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WNK kinase signaling in ion homeostasis and human disease

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Abstract

WNK kinases, along with their upstream regulators (CUL3/KLHL3) and downstream targets (the SPAK/OSR1 kinases and the cation-Cl⁻ cotransporters [CCCs]), comprise a signaling cascade essential for ion homeostasis in the kidney and nervous system. Recent work has furthered our understanding of the WNKs in epithelial transport, cell volume homeostasis, and GABA signaling, and uncovered novel roles for this pathway in immune cell function and cell proliferation.

Introduction

Homeostasis of the intracellular ionic *milieu* is essential for the proper functioning of all cells and a diverse group of cellular processes. The mechanisms responsible for the homeostasis of the intracellular Cl⁻ concentration [Cl⁻]_i, for example, significantly impacts the rate of fluid secretion or absorption across epithelia; how red blood cells counteract potentially damaging osmotic-induced cell swelling or shrinkage; and whether the GABA neurotransmitter excites or inhibits post-synaptic neurons. Accordingly, [Cl⁻]_i is highly regulated by signaling molecules that *sense* changes in intracellular [Cl⁻]_i and *transduce* these signals to the cell membrane to modulate the transport of Cl⁻ transporters and channels (Hoffmann and Dunham, 1995). While the mediators of ion transport are well known, the signaling pathways that dynamically regulate their activities to maintain homeostasis have been relatively unexplored.

Molecular genetics can pinpoint essential genes in complex regulatory pathways, paving the way for novel and unanticipated physiological insights. Over the last 15 years, studies have uncovered a critical role of the with-no-lysine [K] (WNK) serine-threonine kinases, along with their upstream regulators (CUL3/KLHL3) and downstream targets (the SPAK/OSR1 serine-threonine kinases and the *SLC12A* family of electroneutral cation-Cl⁻ cotransporters [CCCs]), in human physiology and disease. Since their identification as disease-causing genes of a Mendelian form of NaCl-sensitive hypertension (i.e., high blood pressure), the WNKs have become recognized as “master regulators” of the CCCs, including the Na⁺-coupled Cl⁻ importers (NCC, NKCC1, and NKCC2) and the K⁺-coupled Cl⁻ exporters (KCC1-KCC4).

Here we briefly review the recent molecular genetics, biochemical, and physiological findings involving the WNK signaling pathway as they relate to human physiology and disease. We will focus on the established roles of the WNK-SPAK-CCC pathway in 1) renal epithelial transport, 2) cell volume homeostasis, and 3) GABA signaling, which have implications for diverse diseases such as essential hypertension, cerebral edema, and neuropathic pain. We will also cover emerging roles for the WNKs in immune function and cell migration, which have relevance for autoimmune diseases and several types of cancer.

Renal epithelial transport

The WNK kinases, first cloned in rat, comprise a serine-threonine kinase subfamily characterized by the lack of a highly-conserved lysine in β strand 3 of kinase subdomain II that usually mediates ATP-binding and the catalysis of phosphoryl transfer in most other kinases (Xu et al., 2000). However, in the WNK kinases, the catalytic activation loop is structured such that a lysine (Lys-233 in WNK1) in β strand 2 executes this function (Min et al., 2004). Mutations in two members of the WNK kinase family, *PRKWNK1* (Chr 12) and *PRKWNK4* (Chr 17), encoding WNK1 and WNK4, respectively, were shown to cause a human monogenic disease, familial hyperkalemic hypertension (FHHT; also known as pseudohypoaldosteronism type 2 or Gordon syndrome) (Wilson et al., 2001). As the disease phenotype involved disordered homeostasis of renal Na^+ , Cl^- , and K^+ transport, the discovery of the WNKs led to the identification of a previously-unknown signaling pathway that modulates renal solute transport and arterial pressure; this pathway has subsequently been termed “a molecular switch” that regulates the balance between renal NaCl reabsorption and K^+ secretion (Alessi et al., 2014; Welling et al., 2010).

At the time of their identification, little was known about WNK kinase function other than that autophosphorylation was increased in response to hypertonic stimuli like NaCl or mannitol (Xu et al., 2000). The FHHt phenotype can often be corrected with thiazide diuretics, drugs that act along the distal convoluted tubule (DCT), suggesting that WNKs modulate NaCl transport along this segment (Mayan et al., 2002). WNK4 is expressed by cells from the thick ascending limb to the collecting duct (McCormick et al., 2014; Ohno et al., 2011). Although WNKs can modulate several transport proteins along these segments, the thiazide-sensitive NaCl cotransporter (NCC, encoded by *SLC12A3*), a member of the *SLC12A* family of cation-Cl⁻ cotransporters, is a primary target. Both work using heterologous expression (Wilson et al., 2003; Yang et al., 2003) and animal models of FHHt (Laloti et al., 2006; Yang et al., 2007) suggests that the primary defect in FHHt is excess transcellular NaCl reabsorption via NCC. While WNK4 expression in heterologous systems typically inhibits NCC activity (Chávez-Canales et al., 2014; Wilson et al., 2003; Yang et al., 2007c), likely by associating with protein phosphatases (Gagnon et al., 2007), WNK4's dominant effect in vivo is to activate NCC (Ohta et al., 2009; Piechotta et al., 2002; San-Cristobal et al., 2009; Vitari et al., 2005; Yang et al., 2007a). WNKs activate NCC by phosphorylating and activating two homologous kinases, SPAK (serine/threonine protein kinase 39, encoded by *STK39*) and *OSR1* (oxidative stress-responsive 1; encoded by *OxSR1*) (Moriguchi et al., 2005); these, in turn, directly phosphorylate NCC along its amino terminal cytoplasmic domain (other CCCs are also targets). For NCC, and for the bumetanide-sensitive Na⁺, K⁺, Cl⁻ transporters (NKCC1 & 2; *SLC12A2* and *A1*), these phosphorylation events activate the transport protein, leading

to increased solute transport, in part by stabilization in the plasma membrane (Figure 1) (Rosenbaek et al., 2014).

Changes in osmolality and Cl^- concentration activate WNK kinases (Xu et al., 2000). When the WNKs are expressed in heterologous systems with NCC, exposure to hypotonic low Cl^- medium therefore increases the abundance of phosphorylated NCC and NCC activity because WNKs activate endogenous SPAK or OSR1 (Pacheco-Alvarez et al., 2006). Chloride binds to the WNK1 catalytic domain, thereby inhibiting autophosphorylation and kinase activity (Piala et al., 2014), effects that are assumed to mediate the sensitivity to chloride. Yet, the physiological relevance of this effect in mammals was unclear, because DCT cells in the renal cortex are typically bathed in extracellular fluid of nearly constant $[\text{Cl}^-]$ and the luminal membrane is nearly water impermeable.

One potential resolution to this conundrum is that WNK Cl^- -responsiveness mediates the DCT's ability to sense extracellular $[\text{K}^+]$ (Terker et al., 2015a; Wade et al., 2015). NCC activity is extraordinarily sensitive to small changes in plasma $[\text{K}^+]$ (Terker et al., 2015b). WNK kinases appear to be essential for this effect, with WNK4 likely playing a primary role (Terker et al., 2015a, 2015c; Wade et al., 2015). According to this model, small changes in plasma $[\text{K}^+]$ affect NCC because they alter intracellular $[\text{Cl}^-]$, thereby modulating WNK and SPAK/OSR1 (Terker et al., 2015c). The unique role of WNK kinases in balancing sodium and potassium excretion stems from the fact that electroneutral NaCl reabsorption (NCC) takes place in the DCT, upstream from the aldosterone-sensitive segments that secrete potassium. **WNK activity is low when plasma $[\text{K}^+]$ is high and aldosterone is secreted, thus NCC is suppressed,** whereas the epithelial Na^+ channel,

ENaC, is stimulated (by aldosterone). Na⁺ reabsorption therefore occurs primarily along the aldosterone-sensitive distal nephron, not along the DCT, and therefore Na⁺ is exchanged for K⁺, which is excreted (Figure 2.). This effect may be enhanced when dietary K⁺ is ingested with organic anions, as the resulting bicarbonate load is delivered to the distal nephron, favoring electrogenic over electroneutral Na⁺ reabsorption. The opposite events occur when plasma [K⁺] is low (Kamel et al., 2014).

