IDENTIFYING NOVEL FUNCTIONS OF THE WNK PATHWAY

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DEDICATION

To my family, friends and mentors, who made my journey possible

# IDENTIFYING NOVEL FUNCTIONS OF THE WNK PATHWAY

by

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DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center

Dallas, Texas

December, 2017

IDENTIFYING NOVEL FUNCTIONS OF THE WNK PATHWAY

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The University of Texas Southwestern Medical Center at Dallas, 2017

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The with no lysine [K] (WNK) pathway consists of WNK kinases, their downstream target kinases, oxidative stress responsive (OSR)1 and SPS/Ste20-related proline-alanine-rich kinase (SPAK), and OSR1/SPAK substrates, cation chloride cotransporters. The pathway regulates ion transport across cell membranes, among other functions, and is implicated in human diseases including hypertension, cancer and neurological diseases. However, the functions of WNK pathway beyond cotransporter regulation have not been extensively studied. The purpose of my work has been to understand novel functions of the WNK pathway. I demonstrated that WNK1, largest and ubiquitously expressed WNK isoform, is an inhibitor of autophagy, an intracellular degradation pathway. WNK1 inhibited the class III phosphatidylinositol 3-kinase (PI3KC3)

complex which acts upstream in the autophagy pathway. In addition, WNK1 inhibited the unc-51–like kinase 1 (ULK1) complex that acts upstream of PI3KC3. WNK1 also inhibited AMP-activated protein kinase (AMPK), the upstream activator of ULK1. The actions of WNK1 on the AMPK-ULK1 axis only partially mediated its effects on autophagy. WNK1 directly bound UV radiation resistance-associated gene (UVRAG) in vitro and had an overlapping localization with it in cells, and autophagy induction led to a decrease in this property. OSR1 had no significant effect on autophagy while SPAK acted as an autophagy inhibitor. Therefore, WNK pathway most likely inhibits autophagy through multiple mechanisms. I also discovered that OSR1 regulates the cellular localization of inward-rectifier potassium channel (Kir) 2.3 that contains an OSR1/SPAK recognition motif, and is activated by WNK. OSR1 promoted Kir2.3 localization to shift towards the cell membrane in the presence of sodium chloride. Similar to OSR1, WNK kinase activity also promoted the change in localization of Kir2.3 elicited by NaCl. Therefore, I suggest that activated WNK induces Kir2.3 channel activity by driving it to the cell membrane.

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| | |
|---|---|
| 5-aza-dc | 5-aza-2'-deoxycytidine |
| ACSM3 | acetyl-CoA medium-chain synthetase 3 |
| ADP | adenosine diphosphate |
| AMP | adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| AP-1 | activator protein-1 |
| AR | androgen receptor |
| AS160 | AKT substrate of 160 kDa |
| ASK | apoptosis signal-regulating kinase |
| ATG | autophagy-related |
| ATM | ataxia-telangiectasia mutated |
| ATP | adenosine triphosphate |
| BAME | N-alpha-benzoyl L-arginine methyl ester |
| BK | large-conductance calcium-activated potassium channel |
| BLAST | Basic Local Alignment Search Tool |
| CaMKK2 | Calcium/calmodulin-dependent protein kinase kinase 2 |
| cAMP | cyclic adenosine monophosphate |
| CCC | cation-chloride cotransporters |
| CCD | cortical collecting duct |
| Cdk | cyclin-dependent kinase |
| CK1 | casein kinase 1 |
| CMV | cytomegalovirus |
| DAPI | 4',6-diamidino-2-phenylindole |
| DAPK | death-associated protein kinase |
| DCT | distal convoluted tubule |
| DFCP1 | double FYVE-containing protein 1 |
| DMEM | Dulbecco's modified Eagle medium |
| DMSO | dimethyl sulfoxide |
| DTT | dithiothreitol |
| EBSS | Earl's balanced salt solution |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EGTA | ethylene glycol tetraacetic acid |
| $E_K$ | $K^+$ equilibrium potential |
| $E_m$ | membrane potential |
| ENaC | epithelial sodium channel |
| ER | estrogen receptor |
| ERK | extracellular signal-regulated kinase |

| | |
|---|---|
| ES | embryonic stem |
| FBS | fetal bovine serum |
| FDA | Food and Drug Administration |
| FIP200 | FAK family kinase interacting protein of 200 kDa |
| GABA | gamma-aminobutyric acid |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GEF | guanine nucleotide exchange factor |
| GFP | green fluorescent protein |
| GPCR | G protein coupled receptors |
| GST | glutathione S-transferase |
| GTP | guanosine triphosphate |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HDAC | histone deacetylase |
| HRP | horseradish peroxidase |
| HSAN | hereditary sensory and autonomic neuropathy |
| HSN | hereditary sensory neuropathy |
| HEK | human embryonic kidney |
| IgA | immunoglobulin A |
| IGF | insulin-like growth factor |
| IPTG | isopropyl β-D-1-thiogalactopyranoside |
| ITSN | intersectin |
| JNK | c-Jun N-terminal kinase |
| $K_{ATP}$ | ATP-sensitive potassium channels |
| $K_{Ca}$ | calcium-activated potassium channels |
| KCC | potassium-chloride cotransporter |
| $K_G$ | G protein gated potassium channels |
| KLHL | kelch-like |
| Kir | inward-rectifier potassium channel |
| $K_V$ | voltage-gated potassium channel |
| Kyn | kynurenine |
| LC3 | light chain 3 |
| LINGO | leucine-rich repeat and Ig domain-containing |
| LKB1 | liver kinase B1 |
| LPA | lysophosphatidic acid |
| MAPK | mitogen-activated protein kinase |
| MAPKAPK | MAPK-activated protein kinase |
| MEK | MAPK/ERK kinase |
| MEKK | MEK kinase |
| MMP | matrix metalloproteinase |
| MO25 | mouse protein 25 |
| mTOR | mammalian target of rapamycin |
| NCC | sodium-chloride cotransporter |
| NDP | nucleotide diphosphate |

| | |
|---|---|
| NEDD4-2 | neural precursor cell-expressed developmentally downregulated gene 4-2 |
| NF-κB | nuclear factor Kappa B |
| NgR | Nogo receptor |
| NKCC | sodium-potassium-2-chloride cotransporter |
| NLS | nuclear localization signal |
| NRBF2 | nuclear receptor binding protein 2 |
| NSCLC | non-small cell lung cancer |
| OSR1 | oxidative stress-responsive 1 |
| PAK | p21 (Rac-1/Cdc42)-activated kinase |
| PAQR3 | progestin and adipoQ receptor family member III |
| PBS | phosphate buffered saline |
| PCNA | proliferating cell nuclear antigen |
| PDAC | pancreatic ductal adenocarcinoma |
| PDK | phosphoinositide-dependent kinase |
| PF2 | PASK/Fray homology 2 |
| PHA | pseudohypoaldosteronism |
| PI | phosphatidylinositol |
| PI3K | phosphatidylinositol 3-kinase |
| PI3KC3 | class III PI3K complex |
| PI3P | phosphatidylinositol 3-phosphate |
| $PIP_2$ | phosphatidylinositol 4,5-bisphosphate |
| PKA | cAMP-dependent protein kinase |
| PKC | protein kinase C |
| PSD95 | post synaptic density 95 |
| PTM | post-translational modifications |
| PVDF | polyvinylidene difluoride |
| RELT | receptor expressed in lymphoid tissues |
| ROMK | renal outer-medullary potassium channel |
| ROS | reactive oxygen species |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SGK | serum- and glucocorticoid-induced protein kinase |
| SH3 | Src homology 3 |
| SLC12A | solute carrier family 12 |
| SNP | single nucleotide polymorphism |
| SPAK | ste20-related proline-alanine-rich kinase |
| SQSTM1 | Sequestosome 1 |
| STK39 | serine/threonine kinase 39 |
| SUR | sulfonylurea receptor |
| TAME | N-alpha p-tosyl L-arginine methyl ester |
| TBC1D4 | Tre-2/BUB2/CDC16 domain family member 4 |
| TCGA | The Cancer Genomic Atlas |
| TCR | T cell receptor |
| TE | Tris-EDTA |

| | |
|---|---|
| TGF | transforming growth factor |
| TLCK | N-alpha p-tosyl L-lysine chloromethyl ketone |
| TMZ | temozolomide |
| TNF | tumor necrosis factor |
| TNFR | tumor necrosis factor receptor |
| TRAIL | tumor necrosis factor-related apoptosis-inducing ligand |
| TRPV | transient receptor potential vanilloid |
| ULK1/2 | unc-51-like kinase 1/2 |
| UVRAG | UV radiation resistance-associated gene |
| VEGFR | vascular endothelial growth factor receptor |
| Vps | vacuolar protein sorting |
| WIPI | WD-repeat protein interacting with phosphoinositides |
| WNK | with no lysine [K] |
| ZEB1 | Zinc finger E-box binding homeobox 1 |

# Chapter 1

## Introduction and the Role of WNK Pathway in Cancer

(Parts of Chapter 1 are derived from "Gallolu Kankanamalage, S., Lee, A. Y., Wichaidit, C., Lorente-Rodriguez, A., Shah, A. M., Stippec, S., Whitehurst, A. W., Cobb, M. H. Multistep regulation of autophagy by WNK1. *Proc Natl Acad Sci U S A*, 2016. 113 (50): p. 14342-14347" and "Gallolu Kankanamalage, S., Lee, A. Y., Wichaidit, C., Lorente-Rodriguez, A., Shah, A. M., Stippec, S., Whitehurst, A. W., Cobb, M. H. WNK1 is an unexpected autophagy inhibitor. *Autophagy*, 2017. 13(5): p. 969-970.")

## Introduction

### The WNK Pathway Overview

The with no lysine [K] (WNK) pathway is a vital signaling pathway that exists in organisms to regulate ion transport across cell membranes. This input to ion transport enables cells to maintain intracellular ionic homeostasis and maintain the cell volume, while making it possible for the cells to respond to varying extracellular osmotic conditions [1, 2]. This pathway is comprised of WNK kinases, their downstream kinases oxidative stress responsive (OSR)1 and SPS/Ste20-related proline-alanine-rich kinase (SPAK, also known as STK39) and downstream cation-chloride cotransporters (CCC) of the solute carrier 12 (SLC12A) family [2]. The activated WNK kinases phosphorylate and activate OSR1 and SPAK [3-5]. Once activated, OSR1 and SPAK phosphorylate and regulate downstream CCCs of the SLC12A family, controlling CCC-mediated ion transport across the cell membrane [6-8] (Figure 1.1).

The WNK pathway regulates physiological functions such as sodium reabsorption in kidney, maintaining blood pressure [2, 9] and GABAergic neuronal function [10]. In addition, WNK pathway components are linked to the regulation of cell proliferation [11, 12], cell death [13], cell migration [14-17], endocytosis [18-21], angiogenesis [16, 22], inflammation [23-26] and spermatogenesis [27]. They also impact multiple signal transduction pathways, including the

ERK1/2 and ERK5 MAP kinase pathways [11, 28]. Many of these functions will be discussed in detail with respect to their roles in cancer.

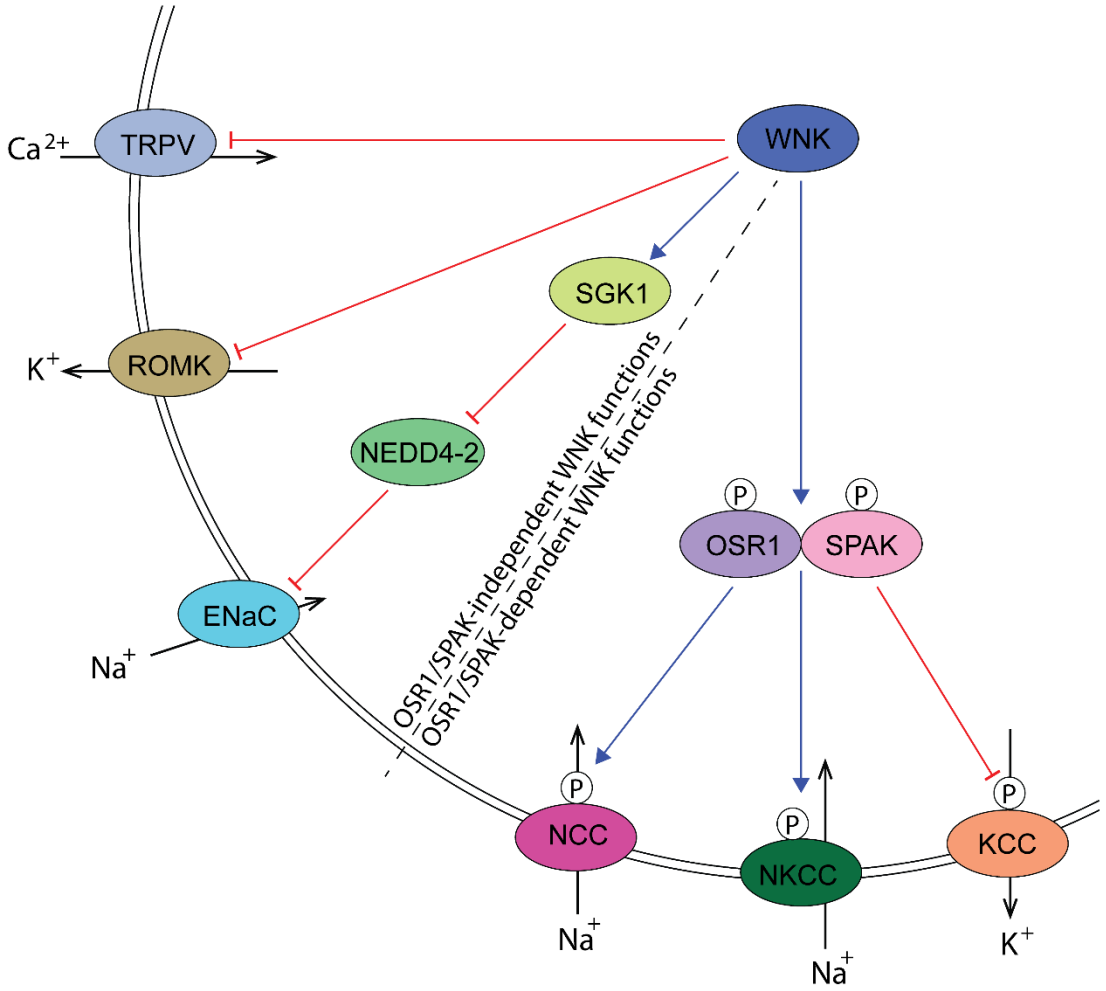



Figure 1.1. The regulation of CCCs and ion channels by the WNK pathway through OSR1/SPAK-dependent and -independent functions.

**WNK Pathway Components in Human Diseases**

Mutations in WNK kinases and their upstream regulators cause increased expression of WNKs 1 and 4, and ion reabsorption defects in kidney that lead to hypertension-related genetic diseases. A large intronic deletion of WNK1 was shown to increase expression of the protein and cause an autosomal-dominant genetic disease called the Gordon's syndrome, also known as

Pseudohypoaldosteronism type II (PHA-II) and familial hyperkalemic hypertension. This syndrome was characterized by hypertension caused by increased kidney salt reabsorption, metabolic acidosis caused by impaired kidney $H^+$ secretion and hyperkalemia which resulted from decreased kidney $K^+$ secretion. Missense mutations of WNK4 in its coding sequence also caused the same genetic disease [29]. Kelch-like 3 (KLHL3) and Cullin-3 which regulate WNK expression in the cells were also mutated in some Gordon's syndrome patients [30-33]. These mutations impair KLHL-Cullin-3-mediated degradation of WNKs, causing their overexpression [34-36]. The mutations in WNK4 also prevented its interaction with KLHL3 [32, 36]. Therefore, increased WNK expression caused by different mechanisms can cause the same genetic disease that results in hypertension.

In addition, mutations in WNK1 can cause a neurological disorder called hereditary sensory and autonomic neuropathy type II (HSAN-II). It is an autosomal recessive genetic disorder that results in loss of peripheral neurons. This neuron loss causes abrogated perception of pain, heat and touch [37, 38]. The mutations causing HSAN-II are located in exon 10 (also called exon HSN2, named for the disease hereditary sensory neuropathy type II) which is located in a WNK1 form expressed in the nervous system [37, 38].

**WNKs**

The WNK protein kinase family is an evolutionarily conserved, atypical group of S/T kinases. Their conserved catalytic domain ATP-binding lysine residue has shifted from beta strand 3 to the glycine-rich loop [39, 40] (Figure 1.2). WNKs are the only kinases in the eukaryotic protein kinase superfamily with this unusual arrangement. This arrangement confers on them unique structural

and functional properties [41]. There are four WNK proteins in mammals, of which WNK1 is the largest, over 2,000 residues, and most widely expressed [40, 42] (Table 1).

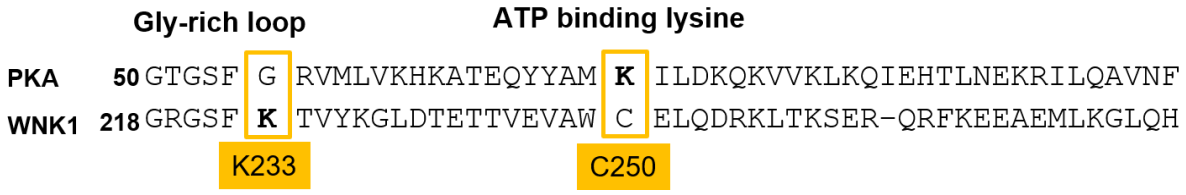



Figure 1.2. Change of position of catalytic lysine residue within the WNK kinase domain. This figure is modified from Figure 1B of Min et al. 2004 [43] and Figure 1A of Huang et al. 2007 [2].

Table 3.1. The mammalian WNK family members

| Gene | Chromosomal Location | Gene Size (bp) | Protein Size (Amino Acids) | Exon Number | Isoforms |
|---|---|---|---|---|---|
| *WNK1* | 12p13.33 | 158,860 | 2382 | 31 | >6 |
| *WNK2* | 9q22.31 | 136,418 | 2297 | 38 | >4 |
| *WNK3* | Xp11.22 | 165,873 | 1800 | 25 | 4 |
| *WNK4* | 17q21.2 | 17,394 | 1243 | 18 | 3 |

The table was generated using information obtained from following online resources. Genecards (http://www.genecards.org/), NCBI Gene (https://www.ncbi.nlm.nih.gov/gene/), UniProt (http://www.uniprot.org/) and Pfam (http://pfam.xfam.org/)

In contrast to ubiquitous expression of WNK1, other WNK isoforms display more tissue-specific expression patterns. WNK2 is largely expressed in brain, heart and colon. WNK3 is primarily expressed in brain, liver and small intestine. WNK4 is expressed primarily in epithelia of kidney, skin, colon, liver and lung [44].

All WNK enzymes contain an N-terminal kinase domain except KS-WNK1, a kidney-specific form translated from an mRNA transcribed from a second initiation site that eliminates much of the kinase domain [44-46]. The kinase domain is followed by an autoinhibitory domain which is also referred to as a PF2-like (PASK/Fray homology (from PASK, an alternative name for SPAK, and Fray, the OSR1/SPAK protein found in fly)) domain, due to its high similarity to the C-terminal PF2 domain of OSR1 and SPAK [44, 45, 47, 48]. The kinase domains among different WNK isoforms show a high degree of similarity [2, 45]. In addition, WNKs contain multiple coiled-coils regions [2, 44] (Figure 1.3) and multiple PXXP motifs [49]. WNK1 contains 24 potential PXXP motifs and two proline-rich regions which concentrate some of those motifs [39]. These motifs facilitate the binding of WNK kinases to SH3 domain-containing proteins such as intersectin (ITSN) [19]. In addition, WNKs contain RFXV motifs which facilitate their binding to the C-terminal PF2 domains of substrate kinases OSR1 and SPAK [8]. Despite the presence of these domains and motifs, a large portion of the WNK structure is predicted to be disordered [44] Pfam (http://pfam.xfam.org/).

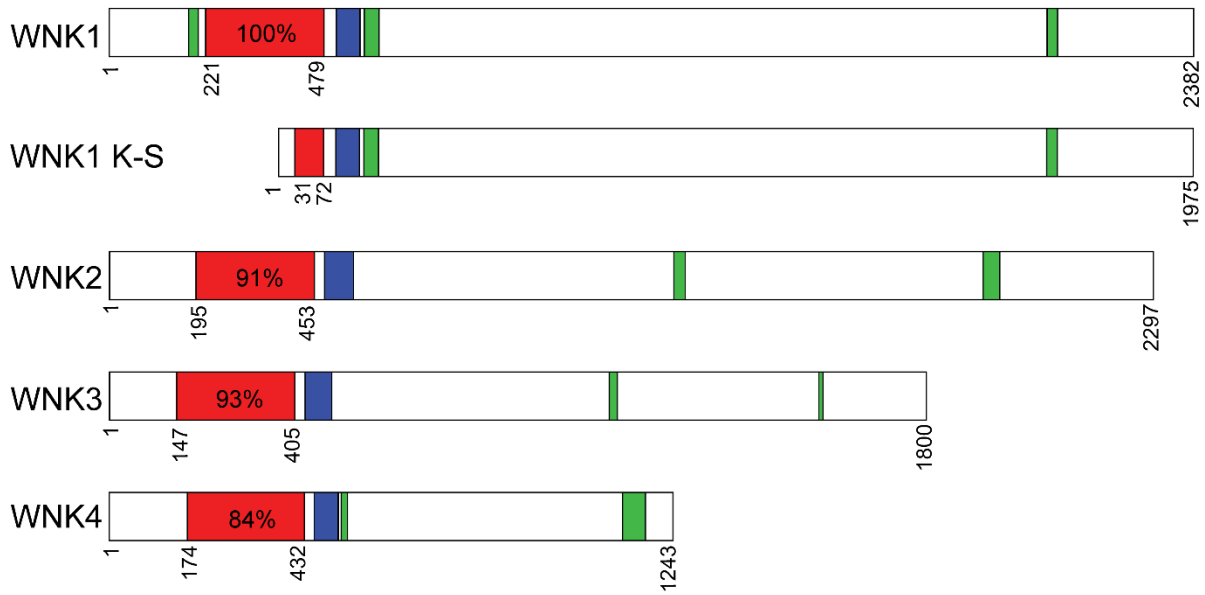


Figure 1.3. Organization of mammalian WNK protein structure. Red - kinase domain, Blue - autoinhibitory domain, Green - coiled-coil regions. This figure was generated using information obtained from following resources - Genecards (http://www.genecards.org/), NCBI Gene (https://www.ncbi.nlm.nih.gov/gene/), UniProt (http://www.uniprot.org/), Pfam (http://pfam.xfam.org/), and adapted from Figure 10 of Gagnon and Delpire 2012 [48] and Figure 1A of Heise et al. 2010 [49]. Percentage identities of kinase domains to WNK1 kinase domain were obtained by Protein BLAST (Align Sequences) - NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The reference sequences used, WNK1 - NP_061852.3, WNK2 - NP_001269323.1, WNK3 - NP_065973.2, WNK4 - NP_115763.2. K-S - Kidney specific

**OSR1/SPAK**

OSR1 and SPAK are S/T kinases which act downstream of WNKs [3-5, 50, 51]. These two proteins are highly similar and thought to have arisen from a gene duplication event [6, 48]. They contain N-terminal kinase domains which share high sequence identity [6]. In addition, they contain C-terminal regulatory regions. The conserved PF2 domain is at the C-terminus of the protein [3, 6]. SPAK structure differs from OSR1 as it contains a proline and alanine-rich region (sometimes called a PAPA box) prior to the kinase domain at the N-terminus. The functional significance of this region is still not clear (Figure 1.4). In addition, SPAK also contains a putative nuclear localization signal (NLS) [6, 48].

OSR1 and SPAK are subjected to common mechanisms of regulation and have some common and some unique physiological functions [4-6, 16, 52]. Both OSR1 and SPAK are widely expressed in multiple tissue types. OSR1 is more abundant in lung, muscle and testis, while SPAK is highly expressed in brain, testis, stomach and salivary glands [53].

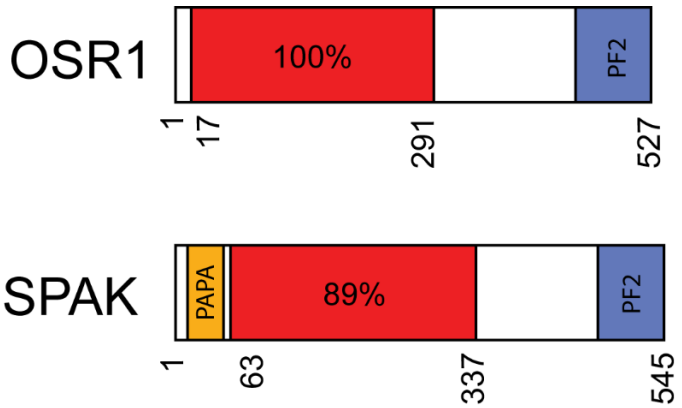



Figure 1.4. Organization of OSR1 and SPAK protein structure. Red – kinase domain. This figure was generated using information obtained from following resources. Genecards (http://www.genecards.org/), NCBI Gene (https://www.ncbi.nlm.nih.gov/gene/), UniProt (http://www.uniprot.org/), Pfam (http://pfam.xfam.org/), Piechotta et al. 2002 [6], Moriguchi et al. 2005 [3] and Gagnon and Delpire 2012 [48]. Percentage identity of SPAK kinase domain to OSR1 kinase domain was obtained by Protein BLAST (Align Sequences) - NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The reference sequences used, OSR1 - NP_005100.1, SPAK - NP_037365.2.

Table 1.4. Information on OSR1 and SPAK

| Gene | Chromosomal Location | Gene Size (bp) | Protein Size (Amino Acids) | Exon Number |
|---|---|---|---|---|
| *OXSR1* | 3p22.2 | 90,400 | 527 | 22 |
| *STK39* | 2q24.3 | 294,122 | 545 | 23 |

The table was generated using information obtained from following online resources. Genecards (http://www.genecards.org/), NCBI Gene (https://www.ncbi.nlm.nih.gov/gene/), UniProt (http://www.uniprot.org/) and Pfam (http://pfam.xfam.org/)

**Activation and Regulation of the WNK Pathway**

WNKs are activated by phosphorylation of the activation loop (S378 and S382 of WNK1) [45, 54]. Autophosphorylation of WNKs on these residues is currently thought to be the primary

means of activation. Activation is induced by hyperosmotic conditions such as elevated sodium chloride or sorbitol [39, 54], and by hypotonic/low chloride conditions [3, 54]. The WNK kinase domain directly binds chloride ions and was suggested to release bound chloride under low-chloride conditions, thereby releasing the restraint on its autoactivation [41]. In addition, apoptosis signal-regulating kinase 3 (ASK3) acts upstream of WNK kinases by inhibiting them. Hypertonic conditions inhibited ASK3, thereby releasing the inhibition of WNK1 activation [55]. Unpublished work from the Goldsmith lab suggests that WNK1 is directly activated by pressure, although the detailed mechanism has not yet been delineated. Other less direct mechanisms are also possible.

