The regulation of salt transport and blood pressure by the WNK-SPAK/OSR1 signalling pathway

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Summary

It has recently been shown that the WNK [with-no-K(Lys)] kinases (WNK1, WNK2, WNK3 and WNK4) have vital roles in the control of salt homeostasis and blood pressure. This Commentary focuses on recent findings that have uncovered the backbone of a novel signal-transduction network that is controlled by WNK kinases. Under hyperosmotic or hypotonic low-Cl^- conditions, WNK isoforms are activated, and subsequently phosphorylate and activate the related protein kinases SPAK and OSR1. SPAK and OSR1 phosphorylate and activate ion co-transporters that include NCC, NKCC1 and NKCC2, which are targets for the commonly used blood-pressure-lowering thiazide-diuretic and loop-diuretic drugs. The finding that mutations in WNK1, WNK4, NCC and NKCC2 cause inherited blood-pressure syndromes in humans highlights the importance of these enzymes. We argue that these new findings indicate that SPAK and OSR1 are promising drug targets for the treatment of hypertension, because inhibiting these enzymes would reduce NCC and NKCC2 activity and thereby suppress renal salt re-absorption. We also discuss unresolved and controversial questions in this field of research.

Key words: Ion co-transporters, SPAK, OSR1, WNK, Protein kinases

Introduction

About one in four adults suffers from high blood pressure (hypertension), a largely asymptomatic condition that is a major risk factor for stroke, congestive heart failure and kidney disease (O'Shaughnessy and Karet, 2006). In 2001, in the USA alone, the cost of treating hypertension and its resulting complications was estimated to have been $54 billion (Balu and Thomas, 2006). Although hypertension can be moderated by lifestyle changes, in most patients the use of various drugs is required to lower blood pressure significantly. Current medications involve the use of thiazide (e.g. bendroflumethiazide) or loop (e.g. furosemide) diuretics, which increase the amount of salt that is excreted by the kidneys. Other classes of commonly prescribed drugs include angiotensin-converting enzyme (ACE) inhibitors (e.g. ramipril), angiotensin-II-receptor antagonists (e.g. losartan), Ca^{2+}-channel blockers (e.g. amlodipine) or alpha-blockers (e.g. doxazosin), which exert their effects by widening and relaxing blood vessels. Beta-blockers (e.g. atenolol) also have efficacy at treating hypertension by reducing heart workload (O'Shaughnessy and Karet, 2006).

A major focus in the hypertension field concerns the physiology and regulation of the ion co-transporters that control salt re-absorption in the kidney. These include the Na^+/Cl^- co-transporter (NCC) and the $Na^+/K^+/2Cl^-$ co-transporters (NKCC1 and NKCC2), which are direct targets for the abovementioned blood-pressure-lowering thiazide-diuretic and loop-diuretic drugs, respectively (Gamba, 2005). NKCC1, NKCC2 and NCC are members of the SLC12 family of electroneutral cation-coupled Cl^- co-transporters, which have vital roles in regulating the cellular secretion of salt and fluid and in protecting cells from damage by osmotic stress (reviewed by Flatman, 2007; Gamba, 2005) (see also Table 1). In humans, loss-of-function mutations of the kidney-specific NCC and NKCC2 co-transporters result in the low-blood-pressure conditions Gitelman's syndrome and Bartter's type I syndrome, respectively (Simon et al., 1996a; Simon et al., 1996b).

It has been appreciated for some time that the activity of SLC12 ion co-transporters is controlled by phosphorylation and the external osmotic environment (Haas et al., 1995; Lytle and Forbush, 1996; Torchia et al., 1992), but little was previously understood about the signalling networks that regulated these enzymes. This changed in 2001, when Richard Lifton and colleagues discovered that pseudohypoaldosteronism type II (PHAII) (also known as Gordon syndrome; a rare Mendelian inherited form of hypertension) was caused by mutations in the genes that encode WNK1 and WNK4, two protein kinases that were then of unknown function (Wilson et al., 2001). PHAII is an autosomal-dominant disorder that is characterised by increased salt re-absorption by the kidney and by hyperkalemia (increased serum K^+), which results from decreased renal K^+ excretion. PHAII-associated mutations in WNK1 are intronic deletions that increase the expression of WNK1 without altering its amino-acid sequence (Wilson et al., 2001). This suggested that a WNK1-containing signalling pathway existed that, when overstimulated through overexpression of WNK1, led to hypertension. Consistent with this hypothesis, heterozygous $WNK1^{-/+}$ mice have reduced blood pressure (Zambrowicz et al., 2003) and polymorphisms within the WNK1 gene in humans have also been associated with variation in blood pressure (Huang et al., 2008; Newhouse et al., 2005; Tobin et al., 2005; Turner et al., 2005). In WNK4, PHAII-causing mutations are point mutations that lie in a highly conserved acidic region of the protein that is located outside the kinase domain (Fig. 1) (Golbang et al., 2005; Wilson et al., 2001). These mutations are not known to alter the expression or intrinsic activity of WNK4 and the molecular mechanism by which they affect WNK4 function is unknown. Importantly, individuals

Table 1. The SLC12 family of electroneutral cation-coupled Cl^- co-transporters

Member*	Co-transport ions	Tissue distribution	Disease
SLC12A1 (NKCC2)	$Na^+/K^+/2Cl^-$	Kidney-specific (TAL)	Bartter's syndrome type I
SLC12A2 (NKCC1)	$Na^+/K^+/2Cl^-$	Ubiquitous	None
SLC12A3 (NCC)	Na^+/Cl^-	Kidney-specific (DCT); bone?	Gitelman's syndrome
SLC12A4 (KCC1)	K^+/Cl^-	Ubiquitous	None
SLC12A5 (KCC2)	K^+/Cl^-	Neuron-specific	None
SLC12A6 (KCC3)	K^+/Cl^-	Widespread	Anderman's syndrome
SLC12A7 (KCC4)	K^+/Cl^-	Widespread	None
SLC12A8 (CCC9)	Unknown	Widespread	Psoriasis
SLC12A9 (CIP)	Unknown	Widespread	None

*Names in parentheses indicate the protein symbol.

with PHAII are unusually sensitive to thiazide diuretics and their blood pressure can be restored with only 20% of the normal dose of these drugs (Mayan et al., 2002). This observation, which indicated that PHAII-causing mutations in WNK kinases increased blood pressure by activating NCC, promoted research to identify the link between WNK isoforms and NCC. These studies are beginning to bear fruit, with recent findings suggesting that WNK kinases regulate ion co-transporters through their ability to activate two related STE20-family protein kinases, termed STE20/SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress-responsive kinase 1 (OSR1).

In this Commentary we discuss what is known about the structure and regulation of WNK isoforms. We describe exciting recent findings that indicate that the ability of WNK1 to regulate blood pressure might, at least in part, be mediated through its phosphorylation and activation of the SPAK and OSR1 protein kinases. We will also outline how SPAK and OSR1 recognise, phosphorylate and activate the kidney-specific NCC and NKCC2 ion co-transporters. The implication of these observations is that SPAK and OSR1 are potential drug targets for the treatment of hypertension, because inhibiting these enzymes would be expected to reduce NCC and NKCC2 activity and thereby reduce renal salt re-absorption, leading to lower blood pressure.

Key features of WNK isoforms

Before WNK1 was identified as a regulator of blood pressure, it had been cloned as an unusual protein kinase that lacked a conserved catalytic Lys residue in subdomain II, which is required for ATP binding, and was therefore termed with-no-K[Lys] protein kinase-1 (WNK1) (Xu et al., 2000). Subsequent work revealed that there are four distinct mammalian isoforms of WNK, all of which lack the conserved Lys residue (Verissimo and Jordan, 2001). Despite this lack, WNK1 is catalytically active (as judged by its ability to autophosphorylate and to phosphorylate the non-specific kinase substrate myelin basic protein) (Xu et al., 2000). The crystal structure of an inactive mutant of WNK1 that was expressed in *Escherichia coli* revealed that another lysine residue that was located in subdomain I of the kinase substitutes for the missing subdomain II Lys residue; this accounts for the observed protein kinase activity of WNK (Min et al., 2004).

