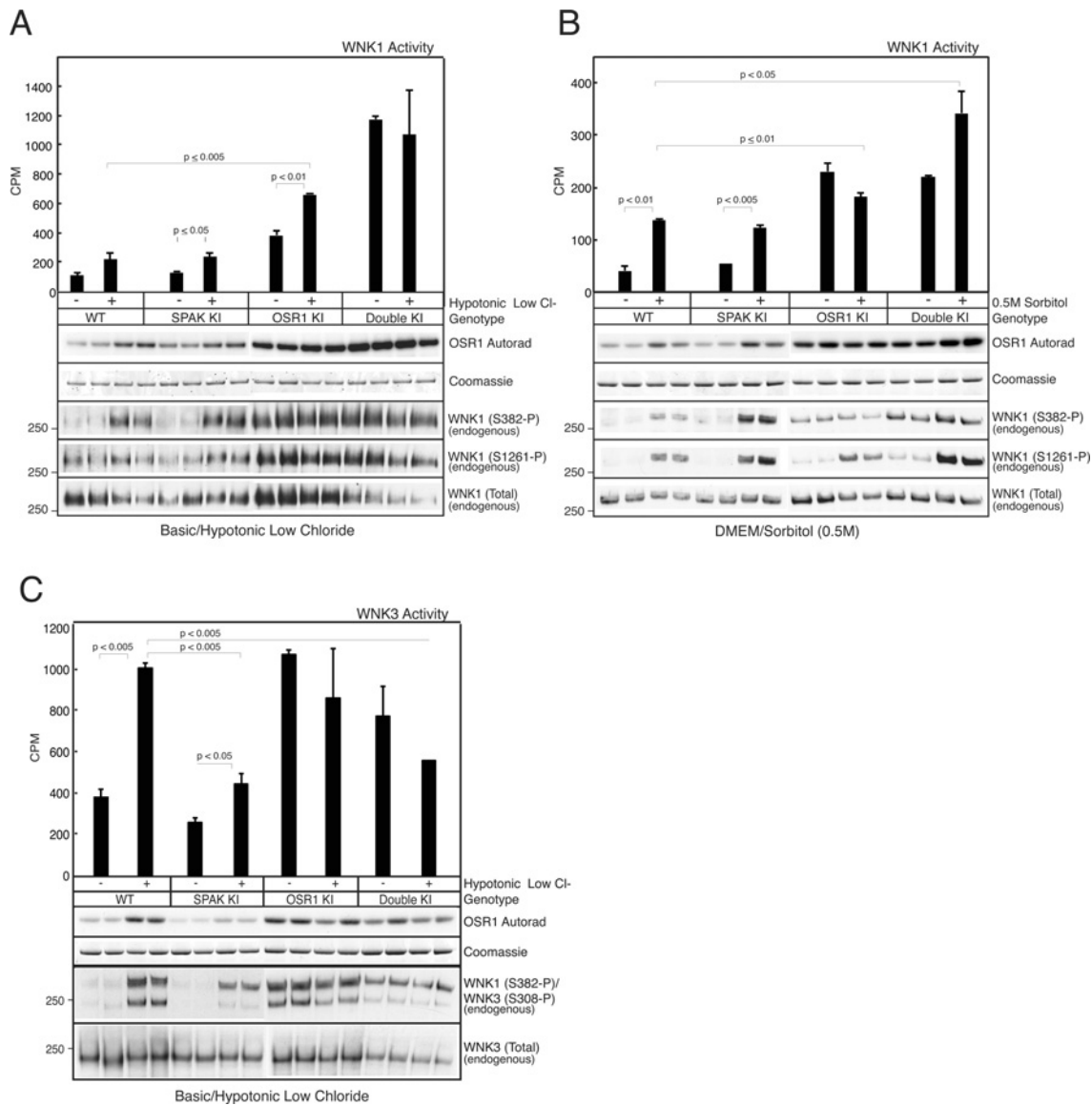
**Figure 2   SPAK/OSR1 suppresses WNK isoforms activity**

(**A**) SPAK/OSR1 ES cells were treated with either basic (−) or hypotonic low-chloride (+) medium for 30 min. WNK1 was immunoprecipitated and its activity was assayed by employing the kinase-inactive (KI) OSR1 (D164A) protein as a substrate. Phosphorylation of OSR1 was determined after electrophoresis followed by autoradiography of the Colloidal-Blue-stained bands corresponding to OSR1. The incorporation of ^{32}P radioactivity was also quantified and results are presented as the mean activity $\pm$ S.E.M. for duplicate samples. WNK1 immunoprecipitates were also immunoblotted with the indicated antibodies. (**B**) Same as (**A**), except that cells were stimulated with isotonic [(−), DMEM] or hyperosmotic [(+), 0.5 M sorbitol] medium. (**C**) Endogenous WNK3 was immunoprecipitated from SPAK/OSR1 ES cells treated with either basic (−) or hypotonic low-chloride (+) medium for 30 min and the activity was assayed employing kinase-inactive OSR1 (D164A) as a substrate. WNK3 immunoprecipitates were also immunoblotted with the indicated antibodies. Statistical analyses were performed using the Student's *t* test. *P* values listed above each bar set refers to the comparison between non-stimulated and stimulated conditions for each ES cell type as well as a comparison of the wild-type stimulated with the stimulated single SPAK, OSR1 and double knockin ES cells. Statistical significance was determined at *P* < 0.05. Molecular masses are indicated in kDa on the left-hand side of the Western blots. WT, wild-type.

lacking OSR1 activity. To verify this, we immunoprecipitated endogenous WNK1 (Figures 2A and 2B) and WNK3 (Figure 2C) from wild-type and knockin ES cells and assessed its activity by monitoring its ability to phosphorylate OSR1 *in vitro*. This revealed that, in the knockin cells lacking OSR1 activity, the basal activity of WNK1 was increased ∼5-fold and that of WNK3 increased ∼2-fold compared with wild-type cells (Figure 2).

Evidence that SPAK/OSR1 do not regulate Ser[1261] phosphorylation of WNK1

Phosphorylation of WNK1 at Ser[1261], which is located next to a CCT domain binding RFXV motif (see the Introduction), is