Although WNK1 can also phosphorylate and activate SPAK/OSR1 *in vitro*, its physiological action along the mammalian distal nephron has been more difficult to resolve; WNK1 deletion is embryonic lethal, owing to defects in heart and vascular development (Xie et al., 2009), and complete deletion of WNK1 in kidney has not been reported. In fact, the predominant WNK1 isoform in the distal nephron, at least at the mRNA level, lacks a kinase domain (O'Reilly, 2003); it is called kidney-specific WNK1 (KS-WNK1), because its pattern of expression is so highly restricted and is driven by an alternate promoter (Delaloy et al., 2003). KS-WNK1 cannot phosphorylate substrates, and instead interacts with, and modulates, the actions of other WNKs, via a carboxyl terminal HQ domain (Chávez-Canales et al., 2014; Yang et al., 2007a). WNK1-associated FHHt, which results from a large deletion within intron 1, leads to 'ectopic' expression of the full length WNK1 isoform in the DCT (Vidal-petiot et al., 2013), thus introducing a kinase-active WNK1 isoform in a segment that normally exhibits little WNK1 kinase activity (Chávez-Canales et al., 2014). As WNK4 deletion nearly abrogates NCC activity (Castaneda-Bueno et al., 2012; Takahashi et al., 2014), the physiological role of renal WNK1 remains uncertain.

The discovery that mutations in two other genes, cullin 3 (*CUL3*) and kelch-like 3 (*KLHL3*) also cause FHHt (Boyden et al., 2012; Louis-Dit-Picard et al., 2012) has broadened understanding of the WNK kinase network. These proteins target WNKs for degradation by the proteasome, by attaching ubiquitin moieties to them. For ubiquitination to occur, an adaptor protein, such as KLHL3, must bring the WNK into association with Cullin 3, part of an E3 ligase that mediates ubiquitination. Disease-causing mutations in *KLHL3* impair either KLHL3 binding to WNK1/4 or KLHL3 binding to Cullin 3 (Ohta et al., 2013). When WNKs cannot associate with Cullin 3 and be ubiquitinated, they are no longer degraded, accumulate in cells, and enhance NCC activity (Figure 3.) (Susa et al., 2014). Interestingly, disease-causing mutations within the acidic domain of WNK4 also disrupt its ability to bind KLHL3 (Ohta et al., 2013), suggesting that the fundamental mechanisms for disease caused by WNK4 and KLHL3 mutations are similar. Mouse models that introduce these mutations in WNK4 or KLHL3 exhibit high NCC activity and increased WNK abundance (Shibata et al., 2013; Wakabayashi et al., 2013).

The mechanism by which Cullin 3 mutations cause FHHt is not as clear. Disease-mimicking *CUL3* mutations (all known mutations delete exon 9) increase WNK4, WNK1, and NCC abundance when introduced into mice (Schumacher et al., 2015), but the mutant protein does not appear to exhibit complete loss of function because deletion of Cullin 3 is embryonic lethal (Singer et al., 1999). Instead, these mutations appear to generate substrate-specific dysfunction (Ibeawuchi et al., 2015; McCormick et al., 2014; Schumacher et al., 2015). The precise nature of these properties, and the reasons for the kidney-specific phenotype, are subjects of active investigation.

Cell volume maintenance

Regulation of cell volume is critical for multiple essential cellular functions and organismal survival. Lacking a rigid cell wall, animal cells combat cell swelling or shrinkage, induced by perturbations in intracellular ion content or extracellular osmolality, by triggering concerted homeostatic counter-responses termed regulatory volume decrease (RVD) or regulatory volume increase (RVI), respectively (Hoffmann, and Dunham, 1995; Hoffmann et al., 2009; Kregenow, 1971, 1981; Lauf and Adragna, 2000). The related NKCC1 and KCC3 cation-Cl⁻ cotransporters, the Na⁺/H⁺ exchangers (e.g., NHE1), the Na⁺/K⁺ pump, and volume-regulated anion channels (VRACs), are important plasmalemmal mediators of ion transport in RVI and RVD (Hoffmann and Dunham, 1995; Hoffmann et al., 2009; Lauf and Adragna, 2012).

The bumetanide-sensitive NKCC1 cotransporter is a relative of NCC in the *SLC12A* family that is ubiquitously expressed, with particularly high expression in secretory epithelia (Haas et al., 2000). SPAK has been shown to physically interact with NKCC1 (and the KCCs – see below) via a specific conserved carboxyl-terminal (CCT) domain (Piechotta et al., 2002; Thastrup et al., 2012; Vitari et al., 2006; Zhang et al., 2015). An Arg-Phe-Xaa-Val/Ile (RFXV/I) domain in the cytoplasmic amino terminal tails of NKCCs and the KCCs, as well as the WNKs, recognizes the CCT motif of SPAK. This CCT-mediated “tethering” function of SPAK to *both* the upstream WNKs and downstream NKCC1 is necessary for cotransporter activity in both isotonic conditions and hyperosmotic (cell shrinkage) conditions (Thastrup et al., 2012; Vitari et al., 2006). The binding of WNKs to SPAK facilitates its phosphorylation of residues located within the T-loop of the SPAK catalytic domain (including Thr233) (Thastrup et al., 2012; Vitari et al., 2006). This event is essential for SPAK’s activation in response to hypertonicity and cell shrinkage, as well

as its ability to physically interact with and phosphorylate NKCC1 at Thr199, Thr201, and Thr206. In turn, NKCC1's activation by SPAK in the context of hypertonicity is an essential component of RVI (Figure 3.).

Despite their physiological importance, the biochemical mechanism of KCC (in)activation had been a mystery until recently, though serine-threonine kinases/phosphatases had long been known to play an essential role (Jennings and al, 1990). Phosphorylation inhibits the KCCs, while dephosphorylation has the opposite effect (Adragna et al., 2004; Altamirano et al., 1988; Dunham et al., 1980; Haas et al., 2000; Jennings and Schulz, 1991; Lytle and Forbush, 1992). KCC1, KCC3, and KCC4 are inactive in isotonic conditions but briskly activated in response to hypotonic or low Cl⁻ cell-swelling conditions (Adragna et al., 2004; Haas et al., 2000; Strange et al., 2006). KCC3 activation by hypotonic cell swelling is prevented by calyculin A, an inhibitor of protein phosphatase 1A (PP1) and PP2 (Lauf and Adragna, 2000). Recognition of these phenomena led to a "2-state mechanism" of KCC3 transport regulation in which KCC3 exists in either in dephosphorylated (active) or phosphorylated (inactive) state (Figure 4). The kinetics of hypotonicity-induced KCC3 activation suggest cell swelling *inactivates* critical regulatory kinases, tipping the balance towards protein dephosphorylation and transporter activation (Jennings and al, 1990).

While the nature of the phosphatase, PP1, was inferred from early inhibitor studies (Jennings and Schulz, 1991), it took almost two decades for the identity of the regulatory kinases and their major phosphorylation sites in the KCCs to be identified. Lifton and colleagues, using titanium dioxide-based phosphopeptide enrichment and techniques allowing for the quantitative assessment of changes in KCC phosphorylation, identified

two phospho-Thr residues in human KCC3, Thr991, and Thr1048, that mediate its inactivation in response to hypertonic cell shrinkage in both epithelial cells and in human red blood cells (Rinehart et al., 2009). These sites are phosphorylated in isotonic (inhibitory) conditions. Homologous sites to KCC3 Thr991 and Thr1048 are phosphorylated in all human KCCs (i.e., “site 1” and “site 2”), including KCC2 (Thr906 and Thr1007, respectively) (de Los Heros et al., 2014). Ala substitution at these Thr residues prevent phosphorylation and result in constitutively-active KCC2 and KCC3 (de Los Heros et al., 2014; Rinehart et al., 2009). SPAK, shown previously to regulate KCC activity in oocytes (Gagnon et al., 2006; Kahle et al., 2005), directly phosphorylates site 2 of the KCCs (de Los Heros et al., 2014).

However, the kinase that *directly* phosphorylates site 1 in the KCCs is unknown (Rinehart et al., 2009). However, a recent functional kinomics study that incorporated a kinome-wide siRNA-phosphoproteomic screen, a high-content kinase inhibitor screen, and a kinase trapping-Orbitrap MS screen revealed the WNK3-SPAK kinase complex to be essential regulator of *both* KCC3 Thr⁹⁹¹ and Thr¹⁰⁴⁸ phosphorylation *in vitro* and *in vivo* (Zhang et al., 2016). Genetic or pharmacological antagonism of WNK3-SPAK facilitates cellular Cl⁻ extrusion by simultaneously decreasing NKCC1 Thr203/Thr207/Thr212 phosphorylation and KCC3 Thr991/Thr1048 phosphorylation. Interestingly, WNK3-SPAK inhibition prevents acute cell swelling in response to osmotic stress, and ameliorates brain swelling after ischemic stroke by simultaneously decreasing the stimulatory phosphorylation of NKCC1 and the inhibitory phosphorylation of KCC3 (Begum et al., 2015; Zhang et al., 2016a; Zhao et al., 2016). These data provide evidence that WNK3-SPAK is an integral component of the long-sought “Cl⁻/volume-sensitive kinase” of the

KCCs, and functions as a molecular rheostat of cell volume (Zhang et al., 2016a) (Figure 4).