The domain structure and known covalent modifications of the four human WNK isoforms are summarised in Fig. 1. These enzymes are relatively large (135-250 kDa) and possess an N-terminal kinase domain that lies between the subfamilies 'sterile 20 kinase' and 'tyrosine-kinase-like' on the human kinome tree (Manning et al., 2002). Apart from the kinase domain and one to three putative coiled-coil domains, no other obvious domains are present in any of the WNK isoforms. WNK1 possesses an auto-inhibitory motif (amino-acid residues 515-569) that suppresses kinase activity and is C-terminal to its catalytic domain; this motif is conserved in other WNK isoforms (Lenertz et al., 2005; Xu et al., 2002). WNK isoforms are also rich in potential PxxP SH3-binding motifs (32 in WNK1, 98 in WNK2, 11 in WNK3 and 37 in WNK4), and PxxP motifs in WNK1 and WNK4 reportedly interact with SH3 domains on intersectin, an endocytosis scaffold protein (He et al., 2007). In a global screen for O-linked β-N-acetylglucosamine (O-GlcNAc)-modified proteins in rat forebrain extracts, WNK1 was shown to contain O-GlcNAc-modified Ser or Thr residues that are located within two C-terminal chymotryptic-digest-derived peptides (Fig. 1) (Khidekel et al., 2004). The role of O-GlcNAc modification of WNK1 remains unknown but warrants further investigation.

WNK1 is widely expressed (Verissimo and Jordan, 2001; Xu et al., 2000) and, of the rat tissues that have been analysed, is most abundant in testis (Vitari et al., 2005). A highly expressed kidney-specific splice variant of WNK1 (termed KS-WNK1), which lacks almost the entire N-terminal kinase domain, has been characterised (Fig. 1) (Delaloy et al., 2003; O'Reilly et al., 2003). This kinase-inactive fragment of WNK1 has been proposed to inhibit full-length WNK1, but further studies are required to establish the importance of this variant (Lazrak et al., 2006; Subramanya et al., 2006; Wade et al., 2006). An interesting recent study has reported that neuronal tissues express two distinct splice variants of WNK1, which possess an inserted sequence within the C-terminal non-catalytic domain that is expressed from distinct exons (termed exon 8b and exon HSN2) (Fig. 1) (Shekarabi et al., 2008). Most importantly, this study reported that, human mutations that lie within exon HSN2 as well as in exon 6 of WNK1 resulted in an early-onset autosomal-recessive loss-of-pain-perception disorder termed hereditary sensory and autonomic neuropathy type II (HSANII) (Shekarabi et al., 2008). The described mutations result in the truncation of a large region of the non-catalytic C-terminal domain of the WNK1 protein; this does not affect the kinase domain (Shekarabi et al., 2008). Further research is needed to understand how HSANII-associated truncations in WNK1 affect its activity and downstream signalling pathways, as well as the ability to sense pain. There are no reports of individuals with HSANII who suffer from high blood pressure (Shekarabi et al., 2008), which indicates that the mutated forms of WNK1 that cause HSANII do not affect WNK1 signalling in the kidney or in other tissues that control blood pressure.

Inspection of the Unigene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene) indicates that *WNK2* mRNA is widely expressed, but that the expression of *WNK3* mRNA is more restricted; it is expressed at high levels in the pituitary gland, in which the expression of *WNK2* and *WNK4* is not observed. It has been reported that *WNK4* is only expressed in the kidney (Wilson

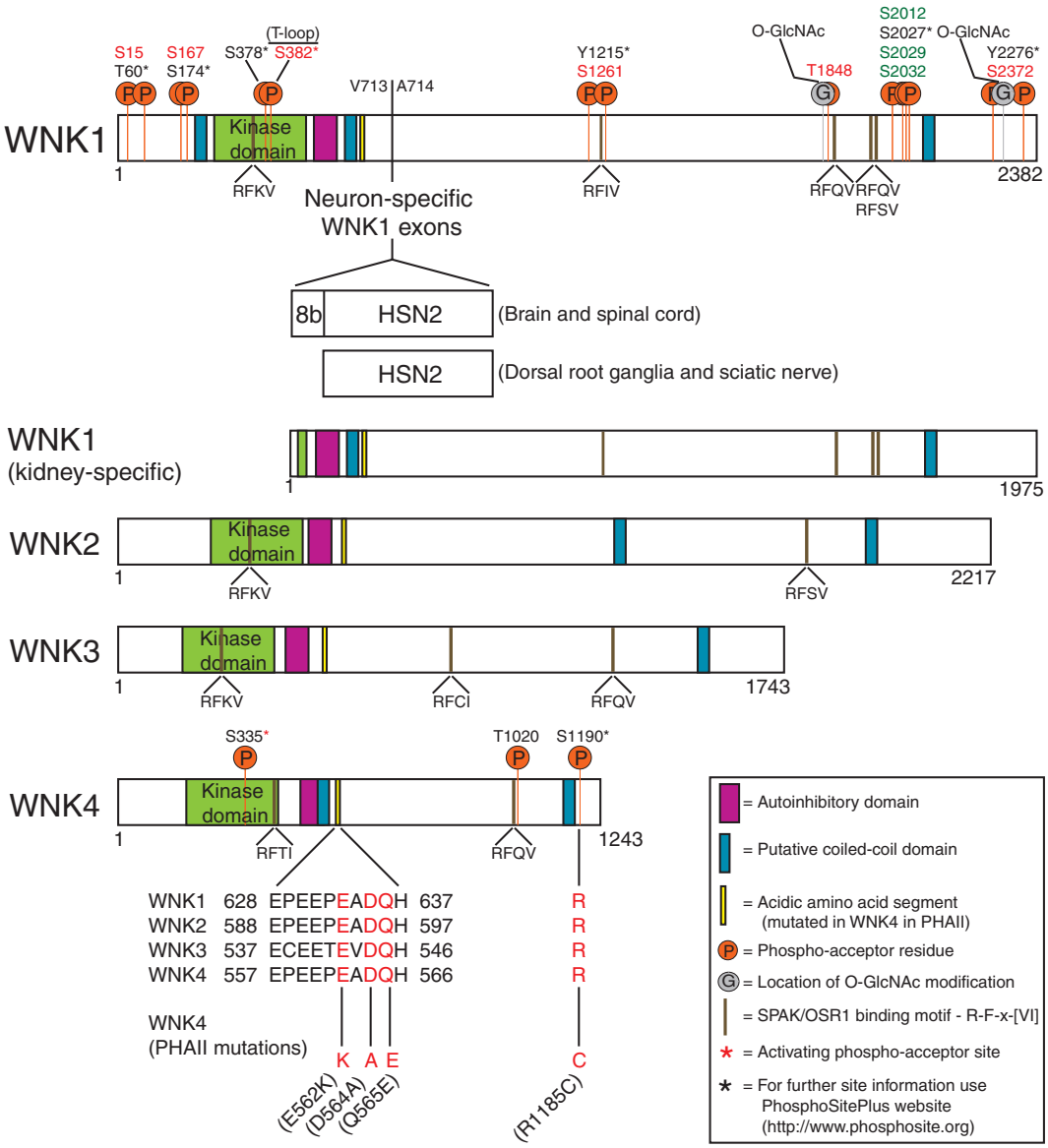

Fig. 1. Domain structure and covalent modifications of human WNK protein kinases. Phosphorylation sites that were identified on endogenous WNK1 isolated from control cells or sorbitol-stimulated cells (Zagorska et al., 2007) are highlighted in green and red, respectively. Further phosphorylation sites are in black and are reported on the PhosphoSitePlus website (http://www.phosphosite.org); the location of additional exons within the neuronal WNK1 splice variants is indicated. In addition, the neuronal isoforms are predicted to lack some of the N-terminal non-catalytic residues. Furthermore, the WNK1 variant that is found in brain and spinal cord reportedly lacks exons 11-12, whereas the variant found in the dorsal root ganglia and sciatic nerve cells lacks exon 11 (Shekarabi et al., 2008). Sequence alignment of the acidic segment of WNK isoforms is illustrated and reported PHAII-associated mutations of WNK4 are highlighted (E562K, D564A, Q565E, R1185C). The positions of domains and residues are drawn approximately to scale in each figure.

et al., 2001), but another study has indicated that it is also expressed in other tissues (Kahle et al., 2004). Consistent with this, the Unigene database suggests that *WNK4* is expressed at high levels in the intestine, eye, mammary gland and prostate. An alternatively spliced isoform of WNK3 that contains a 47-amino-acid extension in the C-terminal domain of the protein has been identified in brain (Holden et al., 2004).