thought to promote dissociation of SPAK/OSR1 from WNK1 after these enzymes are activated by osmotic stress [10,11,15,26]. This would enable the CCT domain of SPAK/OSR1 to interact with substrates such as NKCC1 after these enzymes were activated by WNK1. The kinase that mediates Ser[1261] phosphorylation is unknown, but it has been suggested that SPAK/OSR1 might regulate Ser[1261] phosphorylation, as this would represent a simple system to couple the interaction of SPAK/OSR1 with WNK1 with the activation state of these enzymes [10]. To test this hypothesis, we raised a phosphospecific antibody capable of detecting phosphorylation of WNK1 at Ser[1261]. We found that phosphorylation of Ser[1261] in response to osmotic stress was not affected by loss of SPAK/OSR1 activity in the knockin ES cells
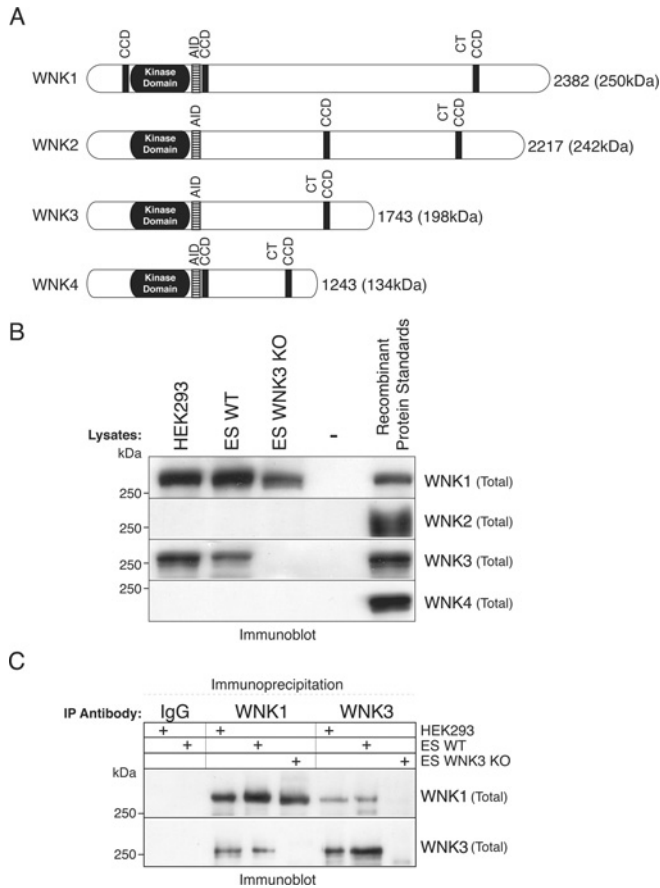
Figure 3   Endogenous WNK1 and WNK3 interact

(**A**) Schematic diagram of WNK isoform structures and the relative placement of their kinase domain, auto-inhibitory domain (AID) and predicted CCDs, including the C-terminal CCD (CT-CCD). (**B**) Lysates from HEK-293, WNK3 wild-type (WT) and WNK3 knockout (KO) gene-trapped ES cells were subjected to immunoblot analysis with the indicated antibodies. Recombinant WNK1–4 proteins were loaded as immunoblotting standards. (**C**) WNK1 and WNK3 were immunoprecipitated from HEK-293, WNK3 wild-type (WT) and WNK3 knock-out (KO) gene-trapped ES cell lysates. The preimmune IgG antibody was used as a negative control for the immunoprecipitation assay. The immunoprecipitates were subjected to immunoblotting using the indicated antibodies. Molecular masses are indicated in kDa on the left-hand side of the Western blots. IP, immunoprecipitation.

(Figures 2A and 2B). This observation suggests that SPAK/OSR1 are not involved in controlling Ser[1261] phosphorylation.

Evidence that endogenous WNK1 and WNK3 interact

Previous studies have shown that overexpressed WNK isoforms interact [20–23]. To demonstrate that endogenous WNK isoforms associate, we raised antibodies specifically recognizing the four WNK isoforms (Supplementary Figures S1 and S2). Using these reagents, we found that endogenous WNK1 and WNK3, but not WNK2 or WNK4, are readily detected in HEK-293 and mouse ES cells (Figure 3B). We also acquired WNK3 knockout ES cells from the Gene Trap Consortium (see the Materials and methods section) and showed that they expressed similar levels of WNK1 as wild-type ES cells and also confirmed the absence of WNK3 in these cells (Figure 3B). We observed that WNK3 was indeed co-immunoprecipitated with endogenous WNK1 in HEK-293 cells and wild-type ES cells, but not in WNK3 knockout ES cells (Figure 3C). Conversely, WNK1 was co-immunoprecipitated with WNK3 in HEK-293 and wild-type ES cells, but not in WNK3 knockout ES cells (Figure 3C).

Identification of key residues required for WNK isoform association

To investigate interaction of WNK isoforms in more detail, we studied the binding of endogenous WNK1 to fragments of the shortest WNK isoform (WNK4) overexpressed in HEK-293 cells (Figure 4A). These experiments revealed that a fragment encompassing the isolated WNK4 C-terminal CCD (residues 1131–1167) interacted with endogenous WNK1 to a similar extent as full-length WNK4. Fragments of WNK4 lacking the C-terminal CCD failed to interact with WNK1 (Figure 4A). To identify the critical residues within the C-terminal CCD required for interaction with WNK1, we undertook an alanine scan, in which the most highly conserved residues within this domain were mutated to alanine. This revealed that mutation of His[1145], Glu[1148] or Gln[1156] markedly reduced interaction with endogenous WNK1 (Figure 4B). Individual mutation of His[1145] or Gln[1156] in full-length WNK4 drastically reduced its ability to interact with endogenous WNK1 (Figure 4C). Moreover, mutation of both His[1145] and Gln[1156] in full-length overexpressed WNK4 abolished interaction with endogenous WNK1 (Figure 4D). His[1145], Gln[1148] and Gln[1156] are conserved in the C-terminal CCDs of all WNK isoforms (Figures 4E and 4F).

## Evidence that WNK isoforms interact via their C-terminal CCD

We next generated HEK-293 cells that stably express full-length forms of wild-type, kinase-inactive or 'HQ mutant' (residues equivalent to His[1145] or Gln[1156] in WNK4 were mutated to alanine) WNK2, WNK3 and WNK4. Using these cells, we confirmed that endogenous WNK1 and WNK3 co-immunoprecipitated with wild-type and kinase-inactive WNK2 (Figure 5A) or WNK4 (Figure 5C). The HQ mutation in WNK2 (Figure 5A) or WNK4 (Figure 5C) abolished interaction with endogenous WNK1 and WNK3. Similarly, the HQ mutation in WNK3 abolished interaction with endogenous WNK1 (Figure 5B). Stimulation of cells with hypotonic low chloride conditions to induce activation of the WNK signalling pathway did not affect association of WNK isoforms (Figure 5).

Gel filtration analysis of endogenous, WNK4 wild-type and WNK4 HQ isoforms

To analyse further the associations of the WNK isoforms, we undertook gel filtration analysis of endogenous WNK1 and WNK3 or stably overexpressed wild-type or HQ mutant WNK4 in HEK-293 cells (Figure 6). Consistent with the association of WNK isoforms, we observed that endogenous WNK1 and WNK3, as well as overexpressed WNK4, eluted at the high molecular mass region of the gel filtration column between the void volume and the 670 kDa marker (Figure 6). Minimal WNK1 and no WNK3 or WNK4 were detected in the fractions in which a ∼250 kDa globular protein would be expected to elute. In contrast, the bulk of the HQ-WNK4 mutant eluted at significantly lower molecular mass fractions of the gel filtration column, namely between the 670 and 158 kDa markers (Figure 6). A fraction of the HQ-WNK4 mutant was found to elute at a high molecular mass region of the column, indicating that HQ-WNK4 can associate with other interactors. We also immunoblotted for endogenous SPAK and OSR1, and observed that these enzymes migrated with a much lower apparent molecular mass, eluting between the 158 kDa and 44 kDa markers.