WNKs form homo- and heterodimers and tetramers [45, 54]. All WNK enzymes except WNK4 phosphorylate WNK1 [56]. WNK1 and WNK4 bind to each other via their kinase domains and through coiled-coils [54, 56, 57]. In addition, WNK1 autoinhibitory domain acted to inhibit the activity of WNK2 and WNK4 [54]. Therefore, these evidences suggest that WNK kinases could form complexes with other WNK family members and might regulate their activity by this mechanism (Figure 1.5).

An important component of WNK regulation comes from degradative mechanisms. WNK kinases bind to KLHL proteins and are ubiquitinated by Cullin-3, an E3 ubiquitin ligase, subsequently driving WNKs to be degraded by the ubiquitin-proteasome pathway [32, 33] (Figure 1.5). In addition, it was shown that WNK expression was regulated by autophagy-mediated lysosomal degradation pathway [58]. Perhaps degradative mechanisms are relied on to control the scaffolding function of WNK proteins.

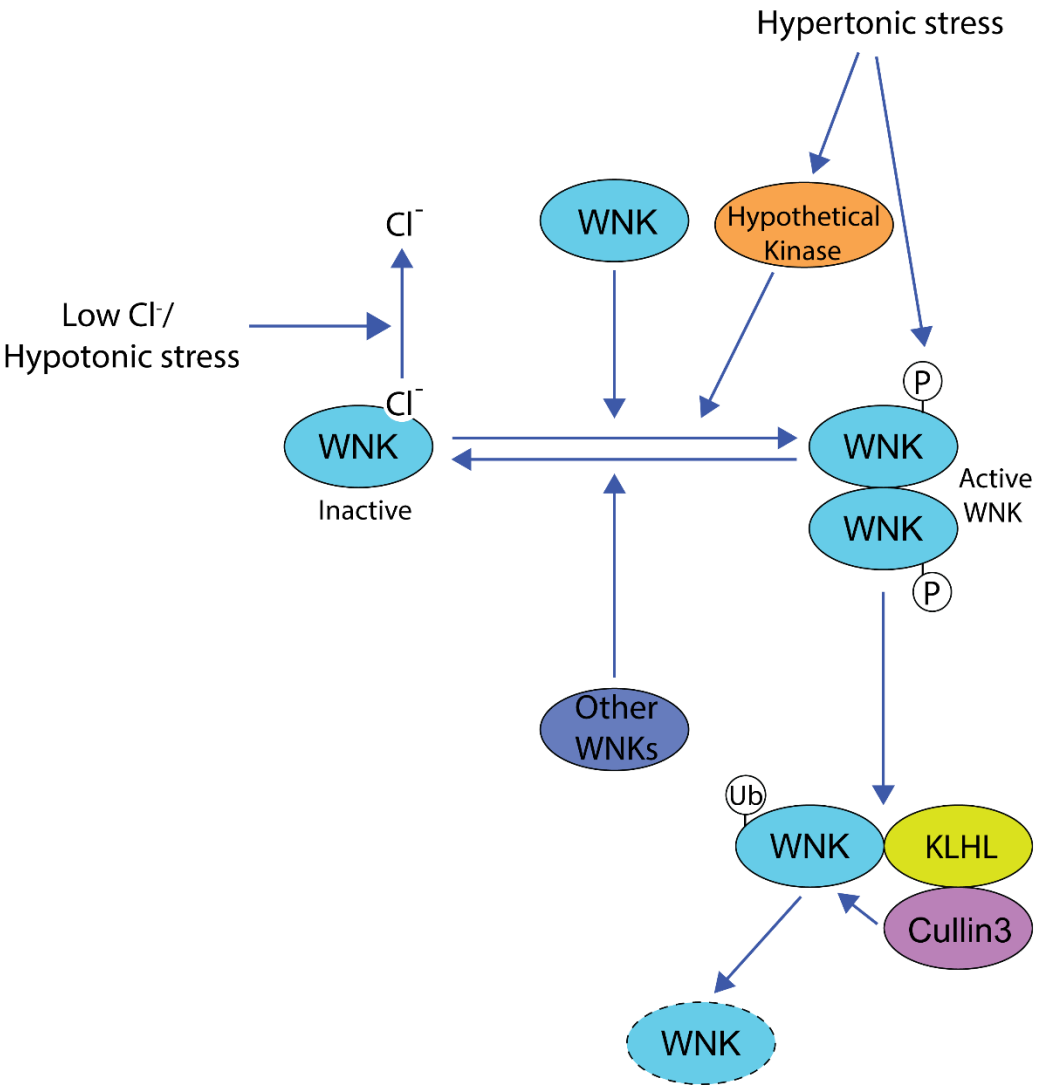



Figure 1.5. The regulation of WNK kinase expression and activity by different stimuli.

WNKs bind their downstream kinases OSR1 and SPAK through the interactions of their RFXV motifs with PF2 domains of OSR1 and SPAK [3, 5, 53]. WNKs can phosphorylate both OSR1 and SPAK, and activate them [3-5, 59-61]. The conserved sites, S380 of SPAK (Rat) and S325 of OSR1 (Mouse) were phosphorylated by WNKs and these phosphorylations increased their activity. T243 of SPAK and corresponding T185 in OSR1 which are located in the activation loop

of kinase domains were phosphorylated by WNKs and were necessary for the activation of these kinases [3-5]. Therefore, WNKs serve as upstream activators of OSR1 and SPAK.

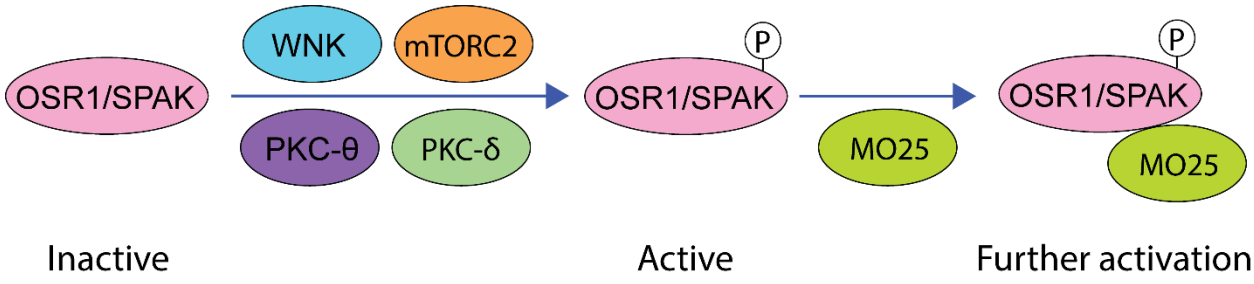



Figure 1.6. The regulation of OSR1/SPAK activity by different proteins through phosphorylation and binding.

In addition, other protein kinases beside WNKs have been shown to activate OSR1 and SPAK. The mammalian target of rapamycin complex 2 (mTORC2) further enhances activation of OSR1 in the presence of osmotic stress by phosphorylation of OSR1 at S339 [62]. SPAK was phosphorylated at S311 by protein kinase C (PKC)-θ, and was activated in a PKC-θ-dependent manner [63]. SPAK was also activated dependent on PKC-δ [64]. Mouse protein 25 (MO25) can also bind and activate OSR1 and SPAK. Both MO25α and β isoforms can increase the activity of OSR1/SPAK by around 100-fold. This activation was suggested to require the WNK-mediated phosphorylation of OSR1 and SPAK [65]. Therefore, OSR1 and SPAK are subjected to activation by multiple proteins (Figure 1.6).

In addition, OSR1 and SPAK form domain-swapped dimers [66, 67]. Therefore, oligomerization could be another regulatory mechanism for OSR1 and SPAK as it allows these proteins to bind to multiple effectors [8].

## Ion Transport Regulation by the WNK Pathway

### Regulation of Cation Chloride Cotransporters

Activated OSR1 and SPAK phosphorylate CCCs of the SLC12A family. The N-terminal regions of these cotransporters contain RFXV motifs that mediate the binding to OSR1 and SPAK through their PF2 domains [5, 6]. Activated OSR1 and SPAK directly phosphorylate sodium-potassium-2-chloride cotransporter (NKCC)1, NKCC2 and sodium-chloride cotransporter (NCC) in their N-terminal regulatory regions, thereby increasing their activity. Most of these phosphorylation sites are similar among the cotransporters [3, 5, 8, 65, 68, 69] (Figure 1.7).

WNKs can inhibit the activities of all potassium-chloride cotransporter (KCC) 1-4 isoforms, and this effect was suggested to be mediated by SPAK [1, 70-73]. As suggested, activated OSR1/SPAK phosphorylate all KCC1-4 proteins at multiple sites. All KCC isoforms were phosphorylated at two sites (one N-terminal and one C-terminal) except KCC3A, which was further phosphorylated at an additional N-terminal site [74].

### Regulation of Ion Channels

#### Renal Outer-Medullary Potassium Channel

WNKs regulate some ion channels by mechanisms that appear to be independent of OSR1 and SPAK. WNK1 and WNK4 inhibit the renal outer-medullary potassium channel (ROMK)/Kir1.1 by enhancing its clathrin-mediated endocytosis. This effect was independent of WNK kinase activity [19, 75-77]. However, this effect was dependent on their binding to ITSN and required PXXP motifs of WNK kinases and SH3 domains of ITSN. The PHA-II causing mutations that increase WNK4 expression enhanced the binding to ITSN and caused increased endocytosis of ROMK [19, 36]. In addition, WNK4 interacted with ROMK and PHA-II causing

mutations enhanced this interaction [19]. Moreover, it was discovered that a kidney specific transcript generating a WNK1 protein lacking enzymatic activity could inhibit the endocytosis of ROMK by counteracting the actions of the long WNK1 isoform [76, 78]. Therefore, the WNK kinases regulate ROMK by multiple mechanisms.

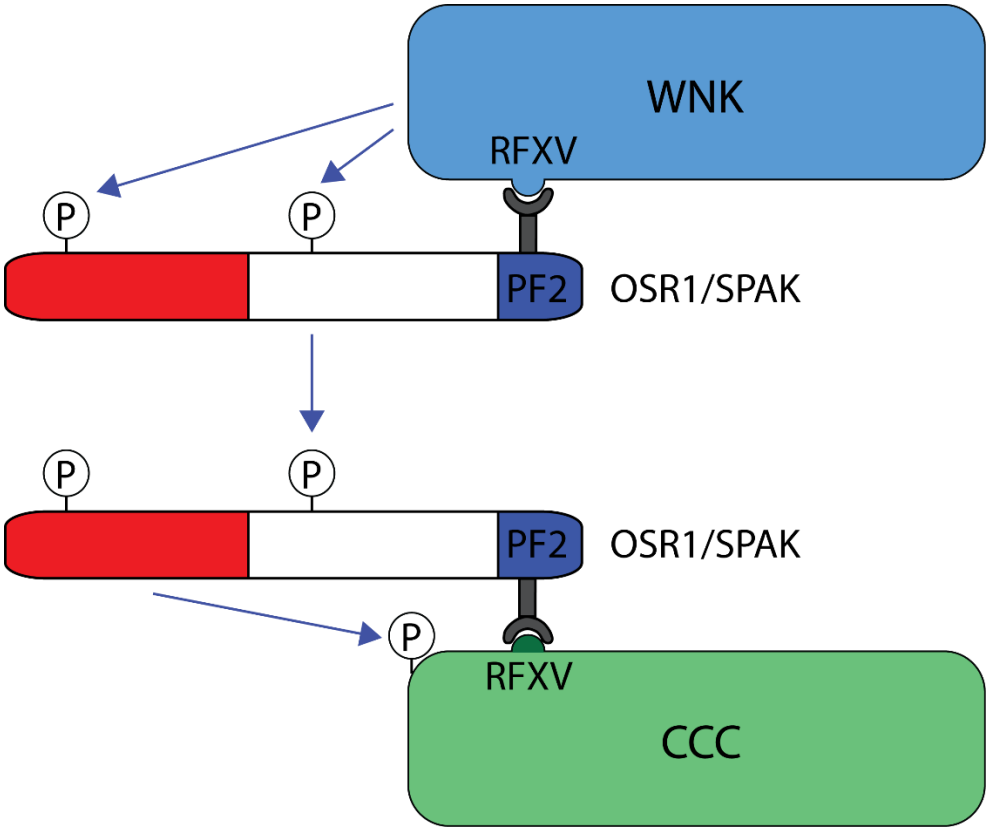



Figure 1.7. The activation of OSR1/SPAK by upstream WNK-mediated phosphorylation and their subsequent phosphorylation of cation chloride cotransporters.

**Epithelial Sodium Channel**

WNK1 activates the epithelial sodium channel (ENaC) via the serum- and glucocorticoid-induced protein kinase (SGK)1. SGK1 bound to the N-terminus of WNK1. The regulation of ENaC by WNK1 was dependent on the E3 ubiquitin ligase NEDD4-2 which is phosphorylated by SGK1. This released ENaC from NEDD4-2 binding and promoted its activity through increased expression on the cell membrane [79, 80]. This action was independent of WNK kinase activity and enhanced by the phosphatidylinositol 3-kinase (PI3K)-AKT- or SGK1-mediated phosphorylation of WNK1 at T58 (corresponding site for human T60) [81]. All four mammalian WNKs activated SGK1 and ENaC [49, 82]. WNK4 was phosphorylated by both SGK1 and AKT at S47 which increased SGK1 activation [49]. Therefore, this suggests a common mechanism for the regulation of ENaC by WNK kinases.

**Transient Receptor Potential Vanilloid Channels**

WNK4 regulates the transient receptor potential vanilloid (TRPV)-5, which mediates calcium reabsorption in kidney [83]. The overexpression of WNK4 inhibited the cell surface localization and activity of TRPV5, which was dependent on residues 444-584 of WNK4. This inhibition was caused by increased caveolae-mediated endocytosis of TRPV5 by WNK4, and was dependent on ITSN. WNK4 increased the dynamic range of TRPV5 activation by PKC [20]. This shows that WNK kinases can inhibit both clathrin and caveolin-mediated endocytosis.

Similar to TRPV5, TRPV4 was also regulated by WNKs. Both WNK1 and WNK4 inhibited TRPV4 by downregulating its cell surface expression. WNK4 kinase activity and a C-terminal region were necessary for the regulation of TRPV4, unlike TRPV5 [84]. Therefore, it seems that WNKs regulate TRPV channels through different mechanisms.

## WNKs in Signaling and Cancer

The involvement of the WNK pathway in human diseases such as hypertension is understood significantly through knowledge of the functions of ion transporters and channels. The last few years have produced a surge in literature that show WNK components use diverse mechanisms to regulate different aspects of cancer formation and progression. Here, I summarize the roles of WNK pathway components in cancer based on their involvement in regulating major cancer-associated signaling pathways, their connections to cancer hallmarks and enabling characteristics [85], and their aberrant expression and mutations in cancer. I focus on WNKs 1-4, OSR1 and SPAK collectively referred to as WNK pathway components.

## Interactions of WNK Pathway Components with Cancer-Related Signaling Pathways

### Cation-Chloride-Cotransporters

The WNK pathway has been shown to regulate survival, proliferation and associated signaling pathways in multiple cell types [44]. Ion cotransporters and channels are important for regulation of cellular volume alterations that play important roles in cancer-related processes such as cell proliferation, migration, apoptosis and drug-resistance [86]. Because the WNK pathway is a well-known regulator of ion channels and cotransporters, it is possible that it regulates these cancer-related cellular processes through the control of ion transport [1, 3-5, 19, 79].

WNK1 expression was upregulated in primary glioma cells and the U87 glioblastoma cell line, together with increases of OSR1 phosphorylation and NKCC1 expression and phosphorylation, showing the activation of WNK pathway in brain cancer. Interfering with the WNK1-OSR1 target NKCC1 with the diuretic bumetanide or by depletion of WNK1 or OSR1 with siRNA impaired the migration of primary glioma cells. In addition, the depletion of WNK1

and OSR1 inhibited the regulatory volume increase and intracellular $K^+$ and $Cl^-$ homeostasis in cells, providing a possible mechanism for the regulation of glioma cell migration by the WNK pathway [15]. Similar findings also implicated WNK3 in glioma [87, 88].

WNKs also inhibit apoptosis in glioblastoma cells, as inhibition of the WNK effector NKCC1 enhanced the temozolomide (TMZ)-induced apoptosis in these cells. This was suggested to have caused by the loss of regulatory volume increase in glioblastoma cells following the inhibition of NKCC1. When NKCC1 was inhibited, TMZ caused apoptotic volume decrease, activation of Caspase-3 and -8, and decrease of $K^+$ and $Cl^-$ in glioblastoma cells which suggested enhanced apoptosis [89].

Evading immune surveillance promotes cancer survival and progression, enabling tumorigenesis [85]. WNK1 inhibited integrin-mediated adhesion of T cells and enhanced T cell migration. The T cell receptor and chemokine receptors activated WNK1 and OSR1 in T cells. The chemokine-induced WNK1-dependent migration of T-cells was dependent on OSR1, SPAK and NKCC1, but T-cell adhesion was not [90].

**ERK5 MAPK**

Mitogen-activated protein kinase (MAPK) pathways are well-known regulators of proliferative and survival signaling cascades that control cancer progression [91-94]. WNK pathway components act as both upstream effectors and downstream targets of MAPK pathways. WNK1 is a positive regulator of extracellular signal-regulated kinase (ERK)5. Overexpression of active but not inactive WNK1 increased the kinase activity of ERK5, suggesting that WNK1 kinase activity is necessary for this effect. The depletion of WNK1 also abolished activation of ERK5 by low/physiological concentrations of epidermal growth factor (EGF) in HeLa cells. The WNK1-

mediated activation of ERK5 required MEK5, the upstream activator of ERK5, and MEKK2 and MEKK3, MAP3Ks in the ERK5 MAPK pathway [95]. WNK1 could co-immunoprecipitate with MEKK2/3, phosphorylate MEKK2/3 in-vitro, and activate MEKK3 in cells. Therefore, WNK1 can activate the ERK5-MAPK pathway. In this study WNK1 had no significant effect on the ERK1/2 MAPK pathway [28].

WNK1 is a positive regulator of ERK5 in hepatocellular carcinoma (HCC). NKCC2 was upregulated in an HCC cell line and acted as an upstream activator of the WNK1-ERK5 axis. Both siRNA-mediated depletion of NKCC2 and treatment with the NKCC antagonist bumetanide decreased WNK1 phosphorylation and ERK5 activation in these cells. They also blocked ERK5-mediated transcription of proliferation-promoting factors such as cyclin D1 and c-Fos [96]. This further confirms the role of WNK1 in promoting ERK5 MAPK signaling and shows that NKCCs can also act upstream of WNK kinases in addition to their role as downstream targets, perhaps by altering intracellular chloride or osmotic pressure.

**ERK1/2 MAPKs**

A recent report suggested an inhibitory function of WNK1 on the ERK1/2 MAPK pathway. WNK1 positively regulated the expression and activity of large-conductance calcium-activated potassium (BK) channel in HEK293 cells. The depletion of WNK1 increased the activation of ERK1/2 MAPK pathway whereas the overexpression of WNK1 inhibited it. This study suggested that WNK1 positively regulated the BK channel by inhibiting its ERK1/2 MAPK-mediated lysosomal degradation [97]. The stable knockdown of WNK1 inhibited proliferation and differentiation of neural progenitor clonal cell lines. WNK1 positively regulated migration and EGF-induced activation of both ERK1/2 and ERK5 MAPK pathways in these cells. This suggests

that WNK1 acts through the MAPK pathways to regulate proliferation, differentiation and migration of neural progenitor cells [98].

WNK2 was identified as a suppressor of the ERK1/2 MAPK signaling pathway in a kinase-dependent manner. The knockdown of WNK2, or the expression of a catalytically inactive WNK2 in HeLa cells, increased the activation of ERK1/2. In addition, the depletion of WNK2 also increased cell cycle progression [11]. WNK2 acted through suppressing the Rho-GTPases-dependent activation of MEK1/2, the upstream kinase activators of ERK1/2. WNK2 suppressed Rac-1 and subsequently, p21 (Rac-1)-activated kinase (PAK) 1, which phosphorylates MEK1/2 [11, 99, 100]. WNK2 also acts as an inhibitor of glioma migration. The depletion of WNK2 in glioma cells enhanced colony formation and increased invasion. This also produced increased Rac-1 activation and decreased RhoA activity, suggesting pro-migratory effects on cells. The re-expression of WNK2 in the A172 glioma cell line, which has lost endogenous WNK2 expression due to promoter methylation, reversed the growth and migration of the cells and decreased the activation of Rac-1. Therefore, it was suggested that WNK2 exerted its tumor suppressor effects on glioma cells also via the control of Rac-1 [17].

WNK2 also acted as a tumor suppressor in pancreatic ductal adenocarcinoma (PDAC) where *WNK2* was epigenetically silenced by hypermethylation. The mRNA and protein expression of WNK2 was lower in human PDAC tumor samples compared to the surrounding tissues. *WNK2* demethylation by 5-aza-2'-deoxycytidine (5-aza-dc), a methylation inhibitor, led to higher expression of WNK2 in pancreatic cancer cell lines and exogenous WNK2 expression in a PDAC cell line led to decreased colony formation. In addition, WNK2 expression negatively correlated with the activation of ERK1/2 [101]. This shows that the inhibitory effect of WNK2 on ERK1/2 MAPK pathway is present in diverse cancer types.

WNK3 acted as a negative regulator of the ERK1/2 MAPK pathway. The overexpression of WNK3 in COS7 cells decreased the activation of the ERK1/2 MAPK signaling in a dose-dependent manner. The depletion of ERK1/2 in cells prevented the WNK3-mediated increase of NCC expression in mouse distal convoluted tubule (mDCT) cells [102]. Therefore, this suggests a tumor suppressor function for WNK3 as an inhibitor of ERK1/2 MAPK signaling.

There are few reports that show a connection between WNK4 and MAPK signaling pathways. In mDCT cells, WNK4 promoted ERK1/2 MAPK signaling, as the overexpression of WNK4 caused dose-dependent activation of ERK1/2, and the siRNA-mediated depletion of WNK4 decreased the NaCl-induced activation of the ERK1/2 MAPK pathway. The PHA-II causing mutations of WNK4 failed to activate the ERK1/2 MAPK signaling. The inhibitory effects of WNK4 on NCC were mediated through the ERK1/2 MAPK pathway [103]. WNK4 overexpression in HEK293 cells could enhance both hypertonicity-induced (NaCl) and growth factor-induced (EGF) activation of ERK1/2 MAPK signaling and p38 MAPK signaling. The PHA-II associated mutants behaved similarly to wild-type WNK4 in this study [104]. These studies agree with a positive regulation of ERK1/2 and p38 MAPK pathways by WNK4. Therefore, it is possible that WNK4 regulates cell proliferation and survival through these MAPK pathways.

Overall, these observations show that WNK kinases are regulators of the ERK1/2-MAPK pathway (Figure 1.8).

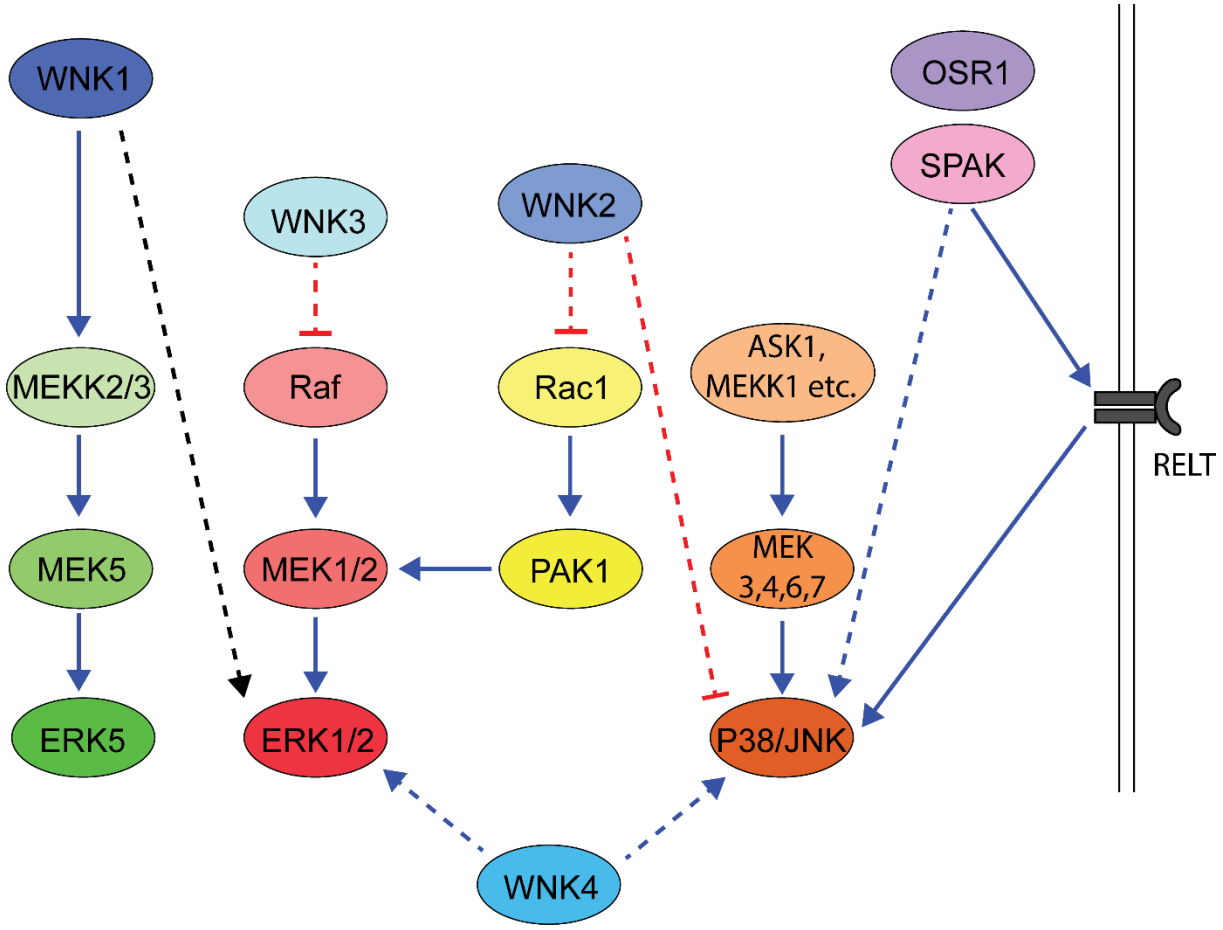



Figure 1.8. The regulation of MAPK pathways by WNK pathway components. This figure was modified from Figure 1 of Raman et al. 2007 [105] and Figure 1A of Kim and Choi 2010 [93].

**p38 and JNK MAPKs**

WNK2 is an inhibitor of matrix metalloproteinase (MMP)2-dependent cell invasion in gliomas. The stable knockdown of WNK2 in a glioma cell line (SW1088) and expression of WNK2 in another glioma cell line in which *WNK2* was silenced (A172) showed that WNK2 inhibited the expression and activity of MMP2. The regulation of MMP2 by WNK2 was partially dependent on the inhibition of c-Jun N-terminal kinase (JNK) activity by WNK2 [106].

WNK4 was phosphorylated in a p38-dependent manner at S575, suggesting that WNK4 acts downstream of the p38 MAPK pathway. The role of this phosphorylation is currently not clear, as it did not alter the WNK4 kinase activity or its binding to KLHL3, an adaptor protein which mediates Cullin-3-dependent WNK degradation [32, 34, 107].