Regulation of WNK isoforms

In cultured cells, WNK1 is activated by either hyperosmotic stress (sorbitol, NaCl or KCl) (Lenertz et al., 2005; Xu et al., 2000; Xu et al., 2002; Zagorska et al., 2007) or hypotonic low-Cl^- conditions (Moriguchi et al., 2005; Richardson et al., 2008). Other signal-transduction-pathway activators, such as growth factors, non-osmotic cellular stresses and protein-phosphatase inhibitors, do not stimulate WNK1 activity (Lenertz et al., 2005; Zagorska et al., 2007). The activation of WNK1 is rapid, occurring within 1 minute of hyperosmotic stress (Zagorska et al., 2007) and within 5 minutes of hypotonic low-Cl^- stress (Richardson et al., 2008). WNK2 is also activated when cells are exposed to hypertonic conditions (Hong et al., 2007).

Hyperosmotic stress (Zagorska et al., 2007) or hypotonic low-Cl^- conditions (Richardson et al., 2008) activate WNK1 by inducing its phosphorylation at Ser382, which is located within a region of the protein kinase catalytic domain that is termed the T-loop or activation-loop [a key motif that regulates the activity of most protein kinases

(Fig. 1)]. Consistent with this observation, mutation of Ser382 to Ala ablated WNK1 activation, whereas its mutation to an acidic Glu residue enhanced basal activity and prevented further activation by hyperosmotic stress (Zagorska et al., 2007). Ser382 and the residues that surround it are conserved in all WNK isoforms, which suggests that other isoforms are activated by similar mechanisms.

A vital unanswered question is how WNK isoforms sense hyperosmotic and hypotonic low-Cl^- stress, and how this leads to increased phosphorylation of the T-loop Ser382 residue. It would be interesting to investigate whether WNK isoforms possess an ion-sensing domain or whether the detection of ions is mediated by a distinct regulator of WNKs. WNK isoforms might possess the intrinsic ability to autophosphorylate their T-loop, because WNK1 when expressed in *E. coli* is phosphorylated at Ser382 (Xu et al., 2002; Zagorska et al., 2007). Co-immunoprecipitation studies have revealed that WNK isoforms can form homo- and heterodimers (Lenertz et al., 2005; Yang et al., 2007a; Yang et al., 2005). The role of dimerisation, and the precise regions of the enzymes that mediate the formation of complexes, require further analysis. Whether the ability of WNK isoforms to form dimers has a role in enabling their autophosphorylation at Ser382 could also be investigated. It cannot be ruled out that a distinct upstream protein kinase (or kinases) exists that phosphorylates WNK1 at Ser382. This notion might be supported by the observation that a catalytically inactive WNK1 mutant, when expressed in HEK 293 cells, is phosphorylated at Ser382 – although it is also possible that endogenous WNK isoforms mediate this reaction (Zagorska et al., 2007). It is also important to identify the protein phosphatase(s) that dephosphorylates Ser382 and to determine whether this enzyme is also regulated by osmotic stress. If osmotic stress inhibits the WNK phosphatase, this could contribute to the enhanced phosphorylation and activation of WNK isoforms that are observed under these conditions.

Hyperosmotic stress also induces a marked translocation of WNK1 to vesicular structures that might be trans-Golgi-network (TGN)-derived vesicles or recycling endosomes, because they display rapid movement and colocalise with adaptor protein complex 1 (AP1) and clathrin but not with the plasma membrane coated-pit marker AP2 nor with endosomal markers (e.g. EEA1, Hrs and LAMP1) (Zagorska et al., 2007). The C-terminal non-catalytic domain of WNK1 mediates its translocation to these TGN or recycling-endosome structures. Recent work suggested that WNK4 also relocalises in a similar manner in hypertonically (NaCl)-treated cells (Shaharabany et al., 2008). The role of this translocation and the proteins that WNK1 and WNK4 interact with and/or control within TGN or recycling endosomes are unknown. The translocation of WNK1 is probably not required for its phosphorylation at Ser382, because mutants that lack the C-terminal non-catalytic domain that is required for translocation are still activated following hyperosmotic stress (Zagorska et al., 2007). Moreover, WNK1 does not translocate to vesicular structures in cells that are subjected to low-Cl^- hypotonic conditions that also activate the enzyme (Eulalia Pozo-Guisado and D.R.A., unpublished observations).

The interaction of SPAK and OSR1 with WNK isoforms

SPAK and OSR1 are related kinases that possess an N-terminal catalytic domain that is similar to those of other members of the STE20 kinase subfamily, and two conserved regions that are known as the serine-motif or S-motif (51 amino-acid residues) (Vitari et al., 2005) and the conserved C-terminal (CCT) domain (92 amino-acid residues) (Vitari et al., 2006) (Fig. 2A). In addition, SPAK possesses a unique 48-amino-acid N-terminal extension that mainly comprises Ala and Pro residues (24 and 12 residues, respectively). SPAK and OSR1 were originally identified through various cloning screens, are widely expressed and arose through gene duplication during vertebrate evolution (Delpire and Gagnon, 2008; Johnston et al., 2000; Tamari et al., 1999; Ushiro et al., 1998).

The link between WNK isoforms and the SPAK and OSR1 protein kinases emerged from screens that had been designed to identify binding partners of WNK1 that was immunoprecipitated from rat testis (Vitari et al., 2005) or from various yeast two-hybrid screens (Anselmo et al., 2006; Gagnon et al., 2006; Moriguchi et al., 2005). These studies revealed that SPAK and OSR1 interact with WNK1 as well as with WNK4, and that binding is mediated by the CCT domain of SPAK and OSR1. The CCT domain interacts with specific RFx[V/I] motifs that are present in WNK1 and WNK4 (Gagnon et al., 2006; Moriguchi et al., 2005; Vitari et al., 2006). All WNK isoforms contain multiple RFx[V/I] motifs (Fig. 1), but further work is required to establish the roles of these motifs in enabling the binding of SPAK or OSR1 and to determine whether WNK-isoforms can simultaneously interact with multiple SPAK or OSR1 molecules. Binding of RFxV-motif-containing peptides to the CCT domain of SPAK and OSR1 is in the high-nanomolar affinity range (Villa et al., 2007; Vitari et al., 2006; Zagorska et al., 2007). Structural analysis has revealed that the CCT domain, when co-crystallised with a WNK4-derived RFxV-motif-containing peptide, forms a novel fold that possesses an elongated deep groove on its surface (Villa et al., 2007). One part of this groove is the 'primary pocket' that forms a web of molecular interactions with the Arg, Phe and Val residues of the RFxV-containing peptide (Fig. 2B). At the other end of the groove lies a deep 'secondary pocket' in which the surface-exposed residues are also highly conserved (Fig. 2B). It is not known whether the secondary pocket interacts with RFxV-motif-containing binding substrates or with distinct molecules. The mutation of conserved residues within the secondary pocket did not significantly inhibit the binding of OSR1 to WNK1 or NKCC1 (Villa et al., 2007).