Effect of HQ mutation on the ability of WNK isoforms to phosphorylate OSR1

We next studied the intrinsic catalytic activity of each WNK isoform by assessing their ability to phosphorylate OSR1 *in vitro*.
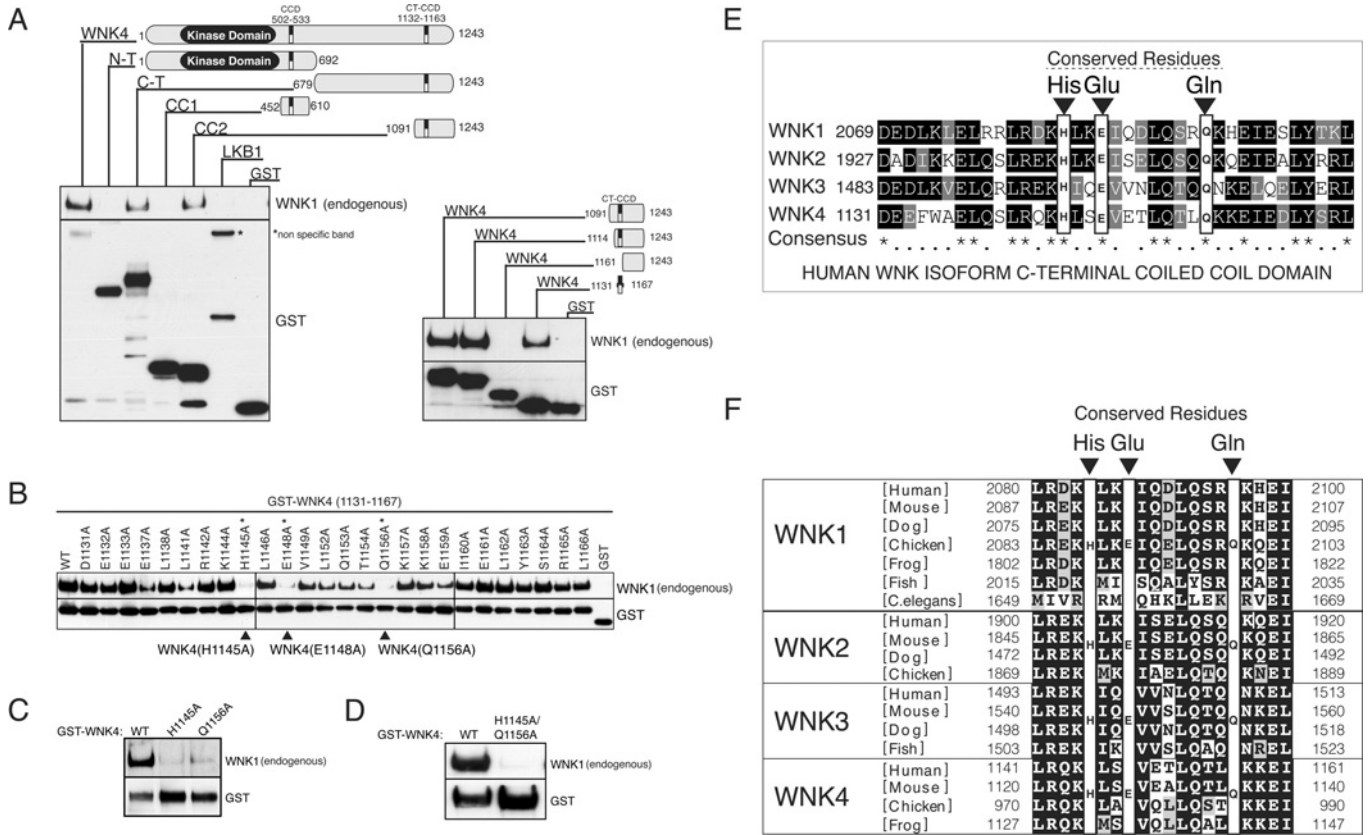
Figure 4   WNK4 C-terminal CCD binds to WNK1

(**A**) HEK-293 cells were transfected with plasmids encoding the indicated GST fusion proteins. At 36 h after transfection, cells were lysed and the clarified lysate was incubated with GSH–Sepharose to purify the GST-fusion proteins. Samples were then immunoblotted with the indicated antibodies. The position of the CCDs are indicated in the WNK4 schematic diagram above the Western blot. (**B**) HEK-293 cells were transfected with GST–WNK4 (1131–1167) wild-type (WT), the indicated alanine residue mutant or GST. At 36 h after transfection, cells were lysed and GST affinity purifications were performed. The purifications were probed for purified GST–WNK4 and co-purifying endogenous WNK1. (**C**) Same as (**B**), except that plasmids encoding full-length GST–WNK4 wild-type or the alanine mutant H1145A and Q1156A were transfected in HEK-293 cells. (**D**) Purified GST–WNK4 wild-type or a double alanine mutant H1145A/Q1156A were transfected in HEK-293 cells. (**E**) Alignment of the C-terminal CCD (CT-CCD) in the individual human WNK isoforms. The arrowheads point at the conserved histidine (His[1145]), glutamic acid (Glu[1148]) and glutamine (Gln[1156]) residues. (**F**) A sequence alignment highlighting the conserved amino acids of the C-terminal CCD of the WNK isoforms in the indicated species; histidine, glutamic acid and glutamine.
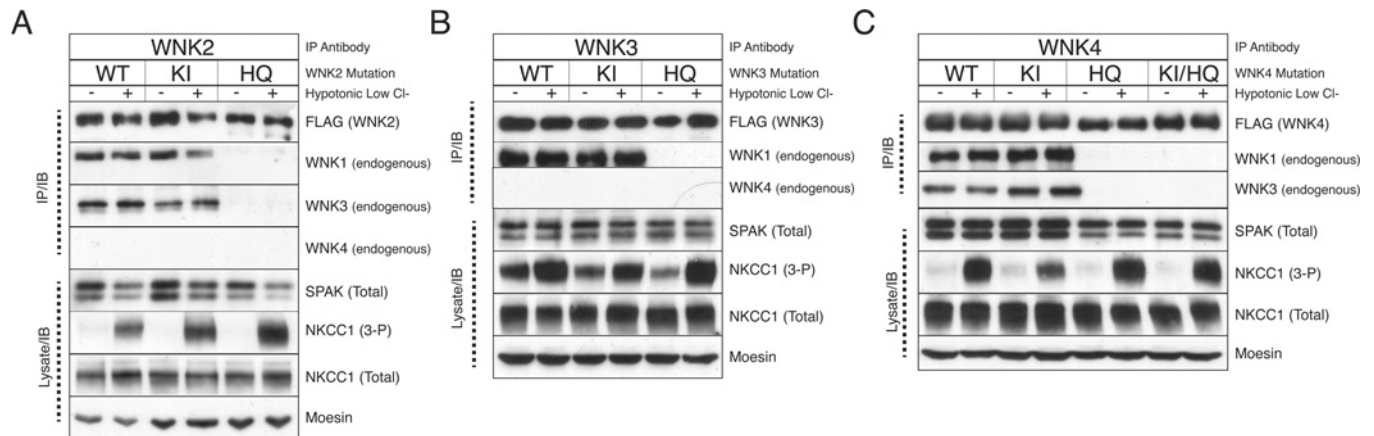
Figure 5   The WNK isoforms dimerize via the C-terminal CCD

