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Review

WNKs of flora and fauna

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ABSTRACT

The with-no-lysine (K) (WNK) family of protein kinases is unique in that they are characterized by a more exposed position of the catalytic lysine in the kinase domain when compared to canonical protein kinases. These kinases have been implicated in regulation of a variety of cellular processes such as salt and water homeostasis, intracellular vesicular trafficking, and neuronal excitability. Mutations in two of the four family members have been linked to Gordon's syndrome which is a genetic hypertensive disease. Recent evidence points to a role of these proteins in several other disease systems including cancer. Newer studies suggest that these proteins are an ancient class with homologs found in unicellular organisms and plants. In this article, we review the different roles of WNK family members in mammals and plants, links with other pathways in the cell and its role in disease.

KEYWORDS: with no lysine, ion cotransporters, vesicular transport, Pseudohypoaldosteronism type II, cancer, cell cycle, plants, *Arabidopsis*, circadian rhythm

ABBREVIATIONS

With-no-lysine (WNK), Mitogen activated protein kinase (MAPK), Mitogen activated protein kinase kinase (MAP2K), Mitogen activated protein kinase kinase kinase (MAP3K), kilodalton (kD), Kidney specific WNK1 (KS-WNK1), Expressed sequence tags (EST), Pseudohypoaldosteronism type II (PHAII), Online Mendelian inheritance in man (OMIM), Sodium chloride cotransporter (NCC), Solute carrier family (SLC), Sodium potassium two chloride cotransporter (NKCC), Oxidative stress responsive kinase 1 (OSR1), Ste-20 related proline alanine rich kinase (SPAK), Renal outer medullary potassium channel (ROMK), Epithelial sodium channel (ENaC), Potassium chloride cotransporter (KCC), Serum and glucocorticoid regulated kinase 1 (SGK1), Phosphatidylinositol-3-kinase (PI3K), Phosphoinositide dependent kinase 1 (PDK1), p21 activated kinase 1 (PAK1), Neurally expressed and developmentally downregulated 4-2 (Nedd 4-2), Gamma amino butyric acid (GABA), Synaptotagmin (Syt), Intersectin 1(ITSN1), Extracellular signal regulated kinase (ERK), Epidermal growth factor (EGF), Distal convoluted tubule (DCT), Protein kinase B (PKB), Phosphatidylinositol 3,4 phosphate (PIP_2), Phospholipase C (PLC), Inositol trisphosphate (IP_3), Diacylglycerol (DAG), Phosphatidylinositol 4 phosphate (PI4P), Mammalian target of rapamycin (mTOR), Transforming growth factor β (TGF-β), Liver kinase B1 (LKB1), Mouse protein 25 (MO-25), Ste-20 related adaptor (STRAD), Spleen tyrosine kinase (SYK), Cystic fibrosis transmembrane conductance receptor (CFTR), (IP(3)) receptor-binding protein released with IP(3) (IRBIT), basolateral solute carrier family 4 member 4 (NBCe1-B), *Arabidopsis* pseudo response regulator (APRR), Abscissic acid (ABA).

INTRODUCTION

The first member of the with-no-lysine (WNK) family of protein kinases was discovered

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serendipitously in our laboratory while searching for homologs of the mitogen-activated protein kinase kinase (MAPKK or MEK). WNKs are a family of four atypical kinases with a non-canonical position of the catalytic lysine [1]. The catalytic lysine involved in the phosphotransferase reaction is more exposed on the surface of the kinase domain in WNKs compared to a more buried position in all other members of the eukaryotic protein kinase superfamily [1, 2]. WNKs are large proteins with molecular weights in mammals ranging from 135-250 kD. Several splice variants of the four family members exist. WNK1 has another isoform that is expressed from an alternate promoter specifically in the kidney (KS-WNK1) and lacks the kinase domain [1-7]. WNKs are complex proteins with only the kinase domain, the autoinhibitory domain and a few sequence motifs conserved among all family members. WNKs have low complexity sequence stretches, putative coiled-coil domains and a number of PxxP and RFXV motifs which serve as binding motifs for interacting partners [4]. There is also a common autoinhibitory domain immediately distal to the kinase domain that inhibits the activity of the kinase domain. Another putative autoinhibitory domain in WNKs lies in the middle of the protein [8]. The expression of the four family members varies. A survey of expressed sequence tags (ESTs) in the Unigene database (http://www.ncbi.nlm.nih.gov/unigene) indicates that WNK1 is ubiquitously expressed in adult organs as well as embryonic tissues. WNK2 is expressed in the brain, testis, uterus, eye, kidney, intestine and a few embryonic tissues. The expression of WNK3 is more restricted with brain and testis being the major organs expressing the proteins. WNK4 is mostly expressed in the kidney, eye, mammary gland and prostate.