Fray, which is the *Drosophila* homolog of OSR1 and SPAK was necessary for maximal activation of JNK following hypertonic-stress induction in S2 cells, yet the human enzyme had little effect on JNK activation in mammalian cells [108]. OSR1 positively regulated the p38 MAPK signaling. The receptor expressed in lymphoid tissues (RELT) is a member of the tumor necrosis factor receptor (TNFR) superfamily [109]. It binds two homologous receptors, RELL-1 and RELL-2. OSR1 bound to and phosphorylated RELT and its two binding partners in-vitro, suggesting that RELT and its homologues were OSR1 substrates [110]. RELL-1 and RELL-2 are activators of p38 MAPK pathway and this effect was blocked when a catalytically-inactive mutant of OSR1 was expressed in the cells, suggesting that RELL-mediated p38 activation requires active OSR1 [111]. These results are called into question by another study that showed OSR1 did not alter the activity of p38 [108].

SPAK was also identified as a kinase that promoted the p38 MAPK signaling [50]. RELT activated the p38 and JNK MAPK pathways and was dependent on SPAK. SPAK interacted with and phosphorylated RELT. The disruption of SPAK-RELT binding by mutating SPAK recognition motif in RELT, and SPAK dependent RELT phosphorylation by expressing a kinase dead mutant of SPAK, prevented the activation of p38 and JNK MAPK signaling by RELT [112]. This suggests that both OSR1 and SPAK regulate RELT by a common mechanism (Figure 1.8). Moreover, SPAK co-immunoprecipitated with p38 showing that these two proteins can complex together. Cellular stress decreased the interaction between SPAK and p38 [53].

A recent study showed that SPAK is overexpressed in non-small cell lung cancer (NSCLC) patient samples and cell lines at both mRNA and protein levels. The expression of SPAK was positively associated with poor cancer prognosis, with high SPAK expression positively correlated with tumor size, tumor stage and advanced lymph node metastasis. The lower SPAK expression correlated with longer overall survival of the patients. SPAK promoted proliferation of NSCLC cell lines. The depletion of SPAK caused cells to significantly accumulate in G0/G1, while decreasing the number of cells in S and G2/M phases. In contrast, overexpression of SPAK decreased the number of cells in G0/G1 phase and increased the number of cells in G2/M phase. These data show that SPAK is a promoter of cell cycle in NSCLC cells. The expression of SPAK also promoted the migration and invasion of these cells, whereas SPAK depletion had opposite effects. In addition, SPAK suppressed apoptosis in these cells. SPAK also promoted tumorigenesis, as NSCLC cells with stable knockdown of SPAK had slower tumor growth, smaller tumors and more tumor apoptosis, when injected into mice. The p38 MAPK pathway was upregulated in these cells and the depletion of SPAK decreased the activation of p38. SPAK was suggested to act through p38 MAPK to exert its oncogenic functions in NSCLC [113]. In addition, SPAK was required for colony formation and tumorigenesis of cervical cancer cell lines through p38 [114].

Inflammation is often an enabling process for tumorigenesis [85]. SPAK is by far the most studied component of the WNK pathway in inflammation. Overexpression of cSPAK, a catalytically-active colon-specific SPAK isoform (cSPAK) which did not contain the N-terminal PAPA box and a part of the kinase domain, activated p38 MAPK and decreased barrier function in Caco2-BBE colorectal carcinoma cells. Treatment of cells with the inflammation-inducing

cytokine interferon-γ enhanced the expression of cSPAK. It was suggested that SPAK may mediate pro-inflammatory signaling in the colon [23].

These findings indicate that WNK pathway components act as both activators and inhibitors of different MAPK pathways (Figure 1.8).

**PI3K-AKT**

PI3K-AKT signaling is a well-studied oncogenic pathway [115, 116]. AKT directly phosphorylated WNK1 at T60 in-vitro. In addition, insulin-like growth factor (IGF)-1 induced phosphorylation of WNK1 at T60 in HEK293 cells, which was blocked by PI3K inhibitors. This effect was also present in mouse embryonic stem (ES) cells. In ES cells lacking phosphoinositide-dependent kinase (PDK)-1 which is required for the activation of AKT, IGF-1 stimulation failed to induce WNK1 T60 phosphorylation, further showing that this phosphorylation is dependent on AKT [117, 118]. This phosphorylation did not affect WNK1 kinase activity or its cellular localization [117]. Another study identified WNK1 as an AKT substrate using a phospho-specific antibody to pull-down AKT substrate phosphorylation motif-containing proteins, following insulin treatment of 3T3-L1 cells. This insulin-stimulated phosphorylation of WNK1 was blocked by the treatment of cells with PI3K inhibitors and siRNA-mediated depletion of AKT, consistent with the findings of the previous report. While the depletion of WNK1 did not affect insulin-induced glucose transport, it increased insulin-induced proliferation of 3T3-L1 preadipocytes [119]. Later it was shown that both AKT and SGK1 phosphorylated rat WNK1 on the comparable residue (T58). This phosphorylation was necessary to mediate the insulin-induced endocytosis of ROMK [21]. In addition, this phosphorylation was necessary for the activation of SGK1 and subsequent activation of ENaC by WNK1 [81]. AKT-dependent WNK1 phosphorylation and enhanced WNK

functions such as phospholipase C-β signaling, angiogenesis and cell migration have been reported by other studies as well [120-124] (Figure 1.9).

Acetyl-CoA medium-chain synthetase 3 (ACSM3) could act as a tumor suppressor in HCC. The overexpression of ACSM3 inhibited migration and invasion of the HCC cell lines HepG2 and SMMC7721, and inhibited cancer metastasis in mouse xenografts. It also decreased the activation of AKT and phosphorylation of WNK1 at T60. Expression of an AKT construct could block the tumor suppressor effects mediated by ACSM3. Therefore, it is possible that AKT-WNK1 axis promotes cell migration and invasion in HCC [124]. In addition, WNK1 was important for the migration of lung cancer cells. Kynurenine (Kyn) which is produced by activated lung fibroblasts helped the migration and growth of lung cancer cells. It could also activate AKT and WNK1 phosphorylation at T60 in lung cancer cell lines. Interestingly, it could also increase the activation of OSR1/SPAK, suggesting WNK1 activation in these cells. The depletion of WNK1 in these cells inhibited their migration, similar to inhibition of AKT. These data show that WNK1 acts as an oncogene in lung cancer and WNK1 kinase activity might be regulated by AKT [123]. A recent report also showed that the PI3K-AKT pathway activated the WNK-OSR1/SPAK-NCC pathway in a hyperinsulinemic db/db mouse model, suggesting AKT-mediated kinase activation of WNK and subsequent phosphorylation of downstream targets [125]. Therefore, the exact contribution of AKT-mediated phosphorylation of WNK1 on its kinase activity is not clear. It should also be noted that a recent study showed that AKT3 acted as a suppressor of WNK1 and WNK3 expression in macrophages [126].

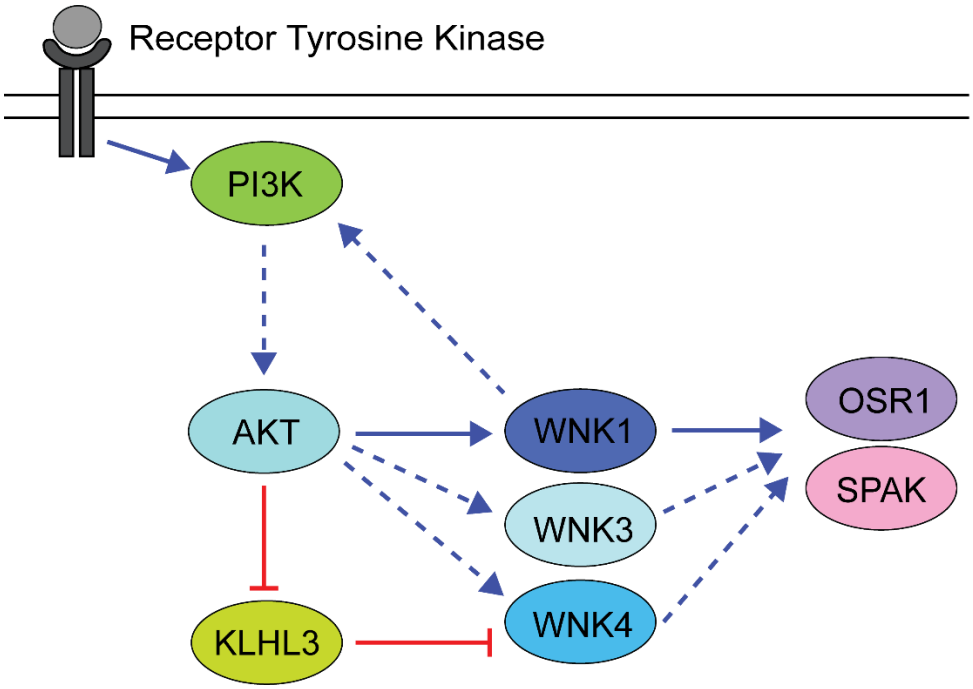



Figure 1.9. The interplay between PI3K-AKT pathway and WNK pathway

In addition to acting as a target of the PI3K-AKT pathway, some evidence implicates WNK1 upstream of AKT. Hypotonic challenge induced WNK1 T60 phosphorylation and proliferation in a rat smooth muscle cell line. The overexpression of WNK1 increased cell proliferation and cell cycle progression already observed with hypotonicity. The siRNA-mediated depletion of WNK1 blocked those effects. In addition, overexpression of WNK1 increased the hypotonicity-induced activation of AKT and PI3K, whereas the depletion of WNK1 had opposite effects. This suggested that hypotonicity-induced cellular effects of WNK1 were mediated through the PI3K-AKT pathway [127] (Figure 1.9).

WNK3 was discovered to undergo EGF-dependent phosphorylation in HEK293 cells and this phosphorylation was blocked by the treatment of wortmannin, which suggested that it was PI3K-AKT dependent. Therefore, WNK3 might also be a direct target of AKT [128].

**G Protein-Coupled Receptors**

WNK pathway components also appear in G protein-regulated events. Lysophosphatidic acid (LPA) induces activation of G protein coupled receptors (GPCR), $LPA_{1-4}$ [129]. LPA induced activation loop phosphorylation and phosphorylation at other sites of WNK1. In addition, the LPA-induced migration of SCC-9 squamous cell carcinoma cells were abolished by the siRNA-mediated depletion of WNK1 [14]. This shows that WNK1 is linked to the GPCR signaling.

The cAMP-dependent protein kinase (PKA) signaling pathway is a known cancer-related signaling pathway [130]. Several studies show that WNK4 is a target of PKA and PI3K-AKT signaling. The phosphorylation of KLHL3 at S433 by PKA and AKT inhibited KLHL3-dependent degradation of WNK4, and increased its cellular expression [131]. In addition, PKA and PKC-dependent phosphorylation of WNK4 at two sites outside the catalytic domain positively modulated WNK4 kinase activity and its downstream signaling [132]. Therefore, it seems that several cancer-related signaling pathways positively regulate WNK4, indicating a possible role for WNK4 in cancer. However, it should be noted that β2-adrenergic receptor/PKA-dependent inhibition of histone deacetylase (HDAC)-8 increased the DNA binding of glucocorticoid receptor in the *WNK4* promoter region which inhibited *WNK4* transcription [133], suggesting that the effect on WNK4 may be of short duration.

**TGF-β**

Transforming growth factor (TGF)-β has both tumor suppressor and tumor promoter activities, as discussed further below. The initial actions are often anti-proliferative [134]. WNKs also intersect with the TGF-β pathway. Both WNK1 and WNK4 can bind and phosphorylate Smad2, a TGF-β downstream effector. Depletion of WNK1 in HeLa cells decreased the total

amount of Smad2, but increased phosphorylated Smad2 in nuclei and increased TGF-β signaling. These findings suggest the potential of WNK1 to influence the tumor-suppressive function of TGF-β [135] (Figure 1.10).

A recent study showed that SPAK mRNA and protein are upregulated in osteosarcoma patient samples. SPAK promoted proliferation of osteosarcoma cell lines. Along with downregulation of proliferating cell nuclear antigen (PCNA) and upregulation of p21, knockdown of SPAK abolished proliferation. Depletion and overexpression experiments indicated that SPAK could also promote invasion of osteosarcoma cell lines. Consistent with data on migration, SPAK depletion also downregulated MMP2, MMP9 and Twist1, and decreased the phosphorylation of smad2/3, suggesting that its loss antagonized the tumor-promoting function of the TGF-β pathway in osteosarcoma [136] (Figure 1.10).

**NF-κB**

Nuclear factor Kappa B (NF-κB) is a well-known tumor promoter [137, 138]. Mouse *WNK1* has a putative NF-κB site in its promoter, suggesting that WNK1 expression in cells may increase following oncogenic activation [139].

cSPAK expression was upregulated in patients with ulcerative colitis, an inflammatory bowel disease. TNF-α induced demethylation of the *STK39* promoter and increased expression of SPAK. The NF-κB bound to the *STK39* promoter was required for upregulated SPAK expression. Increased NF-κB could increase the expression of SPAK in cells [140]. SPAK expression was upregulated by hyperosmotic stress and this effect was dependent on NF-κB and SP1 which show increased binding to the *STK39* promoter following hyperosmotic stress [141]. The toll-like receptor 4 and NF-κB are upstream of SPAK-NKCC1 in rat choroid plexus epithelium [142].

Therefore, SPAK was suggested to mediate the pro-inflammatory functions of the WNK pathway in an NF-κB-dependent manner [114] (Figure 1.11).

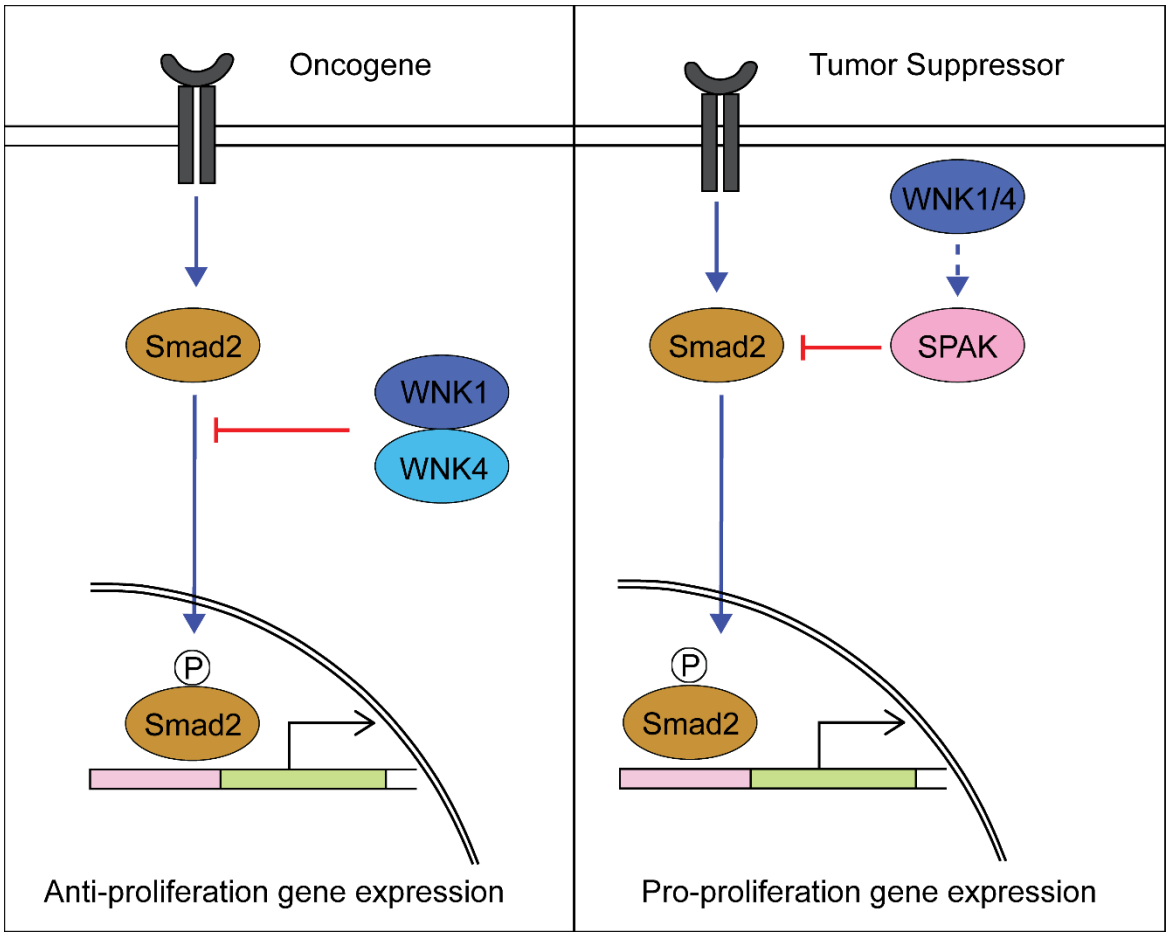



Figure 1.10. The regulation of TGF-β pathway by WNK pathway components.

The potassium chloride cotransporter, KCC3 induced the expression of SPAK in cervical cancer cell lines and in HEK293 cells. KCC3 also upregulated NF-κB and MMP2 in HEK293 cells. It was suggested that NF- κB-induced SPAK activated the p38 MAPK pathway and subsequent activation of MMP2 which promoted the invasion of the cells. Also, the depletion of

SPAK inhibited growth of xenograft tumors dependent on KCC3, supporting the relevance of SPAK in tumor growth [114].

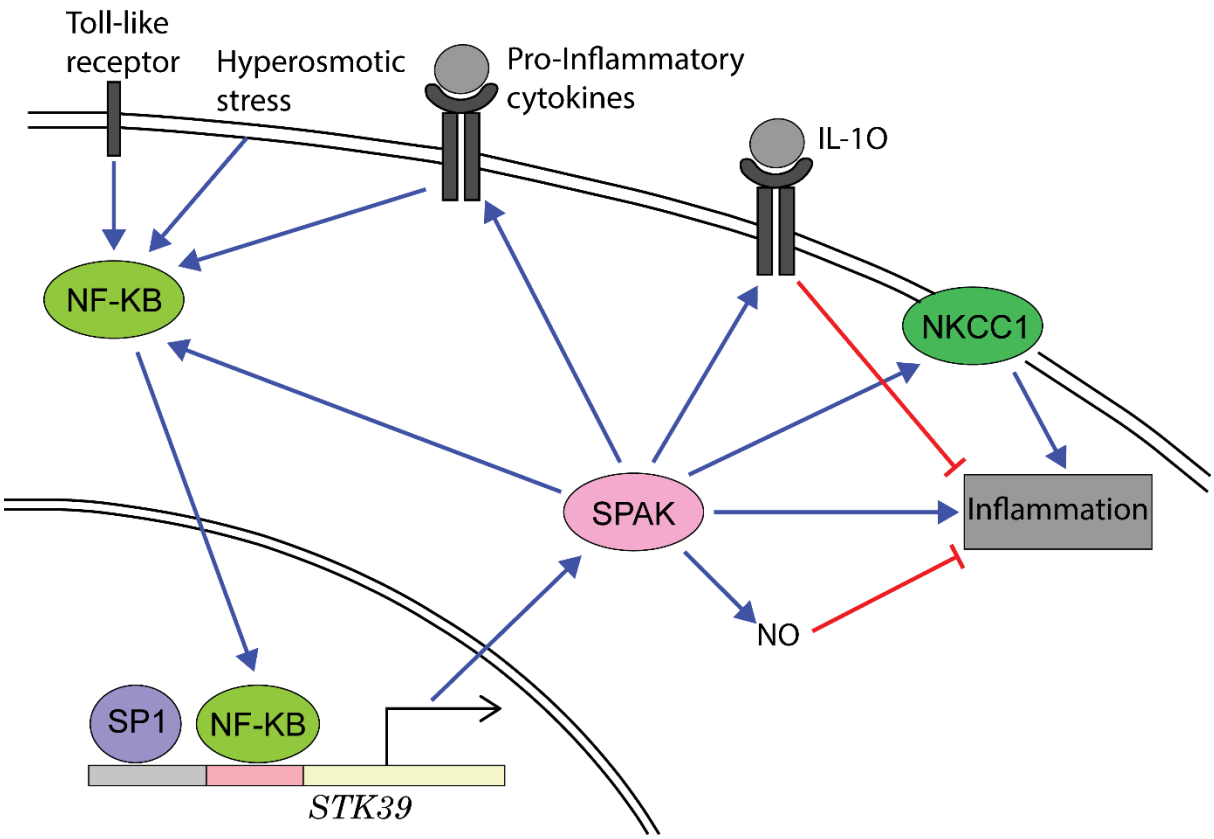



Figure 1.11. Positive and negative effects on inflammation by SPAK

In addition, SPAK functioned not only downstream, but also upstream of NF-κB signaling, as mesangial cells primed with IgA immune complexes of SPAK knockout mice had decreased NF-κB and MAPK signaling [24]. SPAK induced the production of pro-inflammatory cytokines and SPAK knockout mice lacked intestinal and renal inflammation and pro-inflammatory cytokine secretion present in control mice [24-26].

However, SPAK was activated by endotoxin and promoted nitric oxide production in mice [143]. SPAK also increased the production of anti-inflammatory cytokine interleukin 10 in T cells, leaving questions about the inflammatory consequences of SPAK activation [144, 145] (Figure 1.11).

**Other Cancer-Related Pathways**

WNK1 has been linked with the regulation of WNT/β-catenin pathway of which aberrant regulation promotes cancer [146]. The Drosophila WNK acted as a positive regulator of the canonical WNT/β-catenin pathway. This effect was also recapitulated in human HEK293T cells where both WNK1 and WNK2 promoted the WNT/β-catenin pathway activation. Depletion of OSR1 and SPAK, either individually or in combination also decreased the WNT/β-catenin signaling, suggesting that the actions of WNK kinases on this pathway are mediated by OSR1/SPAK [147].

Published work is not in agreement on connections between WNKs and mTOR activities. The shRNA-mediated depletion of WNK2 inhibited mammalian target of rapamycin (mTOR) complex 1 (mTORC1) activity in K562 leukemia cells, increasing their autophagy [148]; yet, a similar study in MCF7 breast cancer cells showed no such effect on mTORC1. WNK2 acted as an autophagy promoter in those cells [149], suggesting that actions of WNK2 on autophagy are cell-type dependent (Figure 1.12). Clearer is work showing that OSR1 can be a downstream target of mTORC2 which phosphorylates OSR1 on S339 and causes a modest increase in its activity [62]. mTORC2 is also involved in cancer, in addition to its related complex mTORC1 [150]. Therefore, it is possible that OSR1 might play a role in cancer through its link with mTORC2.

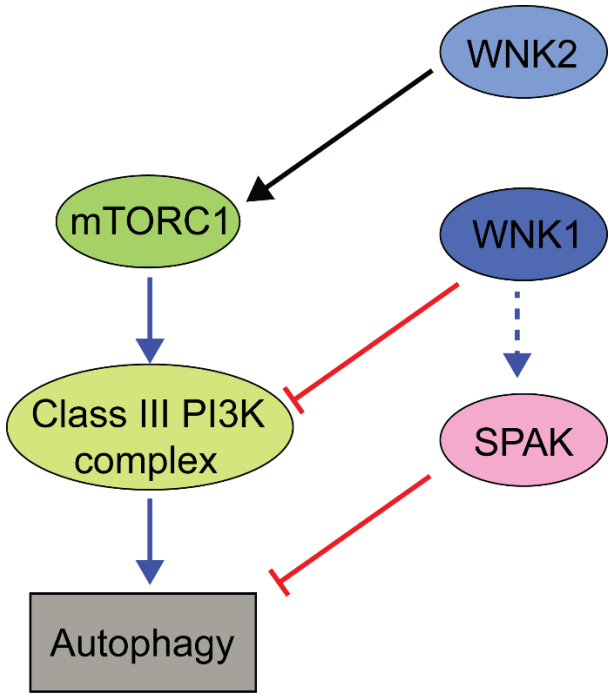



Figure 1.12. Regulation of autophagy by WNK pathway components. This figure was adapted from Figure 5F of Gallolu Kankanamalage et al 2016 [52] and Figure 1 of Gallolu Kankanamalage et al 2017 [151]

Using depletion and to a lesser extent inhibition experiments, I showed that WNK1 is an inhibitor of autophagy in multiple cell lines. It was also an inhibitor of class III PI3K complex (PI3KC3) that promotes autophagy [152]. Our interpretation is that WNK1 inhibits autophagy by suppressing PI3KC3. I also showed that SPAK, but not OSR1, inhibited autophagy. Therefore, some of the action of WNK1 on autophagy may be mediated by SPAK [52] (Figure 1.12).

SPAK interacted with PKC-θ in HEK293 cells and Jurkat T cells, and via yeast two-hybrid assays through its C-terminal region containing the PF2 domain. PKC-θ phosphorylated SPAK primarily on S311. T cell receptor (TCR)/CD28 co-stimulation required PKC-θ and phosphorylation of S311 to activate SPAK. SPAK was necessary for activation of the transcription factor AP-1 in T cells. Therefore, SPAK could mediate PKC-θ-dependent activation of T cells [63]. In Estrogen Receptor (ER) negative breast cancer cell lines, Fra-1, a component of AP-1, is

expressed highly and oncogenic [153-155]. The depletion or inactivation of PKC-θ reduced the expression of Fra-1, while an active mutant of PKC-θ phosphorylated Fra-1, stabilizing it in ER positive breast cancer cells. Depleting SPAK prevented stabilization of Fra-1 partially or completely in BT549 (ER-), MDA-MB-231 (ER-), and MCF7 (ER+) cell lines [156]. Therefore, SPAK seems to regulate the PKC-θ-mediated activation of AP-1 in multiple systems.

Src is a non-receptor tyrosine kinase. Its transforming counterpart v-Src is the oncogene in Rous sarcoma virus which was shown to cause sarcomas in birds more than 100 years ago (Peyton Rous, Nobel Prize) [157, 158]. Src binds WNK4 and phosphorylates multiple sites, thereby blocking the inhibitory effects of WNK4 on ROMK [159].

**Additional Findings Implicating WNKs in Cell Proliferation, Survival and Migration**

WNK1 localized to mitotic spindles during mitosis and its depletion by siRNA resulted in aberrant mitotic spindle formation in HeLa cells. These effects were independent of WNK1 downstream kinase OSR1, suggesting that WNK1 acted through other pathways or acted directly on the mitotic spindle. The cells with depleted WNK1 showed aberrant abscission and decreased survival. These findings show that WNK1 promotes cell proliferation via the regulation cell division [12]. In addition, WNK1 was transcriptionally downregulated by Nogo receptor (NgR) in pheochromocytoma cell line PC12, and WNK1 positively regulated the proliferation and migration of these cells. The inhibitory effects of NgR on cell proliferation and migration were partially rescued by the expression of WNK1. This suggests an oncogenic role for WNK1 in adrenal cancers [160]. Moreover, the depletion of WNK1 by shRNA sensitized the carboplatin-resistant SKOV3 ovarian cancer cells to carboplatin treatment [161].