Stable Flp-In T-REx cell lines expressing wild-type (WT) and mutant forms of WNK2 (**A**), WNK3 (**B**) and WNK4 (**C**), including the kinase-inactive (KI) and HQ forms were stimulated with basic (−) or hypotonic low chloride (+) buffers and lysed. FLAG affinity purifications were performed on the clarified lysates and purifications were immunoblotted with the indicated antibodies. Moesin levels were monitored as a loading control. IB, immunoblot; IP, immunoprecipitation.
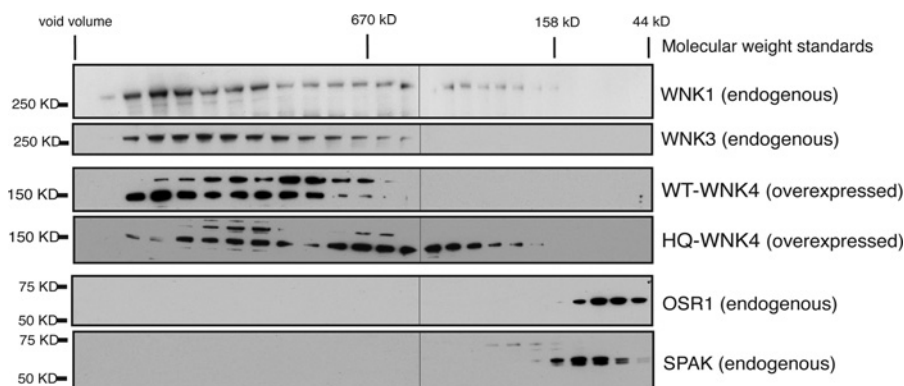
Figure 6   WNK isoforms exist in high molecular mass complexes

HEK-293 cell extract lysates or HEK-293 cell extracts expressing wild-type (WT) WNK4 or HQ mutant WNK4 that has lost ability to associate with other WNK isoforms were chromatographed on a GE Healthcare Superose™ 6 10/300 GL gel-filtration column, and fractions were immunoblotted for the indicated WNK isforms or SPAK and OSR1. Stably overexpressed WNK4 and mutant HQ-WNK4 were detected with an anti-FLAG antibody as these enzymes possessed an N-terminal FLAG tag. The elution positions of the peak fractions of the globular protein standards thyroglobulin (670 kDa), bovine γ-globulin (158 kDa) and chicken ovalbumin (44 kDa) are indicated at the top. Similar results were obtained in two separate experiments. The solid line between immunoblots indicates that the samples were run on separate gels.

This revealed that the HQ mutants of WNK2 (Figure 7A), WNK3 (Figure 7B) as well as WNK4 (Figure 7C) isoforms were active, as they phosphorylated OSR1 to a much greater extent than kinase-inactive mutants (Figure 7). The residual activity observed with the kinase-inactive mutants is likely to be due to association with endogenous isoforms. This is supported by the finding that a double kinase-inactive and HQ mutant of WNK4 failed to significantly phosphorylate OSR1 (Figure 7C). The ability of HQ mutant WNK2 (Figure 7A) and WNK3 (Figure 7B) to phosphorylate OSR1 was modestly reduced compared with the wild-type enzyme, which is likely to result from loss of association with endogenous WNK1 and WNK3.

Osmotic stress promotes WNK1 activation by inducing phosphorylation of the Ser^{382} T-loop residue [9,11]. The Ser^{382} residue as well as the surrounding amino acids are identical on all WNK isoforms, indicating that the previously characterized WNK1 phosphospecific Ser^{382} antibody [11] would recognize the T-loop phosphorylated form of all the WNK isoforms and we have confirmed this experimentally (Supplementary Figure S4 at http://www.BiochemJ.org/bj/441/bj4410325add.htm). To test whether osmotic stress promoted T-loop phosphorylation of other WNK isoforms, we investigated how hypotonic low chloride conditions affected T-loop phosphorylation of WNK isoforms. Hypotonic low chloride conditions induced a marked T-loop phosphorylation of wild-type and HQ mutant WNK2 (Figure 7A), WNK3 (Figure 7B) and WNK4 (Figure 7C). We also observed that the kinase-inactive mutants present an increased basal T-loop phosphorylation, modestly increased by hypotonic low chloride conditions (Figure 7).

Effect of HQ mutation on the ability of WNK isoforms to autophosphorylate

We next assessed the ability of immunoprecipitated WNK isoforms to autophosphorylate when incubated with $[\gamma\text{-}^{32}P]ATP$. This revealed that the HQ mutants of WNK2 (Figure 7A), WNK3 (Figure 7B) and WNK4 (Figure 7C) were phosphorylated to a markedly lower extent than the wild-type enzymes. No autophosphorylation of kinase-inactive WNK2 or WNK3 was observed. As reported previously [26], kinase-inactive WNK4 autophosphorylated to a similar extent as the wild-type protein (Figure 7C). Interestingly, the double kinase-inactive and

HQ mutant WNK4 mutant still became phosphorylated when incubated with $[\gamma\text{-}^{32}P]ATP$ (Figure 7C), suggesting that a distinct kinase activity associates with WNK4 specifically, which has also been suggested by previous work [26].

WNK1, WNK2 and WNK3 but not WNK4 phosphorylate the T-loop of WNK1 *in vitro*

Kinase-inactive WNK1 (1–667, D368A) expressed in *E. coli*, which is not phosphorylated at Ser^{382} [11], was used to test whether immunoprecipitated WNK isoforms can phosphorylate Ser^{382} *in vitro*. We found that wild-type WNK2 (Figure 8A) or WNK3 (Figure 8B) phosphorylated kinase-inactive WNK1 (1–667, D368A) at Ser^{382} *in vitro*. Consistent with this being mediated by WNK isoforms directly rather than a contaminating kinase, we observed that kinase-inactive WNK2 and WNK3 did not phosphorylate the WNK1 (1–667, D368A) at Ser^{382}. Immunoprecipitated endogenous WNK1 also phosphorylated the WNK1 (1–667, D368A) fragment at Ser^{382}, but stimulation with high osmotic stress (0.5 M NaCl or 1 M sorbitol; 1000 m-osM) was required to activate WNK1 in order to observe significant phosphorylation of the WNK1 (1–667, D368A) fragment at Ser^{382} (Figure 8C). In contrast, wild-type WNK4, despite being able to phosphorylate OSR1 (Figure 7C), failed to phosphorylate the kinase-inactive WNK1 (1–667, D368A) fragment at Ser^{382} (Figure 8D). We also observed that the HQ WNK isoform mutants phosphorylated WNK1 (1–667, D368A) at Ser^{382} to a slightly lower extent than wild-type enzyme (Figure 8). This is likely to result from the reduced association of these mutants with other WNK isoforms that can also contribute to phosphorylation of WNK1 (1–667, D368A) at Ser^{382}.