The importance of WNKs was first realized when it was shown that mutations in WNK1 and WNK4 are associated with pseudohypoaldosteronism type II (PHAII), also known as Gordon's syndrome (OMIM #145260) [9]. PHAII is a monogenic disease characterized by hypertension due to increased sodium reabsorption, hyperkalemia and metabolic acidosis, despite normal levels of aldosterone. Intronic deletions that cause an increase in WNK1 expression and missense mutations clustered in a short acidic segment distal to the kinase domain in WNK4 are known to be associated with PHAII. WNK1 is an essential gene and WNK1 knockout mice do not survive beyond embryonic day 13 [10]. Endothelial specific rescue of WNK1 is sufficient to let these mice develop to birth, although they die within the first day of birth [11] suggesting a wider role of WNK1 in maintaining growth and survival.

In this review, we will summarize what is known about the functions of WNKs, explore cross-talks with other pathways and roles of WNKs in other diseases, and provide a brief review on the function and regulation of plant WNKs (Figure1).

WNKs have diverse functions

Regulation of ion transporters: WNK family members are activated by hyperosmotic stresses (e.g. sorbitol and NaCl) [4, 12] and hypotonic low chloride conditions [13-15]. Thiazide diuretics, which inhibit the sodium chloride cotransporter (NCC), solute carrier family 12 member 3 (SLC12A3), have been used to treat the symptoms of PHAII patients. Given the role of WNKs in PHAII, it was hypothesized that regulation of NCC might be one of the functions of WNKs. Indeed, WNKs 1, 3 and 4 were shown to influence NCC activity [13-19]. The regulation of NCC and the related sodium, potassium and two chloride cotransporters (NKCC1 and NKCC2 aka SLC12A2 and SLC12A1) by WNKs is complicated by several apparent contradictory studies using the *Xenopus* system and mouse. This area has been extensively reviewed elsewhere [14, 20-24]. We briefly mention some of the major points in this review.

NCC is predominantly expressed in the distal convoluted tubule. Using *Xenopus laevis* oocytes as a model system, it was demonstrated that coexpression of WNK4 along with NCC had an inhibitory effect on sodium reuptake suggesting that WNK4 inhibited NCC action. PHAII-causing mutations in WNK4 increased the inhibitory action of WNK4 on NCC [14, 15]. WNK1 inhibited the action of WNK4 on NCC while WNK3 was a positive regulator of NCC [18, 25]. This regulation of NCC is further complicated by the presence of the kidney specific isoform of

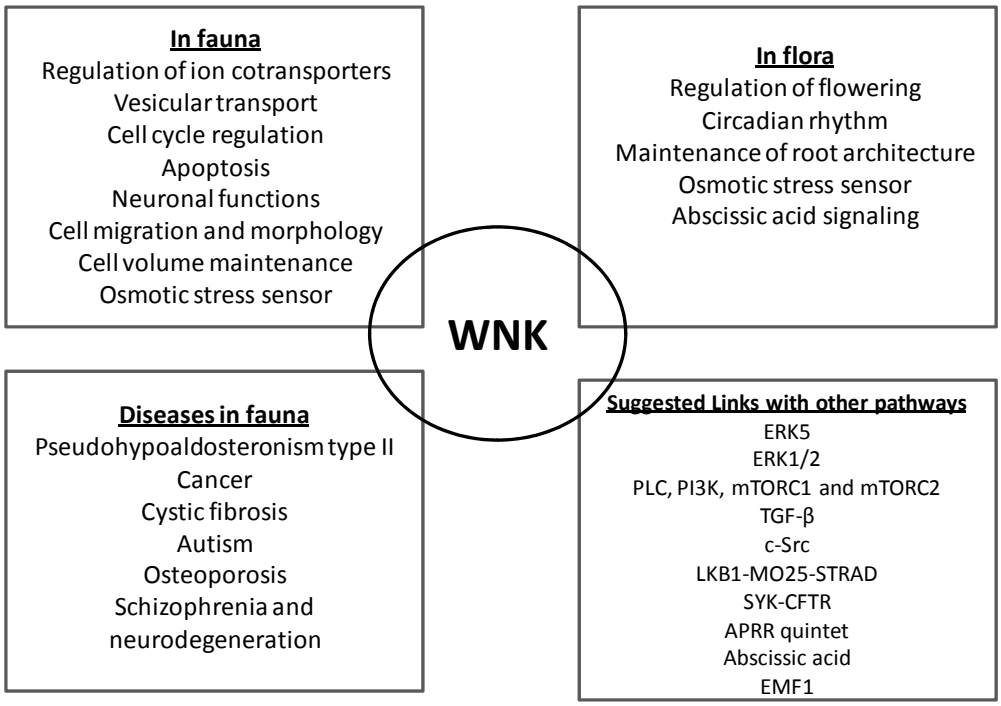

Figure 1. A schematic of the roles of the WNK family members in plants and animals as discussed in this article.