WNK1 promoted invasion and motility of PDAC cell lines. The depletion of Vav3, a guanine nucleotide exchange factor (GEF), inhibited invasion and motility of the PDAC cells although it upregulated WNK1 activity [162]. It is possible that this WNK1 activation could be a feedback mechanism employed by PDAC cells to restore their motility. Moreover, the siRNA-mediated depletion of WNK1 caused decreased invasion of breast cancer cells [163].

An siRNA screen targeting protein and lipid kinases identified WNK4 as a protein that promoted cell proliferation and cisplatin resistance in the DAOY medulloblastoma cell line [164].

SPAK enhanced replication of the hepatitis B virus (HBV), a promoter of liver cancer [165] in HepG2 liver carcinoma cells. SPAK was a target of miRNA26-b which inhibited HBV replication [166].

**WNKs and Cell Death**

WNK3 is a reported suppressor of caspase-3-dependent HeLa cell death and neuronal cell death. Depletion of WNK3 enhanced the HeLa apoptotic response, while its overexpression protected them from cell death [13]. Leucine-rich repeat and Ig domain-containing (LINGO)-1 is a transmembrane receptor which has pro-apoptotic functions. It interacts with WNK3 in rat brain and inhibits its activation. In addition, depletion of endogenous WNK3 or expression of kinase-dead WNK3 enhanced caspase-3 activation and apoptosis in PC12 cells. In addition, WNK3 protected neurons from apoptosis in-vivo [167] (Figure 1.13). Interaction with LINGO-1 may be common among WNKs, as WNK1 also interacts with LINGO-1 and regulates LINGO-1-mediated neurite outgrowth in PC12 cells [168].

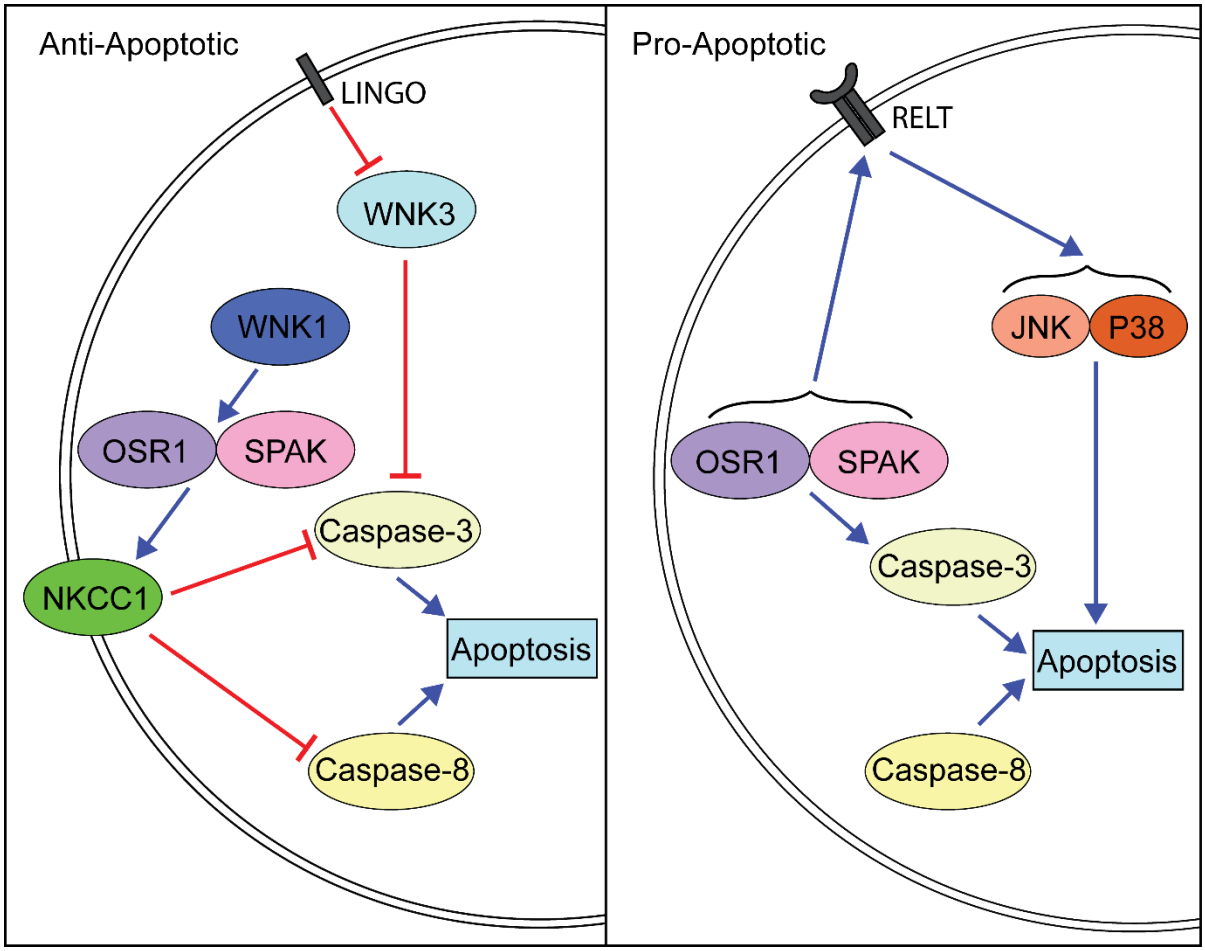



Figure 1.13. Pro and anti-apoptotic functions of WNK pathway components.

SPAK contains a putative caspase-cleavage site. A SPAK truncation that represented the caspase-cleaved form was localized in the nucleus, whereas the full-length protein was primarily cytosolic in COS7 cells [50]. The depletion of SPAK in human B cell lymphoma and B cell precursor leukemia cells prevented DNA double strand break-induced apoptosis in those cells, suggesting an apoptosis-promoting role for SPAK. This pro-apoptotic effect was dependent on caspase-3 [169]. However, the depletion of SPAK in HeLa cells increased sensitivity of cells to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This shows that SPAK may contribute positively or negatively to apoptotic signals by cell type-

dependent processes [170] (Figure 1.13). The same study suggested that TRAIL inhibited SPAK kinase activity and induced its caspase-dependent cleavage at two sites which are different from the putative site identified earlier [50].

**DNA Damage Response**

There is some evidence that WNK pathway components participate in the DNA damage response. OSR1 is apparently phosphorylated by ataxia-telangiectasia mutated (ATM) in fibroblasts exposed to hydrogen peroxide [171], in human osteosarcoma U2OS cells exposed to a different damaging agent [172], and in Epstein-Barr virus transformed B-lymphocytes following ionizing radiation [173]. WNK1 was also phosphorylated at S167 [171]. The functions of these modification in the response to DNA damage are not yet known, but is suggested by work in B cell lymphoma showing that the loss of SPAK enables the tumors to acquire resistance to genotoxic stress [169].

**Angiogenesis**

Enhanced angiogenesis is a hallmark of cancer to maintain blood flow in newly formed tumor tissue [85]. Several components of the WNK pathway have been implicated in angiogenesis. WNK1 knockout mice died before embryonic day 13 and showed gross defects in cardiovascular development [22, 174]. Endothelial-specific WNK1 knockout mice also died mid-gestation with similar cardiovascular defects, indicating that WNK1-induced lethality is a result of failed development of the cardiovascular system and defective angiogenesis [22]. Later, the same group discovered that whole body and endothelial-specific OSR1 knockout mice also failed to develop with similar cardiovascular and angiogenesis defects. In addition, transgenic mice expressing an

active mutant of human OSR1 in a whole-body WNK1 knockout background rescued the cardiovascular defects caused by the loss of WNK1, indicating that WNK1-OSR1 axis is important for cardiovascular development and angiogenesis [175] (Figure 1.14).

Using primary human endothelial cells and cell lines, we showed that WNK1 and OSR1 are required for angiogenesis. The depletion of WNK1 by siRNA inhibited cord formation, an in vitro angiogenesis assay, as well as proliferation and migration. WNK1 depletion inhibited expression of Slug, ZEB1, MMP2 and MMP9, and increased expression of thrombospondin-1, consistent with its pro-migratory effects. The depletion of OSR1 and SPAK, downstream effectors of WNK1, had different effects on endothelial cells. OSR1 was necessary for migration, whereas SPAK was necessary for their proliferation. The expression of Slug in WNK1-depleted cells rescued migration and endothelial cord formation. These findings show that WNK1 acts via its downstream kinases OSR1 and SPAK, and the mesenchymal transcription factor Slug to regulate angiogenesis [16] (Figure 1.14).

WNK1 depletion also blocked angiogenesis in zebrafish. WNK1 mRNA expression could partially rescue angiogenesis aberrations caused by the loss of vascular endothelial growth factor receptor (VEGFR)-2. Mutation of T60, the AKT phosphorylation site in WNK1, prevented this partial rescue. In addition, both VEGFR-2 and VEGFR-3 were found to positively regulate the transcription of *WNK1a*, one of the two major WNK1 isoforms in Zebrafish. This shows that WNK1 is a promoter of angiogenesis in a PI3K-AKT-dependent manner [122] (Figure 1.14).

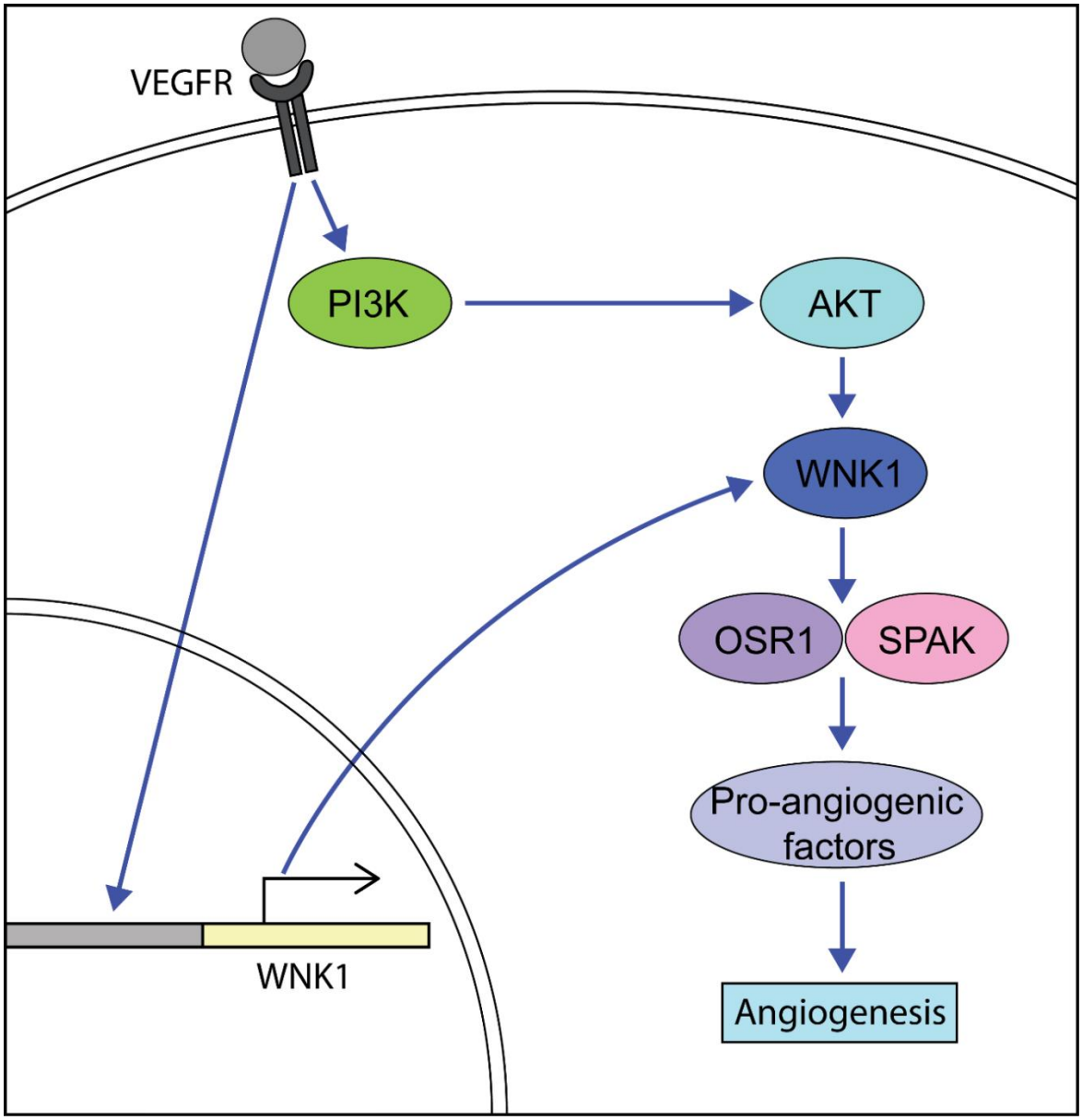



Figure 1.14. Regulation of angiogenesis by the WNK pathway.

**Energy Metabolism**

Aberrant metabolism is a hallmark of cancer [85]. And like many other processes, there is evidence that energy metabolism has not escaped inputs from WNKs. WNK1 binds and phosphorylates AS160 (TBC1D4) which controls insulin-mediated glucose uptake [176-179]. In addition, WNK1 promoted the expression of GLUT-1 at the cell membrane. This effect was

dependent on Rab8A which acted downstream of AS160. The authors suggested that WNK1-mediated phosphorylation of AS160 released active Rab8A to promote trafficking of GLUT-1 to cell surface [176]. In addition, a recent report identified a significant association of WNK1 with several cardiometabolic traits [180].

WNK4 has reported pro-adipogenic activity. It promoted the lipid accumulation and expression of adipogenic genes in 3T3-L1 adipocytes, as well as cell cycle progression. WNK4 knockout mice were less obese on a high-fat diet, suggesting a significant metabolic function [181].

**WNK Components and Aberrations of Genomic Organization**

Multiple studies reveal genomic and/or expression changes of WNK pathway components in different cancers. *WNK1* is amplified with high mRNA expression in stage II/III colorectal tumors [182]. *WNK1* was fused with *B4GALNT3* in papillary thyroid carcinoma in a patient, which led to higher expression of B4GALNT3 [183]. Proteomic analysis of high-grade post-chemotherapy recurrent tumors of ovarian cancer had higher mRNA and protein expression of WNK1 compared with primary tumors. WNK1 was also suggested to be a driver of chronic lymphocytic leukemia [184].

In contrast to *WNK1*, most evidence supports a tumor suppressor function for the *WNK2* gene, which is silenced in multiple types of cancers. In addition to epigenetic silencing in PDAC, as described earlier [101], *WNK2* was aberrantly methylated and its expression was silenced in human gliomas. Treatment of glioma cell lines with a methylation inhibitor restored its expression. Transfection of glioma cell lines with WNK2 decreased their colony forming ability independent of WNK2 kinase activity [185]. *WNK2* is also hypermethylated in meningiomas [186, 187].

Treatment of a meningioma cell line in which *WNK2* was silenced, with 5-aza-dc restored the expression of WNK2. The exogenous expression of WNK2 inhibited colony formation in malignant meningioma cell lines [187]. *WNK2* belongs to a cohort of the twenty most hypermethylated genes in HBV-related HCC, and treatment of two HCC cell lines with 5-aza-dc increased *WNK2* expression [188]. Arsenic trioxide which induces apoptosis in the promyelocytic leukemia cell line NB-4 upregulated transcription of WNK2 [189]. Together, these findings show that WNK2 acts as a tumor suppressor in different types of cancers and epigenetic silencing by hypermethylation seems to be the major mechanism of its inactivation.

OSR1 can be a tumor suppressor in osteosarcoma and ovarian cancer. *OXSR1* was associated with copy number loss and underexpression in 35% or more of tumors from osteosarcoma patients, and was suggested as a candidate tumor suppressor gene in osteosarcoma [190]. In high-grade platinum-resistant ovarian tumor samples, the *OXSR1* promoter was methylated. Depletion of OSR1 from ovarian cell lines led to carboplatin resistance [191].

Aberrant expression of SPAK is linked to different types of cancers. *STK39* is epigenetically silenced in B cell lymphoma by promoter hypermethylation [169]. *STK39* was downregulated in metastatic prostate tumors compared with normal prostate and primary prostate tumors, and was suggested as a predictor of metastasis [192].

These findings are matched by a number of reports, suggesting that SPAK is an oncogene. SPAK is linked to NSCLC, where *STK39* contained the top two of five major single nucleotide polymorphisms (SNPs) associated with the poor overall survival of NSCLC patients [193]. Androgens increased SPAK expression in LNCaP prostate cancer cells in an androgen receptor (AR)-dependent manner [194]. AR promotes tumorigenesis in prostate cancers [195, 196]. Therefore, it is possible that SPAK participates in oncogenic functions of AR. In line with this

hypothesis, the basal and androgen-induced expression of SPAK in LNCaP cells were inhibited by genistein, an isoflavone chemical suggested as a potential therapeutic agent in prostate cancer. The same study showed that 17 β-estradiol also induced the expression of SPAK in LNCaP cells and it was blocked by genistein [197]. Another anti-androgenic compound, DIMN-26 also downregulated the expression of SPAK in LNCaP-LN3 cells [198].

**Conclusions and Future Directions**

The effect of WNK pathway components in cancer varies depending on the type of cancer and stage of cancer. Most of the frequently occurring cancers such as lung, breast, prostate and brain cancers are affected by aberrations in WNK pathway components. Based on the available literature, several conclusions can be drawn. WNK kinases, OSR1 and SPAK may both promote and inhibit cancer. Activation or inhibition of WNK pathway components may provide cancers with growth and survival advantages and may influence their survival by affecting processes discussed above (i.e., inflammation, altered metabolism, etc.) that enable cancer success. Numerous examples of pro-oncogenic activities of WNK1 are known, e.g., enhanced migration, in contrast to relatively few examples of tumor suppressor functions. The Cancer Genomic Atlas (TCGA) cBioPortal (http://www.cbioportal.org/) reported that *WNK1* was amplified in testicular germ cell cancer, breast cancer, ovarian cancer and prostate cancer. It was either amplified or mutated in lung squamous cell carcinomas and cholangiocarcinomas. These changes were present in at least 10% of the total samples available in those studies.

In contrast to WNK1, WNK2 has consistently demonstrated tumor suppressor activity (not clear whether or not amplification in prostate and breast cancers (TCGA cBioPortal) is consistent with tumor suppression). This observation is further supported by the mutations of *WNK2* in

desmoplastic melanoma, cutaneous melanoma, uterine carcinosarcoma and lung adenocarcinoma in at least 10% of the total samples (TCGA cBioPortal). Some of these mutations could cause inactivation of WNK2. However, multiple reports that I have reviewed here suggest that epigenetic inactivation is a more prevalent mechanism of silencing WNK2 in cancers. Despite being structurally similar and involved in similar physiology [3-5, 59] (Figure 1.3), WNK1 and WNK2 tend to act in opposite directions in cancer. WNK3 and WNK4 seem to perform oncogenic functions in most cancers, similar to WNK1.

In spite of the similarity in substrate specificity and regulation of OSR1 and SPAK [3, 4], the majority of data on OSR1 indicate that it is a tumor suppressor. In contrast, SPAK seems to project oncogenic functions in most cancer types. SPAK promotes both oncogenic and tumor suppressor functions in prostate cancers [169, 192, 194], and this is most likely tumor stage-dependent. SPAK has both pro- and anti-inflammatory activities which may also be context-dependent.

WNK pathway components act via the WNK-OSR1/SPAK-CCC axis and by other less-characterized pathways to inflict their effects on cancers. Most of these noncanonical pathways cross-talk with major signaling pathways such as MAPK or PI3K-AKT pathways with known linkages to cancer [91, 92, 115, 116]. In the case of WNK1, there are some questions that needs to be addressed as to how it affects and get affected by these pathways. There are conflicting reports about the role of WNK1 on the activation of ERK1/2 MAPK pathway and it is possible that this effect might be context-dependent. In addition, there are conflicting reports on the effect of AKT-mediated phosphorylation at T60 on the activation of WNK1. The biochemical actions of T60 phosphorylation need to be defined.

A shortcoming of many of these studies on crosstalk is lack of molecular mechanisms. These include WNK interactions with PI3K/AKT and the WNT-β-catenin pathways. More in-depth study

should clarify cross-talk between SPAK and transcriptional regulation by NF-κB and regulation of the MAPK p38.

The six WNK pathway proteins we analyzed are kinases. Therefore, it is important to characterize whether or not the effects exerted by WNK components in cancer are kinase-dependent. This will be useful not only to understand the biology behind those WNK-dependent effects, but also to determine whether those events are druggable in the treatment of those cancers. In addition, it is important to differentiate between WNK functions that are cancer-specific and those that do not affect the physiology of normal cells.

Currently, there are no WNK pathway targeting drugs used in clinic to treat cancer. However, the WNK pathway presents several attractive drug targets and could be utilized for cancer therapy, possibly in combination with other cancer therapeutics [10, 199]. In contrast to conventional kinase targeting drugs, the WNK kinase inhibitors have the potential to achieve higher specificity because the kinase domain of WNK kinases are structurally unique and different from other kinases. Therefore, WNK kinase inhibitors have the potential to have fewer off-target effects on other kinases, potentially minimizing drug-related side effects [10, 199]. However, because different WNK kinase isoforms could have opposite effects in the same cancer type, the pan-WNK inhibitors targeting all WNK kinases may not be ideal, and may cause detrimental effects. Therefore, it will be important to develop WNK inhibitors that target individual WNK kinases for cancer therapy.

In addition to inhibitors that catalytically inactivate WNKs, inhibitors that disrupt the binding of WNKs with OSR1/SPAK or that inhibit OSR1 or SPAK could also be used as pathway therapeutics [10, 199]. In fact, several of these inhibitors have already been developed, but with insufficient characterization [200-202]. Approaches targeting ion cotransporters such as NKCCs

present promising strategies to treat cancer [87, 128]. These already have several FDA-approved drugs, e.g., bumetanide and furosemide, used to treat human diseases such as hypertension [203-205]. As highlighted by this review, these drugs may successfully inhibit cancerous characteristics in cell lines and animal models. Therefore, the effects of these drugs should be tested in clinical trials. It seems that promoter methylation is a major mechanism of silencing WNK2 and OSR1 in cancer. In such cancers, methylation inhibitors may prove useful along with other cancer therapeutics.

# Chapter 2

## Multistep Regulation of Autophagy by WNK1

(Chapter 2 is derived from "Gallolu Kankanamalage, S., Lee, A. Y., Wichaidit, C., Lorente-Rodriguez, A., Shah, A. M., Stippec, S., Whitehurst, A. W., Cobb, M. H. Multistep regulation of autophagy by WNK1. *Proc Natl Acad Sci U S A*, 2016. 113 (50): p. 14342-14347" and "Gallolu Kankanamalage, S., Lee, A. Y., Wichaidit, C., Lorente-Rodriguez, A., Shah, A. M., Stippec, S., Whitehurst, A. W., Cobb, M. H. WNK1 is an unexpected autophagy inhibitor. *Autophagy*, 2017. 13(5): p. 969-970.")


### Abstract

I report that WNK1 inhibits autophagy, an intracellular degradation pathway implicated in several human diseases. Using small-interfering RNA mediated WNK1 knockdown, I show autophagosome formation and autophagic flux are accelerated. In cells with reduced WNK1, basal and starvation-induced autophagy is increased. I also show that depletion of WNK1 stimulates focal class III phosphatidylinositol 3-kinase complex (PI3KC3) activity, which is required to induce autophagy. Depletion of WNK1 increases the expression of the PI3KC3 upstream regulator unc-51-like kinase 1 (ULK1), its phosphorylation, and activation of the kinase upstream of ULK1, the AMP-activated protein kinase. In addition, the N-terminal region of WNK1 binds to the UV radiation resistance-associated gene (UVRAG) in vitro and WNK1 partially colocalizes with UVRAG, a component of a PI3KC3 complex. This colocalization decreases upon starvation of cells. Depletion of the SPS/STE20-related proline-alanine-rich kinase, a WNK1-activated enzyme, also induces autophagy in nutrient-replete or -starved conditions, but depletion of the related kinase and WNK1 substrate, oxidative stress responsive 1, does not. These results indicate that WNK1 inhibits autophagy by multiple mechanisms.

## Introduction

### Overview of the Autophagy Degradation Pathway

Autophagy is a process conserved throughout evolution that degrades intracellular materials to remove damaged and outdated components and to supply cells with nutrients and building blocks [206-209]. Autophagy is induced by cellular stress and protects against infections by pathogens [210-216]. This process is initiated by the formation of phagophores/isolation membranes, which expand to become double-membraned vesicles called autophagosomes, while engulfing the cellular materials needed to be degraded by autophagy. This initiation and expansion are catalyzed by association of different proteins including autophagy-related (ATG) 9, double FYVE-containing protein 1 (DFCP1), WD-repeat protein interacting with phosphoinositides (WIPI) proteins and light chain 3 (LC3)-II in the isolation membranes [207, 217]. LC3 is processed and modified by conjugation to a phosphatidylethanolamine tail to become LC3-II, which is anchored in the autophagosome membrane. This is catalyzed by the ATG4, ATG7, ATG3 and ATG5-12-16L conjugation system. In addition, formation of the ATG5-12-16L complex is catalyzed by ATG7 and ATG10 [207, 217, 218]. Once formed, autophagosomes fuse with cellular lysosomes and generate vesicles called autolysosomes. The engulfed cellular materials degrade inside autolysosomes because of the action of lysosomal hydrolases. Alternatively, autophagosomes can also fuse with cellular endosomes to generate intermediate vesicles called amphisomes. They subsequently fuse with lysosomes, producing autolysosomes [217, 219, 220] (Figure 2.1). Critical to maintain intracellular homeostasis, autophagy has roles in diseases such as neurodegeneration, metabolic diseases, autoimmune and inflammatory diseases, infectious diseases, cardiovascular diseases and cancer [218, 221-224].