DISCUSSION

To our knowledge, we have generated the first cell line that lacks SPAK/OSR1 activity and used these to demonstrate that SPAK/OSR1 activity does indeed play a crucial role in controlling NKCC1 phosphorylation. This is emphasized by the complete loss of phosphorylation of NKCC1 and the ability of the OSR1-deficient knockin cells to uptake rubidium in a bumetanide-dependent manner (Figure 1). These results provide the first genetic evidence in mammalian cells that SPAK/OSR1 are
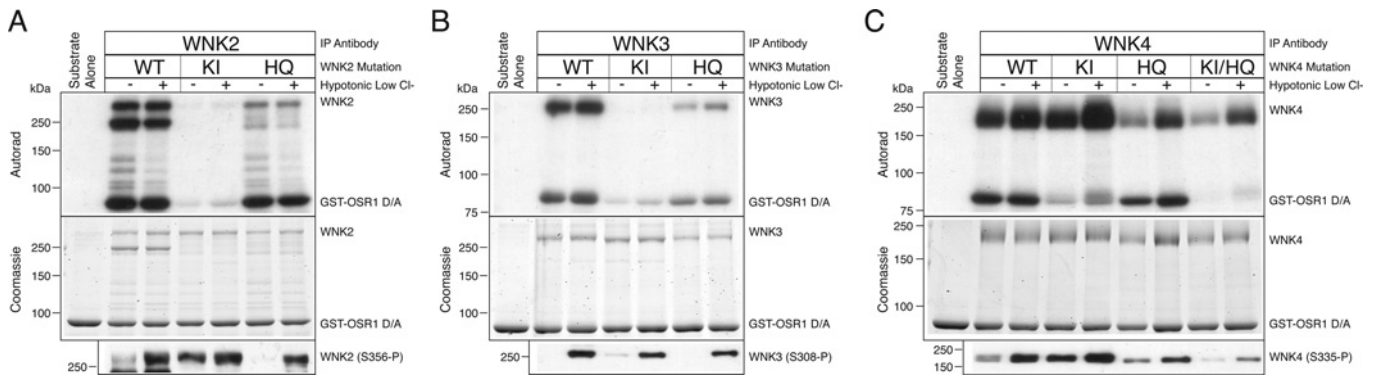
Figure 7   Dimerization is important for autophosphorylation
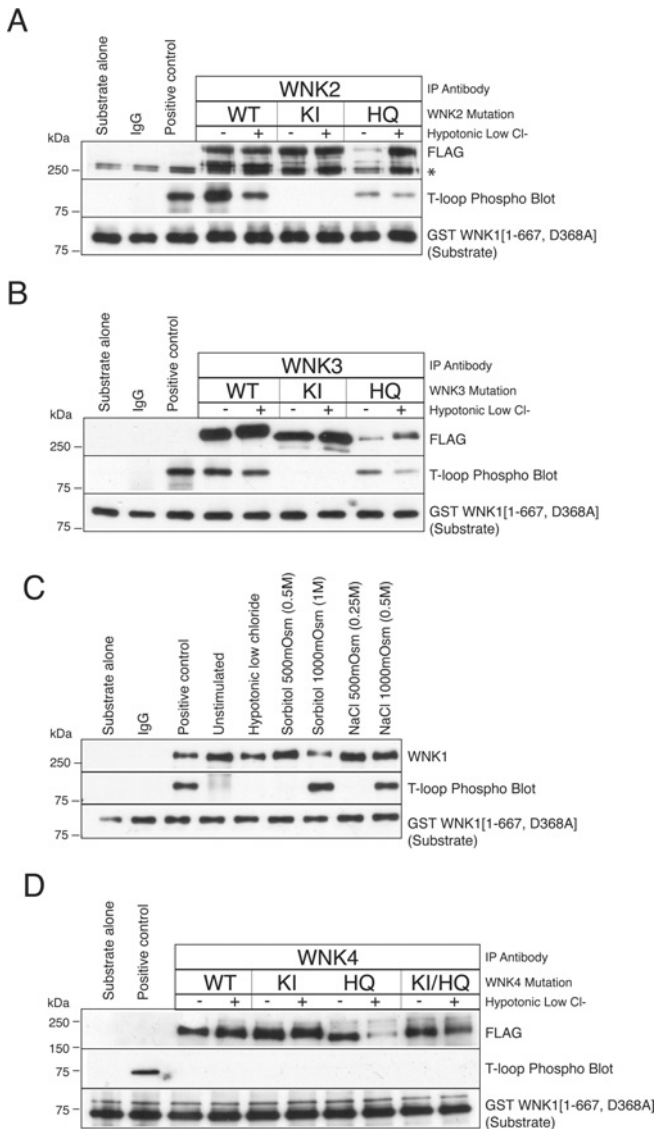
Stable Flp-In T-Rex cell lines expressing wild-type (WT) and mutant forms of WNK2 (**A**), WNK3 (**B**) and WNK4 (**C**) including the kinase-inactive (KI) and HQ forms, were stimulated with basic (−) or hypotonic low chloride (+) buffers and lysed. The FLAG–WNK isoforms were affinity-purified and assayed for their activity towards the full-length kinase-dead OSR1 (D164A) (D/A) protein. The top two panels are the resulting autoradiography and Coomassie Blue-stained gel labelled accordingly with the respective bands for the WNK isoforms and the GST–OSR1 substrate. In the bottom panels, FLAG affinity purifications for the aforementioned assays were immunoblotted with the anti-WNK1 phospho-Ser[382] antibody that recognizes the equivalent T-loop residue of WNK2, WNK3 and WNK4. Molecular masses are indicated in kDa on the left-hand side of the Western blots. IP, immunoprecipitation.