Activation of SPAK and OSR1 by WNK isoforms

WNK1 and WNK4 phosphorylate SPAK and OSR1 at two conserved residues, namely a T-loop Thr residue (Thr233 in SPAK and Thr185 in OSR1) and a Ser residue in the S-motif (Ser373 in SPAK and Ser325 in OSR1) (Fig. 2A) (Vitari et al., 2005). The phosphorylation of the T-loop residue mediates the activation of SPAK and OSR1, as its mutation to Ala prevented activation (Vitari et al., 2005; Zagorska et al., 2007). WNK1 phosphorylated SPAK and OSR1 in vitro, to a much greater stoichiometry at the S-motif than at the T-loop residue. However, the role of S-motif phosphorylation is unknown, as its mutation does not affect activation (Vitari et al., 2005; Zagorska et al., 2007). In vitro, WNK1 phosphorylated SPAK and OSR1 more efficiently than WNK4 (Vitari et al., 2005). Consistent with the hypothesis that SPAK and OSR1 are physiological substrates for WNK1, conditions that stimulate WNK1 activation in cells induced the activation of endogenous SPAK and OSR1 (Anselmo et al., 2006; Moriguchi et al., 2005; Richardson et al., 2008; Zagorska et al., 2007). Activation of SPAK and OSR1 was accompanied by phosphorylation of the T-loop- and S-motif residues that are targeted by WNK1 in vitro (Anselmo et al., 2006; Moriguchi et al., 2005; Richardson et al., 2008; Zagorska et al., 2007). The siRNA-mediated knockdown of WNK1 expression partially inhibited SPAK and OSR1 activity as well as phosphorylation of their T-loop and S-motif residues (Anselmo et al., 2006; Zagorska et al., 2007). Consistent with a role for WNK1 in regulating SPAK and OSR1, time-course studies

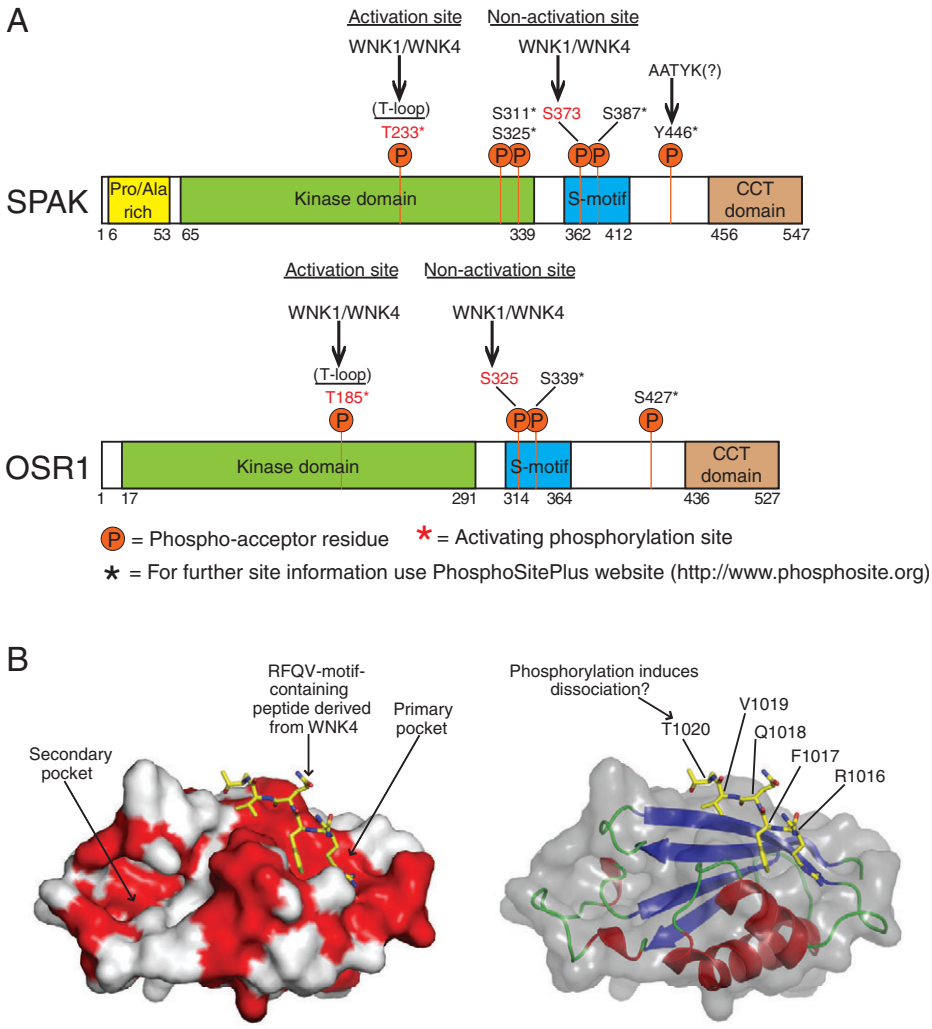

Fig. 2. (A) Domain structure and covalent modifications of human SPAK and OSR1 protein kinases. Residues on SPAK and OSR1 that are phosphorylated by WNK1 and WNK4 are highlighted in red. Further phosphorylation sites are in black and are reported on the PhosphoSitePlus website (http://www.phosphosite.org). (B) Crystal structure of the CCT domain of OSR1 in complex with a WNK4-derived RFxV-containing peptide. (Left panel) Sequence conservation of the protein surface of the CCT domain of OSR1 in orthologues from *C. elegans* to human. Grey represents non-conserved residues and red represents identical residues. The location of the primary pocket, in which the RFxV motif (peptide stick representation with yellow carbon atoms) is bound, and the surface-exposed secondary groove (of unknown function) are labelled. (Right panel) Molecular interactions of the CCT domain of OSR1 (α-helices are in red and β-strands in blue) and the WNK4-derived RFQV-containing peptide. The RFQV motif (residues 1016-1019) and residue Thr1020 are labelled. The arrow indicates that phosphorylation of Thr1020 would cause a steric clash with the backbone of the CCT domain, which could inhibit binding of the RFQV motif. Note that Thr1020 is equivalent to the residue termed Thr1008 in the original structural study (Villa et al., 2007), which was wrongly numbered .

revealed that the phosphorylation and activation of SPAK and OSR1 occurred with slower kinetics than WNK1 activation in cultured cells (Richardson et al., 2008).

Regulation of SLC12 family members by WNK-SPAK/OSR1 signalling

The role of SPAK and OSR1 in the regulation of NKCC1 activity

The first binding partner and substrate for SPAK and OSR1 to be identified was the SLC12-family ion co-transporter NKCC1 (Dowd and Forbush, 2003; Piechotta et al., 2003; Piechotta et al., 2002). Human NKCC1 is a glycosylated 1212-residue protein that possesses 12 transmembrane domains that are flanked by N-terminal and C-terminal cytoplasmic domains (Fig. 3A) (Delpire et al., 1994; Gerelsaikhan and Turner, 2000; Xu et al., 1994). NKCC1 is ubiquitously expressed and, through its ability to take up Na^+, K^+ and Cl^- ions, controls cell volume and osmotic balance between the external and internal cellular environments (Flatman, 2007; Gamba, 2005). The finding that hyperosmotic stress stimulated WNK1 as well as SPAK and OSR1 led to the suggestion that the WNK pathway functions to regulate NKCC1 via SPAK and OSR1 (Vitari et al., 2005), and recent research has confirmed that this, indeed, is the case. In vitro studies have revealed that SPAK and OSR1 efficiently phosphorylate a cluster of conserved Thr residues that are located in the N-terminal cytosolic domain of NKCC1 (Gagnon et al., 2007a; Moriguchi et al., 2005; Vitari et al., 2006). SPAK and OSR1 are likely to directly phosphorylate Thr203, Thr207, Thr212 and Thr217 on human NKCC1, and the equivalent residues in mouse and shark NKCC1 (Fig. 3B). The activation of NKCC1 that results from the overexpression of SPAK or from hyperosmotic stress or hypotonic low-Cl^- conditions is inhibited by the mutation of Thr212 or Thr217 (Darman and Forbush, 2002; Dowd and Forbush, 2003; Gagnon et al., 2007a).