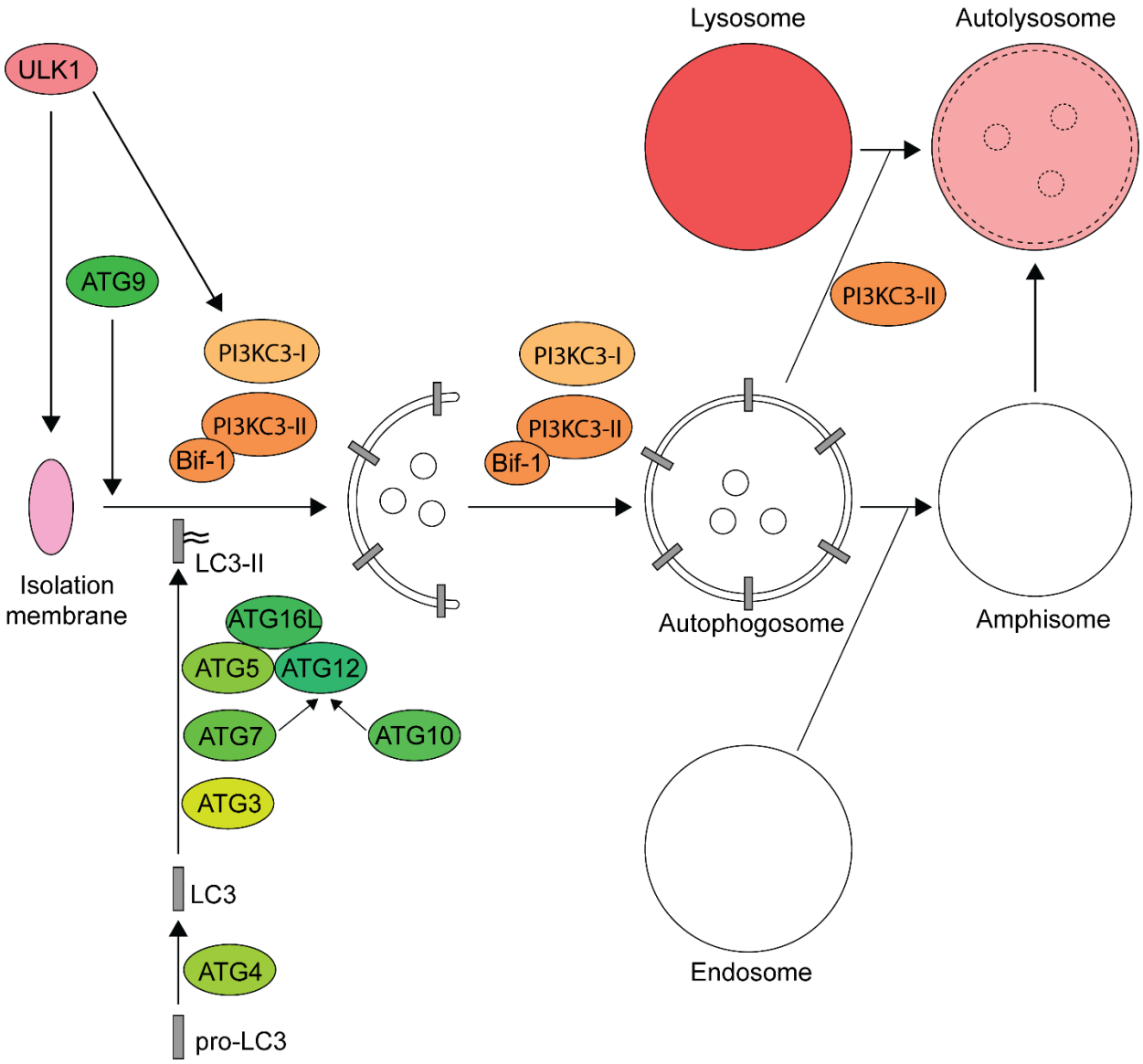



Figure 2.1. The overview of autophagy pathway and its regulation by protein complexes. This figure is inspired by Figure 2 of Mizushima and Komatsu 2011 [207], Figure 1 of Lamb et al. 2013 [217], Figure 1 of Eskelinen and Saftig 2009 [219], Figure 1 of Cook et al. 2011 [225] and information from Wong et al. 2011 [226].

**Unc-51–Like Kinase 1 Protein Complex**

The initiation step of autophagy is catalyzed by the unc-51–like kinase 1 (ULK1) protein complex, which activates class III phosphatidylinositol 3-kinase (PI3KC3) complex, thereby inducing autophagy [227, 228]. In addition, the ULK1 complex has been shown to regulate the

cellular localization of ATG9 [229]. ULK1 complex is comprised of ULK1, ATG13, FAK family kinase interacting protein of 200 kDa (FIP200) and ATG101. ULK1 is the catalytic subunit of this complex [230-234].

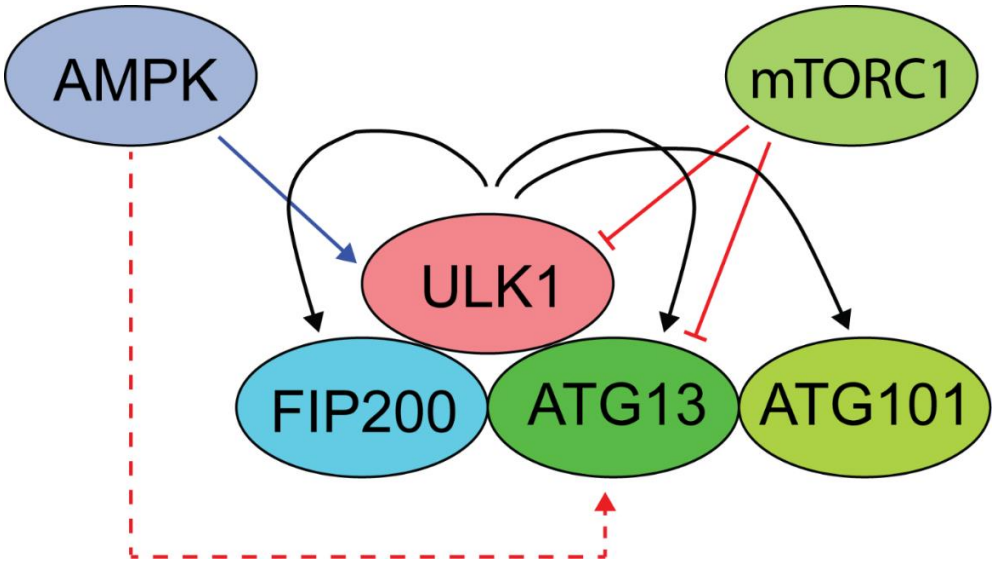



Figure 2.2. The organization of the ULK1 complex and its regulation by phosphorylation. This figure is inspired by figure 2.A of Papinski and Kraft 2016 [235], Figure 4.G of Egan et al. 2011 [236] and Figure 1 and information of Lin and Hurley 2016 [230].

ULK1 activity is controlled by the upstream kinases, the mammalian target of rapamycin complex 1 (mTORC1) and the AMP-activated protein kinase (AMPK). AMPK phosphorylates ULK1 at S317, S467, S555, T574, S637, S659 and S777. These phosphorylation events are important for activation of ULK1, regulating mitochondrial homeostasis or controlling ATG9 localization [229, 230, 236-238]. ULK1 phosphorylation by mTORC1 at S637 and S757 inhibit ULK1 under nutrient-rich conditions [230, 236-238]. In addition, AMPK and mTORC1 phosphorylate ATG13 at S224 and S258, respectively, and these phosphorylations were suggested to inhibit the activity of the ULK1 complex [230, 239]. (Figure 2.2).

In addition to other kinases, ULK1 also phosphorylates other members of the ULK1 complex. ULK1 phosphorylates ATG13 at S389, and ATG101 at S11 and S203. It also phosphorylates FIP200 at S943, S989 and S1323. However, the functional significance of these phosphorylation events is still not clear [230, 240] (Figure 2.2).

**Class III Phosphatidylinositol 3-Kinase Complex**

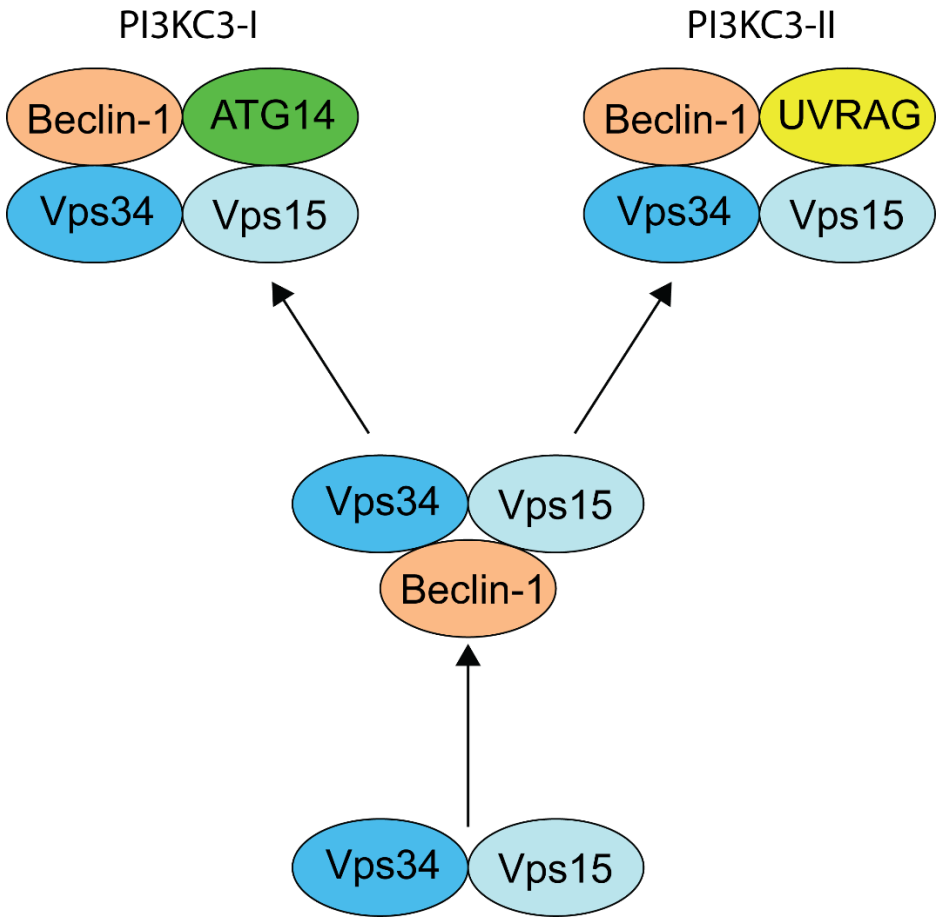



Figure 2.3. The formation of autophagy-related PI3KC3 complexes. This figure is modified from figures 4 and 5 of Backer 2016 [152] and Figure 7.F of Kim et al. 2013 [241].

The PI3KC3 complex initiates autophagy upon activation [152, 242]. This complex contains vacuolar protein sorting (Vps) 34 as its catalytic subunit, which converts cellular

phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PI3P) to assist in autophagosome formation. Vps34 normally associates with Vps15 (p150), which is thought to be a pseudokinase, to form a complex. Vps15 is required for the catalytic activity of Vps34 [152]. The Vps34-Vps15 complex can associate with Beclin-1 to form a trimeric complex. The autophagy-associated PI3KC3 complexes require either ATG14 or UV radiation resistance-associated gene (UVRAG) to associate with this trimeric complex to generate tetrameric complexes [152, 243, 244]. The PI3KC3 complex containing ATG14 is called PI3KC3-I, while the PI3KC3 complex containing UVRAG is called PI3KC3-II [152] (Figure 2.3). Additional scaffold proteins such as nuclear receptor binding protein (NRBF) 2 and progestin and adipoQ receptor family member III (PAQR3) are important for the formation of PI3KC3-I [152, 245-248]. PI3KC3-I is required for the initiation step of autophagy, where autophagosomes are formed, while PI3KC3-II is required for autophagosome-lysosome fusion [217, 249]. Some evidence shows that the UVRAG-containing PI3KC3-II complex also regulates the initiation of autophagy [226, 244] (Figure 2.1).

Different upstream kinases regulate the activity of PI3KC3. The ULK1 complex phosphorylates Beclin-1 at S14, thereby activating it under amino acid starvation [228]. In addition, AMPK phosphorylates Beclin-1 at S91 and S94, also activating it. The phosphorylation of non-autophagy associated Vps34 at T163 and T165 by AMPK inhibits it [152, 241]. In addition, phosphorylation of Beclin-1 by MAPK-activated protein kinase (MAPKAPK) 2/3 and death-associated protein kinase (DAPK) activate the PI3KC3 complex in starved cells [152, 250, 251].

In nutrient-rich and growth factor stimulated cells, a number of PI3KC3 complex phosphorylations regulate their activity. The mTORC1-mediated phosphorylation of UVRAG at S498 inhibits PI3KC3, while phosphorylation at S550 and S571 activate this complex [252, 253]. Epidermal growth factor receptor (EGFR), casein kinase 1 (CK1) and AKT-mediated

phosphorylations of Beclin-1 inhibit it [152, 254-256]. Several sites of ATG14 are phosphorylated and ATG14 is inhibited by mTORC1 [257]. Moreover, cyclin-dependent kinase (Cdk) 1 and Cdk5 phosphorylate and inhibit Vps34 [258]. Therefore, the PI3KC3 complex is subjected to rigorous regulation by different upstream kinases to control its activity [152] (Figure 2.4).

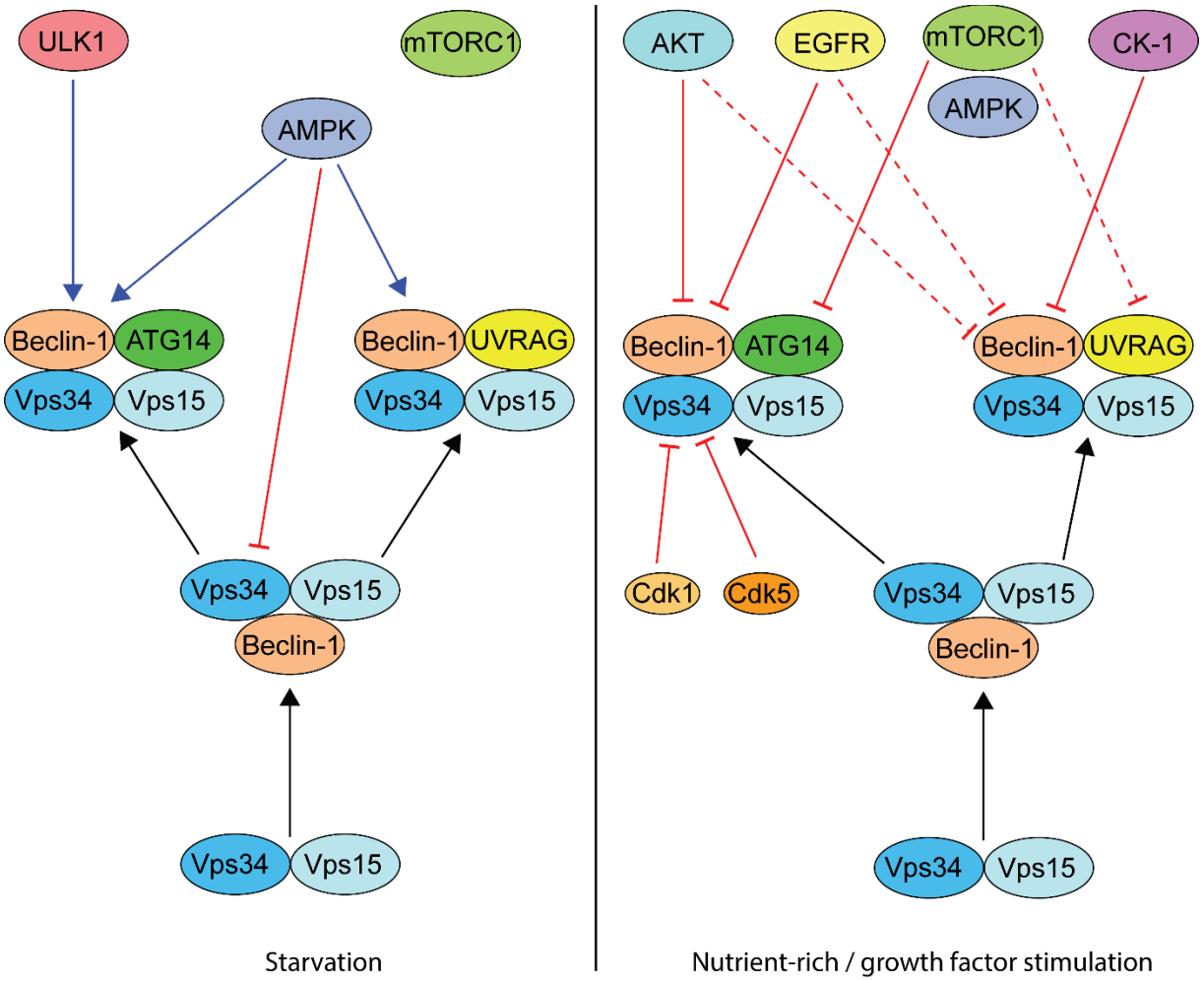
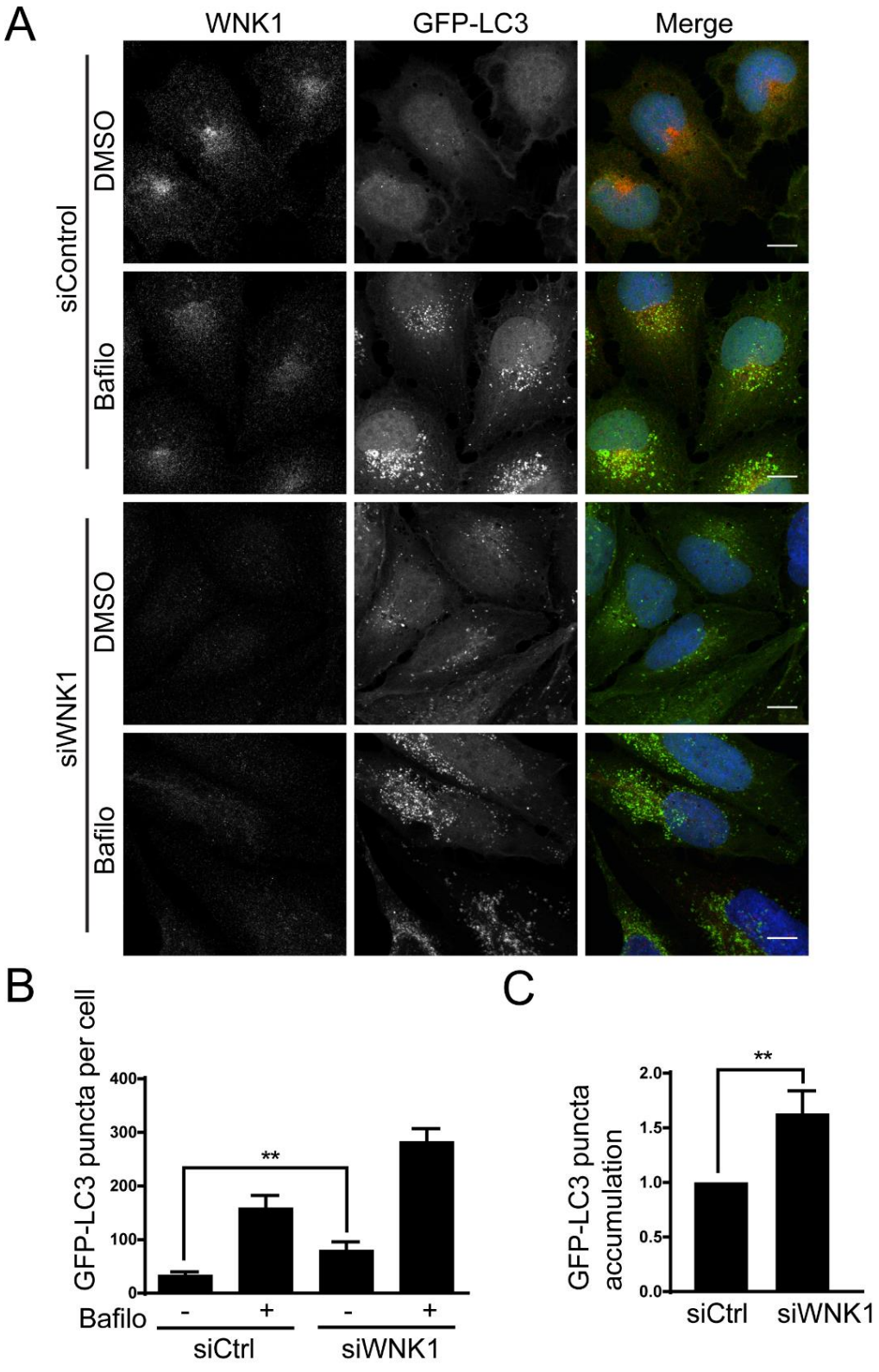



Figure 2.4. The regulation of PI3KC3 complexes under starvation and nutrient-replete/growth factor-containing conditions by phosphorylation events. This figure is modified from figures 4 and 5 of Backer 2016 [152] and Figure 7.F of Kim et al. 2013 [241].

## Results

### WNK1 Depletion Increases Autophagy

To analyze its role in autophagy, WNK1 was knocked down with small interfering RNA (siRNA) in U2OS cells stably expressing green fluorescent protein tagged LC3 (GFP-LC3) [208, 259]. WNK1 depletion increased the number of GFP-LC3 puncta (Fig. 2.5 A and B), showing that reducing the amount of WNK1 increased autophagosome formation. Bafilomycin, which inhibits autophagosome-lysosome fusion, increased the accumulation of GFP-LC3 puncta in WNK1-depleted cells (Fig. 2.5 A and C), indicating that the knockdown of WNK1 increased autophagic flux. The accumulation of LC3-II, a marker of autophagy [260], in bafilomycin-treated cells increased with WNK1 depletion (Fig. 2.6 A and B), consistent with the conclusion that reduced WNK1 expression accelerated autophagy. I also depleted WNK1 from HeLa cells and found a decrease in the selective autophagy substrate p62 (SQSTM1) [261] and an increase in LC3-II in both nutrient-replete and nutrient-starved cells (Fig. 2.6 C and D). Similar increases were found in autophagy, following WNK1 knockdown using multiple siRNA oligonucleotides and also in HEK293T and A549 cells (Figure 2.7 and Table 2.1). I conclude that WNK1 inhibits basal and starvation-induced autophagy in multiple systems.


A
WNK1
GFP-LC3
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siControl
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2.0
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siCtrl
siWNK1

Figure 2.5. WNK1 depletion increases autophagy - Immunofluorescence analysis. (A) U2OS GFP-LC3 cells were treated with 20 nM siControl or siWNK1 oligonucleotides. After three days, fresh medium was added containing DMSO or bafilomycin for four hours. Then, cells were analyzed by immunofluorescence. The images were deconvoluted, opened in Imaris 8 (Bitplane), and were subjected to background subtraction (filter width, 26.6 μm). The DAPI channel was masked and output was generated for GFP channel to remove the nuclear GFP from the analysis. The puncta-size threshold was set at 0.3 μ and puncta was selected by adjusting the quality in the spots. Then, the puncta were automatically counted by the software. At least 40 cells were selected per condition in each experiment. (Scale bars, 12 μm.) (B) Quantitation of change in number of GFP-LC3 puncta per cell in A. (C) Quantitation of relative change in GFP-LC3 puncta in bafilomycin-treated cells in A. **P < 0.01.

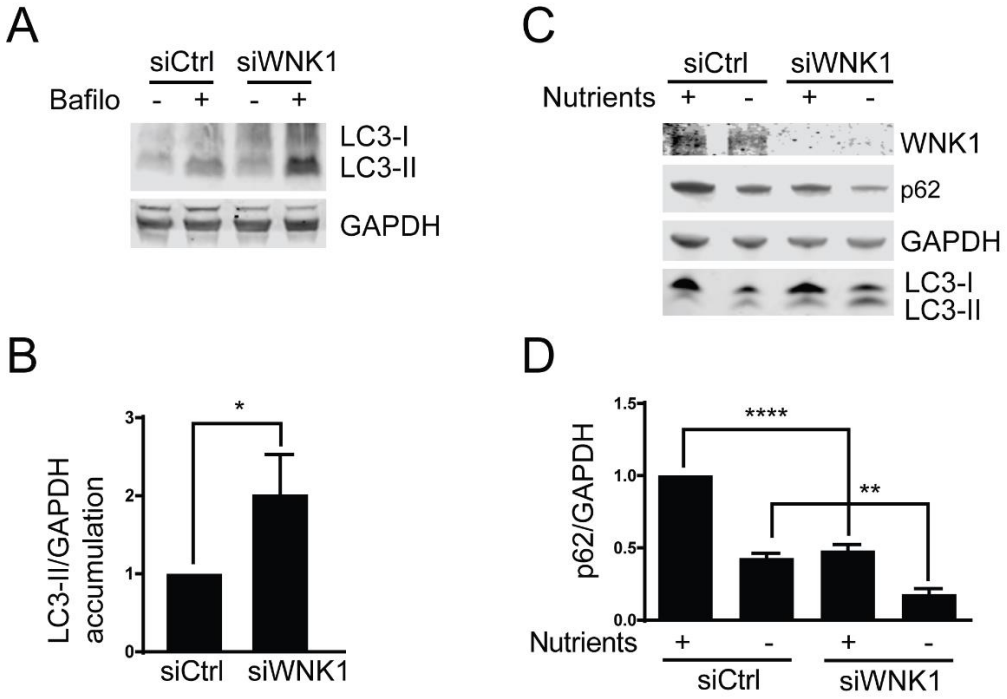


Figure 2.6. WNK1 depletion increases autophagy – Immunoblotting analysis. (A) WNK1 was knocked down in U2OS GFP-LC3 cells and treated with bafilomycin as described in Figure 2.5. Immunoblots are shown. (B) Quantitation of relative change of LC3-II in bafilomycin-treated cells in A. (C) HeLa cells were treated with 20 nM siControl or siWNK1 #2 for either three days or repeated after two days. Before lysis, cells were incubated in medium without or with nutrients for four hours. Proteins were analyzed by immunoblotting. (D) Quantitation of relative change of p62 in C. *P < 0.05, **P < 0.01, ****P < 0.0001.

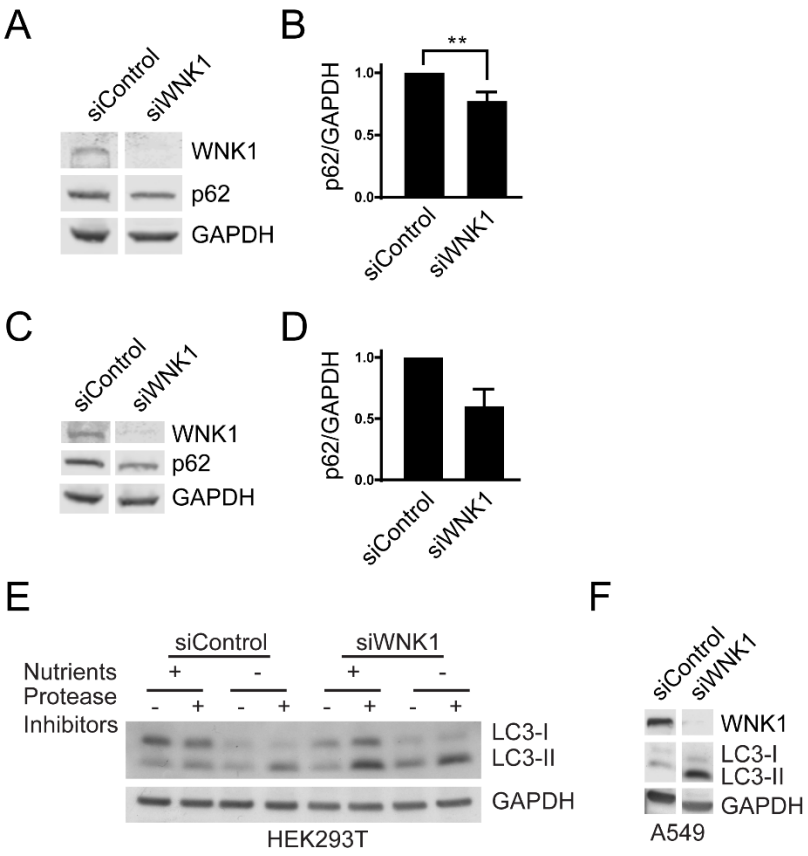


Figure 2.7. WNK1 inhibits autophagy in multiple cell lines. (A) HeLa cells were treated with 20 nM siControl or siWNK1 #3 oligonucleotides. After three days, the medium was replaced with fresh medium for four hours. Cells were then lysed, and proteins analyzed by immunoblotting. (B) Quantitation of the relative change of p62 in A. **P < 0.01. (C) HeLa cells were treated with 20 nM siControl or siWNK1 #4 oligonucleotides. After three days, the medium was replaced with fresh medium for four hours. Cells were lysed, and proteins analyzed by immunoblotting. n = 2. (D) Quantitation of the relative change of p62 in C. (E) HEK293T cells were treated with 10 nM siControl or siWNK1 #2 oligonucleotides. After three days, cells were treated with E-64d/pepstatin A or DMSO for four hours. Cells were then lysed in (150 mM NaCl, 10 mM Tris pH 7.4, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 0.5% Nonidet P-40 with 1% protease inhibitors), mixed with Laemmli sample buffer, and proteins were analyzed by immunoblotting. Secondary antibodies conjugated with horseradish peroxidase were used for blotting by enhanced chemiluminescence. Other WNK1 knockdown experiments in this cell line yielded similar results. (F) A549 cells were treated with 10 nM siControl or siWNK1 #2 oligonucleotides. After three days, cells were lysed, and proteins analyzed by immunoblotting as in E. Experiments in E and F were performed by A-Young Lee, Ph.D.