key regulators of NKCC1 function. We also observed that WNK1 and WNK3 basal activity was markedly elevated in the SPAK/OSR1-deficient ES cells as judged by significantly increased phosphorylation of OSR1 at its S-motif Ser[325] residue (Figure 1) and increased kinase activity of immunoprecipitated WNK1 and WNK3 (Figure 2). These observations provide the first evidence that SPAK/OSR1 control WNK1 activity. One possibility is that this is mediated through a regulatory negative feedback network that is a common feature of many signal transduction pathways. For example, in the MAPK (mitogen-activated protein kinase) pathway, ERK1 (extracellular-signal-regulated kinase 1)/ERK2 phosphorylate the upstream MEK (MAPK/ERK kinase) [27], whereas RSK (ribosomal S6 kinase), a kinase that is activated by ERK, phosphorylates and inhibits mSOS (mammalian son of sevenless), the guanine nucleotide exchange factor for Ras [28]. In the PI3K (phosphoinositide 3-kinase) pathway, S6K1 (S6 kinase 1) phosphorylates upstream components such as IRS (insulin receptor substrate) adaptors [29] and Rictor [30] to modulate overall PI3K pathway activity. In the Parkinson's disease LRRK2 (leucine-repeat-rich kinase 2) pathway, a downstream component of this network controls LRRK2 phosphorylation, 14-3-3 binding and localization [31,32]. These regulatory feedback loops are inherent features of signal transduction pathways and play crucial roles in modulating the extent and duration of signalling networks. It is also possible that the lack of NKCC1 activity in the SPAK/OSR1 knockin cells results in these cells being inherently osmotically stressed, leading to the constitutive activation of WNK1. In future studies it will be important to define the molecular mechanism by which WNK isoforms are controlled by SPAK/OSR1, as this will shed more light into how the activity of the WNK enzymes are regulated. However, our results emphasize that modulating activity and/or expression of SPAK and OSR1 can have a major impact on activity of their upstream WNK activators. This is also important, as whole-genome association studies have identified mutations that predispose to hypertension that link to the SPAK gene and increase SPAK expression [33]. It would be interesting to see whether these mutations also affected WNK isoform activity and hence perhaps other downstream targets of these enzymes. Pharmaceutical companies are developing inhibitors of SPAK/OSR1 for the treatment of high blood pressure and the prediction is that these drugs might elevate WNK isoform activity.

It would be important to appreciate the consequences of this in future work.

We have extended previous work [20–23] by demonstrating that endogenous WNK isoforms interact, and that the C-terminal CCD mediates this association. Our findings are most consistent with two of the earlier studies, namely that a fragment of WNK4 (residues 399–1222, containing the C-terminal CCD) interacts with full-length WNK4 in a yeast two-hybrid system [22] and that a fragment of WNK4 (residues 445–1175, containing the C-terminal CCD) bound a C-terminal fragment of WNK3 (residues 1243–1743, containing the C-terminal CCD) [23]. We have also shown that endogenous WNK1 and WNK3 as well as overexpressed WNK4 migrate on gel filtration with an apparent molecular mass of >670 kDa (Figure 6), in agreement with the notion that these enzymes can stably associate with each other. Consistent with this, we also find that the bulk of the HQ-WNK4 mutant, which is unable to interact with other WNK isoforms, migrates on gel filtration with a markedly lower apparent molecular mass than wild-type isoforms (Figure 6). The finding that a small fraction of the HQ-WNK4 mutant eluted at an similar region to that of wild-type WNK4 indicates that WNK isoforms may bind to other interactors in a manner not influenced by the HQ mutation. The use of HQ mutants might simplify future work to identify other interactors of WNK isoforms. In future studies it would be important to identify these components. Interestingly, despite SPAK and OSR1 binding to RFXV motifs of WNK isoforms [15,25], we observed that SPAK as well as OSR1 eluted at a much lower apparent molecular mass region of the gel filtration column than WNK isoforms (Figure 6). This demonstrates that either the bulk of cellular SPAK/OSR1 is not associated with WNK isoforms and/or the interactions between WNK isoforms and SPAK/OSR1 are not sufficiently robust to withstand the conditions of cell lysis and gel filtration.

The discovery of the HQ mutation, which abolishes association of WNK isoforms, will be useful in future work to investigate further the physiological importance of this interaction. Our initial findings suggest that the interaction of WNK isoforms is not rate-limiting in controlling the activation of WNK isoforms that have been overexpressed in HEK-293 cells, as the non-associating HQ mutants are activated in response to osmotic stress, albeit at a moderately reduced level compared with wild-type (Figure 7). The high degree of conservation of the C-terminal CCD in

Figure 8   WNK2 and WNK3 can phosphorylate the T-loop of WNK1

Stable Flp-In T-REx cell lines expressing wild-type (WT) and mutant forms of WNK2 (**A**), WNK3 (**B**) and WNK4 (**D**), including the kinase-inactive (KI) and His/Gln (HQ) forms, were stimulated with basic (−) or hypotonic low chloride (+) buffers and lysed. The FLAG–WNK isoforms were affinity purified and their activity towards Ser^{382} in GST–WNK1 (1–667, D368A) was assayed. The reactions were immunoblotted with the antibodies indicated. The T-loop phospho blot was undertaken using the anti-WNK1 phospho-Ser^{382} phosphospecific antibody that cross-reacts with the phosphorylated T-loop of all WNK isoforms (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/441/bj4410325add.htm). The asterisk (*) marks an unspecified band. (**C**) HEK-293 cells were either untreated or stimulated with hypotonic low chloride, 500 m-osM (0.5 M) or 1000 m-osM (1 M) sorbitol, 500 m-osM (0.25 M) or 1000 m-osM (0.5 M) NaCl for 30 min. Endogenous WNK1 was immunoprecipitated from the lysates and then subsequently assayed using a recombinant WNK1 (1–667, D368A) fragment as substrate. The reaction was immunoblotted with the WNK1 phospho-Ser^{382} and GST antibodies. The positive control was 1 ng of wild-type GST–WNK1 (1–667, D368A) expressed in *E. coli*, which previous work has shown is constitutively phosphorylated at Ser^{382} [11]. Molecular masses are indicated in kDa on the left-hand side of the Western blots. IP, immunoprecipitation.

all WNK isoforms and orthologues, including *Caenorhabditis elegans* (Figures 4E and 4F), strongly suggests that this interaction plays an important role. It should also be noted that our analysis of the activation of the non-associating HQ-mutant WNK isoforms was undertaken with overexpressed proteins. Endogenous levels of WNK isoforms in cells are expressed at relatively low

levels and therefore their ability to interact with each other may serve to enhance the efficiency in which they can trans-autophosphorylate each other in response to osmotic stress. Perhaps the best way to define the role these interactions play would be to generate HQ-WNK mutant knockin mice or cells, and investigate the impact on activation of WNK isoforms, SPAK/OSR1 and blood pressure.

The activity of WNK isoforms is normally assessed after their immunoprecipitation and the quantification of their ability to phosphorylate a substrate such as OSR1. Alternatively, the ability of immunoprecipitated WNK isoforms to autophosphorylate has also been widely used as a reporter of WNK activity. The finding that WNK isoforms associate complicates such *in vitro* analysis of immunoprecipitated WNK activity, as the co-immunoprecipitated associated WNK isoforms will also be capable of phosphorylating substrates. Consistent with this, the HQ-WNK mutants displayed reduced ability to phosphorylate OSR1 (Figure 7) as well as the WNK1 (1–667, D368A) fragment at Ser^{382} (Figure 8). Many groups have also investigated the ability of WNK isoforms to undergo autophosphorylation when incubated with [γ-^{32}P]ATP [4,6,19,20,34]. As the ability of HQ mutants of WNK isoforms to autophosphorylate is markedly diminished compared with the wild-type protein (Figure 7), most of the phosphorylation observed under these conditions probably results from the association of WNK isoforms.