WNK1 which is expressed in the renal tubules exclusively [5]. KS-WNK1 acts as a dominant negative and inhibits the action of the long form of WNK1 on NCC [26]. A KS-WNK1 knockout mouse shows sodium retention, high blood pressure and increased the surface expression of NCC and its related cotransporters NKCCs in the renal tubule [27]. The level of expression of KS-WNK1 influences its action although the exact mechanism is still unknown. Caution is warranted in interpreting the results of studies that used overexpression and heterologous model systems without reinvestigation. Other findings which bear on these conclusions are discussed below.

There are two ways by which WNK4 has been shown to regulate NCC, which may seem to contradict the findings above. First, WNK4, like the other WNKs, phosphorylates oxidative stress responsive kinase 1 (OSR1) and its homolog Ste-20 related proline alanine rich kinase (SPAK). OSR1 and SPAK in turn phosphorylate NCC and the related cotransporters NKCC1 and NKCC2 [28-32] on conserved serine and threonine residues. All four WNKs can phosphorylate and activate OSR1 to the same extent ([15] and Sengupta *et al.*, submitted). Phosphorylation by these kinases activates the transporters, which was established for NKCCs prior to their links to WNKs [33-35]. Interestingly, new data suggest that SPAK has divergent effects on NCC and NKCC [29, 36]. This may be due to the expression of different isoforms of SPAK in the kidney. A recent study suggests that SPAK has a kidney specific isoform that can act as a dominant negative to the full length isoform and is generated in response to dietary sodium intake [37]. To get a clearer picture of ion transporter regulation, it is pertinent to take into account expression of the different isoforms of WNK and SPAK in future studies.

A second suggested mechanism of transporter activation by WNKs was through regulating trafficking to the membrane [14, 15]. Some studies show that coexpression of WNK4 along with NCC in mammalian models causes inhibition of Na^+ flux through inhibition of surface expression of the transporter in a dynamin-independent manner [38]. WNK4 inhibits NCC by

inhibiting its delivery to the membrane without affecting the net rate of internalization [39]. This is done by targeting NCC to the lysosome by interaction with the adaptor protein AP-3 which marks cargo destined for lysosomes. Sortilin is a lysosomal sorting receptor similar to the yeast vacuolar protein Vps10p. It was determined that sortilin mediates the degradation of NCC brought about by WNK4 [40]. These apparently conflicting ideas concerning regulation of NCC by WNKs still need to be reconciled.

An additional complication is that all four WNKs can interact with each other via a C-terminal coiled-coil region and can phosphorylate each other. WNKs autophosphorylate and can phosphorylate each other on an activation loop residue [4, 41]. Thus, WNK family members have the potential to form an autoactivating signaling complex and their hetero-oligomerization may be affected by overexpression of single forms. Yet, clear evidence showing that they actually function as a complex *in vivo* is lacking.

WNKs 1, 3 and 4 have also been shown to inhibit the potassium chloride cotransporters (KCC1-4) which are also members of the SLC12 family [42]. Whether this is brought about by activation of OSR1 and SPAK remains to be determined but seems likely. WNK4 also phosphorylates claudins 1-4, which are tight junction proteins involved in paracellular Cl^- flux [43]. WNKs also regulate the chloride channel SLC12A9 [44]. A study suggested that the inositol-1,4,5-trisphosphate (IP(3)) receptor-binding protein released with IP(3) (IRBIT) was involved in mediating fluid and bicarbonate secretion by the CFTR and basolateral solute carrier family 4 member 4 (NBCe1-B) by antagonizing the effects of the WNK-SPAK pathway [45], thereby regulating chloride, bicarbonate and fluid secretion in epithelial cells. Using *Caenorhabditis elegans* as a model system, groups have shown that the WNK isoform expressed in these organisms can bind to and activate the OSR1 relative Gck-3 and thereby regulate the ClC anion channel which has multiple roles in volume regulation during development, cell cycle control, osmotic homeostasis and ovulation in these organisms [46, 47].

The renal outer medullary potassium channel (ROMK) is also regulated by the WNK kinases. WNKs 1, 3 and 4 inhibit ROMK by decreasing its surface expression [3, 24, 48, 49]. KS-WNK1 inhibits WNK1-mediated ROMK inhibition and interestingly PHAII mutations in WNK4 cause increased inhibition of ROMK (see below).

WNKs also regulate the epithelial sodium channel (ENaC) in a kinase-independent manner. All four WNKs interact with the sodium- and glucocorticoid-regulated kinase (SGK1) via regions of their N-termini that precede the kinase domain [50]. Interaction with this N-terminal region is sufficient to activate SGK1. SGK1 can then be translocated to the membrane by an unidentified mechanism where it is phosphorylated on its activation loop by phosphoinositide-dependent kinase (PDK1) in a phosphatidylinositol-3-kinase (PI3K) dependent manner. Active SGK1 phosphorylates Nedd4-2 (neurally expressed and developmentally downregulated), an E3 ligase one of whose target is the epithelial sodium channel [51-54]. Phosphorylation by SGK1 creates a 14-3-3 binding site on Nedd4-2 resulting in its sequestration. Thus, ENaC is retained in the plasma membrane, where it has a high open probability, facilitating sodium reabsorption. Although thiazides have been most useful in treating PHAII patients, symptoms of some patients are improved with spironolactone, an aldosterone antagonist. Aldosterone stimulates ENaC biosynthesis; thus, the aldosterone receptor antagonist decreases the amount of ENaC expressed.