**WNK1 Depletion Increases the Activity of PI3KC3**

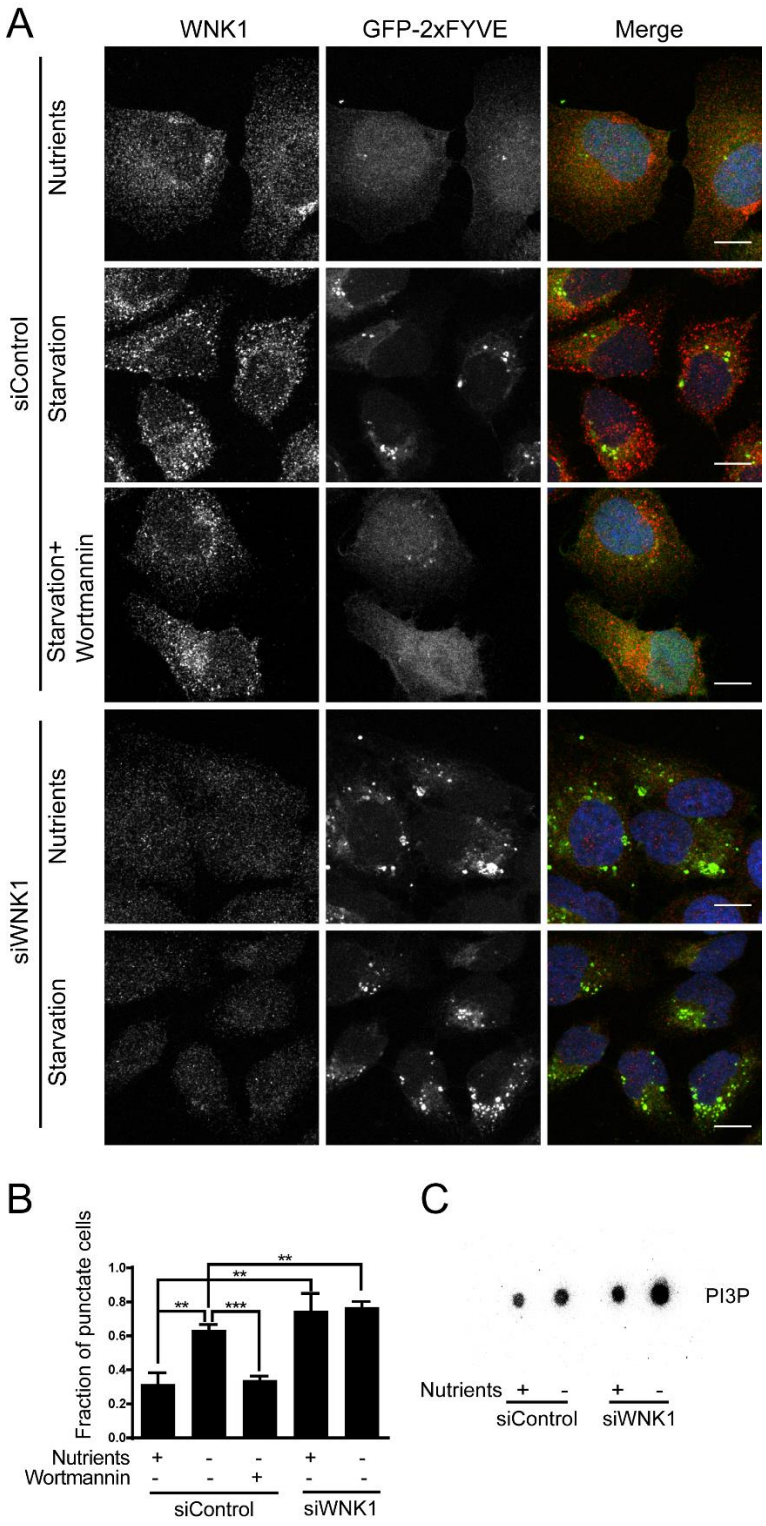

Figure 2.8. WNK1 depletion increases the activity of PI3KC3. (A) WNK1 was knocked down with 20 nM siRNA in U2OS cells. After one day, the cells were transfected with a GFP-2xFYVE domain-expressing plasmid. After two days, cells were placed in normal or starvation medium containing DMSO or 150 nM wortmannin for four hours and then analyzed by immunofluorescence. The images were opened in Imaris 8 (deconvoluted) or ImageJ; images opened in Imaris 8 were subjected to background subtraction (filter width 26.6 μm). Cells were categorized as having obvious GFP-2xFYVE puncta or with diffuse GFP-2xFYVE expression. At least 100 cells were selected per condition in each experiment. (Scale bars, 12 μm.) (B) Quantitation of the fraction of GFP-2xFYVE punctate cells in A. **P < 0.01 and ***P < 0.001. (C) WNK1 was knocked down by 20 nM of siRNA in HeLa cells and after three days, the media was replaced by normal media or starvation media for four hours. Then the cells were lysed with Immunoprecipitation lysis buffer and immunoprecipitated by Beclin-1 antibody. Part of the immunoprecipitate was used for a Vps34 lipid kinase assay and PI3P production was analyzed. Lipid kinase assay was performed by Hashem Dbouk, Ph.D.

Because PI3P binds to FYVE domain-containing proteins, a localized increase in PI3KC3 activity will be detected as an increase in FYVE domain puncta [262]. I expressed a GFP-2xFYVE domain construct in U2OS cells and validated its ability to detect PI3KC3 activity using starvation to induce autophagy. As expected, starvation increased the fraction of cells that displayed dominantly punctate GFP-2xFYVE staining (Fig. 2.8 A and B). Wortmannin, which inhibits PI3KC3 [263], converted the staining pattern from punctate to diffuse (Fig. 2.8 A and B). Similar to starvation, depletion of WNK1 increased the fraction of cells in which GFP-2xFYVE staining was punctate, whether in nutrient-rich or nutrient-poor medium (Fig. 2.8 A and B). Similar effects of WNK1 depletion on PI3KC3 were also observed in HeLa cells (Fig. 2.9). I also knocked down WNK1 and co-immunoprecipitated Vps34 from HeLa cells using a Beclin-1 antibody. Then, a Vps34 lipid kinase assay was performed and it was observed that depletion of WNK1 increased the kinase activity of immunoprecipitate associated with Beclin-1. This effect was present under both nutrient-rich treatment and cellular starvation (Figure 2.8 C). These data support the conclusion that WNK1 knockdown increases PI3KC3 complex activity.

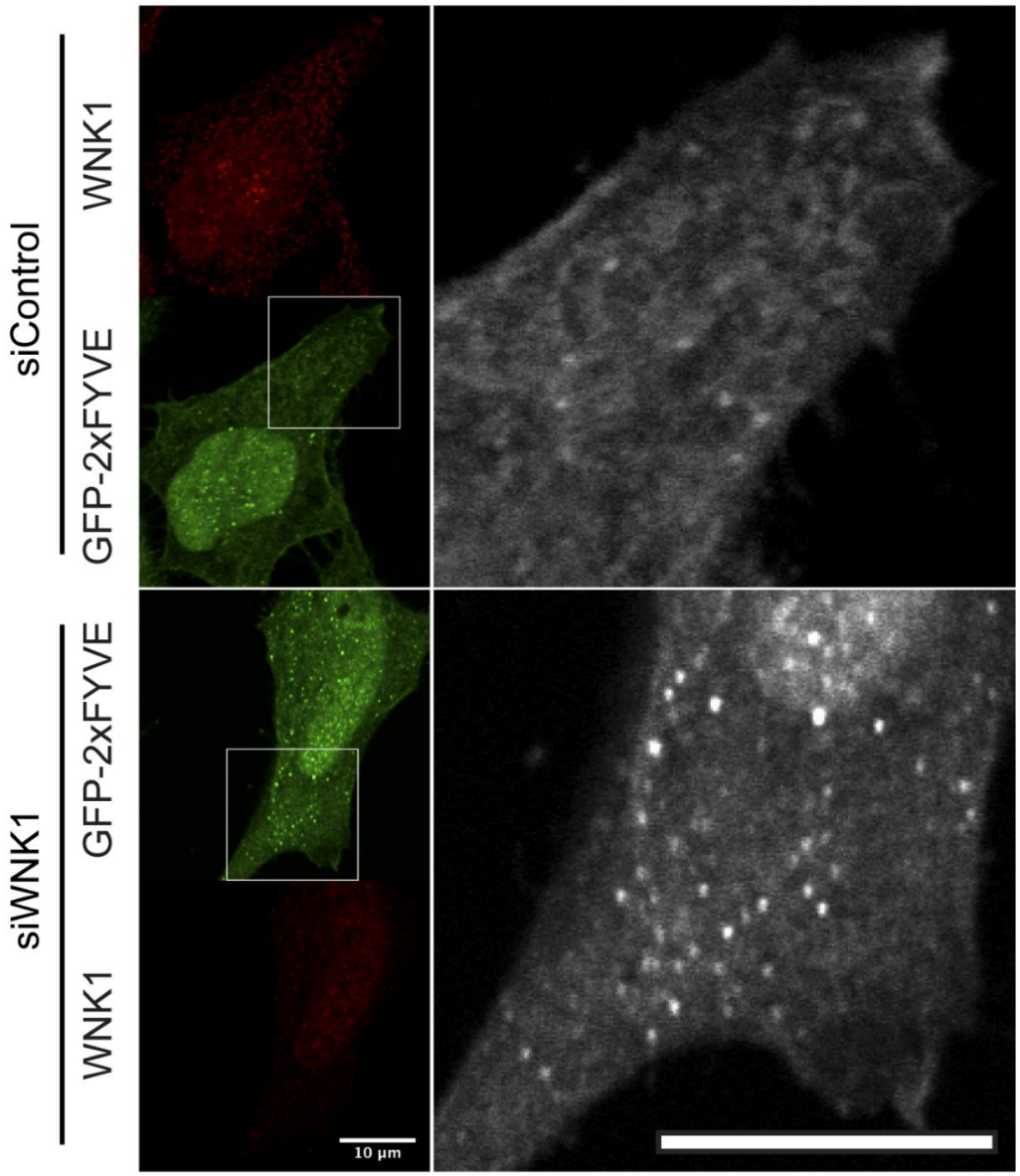


Figure 2.9. WNK1 inhibits the activity of PI3KC3 in HeLa cells. WNK1 was knocked down from HeLa cells by 20 nM siRNA. The next day, cells were transfected with a plasmid expressing a GFP-2xFYVE domain. After two more days, the medium was replaced by fresh medium for four hours. Then the cells were analyzed by immunofluorescence on a Zeiss LSM 780. Images shown on the right are enlargements of boxed portions of images on the left. Images shown here are selected from the mid plane of the Z stack. (Scale bars, 10 µm.). Imaging in this experiment was performed by Andres Lorente-Rodriguez, Ph.D.

**WNK1 Depletion Increases the Amount and Phosphorylation of Unc-51–Like Kinase 1**

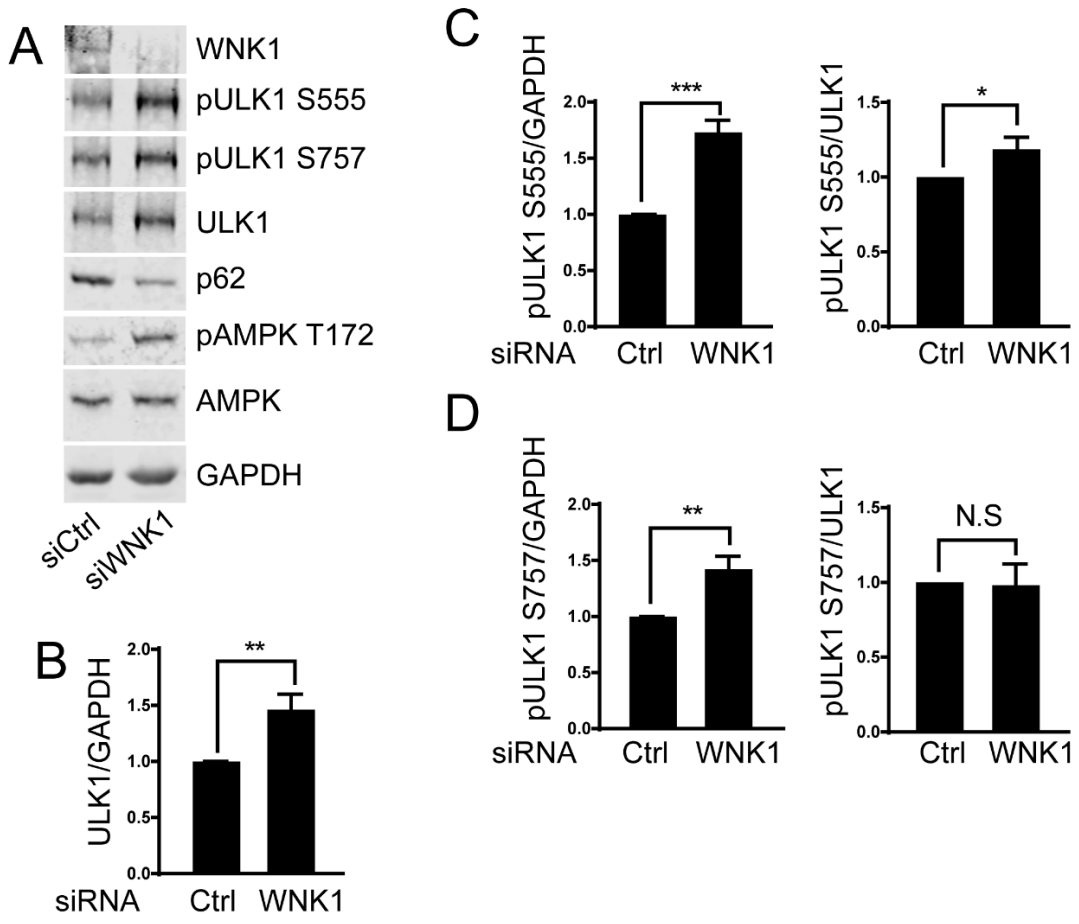


Figure 2.10. WNK1 depletion increases the amount and phosphorylation of ULK1. (A) HeLa cells were treated with 20 nM siControl or siWNK1, plus an additional 20 nM siControl. After three days, cells were placed in fresh medium for four hours, lysed, and proteins were analyzed by immunoblotting. (B) Quantitation of relative change of ULK1 expression in A. (C) Quantitation of relative change in total ULK1 pS555 and ULK1 pS555/total ULK1 in A. (D) Quantitation of relative changes in total ULK1 pS757 and ULK1 pS757/total ULK1 in A. *P < 0.05, **P < 0.01, ***P < 0.001, and NS - not significant.

As stated in the introduction, phosphorylation of ULK1 by AMPK at S555 activates ULK1, whereas ULK1 phosphorylation by mTORC1 at S757 inhibits ULK1 [236, 237]. Depletion of WNK1 increased phosphorylation of S555 (pS555) as well as the total amount of ULK1, resulting

in a net increase in the ratio of pS555 ULK1/ULK1 (Figure 2.10 A, B and C). pS757 ULK1 also increased with WNK1 depletion; however, the ratio of pS757 ULK1/ULK1 did not change (Figure 2.10 A and D).

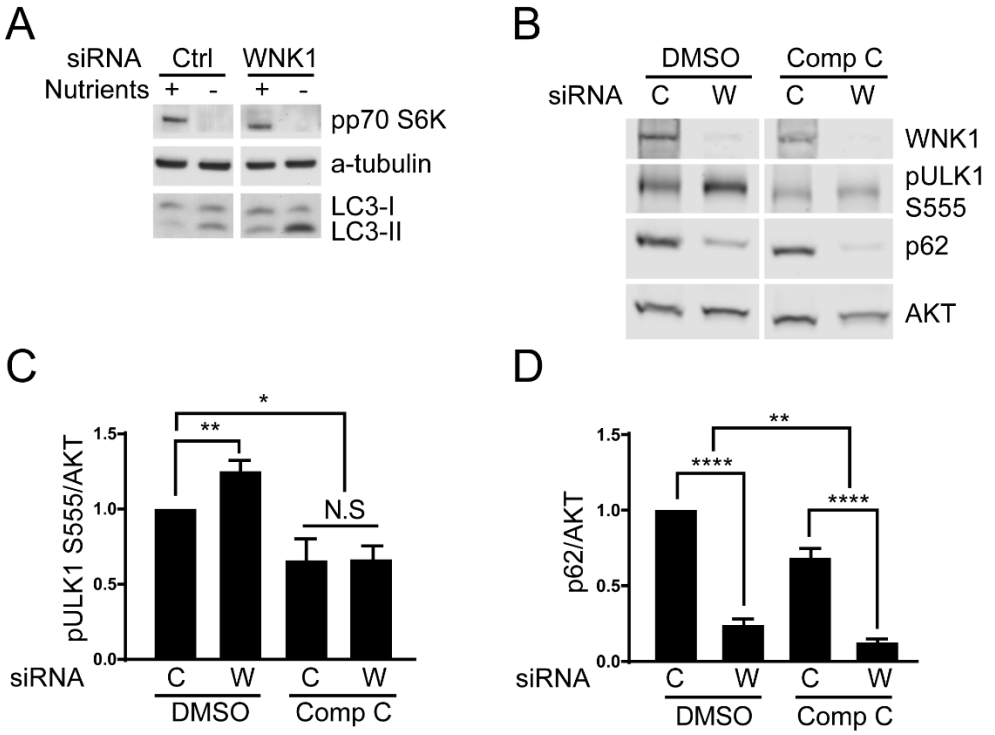


Figure 2.11. WNK1 action on autophagy is independent of mTORC1 and partially dependent on its action on ULK1 (A) HeLa cells were treated with 10 nM siControl or siWNK1 #2 for three days, placed in normal or starvation medium for two hours, lysed (see Figure 2.7 for lysis buffer) and proteins were analyzed by immunoblotting; secondary antibodies conjugated with horseradish peroxidase were detected by enhanced chemiluminescence. This experiment was performed by A-Young Lee, Ph.D. (B) WNK1 was knocked down by 20 nM siRNA in HeLa. After three days, cells were treated with DMSO (control) or 10 μM compound C for four hours, lysed and proteins were analyzed by immunoblotting. (C) Quantitation of ULK1 pS555 relative change in B. (D) Quantitation of relative change in p62 in B, n = 4. Compound C decreased p62 in siControl, $P < 0.0001$ and with siWNK1, $P < 0.01$. Despite the fact that compound C inhibits AMPK, it has been shown previously to induce autophagy [264]. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$. NS, not significant.

The mTORC1 activity was assessed independently by measuring phosphorylation of p70 S6 kinase [265] and it was found that the activity of mTORC1 was unchanged by WNK1 depletion (Figure 2.11 A). In contrast, the loss of WNK1 increased phosphorylation of the activating site T172 on AMPK, suggesting enhanced AMPK activation (Figure 2.10 A), and consistent with increased ULK1 pS555. Treatment of cells with the AMPK inhibitor compound C decreased ULK1 pS555 and prevented the increase caused by WNK1 depletion (Figure 2.11 B and C). Compound C had relatively little effect on the reduction of p62 caused by WNK1 depletion (Figure 2.11 B and D). The AMP/ATP ratio in cells depleted of WNK1 was unchanged (Figure 2.12). From these data, I suggest that autophagy inhibition exerted by WNK1 is partially dependent on its effect on ULK1 but depends more on other mechanisms.

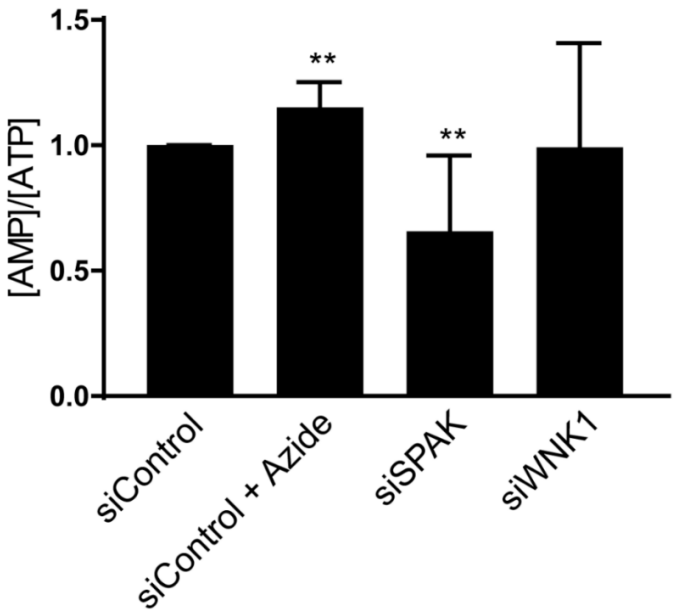


Figure 2.12. Effect of WNK1 and SPAK knockdown on AMP/ATP ratio. HeLa cells were treated with 20 nM siControl, siWNK1 and siSPAK for three days. Before lysis, half of siControl cells were treated with 5–10 mM $NaN_3$ for two hours as a positive control to decrease ATP. Cells were then lysed, and their adenosine nucleotide content was measured, as stated in Materials and Methods. n = 7. For $NaN_3$ treatment, n = 3. **P < 0.01. This experiment was performed with graduate student Akansha M. Shah.

**WNK1 Colocalizes and Interacts with UV Radiation Resistance-Associated Gene**

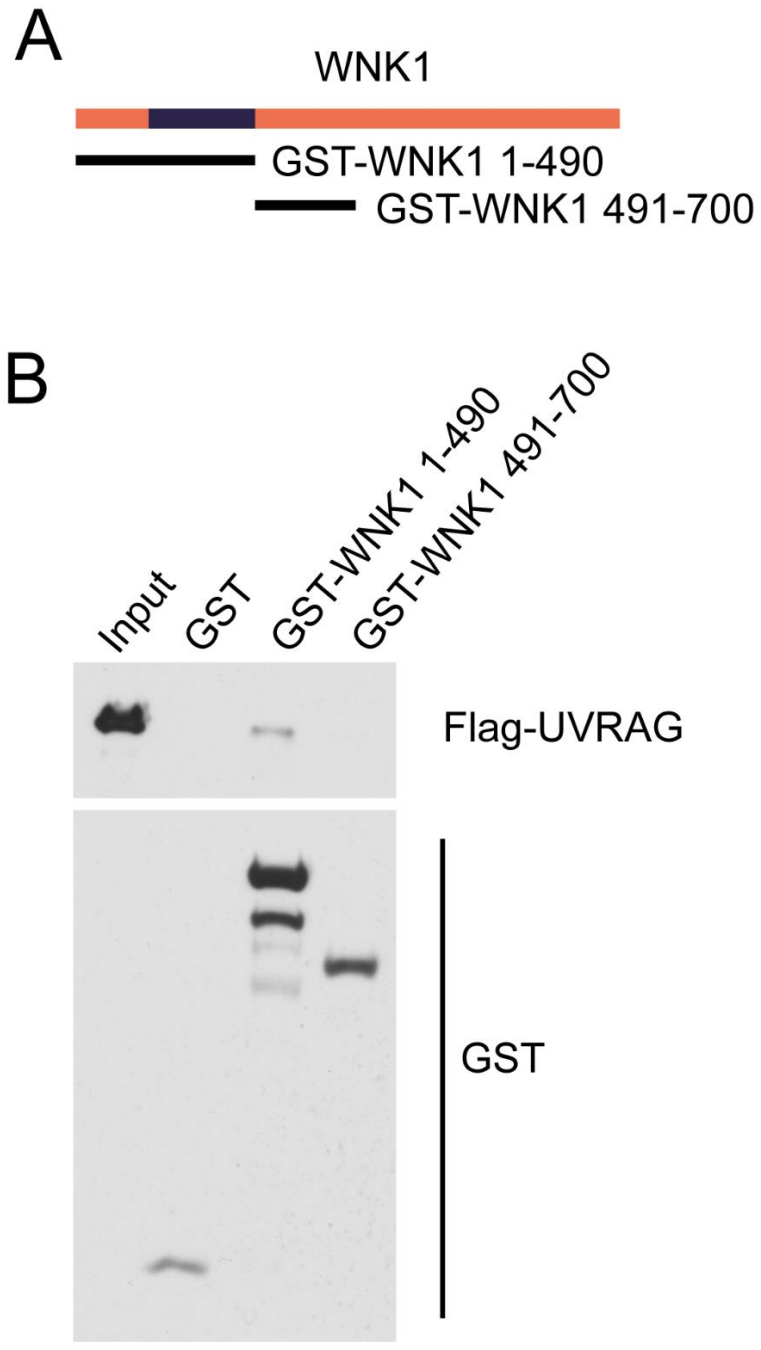


Figure 2.13. WNK1 interacts with UVRAG in vitro. (A) Rat WNK1 fragments. WNK1 1–490 encompasses the kinase domain, residues 193–490. (B) Recombinant Flag-UVRAG proteins were captured in vitro with the indicated GST proteins. Binding was detected by immunoblotting and chemiluminescence as in Figure 2.7 E. The experiment shown here was performed by A-Young Lee, Ph.D.