Interestingly, a human patient was recently discovered to carry a *de novo* gain-of-function mutation that substitutes an Ala at KCC3-Thr991, thereby disrupting phosphorylation and regulation of the transporter (Kahle et al., 2016a). Work performed using the patient's fibroblasts and with fibroblasts isolated from a mouse model reproducing this mutation confirmed constitutive KCC3 activity in normal isotonic conditions. The patient suffers from a CMT2 (Charcot-Marie-Tooth 2)-like neuropathy, characterized by an axonal, non-demyelinating, peripheral neuropathy leading to distal muscle weakness and atrophy, mild sensory loss, and normal-near normal nerve conduction velocities (Kahle et al., 2016a). Notably, loss-of-function mutations in KCC3 cause peripheral neuropathy associated with agenesis of the corpus callosum (ACCPN; OMIM# #218000), an autosomal recessive severe sensorimotor neuropathy associated with mental retardation, dysmorphic features and complete or partial agenesis of the corpus callosum (Boettger et al., 2003; Howard et al., 2002; Uyanik et al., 2006). In this case, however, with the exception of one missense mutation (R207C), all other *SLC12A6* mutations lead to truncation of KCC3, thereby affecting KCC3 expression and/or function at the plasma membrane. Interestingly, these patients also suffer from a severe, early onset neuropathy with sensory and motor deficits. KCC3 knockout mice recapitulate the locomotion and neuropathy phenotypes (Ding and Delpire, 2014; Howard et al., 2002; Shekarabi et al., 2012) and demonstrate axonal swelling (Byun and Delpire, 2007). Together, these results suggest that the function of the peripheral nervous system depends on finely-tuned,

kinase-regulated KCC3 activity and implicate abnormal cell volume homeostasis as a previously unreported mechanism of axonal degeneration.

Neuronal GABA signaling

GABA is the major inhibitory neurotransmitter of the mature central nervous system (CNS). The GABA_A receptor (GABA_AR), which mediates fast synaptic hyperpolarization, is a ligand-gated [Cl⁻] channel. In most mature neurons, intraneuronal [Cl⁻]_i is low, and opening of the GABA_AR channel results in flow of Cl⁻ ions into the cell and membrane hyperpolarization, decreasing the propensity for the neuron to fire and thereby establishing GABA as the major inhibitory neurotransmitter of the mature CNS. However, in immature neurons and certain pathologic states, [Cl⁻]_i is elevated, reducing or reversing Cl⁻ inflow when GABA_AR is activated and attenuating or inverting the hyperpolarization of the post-synaptic membrane. Dynamic neuronal regulation of [Cl⁻]_i enables tuning of the response to GABA, and consequently, neuronal excitability. Indeed, a developmentally-regulated decrease in [Cl⁻]_i drives the “switch” of GABA from an excitatory to inhibitory neurotransmitter, a highly-conserved feature of mammalian neurodevelopment (Figure 5.) (Ben-Ari, 2002).

Similar to other cells, intraneuronal [Cl⁻]_i is modulated by the regulated activities of the CCCs. Early in development, Cl⁻ is imported via NKCC1. Conversely, Cl⁻ is exported via KCC2, a transporter related to KCC3 that is expressed exclusively in neurons (Gagnon and Delpire, 2013). KCC2 displays a developmentally-regulated increase in activity coinciding with depletion of neuronal [Cl⁻]_i and the establishment of the normal hyperpolarizing response to GABA (Rivera et al., 1999). As WNK kinases reciprocally regulate the activities of NKCC1 and KCCs via phosphorylation in other tissues (see

above), WNKs have been compelling candidates for regulators of CCC-dependent GABAergic neurotransmission. Certain WNK isoforms exhibit specificity in their expression to the CNS (Shekarabi et al., 2013), and mutations in WNK1/HSN2, an isoform of WNK1 largely restricted to the central nervous system (including dorsal horn spinal cord neurons) cause hereditary sensory neuropathy type II (HSN2), a severe autosomal recessive disease in humans characterized by congenital pain insensitivity [(OMIM #201300;(Shekarabi et al., 2008)], suggesting an important role for WNK kinases in the human CNS.

Direct evidence for WNK regulation of neuronal Cl⁻ homeostasis via the CCCs has only recently emerged. Inoue and colleagues demonstrated that overexpression of WNK1 in embryonic rat cortices resulted in KCC2 phosphorylation at Thr906 and Thr1007, causing KCC2 inhibition (Inoue et al., 2012). Note that these sites in KCC2 are homologous to those in KCC3 regulated by the WNKs in the context of cell swelling and shrinkage in RBCs. Friedel and colleagues recently extended these results by showing that WNK1 forms a physical complex with KCC2 in the developing mouse brain, and is required for normal inhibitory phosphorylation of KCC2 at Thr906 and Thr1007 (Friedel et al., 2015). In immature neurons, phosphorylation at these sites suppresses KCC2-mediated Cl⁻ efflux and maintains elevated [Cl⁻]_i early in development – suggesting that WNK regulation of KCC2 may play a role in the GABA developmental switch secondary to changes in high to low [Cl⁻]_i (Friedel et al., 2015). Interestingly, Kahle et al. determined that WNK1/HSN2 contributes to a maladaptive decrease in KCC2 activity via inhibitory phosphorylation at Thr906/Thr1007; this elevates [Cl⁻]_i in dorsal horn neurons, disrupts GABA-mediated inhibition, and contributes to neuropathic pain in the spared nerve injury

(SNI) model (Kahle et al., 2016b). Although WNK1 kinase activity is required for KCC2 phosphorylation at these residues, WNK1 itself does not directly phosphorylate KCC2; instead, it phosphorylates SPAK, which phosphorylates KCC2 at Thr1007, and signals via a yet unknown kinase to phosphorylate KCC2 Thr906 (Friedel et al., 2015) (Figure 3).

In addition to providing mechanistic insights into the normal ontogenesis of GABA inhibition in the nervous system, these data also suggest WNK kinases may be promising therapeutic targets for disorders featuring neuronal hyperexcitability due to genetically-encoded or secondary maladaptive “GABAergic disinhibition.” These disorders, such as neuropathic pain (Kahle et al., 2014), neonatal seizures (Khanna et al., 2013), temporal lobe epilepsy (Cohen and Navarro, 2002), tumor-associated seizures (Campbell et al., 2015), motor spasticity (Boulenguez et al., 2010), morphine-induced hyperalgesia (Ferrini et al., 2013), some forms of autism spectrum disorders (Cellot and Cherubini, 2014), and schizophrenia (Hyde et al., 2011), are phenotypically diverse but share a common pathologic signature that involves elevated $[Cl^-]_i$ in distinct neuronal populations, and accordingly, impaired inhibition. In such pathological settings, WNK inhibition might be expected to decrease Cl^- influx via NKCC1 and simultaneously stimulate Cl^- efflux via KCC2, thereby promoting a therapeutic Cl^- extrusion. Early preclinical work has corroborated this hypothesis; for example, genetic deletion or pharmacologic inhibition of WNK1 prevents the development of neuropathic pain following peripheral nerve injury in mice (Kahle et al., 2016b). Further work into the development of WNK inhibitors for use in humans is underway (Yamada et al., 2016).

Non-renal epithelial transport

The WNK-SPAK pathway has only recently been explored in the regulation of ion transport across secretory epithelia in tissues other than the kidney, such as the skin, pancreas, and intestine. This investigation has stemmed in part from the original observations that, outside the kidney, WNK1 and WNK4 predominantly localized to polarized epithelia, including those lining the lumen of the hepatic biliary ducts, pancreatic ducts, sweat ducts, and colonic crypts (Choate et al., 2003; Kahle et al., 2004). Epithelia in these tissues express channels and transporters that are responsible for transcellular Cl^- and/or HCO_3^- ion movement from the blood, across the epithelial cell basolateral and apical membranes, and into the tissue lumen (e.g., sweat duct, pancreatic duct, or intestinal lumen). In doing so, these secretory epithelial cells therefore produce and maintain the homeostasis of sweat, pancreatic juice, intestinal mucus, and other bodily fluids. So far, the primary transport molecules in these tissues identified as targets of the WNKs-SPAK pathway include the $\text{Na}^+/\text{HCO}_3^-$ transporter NBCe1 (electrogenic sodium bicarbonate cotransporter 1); the $\text{Cl}^-/\text{HCO}_3^-$ exchanger family *SLC26A*; and the Cl^- channel CFTR (cystic fibrosis transmembrane conductance regulator) (Hong et al., 2014, 2013; Mendes et al., 2011; Yang et al., 2007, 2011).