SPAK and OSR1 interact with NKCC1 through their CCT domain. Two RFxV motifs are located within the N-terminal cytosolic domain of NKCC1 (Fig. 3A) (Gagnon et al., 2007a) and the mutation of these motifs inhibited the binding of SPAK and OSR1 as well as the activation of NKCC1 by the overexpression of SPAK or by hyperosmotic stress (Gagnon et al., 2007a). Thus, SPAK and OSR1 utilise their CCT domain to interact with WNK kinases as well as with substrates such as NKCC1. A complication of this is that there must be a mechanism to dissociate SPAK or OSR1 from WNK isoforms once SPAK or OSR1 have been activated. This would be essential to enable activated SPAK or OSR1 to interact with and phosphorylate substrates. Recent analysis suggests that hyperosmotic stress induces the phosphorylation of WNK1 at Ser1261 (Fig. 1), a residue that is immediately C-terminal to a CCT-domain-binding RFxV motif (Zagorska et al., 2007). Binding studies revealed that the phosphorylation of Ser1261 on a WNK1-derived peptide that encompassed this RFxV-motif ablated its interaction with SPAK

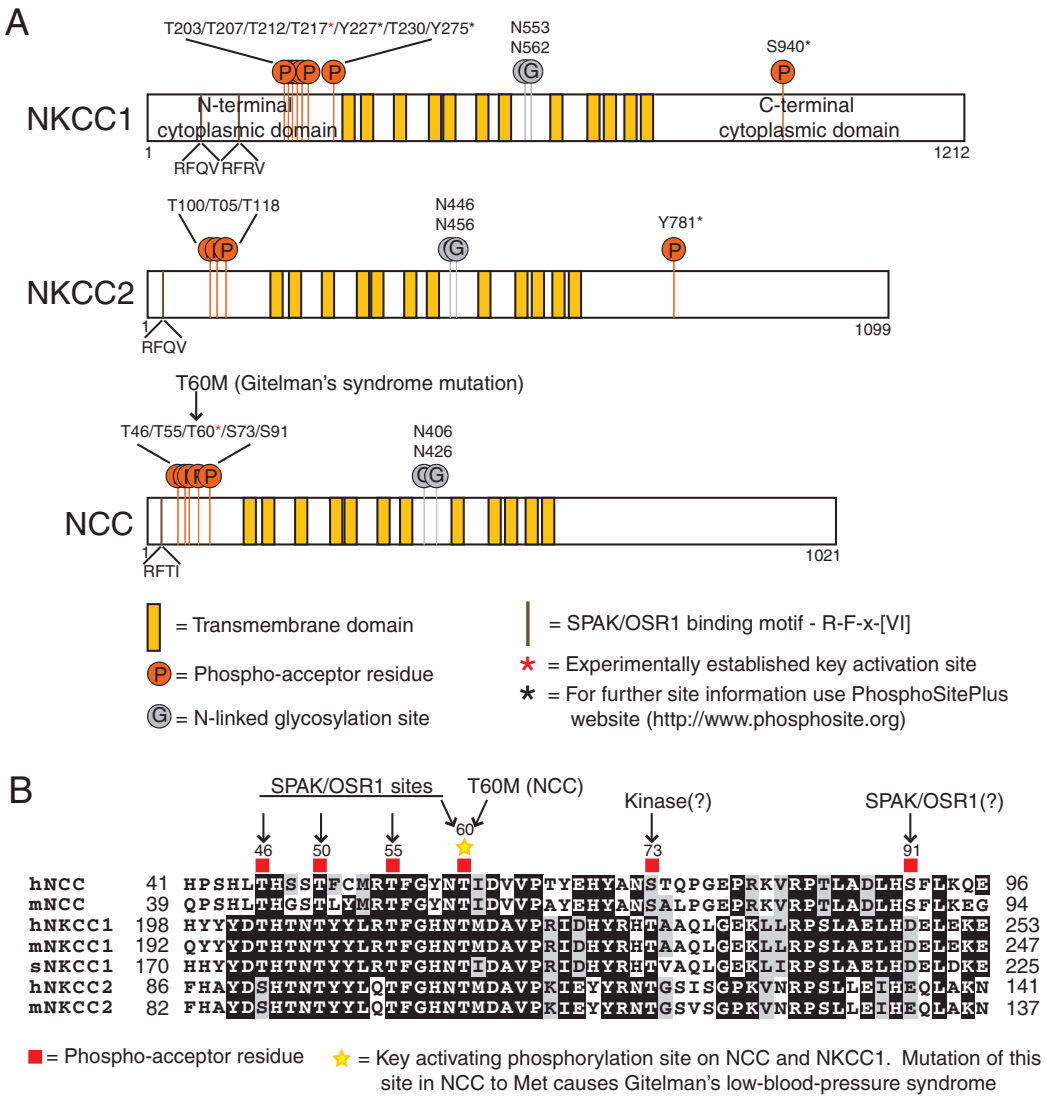

Fig. 3. (A) Domain structure and covalent modifications of the human SLC12 co-transporters NKCC1, NKCC2 and NCC. (B) Sequence alignment of the N-terminal region of NCC, NKCC1 and NKCC2, which is regulated by phosphorylation. Identical residues are highlighted in black and similar residues are in grey. Numbering above symbols indicates residue number on human NCC. h, human; m, mouse; s, shark.

and OSR1 (Zagorska et al., 2007). Similarly, the RFxV-containing peptide derived from WNK4 that was co-crystallised with the CCT domain is N-terminal to a phosphorylatable residue (Thr1020) and phosphorylation of this peptide prevented binding to the CCT domain (Villa et al., 2007). Modelling analysis indicated that phosphorylation of Thr1020 (Fig. 1) induces a steric clash with the backbone of the CCT domain that could trigger dissociation (Fig. 2B). Whether Thr1020 is phosphorylated in vivo has not been established, but it is noteworthy that most CCT-binding RFx[V/I] motifs found in WNK isoforms, SLC12 ion co-transporters and other potential SPAK- and OSR1-binding proteins, such as apoptosis-associated tyrosine kinase (AATYK) isoforms, are N-terminal to a Ser or Thr residue (Delpire and Gagnon, 2007). This suggests that the phosphorylation of residues that are C-terminal to and/or lie close to RFxV motifs is a general mechanism to control the association of interactors with the CCT domain. We suggest that WNK isoforms form a complex with SPAK or OSR1, and that dissociation is triggered by the phosphorylation of the Ser or Thr residue that is C-terminal to or lies close to the RFxV motif of WNKs. The upstream kinase(s) that mediates this phosphorylation is unknown. However, it is tempting to speculate that SPAK and OSR1 may themselves control RFxV-motif phosphorylation on WNKs, because this could provide a simple mechanism to dissociate SPAK or OSR1 from WNK isoforms once they become activated.

NKCC1 interacts with protein phosphatase 1 (PP1) through a RVxF binding motif on NKCC1 that, intriguingly, lies within a RFRVNF sequence that overlaps with a CCT-binding RFxV motif (Darman et al., 2001). It would be interesting to study the role of the binding of PP1 in the regulation of NKCC1 by the WNK1-SPAK/OSR1 signalling pathway. Via their CCT domains, SPAK or OSR1 also associate with isoforms of AATYK that reportedly bind to PP1 (Piechotta et al., 2003). One study has suggested that the binding of SPAK or OSR1 to a complex of AATYK1 and PP1 inhibits the ability of SPAK to activate NKCC1 (Gagnon et al., 2007b). Caution is needed when interpreting these findings,

however, as the overexpression of AATYK1 or any other binding partner might artificially sequester SPAK and OSR1, which may inhibit their activation by WNKs or their interaction with substrates. It would also be interesting to study whether AATYK isoforms phosphorylate SPAK or OSR1, or vice versa. Global phosphoproteomic analysis indicated that SPAK is phosphorylated at Tyr446 in cultured cells (http://www.phosphosite.org), and it might prove worthwhile to investigate whether SPAK is directly phosphorylated by AATYK at this residue (Fig. 2A).

