



Transworld Research Network
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Emerging Signaling Pathways in Tumor Biology, 2010: 43-70
ISBN: 978-81-7895-477-6 Editor: Pedro A. Lazo

3. WNK kinase signalling in cancer biology

Sónia Moniz and Peter Jordan

*Departamento de Genética Humana, Instituto Nacional de Saúde 'Dr. Ricardo Jorge'
Lisboa, Portugal*

Abstract. The subfamily of WNK (With No K [lysine]) protein kinases is characterised by a unique sequence variation in the catalytic domain: a conserved lysine residue that is essential for catalytic activity in most eucaryotic protein kinases is located in an alternative position within the catalytic domain and this variation may result in unique substrate binding properties. The human genome contains four *WNK* genes, with different tissue-specific expression patterns. Mutations in *WNK1* or *WNK4* cause a hereditary hypertension syndrome due to increased renal salt retention. At the molecular level, WNK1, WNK3 or WNK4 have been shown to regulate different ion transporters in both the kidney and extrarenal tissues. Growing evidence has also revealed additional roles for WNK kinases in multiple signalling cascades related to tumour biology. There is strong evidence for a role as upstream regulators of MAPK cascades involved in cell proliferation control. In addition, a requirement of some WNK members for cell survival has been demonstrated. Here, we review the experimental evidence linking WNK kinases to tumorigenesis and discuss their role in major aspects of tumour biology: G1/S cell cycle progression, metabolic tumour cell adaptation, evasion of apoptosis, and metastasis.

Correspondence/Reprint request: Dr. Peter Jordan, Departamento de Genética Humana, Instituto Nacional de Saúde 'Dr. Ricardo Jorge', Avenida Padre Cruz, 1649-016 Lisboa, Portugal
E-mail: peter.jordan@insa.min-saude.pt

Introduction

The phosphorylation of cellular proteins alters their function and is catalysed by protein kinases in response to extra- or intracellular stimuli. A superfamily of protein kinases has been recognized based on conserved sequence elements in their catalytic domains and human genome-wide analyses have concluded that there are 518 different protein kinase genes [1-3]. Of these, 478 genes present the classical eukaryotic protein kinase catalytic domain [4], while 40 are atypical kinases. Of the 478 classical kinases, 428 possess known or likely kinase activity, while the remaining 50 proteins lack conserved key sequence elements. Among the 428 classical kinases, 365 fall into seven major families (TK, CAMK, AGC, CMGC, STE, TKL, and CK1), whereas 63 kinases present sequence variations in their catalytic domains and have been classified separately as 'Other' [3]. This group contains unique kinase genes or small subfamilies, including the WNK (With No [K] = lysine) subfamily. Phylogenetically, WNKs define a separate protein kinase branch, most closely related to the STE (mammalian homologs of the *Saccharomyces cerevisiae* STE families of serine/threonine kinases) and TKL (tyrosine kinase-like) family branches [1, 3].

The unique sequence variation that characterises WNK protein kinases [5, 6] is the lack of a highly conserved catalytic lysine in subdomain II, important for the correct positioning of adenosine triphosphate (ATP) within the classical catalytic domain [4]. The crystal structure for the recombinant WNK1 kinase domain has been determined [7] and revealed that an alternative lysine from subdomain I reaches into the position normally occupied by the conserved lysine residue from subdomain II and therefore confers catalytic activity [5] (see Figure 1).

The sequence variation in the catalytic domain can be expected to induce conformational changes responsible for unique ATP and substrate binding properties of WNK kinases. Otherwise, the overall structure and folding of the WNK1 catalytic domain resembles that of other protein kinases with typical bilobal domain architecture [8].

WNK proteins are serine/threonine kinases that undergo autophosphorylation and require phosphorylation of at least one serine residue within the WNK activation loop, S³⁸² in WNK1, to become active [9, 10]. This serine is part of an activation site motif (S³⁷⁸FAKS³⁸²) that is conserved in MEK family kinases. Sequence alignment identifies homologue serine residues in positions 356, 308 and 332 for WNK2, 3 and 4, respectively.

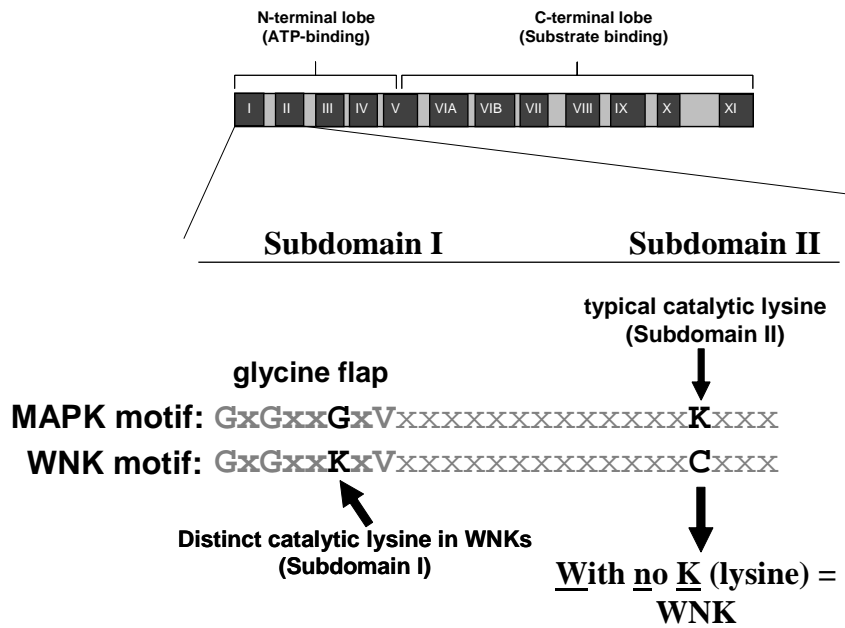


Figure 1. Sequence variation in the catalytic domain that characterises the WNK subfamily of protein kinases. The typical protein kinase catalytic domain is subdivided into 12 subdomains. A conserved lysine in subdomain II, which binds ATP, is absent in WNK kinases and functionally substituted by another lysine located in subdomain I [5, 7], as indicated.

Another primary level in the regulation of WNK kinase activity can be provided by the autoinhibitory domain encompassing about 70 residues just C-terminal to the catalytic domain and these are conserved in WNK protein kinases across species [9, 11].

The characteristic catalytic lysine in subdomain I together with 7 other adjacent amino acid residues form an invariant WNK signature sequence [6] (see Figure 2). A database search using the WNK signature sequence revealed the existence of a variable number of *WNK* genes in animals and plants that are absent in uni-cellular organisms. The number of *WNK* genes increases from one *WNK* gene in *C. elegans* or *Drosophila melanogaster* to four different *WNK* genes in mouse and man, while the higher plant *Arabidopsis thaliana* has 9 *WNK* genes [6, 12, 13].

The human genome contains four *WNK* genes with chromosomal locations at 12p13.3 (WNK1), 9q22.31 (WNK2), Xp11.23-p11.21 (WNK3) and 17q21-q22 (WNK4). The human WNK1 gene contains 28 exons and extends over more than 150 kb, WNK2 is encoded by 31 exons that span 136 kb, WNK3 contains 24 exons and spans 165 kb, whereas WNK4 is encoded by 19 exons contained within 16 kb of genomic DNA [6, 14, 15]. These genes differ in their tissue specific expression patterns. WNK1 is expressed in most human foetal and adult tissues; WNK3 is mainly expressed in brain,

liver and small intestine, WNK2 is mainly present in brain, heart and colon crypt, and WNK4 predominantly in the kidney, colon, skin, liver, and lung [5, 6, 14-19]. Expression analysis of WNK kinases has also revealed the existence of tissue-specific alternative promoter usage, alternative splicing and polyadenylation variants [6, 14, 15, 19-21].

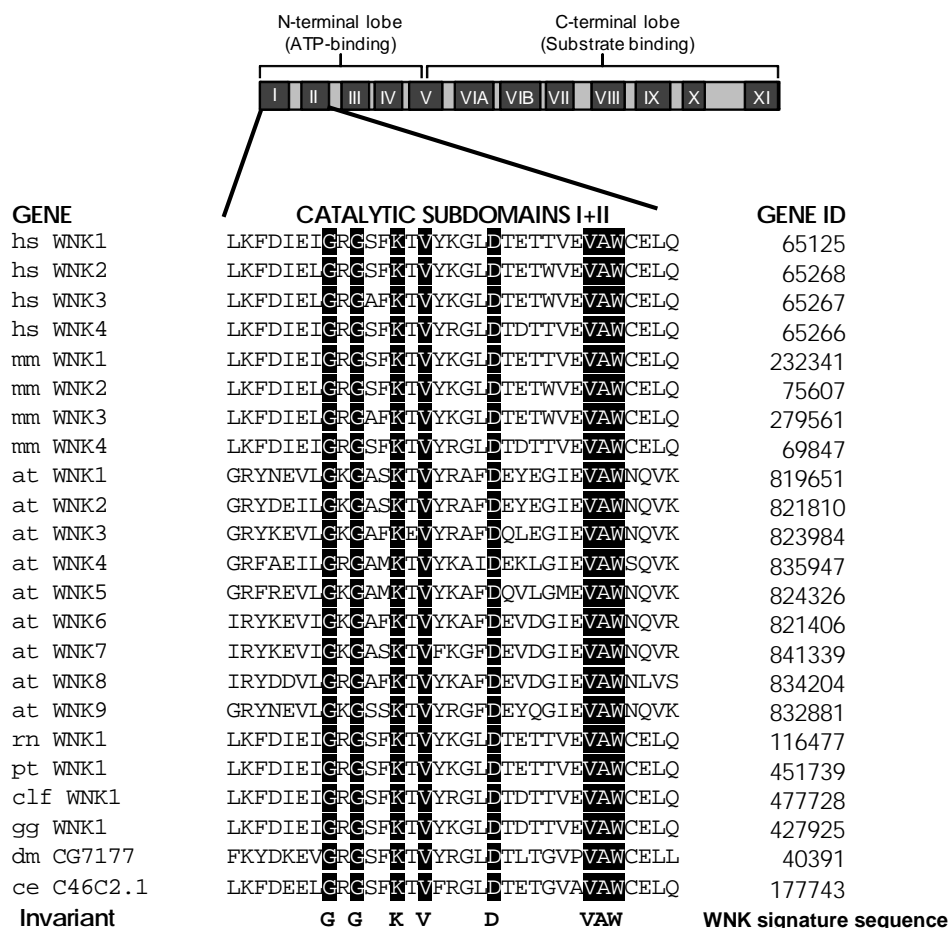


Figure 2. Alignment of subdomains I and II of the catalytic domains of some WNK kinases from various species. The invariant WNK signature sequence is indicated below. Species abbreviations are: hs, *Homo sapiens*; mm, *Mus musculus*; at, *Arabidopsis thaliana*; rn, *Rattus norvegicus*; pt, *Pan troglodytes*; clf, *Canis lupus familiaris*; gg, *Gallus gallus*; dm, *Drosophila melanogaster*; ce, *Caenorhabditis elegans* (updated from Verissimo and Jordan, 2001); NCBI sequence IDs are also given.