For example, the exocrine gland of the pancreas secretes a pancreatic juice rich in Cl^- and HCO_3^- that also contains enzymes to digest dietary carbohydrates, proteins, and fats. WNK1-SPAK phosphorylation of NBCe1 and CFTR significantly inhibits ductal HCO_3^- secretion by reducing the plasma membrane expression of both NBCe1 and CFTR (Yang et al., 2009, 2011). Consistent with this, knock-down of several different WNK kinases in pancreatic ducts increases NBCe1 and CFTR-dependent ductal secretion. Interestingly, the NBCe1-B/CFTR activator inositol-1,4,5-trisphosphate (IP(3)) receptor-

binding protein released with IP(3) (IRBIT) antagonizes the effects of the WNKs and SPAK on NBCe1 and CFTR by recruiting PP1 to the complex to dephosphorylate CFTR and NBCe1-B and stimulate their activities (Yang et al., 2011). Given that the regulatory modalities in a conserved domain of NBCe1 may be present in CFTR and other transporters like the Slc26a6 sulfate transporter (Hong et al., 2013), and multiple ion transport proteins in secretory epithelia are regulated by PP1 and/or calcineurin, the WNK-SPAK and IRBIT-PP1 regulatory pathways of Cl^- and HCO_3^- transport may serve to precisely tune the rate of epithelial secretion in response to physiological demands or pathological stimuli in numerous epithelia (Hong et al., 2014). The relevance of this pathway for human physiology and disease was recently demonstrated in a large scale human genetic study. CFTR variants that disrupt the WNK1-SPAK activation are associated with a selective, HCO_3^- defect in CFTR channel function and in turn affects organs that utilize CFTR for bicarbonate secretion (e.g. the pancreas), but do not cause typical CF (LaRusch et al., 2014; Park et al., 2010).

The colonic epithelium secretes mucus that is also rich in HCO_3^- and Cl^- . Inflammatory bowel diseases (IBDs), including Crohn's disease and ulcerative colitis, are characterized by impaired immune regulation and epithelial barrier disruption. The mechanisms of the WNK-SPAK pathway in the regulation of colonic transport are less well characterized than in the pancreas. Targeted expression of SPAK has been shown to increase colonic epithelial permeability, and proinflammatory cytokines, which are elevated in induced experimental colitis, exacerbate this effect (Yan et al., 2007, 2011). In contrast, SPAK knockout mice exhibit higher intestinal barrier function and lower cytokine production in induced experimental colitis (Zhang et al., 2013). The correlated expression of SPAK with

colon osmolality and the production of proinflammatory cytokines has been linked to SP1 and NF- κ B binding sites in the SPAK promoter (Yan et al., 2008). These studies highlight the shared mechanisms and roles of the WNKs in regulating ion homeostasis in different tissues, and have implications for our understanding of CF and IBD, both of which are associated with abnormal epithelial transport.

The WNK-SPAK pathway was also implicated in the regulation of glucose reabsorption by sodium-glucose cotransporters (SGLTs) in the small intestine (mainly by SGLT1). Current findings suggest that SPAK mitigates SGLT1 activity when co-expressed in oocytes by lowering its plasma membrane insertion (Elvira et al., 2014). In addition, insulin was shown to enhance the WNK-SPAK-NCC pathway in a leptin signaling deficient mouse model of hyperinsulinemic metabolic syndrome by a mechanism involving the PKA (protein kinase A)/PKC (protein kinase C)-mediated phosphorylation of NCC (Fujita, 2014; Nishida et al., 2012). Insulin stimulates NCC activity by PKA-mediated phosphorylation and inactivation of KLH3, which leads to a decrease in WNK4 degradation (Shibata et al., 2014; Yoshizaki et al., 2015). In addition, the insulin mediated phosphorylation of SGLT2 links glucose reabsorption with the WNK-SPAK-NCC pathway (Shojaiefard et al., 2007), and insulin mediates the phosphorylation of WNK1 by the PI3K/Akt/PKB pathway (Jiang et al., 2005; Sale et al., 2006). In addition to inhibition of SGLTs by gliflozins such as canagliflozin, this data suggests mechanisms by which a new class of drugs could be developed that specifically targets the WNK4-mediated activation of NCC.

Immune function and cell migration

The role of the WNK pathway in the regulation of cellular functions not classically associated with ion transport has recently emerged. However, even in these processes, such as immune function and cell proliferation and migration, WNK-dependent modulation of ion transport is being shown as a common mechanism (Figure 6). For example, previous work had demonstrated that WNK1 is strongly expressed in the thymus and spleen of newborn and adult mice (Shekarabi, et al., 2013). Köchl et al. utilized an RNAi screen to identify WNK1 as a regulator of both integrin-mediated adhesion and T cell migration (Köchl et al., 2016). WNK1 decreases activated T-cell adhesion to endothelial cells and stimulates the migration of Jurkat T cells *in vitro* and primary CD4⁺ T-cells in lymph nodes and spleen during T-cells recirculation (Köchl et al., 2016). When WNK1-deficient primary T-cells are activated by the chemokine receptor CXCR4 or TCRs (T cell antigen receptor), they are significantly more adherent to ICAM1 (intercellular adhesion molecule 1), a ligand for LFA-1 (lymphocyte function-associated antigen 1, an integrin adhesion molecule on T-cells, $\alpha_1\beta_2$) and to endothelial cell monolayers. Furthermore, the NKCC1 inhibitor bumetanide decreased the chemotaxis effect of CCL21, a chemokine, in WT T-cells. These results are reminiscent of how WNKs modulate glioma cell migration through the regulation of focal adhesion dynamics, cell contractility, and cell volume via NKCC1-dependent changes in ion transport across the leading edge of cells (Algharabil, et al. 2012; Garzon-Muvdi et al., 2012; Haas and Sontheimer, 2010; Haas et al., 2011; Zhu et al., 2014). These findings are significant, since leukocytes utilize this migratory mechanism to exit from the vessel lumen and transmigrate between endothelial cells into the subendothelial matrix. Collectively, this work demonstrated a novel link between the WNKs-SPAK-CCCs pathway and T-cell and

endothelial and adhesion-mediated cytoskeleton reorganization. These mechanisms of adhesion and migration likely have to do with changes in cell shape and volume, and may be examples of how the same transport system can be utilized for different though partially overlapping cellular processes depending on cell type and physiological need.

Downstream targets of the WNK family have also been implicated in immune system function. A high NaCl diet has been linked to higher SGK1 (serum- and glucocorticoid - induced kinase 1) expression and generation of pathogenic T_H-17 cells ([interleukin-17 (IL-17)-producing helper T cells] *in vitro* and *in vivo* (Kleinewietfeld et al., 2013). Pathogenic T_H-17 cell activation is critical for the development of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, and genetic risk factors associated with MS are related to T_H-17 cell activation. SGK1 has a critical role in regulating IL-23 receptor expression and stabilizing the T_H-17 cell phenotype, suggesting a role for this kinase in both autoimmune and inflammatory disorders (Wu et al., 2013). SGK1 is a key regulator of ENaCs and other NaCl transport systems, and is activated by WNK1 and WNK4 in a kinase-independent fashion (Heise et al., 2010; Xu et al., 2005a, 2005b). SGK1 and AKT1 also phosphorylate WNK1 and WNK4 to inhibit ROMK (renal outer medullary K⁺ channel) (Cheng and Huang, 2011). The expression of the N-termini of all four WNKs results in modest to strong activation of SGK1. These findings suggest the WNK-SGK pathway may maladaptively induce TH-17 cell activation to promote autoimmunity in specific disease contexts via increases in dietary NaCl intake. This is fascinating, since increased dietary NaCl impacts blood pressure through the same WNK-regulated pathway in the distal nephron.