The role of SPAK and OSR1 in regulating NCC, NKCC2 and blood pressure

The SLC12 ion co-transporters NCC and NKCC2 are related to NKCC1 and possess similar domain architecture (Fig. 3A) (Gamba et al., 1994; Gamba et al., 1993; Payne and Forbush, 1994). Unlike NKCC1, however, NCC and NKCC2 are expressed only in the kidney: NCC is predominantly expressed in the distal convoluted tubule (DCT), whereas NKCC2 is expressed in the thick ascending limb (TAL) (Table 1). NCC and NKCC2 are the targets for the commonly used blood-pressure-lowering thiazide-diuretic- and loop-diuretic drugs, respectively, and have vital roles in controlling renal salt re-absorption and, hence, blood pressure (Flatman, 2007; Gamba, 2005). Sequence alignments indicate that SPAK and OSR1 phosphorylation sites on NKCC1 are conserved in human and mouse NCC and NKCC2 (Fig. 3B), which suggests that the WNK-SPAK/OSR1 pathway also activates these co-transporters. This would explain why increased expression of WNK1 in PHAII patients can lead to hypertension: WNK1 could induce the overactivation of NCC through the SPAK and OSR1 kinases. Moreover, this would account for the increased sensitivity of PHAII patients to thiazide diuretics that inhibit NCC. Recent studies suggest that SPAK and OSR1 directly phosphorylate and activate NCC (Richardson et al., 2008). Evidence suggests that SPAK and OSR1 directly phosphorylate human NCC at three sites (Thr46, Thr55 and Thr60) that are equivalent to residues in human NKCC1 that are phosphorylated by SPAK and OSR1 (Fig. 3B) (Richardson et al., 2008). NCC also interacts with SPAK and OSR1 through a single N-terminal RFxI motif (Fig. 3A); this binding is necessary for maximal phosphorylation as well as for the activation of NCC in cultured HEK 293 cells (Richardson et al., 2008). Importantly, the mutation of Thr60 (equivalent to Thr217 in human NKCC1) to a non-phosphorylatable Ala residue, prevented activation of NCC in response to hypotonic low-Cl^- conditions both in mammalian cells (Richardson et al., 2008) and in *Xenopus laevis* oocytes (Pacheco-Alvarez et al., 2006). Moreover, the Thr60Ala mutation also markedly suppressed phosphorylation of Thr46 and Thr55, which indicates that phosphorylation of Thr60 is essential for enabling and/or maintaining the phosphorylation of other residues (Richardson et al., 2008). The importance of Thr60 in mediating NCC activation is further highlighted by the discovery of a Thr60Met NCC mutation in Asian patients with Gitelman's syndrome (Lin et al., 2005; Maki et al., 2004; Shao et al., 2008). The Thr60Met mutation in NCC might be more prevalent in Asian populations, because it has so far not been reported in other ethnic groups with Gitelman's syndrome (Ji et al., 2008).

In addition to inducing phosphorylation of NCC at Thr46, Thr55 and Thr60, hypotonic low-Cl^- stress induced the phosphorylation of NCC at Ser91, which was inhibited by mutation of the CCT-domain-binding RFxI motif on NCC. In vitro, however, SPAK or OSR1 did not phosphorylate NCC at Ser91, which indicates that other kinases might phosphorylate this residue (Richardson et al., 2008). Increased phosphorylation of another residue of NCC (Ser73 in human; Ser71 in mouse) was also reported in mice expressing a WNK4[D561A] PHAII-associated mutation (Yang et al., 2007b). However, SPAK and OSR1 do not appear to phosphorylate Ser73 directly in vitro, nor was phosphorylation of this site detected by mass spectrometry analysis of NCC that had been overexpressed in HEK 293 cells (Richardson et al., 2008). Further work is required to define the significance of the Ser73 site and to determine which kinase(s) regulates its phosphorylation. Until these questions are answered, we suggest that the regulation of NCC by the WNK pathway be monitored by analysing phosphorylation of the SPAK- and OSR1-dependent phospho-acceptor residues (Thr46, Thr55 and Thr60) rather than just that of Ser73.

The region of NKCC1 and NCC that is phosphorylated by SPAK and OSR1 is highly conserved in NKCC2 (Fig. 3B), indicating that the WNK pathway may also regulate NKCC2 via SPAK and OSR1. Consistent with this hypothesis, NKCC2 interacted with SPAK and OSR1 in a yeast two-hybrid screen (Piechotta et al., 2002) and an N-terminal fragment of NKCC2 was phosphorylated by SPAK and OSR1 in vitro, although the sites of phosphorylation were not mapped (Moriguchi et al., 2005). Hypertonic conditions stimulate phosphorylation of NKCC2 at the residues that correspond to Thr100 and Thr105 in human NKCC2 (equivalent to Thr212 and Thr217 in human NKCC1 and Thr55 and Thr60 in human NCC) (Gimenez and Forbush, 2005; Rinehart et al., 2005). It has been reported that combined mutation of three of the putative phosphorylation sites on NKCC2 (which correspond to Thr100, Thr105 and T118 on human NKCC2) is required to inhibit the activation of NKCC2 in response to osmotic stress (Gimenez and Forbush, 2005; Ponce-Coria et al., 2008); by contrast, and as discussed above, mutation of a single Thr residue in NCC and NKCC1 prevents activation. A recent study has also demonstrated that overexpression of WNK3 in *X. laevis* oocytes leads to the activation of NKCC2; this is dependent upon the interaction of SPAK and OSR1 with one of the three RFx[V/I]-motifs that are present on WNK3 (centred around Phe1290 in human WNK3) (Fig. 1) (Ponce-Coria et al., 2008).

Although it is not known how the phosphorylation of NCC or other related ion co-transporters stimulates their co-transporter activity, one possibility is that phosphorylation by SPAK or OSR1 enhances activity by stimulating the translocation of the co-transporters from intracellular vesicles to the plasma membrane. Consistent with this idea, several reports have correlated NCC activity with the level of this enzyme at the plasma membrane (Cai et al., 2006; Golbang et al., 2005; Wilson et al., 2003; Yang et al., 2003). It is also possible that the phosphorylation of ion co-transporters by SPAK and OSR1 induces a conformational change that directly activates their ability to transport ions. The mechanism by which phosphorylation of SPAK and OSR1 control ion co-transporter activity and trafficking deserves more detailed study.

The regulation of NCC by WNK4

Numerous studies have investigated the role of WNK4 in the regulation of NCC by overexpressing heterologous WNK4 variants in *X. laevis* oocytes and evaluating the effects on NCC activity and membrane localisation (reviewed by Kahle et al., 2008). These experiments have revealed that the overexpression of wild-type WNK4, but not catalytically inactive WNK4 or PHAII-associated WNK4 mutants, inhibits rather than activates NCC. These

observations have been widely interpreted as evidence that wild-type WNK4 operates as a negative regulator of NCC (Kahle et al., 2008). Further work is required to establish the mechanism by which WNK4 inhibits NCC, and whether this is mediated by SPAK and OSR1. Caution is required when interpreting the results of overexpression studies, as it is possible that expression of WNK4 at highly supraphysiological levels in cells can inhibit NCC by sequestering the SPAK and OSR1 kinases, and/or other components that regulate NCC activity and localisation. It is also possible that, following overexpression, the forced heterodimerisation of WNK4 with other WNK isoforms alters their ability to phosphorylate and activate SPAK and OSR1. For future studies, we recommend that the effects of WNK4 overexpression be complemented with studies that examine siRNA-mediated knockdown of WNK4 expression, and also measurements of SPAK and OSR1 phosphorylation, activity and localisation. It is also crucial to monitor the phosphorylation status of NCC at the SPAK and OSR1 phosphorylation sites by employing phosphospecific antibodies.