The four human WNK kinases are large proteins containing 2382, 2297, 1743 or 1243 amino acids with predicted molecular weights of 251, 243, 192 and 135 kDa, for WNK1, 2, 3 and 4 respectively [6, 14, 15, 21]. They share high homology within their kinase domains and the adjacent auto-inhibitory domain [9], however, homology outside their catalytic domains is low except for the presence of three short WNK homology regions (Figure 3).

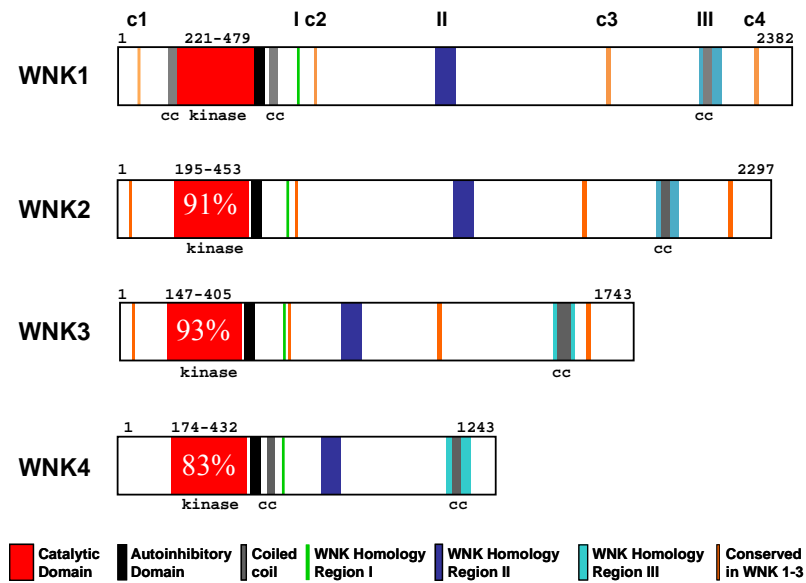


Figure 3. Schematic representation of the four human WNK proteins. Catalytic, autoinhibitory and coiled-coil domains are shown as well as conserved regions of homology between WNK kinases. The white numbers indicate sequence identity of the respective catalytic domain with regard to the WNK1 sequence.

The most C-terminal homology region III contains a coiled-coil domain and WNK1 and WNK4 have at least one further coiled-coil domain outside the conserved regions. In addition, four short regions of unknown function are conserved between WNK1, WNK2 and WNK3. Multiple PXXP motifs, the typical binding sites for Src-homology 3 (SH3) domains [22], are also present in WNK protein kinases, suggesting a potential scaffolding function for these proteins [6, 23].

In this chapter we will address the experimental evidence linking WNK kinases to tumorigenesis and discuss four main aspects: their roles in G1/S cell cycle progression, metabolic tumour cell adaptation, evasion of apoptosis and, finally, invasion and metastasis.

1. Role of WNK kinases in cell cycle progression

Normal cells require extracellular growth stimulatory signals in order to leave the quiescent and enter a proliferative state. In epithelial cells the stimulation of cell proliferation is intimately linked to the receptor-mediated activation of the mitogen-activated protein kinase (MAPK) cascades.

1.1. WNKs regulate MAP kinase cascades

The best characterized MAPK pathways in mammals involve the extracellular signal-regulated kinases 1 and 2 (ERK1/2), the p38 isoforms,

the c-jun N-terminal kinases (JNK1-3), and ERK5. In general, MAPK cascades consist of three protein kinase layers: MAPKs are phosphorylated and activated by MAPK kinases (MAP2Ks) which are in turn activated by phosphorylation by MAPK kinase kinases (MAP3Ks). Other kinases, including p21-activated protein kinases (PAKs), can act as upstream regulators (MAP4Ks) for these MAPK cascades and connect them with additional signalling pathways. MAPK signalling cascades regulate fundamental biological functions such as cell growth, proliferation, differentiation, migration and apoptosis and are found to be deregulated in approximately one third of all human cancers [24].

Sequence alignments using the catalytic domain of WNK1 showed that the closest human homologous kinases are the MAP3Ks MEKKs or Rafs and the MAP4K PAKs, which all display around 30 % sequence identity and 50 % sequence homology [6]. It is therefore not surprising that effects of WNK kinases on various MAPK cascades have been observed. WNK1 was reported to be required for EGF-dependent stimulation of ERK5 but the activation of ERK1/2, JNK or p38 MAP kinases was not significantly affected. *In vitro*, WNK1 interacts with and phosphorylates MEKK2 and MEKK3, however, this phosphorylation does not seem to stimulate MEKK2/3 activity. In contrast, transfection of cells with either WNK1 or its kinase-dead mutant WNK1K233M stimulates MEKK3 autophosphorylation and activity towards its substrate MEK6. Apparently, WNK1 acts by protein-protein interaction to assemble an ERK5 activation complex and thus acts as an upstream regulator of the ERK5 pathway (Figure 4). WNK1 was required for activation of ERK5 by EGF, in HeLa cells, but in the presence of high concentrations of EGF, this effect became less pronounced [25]. In agreement with these findings, the down-regulation of WNK1 in C17.2 mouse neural progenitor cells also suppressed activation of ERK5 and greatly reduced cell growth [26].

In contrast to WNK1, human WNK2 had no effect on ERK5 but modulated activation of ERK1/2. Experimental depletion of WNK2 or overexpression of a kinase-dead WNK2K207M mutant led to increased phospho-ERK1/2 levels but only when a basal ERK stimulation was present and not in serum-free culture conditions [19]. This increase in ERK1/2 activation promoted cell cycle progression through G1/S and sensitized cells to respond to lower concentrations of EGF. From these data one might predict that loss of WNK2 expression can promote cell cycle progression in tumour cells. Interestingly, WNK2 expression is silenced in a significant percentage of human gliomas [27, 28, see section 5 below] suggesting that this pathway may be used in some tumour types to promote cell proliferation. The mechanism by which a reduction in WNK2 expression increased

ERK1/2 activation involves phosphorylation of MEK1 at serine 298, a modification that increases MEK1 affinity towards ERK1/2. Apparently, WNK2 affects Rac1 activation with subsequent stimulation of the Rac-effector PAK1, the kinase responsible for MEK1 S298 phosphorylation [29] (Figure 4). Overexpression of WNK4 was also claimed to increase the phosphorylation of ERK1/2 and p38 MAPKs following EGF stimulation or hyperosmotic stress [30]. However, the mechanism of WNK4 involvement remains unclear and the corresponding effect of endogenous WNK4 was not addressed.

Together, these data demonstrate that WNK kinases can act upon different MAPK cascades and modulate cell proliferation. It may also be anticipated that this modulation is concerted because WNK kinases appear to regulate each others activities. For example, the recombinant kinase domain of WNK1 was shown to phosphorylate recombinant WNK2 and WNK4 catalytic domains *in vitro*, in particular WNK4 on serine 332 in its activation loop [31]. WNK4 can also phosphorylate the catalytic domain of WNK1 and expression of the autoinhibitory domains of WNK1 and WNK4 are able to inhibit each others catalytic activities [11, 31]. Gel filtration profiles further indicate that endogenous WNK1 exists as a tetramer [31]. Although the functional role of oligomerization is still unclear, there is some evidence that the autoinhibitory domain of one WNK1 molecule may inhibit the catalytic activity of another WNK1 molecule within the tetramer [9, 31]. Additionally, given the presence of conserved coiled-coil protein interaction domains in all

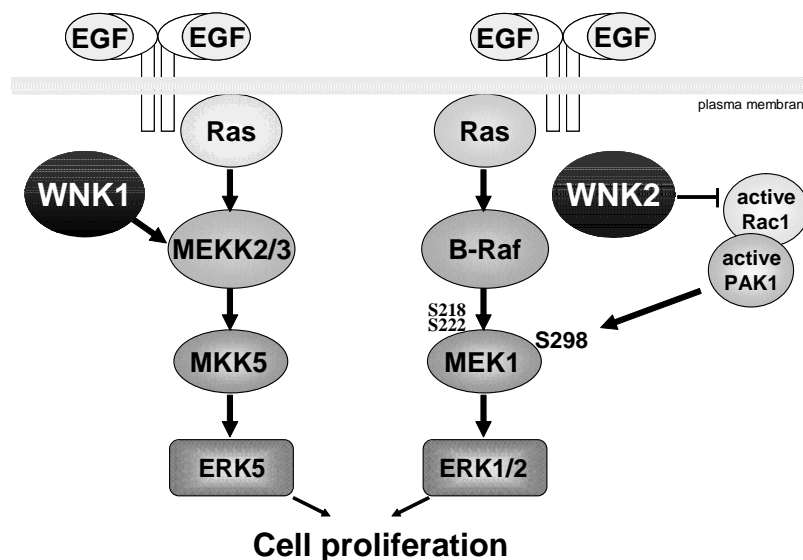


Figure 4. Schematic representation of the role of human WNK1 and WNK2 in the cellular response to EGF. (Left side) Physiological activation of the ERK5 pathway is enhanced in the presence of WNK1. (Right side) Expression of WNK2 controls a Rho GTPase/PAK-mediated signalling cross talk that can increase ERK1/2 activation.

four human WNK proteins, it is likely that hetero-oligomers form under certain conditions. In this way, the activity of different WNK proteins on parallel MAPK cascades might be coordinated within a single complex [23].