Potential therapeutic strategies targeting the WNK pathway

In addition to providing insight into the basic physiological mechanisms of ionic and volume homeostasis, the body of work reviewed herein also points to novel therapeutic strategies for a diverse array of human disorders by targeting the WNK-SPAK-CCC cascade. Indeed, some of these nodes have already begun to be tested. For example, WNK-SPAK inhibition would be expected to potently increase natriuresis, cause diuresis, and consequently lower systolic blood pressure. This approach may have several potential advantages over the use of thiazides, which are usually insufficient as monotherapy and have multiple off-target side effects such as impaired glucose tolerance, photosensitivity, or hyperuricemia. WNK-SPAK inhibition may be more potent antihypertensives than thiazides (or loop diuretics) alone, as they target multiple transporters, including NCC and NKCC2, simultaneously, and would avoid the off-target effects of thiazides. The natriuretic and antihypertensive effects of WNK inhibition were recently demonstrated in rodents, affirming the candidacy of WNK inhibition as a diuretic antihypertensive (Yamada, et al., 2016).

In the nervous system, regulation of $[Cl^-]_i$ and the postsynaptic response to GABA by the WNK-SPAK-CCC pathway has exciting implications for the numerous neurological disorders characterized by pathologically elevated $[Cl^-]_i$ resulting in so-called “GABAergic disinhibition.” For these conditions, which include neuropathic pain, neonatal seizures, certain forms of epilepsy, motor spasticity, and even psychiatric disorders such as some forms of autism and schizophrenia, inhibition of WNK-SPAK signaling may attenuate the pathologic elevations of $[Cl^-]_i$ via simultaneous activation of KCCs and inhibition of NKCC. Preclinical work has supported this hypothesis; for example, treatment of spinal cord slices from mice with neuropathic pain from sciatic nerve injury with the WNK-SPAK

inhibitor STOCK1S-50699 normalizes $[Cl^-]_i$ and restores the GABA-induced current (Kahle et al., 2016b). Thus, WNK-SPAK inhibitors may be novel, non-addictive analgesics in neuropathic pain. They may also be useful in the myriad other neurologic disorders of GABAergic disinhibition.

WNK-SPAK-CCC regulation of cellular ionic homeostasis also modulates cell volume, and is implicated in disorders of cell volume such as sickling of erythrocytes in sickle cell disease (Rinehart et al., 2009) and cerebral edema in ischemic stroke or hyponatremia (Kahle et al., 2015). For example, in ischemic cerebral edema, energetic failure results in pathologic increase in $[Na^+]_i$ and $[Cl^-]_i$, causing cytotoxic cell swelling. Inhibition of the WNK-SPAK-CCC cascade in this setting would be expected to simultaneously inhibit ionic import via NKCC and stimulate ionic export by KCCs to alleviate cellular osmotic stress. Zhang and colleagues recently demonstrated this effect in mice by showing that WNK3-knockout mice exhibited less cytotoxic edema after middle cerebral artery occlusion (Zhao et al., 2016).

Finally, emerging roles of WNK-SPAK signaling in autoimmunity, certain cancers, inflammatory bowel disease, and other disorders discussed here point to additional possible applications of WNK-SPAK modulation in human disease. The development of WNK-SPAK inhibitors with favorable pharmacokinetics for clinical use is underway. Exploiting the unique structure of WNK kinases to enhance kinase specificity is a promising strategy (Yamada et al., 2016b; Zhang et al., 2016b).

Conclusions

The WNK-SPAK-CCC pathway is essential for normal ion homeostasis in multiple tissues, and genetic mutations in several of its members cause human diseases characterized by impaired ion transport in the kidney and nervous system. The established roles of the WNK-SPAK-CCC pathway in epithelial transport, cell volume homeostasis, and GABA signaling have implications for diverse diseases such as essential hypertension, cerebral edema, and neuropathic pain. Exciting emerging roles for the WNKs in immune cell function and cell migration are also related their major function of ion transport homeostasis, and have relevance for autoimmune diseases and types of cancer, including glioma (Table 1). The importance of the WNK-SPAK-CCC pathway for human physiology, coupled with unique structural and biochemical characteristics of its signaling mechanisms (Yamada, et al., 2016a; Yamada et al., 2016b), suggest an improved understanding of this pathway may yield novel opportunities to manipulate cellular ion gradients for therapeutic benefit.

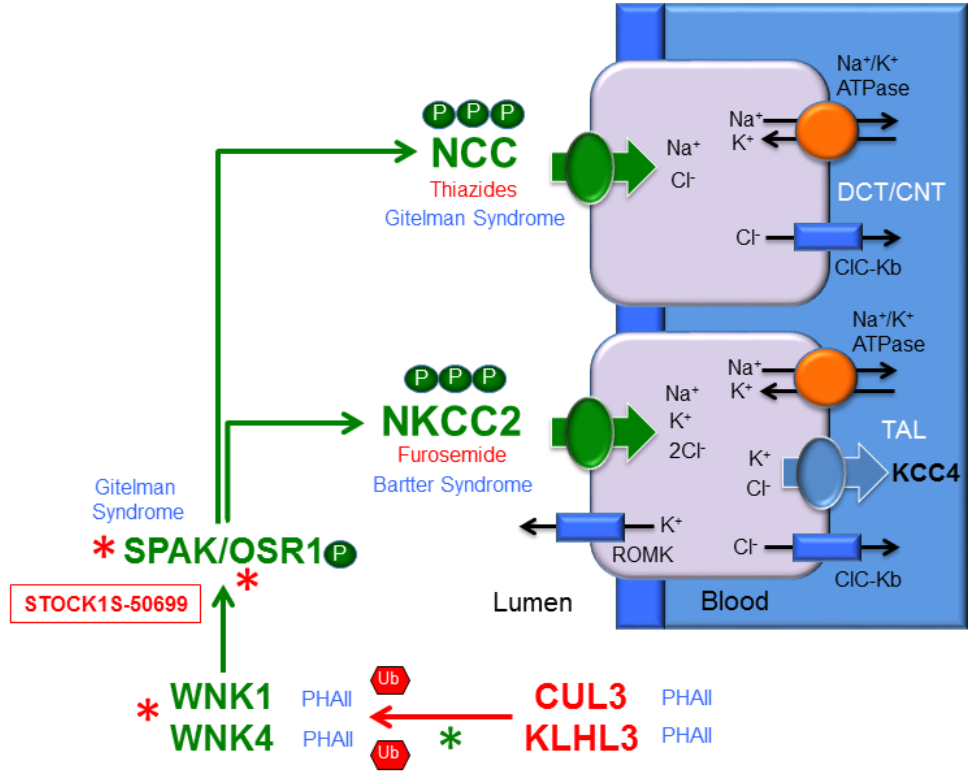


Figure 1. WNKs regulate NCC and NKCC2 through the kinases SPAK and OSR1 to achieve blood pressure and K⁺ homeostasis in humans. WNK1 and WNK4 are abundant in the kidney. Inhibition of WNKs in the kidney is predicted to elicit a K⁺-sparing, antihypertensive effect by reducing the reabsorption of NaCl by NCC in the distal collecting and connecting tubules (DCT/CNT) and by NKCC2 in the thick ascending limb (TAL). Red asterisks depict nodes in the signaling pathway where inhibition would be expected to decrease blood pressure. The green asterisk depicts a node where stimulation would be expected to decrease blood pressure. Mendelian diseases labeled in blue are those resulting from mutation of the indicated gene in humans. STOCK1S-50699, a recently developed WNK-SPAK/OSR1 inhibitor.