Maintenance of neuronal cell volume and excitatory properties are dependent on regulation of ion balance inside and outside of the neuronal cell. Since WNK3 is expressed highly in neurons, and WNKs can regulate ion balance, it was logical that WNK3 could be responsible for maintaining ion balance in neuronal cells. WNK3 co-localizes with NKCC1 and KCC1/2 in gamma amino butyric acid (GABA) receptor expressing neurons [55]. It helps regulate transporter activity and maintain cell volume in response to osmotic stress. Recently, it was shown that WNK2 is expressed in adult and fetal brains, especially in the neocortical pyramidal cells, thalamic relay cells, and cerebellar granule and Purkinje cells. WNK2 forms a complex with SPAK in these neuronal cells and regulates the cation chloride transporters NKCC and KCC [56].

Involvement in vesicular transport: As stated above, several of the ion transporters are regulated by WNKs 1 and 4 via changes in surface expression of the transporters. To determine the mechanism involved, we used a yeast two hybrid screen to determine whether WNK1 could interact with any of the players involved in vesicular transport. We found that WNK1 interacts with synaptotagmin2 (Syt2) [57]. Synaptotagmins regulate membrane fusion and vesicle transport in neuronal and neuroendocrine cells. Syt2 is similar in function to Syt1 which has been extensively studied. Syts have calcium sensing domains known as C2 domains. Through their C2 domains Syts can bind to membranes upon Ca^{2+} influx and enhance membrane fusion events. WNK1 can bind to and phosphorylate the C2 domain of Syt2 causing a change in the calcium requirement for the membrane binding activity of the Syt2 C2 domain. This alteration of calcium requirement suggests that WNK1 might be regulating the calcium sensing properties of Syt2 and thereby regulating its roles in mediating membrane fusion.

WNKs1 and 4 inhibit the renal outer medullary potassium channel (ROMK) by decreasing the surface abundance of the channel via a clathrin and dynamin dependent mechanism [58]. This is independent of the kinase activity of WNK1 and is dependent on the interaction of WNKs 1 and 4 with the endocytic scaffold protein intersectin (ITSN1). WNKs have several proline rich PxxP motifs that can interact with SH3 domains. SH3 domains of ITSN1 were found to interact with N-terminal PxxP motifs on WNK. There are three PxxP motifs in the N-terminal region of WNK1 and mutation of all three completely abolishes interaction with ITSN1. PHAII missense mutations on WNK4 increase the affinity for interaction with ITSN1 and ROMK, leading to enhanced ROMK internalization and decreased potassium uptake. We also have evidence suggesting that WNKs are involved in membrane trafficking (Tu *et al.*, manuscript in preparation).

WNK3 and apoptosis: A study showed that WNK3 interacts with caspase-3 in HeLa cells. Overexpression of WNK3 inhibits while downregulation of WNK3 accelerates apoptosis in a caspase-3 dependent manner [59]. WNK3 translocates to the nucleus upon apoptotic stimuli. The details of the mechanism by which WNK3 can regulate apoptosis need to be elucidated. Roles of other WNKs in apoptosis also remain to be determined.

WNK1 and cell cycle regulation: A study showed an effect of WNK1 in regulation of cell proliferation and migration in multipotent C17.2 neuronal stem cells from mouse [60]. Upon downregulation of WNK1 by RNA interference, the authors showed altered cell morphology, reduction in cell migration and an impairment of progression of the cell cycle. WNKs have been identified in screens of proteins involved in development, cancer, survival and proliferation [61-63]. Our lab has recently found evidence that WNK1 is involved in regulation of the mammalian cell cycle and survival. WNK1 localizes in a punctuate manner in cells and co-localizes with the mitotic spindle. Knocking down endogenous WNK1 in HeLa cells causes multipolar spindle formation and results in multinuclear daughter cells. Further, the cells show highly elongated bridges between the daughter cells and aberrant abscission. This phenotype can be rescued by overexpressing full-length rat WNK1. This indicates that this phenomenon is specific to WNK1 knockdown. This effect is independent of the WNK substrate OSR1 [64].