Recent studies have succeeded in recapitulating the PHAII phenotype of hypertension and hyperkalemia in animal models, by generating WNK4[Q562E] transgenic mice (Lalioti et al., 2006) and WNK4[D561A] knock-in mice (Yang et al., 2007b). Importantly, the phenotype of the PHAII WNK4[Q562E] transgenic mice was reversed by crossing them with genetically NCC-deficient mice (Lalioti et al., 2006). This suggests that NCC is a key mediator of the pathway by which the WNK4[Q562E] mutation increases blood pressure. Blood pressure and hyperkalemia was also reduced by treating WNK4[D561A] mice with thiazides (Yang et al., 2007b). Taken together, these findings strongly indicate that WNK4 mutations do indeed exert their hypertensive and hyperkalemic effects through NCC. Notably, increased phosphorylation of SPAK and OSR1 was observed in WNK4[D561A] mice, which suggests that the effects of WNK4 on NCC can be mediated through SPAK and OSR1 rather than through other, more complex, mechanisms that involve heterodimerisation and kinase-dependent and -independent roles of WNK isoforms – as recently proposed (Yang et al., 2007a). It has also been observed that overexpression of wild-type WNK4 in mice inhibited NCC; this led to a Gitelman's-like syndrome that was accompanied by hypoplasia of the distal convoluted tubule, hypotension, hypokalemia and metabolic alkalosis, which is consistent with the hypothesis that WNK4 functions as a negative regulator of NCC (Lalioti et al., 2006). It would be interesting to investigate how overexpression of wild-type WNK4 might affect activation of SPAK and OSR1 in the distal convoluted tubule of these mice.

The regulation of KCCs by WNK-SPAK/OSR1 signalling

In addition to NKCC1, NKCC2 and NCC, the SLC12 family of co-transporters comprises four K^+/Cl^- co-transporters (KCC1-KCC4) (Table 1) that also have vital roles in maintaining cell volume and blood-pressure regulation (Kahle et al., 2006). KCC1, KCC3 and KCC4 are widely expressed, whereas KCC2 is found predominantly in neuronal tissues. In contrast to Na^+ co-transporters, which catalyse ion uptake, KCCs catalyse the efflux of K^+ and Cl^- from the cell. KCC isoforms are inhibited rather than activated by the hyperosmotic stress conditions that stimulate the WNK pathway. Inhibition of KCC isoforms enables cells to retain Cl^- that is imported into cells by Na^+ co-transporters (Kahle et al., 2006). Recent work suggests that the overexpression of WNK3 and WNK4 in oocytes leads to the inhibition of KCC isoforms (de Los Heros et al., 2006; Garzon-Muvdi et al., 2007; Kahle et al., 2005). It would be interesting to investigate whether SPAK and OSR1 phosphorylate and inhibit KCCs, although it should be noted that in one study the authors concluded that overexpression of SPAK in oocytes does not inhibit KCCs (Garzon-Muvdi et al., 2007). The inhibition of KCCs would enhance the ability of the WNK pathway to retain cellular Cl^- by stimulating ion influx (through the activation of SLC12 Na^+ co-transporters) and simultaneously suppressing ion efflux (by inhibiting the activity of SLC12 K^+ co-transporters) (Fig. 4).

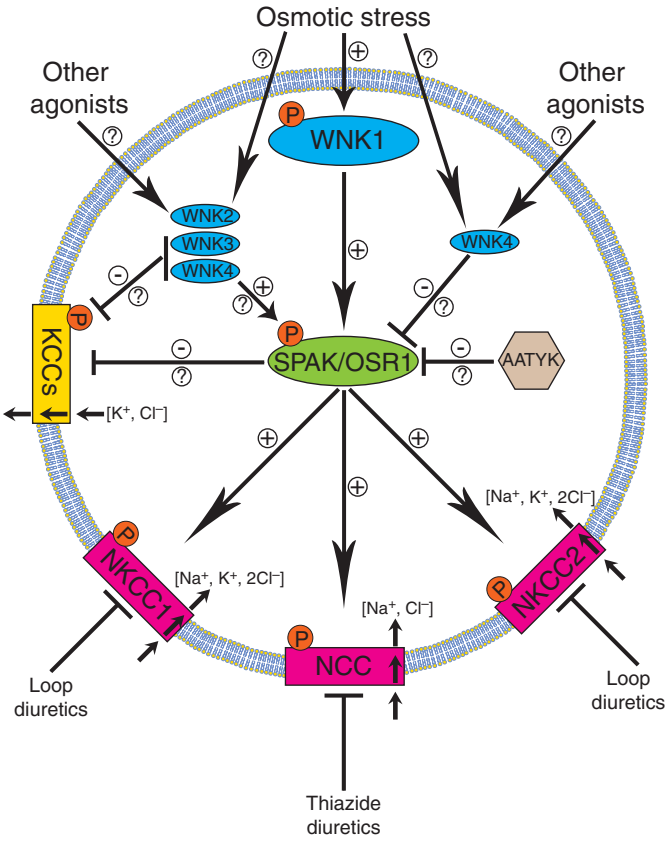

Fig. 4. Proposed mechanism by which the WNK-SPAK/OSR1 signalling pathway regulates salt re-absorption and blood pressure. Although there is clear evidence that WNK1 activates NCC via SPAK and OSR1, further work is required to determine whether other WNK isoforms regulate ion co-transporters through SPAK and OSR1. It should also be noted that most studies that employ overexpression systems have reported that WNK4 negatively regulates NCC. Whether this inhibitory effect is mediated through SPAK or OSR1 has not been studied.

Two splice variants of human KCC, termed KCC2a and KCC3a, possess potential SPAK- and OSR1-binding RFxV motifs and, interestingly, KCC3a has been reported to interact with SPAK and OSR1 in a yeast two-hybrid screen, which indicates that it might indeed be a substrate of SPAK and OSR1 (Piechotta et al., 2002). However, the cluster of Thr residues that are phosphorylated by SPAK and OSR1 in NCC and NKCC1 is not conserved in KCC isoforms, which suggests that phosphorylation would occur at a distinct region. Intriguingly, there are two SLC12 co-transporters, termed cation Cl^- co-transporter-9 (CCC9) and co-transporter-interacting protein (CIP) that do not contain RFx[V/I]-motifs; their physiological roles have not, however, been defined (Gamba, 2005) (Table 1). It would also be interesting to study whether the recently identified neuronal HSANII-associated isoforms of WNK1 (Shekarabi et al., 2008) have

Box 1. Major outstanding questions

- How are hyperosmotic and hypotonic low-Cl^- conditions sensed by WNK isoforms?
- What are the identities of the kinase and phosphatase that regulate the phosphorylation of Ser382 on WNK1?
- Do the four isoforms of WNK possess distinct functions?
- What are the roles of the auto-inhibitory domain, heterodimerisation and O-GlcNAc modification of WNK isoforms?
- What purpose is served by the translocation of WNK isoforms to intracellular vesicles and how is this controlled?
- What is the mechanism by which WNK4 might negatively regulate NCC and does this involve SPAK and OSR1?
- What is the role of the acidic segment on WNK4 and how do PHAII-associated mutations increase blood pressure?
- What is the function of the short kinase-inactive kidney-specific isoform of WNK1? Does it inhibit activation of SPAK and OSR1 and phosphorylation of NCC and NKCC2?
- Do WNK isoforms regulate processes independently of SPAK and OSR1 and, if so, what are the mechanisms?
- What are the kinases that phosphorylate Ser and Thr residues that lie C-terminal to the CCT-binding RFxV motifs and promote the dissociation of SPAK and OSR1 from WNK isoforms? How are these kinases regulated?
- Is the WNK-SPAK/OSR1 pathway controlled by blood-pressure-controlling hormones such as angiotensin II and aldosterone?
- How does the phosphorylation of sodium ion co-transporters by SPAK and OSR1 activate these enzymes and/or stimulate their trafficking to the plasma membrane?
- How does the WNK pathway inhibit KCC isoforms, and are SPAK and OSR1 involved?
- Do SPAK and OSR1 phosphorylate and regulate targets other than SLC12 ion co-transporters?
- What is the role of the deep secondary pocket on the CCT domain of OSR1 and what does it interact with?
- How does AATYK influence SPAK and OSR1 function, and vice versa?
- What is the role of interaction of protein phosphatases with SLC12 ion co-transporters and/or AATYK isoforms?
- Is there crosstalk between the WNK-SPAK/OSR1 ion–co-transporter pathway and other signalling networks?
- How do mutations that truncate the C-terminal non-catalytic region of neuronal WNK1 isoforms lead to the pain perception disorder HSANII? Are SPAK, OSR1 and ion co-transporters such as KCCs involved?
- Would chemical inhibitors or genetic manipulations (in mouse) of WNK1 and/or SPAK and OSR1 affect pain perception?

a role in the control of KCC isoforms, and whether this is connected with the ability of the WNK pathway to control pain perception.