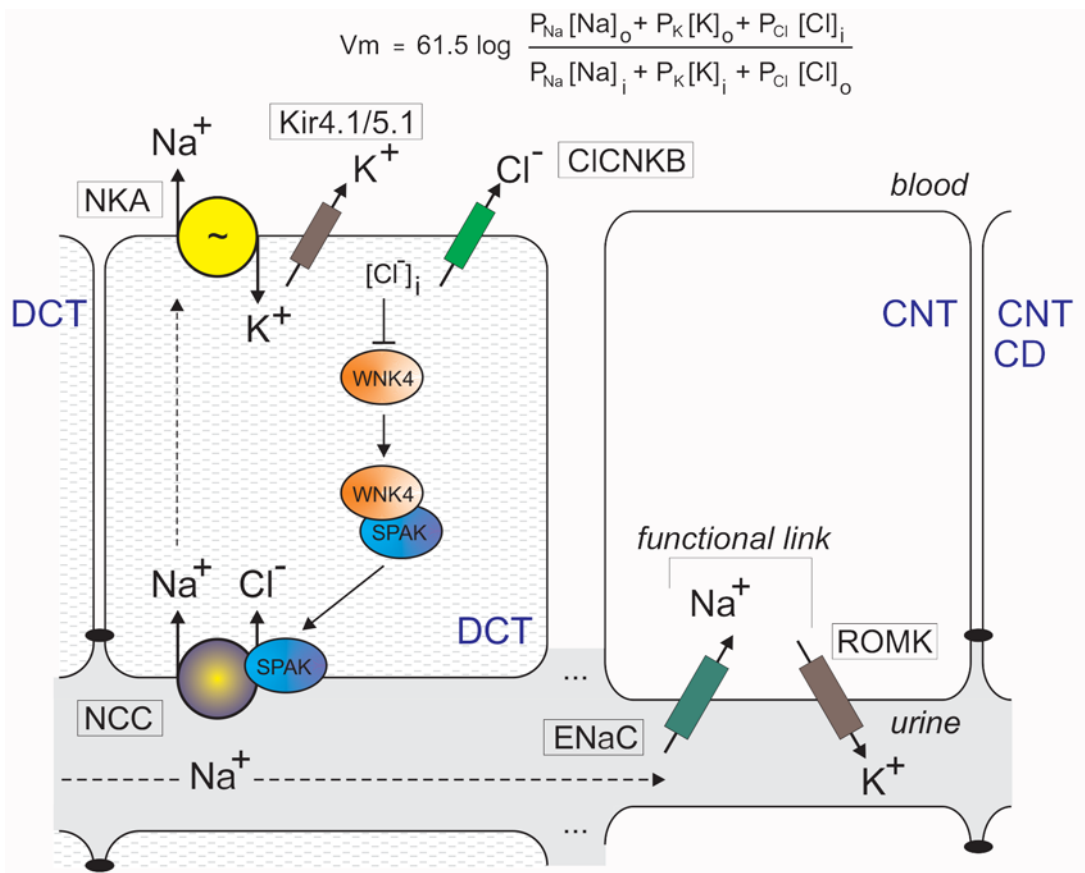


Figure 2. Role of the Distal Convoluted Tubule in Na⁺ reabsorption and in sensing plasma K⁺.

Na⁺ is reabsorbed in distal nephron through the Na-Cl cotransporter - NCC (DCT) and the epithelial Na⁺ Channel - ENaC (CNT). The driving force for Na⁺ reabsorption is provided by the basolateral Na⁺/K⁺-ATPase (NKA). NCC function is activated by a cascade of protein kinases, WNK4 and SPAK. A decrease of plasma K⁺ will result in a decrease in intracellular Cl⁻ (linked by Membrane potential V_m in Goldman Equation).

Decrease in intracellular Cl⁻ stimulates WNK4 which phosphorylates and activates SPAK, which phosphorylates and activates NCC. Decrease in plasma K⁺ results in increase Na⁺ reabsorption and thus decrease Na⁺ delivery to downstream CNT/CD segments leading to conservation of K⁺. Note that the same basolateral transporters exist in CNT and CD segments but are not shown for clarity.

DCT = Distal Convoluted Tubule; CNT = Connecting Tubule; CD = Collecting duct.

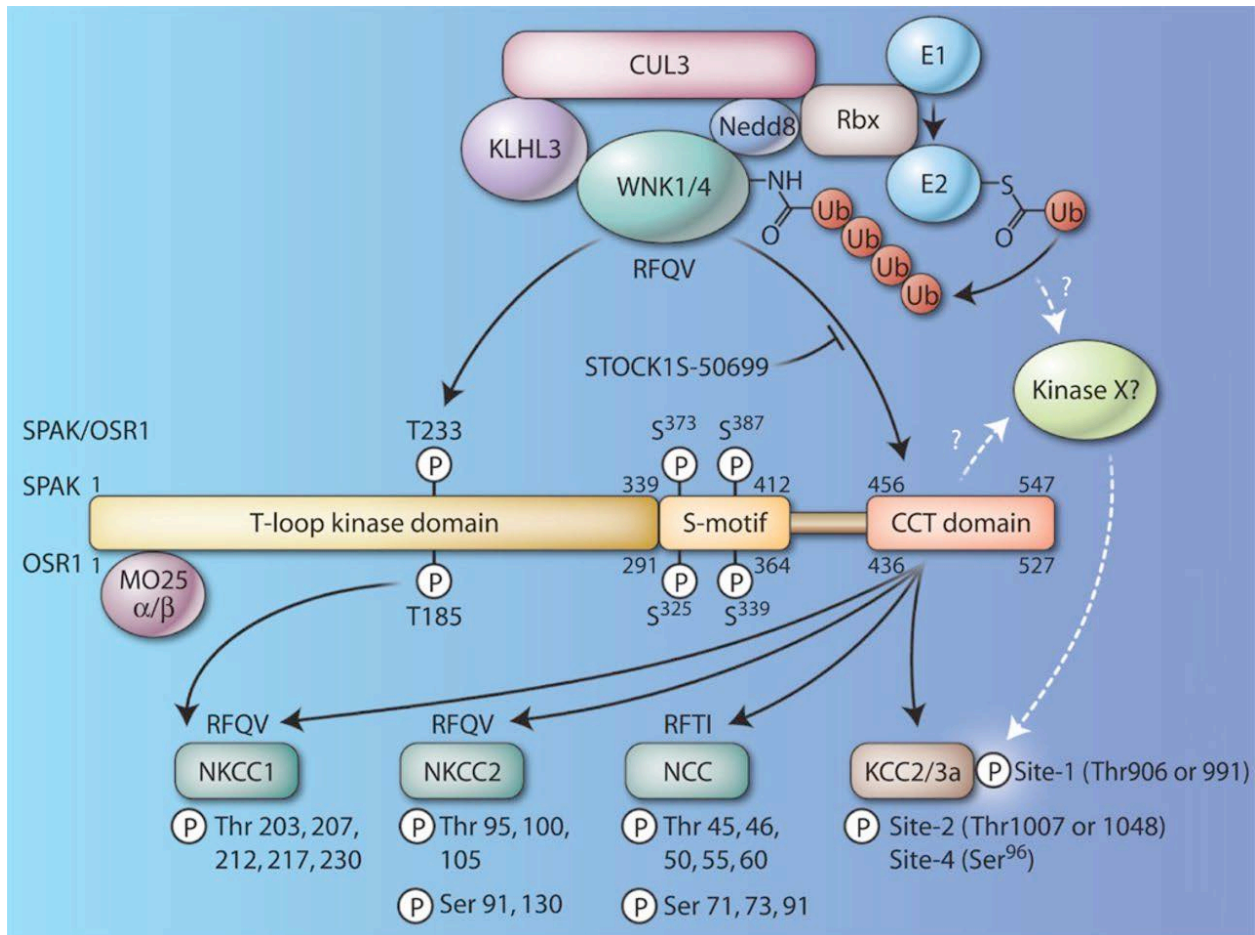


Figure 3. The WNK-SPAK-CCC signaling pathway (adapted from (Alessi et al., 2014)). Domains and sites important for regulation of and signaling through the WNK-SPAK/OSR1 pathway. Proteins with slashes indicate that multiple isoforms have the same properties. For SPAK/OSR1, the residue numbering above the protein represents SPAK, and the residue numbering below represents OSR1. Kinase X refers to a yet unidentified kinase that is regulated by WNKs and mediates the direct phosphorylation and inhibition of Site-1 on the KCCs. Rbx and Nedd8 are part of the ubiquitin ligase complex. E1 and E2 represent the two enzymes involved in transfer of ubiquitin (Ub) onto itself to form polyubiquitin chains. STOCK1S-50699 is a small-molecule inhibitor that blocks the interaction between SPAK/OSR1 and WNK by binding to the CCT domain.