WNKs also regulate upstream activators of MAPK pathways (MAP4Ks), such as those belonging to the STE20-related protein kinases. These consist of 10 subfamilies that include PAK and the germinal centre kinases (GCKs). The proline-alanine-rich kinase (SPAK) and oxidative-stress responsive 1 (OSR1, also referred to as OXSR1) are members of the GCK VI subfamily and primarily known for their role in cell volume sensing and osmotic homeostasis. Given that cell size and volume are often linked to cell cycle progression, it is not surprising that these kinases have roles as upstream activators of MAPK pathways (MAP4Ks) [32, 33]. For example, SPAK is a known activator of the p38 and JNK cascades [34-36] and OSR1 acts upstream of CaMKII and p38 in *C. elegans* [37].

OSR1 and SPAK interact with WNK1, WNK3 and WNK4 [38-44] and WNK1 and WNK4 phosphorylate these proteins on two residues: T233 and S373 on SPAK and T185 and S325 on OSR1. Residues T233 or T185 are located in the activation loops of these kinases' catalytic domains and mediate their activation [38]. The molecular details of how the phosphorylation of SPAK and OSR1 by WNK kinases relates to cell proliferation have not yet been investigated.

As mentioned above, WNK2 also acts upon another STE20 member, PAK1, but direct phosphorylation does not seem to be involved. Rather, WNK2 affects activation of Rac1, a known upstream stimulator of PAK1 [29] and active PAK1 phosphorylates MEK1 on S298, leading to increased activity of ERK1/2 MAPK [45-49].

1.2. Effect of WNKs on receptor traffic

MAP kinases are activated in response to growth factor binding to their cell surface receptors and can either stimulate or inhibit cell proliferation. After ligand binding, most receptors become internalized into signalling-competent endosomes and then either recycle back to the cell surface or become degraded in lysosomes as a mechanism to downregulate receptor signalling in cells [50, 51]. Vesicular traffic can therefore modulate cellular signalling intensity and WNK4 has been proposed to promote cargo delivery to lysosomes. Expression of WNK4 inhibited the activity of the sodium chloride cotransporter NCC by diverting the channel to lysosomal degradation [52, 53] and because this effect involved a general adaptor protein (AP) 3-dependent endosomal sorting mechanism, WNK4 may also modulate the ratio of degradation of endocytosed growth factor receptors. This mechanism

is known to downregulate receptor signalling in cells and can have consequences for cell proliferation [50].

Similarly, WNK1 and WNK4 have been shown to stimulate clathrin-dependent endocytosis of the renal outer medullar potassium 1 channel (ROMK1), thus inhibiting potassium secretion [54-56]. This process involves the interaction of WNK1 and WNK4 with the scaffold protein intersectin 1 (ITSN1), which binds to specific proline-rich WNK motifs via its SH3 domains and does not require WNK kinase activity [56].

Additional support of a role for WNKs in endocytosis comes from a genome-wide RNAi screen to determine the effect of human protein kinases on endocytosis. In this screen virus entry was used as a measure of clathrin-mediated (vesicular stomatitis virus- VSV) or caveolin-dependent (simian virus- SV40) endocytosis and it was found that WNK4 interfered with VSV entrance whereas WNK2 inhibited caveolin-mediated SV40 uptake [57, Table S1].

1.3. WNKs as targets of insulin signalling and the PI3K/Akt pathway

There is substantial evidence that many tissues and derived cancer cells express insulin and insulin-like growth factor 1 (IGF1) receptors and that elevated circulating levels of IGF1 and insulin reflect a nutritional energy imbalance with increased cancer risk [58]. Insulin and IGF1 receptors are important activators of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling network and increase the proliferation of neoplastic cell lines.

Following IGF1 treatment of cells, Akt is activated downstream of PI3K and phosphorylates WNK1 in its threonine residue 60, although this phosphorylation does not appear to affect WNK1 kinase activity, or its subcellular localization [59-63]. This WNK1 phosphorylation is dependent on PDK1, which phosphorylates Akt within its activation loop [64], and is blocked by PI3K inhibitors. IGF1 also activates the Akt-homologous serum- and glucocorticoid-induced protein kinase SGK1. While SGK1 is known to regulate sodium transport in response to aldosterone in the kidney, it also promotes degradation of the cyclin-dependent kinase inhibitor protein p27^{kip} in other cell types [65].

Xu and collaborators proposed that WNK1 phosphorylation by PKB/Akt contributes to SGK1 activation and that WNK1 is required for SGK1 activation by IGF1 [61]. There is evidence that SGK1 may in turn phosphorylate WNK1 on Thr60 and/or other sites and create a positive feedback [62].

When 3T3-L1 adipocytes were treated with insulin, an increased WNK1 phosphorylation was observed, and RNAi-induced depletion of WNK1 enhanced insulin-stimulated thymidine incorporation by about 2-fold without significantly affecting insulin-stimulated glucose transport in these cells [60]. This observation suggests a negative regulatory role for WNK1 in insulin-mediated proliferation control.

SGK1 apparently also phosphorylates WNK4 on serine residue 1169 in response to aldosterone [66] but a role related to cell proliferation has not been investigated yet.

1.4. WNKs in transforming growth factor β signalling

The transforming growth factor (TGF) β transduces signals by stimulating the formation of heteromeric complexes of type I and type II serine/threonine kinase receptors which then phosphorylate and activate cytoplasmic transcription activators of the Smad family. Signalling from the TGF β -Smad pathway often plays an anti-proliferative role in many cell types, acting as a tumour suppressor [67].

WNK1 and WNK4 kinase domains both interacted with and phosphorylated Smad2 *in vitro*. The siRNA-mediated knockdown of WNK1 reduced overall Smad2 levels but increased Smad2/3-dependent transcriptional responses [68]. This suggests that WNK1 imposes an inhibitory constraint on Smad2 and TGF β signalling. Interestingly, a systematic mapping of the TGF β receptor interactome revealed a link to another WNK1 substrate, the OSR1 protein kinase [69].

1.5. WNKs in calcium signalling

An increase in cytoplasmic calcium serves as a second messenger for calmodulin- and calcineurin-activated signalling pathways that promote cancer cell proliferation [70, 71].

One mechanism of increasing cytoplasmic calcium is uptake from the extracellular medium by calcium-channels, such as the transient receptor potential vanilloid (TRPV) channels and TRPV6 overexpression has been reported in carcinomas of the colon, breast, thyroid, and ovary [72]

Expression of WNK4 specifically enhanced TRPV5-mediated calcium uptake, which correlates with increased membrane expression of the channel [73], whereas WNK3 stimulated TRPV5 and TRPV6-mediated transport [74]. In contrast, WNK4 and WNK1 decreased cell surface expression of TRPV4, which plays a role in osmoregulation following hypotonic cell swelling [75].

Additionally, WNK1 was also implicated in intracellular calcium sensing, because it selectively binds synaptotagmin 2 and phosphorylates the C2 domains, involved in calcium binding. WNK1 and synaptotagmin 2 co-localize in secretory vesicles in an insulin secreting pancreatic β cell line and phosphorylation by WNK1 increased the amount of calcium necessary for synaptotagmin 2 binding to phospholipid vesicles [76].

Together, these results show that WNK kinases can affect multiple signalling pathways related to cell proliferation; however, at present their best defined roles are as upstream regulators of MAPK cascades.

2. WNK kinases and metabolic adaptation of tumour cells

Cell adaptation to the extracellular environment is required during the initiation of tumour formation when cells start to proliferate and in consequence experience suboptimal supply of oxygen and nutrients due to diffusion limits. In response, cancer cells upregulate the production of glycolytic energy leading to the generation of lactate and acidosis of the extracellular medium. Tumour cells have to compensate for this acid-induced toxicity in order to remain viable and continue to grow [77, 78].

2.1. WNKs and glucose uptake

Malignant cells have accelerated metabolism and the increased requirements for ATP production are satisfied by aerobic glycolysis [79]. One adaptation is to increase glucose transport into malignant cells by overexpression of glucose transporters (GLUTs) [80] and elevated expression of GLUT1 has been described in many cancers.

In 3T3L1 adipocytes, the syntaxin 4-regulatory protein Munc18c functions in insulin-stimulated GLUT4 vesicle translocation and fusion [81]. A role for WNK1 has been reported in vesicle/granule exocytosis, because it forms a complex with Munc18c. WNK1 binds Munc18c via its kinase domain but does not phosphorylate it, suggesting a scaffolding function for WNK1 in recruiting Munc18c as a substrate for another kinase or phosphatase [82]. Interestingly, increased glucose supply decreased the expression of WNK4 in mouse kidneys, indicating the existence of a regulatory mechanism that links WNK expression to extracellular glucose concentrations [83].

Because synaptotagmins and Munc18c are implicated in a variety of membrane trafficking and vesicle fusion events, their interaction with WNK1 provides a putative mechanism for how WNKs may regulate retention or insertion of plasma membrane proteins [76].

2.2. Regulation of extracellular pH and the plasma membrane conductances

Mutations in the *WNK1* and *WNK4* genes were initially discovered to cause pseudo-hypoaldosteronism type II (or Gordon's syndrome), a rare familial form of hypertension [14] characterised by increased renal salt reabsorption accompanied by hyperkalemia and metabolic acidosis due to impaired K^+ and H^+ excretion. Subsequently, a huge body of evidence has shown that WNK1, WNK3 and WNK4 are involved in the regulation of a variety of renal but also extra-renal ion channels (for WNK2 no such studies have been reported yet). Summarizing these studies, evidence links WNKs to the regulation of cell surface expression or channel activity of the cotransporters NCC, NKCC and KCC, the ROMK and Kir1.1 potassium channels, the CFTR chloride channel, the Cl^-/HCO_3^- exchangers SLC26A6 and A9, the epithelial sodium channel (ENaC), and the TRPV4 and TRPV5 calcium channels [reviewed in 18, 84-86]. These data indicate that WNK kinases are important to maintain fundamental cell functions related to electrolyte homeostasis.