Osmotic stress induces the activation of WNK1 by inducing its phosphorylation at Ser^{382} [6,11]. The results from the present study demonstrate that osmotic stress also induces phosphorylation of the equivalent T-loop residue of WNK2, WNK3 and WNK4 isoforms (Figure 7). An unanswered question is: how can cells sense osmotic stress and how does this promote T-loop phosphorylation of the WNK isoforms? Studies undertaken to date suggest that WNK1 possesses intrinsic ability to autophosphorylate its own T-loop, as fragments of WNK1 that encompass the kinase domain when expressed in *E. coli* are constitutively phosphorylated at Ser^{382} [6,11]. Consistent with this, kinase-inactive WNK1 is not phosphorylated at Ser^{382} when expressed in bacteria [11]. In the present study we provide more evidence for this model by showing that three full-length WNK isoforms (WNK1, WNK2 and WNK3) possess the intrinsic ability to phosphorylate WNK1 at Ser^{382} *in vitro* (Figures 8A–8C). These experiments provide further evidence that WNK isoforms are capable of trans-autophosphorylating their own T-loop residue. Kinase-inactive WNK1, when expressed in HEK-293 cells, is phosphorylated normally at Ser^{382} in response to osmotic stress [11], suggesting that overexpressed WNK1 can be trans-autophosphorylated on its T-loop by endogenous WNK isoforms. To date, no specific inhibitors of WNK isoforms have been reported, although if such compounds were generated, it would be fascinating to study how these affected the T-loop phosphorylation and activation of the WNK isoforms. We also observed that WNK4 was unable to phosphorylate the T-loop residue of WNK1, despite being judged as active based on its ability to phosphorylate SPAK/OSR1 (Figure 8D). Further work is required to establish the physiological significance of this observation.

It is vital that cells respond rapidly to osmotic stress before they are irreversibly damaged. Indeed, WNK1 is phosphorylated at Ser^{382} and activated within 30 s of cells being treated with various osmotic shocks [11]. Perhaps the ability of WNK isoforms to regulate their activity by autophosphorylation serves as a simple and efficient system to enable WNK isoforms to be switched on very quickly in response to osmotic stress and thereby trigger responses that protect cells from becoming damaged. More work is warranted to study this and to define how osmotic stress

is detected and promotes trans-autophosphorylation of WNK isoforms. WNK isoforms possess a long C-terminal region of unknown function and it would be interesting to investigate whether this domain is involved in sensing osmotic stress. It is also possible that osmotic stress may inhibit the protein phosphatase that acts on the T-loop of WNK isoforms. If WNK isoforms auto-activated at a constant rate and osmotic stress inhibited WNK phosphatase activity, this could also represent a simple mechanism by which WNK isoforms could be rapidly switched on. In future studies, it would be interesting to explore what protein phosphatase(s) act on WNK isoforms, perhaps using a focused phosphatase catalytic and regulatory subunit siRNA (small interfering RNA)-based screening approach.

In summary, we have generated the first cell line that lacks SPAK and OSR1 catalytic activity and use these cells to demonstrate the vital role that SPAK/OSR1 play in regulating NKCC1 activity. We also observe markedly increased WNK1/WNK3 activity in the cells lacking SPAK/OSR1 activity, indicating these STE20 kinases suppresses WNK isoform activity either via a negative feedback loop or indirectly through inhibition of NKCC1 activity, resulting in cells becoming osmotically stressed. Our findings also provide further insights into the molecular mechanism by which WNK isoforms are controlled. We define how WNK isoforms interact and identify a simple HQ-mutation that disrupts the self-association of WNK isoforms. Studying the regulation and function of HQ-WNK mutants will be useful in establishing the physiological role that this interaction plays. Moreover, we provide further evidence that WNK isoforms are regulated by trans-autophosphorylation by demonstrating for the first time that WNK1, WNK2 and WNK3, but not WNK4, can directly phosphorylate the T-loop of WNK1 *in vitro*. To date, only three physiological substrates of SPAK/OSR1 have been identified (NKCC1, NCC and NKCC2), and it is likely that there will be many more substrates of these enzymes. No specific inhibitors of SPAK/OSR1 or WNK isoforms have been developed thus far, making it very hard to evaluate the physiological functions of this signal transduction network. Identifying new substrates of SPAK/OSR1 is important as it may reveal new roles for the WNK signalling pathway. The availability of ES cells lacking SPAK/OSR1 activity will be very useful in helping to identify and validate new targets of the WNK–SPAK/OSR1 signalling network.

AUTHOR CONTRIBUTION

Jacob Thastrup helped with the experiments shown in Figure 3 and undertook most of the experimentation shown in Figures 5, 7 and 8, and Supplementary Figures S1 and S2. Fatema Rafiqi undertook the experiments shown in Figures 1 and 2, and made a substantial contribution towards helping generate data for other Figures. Alberto Vitari undertook the experiments shown in Figure 4. Eulalia Pozo-Guisado contributed to the experiments shown in Figure 8. Maria Deak undertook the cloning required for this study. Youcef Mehellou undertook the experiments shown in Figure 6 and Supplementary Figure S4. All authors were involved in planning and analysing the experimental data. Dario Alessi wrote the paper with help from all of the other authors.

ACKNOWLEDGEMENTS

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SPAK/OSR1 regulate NKCC1 and WNK activity: analysis of WNK isoform interactions and activation by T-loop trans-autophosphorylation

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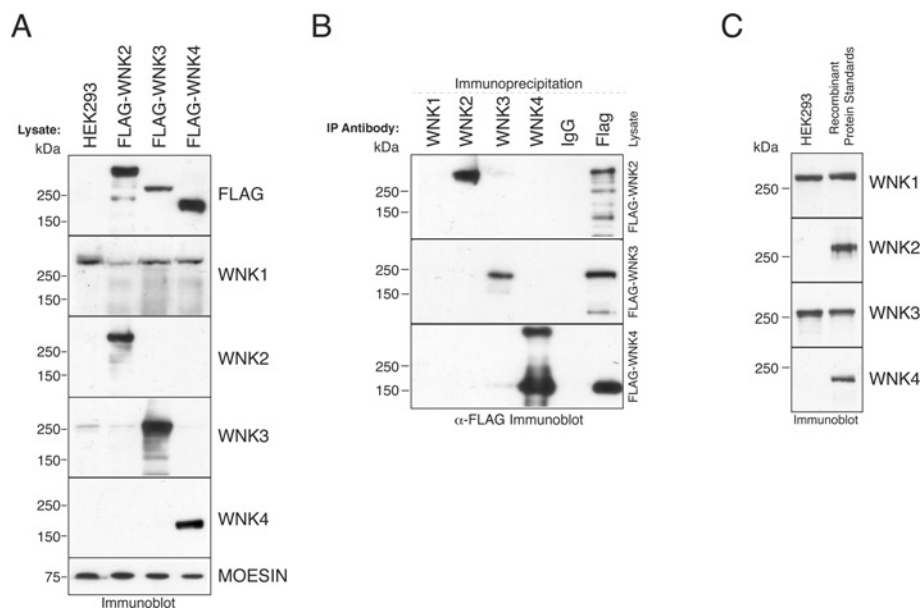
Figure S1   Specificity of human WNK isoform antibodies

(**A**) HEK-293 cells were untransfected or transfected with FLAG-tagged human WNK2, WNK3 and WNK4. Lysates (20 μg) were subjected to immunoblot analysis with the indicated antibodies. Moesin levels were monitored as a loading control. (**B**) Using the human FLAG–WNK-transfected cell lysates, immunoprecipitations with the individual antibodies were performed on each lysate as indicated. The immunoprecipitations were next subjected to immunoblot analysis with the anti-FLAG antibody to check the specificity of the individual isoform immunoprecipitations. (**C**) HEK-293 lysates (20 μg) were subjected to immunoblot analysis with the indicated isoform-specific antibodies. Lysates expressing the individual FLAG-tagged WNK isoforms were employed as a positive control. Molecular masses are indicated in kDa on the left-hand side of the Western blots. IP, immunoprecipitation.