Connections with other pathways

MAPK pathways: We isolated cDNAs encoding WNK1 and the kinase domain of WNK2 while searching for homologues of the mitogen-activated protein kinase kinase (MAPKK or MAP2K) also known as MEK (we called it W-MEK for a while). It was therefore sensible for us to test whether WNK1 could activate the different MAPK pathways. The MAPK cascade consists of a three kinase core cascade which may be regulated by a small G protein or an additional protein kinase (sometimes called MAP4K), or may be activated by oligomerization. The first kinase in the core cascade is the MAP kinase kinase kinase (MAPKKK, MAP3K, or MEKK). The MAP3K then phosphorylates and activates its downstream substrate, the MAP2K which in turn activates the MAPK. The best studied MAPK pathways are the extracellular signal-regulated kinase (ERK1/2) pathway, the c-Jun N-terminal kinase (JNK)

pathway, the p38 pathway and the ERK5 pathway. We found that WNK1 was unable to activate ERK1/2 or JNK. It had only a marginal effect on p38. However, overexpressed WNK1 could activate the ERK5 pathway [65]. ERK5 is activated in response to growth factors and stress conditions including osmotic and oxidative stresses and is implicated in cell survival and proliferation. Upstream regulators of ERK5 include the MAP3Ks, MEKK2 and MEKK3. We showed that WNK1 can bind to and phosphorylate MEKK3. However, this phosphorylation is dispensable for activation of MEKK3. The effect of phosphorylation of MEKK3 on its activation and ability to activate the ERK5 pathway is yet to be defined.

WNK2, on the other hand, was shown to regulate the ERK1/2 pathway via MEK1 and Rho GTPase [66, 67]. Knockdown of WNK2 in HeLa cells caused phosphorylation of ERK1/2 and phosphorylation of MEK1 at S298 in response to epidermal growth factor (EGF) stimulation. Silencing WNK2 caused an increase in Rac GTP loading and concomitantly a decrease in the amount of Rho-GTP. WNK2 cannot directly phosphorylate MEK or PAK1. However, depletion of endogenous WNK2 causes upregulation of phospho-PAK1. WNK2 interacts with RhoA and localizes to the membrane. This suggests that WNK2 might have a negative regulatory effect on ERK stimulation in HeLa cells in response to growth factors. Depletion of WNK2 caused more cells to enter the S-phase of cell cycle, thereby implicating WNK2 in growth and survival. However, whether phosphorylation of ERK1/2 in this situation actually activates the kinase was not measured using substrate phosphorylation assays. It was also not determined whether the phosphorylation of MEK at S298 in this system is directly through PAK1. Whether this effect of WNK2 is cell or tissue type specific should also be addressed.

In another recent study, WNK4 was implicated in regulation of NCC expression which was suggested to occur through the ERK1/2 pathway [68]. WNK4 overexpression in a mouse distal convoluted tubule (DCT) cell line was shown to increase ERK1/2 activation modestly in a dose dependent manner. PHAII mutations significantly decreased ERK1/2 activity. Knocking down WNK4 or ERK1/2 resulted in increased surface expression of NCC. Mechanistic links, however, were not identified. Because of the use of overexpression system, some additional control experiments would have made the study more convincing. The indication that WNKs 2 and 4 cross-talk with the ERK1/2 pathway, while WNK1 does not, suggests that the four family members might have divergent functions in mammals. Additionally, cross-talk with the essential cell growth, proliferation and survival pathways indicates a wider range of physiologic functions of WNKs.

PLC, PI3K and mTOR: WNK1 was shown to be phosphorylated by protein kinase B/Akt downstream of insulin and insulin like growth factor (IGF1) stimulation. Akt phosphorylates WNK1 specifically at an N-terminal threonine residue (T60 in human WNK1) in response to IGF1 stimulation *in vitro* which can be inhibited using phosphatidylinositol 3- kinase (PI3K) inhibitors. Cells lacking phosphoinositide dependent kinase (PDK1) which phosphorylates and activates Akt reveal that this activation of WNK1 is dependent on Akt [8, 54, 69, 70]. Phosphorylation of WNK1 by Akt does not affect its kinase activity as measured by phosphorylation of myelin basic protein (MBP). ENaC and ROMK surface expression is inhibited by WNKs and phosphorylation of WNKs by PKB mediates it [71]. One of the consequences of phosphorylation of WNK1 by Akt could be a change in the conformation of WNK leading to change in its binding properties to different proteins.

A recent study implicates WNK1 as a novel regulator of phospholipase C β (PLCβ) activity. PLCβ hydrolyzes phosphotidylinositol 4, 5 bisphosphate (PIP_2) to generate second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3) downstream of signals from G-protein coupled receptors. The concentration of PIP_2 in the membrane is rate limiting for PLCβ action. This study shows that WNK1 promotes the synthesis of PIP_2 by stimulating phosphatidylinositol 4 kinase type IIIα (PI4K IIIα), which converts phosphatidylinositol-4 phosphate to PIP_2 [72]. WNK1 activity is essential for PLCβ function downstream of Gq coupled receptors. Insulin and IGF1, which activate the PI3K-Akt pathway,

potentiate the signaling downstream of Gq, possibly via phosphorylating WNK1. This study implicates WNK1 as a coordinator and mediator of two distinct pathways.