The regulation of electrolyte homeostasis is important for tumour cells and one relevant aspect is pH regulation. The extracellular environment in the tumour is more acidic than the intracellular pH (6.2–6.9 compared with 7.3–7.4). The increased production and export of glycolytic lactate together with overexpression of the Na^+/H^+ exchanger NHE1, create a reversed pH gradient across the tumour cell membrane. Through NHE1 action, the inwardly directed sodium gradient can drive the uphill extrusion of protons, alkalize intracellular pH and further acidify the extracellular pH. A supporting activity in pH regulation is provided by the chloride/bicarbonate (Cl^-/HCO_3^-) anion exchangers of the SLC26A family [87]. The combined transport activities of NHE and SLC26A channels lead to electroneutral NaCl absorption with net H^+ secretion. Recent findings suggest that the expression of the SLC26A family is regulated by WNKs. When expressed in *Xenopus* oocytes, WNK4 inhibits the expression at the plasma membrane of SLC26A6 [17] and WNK1, WNK3 and WNK4 inhibit the expression of SLC26A9 [88], consequently decreasing channel activities.

Another aspect is the electrochemical gradient across the plasma membrane which most cells require to sustain nutrient uptake or release of metabolic products. For example, the electrochemical gradient for Na^+ drives the uptake of nutrients and is in part maintained by the negative membrane potential. In case of enhanced Na^+/H^+ exchanger activity, as observed in tumour cells, the intracellular Na^+ concentration increases so that the ATP-consuming Na^+/K^+ ATPase becomes activated to exchange cytosolic Na^+ for

K^+ . In consequence, K^+ ions need to recycle through membrane potassium channels in order to keep the Na^+/K^+ ATPase going. A solid body of evidence documents that various types of tumours overexpress voltage-gated K^+ channels, in particular the *ether-a-go-go* (EAG) and BK (Ca^{2+} -activated K^+ channels) subfamilies. These channels open in response to a depolarization of the cell membrane, thus allowing an efflux of K^+ ions. Experimental overexpression of these channels promotes tumour cell proliferation, probably because it helps to maintain the membrane potential [89].

Although WNK kinases have not yet been linked to the direct regulation of voltage-gated K^+ channels, their known effects on channels involved in Na^+ , K^+ , Cl^- and HCO_3^- homeostasis already indicate that these proteins play at least an overall regulatory role on electrochemical cell homeostasis and lead us to suggest that these channels may represent a further link between WNKs and tumour biology that should be explored.

3. Role of WNK kinases in the evasion of apoptosis

The apoptotic program is a major barrier to tumour development that must be inactivated to achieve net tumour cell proliferation. A complex interplay between different pro- or anti-apoptotic factors determines whether cells survive or activate the cell death programme [90].

3.1. WNK3 modulates caspase 3

Experimental data and mathematical models have suggested that apoptosis activation depends on molecular threshold values that trigger either the cell death pathway or maintain cell survival [91, 92]. Thus, either overproduction of anti-apoptotic factors, or loss of expression of pro-apoptotic factors, or changes in their activation status can shift the balance towards cell survival. WNK3 has been shown to act on this balance by promoting cell survival in a caspase-3-dependent pathway [21]. Suppression of endogenous WNK3 by RNA interference accelerated the apoptotic response of HeLa cells and promoted the activation of caspase-3. The mechanism of WNK3 action involves interaction with procaspase-3 and heat-shock protein 70. The prosurvival role was only in part dependent on the catalytic activity of WNK3 because a kinase-dead WNK3K159M mutant also interacted with procaspase-3 and increased cell survival to some extent. This indicates an adaptor or scaffold function for WNK3 within a protein complex that controls procaspase-3 activation. Accordingly, the level of WNK3 expression or activity may determine sensitivity or tolerance of cells towards apoptotic stimuli.

3.2. Genome-wide screening results

The role of protein phosphorylation in apoptosis induction was recently studied by systematic depletion of each human protein kinase or phosphatase in HeLa cells using RNA interference [93]. This approach identified 73 kinases whose suppression increased the level of apoptosis by at least twofold over control, defining them as survival kinases. In this screen WNK1 and WNK3 kinases also scored positive, albeit below the threshold value (WNK1- 1.98 fold; WNK3- 1.58 fold), whereas WNK2 and WNK4 had no effect on cell survival [J. Blenis, Boston, personal communication]. These results are further supported by a genome wide screen for cell survival factors in *Drosophila melanogaster*. The single *Drosophila* WNK gene (designated *CG7177*; NM_141072) is most homologous to WNK3 and WNK1, and its depletion by RNAi affected cell survival in fly S2R⁺ cells [94].

Although WNK1 and WNK3 were detected in these screens, they did not stand out as essential genes for sustaining cell survival. However, these screens scored for spontaneous induction of apoptosis and a different physiological response can be expected when cells are exposed to conditions that challenge cell survival. For example, the single *Caenorhabditis elegans* WNK kinase (designated C46C2) was found essential for worm survival but only following hyperosmotic stress conditions [13]. It can be expected that the roles of WNK1 and WNK3 in cell survival will become more evident when cells face metabolic stress situations, including the above mentioned glycolytic acidosis and cell volume regulation.

3.3. Putative role of WNK substrates OSR1 and SAPK

Which candidate signalling pathways could be involved in the anti-apoptotic function of WNK kinases? The survival under osmotic stress depends on OSR1 [37]. As mentioned previously, OSR1 and SPAK interact with WNK1, WNK3 and WNK4 (see section 1.2) and are key regulators of the NCC, NKCC and KCC ion channels involved in the regulation of ion homeostasis and volume control in mammalian cells [95, 96]. Cells under hyperosmotic stress conditions are challenged to induce an apoptotic response and such conditions, including high concentrations of NaCl, KCl, glucose, sucrose, mannitol or sorbitol, provoke a marked and reproducible increase in WNK1 activity [5, 9, 31] including towards its substrate OSR1 [10]. Thus, the expression or activity levels of WNK1 and WNK3 may determine the cellular capacity to cope with osmotic or acid-related stress.

In a yeast two hybrid screen with the SPAK C-terminal substrate binding domain, the apoptosis-associated tyrosine kinase (AATYK) was identified as

an interacting protein [97], and in myeloid precursor [98] or cerebellar granule cells [99], AATYK expression promotes apoptosis.

In addition, SPAK and OSR1 participate in other signalling pathways related to cell survival. Overexpression of the TNF receptor RELT led to activation of both the p38 and JNK survival pathways in 293 cells [35] and this was dependent on SPAK kinase activity, which is regulated by WNKs. Also, following extrinsic pro-apoptotic stimuli such as TRAIL receptor binding, SPAK is cleaved at two distinct sites by a caspase 3-like protease, which removes its substrate-binding domain. Accordingly, experimental depletion of SPAK expression by siRNA increased the sensitivity of HeLa cells to TRAIL-induced apoptosis [100]. Thus, down-regulation of SPAK activity is an important target to enhance the apoptotic effect of TRAIL, where WNK kinases may influence cell survival.

4. WNK kinases in invasion and metastasis

Cancer cells can escape from the constraints of their tissue of origin and enter into the circulation to reach distant organs and eventually form secondary tumours, called metastases.

4.1. Epithelial–mesenchymal transition

In many solid tumour types, the epithelial tumour cells switch to a highly motile fibroblastoid or mesenchymal phenotype, a process called epithelial–mesenchymal transition (EMT). A well characterised inducer of EMT is TGF β signalling [101, 102] that uses Smad-mediated gene expression to induce the transcription factors Snail and Slug. These repress expression of the E-cadherin gene required for epithelial cell adhesion, a hallmark phenotype of EMT [103]. Since WNK1 was shown to phosphorylate Smad2 *in vitro* and apparently negatively controls Smad2/3-dependent transcriptional responses and TGF β Signalling [68], this suggests that loss of expression or inactivating WNK1 mutations could promote EMT of epithelial tumour cells.

4.2. Neuronal cell invasion

The ganglioside GD3 is a sialic acid-containing glycosphingolipid normally expressed during development but also in pathological conditions such as cancer. In neural tumour cells, WNK1 expression was found to correlate with that of GD3 [104]. In particular, experimental suppression of the GD3-synthase gene in F-11 tumour cells led to a reduced rate of cell migration and invasiveness [105], conditions under which a dramatic

decrease in WNK1 expression was observed. This suggests that WNK1 may contribute to the invasive phenotype of F-11 cells but further mechanistic details are not yet available.

4.3. Role of WNK-regulated Rho GTPases

Rho GTPases control the dynamics of the actin cytoskeleton and are important for cell migration and invasiveness [106]. WNK2 controls, through a yet unknown mechanism, the activation of the small GTPase RhoA, which in a reciprocal way regulates activation of Rac1 [29]. The suppression of WNK2 in cell lines leads to reduced RhoA, but increased Rac1 activation. It remains to be determined whether changes in WNK2 expression or activity affect RhoA/Rac1 activation in tumours, but in this sense, the reported epigenetic silencing of WNK2 in infiltrative gliomas [27] is highly suggestive.

A recent report also links WNK1 to Rho GTPases. In neuronal cells WNK1 can be isolated in a complex with Rho-GDI and was proposed to mediate the regulation of Nogo66-induced RhoA activation and neurite outgrowth [107].

Interestingly, a reciprocal relation between RhoA and Rac1 signalling has also been reported to mediate NHE1-dependent changes in motility and invasion of breast cancer cells [107]. For example, NHE1 has a crucial role in driving invasion, possibly by acting as an integrator protein that links the cytoskeleton and various metastasis-specific signalling complexes that mediate invasive activity. NHE1 can directly regulate cytoskeletal dynamics independently of its ion-transporting capabilities because it binds the ezrin, radixin and moesin (ERM) family of cytoskeleton regulators [109]. This feature of actin cytoskeleton remodeling has also been reported downstream of other ion channels [110], including some that are regulated by WNKs.