Genetic analysis of the WNK signalling pathway

Caenorhabditis elegans contains a single homologue of WNK1 (WNK-1) and SPAK and OSR1 (GCK-3). Recent studies have established that a major role of WNK-1 in *C. elegans* is to activate GCK-3 by phosphorylating a Thr residue (Thr280) within its T-loop (Hisamoto et al., 2008). Activated GCK-3 phosphorylates and inhibits the CIC anion channel CLH-3, which has a role in regulating *C. elegans* canal formation and fertility (Denton et al., 2005; Hisamoto et al., 2008). Interestingly, the activation of GCK-3 by WNK-1 required the presence of Thr280 but not the S-motif residue (Ser419), which was also efficiently phosphorylated by WNK-1 (Hisamoto et al., 2008). Moreover, the regulation of CLH-3 by the WNK-1 pathway is dependent on an interaction between the CCT domain of GCK-3 and an RFxI motif on the C-terminal tail of CLH-3 (Denton et al., 2005; Hisamoto et al., 2008). The siRNA-mediated knockdown of GCK-3 expression also impaired the ability of *C. elegans* to tolerate hyperosmotic stress (Choe and Strange, 2007). The *C. elegans* WNK-1 signalling module (WNK-1–GCK-3–CIC anion channel) is analogous to the mammalian WNK-SPAK–SLC12 co-transporter pathway. This evolutionary conservation of the WNK signalling pathways between *C. elegans* and humans emphasises the importance of this network in regulating osmotic homeostasis.

SPAK and OSR1 – new drug targets for the treatment of hypertension?

A major implication of the findings described in this review is that the WNK-SPAK/OSR1 pathway represents a system that is amenable to drug treatment and could be exploited to treat high blood pressure by inhibiting salt re-absorption in the kidney. Targeting WNKs might be more challenging than targeting SPAK and OSR1, as it is unclear which combination of the four WNK isoforms would need to be inhibited to reduce the activity of NCC and NKCC2 in the kidney. As WNK4 might negatively regulate NCC, it is possible that inhibition of WNK4 activates NCC. To target WNKs it might, therefore, be necessary to generate drugs that inhibit WNK1, WNK2 and WNK3, but not WNK4, which could be challenging given the high degree of sequence identity of the catalytic domains of these enzymes. However, one potential advantage of targeting WNK isoforms is the unusual position of the catalytic Lys residue on WNK isoforms compared with other kinases – a feature that might be exploited to develop WNK-specific ATP-competitive inhibitors.

SPAK and OSR1 are arguably simpler enzymes to target than WNK isoforms. Inhibiting SPAK and OSR1 should reduce the activity of NCC and NKCC2, thereby suppressing renal salt re-absorption and blood pressure. SPAK and OSR1 are likely to have redundant roles in the regulation of NCC and NKCC2, so a drug that inhibited both isoforms might be more efficient at lowering blood pressure. The kinase domains of SPAK and OSR1 are 89% identical, which suggests that many inhibitors target both isoforms. The analysis of mice that are deficient in SPAK and/or OSR1 would also be useful to validate the role of these enzymes in regulating the activity of NCC and NKCC2, as well as blood pressure. A key advantage of a drug that inhibited SPAK and/or OSR1 is that it would probably reduce the activity of both NCC and NKCC2. The partial inhibition of both NCC and NKCC2 through the use of an inhibitor of SPAK and OSR1 might have a greater blood-pressure-lowering effect than the inhibition of only one of these co-transporters with thiazide or loop diuretics.

Other WNK-regulated pathways

In this Commentary we have focussed on SPAK and OSR1 as the main effectors of WNKs. However, WNK kinases are likely to have functions beyond the regulation of ion transport, and these might be independent of SPAK and OSR1. WNK isoforms have been

implicated in interacting with, phosphorylating and/or controlling the function of several other targets, which include the epithelial sodium channel (ENaC), the renal outer medullary K^+ channel (ROMK), serum- and glucocorticoid-induced kinase (SGK), the transient receptor potential vanilloid-4/5 Ca^{2+}-channel (TRPV4/5), the cystic fibrosis transmembrane conductance regulator (CFTR), claudin isoforms, synaptotagmin 2 and the Cl^--base exchanger SLC26A6 (CFEX) (reviewed by Huang et al., 2007; Kahle et al., 2008). Much of the evidence that supports these observations is based upon overexpression studies and more work is therefore required to validate their importance and impact on the regulation of blood pressure.

Other reports have suggested that WNK isoforms regulate proliferation and apoptosis, perhaps through caspase 3 (Moniz et al., 2007; Verissimo et al., 2006). Furthermore, in the majority of infiltrative gliomas WNK2 expression was reportedly silenced by methylation, and deletions within the *WNK2* gene were found at low frequency (Hong et al., 2007). The overexpression of wild-type or kinase-inactive WNK2 inhibited colony formation in glioma cells, which suggests that WNK2 functions as a kinase-independent suppressor of cell growth (Hong et al., 2007). *D. melanogaster WNK1* has been found in a genome-wide RNAi screen for genes that regulate cell survival (Boutros et al., 2004). The overexpression of WNK1 has been proposed to activate ERK5 protein kinase, which is vital for organ development (Xu et al., 2004). The lethal phenotype of WNK1-knockout mice, which die at around embryonic day 13, also points to developmental roles for the WNK network (Zambrowicz et al., 2003). WNKs have also been proposed to modulate TGFβ-Smad signalling by inhibiting transcriptional processes that are mediated by Smad2 (Lee et al., 2007). It will be important to unravel the mechanism by which WNK isoforms control cell proliferation, survival and development and to determine whether SPAK, OSR1 or SLC12 ion co-transporters are involved.

Conclusions and perspectives

The discovery that WNK kinases are upstream activators of SPAK and OSR1, coupled with recent studies that demonstrate that the activity of renal ion co-transporters such as NCC is controlled by SPAK or OSR1, has demonstrated that the WNK-SPAK/OSR1 signalling pathway is an important component of blood-pressure regulation. These data suggest that the WNK kinases are the master sensors of hyperosmotic as well as hypotonic low-Cl^- conditions and that they regulate cellular ion content by activating SLC12-family Na^+ transporters, and perhaps by inhibiting SLC12-family K^+ co-transporters, via the kinases SPAK and OSR1 (Fig. 4). An important implication of recent studies is that inhibitors of SPAK, OSR1 or WNK isoforms have the potential to reduce salt re-absorption in the kidney by suppressing the activity of the NCC and NKCC2 co-transporters and, therefore, lower blood pressure. There are still many unanswered questions as to how the WNK pathway operates and is regulated, and we have summarised these in Box 1. It is also our opinion that, in the future, more will be learned from rigorous analysis of knockout or knock-in mice or of cells that are defective in specific components of the WNK-SPAK/OSR1 ion co-transporter pathway than from overexpression studies alone.

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