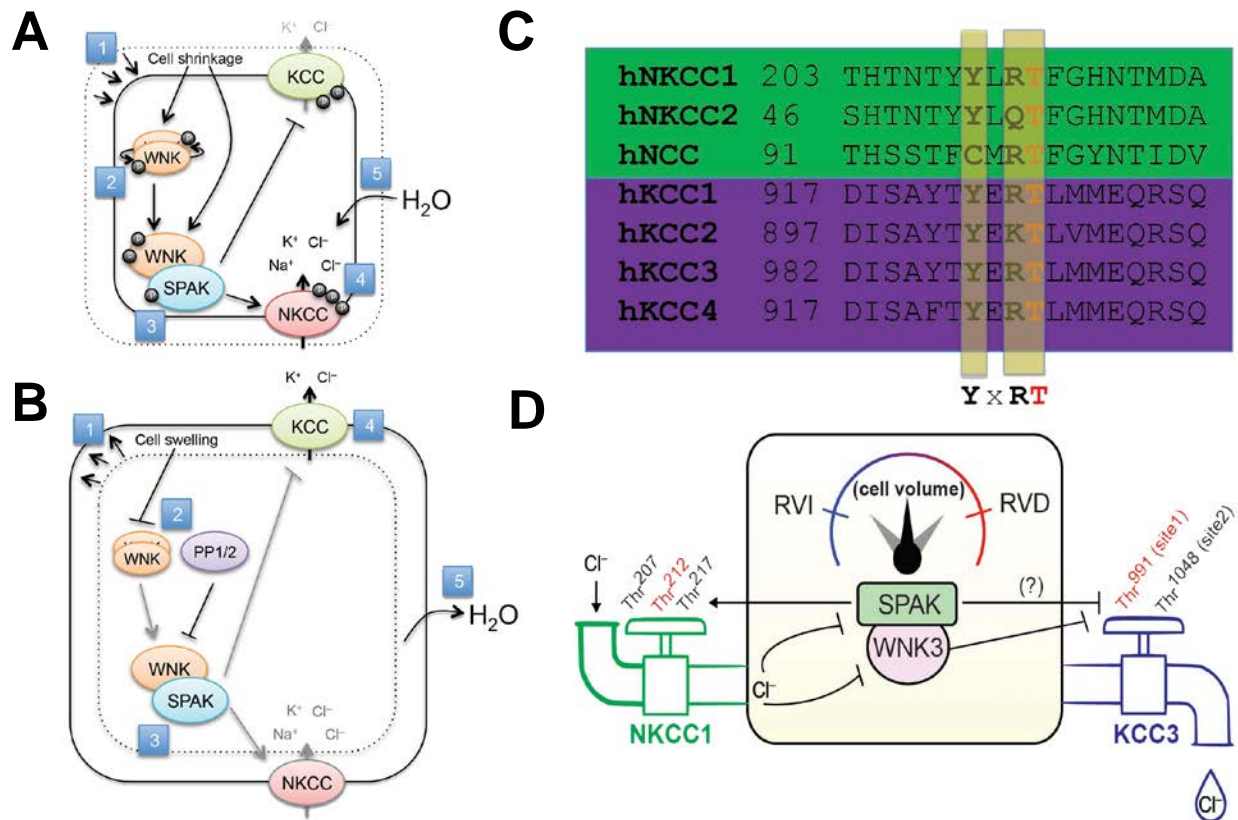


Figure 4. Models depicting the cell volume regulation by the WNK and SPAK kinases and their effectors NKCC1 and KCC3; a molecular rheostat of cell volume [adapted from (Zhang et al., 2016a)]. (A) Cell volume is tightly controlled by WNK-SPAK regulation of NKCC and KCC through a system that *senses* changes in cell volume and *transduces* these signals to affect ionic transport at the cell membrane. Water moves across the membrane according to resulting changes in osmotic gradients. Cell shrinkage (1) is detected via a yet unknown mechanism, and results in activation of the WNK kinases (2). Hyperosmolar stress increases WNK autophosphorylation. Autophosphorylation at a specific serine residue within the WNK activation loop (Ser382 in Wnk1) is required for WNK activation. Autophosphorylation at another serine residue (Ser378 in Wnk1) increases activity. WNK is likely further phosphorylated by other kinase(s) on other sites. (3) Activated WNK binds to and phosphorylates SPAK at residues located within the T-loop of the SPAK catalytic domain. (4) Phosphorylation activates SPAK and results in phosphorylation of multiple residues on both NKCC and KCC. Phosphorylation has opposite effects on NKCC and KCC; while NKCC is activated by phosphorylation, KCC is inhibited. Thus, phosphorylation of both NKCC and KCC results in net Na^+ , K^+ , and Cl^- influx into the cell via activated NKCC. Obligatory water influx increases cell volume (5), resulting in “regulatory volume increase (RVI)”. (B) Cell swelling (1) is detected via a yet unknown mechanism and inhibits WNK activation. (2) Autophosphorylation is decreased, and protein phosphatases dephosphorylate WNKs to

reduce activating phosphorylation. (3) SPAK is not phosphorylated and remains inactive. (4) NKCC and KCC are not phosphorylated by SPAK, and protein phosphatases (such as PP1 and PP2A) dephosphorylate NKCC and KCC. Dephosphorylation decreases the activity of NKCC and reciprocally activates KCC. Thus, dephosphorylation of both NKCC and KCC results in net K^+ , and Cl^- efflux out of the cell via activated KCC. Obligatory water exit decreases cell volume (5), resulting in “regulatory volume decrease (RVD)”. (C) A proposed phosphorylation motif in SLC12A family NKCC1 and the KCC cotransporters, including KCC3, is shown in a segment of the human KCC1-4 C terminus aligned with a segment of the NKCC1, NCC and NKCC2 N-terminus from human (h) [Revised from (Rinehart et al., 2009)]. The threonine (T) highlighted in yellow indicates a single phosphorylation site that is common to all the transporters. With nearby shared tyrosine (Y) and arginine (R) residues separated by any amino acid residue (X), a candidate SLC12A family regulatory phosphorylation motif is suggested. In KCC3, the highlighted Thr in yellow is Thr991. Phosphorylation at Thr212 in human NKCC1 (Thr184 in shark) by WNK1-SPAK kinase signaling is a key event (along with Thr203 and Thr207) required for NKCC1 activation in conditions that simultaneously promote the inhibitory phosphorylation of KCC3 Thr991 (Darman and Forbush, 2002; Dowd and Forbush, 2003; Vitari et al., 2005). KCC3 Thr991 (and homologous sites in other KCCs) and NKCC1 Thr212 may be part of a phospho-motif “YXRT” that is important for the coordinated control of NKCCs and the KCCs by the WNK3-SPAK kinase complex (as in D). (D) Coupling of the WNK3-SPAK kinase complex to NKCC1 and KCC3 could comprise a “molecular rheostat” of cell volume regulation. The WNK3-SPAK kinases may have dual functions as sensors of both cell volume and $[Cl^-]_i$, as well as transducers that communicate changes of these parameters to plasmalemmal ion transport proteins. NKCC1 (“in-flow”) is activated and KCC3 (“out-flow”) is inhibited by WNK3-SPAK-dependent phosphorylation at the indicated sites, leading to regulatory volume increase (RVI, in blue to left of rheostat) that mediates net accumulation of intracellular solute – as would occur in response to prior cell shrinkage. In the opposite scenario, NKCC1 is inhibited and KCC3 is activated by WNK3-SPAK inhibition and by activation of protein phosphatases, leading to decreased NKCC1/KCC3 phosphorylation. The resulting regulatory volume decrease (RVD, in red to right of rheostat) and regulatory volume decrease (RVI, in blue to left of rheostat) mediates net reduction of intracellular solute – as would occur in response to cell swelling. Therefore, the WNK3-SPAK complex might function as a “sensor-transducer” of cell volume perturbations that, via a physical and functional coupling to NKCC1 and KCC3, comprises a molecular rheostat of cell volume.