5. Mutational mechanisms affecting WNK genes in tumours

If WNK signalling were important for tumour development, one can expect that genetic alterations affect either WNK gene expression or their coding sequence with consequences for protein activity.

5.1. Changes in gene expression

The strongest evidence for such alterations was reported for WNK2. Expression of the human *WNK2* gene is silenced in a large percentage of human gliomas due to extensive methylation in the CpG island encompassing the 5' end of this gene [27]. Likewise, the *WNK2* promoter was hypermethylated in 83% and 71% of grade II and III meningiomas, respectively and this was associated with decreased WNK2 expression in primary

tumours. In contrast, promoter methylation was rare in a total of 209 tumours from thirteen other tumour types [28].

This finding makes *WNK2* a candidate tumour suppressor gene in gliomas. *WNK2* indirectly inhibits MEK1 and restrains growth-promoting signals through the EGF receptor. Thus, it is possible that the epigenetic silencing of *WNK2* interacts on a functional level with genetic alteration of EGFR signalling, a common abnormality in glioblastomas especially due to EGFR gene amplifications [111]. It is interesting to note that colon tumours also show frequent hyper-stimulation of the EGFR signalling pathway and that a partial clone of *WNK2* has previously been isolated as the colon cancer antigen SDCC43 [112]. An incomplete *WNK2* fragment was also isolated as a T-cell recognized pancreatic cancer cell antigen P/OKcl13 [113].

The other three human *WNK* genes share with *WNK2* the presence of large CpG islands surrounding their promoter regions and extending into the first exons [6, 20, 15, 27]. This observation suggests some common mechanism of regulation under physiological conditions but also indicates that hyper-methylation of the *WNK1*, *WNK3* or *WNK4* promoters may exist in other tumour types.

In future studies, the presence of genomic deletions in *WNK* genes should also be explored because one mutation type that predisposes patients to Gordon syndrome is a deletion within intron 1 of *WNK1*. This is a very large intron of 58 kb which contains a kidney-specific repressor element [114] and its deletion results in *WNK1* overexpression [14]. The structure of the *WNK2* and *WNK3* genes is conserved with respect to a large intron 1 (44 kb and 22 kb, respectively), raising the possibility that a similar mutational mechanism may lead to their overexpression. However, a homology search using the kidney-specific *WNK1* repressor element C1 [114] did not identify any homologous sequences in introns 1 from the *WNK2* and *WNK3* genes [unpublished observation].

WNK expression studies will further need to test for the presence of alternative promoters. In *WNK1* intron 4 an intragenic promoter generates a kidney-specific isoform (KS-*WNK1*) lacking kinase activity, which is thus functionally different from the long ubiquitous isoform (L-*WNK1*). Epigenetic changes could shift promoter usage in tumours and thus generate dominant-negative *WNK* isoforms, a possibility that remains to be explored as a potential mutagenic mechanism.

An additional avenue that requires more attention is the role of *WNK* alternative splicing variants. For example, one *WNK1* variant lacks exons 11 and 12 [6], two *WNK2* transcripts exist that differ in their C-terminal exon with different C-terminal sequences [19], and for *WNK3* two variants differ in usage of mostly brain-specific exons [15, 21]. Alternative splicing variants

can affect transcript turnover or encode protein isoforms. At present no simple structure/function relation exists to predict functional differences between two splicing variants from the same gene, however, many examples of variants have been documented that significantly differ regarding regulation or signalling activities [115-118]. Indeed, a recent report revealed that the two WNK3 variants have opposite effects on expression of the ion channel NCC [119].

5.2. WNK point mutations in tumours

In the last years, large-scale cancer genome sequencing efforts were conducted to determine the full spectrum of mutations in a given tumour. Among the provided lists of mutated genes a variety of point mutations in several *WNK* genes were identified in breast, colon, lung or brain tumours (Figure 5) [120-124].

No experimental evidence exists, however, showing whether the resulting missense or frameshift mutations have a functional impact on the corresponding WNK protein. It is possible that these mutations represent just 'passenger' mutations in a genetically unstable cancer cell genome that are not causally implicated in oncogenesis [120].

Tissue	Histology/Type	Gene	Zygoty	cDNA	Protein	Mutation	Reference
Breast	IDC	WNK1	Het	c.5395C>G	p.Q1799E	Missense	2,3,4
Breast	pleomorphic lobular ca	WNK1	Het	c.1255G>C	p.E419Q	Missense	2,4
Breast	pleomorphic lobular ca	WNK1	Het	c.6569C>G	p.S2190C	Missense	2,4
Lung	adenocarcinoma	WNK1	Hom	c.7086C>A	p.F2362L	Missense	1,4
Ovary	serous carcinoma	WNK1	Het	c.2829C>T	p.Y943Y	Silent	4
Colon	colorectal	WNK1	Het	c.3596A>G	p.E1199G	Missense	3
Brain	glioblastoma	WNK1	Het	c.5293G>A	p.G1765S	Missense	5
Brain	glioblastoma	WNK2	Het	c.3799G>A	p.A1267T	Missense	5
Colorectal	adenocarcinoma	WNK2	Het	c.1922delC	p.P641fs*2	Frameshift deletion	4
Stomach	adenocarcinoma	WNK2	Het	c.4116delC	p.S1373fs*5	Frameshift deletion	4
Lung	adenocarcinoma	WNK2	Het	c.5933G>T	p.S1978I	Missense	1,4
Lung	neuroendocrine carcinoma	WNK2	Het	c.4856G>A	p.G1619E	Missense	1,4
Ovary	serous carcinoma	WNK2	Het	c.1486G>T	p.V496L	Missense	4
Ovary	Mucinous carcinoma	WNK2	Het	c.6642delC	p.T2215fs*31	Frameshift deletion	4
Glioma	glioblastoma	WNK3	Het	c.2784C>T	p.H928H	Silent	4
Lung	squamous cell carcinoma	WNK3	Het	c.2561C>G	p.S854C	Missense	4
Lung	large cell carcinoma	WNK3	Het	c.4599G>T	p.L1533F	Missense	4
Kidney	clear cell carcinoma	WNK3	Het	c.3809C>A	p.T1270N	Missense	4
Kidney	clear cell carcinoma	WNK3	Het	c.4900T>C	p.S1634P	Missense	4
Stomach	adenocarcinoma	WNK4	Het	c.1786delG	p.V596fs*53	Frameshift deletion	4
Melanoma	metastatic	WNK4	Het	c.1402C>T	p.L468L	Silent	4
Melanoma		WNK4	Het	c.2974C>T	p.P992S	Missense	4
Melanoma		WNK4	Het	c.3154C>T	p.P1052S	Missense	4
Ovary	Mucinous carcinoma	WNK4	Het	c.1302C>G	p.D434E	Missense	4
Breast	IDC	WNK4	Het	c.441C>G	p.F147L	Missense	4

References: 1= Davies et al., 2005; 2= Stephens et al., 2005; 3=Sjöblom et al., 2006; 4= Greenman et al., 2007; 5=Parsons et al., 2008

Figure 5. List of WNK mutations identified in different unbiased cancer genome sequencing efforts (corresponding references are indicated).

6. Summary and perspective

Considering the reviewed data, the evidence for a role of WNK kinases in cancer cell signalling is just beginning to emerge. At present, experimental evidence is strongest concerning their role as upstream regulators of MAPK cascades, as modulators of Rho-GTPases and inhibitors of apoptosis (see Figure 6). The next few years will certainly shed light on further aspects of cancer biology that are affected by WNKs. It will be of particular interest to elucidate how their abundantly documented role on the regulation of activity or cell surface expression of ion channels can be related to tumour development. In order to understand the underlying signalling network, it will be important to systematically identify WNK interacting proteins and physiological substrate proteins, cellular phenotypes following WNK suppression, and the genetic changes affecting WNK genes during tumorigenesis.

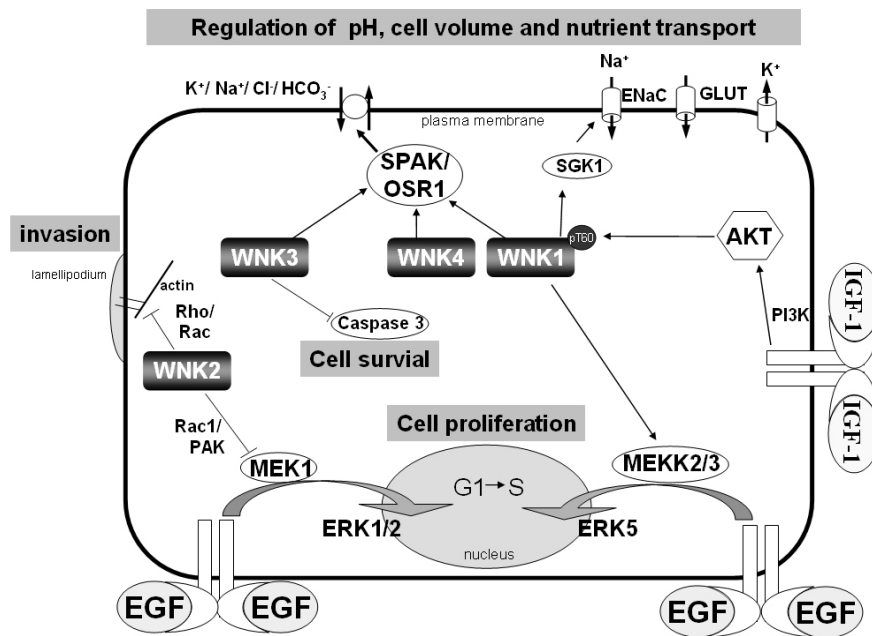


Figure 6. Schematic summary of major WNK-regulated cellular processes with a role in cancer cell biology. Arrows symbolise stimulation and t-shaped lines inhibition.

Acknowledgements

The authors wish to thank Drs. Fátima Veríssimo (Heidelberg), Karl Kunzelmann (Regensburg) and Jonathan Morris (London) for their helpful suggestions on the manuscript. Work in the authors' laboratory was supported by the Fundação para a Ciência e a Tecnologia, Portugal (Programa de Financiamento Plurianual do CIGMH, grants POCTI/33221/99, POCTI/56294/04 and fellowship BD 11180/02 to S.M.).