Akt and SGK1 are also substrates of the mammalian target of rapamycin complex 2 (mTORC2). mTOR is a metabolically regulated kinase implicated in regulating a staggeringly wide array of cellular activities [73]. mTOR forms at least two functional complexes, mTORC1 and mTORC2 [74-78]. mTORC1 is involved in regulation of translation, autophagy and metabolic processes downstream of nutrient signals. mTORC2 phosphorylates the hydrophobic motif of several AGC kinases including Akt and SGK1 [51, 79]. This phosphorylation is essential for activation and stability of these proteins. Akt and SGK1 both are implicated upstream of WNK1. WNK1 is important for the activation of SGK1 [53, 54, 80]. In a phosphoproteomic analysis to determine proteins downstream of mTOR, two groups independently identified peptides corresponding to WNK1 whose phosphorylation was deregulated by mTOR inhibitors rapamycin and KU0063794 [81, 82]. The cross-talk between mTOR and WNK pathway could reveal important facets of functions of WNK proteins.

TGF-β pathway: The transforming growth factor β (TGF-β) pathway is important in the control of a wide variety of physiological processes such as embryogenesis, differentiation, proliferation and apoptosis. The TGF-β receptor is a serine-threonine protein kinase. Binding of TGF-β to it stimulates the formation of heteromeric complexes of type I and type II serine-threonine kinase receptors and subsequent trans-autophosphorylation of these receptor complexes. Activated type I receptors activate specific receptor-activated Smads (R-Smads) which leads to interaction with the co-mediator Smad. The dimerization of these transcription factors mediates signal relay to the nucleus where downstream genes are regulated. In a yeast two hybrid screen, we identified Smad2 as an interacting partner for WNK1 [83]. WNK1 can phosphorylate Smad2 *in vitro*. Interestingly, reduction in WNK1 levels in HeLa cells caused reduction in Smad2 expression subsequent to its activation and nuclear translocation, and thereby increased signaling through this pathway suggesting that WNK1 acts as a negative regulator. Interestingly, OSR1 was reported to interact with the TGF-β receptor, further suggesting a functional interaction of WNK1 with this pathway [84]. The TGF-β-Smad pathway is implicated in several cancers due to their roles in controlling proliferation and apoptosis. Implication of WNK1 as a negative regulator of this pathway warrants the suggestion that WNK1 is implicated in tumorigenesis.

c-Src: The Src family of protein tyrosine kinases are well known for their roles as proto-oncogenes and their widespread mutations associated with various forms of cancers. It was shown that overexpression of c-Src along with ROMK in *Xenopus* oocytes decreased the activity of the channel as measured by barium sensitive currents in a patch clamp study. Inhibition of the protein tyrosine phosphatases reversed this effect [85]. This effect of Src was enhanced when WNK4 was coexpressed [86]. Src was shown to affect the SGK1 mediated phosphorylation on WNK4. This links WNKs to yet another important cellular signaling pathway which has implications in cancer biology.

LKB1-MO25-STRAD: Recently, the mouse protein25A (MO25α) was shown to regulate OSR1 and SPAK activation. Overexpression of MO25α along with OSR1 and SPAK stimulated the activity of the kinases. Conserved MO25α binding sites were identified within OSR1 and SPAK [87]. MO25α is an adaptor that regulates the activity of the tumor suppressor kinase LKB1 through the Ste20-related adaptor Strad, a homolog of OSR1 and SPAK [74, 87]. The exact mechanism of MO25α on the OSR-NCC pathway needs to be clarified.

CFTR channel: WNKs 1 and 4 regulate the surface expression of the cystic fibrosis transmembrane conductance regulator (CFTR). Recent evidence suggests that this effect, at least in the case of WNK4, is linked to the action of the spleen tyrosine kinase (Syk) [88]. Biochemical and physiological assays reveal that Syk phosphorylates a tyrosine residue (Y512) on CFTR and ablation of this phosphorylation by site directed mutagenesis alters the surface expression of the channel. WNK4 can interact with and inhibit Syk in a kinase independent manner. WNK4 and Syk play

antagonistic roles in maintaining homeostatic levels of expression of the channel. It would be interesting to determine the mechanism by which WNK4 inhibits Syk function and whether PHAII causing mutations in WNK4 alter this activity. It would also be beneficial to determine if CFTR patients experience overlapping mutations in WNK4 and how other WNKs affect the channel expression and activity.

Upstream regulation

Much effort has gone into understanding the downstream effectors of WNKs, while very little is known about the upstream regulators of these proteins. WNKs are activated by volume changes in the cell, but whether they are the actual sensors of the osmotic stress is not known. WNK4 has been shown to respond to angiotensin signaling [89] suggesting that they may be downstream of other effectors that respond to hypovolemia. A recent study has shown that the micro RNA miR-192 has a target sequence in the 3' untranslated region of WNK1 gene. miR-192 can regulate WNK1 expression in a tissue culture system and aldosterone can regulate the expression of this micro-RNA [90]. Whether this and other small RNAs regulate WNK1 expression and activity under physiological circumstances remains to be seen.