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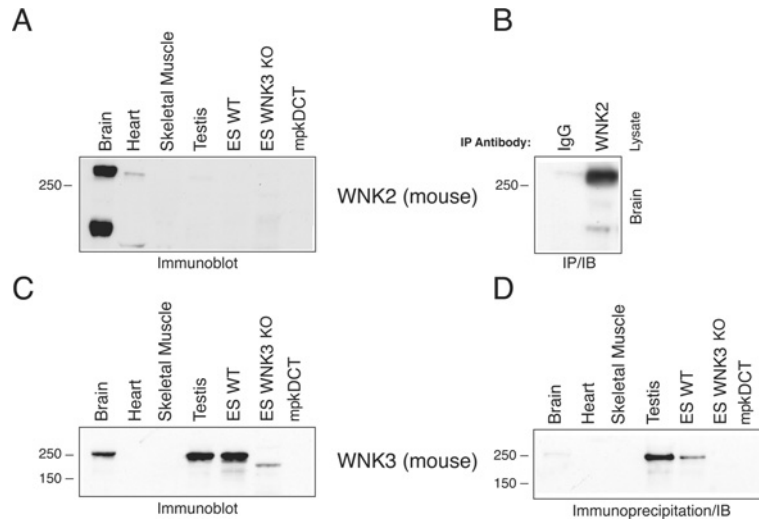
Figure S2   Characterization of the mouse WNK2 and WNK3 antibodies

(**A**) Lysates (40 μg) from a panel of mouse tissues and mouse cell lines were subjected to immunoblot analysis with the anti-WNK2 mouse antibody. (**B**) WNK2 was immunoprecipitated from mouse brain lysates and probed with the anti-WNK2 mouse antibody. Immunoprecipitation with the preimmune IgG was used as a negative control. (**C**) Same as (**A**), except that the lysate panel of mouse tissues and cell lines was immunoblotted with the anti-WNK3 mouse antibody. (**D**) WNK3 was immunoprecipitated using the mouse-specific WNK3 antibody from the indicated mouse tissues and cell lines. The immunoprecipitates were subjected to immunoblot analysis with the WNK3 mouse-specific antibody. Molecular masses are indicated in kDa on the left-hand side of the Western blots. IB, immunoblot; IP, immunoprecipitation; KO, knockout ES cells derived from the Gene Trap Consortium; mpkDCT, mouse-derived kidney distal-convoluted-tubule-derived cells; WT, wild-type.
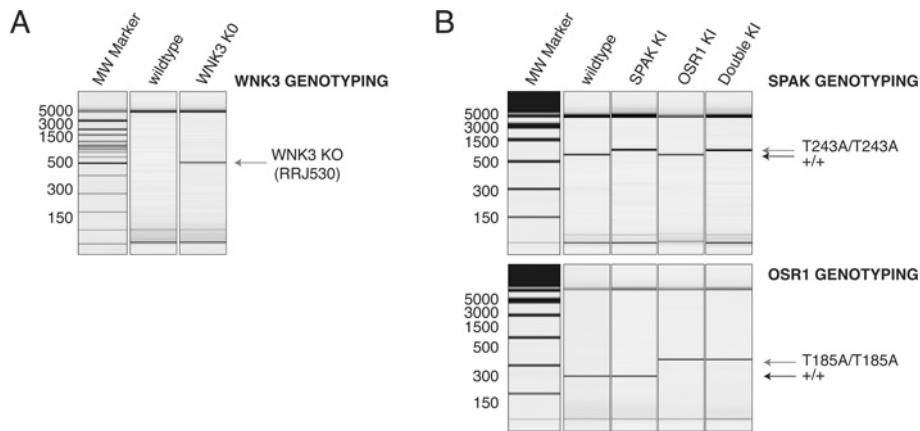
Figure S3   ES cell genotyping

(**A**) Genomic DNA from the WNK3 wild-type and knockout ES cells was PCR amplified with the WNK3 primers described in the Materials and methods section of the main text. For the WNK3 knock-out, a 500 bp insert product used to disrupt the *WNK3* gene is detected. (**B**) Genomic DNA from each SPAK/OSR1 ES wild-type, single and double cell line was PCR amplified with previously reported SPAK and OSR1 primers [17]. For SPAK, the wild-type allele generates a 679 bp product, whereas the knockin allele generates a 587 bp product. For OSR1, the wild-type allele generates a 290 bp product, whereas the knockin allele generates a 337 bp product. The larger knockin allele product is due to the presence of the 92 bp *FRT* site and flanking region, which remains in an intronic region following Flp-mediated excision of the neomycin selection cassette. Molecular size (MW) markers are indicated in kb on the left-hand side of the gels.
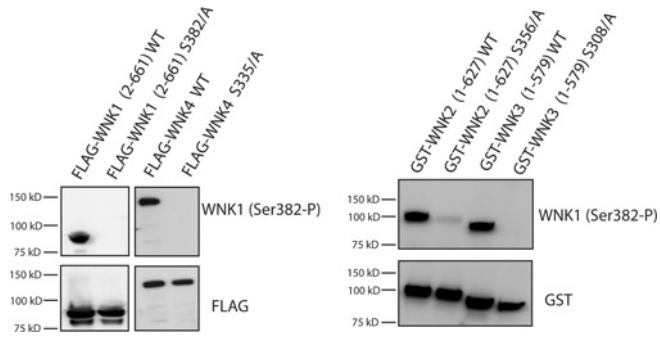
Figure S4   Characterization of the T-loop WNK isoform-specific antibody

HEK-293 cells were transfected with the indicated FLAG-tagged or GST-tagged WNK isoform constructs encoding the wild-type (WT) and T-loop mutant in which the residue equivalent to Ser^{382} in WNK1 is changed to an alanine residue to prevent activation (Ser^{356} in WNK2, Ser^{308} in WNK3 and Ser^{335} in WNK4). At 36 h post-transfection, the cells were lysed and whole cell lysates (20 μg) were subjected to immunoblot analysis with the indicated antibodies. Molecular masses are indicated in kDa on the left-hand side of the Western blots.