References

1. Manning, G., Whyte, D.B., Martinez, R., Hunter, T., Sudarsanam, S. 2002, The protein kinase complement of the human genome. *Science.*, 298, 1912-34.
2. Kostich, M., English, J., Madison, V., Gheyas, F., Wang, L., Qiu, P., Greene, J., Laz, T.M. 2002, Human members of the eukaryotic protein kinase family. *Genome Biol.*, 3, RESEARCH0043.
3. Hanks, S.K. 2003, Genomic analysis of the eukaryotic protein kinase superfamily: a perspective. *Genome Biol.*, 4, 111.1-111.7.
4. Hanks, S.K., Hunter, T. 1995, The eucaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.*, 9, 576-96.
5. Xu, B.E., English, J.M., Wilsbacher, J.L., Stippec, S., Goldsmith, E.J., Cobb, M.H. 2000, WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. *J Biol Chem.*, 275, 16795-801.
6. Verissimo, F., Jordan, P. 2001, WNK kinases, a novel protein kinase subfamily in multi-cellular organisms. *Oncogene.*, 20, 5562-9.
7. Min, X., Lee, B.H., Cobb, M.H., Goldsmith, E.J. 2004, Crystal structure of the kinase domain of WNK1, a kinase that causes a hereditary form of hypertension. *Structure.*, 12, 1303-11.
8. Knighton, D.R., Zheng, J.H., Ten Eyck, L.F., Ashford, V.A., Xuong, N.H., Taylor, S.S., Sowadski, J.M. 1991, Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase, *Science.*, 253, 407-14.
9. Xu, B.E., Min, X., Stippec, S., Lee, B.H., Goldsmith, E.J., Cobb, M.H. 2002, Regulation of WNK1 by an autoinhibitory domain and autophosphorylation. *J Biol Chem.*, 277, 48456-62.
10. Zagórska, A., Pozo-Guisado, E., Boudeau, J., Vitari, A.C., Rafiqi, F.H., Thastrup, J., Deak, M., Campbell, D.G., Morrice, N.A., Prescott, A.R., Alessi, D.R. 2007, Regulation of activity and localization of the WNK1 protein kinase by hyperosmotic stress. *J Cell Biol.*, 176, 89-100.
11. Wang, Z., Yang, C.L., Ellison, D.H. 2004, Comparison of WNK4 and WNK1 kinase and inhibiting activities, *Biochem Biophys Res Commun.*, 317, 939-44.
12. Nakamichi, N., Murakami-Kojima, M., Sato, E., Kishi, Y., Yamashino, T., Mizuno, T. 2002, Compilation and characterization of a novel WNK family of protein kinases in *Arabidopsis thaliana* with reference to circadian rhythms. *Biosci Biotechnol Biochem.*, 66, 2429-36.
13. Choe, K., Strange, K. 2007, Evolutionarily conserved WNK and Ste20 kinases are essential for acute volume recovery and survival following hypertonic shrinkage in *Caenorhabditis elegans*. *Am J Physiol Cell Physiol.*, 293, C915-27.
14. Wilson, F.H., Disse-Nicodeme, S., Choate, K.A., Ishikawa, K., Nelson-Williams, C., Desitter, I., Gunel, M., Milford, D.V., Lipkin, G.W., Achard, J.M. 2001, Human hypertension caused by mutations in WNK kinases. *Science.*, 293, 1107-12.
15. Holden, S., Cox, J., Raymond, F.L. 2004, Cloning, genomic organization, alternative splicing and expression analysis of the human gene WNK3 (PRKWNK3). *Gene.*, 335, 109-19.

16. Choate, K.A., Kahle, K.T., Wilson, F.H., Nelson-Williams, C., Lifton, R.P. 2003, WNK1, a kinase mutated in inherited hypertension with hyperkalemia, localizes to diverse Cl⁻-transporting epithelia. *Proc Natl Acad Sci U S A.*, 100, 663-8.
17. Kahle, K.T., Gimenez, I., Hassan, H., Wilson, F.H., Wong, R.D., Forbush, B., Aronson, P.S., Lifton, R.P. 2004, WNK4 regulates apical and basolateral Cl⁻ flux in extrarenal epithelia. *Proc Natl Acad Sci U S A.*, 101, 2064-69.
18. Kahle, K.T., Wilson, F.H., Lalioti, M., Toka, H., Qin, H., Lifton, R.P. 2004, WNK kinases: molecular regulators of integrated epithelial ion transport. *Curr Opin Nephrol Hypertens.*, 13, 557-62.
19. Moniz, S., Veríssimo, F., Matos, P., Brazão, R., Silva, E., Kotelevets, L., Chastre, E., Gespach, C., Jordan, P. 2007, Protein kinase WNK2 inhibits cell proliferation by negatively modulating the activation of MEK1/ERK1/2. *Oncogene.*, 26, 6071-81.
20. Delaloy, C., Lu, J., Houot, A.M., Disse-Nicodeme, S., Gasc, J.M., Corvol, P., Jeunemaitre, X. 2003, Multiple promoters in the WNK1 gene: one controls expression of a kidney-specific kinase-defective isoform. *Mol Cell Biol.*, 23, 9208-21.
21. Veríssimo, F., Silva, E., Morris, J.D., Pepperkok, R., Jordan, P. 2006, Protein kinase WNK3 increases cell survival in a caspase 3-dependent pathway. *Oncogene.*, 25, 4172-82.
22. Kay, B.K., Williamson, M.P., Sudol, M. 2000, The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *FASEB J.*, 14, 231-41.
23. Xu, B.E., Lee, B.H., Min, X., Lenertz, L., Heise, C.J., Stippec, S., Goldsmith, E.J., Cobb, M.H. 2005, WNK1: analysis of protein kinase structure, downstream targets, and potential roles in hypertension. *Cell Res.*, 15, 6-10.
24. Dhillon, A.S., Hagan, S., Rath, O., Kolch, W. 2007, MAP kinase signalling pathways in cancer. *Oncogene.*, 26, 3279-90.
25. Xu, B.E., Stippec, S., Lenertz, L., Lee, B.H., Zhang, W., Lee, Y.K., Cobb, M.H. 2004, WNK1 activates ERK5 by an MEKK2/3-dependent mechanism. *J Biol Chem.*, 279, 7826-31.
26. Sun, X., Gao, L., Yu, R.K., Zeng, G. 2006, Down-regulation of WNK1 protein kinase in neural progenitor cells suppresses cell proliferation and migration. *J Neurochem.*, 99, 1114-21.
27. Hong, C., Moorefield, K.S., Jun, P., Aldape, K.D., Kharbanda, S., Phillips, H.S., Costello, J.F. 2007, Epigenome scans and cancer genome sequencing converge on WNK2, a kinase-independent suppressor of cell growth. *Proc Natl Acad Sci USA.*, 104, 10974-79.
28. Jun, P., Hong, C., Lal, A., Wong, J.M., McDermott, M.W., Bollen, A.W., Plass, C., Held, W.A., Smiraglia, D.J., Costello, J.F. 2008, Epigenetic silencing of the kinase tumor suppressor WNK2 is tumor-type and tumor-grade specific. *Neuro Oncol.*, in press.
29. Moniz, S., Matos, P., Jordan, P. 2008, WNK2 modulates MEK1 activity through the Rho GTPase pathway. *Cell Signal.*, 20, 1762-8.

30. Shaharabany, M., Holtzman, E.J., Mayan, H., Hirschberg, K., Seger, R., Farfel, Z. 2008, Distinct pathways for the involvement of WNK4 in the signaling of hypertonicity and EGF. *FEBS J.*, 275, 1631-42.
31. Lenertz, L.Y., Lee, B.H., Min, X., Xu, B.E., Wedin, K., Earnest, S., Goldsmith, E.J., Cobb, M.H. 2005, Properties of WNK1 and implications for other family members. *J Biol Chem.*, 280, 26653-8.
32. Dan, I., Watanabe, N.M., Kusumi, A. 2001, The Ste20 group kinases as regulators of MAP kinase cascades. *Trends Cell Biol.*, 11, 220-30.
33. Strange, K., Denton, J., Nehrke, K. 2006, Ste20-type kinases: evolutionarily conserved regulators of ion transport and cell volume. *Physiology.*, 21, 61-8.
34. Johnston, A.M., Naselli, G., Gonez, L.J., Martin, R.M., Harrison, L.C., Deaizpurua, H.J. 2000, SPAK, a Ste20/SPS1-related kinase that activates the p38 pathway. *Oncogene.*, 19, 4290-97.
35. Polek, T.C., Talpaz, M., Spivak-Kroizman, T. 2006, The TNF receptor, RELT, binds SPAK and uses it to mediate p38 and JNK activation. *Biochem Biophys Res Commun.*, 343, 125-34.
36. Yan, Y., Nguyen, H., Dalmaso, G., Sitaraman, S.V., Merlin, D. 2007, Cloning and characterization of a new intestinal inflammation-associated colonic epithelial Ste20-related protein kinase isoform. *Biochim Biophys Acta.*, 1769, 106-16.
37. Solomon, A., Bandhakavi, S., Jabbar, S., Shah, R., Beitel, G.J., Morimoto, R.I. 2004, *Caenorhabditis elegans* OSR-1 regulates behavioral and physiological responses to hyperosmotic environments. *Genetics.*, 167, 161-70.
38. Vitari, A.C., Deak, M., Morrice, N.A., Alessi, D.R. 2005, The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. *Biochem. J.*, 391, 17-24.
39. Moriguchi, T., Urushiyama, S., Hisamoto, N., Iemura, S., Uchida, S., Natsume, T., Matsumoto, K., Shibuya, H. 2005, WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. *J Biol Chem.*, 280, 42685-93.
40. Anselmo, A.N., Earnest, S., Chen, W., Juang, Y.C., Kim, S.C., Zhao, Y., Cobb, M.H. 2006, WNK1 and OSR1 regulate the Na⁺, K⁺, 2Cl⁻ cotransporter in HeLa cells. *Proc Natl Acad Sci USA.*, 103, 10883-8.
41. Gagnon KB, England R, and Delpire E. 2006, Volume sensitivity of cation-chloride cotransporters is modulated by the interaction of two kinases: SPAK and WNK4. *Am J Physiol Cell Physiol* 290: C134-C142.
42. Vitari, A.C., Thastrup, J., Rafiqi, F.H., Deak, M., Morrice, N.A., Karlsson, H.K., Alessi, D.R, 2006, Functional interactions of the SPAK/OSR1 kinases with their upstream activator WNK1 and downstream substrate NKCC1. *Biochem J.*, 397, 223-31.
43. Richardson, C., Rafiqi, F.H., Karlsson, H.K., Moleleki, N., Vandewalle, A., Campbell, D.G., Morrice, N.A., Alessi, D.R. 2008, Activation of the thiazide-sensitive Na⁺-Cl⁻ cotransporter by the WNK-regulated kinases SPAK and OSR1. *J Cell Sci.*, 121, 675-84.