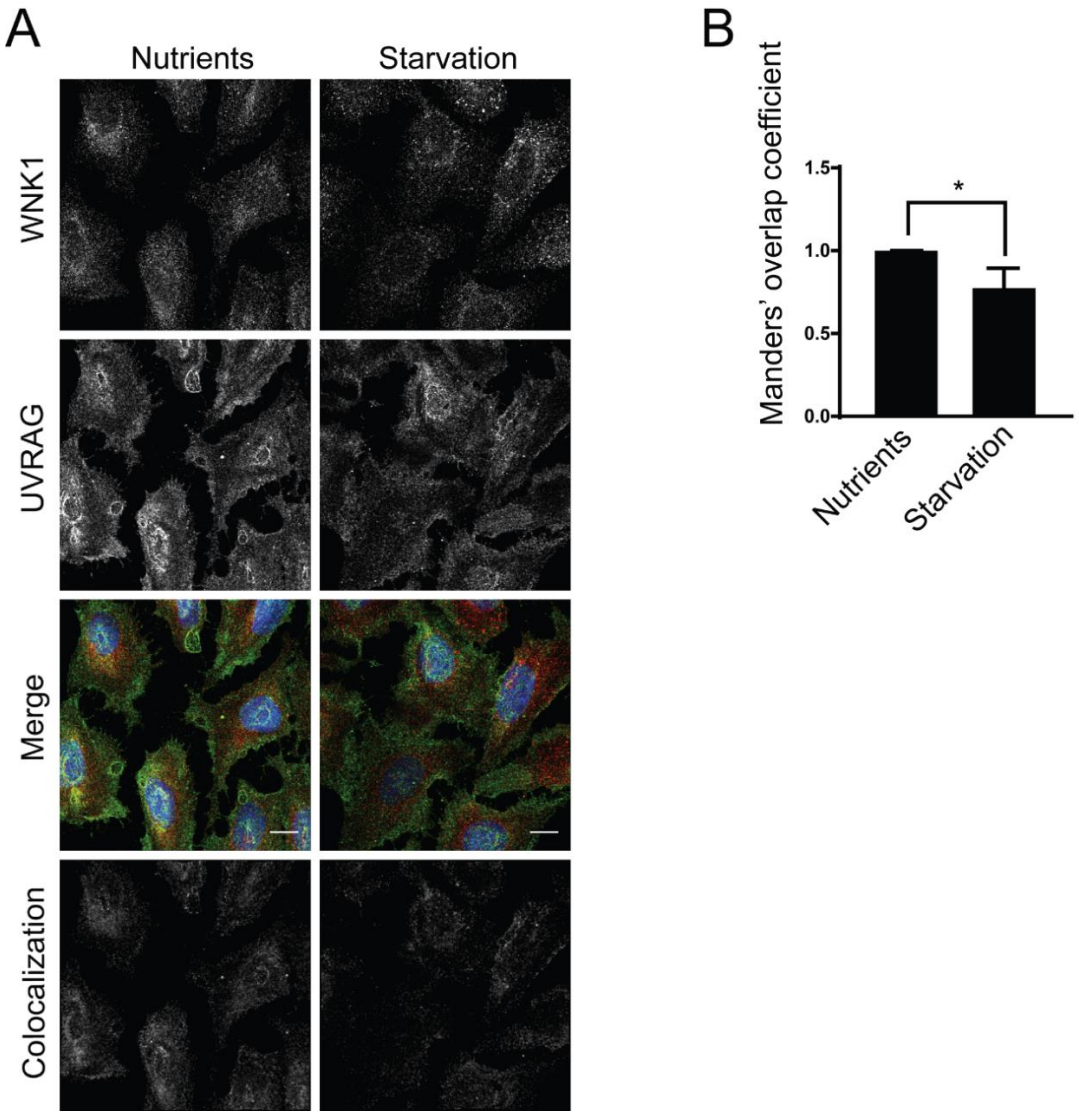


Figure 2.14. WNK1 colocalizes with UVRAG. (C) U2OS cells were plated on glass coverslips. After three days, fresh normal or starvation medium was added for four hours. Cells were analyzed by immunofluorescence. Images were deconvoluted and corrected for background as in Fig. 2.8 A. Colocalization thresholds for WNK1 and UVRAG channels were calculated and a colocalization channel was generated by the Imaris 8 software. The colocalization image shown was captured by the software. (Scale bars, 12 μm.) (D) Quantitation of the change of colocalization between WNK1 and UVRAG in A, using the relative Manders' coefficient. *$P < 0.05$.

Next, WNK1 binding to components of the PI3KC3 complex was evaluated as a possible mechanism by which it exerts additional inhibitory effects on autophagy. Of several components tested, WNK1 bound to UVRAG in vitro using a GST pull-down assay with recombinant Flag-UVRAG and GST-WNK1 fragments (Figure 2.13 A). Flag-UVRAG bound GST-WNK1 1–490 but not GST-WNK1 491–700 or GST alone (Figure 2.13 B). These data indicate that UVRAG can interact with the N-terminal region of WNK1.

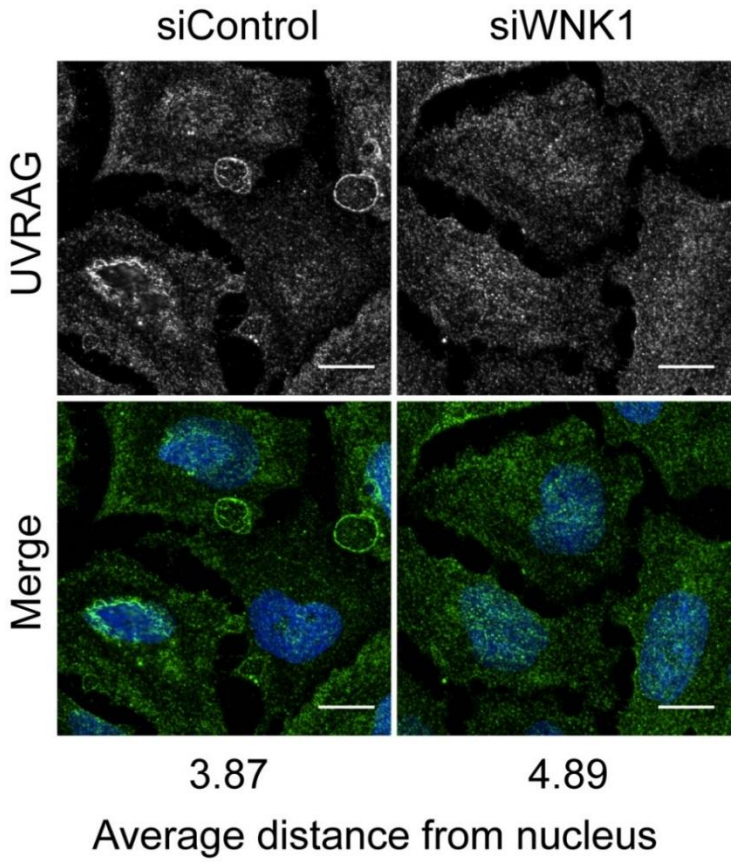


Figure 2.15. WNK1 depletion re-localizes UVRAG. WNK1 was knocked down by 20 nM siRNA in U2OS cells. After three days, the cells were treated with fresh medium for four hours. Then the cells were analyzed by immunofluorescence. The UVRAG cellular distribution was calculated using CellProfiler software [266] by Pearl Wichaidit, Ph.D. For each cell, the median Euclidean distance between UVRAG-staining speckles and the center of the nucleus was obtained and normalized to median cell radius. The resulting value was taken as the distance of UVRAG from the nucleus. (Scale bar, 150 pixels)

To determine whether these proteins were in proximity in cells, I assessed the localization of endogenous WNK1 and UVRAG by immunofluorescence. WNK1 is found in a punctate pattern throughout the cytoplasm of many cells [12, 267, 268] and displayed some colocalization with UVRAG (Figure 2.14 A and B). Activation of autophagy by starvation decreased the colocalization between the two proteins (Figure 2.14 A and B). Depletion of WNK1 in U2OS cells changed the distribution of UVRAG, as observed by immunofluorescence, indicating that WNK1 may influence the cellular localization of UVRAG (Figure 2.15). Therefore, I suggest that WNK1 also affects the PI3KC3 complex via its component UVRAG.

**The Depletion of SPAK in Cells Increases Autophagy**

Because most of the known functions of WNKs are mediated by their downstream targets OSR1 and SPAK, I compared their effects on autophagy. Depletion of SPAK, but not OSR1, caused a reduction of p62 (Figure 2.16) and increased the ratio of LC3-II/LC3-I (Figure 2.16 C and E). These data show that the WNK1 downstream kinase SPAK also inhibits autophagy under nutrient-rich and -starved conditions, and may serve as a mediator of WNK1 actions on autophagy, whereas its relative OSR1 has no apparent effect on this process.

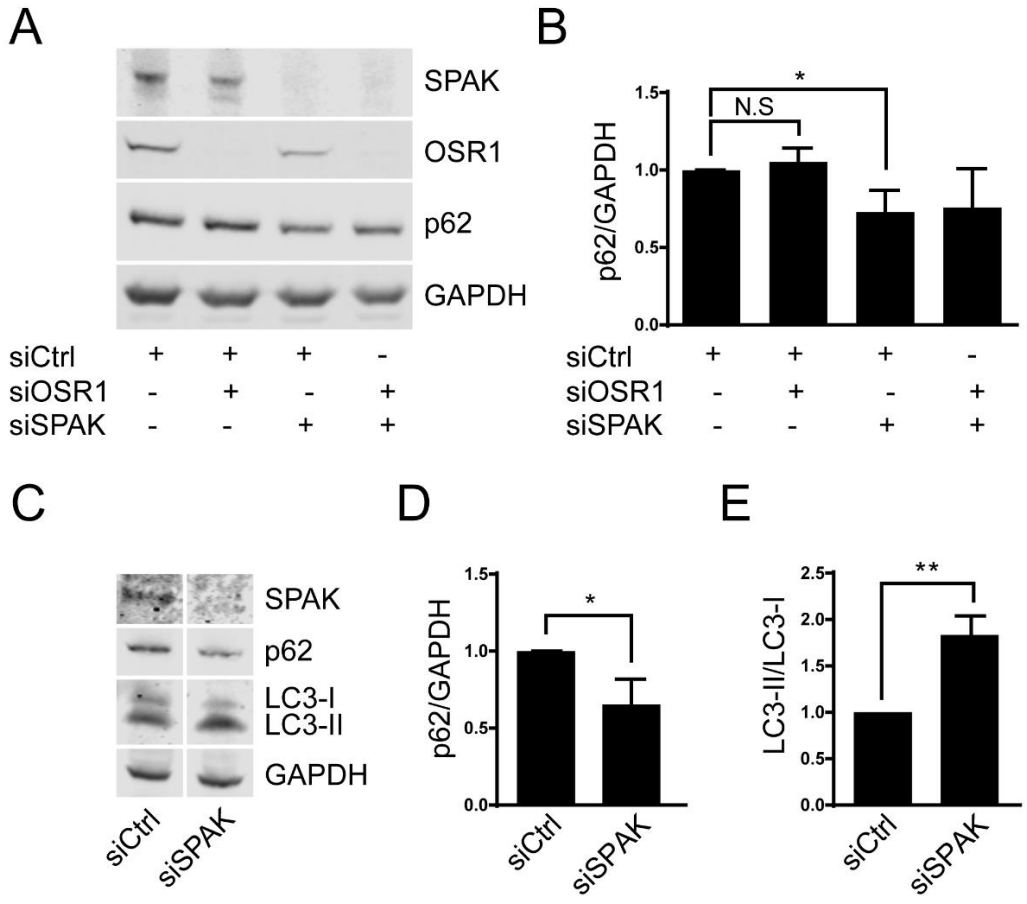


Figure 2.16. SPAK depletion increases autophagy. (A) HeLa cells were treated with 30 nM siRNA for OSR1 and SPAK, individually or in combination (total 60 nM), as indicated. After three days, fresh medium was added for four hours before harvest and protein immunoblotting. (B) Quantitation of the relative p62 change in A. *P < 0.05. NS, not significant. (C) HeLa cells were treated with 20 nM siRNA for SPAK and after three days, cells were starved for four hours. Lysate proteins were immunoblotted. (D) Quantitation of the relative p62 change in C. (E) Quantitation of the relative change of LC3-II/LC3-I ratio in C. *P < 0.05 and **P < 0.01.

**Discussion**

In this study, I show that reduced expression of WNK1 accelerates autophagy in diverse cell types, leading to our conclusion that a normal function of WNK1 is to suppress autophagy. Previously, WNK2 has been implicated in autophagy, both as an autophagy activator and inhibtior [148, 149].

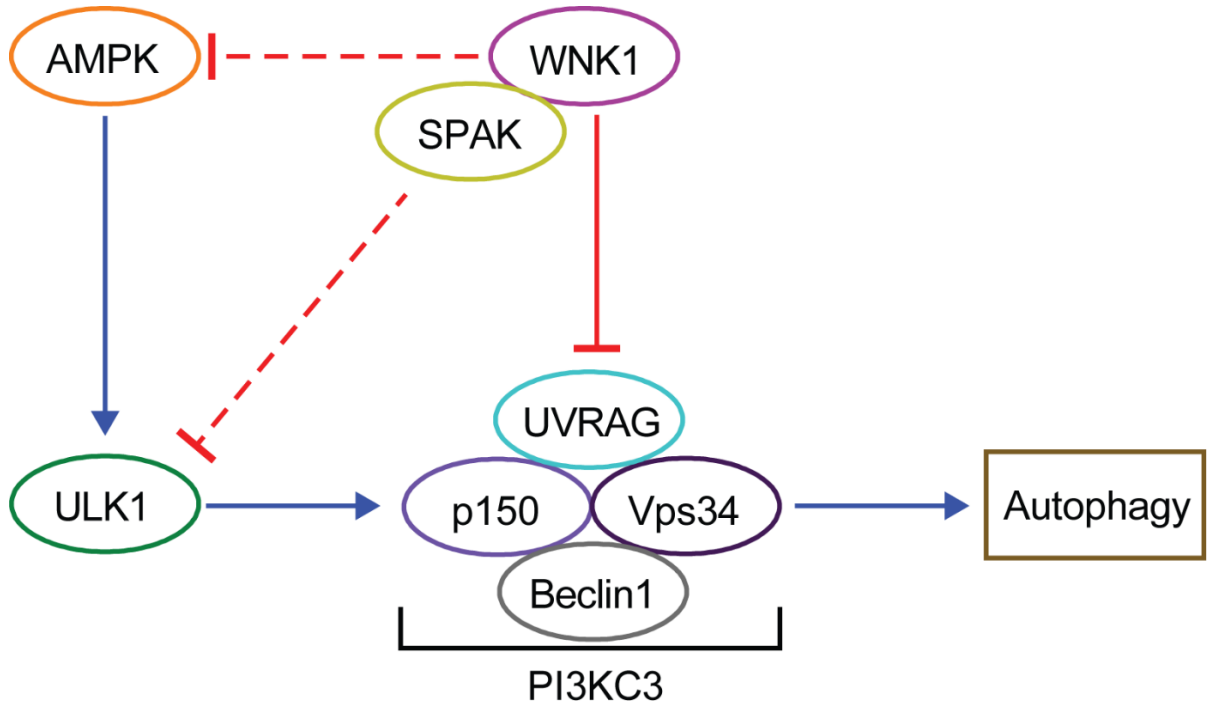


Figure 2.17. A model of autophagy regulation by WNK1.

WNK1 appears to restrain autophagy by acting at multiple levels in the process (Figure 2.17). Increased phosphorylation of AMPK and its substrate ULK1 in cells with reduced WNK1 expression indicates an inhibitory effect of WNK1 at the initial autophagy-inducing steps of the pathway. The fact that compound C has only a minor effect on autophagy induction by WNK1 depletion is evidence that this step contributes relatively little to the action of WNK1 on autophagy. In contrast to several autophagy regulators, WNK1 works independently of mTORC1, based on

the lack of change in mTORC1 activity using phosphorylation of ULK1 and p70S6 kinase as readouts. Autophagy regulation by WNK1 action on PI3KC3 complexes is most clearly demonstrated by changes in PI3KC3 activity and a mechanism may be the interaction of WNK1 with UVRAG. Reduced WNK1 increases PI3KC3 activity deduced from cellular reporter and lipid kinase assays, indicating that the presence of WNK1 suppresses PI3KC3. WNK1 colocalization with UVRAG is reduced upon induction of autophagy, consistent with the idea that the interaction of WNK1 with UVRAG inhibits autophagy. UVRAG is a component of the PI3KC3 complex that enhances autophagosome formation and maturation [244, 249]. The interaction with WNK1 may redirect UVRAG away from its role in autophagy. For example, WNK1 may extract UVRAG from the PI3KC3 complex or divert UVRAG toward endocytic trafficking, another function of the UVRAG–PI3KC3 complex [269-271]. WNK1 itself has been associated with endocytic trafficking (24–27). Based on our current findings, the UVRAG–PI3KC3 step accounts for the most substantial effect of WNK1 on autophagy. Previous studies using gene disruption in mice suggested that OSR1 and WNK1 have some overlapping functions in endothelial cells during cardiovascular development [175], and further in vitro studies suggested different functions of OSR1 and SPAK [16]. In examining the possibility that OSR1 or SPAK mediate the effects of WNK1 on autophagy, I found that depletion of SPAK, but not OSR1, stimulated autophagy. From side-by-side comparison in multiple experiments, depletion of SPAK causes only a fraction of the increase in autophagy caused by decreased WNK1 expression. This is added evidence suggesting that there is more than one mechanism through which WNK1 slows autophagy.

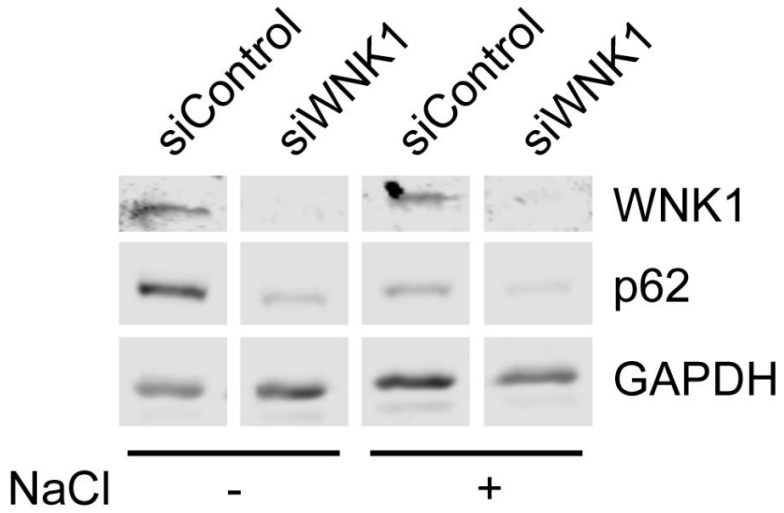


Figure 2.18. Hyperosmotic stress-induced autophagy is independent of WNK1. WNK1 was knocked down by 20 nM siRNA in HeLa cells. After three days, the cells were treated with medium containing 0.05-0.2 M NaCl (Also with 0.1% DMSO) for four hours. Then the cells were analyzed by immunoblotting. n = 2.

Osmotic stress has been shown to induce autophagy [272, 273]. Because WNK1 is activated by osmotic stress, I tested the idea that the osmotic response occurs via WNK1. If so, loss of WNK1 should have prevented any further activation of autophagy by hypo- or hyperosmotic stress. I found that autophagy was further stimulated by exposure to either type of osmotic challenge even in cells with little WNK1 expression (Figure 2.18). Thus, I was unable to make a connection between osmotic stress-induced autophagy and WNK1 and expect that the osmotic response is mediated by some other pathway, such as the p38 pathway [274].

Some mechanisms have been identified for regulating the amount of the autophagy kinase ULK1 [275, 276]. One of these involves the kelch-like adaptor, KLHL20, which connects ULK1 to Cullin-3 for degradation by an F-box E3 ligase. WNKs are also regulated by binding kelch-like adaptors, KLHL2 and KLHL3, which target them for degradation [29, 30, 32-34]. Concentrations of WNKs can be changed relatively rapidly in cells by this type of degradative process with pathophysiological implications [58]. Changing the WNK1 amount through degradation, distinct

from changing WNK1 activity, may be a physiologically significant mechanism to induce autophagy.

I demonstrate here that WNK1 can exert inhibitory control over autophagy that is sensitive to WNK1 expression. Among our findings, the connection between WNK1 and regulation of PI3KC3, although not fully defined mechanistically, may have implications for other processes mediated by products of this lipid kinase.

WNK1 is widely expressed, yet little is known about its actions beyond its well-studied regulation of ion transport proteins [5, 8]. Our finding that WNK1 suppresses autophagy in multiple cell types is indicative of WNK1 actions outside of ion transport. Because autophagy is a complex cellular pathway with many regulatory inputs, identifying steps influenced by WNK1 may be of value to better understand how autophagy is controlled.

## Materials and Methods

### Materials

The following antibodies were used: WNK1, SPAK, OSR1, ULK1, ULK1 pS555, ULK1 pS757, AKT1 (Cell Signaling Technology); p62, GAPDH, GST (Santa Cruz); LC3 (MBL); UVRAG, Flag M2 (Sigma-Aldrich); HRP-conjugated secondary antibodies (MBP). The following siRNAs were obtained from Life Technologies/Ambion: siOSR1 (Silencer select validated s19303), siSPAK (Silencer select validated s26208); WNK1 siRNAs are in Table 2.1. The following plasmids were used: pFlag-UVRAG-11d, [hUVRAG cloned into pFlag-11d, made by combining pFlag-CMV2 (Sigma) and pET-11d (Stratagene)], pGEX-KG-WNK1 1–490, pGEX-KG-WNK1 491–700 [79]. The plasmids were generated by Steve Stippec and A-Young Lee, Ph.D.

Chemicals were obtained as indicated: compound C (Calbiochem), bafilomycin (LC Laboratories), wortmannin (Sigma-Aldrich).

Table 2.1. siRNA oligonucleotides for WNK1 knockdown

| siRNA Name | Type | Sequence (5′–3′)/Information |
|---|---|---|
| siWNK1 | Silencer select custom | CAGACAGUGCAGUAUUCACtt |
| siWNK1 #2 | Silencer select custom | GGAUCAAGUGCGAGAAAUUtt |
| siWNK1 #3 | Silencer select validated | (s35233) |
| siWNK1 #4 | Silencer select validated | (s35234) |

**Cell Culture and Transfection**

HeLa, U2OS, and U2OS GFP-LC3 were grown in Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) FBS, 1% (vol/vol) L-glutamine at 37 °C, 5% $CO_2$. Earl's balanced salt solution (EBSS) was the starvation medium. Knockdown was for three days, except in indicated experiments in which it was repeated with a two-day interval. When plated, cells were transfected with the indicated concentrations of siRNA using Lipofectamine RNAiMAX reagent (Life Technologies) according to the manufacturer's instructions. The medium was changed the day after transfection. Plasmid transfection was with Lipofectamine 2000 (Life Technologies), according to manufacturer's instructions. The Lipofectamine 2000 to DNA ratio used was 2 μl: 1 μg. In some cases, DMSO was included as a solvent control.

**Immunofluorescence and Image Representation**

Unless stated otherwise, the following protocol was used for immunofluorescence. Cells on glass coverslips were fixed with 4% (vol/vol) paraformaldehyde in PBS for 15 minutes at room temperature followed by permeabilization with 0.1% of Triton X-100 in PBS for 4.5 minutes and blocking with 10% (vol/vol) normal goat serum (Invitrogen). Incubation with primary antibodies for one hour at room temperature or overnight at 4 °C was followed by fluorescently labeled Alexa Fluor secondary antibodies diluted 1:500 for one hour at room temperature. Coverslips were mounted on DAPI Fluoromount-G (Southern Biotech) medium. Fluorescent images were obtained with a Zeiss LSM880 laser scanning confocal microscope as z-stacks through 40x oil objective lense. The deconvoluted z-stacks were opened in FIJI (ImageJ) and compressed into 2D z-projections of maximum intensity [277]. The brightness and contrast of each channel of the image panels were adjusted similarly across experimental conditions.

**Image Deconvolution**

Where indicated, the images were deconvoluted by the software Autoquant X3 (Bitplane). Images were opened in Autoquant X3 and the output file format was selected as .ims (Imaris file type) with 16 bits. Then they were deconvoluted using the 3D blind deconvolution option.

**SDS-PAGE and Immunoblotting Analysis**

The following protocol was used unless otherwise noted. Cells were lysed with 2× Laemmli SDS sample buffer [250 mM Tris HCl, pH 6.8, 10% (wt/vol) SDS, 30% (vol/vol) glycerol], heated and sonicated or passed through a 25-gauge needle, followed by addition of 5% (vol/vol) β-mercaptoethanol and 0.02% bromophenol blue. Equal amounts of protein were resolved on SDS

8% (wt/vol) polyacrylamide gels or 15% (wt/vol) Tricine gels and transferred onto PVDF membranes. Membranes were blocked with Odyssey blocking buffer (#927-40000) diluted in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.1 % Tween 20. Membranes were incubated with primary antibodies diluted in the above solution overnight at 4 °C. After washing, they were incubated with Odyssey IRDye secondary antibodies diluted 1: 5,000 at room temperature for one hour, followed by imaging with a LI-COR Odyssey imager. The immunoblots were quantified using Image Studio software (LI-COR).

**Recombinant Protein Expression and Purification**

Flag-UVRAG was expressed in *Escherichia coli* Rosetta2 cells. Expression of recombinant proteins was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for three hours. The bacterial pellet was sonicated after addition of 80 mM Pipes, pH 6.8, 20% (vol/vol) glycerol, 1 mM $MgCl_2$, 1 mM EGTA, 0.2 mM EDTA, 0.5 M NaCl, 0.5 mM ATP, 0.1% Nonidet P-40, protease inhibitors. After sedimentation, M2 agarose beads in 80 mM Pipes, 20% (vol/vol) glycerol, 0.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM ATP, 1 mM DTT with protease inhibitors were added followed by rotation overnight at 4 °C. The beads were sedimented, washed with the buffer above containing 300 mM KCl plus 0.1% Nonidet P-40, and again without Nonidet P-40. Beads were transferred to a spin column and recombinant proteins were eluted with 0.2 mg/mL Flag peptide in the above buffer with 100 mM KCl and 0.03% Nonidet P-40. This was performed by A-Young Lee, Ph.D.

**GST Pull-Down Assay**

The plasmid pGEX-KG that expresses GST and GST-tagged WNK1 fragments were expressed in *E. coli*. Then the cells were lysed and sonicated (See above) (This was performed by A-Young Lee, Ph.D.) Then, Pierce glutathione agarose beads (Thermo Scientific) were added to the lysates, rotated at 4 °C and obtained by centrifugation. The beads were washed three times with lysis buffer, twice with wash buffer with 0.1 M KCl, and mixed with recombinant Flag-UVRAG proteins in vitro at 4 °C for one hour. The beads were then sedimented, washed, and proteins eluted with Laemmli buffer with 5% (vol/vol) β-mercaptoethanol. Protein association was assessed by immunoblotting.

**Immunoprecipitation and Lipid Kinase Assay for Vps34**

After subjecting for experimental conditions, samples were lysed in Immunoprecipitation lysis buffer (50 mM HEPES pH 7.7, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 % Glycerol, 0.2 mM $Na_3VO_4$, 100 mM NaF, 50 mM β-glycerophosphate, 0.1% protease inhibitor and 0.1% Nonidet P-40), flash-frozen in liquid nitrogen and stored in -80 ºC. Then they were thawed on ice and rotated for 30 minutes, and sedimented at maximum speed for 10 minutes. The supernatant was obtained for immunoprecipitation. Then, a Beclin-1 antibody was added to the lysates (1 µg per 250-300 µl) and they were rotated for two hours. Then, Protein A/G-Plus Agarose beads (Santa Cruz) were added to the samples and rotated again for one hour. Beads were sedimented and washed 3 times with PBS containing 1% Nonidet P-40. The beads were washed three times with 500 mM LiCl in 50 mM Tris (pH 7.4), twice in TE solution (20 mM Tris (pH 7.5), 100 mM NaCl and 1 mM EDTA) and resuspended in 25 µl of the TE solution. Volumes of 10 µl of 100 mM $MnCl_2$, 10 µl of lipid vesicles (final concentration – 100 µM PI) and 5 µl of ATP mix (1 µl hot

ATP + 1 µl cold ATP (100 µM stock) and 3 µl water) were added per each reaction. Then they were shaken at room temperature for 15 minutes. The reactions were stopped with 5 µl of 500 µM EDTA. Then the reactions were spotted on nitrocellulose membrane (0.2 µm pores) and allowed to air dry. The membranes were washed five times in 1 M NaCl with 1% phosphoric acid and allowed to dry. The radioactivity of the spots was analyzed by the phosphoimager or scintillation counting. Lipid kinase assay was performed by Hashem Dbouk, Ph.D.