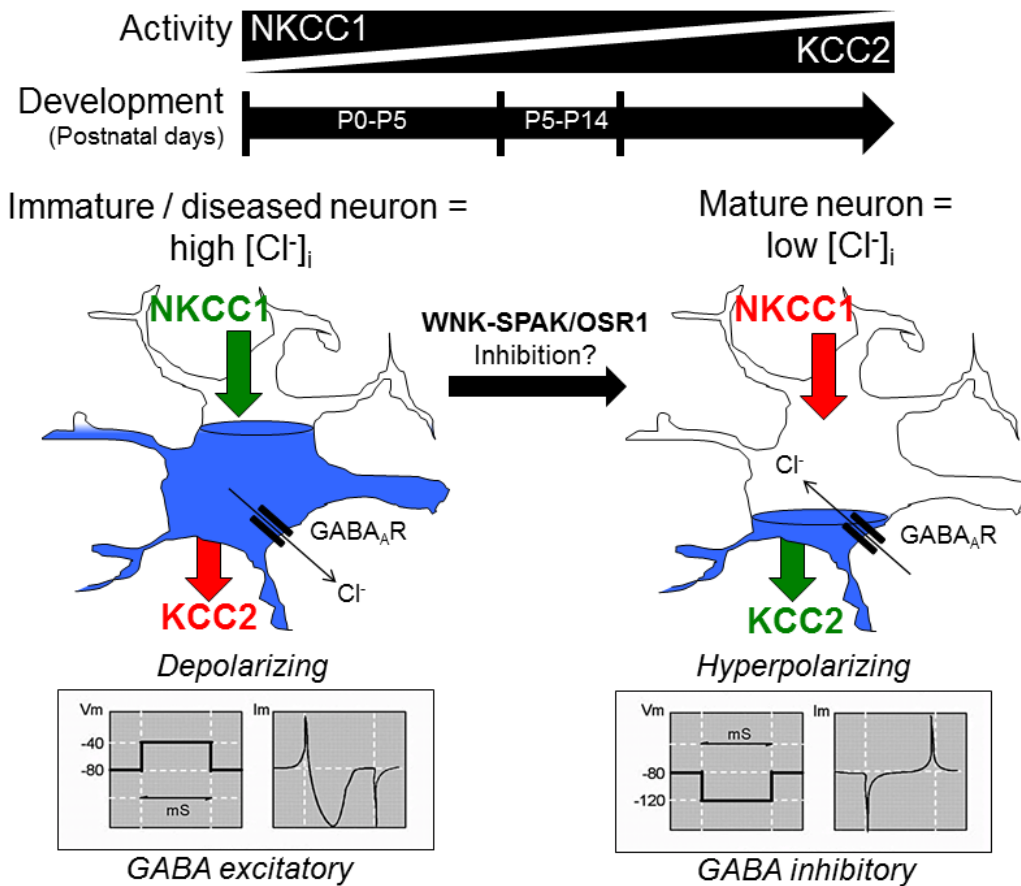


Figure 5. A strategy to facilitate neuronal Cl^- extrusion by inhibiting the WNK-SPAK/OSR1 pathway. Top shows the switch in the abundance of NKCC and KCC2 that occurs during postnatal development. This switch converts the GABAergic signal from depolarizing to hyperpolarizing. Left middle shows that in developing neurons and in some diseased neurons, $[Cl^-]_i$ (blue fill) is increased due to high NKCC1 activity, low KCC2 activity, or both. Activation of GABAAR results in Cl^- efflux, depolarization, and excitation. Left lower shows neuronal depolarization in response to GABA activation of GABAAR. Right middle shows that in healthy mature neurons, $[Cl^-]_i$ is low because KCC2 activity predominates and GABAAR activation results in Cl^- influx and hyperpolarization. Right lower shows neuronal hyperpolarization in response to GABA activation of GABAAR.

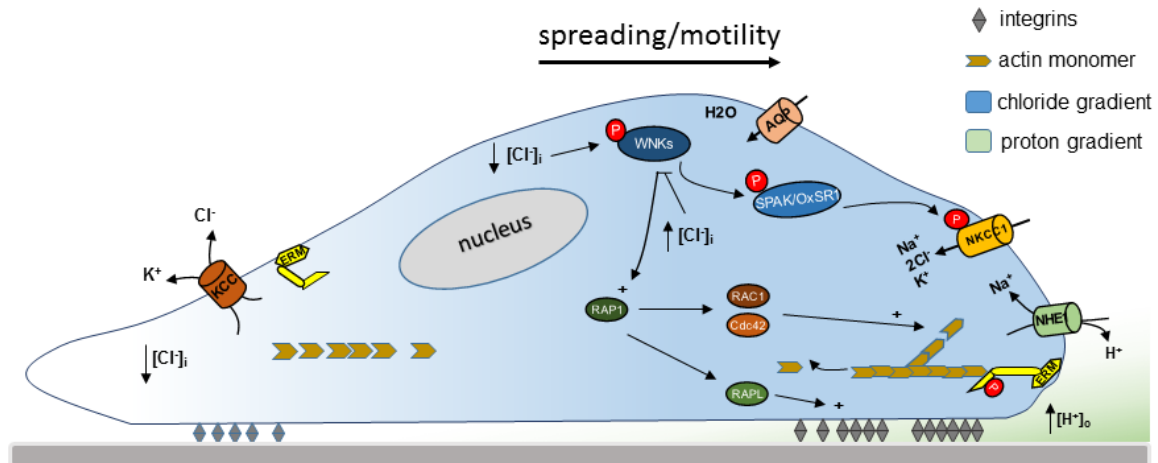


Figure 6. Contribution of the WNK-SPAK pathway and CCCs to cell adhesion and motility. A model illustrates asymmetrical chloride extrusion (efflux) in the trailing edge and chloride entry (influx) in the leading edge of a cell by KCCs and NKCC1, respectively, which leads to water influx and increase in volume of the leading edge. Lower $[Cl^-]_i$ initiates a cascade involving the WNK-SPAK kinases which activates NKCC1 to influx more Cl^- . Higher $[Cl^-]_i$ in the leading edge mitigates the WNKs activity but also enhances RAP1 (Ras-related protein 1) GTPase activity on RAC1 and Cdc42 Rho GTPases to facilitate actin polymerization and induces the formation of protrusions. This also increases integrins adhesion to the extracellular matrix (here) or to another cell. In addition to chloride, increase in proton efflux by Na^+/H^+ exchanger 1 (NHE1) in the leading edge, engages ERM (ezrin/radixin/moesin) proteins and stabilizes actin and its binding to the plasma membrane creating a tension required for moving forward. Higher proton concentrations also facilitates integrins activation and binding to the extracellular matrix. (Garzon-Muvdi et al., 2012; Haas et al., 2011; Köchl et al., 2016; Schwab et al., 2012)

The WNK pathway	Tissue/Cells	Contribution	References
Kidney			
WNK1/3/4-SPAK-NCC	DCT, CNT	Na ⁺ reabsorption	Chavez-Canales et al., 2014; Grimm et al., 2012; *Moriguchi et al., 2005; Ohta et al., 2009; *Rinehart et al., 2005; *Yang et al., 2003; *Wilson et al., 2003; Takahashi et al., 2014; *Yang et al., 2007; Zhang et al. 2015.
WNK1/3/4-SPAK-NKCCs	TAL2	Na ⁺ reabsorption	*Anselmo et al., 2006; *Gagnon et al., 2006; McCormick et al., 2011; *Moriguchi et al., 2005; Richardson et al., 2011; *Rinehart et al., 2005; Zhang et al. 2015.
WNK1/4-SGK1-ROMK	CNT, CCD	K ⁺ secretion	*Cheng and Huang, 2011; Lazrak et al., 2006; *Ring et al., 2007b; *Kahle et al., 2003.
WNK1/4-SGK1-BKCa	CNT, CCD	K ⁺ secretion	Webb et al., 2016; Yue et al., 2013.
WNK1/4-SGK1-ENaC	CNT, CCD, DCT2	Na ⁺ reabsorption	Ring et al., 2007a; Ring et al., 2007b; Xu et al., 2005a; Xu et al., 2005b.
WNK3/4-TRPV4/5/6	DTL, ATL, DCT	Ca ²⁺ absorption	*Fu et al., 2006; *Zhang et al., 2008; *Jiang et al. 2007.
Nervous system			
WNK3-SPAK-NKCC1	cortex	cerebral edema	Begum et al., 2015; Zhao et al., 2016.
WNK3-SPAK-NKCC1	astrocytes, endothelial cells	cell volume control	Zhang et al., 2016b.
WNK1-SPAK-KCC2	cortical neurons, spinal cord	GABA function	Friedel et al., 2015; Inoue et al., 2012; Kahle et al., 2016b.
WNK1-SPAK-NKCC1	spinal cord	GABA function	Friedel et al., 2015; Kahle et al., 2016b.
Non-renal epithelial			
WNK1-SPAK-NBCe1/ CFTR	secretory epithelial	HCO ₃ ⁻ ductal secretion	*Hong et al., 2013; *Yang et al., 2007; Yang et al., 2011.
WNK3-TRPV6	small intestine*	intestinal Ca ²⁺ absorption	*Zhang et al., 2008.
SPAK-SGLT1	small intestine	glucose reabsorption	*Elvira et al., 2014.
Non epithelial			
WNK1/SPAK/KCC3	fibroblast	cell volume control	Kahle et al., 2016a.
WNK1/SPAK/NKCC1	HEK293	cell volume control	Vitari et al., 2006.
WNK1/SPAK/NKCC1	T-cells, cell lines	T-cell and cell lines migration	Garzon-Muvdi et al., 2012; Haas et al., 2011; Kochl et al., 2016; Zhu et al., 2014.
WNK3/SPAK/NKCC1	cell lines, tumor cells	cell migration, tumors invasion	Haas et al., 2011.
WNK1/SPAK	cell lines	autophagy	*Gallolu Kankanamalage et al., 2016

Table 1. A summary of the functional contribution of the WNK pathway in different tissues and cells based on the current findings. DCT: distal convoluted tubule; CNT: connecting tubules; TAL: thick ascending limb of Henle's loop; CCD: cortical collecting duct; Large Conductance Ca²⁺-Dependent K⁺; ROMK: renal outer medullary K⁺ channel; DTL: descending thin limbs and ATL: ascending thin limbs of Henle's loops. * indicates that the pathway was tested in vitro .

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