44. Ponce-Coria, J., San-Cristobal, P., Kahle, K.T., Vazquez, N., Pacheco-Alvarez, D., de Los Heros, P., Juárez, P., Muñoz, E., Michel, G., Bobadilla, N.A., Gimenez, I., Lifton, R.P., Hebert, S.C., Gamba, G. 2008, Regulation of NKCC2 by a chloride-sensing mechanism involving the WNK3 and SPAK kinases. *Proc Natl Acad Sci USA.*, 105, 8458-63.
45. Frost, J.A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P.E., Cobb, M.H. 1997, Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *EMBO J.*, 16, 6426-38.
46. Coles, L.C., Shaw, P.E. 2002, PAK1 primes MEK1 for phosphorylation by Raf-1 kinase during cross-cascade activation of the ERK pathway. *Oncogene.*, 21, 2236-44.
47. Eblen, S.T., Slack, J.K., Weber, M.J., Catling, A.D. 2002, Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. *Mol Cell Biol.*, 22, 6023-33.
48. Slack-Davis, J.K., Eblen, S.T., Zecevic, M., Boerner, S.A., Tarcsfalvi, A., Diaz, H.B., Marshall, M.S., Weber, M.J., Parsons, J.T., Catling, A.D. 2003, PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. *J Cell Biol.*, 162, 281-91.
49. Park, E.R., Eblen, S.T., Catling, A.D. 2007, MEK1 activation by PAK: A novel mechanism. *Cell Signal.*, 19, 1488-496.
50. Sorkin, A., von Zastrow, M. 2002, Signal transduction and endocytosis: close encounters of many kinds. *Nat Rev Mol Cell Biol.*, 3, 600-14.
51. González-Gaitán, M. 2003, Signal dispersal and transduction through the endocytic pathway. *Nat Rev Mol Cell Biol.*, 4, 213-24.
52. Cai, H., Cebotaru, V., Wang, Y.H., Zhang, X.M., Cebotaru, L., Guggino, S.E., Guggino, W.B. 2006, WNK4 kinase regulates surface expression of the human sodium chloride cotransporter in mammalian cells. *Kidney Int.*, 69, 2162-70.
53. Subramanya, A.R., Liu, J., Ellison, D.H., Wade, J.B., Welling, P.A. 2009, WNK4 diverts the thiazide-sensitive NaCl cotransporter to the lysosome and stimulates AP-3 interaction. *J Biol Chem.*, in press.
54. Kahle, K.T., Wilson, F.H., Leng, Q., Lalioti, M.D., O'Connell, A.D., Dong, K., Rapson, A.K., MacGregor, G.G., Giebisch, G., Hebert, S.C., Lifton, R.P. 2003, WNK4 regulates the balance between renal NaCl reabsorption and K⁺ secretion. *Nat Genet.*, 35, 372-6.
55. Cope, G., Murthy, M., Golbang, A.P., Hamad, A., Liu, C.H., Cuthbert, A.W., O'Shaughnessy, K.M. 2006, WNK1 affects surface expression of the ROMK potassium channel independent of WNK4. *J Am Soc Nephrol.*, 17, 1867-74.
56. He, G., Wang, H.R., Huang, S.K., Huang, C.L. 2007, Intersectin links WNK kinases to endocytosis of ROMK1. *J Clin Invest.*, 117, 1078-87.
57. Pelkmans, L., Fava, E., Grabner, H., Hannus, M., Habermann, B., Krausz, E., Zerial, M. 2005, Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature.*, 436, 78-86.
58. Pollak, M. 2008, Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer.*, 8, 915-28.
59. Vitari, A.C., Deak, M., Collins, B.J., Morrice, N., Prescott, A.R., Phelan, A., Humphreys, S., Alessi, D.R. 2004, WNK1, the kinase mutated in an inherited

- high-blood-pressure syndrome, is a novel PKB (protein kinase B)/Akt substrate. *Biochem. J.*, 378, 257-68.
60. Jiang, Z.Y., Zhou, Q.L., Holik, J., Patel, S., Leszyk, J., Coleman, K., Chouinard, M., Czech, M.P. 2005, Identification of WNK1 as a substrate of Akt/protein kinase B and a negative regulator of insulin-stimulated mitogenesis in 3T3-L1 cells. *J Biol Chem.*, 280, 21622-28.
 61. Xu, B.E., Stippec, S., Chu, P.Y., Lazrak, A., Li, X.J., Lee, B.H., English, J.M., Ortega, B., Huang, C.L., Cobb, M.H. 2005, WNK1 activates SGK1 to regulate the epithelial sodium channel. *Proc Natl Acad Sci USA.*, 102, 10315-20.
 62. Xu, B.E., Stippec, S., Lazrak, A., Huang, C.L., Cobb, M.H. 2005, WNK1 activates SGK1 by a phosphatidylinositol 3-kinase-dependent and non-catalytic mechanism. *J Biol Chem.*, 280, 34218-23.
 63. Sale, E.M., Hodgkinson, C.P., Jones, N.P., Sale, G.J. 2006, A new strategy for studying protein kinase B and its three isoforms. Role of protein kinase B in phosphorylating glycogen synthase kinase-3, tuberlin, WNK1, and ATP citrate lyase. *Biochemistry.*, 45, 213-23.
 64. Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., Cohen, P. 1997, Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol.*, 7, 261-9.
 65. Hong, F., Larrea, M.D., Doughty, C., Kwiatkowski, D.J., Squillace, R., Slingerland, J.M. 2008, mTOR-raptor binds and activates SGK1 to regulate p27 phosphorylation. *Mol Cell.*, 30, 701-11.
 66. Ring, A.M., Leng, Q., Rinehart, J., Wilson, F.H., Kahle, K.T., Hebert, S.C., Lifton, R.P. 2007, An SGK1 site in WNK4 regulates Na⁺ channel and K⁺ channel activity and has implications for aldosterone signaling and K⁺ homeostasis. *Proc Natl Acad Sci USA.*, 104, 4025-9.
 67. Massagué, J., Blain, S.W., Lo, R.S. 2000, TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell.*, 103, 295-309.
 68. Lee, B.H., Chen, W., Stippec, S., Cobb, M.H. 2007, Biological cross-talk between WNK1 and the transforming growth factor beta-Smad signaling pathway. *J Biol Chem.*, 282, 17985-96.
 69. Barrios-Rodiles, M., Brown, K. R., Ozdamar, B., Bose, R., Liu, Z., Donovan, R. S., Shinjo, F., Liu, Y., Dembowy, J., Taylor, I. W., Luga, V., Przulj, N., Robinson, M., Suzuki, H., Hayashizaki, Y., Jurisica, I., and Wrana, J. L. 2005, High-throughput mapping of a dynamic signaling network in mammalian cells. *Science.*, 307, 1621-25.
 70. Monteith, G.R., McAndrew, D., Faddy, H.M., Roberts-Thomson, S.J. 2007, Calcium and cancer: targeting Ca²⁺ transport. *Nat Rev Cancer.*, 7, 519-30.
 71. Roderick, H.L., Cook, S.J. 2008, Ca²⁺ signalling checkpoints in cancer: remodelling Ca²⁺ for cancer cell proliferation and survival. *Nat Rev Cancer.*, 8, 361-75.
 72. Prevarskaya, N., Zhang, L., Barritt, G. 2007, TRP channels in cancer. *Biochim Biophys Acta.*, 1772, 937-46.
 73. Jiang, Y., Ferguson, W.B., Peng, J.B. 2007, WNK4 enhances TRPV5-mediated calcium transport: potential role in hypercalciuria of familial hyperkalemic