Roles in other diseases

Relevant to suggestions about WNK1 and tumorigenesis, WNK2 and WNK3 have been implicated in cancer (reviewed elsewhere [91]). WNK3 regulates neuronal cell volume and has been implicated in regulating metastatic potential of certain gliomas [92]. WNK2 has been shown to be silenced epigenetically in different types and grades of tumors [93].

Further, familial microdeletions in the WNK3 gene have been shown to be linked to some forms of autism [94]. It was reported that OSR1 and WNK3 were highly overexpressed in patients with schizophrenia when examined postmortem [95]. Perhaps a change in expression pattern and/or kinase activity of WNK and OSR might affect chloride transport through NKCC and KCC in schizophrenia patients and thereby affect GABA-ergic neuronal function.

A very interesting genome wide search showed that WNK1 was downregulated in circulating B cells in women with postmenopausal osteoporosis [96]. This opens up a new and exciting avenue for the study of WNKs in regulating bone structure and mineral deposition. This also suggests the WNKs might be regulated downstream of estrogen and progesterone. This also begs a comment about the knowledge gap in understanding upstream regulators of WNKs; this is an important and poorly examined field of study.

WNKs in unicellular organisms and plants

Originally thought to be expressed only in multicellular eukaryotes, WNKs are present in unicellular eukaryotes such as *Chlamydomonas reinhardtii, Giardia lamblia* and *Phytophthora sojae* [97]. These unicellular WNKs are similar in size and domain organization to the WNKs found in multicellular plants such as mustard (*Arabidopsis thaliana)* and rice (*Oryza sativa)*. The kinase and the autoinhibitory domains are conserved among unicellular and multicellular plants and mammals. Discovery and phylogenetic analyses of plant and unicellular WNKs indicate that WNKs represent an ancient class of proteins whose importance has been underrated (Figure 2).

Plant WNKs have not had much attention until recently and only two model systems namely *Arabidopsis* and rice (*Oryza sativa*) have been used to study them. The *Arabidopsis* genome encodes 11 WNK kinases and the *Oryza* genome encodes for five of them [97]. The first *Arabidopsis* WNK was found as an interacting partner of the circadian clock protein APRR3 (*Arabidopsis* pseudo response regulator 3) in a yeast two hybrid analysis [98]. APRR3 is a part of a quintet of proteins whose transcription is regulated in response to the circadian rhythm and these have been thought to be the regulators of the clock genes. Each of the APRR transcripts starts to accumulate within 2h intervals after dawn in the sequence APRR9-APRR7-APRR5-APRR3-APRR1 [99, 100]. The *Arabidopsis* WNK1 (AtWNK1) can interact with APRR3 and phosphorylate it. It can also associate with APRR5, but not with other APRR proteins. Expression of AtWNK1 is also regulated in tandem with APRR3 in response to the circadian rhythm [98, 101]. Similarly, expression of the rice

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Mus             SQQQDDIEELETKAVGMSNDGRFLKFDIEIGRGSFKTVYKG 238
Homo            SQQQDDIEELETKAVGMSNDGRFLKFDIEIGRGSFKTVYKG 238
Glycine         MYKGRFGGKAELGYVETDPSGRYGRFRDILGKGAVKVVYRA 41
Arabidopsis     MNNLSYLEPDYSEFVEVDPTGRYGRYNEVLGKGASKTVYRA 41
                :          *  .  **: ::   :*:*: *.**:.
Mus             LDTETTVEVAWCELQDRKLTKSER--QRFKEEAEMLKGLQHPNIVRFYDSWESTVKGKKC 296
Homo            LDTETTVEVAWCELQDRKLTKSER--QRFKEEAEMLKGLQHPNIVRFYDSWESTVKGKKC 296
Glycine         FDEVLGREVAWNQVKLGDVFHSPDLLPRLYSEVHLLKNLEHDSIMTFHDSWIDVHC--RT 99
Arabidopsis     FDEYEGIEVAWNQVKLYDFLQSPEDLERLYCEIHLLKTLKHKNIMKFYTSWVDTAN--RN 99
                :*     **** :::  .. :*     *:  * .:** *:* .*: *: ** ..    :
Mus             IVLVTELMTSGTLKTYLKRFKVMKIKVLRSWCRQILKGLQFLHTRTPPIIHRDLKCDNIF 356
Homo            IVLVTELMTSGTLKTYLKRFKVMKIKVLRSWCRQILKGLQFLHTRTPPIIHRDLKCDNIF 356
Glycine         FNFITELFTSGTLREYRKKYQRVDIRAVKNWARQILSGLEYLHSHDPPVIHRDLKCDNIF 159
Arabidopsis     INFVTELFTSGTLRQYRLRHKRVNIRAMKHWCRQILRGLHYLHSHDPPVIHRDLKCDNIF 159
                : ::***:*****: *  :.: :.*:.:: *.**** **.:**:: **:**********
Mus             ITGPTGSVKIGDLGLATLKRAS-FAKSVIGTPEFMAPEMYEEKYDESVDVYAFGMCMLEM 415
Homo            ITGPTGSVKIGDLGLATLKRAS-FAKSVIGTPEFMAPEMYEEKYDESVDVYAFGMCMLEM 415
Glycine         INGHLGQVKIGDLGLAAILRGSQHAHSVIGTPEFMAPELYEEEYNELVDIYSFGMCMIEI 219
Arabidopsis     VNGNQGEVKIGDLGLAAILRKS-HAAHCVGTPEFMAPEVYEEAYNELVDIYSFGMCILEM 218
                :.*  *.*********:: * * .*   :*********:*** *:* **:*:****::*:
Mus             ATSEYPYSECQNAAQIYRRVTSGVKPASFDKVAIPEVKEIIEGCIRQNKDERYSIKDLLN 475
Homo            ATSEYPYSECQNAAQIYRRVTSGVKPASFDKVAIPEVKEIIEGCIRQNKDERYSIKDLLN 475
Glycine         FTSEFPYSECSNPAQIYKKVTSGKLPEAYYRIHDLEAQKFVGKCSAN-VSERLSAKELLL 278
Arabidopsis     VTFDYPYSECTHPAQIYKKVMSGKKPDALYKVKDPEVKCFIEKCLAT-VSLRVSARELLD 277
                 * ::***** :.****::* **  * :  ::   *.: ::  *     . * * ::**
Mus             HAFFQEET
Homo            HAFFQEET
Glycine         DPFLATEQ
Arabidopsis     DPFLRIDD
                ..*:  :
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Figure 2a. Alignment of the kinase domains of WNK1 isoform 1 of mouse (*Mus musculus*), WNK1 isoform 1 of humans (*Homo sapiens*), soybean (*Glycine max*) and mustard (*Arabidopsis thaliana*) showing remarkable conservation among the different species.