- hypertension caused by gene mutation of WNK4. *Am J Physiol Renal Physiol.*, 292, F545–F554.
74. Zhang, W., Na, T., Peng, J.B. 2008, WNK3 positively regulates epithelial calcium channels TRPV5 and TRPV6 via a kinase-dependent pathway. *Am J Physiol Renal Physiol.*, 295, F1472-84.
 75. Fu, Y., Subramanya, A., Rozansky, D., Cohen, D.M. 2006, WNK kinases influence TRPV4 channel function and localization. *Am J Physiol Renal Physiol* 290: F1305–F1314,
 76. Lee, B.H., Min, X., Heise, C.J., Xu, B.E., Chen, S., Shu, H., Luby-Phelps, K., Goldsmith, E.J., Cobb, M.H. 2004, WNK1 phosphorylates synaptotagmin 2 and modulates its membrane binding. *Mol. Cell.*, 15, 741-51.
 77. Gatenby, R.A., Gillies, R.J. 2008, A microenvironmental model of carcinogenesis. *Nat Rev Cancer.*, 8, 56-61.
 78. Denko, N.C. 2008, Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nat Rev Cancer.*, 8, 705-13.
 79. Gatenby RA and Gillies RJ. 2004, Why do cancers have high aerobic glycolysis? *Nat Rev Cancer.* 4(11):891-9.
 80. Macheda, M.L., Rogers, S., Best, J.D. 2005, Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol.*, 202, 654-62.
 81. Thurmond, D.C., Pessin, J.E. 2001, Molecular machinery involved in the insulin-regulated fusion of GLUT4-containing vesicles with the plasma membrane. *Mol Membr Biol.*, 18, 237-45.
 82. Oh, E., Heise, C.J., English, J.M., Cobb, M.H., Thurmond, D.C. 2007, WNK1 is a novel regulator of Munc18c-syntaxin 4 complex formation in SNARE-mediated vesicle exocytosis. *J Biol Chem.*, 282, 32613-22.
 83. Song, J., Hu, X., Riazi, S., Tiwari, S., Wade, J.B., Ecelbarger, C.A. 2006, Regulation of blood pressure, the epithelial sodium channel (ENaC), and other key renal sodium transporters by chronic insulin infusion in rats. *Am J Physiol Renal Physiol.*, 290, F1055-64.
 84. Gamba G. (2005). Role of WNK kinases in regulating tubular salt and potassium transport and in the development of hypertension. *Am J Physiol Renal Physiol.*, 288, F245-F252.
 85. Kahle, K.T., Rinehart, J., Ring, A., Gimenez, .I, Gamba, G., Hebert, S.C., Lifton, R.P. 2006, WNK protein kinases modulate cellular Cl⁻ flux by altering the phosphorylation state of the Na-K-Cl and K-Cl cotransporters. *Physiology (Bethesda).*, 21, 326-35.
 86. Kahle, K.T., Ring, A.M., Lifton, R.P. 2008, Molecular physiology of the WNK kinases. *Annu Rev Physiol.*, 70, 329-55.
 87. Cardone, R.A., Casavola, V., Reshkin, S.J. 2005, The role of disturbed pH dynamics and the Na⁺/H⁺ exchanger in metastasis. *Nat Rev Cancer.*, 5, 786-95.
 88. Dorwart, M.R., Shcheynikov, N., Wang, Y., Stippec, S., Muallem, S. 2007, SLC26A9 is a Cl⁻ channel regulated by the WNK kinases. *J Physiol.*, 584, 333-45.
 89. Kunzelmann, K. 2005, Ion channels and cancer. *J Membr Biol.*, 205, 159-73.

90. Igney, F.H., Krammer, P. 2002, Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer.*, 2, 277-88.
91. Bagci, E.Z., Vodovotz, Y., Billiar, T.R., Ermentrout, G.B., Bahar, I. 2006, Bistability in apoptosis: roles of bax, bcl-2, and mitochondrial permeability transition pores. *Biophys J.*, 90, 1546-59.
92. Dogu, Y., Díaz, J. 2009, Mathematical model of a network of interaction between p53 and Bcl-2 during genotoxic-induced apoptosis. *Biophys Chem.*, 143, 44-54.
93. MacKeigan, J.P., Murphy, L.O., Blenis, J. 2005, Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. *Nat Cell Biol.*, 7, 591-600.
94. Boutros, M., Kiger, A.A., Armknecht, S., Kerr, K., Hild, M., Koch, B., Haas, S.A., Paro, R., Perrimon, N., Heidelberg Fly Array Consortium. 2004, Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science.*, 303, 832-835.
95. Delpire, E., Gagnon, K.B. 2008, SPAK and OSR1: STE20 kinases involved in the regulation of ion homeostasis and volume control in mammalian cells. *Biochem J.*, 409, 321-31.
96. Richardson, C., Alessi, D.R. 2008, The regulation of salt transport and blood pressure by the WNK-SPAK/OSR1 signalling pathway. *J Cell Sci.*, 121, 3293-304.
97. Piechotta, K., Garbarini, N., England, R., Delpire, E. 2003, Characterization of the interaction of the stress kinase SPAK with the Na⁺-K⁺-2Cl⁻ cotransporter in the nervous system: evidence for a scaffolding role of the kinase. *J Biol Chem.*, 278, 52848-56.
98. Gaozza, E., Baker, S.J., Vora, R.K., Reddy, E.P. 1997, AATYK: a novel tyrosine kinase induced during growth arrest and apoptosis of myeloid cells. *Oncogene.*, 15, 3127-35.
99. Tomomura, M., Furuichi, T. 2005, Apoptosis-associated tyrosine kinase (AATYK) has differential Ca²⁺-dependent phosphorylation states in response to survival and apoptotic conditions in cerebellar granule cells. *J Biol Chem.*, 280, 35157-63.
100. Polek, T.C., Talpaz, M., Spivak-Kroizman, T.R. 2006, TRAIL-induced cleavage and inactivation of SPAK sensitizes cells to apoptosis. *Biochem Biophys Res Commun.*, 349, 1016-24.
101. Thiery, J.P. 2003, Epithelial–mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol.*, 15, 740-6.
102. Padua, D., Massagué, J. 2009, Roles of TGFβ in metastasis. *Cell Research.*, 19, 89-102.
103. Thuault, S., Tan, E.J., Peinado, H., Cano, A., Heldin, C.H., Moustakas, A. 2008, HMGA2 and Smads co-regulate SNAIL1 expression during induction of epithelial-to-mesenchymal transition. *J Biol Chem.*, 283, 33437-46.
104. Zeng, G., Gao, L., Xia, T., Gu, Y., Yu, R.K. 2005, Expression of the mouse WNK1 gene in correlation with ganglioside GD3 and functional analysis of the mouse WNK1 promoter. *Gene.*, 344, 233-9.
105. Zeng, G., Gao, L., Yu, R.K. 2000, Reduced cell migration, tumor growth and experimental metastasis of rat F-11 cells whose expression of ganglioside GD3 was suppressed. *Int J Cancer.*, 88, 53–57.

106. Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., Horwitz, A.R. 2003, Cell migration: integrating signals from front to back. *Science.*, 302, 1704-9.
107. Zhang, Z., Xu, X., Zhang, Y., Zhou, J., Yu, Z., He, C. 2009, LINGO-1 interacts with WNK1 to regulate Nogo-induced inhibition of neurite extension. *J Biol Chem.*, 284, 15717-28.
108. Paradiso, A., Cardone, R.A., Bellizzi, A., Bagorda, A., Guerra, L., Tommasino, M., Casavola, V., Reshkin, S.J. 2004, The Na⁺-H⁺ exchanger-1 induces cytoskeletal changes involving reciprocal RhoA and Rac1 signaling, resulting in motility and invasion in MDA-MB-435 cells. *Breast Cancer Res.*, 6, R616-28.
109. Denker, S.P., Huang, D.C., Orlowski, J., Furthmayr, H., Barber, D.L. 2000, Direct binding of the Na-H exchanger NHE1 to ERM proteins regulates the cortical cytoskeleton and cell shape independently of H(+) translocation. *Mol. Cell.*, 6, 1425-36.
110. Denker, S.P., Barber, D.L. 2002, Ion transport proteins anchor and regulate the cytoskeleton. *Curr Opin Cell Biol.*, 14, 214-20.
111. Ekstrand, A.J., James, C.D., Cavenee, W.K., Seliger, B., Pettersson, R.F., Collins, V.P. 1991, Genes for epidermal growth factor receptor, transforming growth factor α , and epidermal growth factor and their expression in human gliomas in vivo. *Cancer Res.*, 51, 2164-72.
112. Scanlan, M.J., Chen, Y.T., Williamson, B., Gure, A.O., Stockert, E., Gordan, J.D., Türeci, O., Sahin, U., Pfreundschuh, M., Old, L.J. 1998, Characterization of human colon cancer antigens recognized by autologous antibodies. *Int J Cancer.*, 76, 652-8.
113. Ito, M., Shichijo, S., Tsuda, N., Ochi, M., Harashima, N., Saito, N., Itoh, K. 2001, Molecular basis of T cell-mediated recognition of pancreatic cancer cells. *Cancer Res.*, 61, 2038-46.
114. Delaloy, C., Elvira-Matlot, E., Clemessy, M., Zhou, X.O., Imbert-Teboul, M., Houot, A.M., Jeunemaitre, X., Hadchouel, J. 2008, Deletion of WNK1 first intron results in misregulation of both isoforms in renal and extrarenal tissues. *Hypertension.*, 52, 1149-54.
115. Schwerk, C., Schulze-Osthoff, K. 2005, Regulation of apoptosis by alternative pre-mRNA splicing. *Mol Cell.*, 19, 1-13.
116. Venables, J.P. 2006, Unbalanced alternative splicing and its significance in cancer. *BioEssays.*, 28, 378-86.
116. Venables, J.P. (Ed). 2006, Alternative Splicing and Cancer, Transworld Research Network, Kerala (India), (ISBN: 81-7895-235-1).
118. Srebrow, A., Kornblihtt, A.R. 2006, The connection between splicing and cancer. *J Cell Sci.*, 119, 2635-41.
119. Glover, M., Zuber, A.M., O'Shaughnessy, K.M. 2009, Renal and brain isoforms of WNK3 have opposite effects on NCCT expression. *J Am Soc Nephrol.* 20, 1314-22.
120. Davies, H., Hunter, C., Smith, R., Stephens, P., Greenman, C., Bignell, G., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W. et al. 2005, Somatic mutations of the protein kinase gene family in human lung cancer. *Cancer Res.*, 65, 7591-5.

121. Stephens, P., Edkins, S., Davies, H., Greenman, C., Cox, .C, Hunter, C. Bignell G, Teague, J., Smith, R., Stevens, C. et al. 2005, A screen of the complete protein kinase gene family identifies diverse patterns of somatic mutations in human breast cancer. *Nat Genet.* 37, 590-2.
122. Sjöblom, T., Jones, S., Wood, L.D., Parsons, D.W., Lin, J., Barber, T.D., Mandelker, D., Leary, R.J., Ptak, J., Silliman, N., et al. 2006, The consensus coding sequences of human breast and colorectal cancers. *Science.*, 314, 268-74.
123. Greenman, C., Stephens, P., Smith, R., Dalgliesh, G.L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C., et al. 2007, Patterns of somatic mutation in human cancer genomes. *Nature.*, 446, 153-8.
124. Parsons, D.W., Jones, S., Zhang, X., Lin, J.C., Leary, R.J., Angenendt, P., Mankoo, P., Carter, H., Siu, I.M., Gallia, G.L., et al., 2008, An integrated genomic analysis of human glioblastoma multiforme. *Science.*, 321, 1807-12.