Figure 2b. Schematic of the domain organization of mammalian and plant WNKs showing the similarities and differences between the two.

WNK1 (OsWNK1) is also regulated in response to the light-dark cycle [102].

Subsequent to this study, the different plant WNKs were mapped within the *Arabidopsis* genome and the roles of other plant WNKs studied. The plant WNKs were originally classified as part of the mitogen activated protein kinase (MAPK) family. However, sequence comparison revealed that they are lacking the catalytic lysine in subdomain II which is a hallmark of WNKs. In plant WNKs, this lysine is most often replaced by an arginine residue (asparagine in *Phycomyces*) while in

mammalian WNKs, a cysteine residue replaces the catalytic lysine [97, 101].

WNKs have also been implicated in regulating flowering time in *Arabidopsis* by interacting with embryonic flower 1 (EMF1) [103]. AtWNK1 knockouts have delayed flowering while knockout mutants in AtWNK2, AtWNK5 and AtWNK8 genes cause early flowering [104] suggesting that the different AtWNKs have a complicated and concerted action in regulating the photoperiod and therefore flowering times. In addition, AtWNK8 interacts with and phosphorylates the subunit C of the vacuolar ATPase of *Arabidopsis* (VHA-C) *in vitro*. This implicates AtWNK in the regulation of energy and pH balance in plants [97].

Similar to the roles of mammalian WNKs, the soybean (*Glycine max*) WNK1 (GmWNK1) has been implicated in regulating lateral root growth and maintaining the root architecture in response to osmotic stresses and downstream of abscisic acid (ABA) signaling [105]. Using a transgenic plant constitutively expressing GmWNK1, the same group showed that plant WNKs are also involved in maintaining salt balance and homeostasis in plants. These transgenic plants show altered ABA levels and altered expression of ABA responsive genes and they are more tolerant to osmotic stresses during seed germination and seedling development [106]. Thus, plant and animal WNKs are evolutionarily conserved proteins with some similar functions in regulating salt homeostasis among other more divergent functions specific to the kingdom.

CONCLUSIONS AND PERSPECTIVES

Despite the discovery of WNK1 more than a decade ago, the functions and properties of this unique family of proteins are still being determined. This is due in part to the difficulty in working with these proteins. WNKs are large proteins whose expression and purification still remains a challenge. As such, most studies with WNKs have been carried out using transient and heterologous overexpression systems using fragments. It remains to be seen whether the functions of the full length proteins differ from those of the fragments and whether endogenous functions differ from those of overexpressed proteins in heterologous systems.

Several exciting studies implicate WNKs in a wide variety of cellular processes suggesting that this family of proteins will have many as yet unknown functions. Implication of WNKs in several disease systems also attests to a wider role of these proteins. Insights from plant WNKs suggest a role for WNKs in maintaining circadian rhythms and flowering times. WNKs are now known to be expressed in ancient unicellular organisms suggesting that WNKs date further back in the evolutionary timescale.

All of these studies point to the fact that the with-no-lysine family of proteins has come a long way from their humble beginnings. They are now implicated in a much wider array of cellular physiology and our appreciation of their importance is only going to increase with time.

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