**Protease Inhibitor Mixture Preparation**

Protease inhibitors were purchased from Sigma. Pepstatin A (25 mg), leupeptin (25mg), TAME (N-alpha p-tosyl L-arginine methyl ester; 250 mg), TLCK (N-alpha p-tosyl L-lysine chloromethyl ketone; 250 mg), BAME (N-alpha-benzoyl L-arginine methyl ester; 250 mg), and soybean trypsin inhibitor (250 mg) were dissolved in 62.5 mL of DMSO and stored at −20 °C. This was performed by Steve Stippec.

**Measurement of AMP and ATP Concentrations**

AMP and ATP were measured using the ATP/ADP/AMP Assay Kit (Biomedical Research Service and Clinical Application) according to the manufacturer's instructions. In brief, the cells were washed twice with cold 1× PBS, collected and sedimented at 12,000 × g for 10 min. The cell pellets were resuspended in 0.1 mL of cold water, vortexed vigorously, and boiled for 10 min and sedimented at 21,130 × g for 5 min. Measurements were made on the supernatants according to the manufacturer's protocol. Luminescence was measured using a plate reader.

**Statistical Analysis**

Prism software (GraphPad) was used for statistical analysis and the Student's t test was used to determine the statistical significance of the changes between the conditions. Results are presented as the mean of three or more independent experiments unless specified otherwise. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

# Chapter 3

## Regulation of Kir2.3 Localization by the WNK Pathway


### Abstract

The inward-rectifier potassium (Kir) channel family is important for the regulation and maintenance of cell membrane potential and cellular electrical conductance through $K^+$ transport across cell membranes. Some Kir channels, including all members of the Kir2.X family, contain OSR1/SPAK recognition motifs and we have shown that OSR1 activates Kir2.1 and Kir2.3. Here, I show that Kir2.3 changes its cellular localization from perinuclear toward the cell membrane following NaCl treatment. The depletion of OSR1 with small-interfering RNA prevents the NaCl-induced change in Kir2.3 localization. Inhibition of WNK kinase activity with a small molecule inhibitor also decreases the NaCl-induced change in Kir2.3 localization. Therefore, I propose that the WNK-mediated activation of OSR1/SPAK in the presence of hyperosmotic stress drives this and perhaps other Kir channels to cell membranes, where they are active.

## Introduction

### Potassium Channels

Potassium channels are vital for cellular physiology. By transporting potassium ions across the cell membrane, they enable cells to perform diverse functions and respond to different stimuli [278-282]. Their aberrant expression and/or regulation has been linked to numerous human diseases including cancer and neuromuscular and cardiac diseases [278-282]. In cancer, they have been proposed as promising therapeutic targets because they localize on the cell membrane, are targeted by approved drugs, and have high structural diversity [278-280].

Potassium channels have intracellular domains, transmembrane domains and extracellular domains which contain P-loops that form the potassium transporting passage of the channels. Four P-loops form this pore [278, 281, 282]. Potassium channels can be divided into four main classes depending on their structure, conductance properties and upstream stimulation [278]. Voltage-gated potassium channels ($K_V$) are activated by changes in membrane potential and facilitate outward flow of $K^+$. Calcium-activated potassium channels ($K_{Ca}$) are activated by the changes in intracellular calcium concentration to drive $K^+$ out of the cell. Two-pore-domain potassium channels, which contain two P-loops, constitutively transport $K^+$ out of cell, and are thereby known as leak channels. Inward-rectifier potassium channels (Kir) can transport $K^+$ in either an inward or outward direction, depending on the membrane potential and the $K^+$ equilibrium [278-282].

### Inward-rectifier Potassium Channels

Kir channels are important for maintaining and regulating resting membrane potential and action potential of different types of excitable cells. They were identified to permit the flow of $K^+$ in the inward direction [283, 284]. When the membrane potential ($E_m$) of cells is more negative

than the $K^+$ equilibrium potential ($E_K$), Kir channels move $K^+$ inward, and had a higher conductance. $K^+$ flowed out of cells, and had lower conductance when $E_m$ was positive to $E_K$ [283-287]. However, they are activated independent of membrane voltage unlike $K_V$ channels [283].

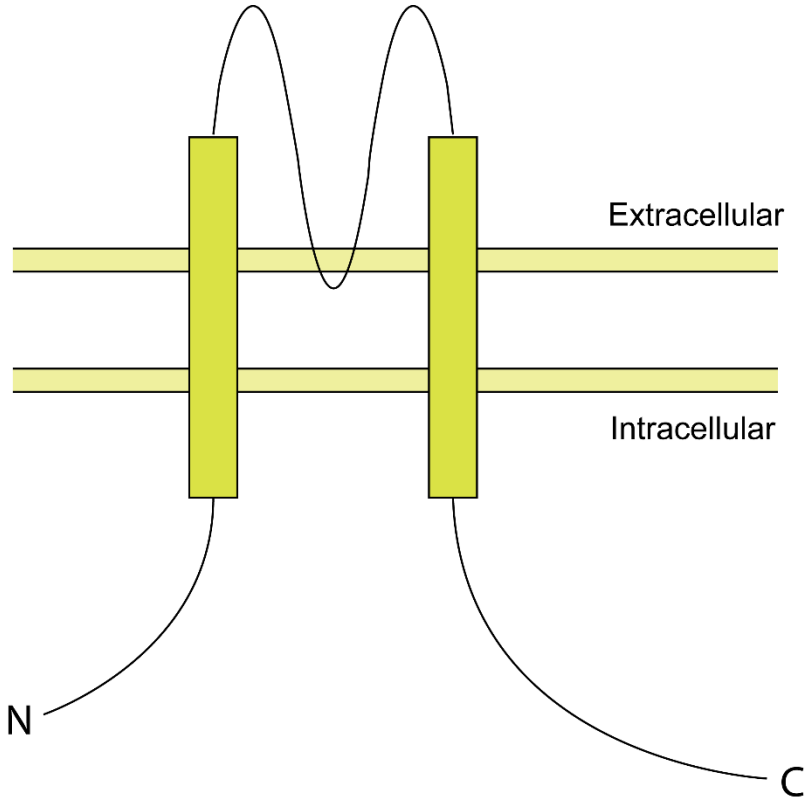


Figure 3.1. The basic structure of the Kir channel subunit. This image was adapted from Figure 2.A of Hibino et al 2010 [283].

The basic structure of the Kir channel subunit consists of two transmembrane domains (TM1 and TM2), a P-loop that participates in the construction of the extracellular pore (H5) and cytoplasmic N- and C-terminal regions [283, 284] (Figure 3.1). Tetramers, either homo or hetero-tetramers, are the functional units. [283, 284, 288, 289] (Figure 3.2). Kir channels maintain cell

membrane polarization, amplify cell membrane hyperpolarization, regulate cell-cell communication and control vasodilation [284, 290].

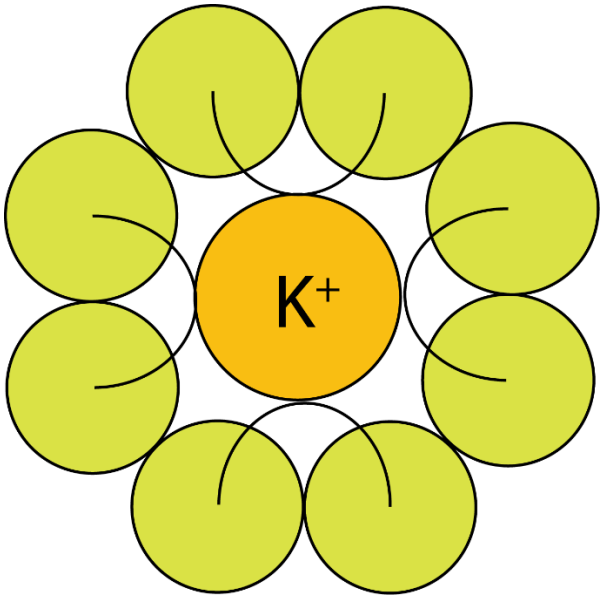


Figure 3.2. The tetrameric organization of Kir channels. This figure shows a top view of a Kir channel. This was adapted from Figure 1.B of Pardo and Stuhmer 2014 [278].

**Kir Family Subcategories**

The Kir channel family consists of sixteen subunits in humans and they belong to seven subfamilies [283, 291]. The Kir2.X and 3.X subfamilies are strong inward rectifiers. The Kir4.X subfamily is an intermediate inward rectifier, whereas Kir1.1 and Kir6.X are weak inward rectifiers [283]. Kir channels are further divided into four functional subcategories based on their mode of activation and strength of inward rectification [283] (Table 3.1).

Table 3.1. Different subcategories of Kir channels and their composition

| Subcategory | Gene Name | Protein Name |
|---|---|---|
| Classical Kir channels | *KCNJ2* | Kir2.1 |
| | *KCNJ4* | Kir2.3 |
| | *KCNJ12* | Kir2.2 |
| | *KCNJ14* | Kir2.4 |
| | *KCNJ18* | Kir2.6 |
| Potassium transport channels | *KCNJ1* | Kir1.1 (ROMK) |
| | *KCNJ10* | Kir4.1 |
| | *KCNJ15* | Kir4.2 |
| | *KCNJ16* | Kir5.1 |
| | *KCNJ13* | Kir7.1 |
| G protein gated potassium channels | *KCNJ3* | Kir3.1 |
| | *KCNJ6* | Kir3.2 |
| | *KCNJ9* | Kir3.3 |
| | *KCNJ5* | Kir3.4 |
| ATP-sensitive potassium channels | *KCNJ8* | Kir6.1 |
| | *KCNJ11* | Kir6.2 |

This table was constructed using information from Hibino at al. 2010 [283] and Ryan et al. 2010 [291].

**Classical Kir Channels**

This category consists of the Kir channels of the 2.X subfamily. They retain the basic structural organization and regulation of Kir channels [283]. They are highly expressed in cardiac myocytes and controls their electrical excitation [283]. They are also expressed in vascular endothelial cells and vascular smooth muscle cells, and regulate the functions of blood vessels [283, 292, 293]. In addition, these channels are important for the regulation of skeletal muscle

function [283, 294, 295]. Kir2.3 is localized in the basolateral membrane of the kidney cortical collecting duct (CCD) and is suggested to regulate directional $K^+$ flow [283, 296, 297]. Autosomal dominant mutations in Kir2.1 cause Andersen's syndrome, which is characterized by cardiac arrhythmias, periodic paralysis and dysmorphic bone features [298, 299].

**G Protein Gated Potassium Channels ($K_G$ Channels)**

This category consists of the Kir3.X channels which are activated by intracellular GTP [283, 300]. Gβγ but not Gα subunits are responsible for the activation of $K_G$ channels [301-303]. Gβγ subunits can directly interact with both N- and C-terminal regions of Kir3.X channels [283, 304, 305]. These channels are important for the function of heart, neurons and pancreas [283].

**ATP-sensitive Potassium Channels ($K_{ATP}$ Channels)**

The opening of these channels is activated by nucleotide diphosphates (NDP) and inhibited by ATP [283, 306, 307]. In contrast to other subcategories, $Kir_{ATP}$ channels are octamers with four Kir6.X channel subunits and four sulfonylurea receptors (SURx), which act as auxiliary subunits. The Kir6.X subunits regulate the ATP-mediated inhibition of the channels, whereas SURx proteins regulate the NDP-mediated activation of the channels [283]. These channels are important for the function of pancreas, heart, smooth muscles and brain [283].

**Potassium Transport Channels**

Kir1.X, Kir4.X, Kir5.X and Kir7.X belong to this subcategory. Kir1.1, Kir4.1 and Kir5.1 are important for the regulation of ion transport and reabsorption in kidney [283]. Kir4.X and Kir5.X

channels are important for the function of stomach, cochlea and glial cells [283]. Kir7.X channels are expressed in epithelial cells and their physiological functions are still not clear [283].

**Regulation of Kir Channels**

Kir channels are regulated by binding of polyamines and $Mg^{2+}$ in their transmembrane and cytoplasmic domains. This blocks the outward flow of $K^+$ from the channels [308, 309]. In addition, phosphatidylinositol 4,5-bisphosphate ($PIP_2$) is necessary for the function of Kir channels. $PIP_2$ binds Kir channels via the positively charged C-terminal residues [310-312]. Cholesterol also binds and regulates Kir2.X channels. High cholesterol in membranes inhibits, whereas low cholesterol activates these channels [284, 313-315].

In addition, Kir channels are regulated by post-translational modifications. PKA phosphorylates Kir2.3 and regulates its binding to PSD95 [316, 317]. Multiple effects of PKA phosphorylation on Kir2.1 channel activity have been reported and may be context dependent [284]. In addition, numerous reports indicate that PKA and PKC regulate Kir2.X channels [318-321]. PKA phosphorylation of Kir6.1 and associated subunit were shown to increase the channel activity [322]. Both PKC-mediated and SGK1/PKA phosphorylations of Kir1.1 increased its surface expression [323, 324]. Moreover, a tyrosine kinase phosphorylation site has also been identified in Kir2.X channels, although the effect of this phosphorylation is not clear [284, 325, 326]. Nitric oxide induces S-nitrosylation of Kir2.1, thereby increasing its activity [327].

**Findings on Kir Channels in Cancer**

Several Kir family members are implicated in different types of cancer. Kir2.1 was overexpressed in small cell lung cancer and mediated multi-drug resistance in cancer cells. Kir2.1

expression was dependent on the ERK1/2 MAPK pathway and miR-7 in those cells [328]. The depletion of Kir2.2 increased reactive oxygen species (ROS)-mediated cellular senescence in multiple cell types and reduced tumorigenesis [329]. In addition, increased Kir2.2 expression resulted in increased proliferation of prostate cancer cells. This effect was due to activation of the NF-κB pathway by RelA and independent of channel activity of Kir2.2 [330]. Moreover, Kir3.1 expression was positively correlated with lymph-node metastasis in breast cancer [331], while Kir4.2 promoted the migration of glioblastoma cells [332].

**Identification of Kir Channels As Potential WNK Pathway Interactors**

Several members of the Kir family contain an OSR1/SPAK recognition motif and they are among the top hits in a bioinformatics screen to identify potential OSR1/SPAK binding partners (Clinton A. Taylor, Ph.D. Dissertation, UT Southwestern). When an activated mutant of OSR1 that mimics activation of the WNK pathway is co-expressed with Kir2.1 which has the OSR1/SPAK binding motif, the activity of Kir2.1 is increased. However, when the same OSR1 mutant is co-expressed with Kir4.1, which does not contain the OSR1/SPAK binding motif, the activity of Kir4.1 is not altered, showing that the OSR1/SPAK recognition motif is important for the regulation of Kir channels by OSR1 (Clinton A. Taylor, Ph.D., Sung-Wan An, Ph.D. (Chou-Long Huang lab) unpublished) (Figure 3.3).

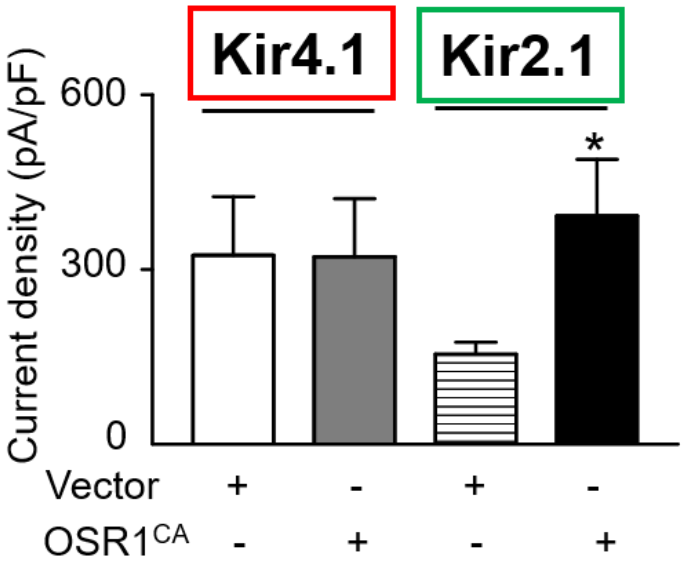


Figure 3.3. The effect of constitutively active OSR1 on Kir2.1 and Kir4.1 activity. The activities of the channels were measured as current density readings. Unpublished data from Sung-Wan An, Ph.D. from Chou-Long Huang lab, obtained with permission from Dr. Chou-Long Huang.

In addition, when Kir2.1 is co-expressed with wild-type OSR1 and WNK1, its activity is increased similar to co-expression with the active OSR1 mutant, showing that the WNK pathway is responsible for this regulation (Clinton A. Taylor, Ph.D., Sung-Wan An, Ph.D. (Chou-Long Huang lab) unpublished) (Figure 3.4). The activity of Kir2.3, which also contains the OSR1/SPAK recognition motif, is also increased when it is co-expressed with the active OSR1 mutant. Importantly, mutation of the OSR1/SPAK recognition motif abolishes this effect by the OSR1 mutant, showing that this motif is necessary for the regulation of Kir channels by the WNK pathway (Clinton A. Taylor, Ph.D., Sung-Wan An, Ph.D. (Chou-Long Huang lab) unpublished) (Figure 3.5).

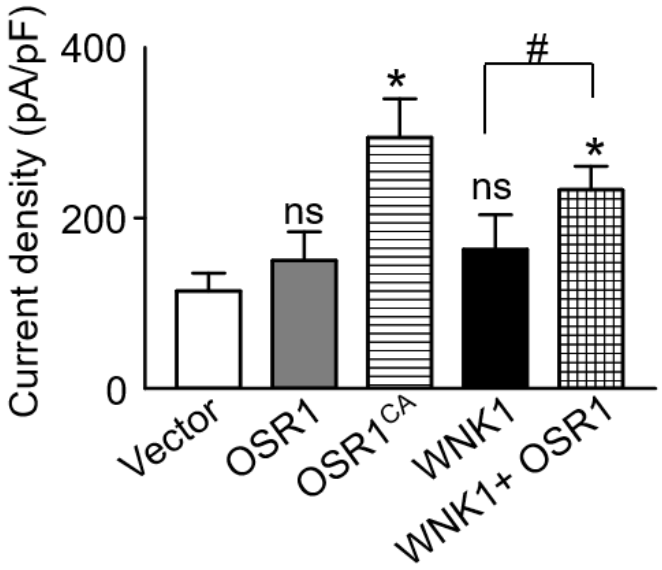


Figure 3.4. The effect of WNK pathway activation on Kir2.1 activity. The activity of the channel was measured as current density readings. Unpublished data from Sung-Wan An, Ph.D. from Chou-Long Huang lab, obtained with permission from Dr. Chou-Long Huang.

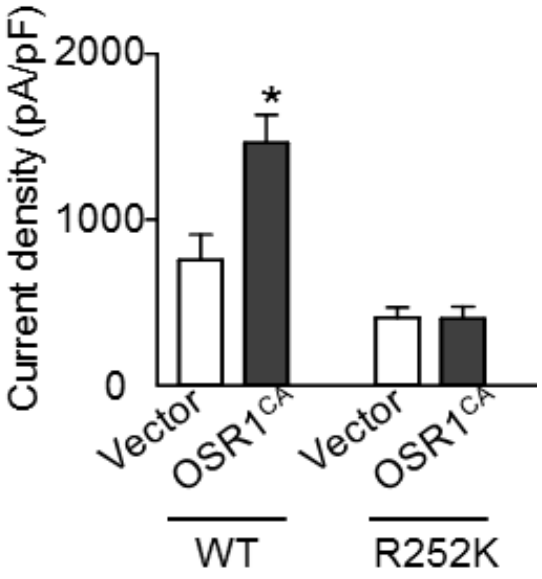


Figure 3.5. The dependence of Kir2.3 on OSR1/SPAK recognition motif for its activation. The activity of the channel was measured as current density readings. Unpublished data from Sung-Wan An, Ph.D. from Chou-Long Huang lab, obtained with permission from Dr. Chou-Long Huang.

## Results

### OSR1 Regulates the Cellular Localization of Kir2.3

To analyze whether OSR1 regulates the activity of Kir channels by controlling its membrane localization, I depleted OSR1 in HeLa cells by siRNA-mediated gene knockdown. Then I transiently expressed Kir2.3 in the cells and treated them with or without NaCl to stimulate WNK. Following the treatment, I analyzed the cells by immunofluorescence. Under control conditions, Kir2.3 was largely localized in the perinuclear region of the cells, consistent with previous studies [333, 334]. OSR1 was localized throughout the cytoplasm. With NaCl, Kir2.3 localization was largely shifted towards the plasma membrane or dispersed throughout the cytoplasm in a fraction of the cells. NaCl also drove OSR1 towards the plasma membrane in a fraction of the cells. OSR1 depletion alone did not alter the localization of Kir2.3; however, it prevented the NaCl-induced membrane association of Kir2.3 and decreased the number of cells with a dispersed Kir2.3 localization. This shows that OSR1 is necessary for the NaCl-induced re-localization of Kir2.3 (Figure 3.6A-D).

### OSR1 Does Not Alter the Cellular Amount of Kir2.3

To analyze whether the re-localization of Kir2.3 was affected by changes in its cellular protein amount, I performed a similar experiment as described in Figure 3.6, in which I analyzed protein amount in cells by immunoblotting, instead of immunofluorescence. Neither OSR1 depletion nor NaCl changed the amount of Flag-Kir2.3 considerably, indicating that its localization changes are not due to a change in its protein amount (Figure 3.7A and B).

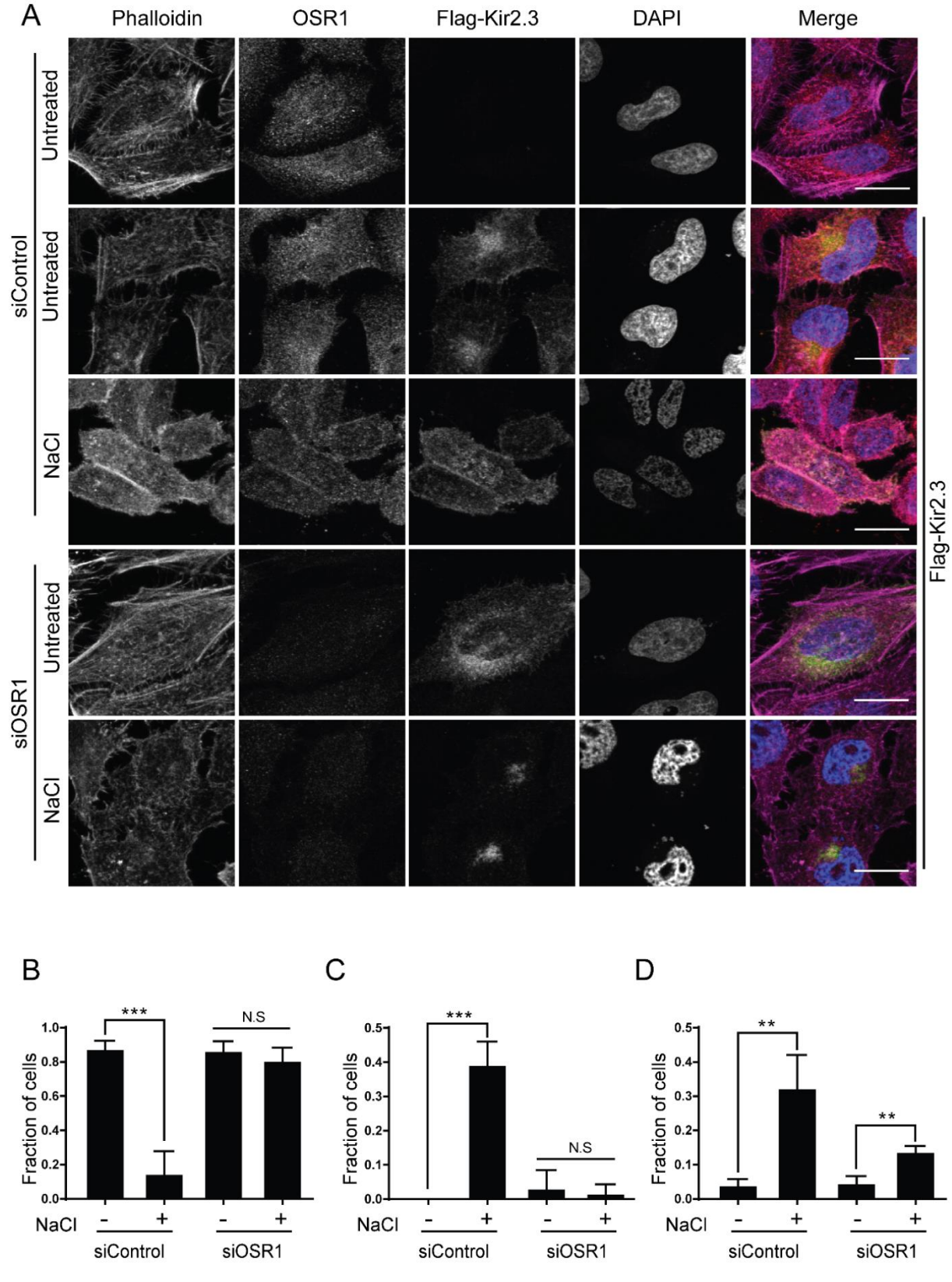
A
Phalloidin
OSR1
Flag-Kir2.3
DAPI
Merge
siControl
siOSR1
Untreated
NaCl
Flag-Kir2.3
B
C
D
Fraction of cells
***
N.S
**
NaCl
siControl
siOSR1

Figure 3.6. OSR1 regulates the localization of Kir2.3 in the presence of NaCl (A) HeLa cells were plated on coverslips and treated with 20 nM siControl or siOSR1 oligonucleotides. The following day, a plasmid encoding 3x-Flag(A95-A96)-Kir2.3, or a control plasmid was transfected into cells. After two more days, the medium was replaced with fresh medium with or without 150 mM NaCl for three hours. Cells were then analyzed by immunofluorescence and the images were deconvoluted. (B-D) Quantitation of fractions of cells with distinct Flag-Kir2.3 localization phenotypes in A. The deconvoluted cell images were opened in Imaris 8 (Bitplane). Cells were categorized as (B) Having a predominantly perinuclear localization of Flag-Kir2.3 (C) Having a predominantly cell membrane-associated localization of Flag-Kir2.3 and (D) Having Flag-Kir2.3 dispersed throughout the cytoplasm, based on their Flag-Kir2.3 localization phenotype. The cells with high expression of Flag-Kir2.3 were omitted from the analysis. (Scale bars, 18 μm) The changes analyzed were between NaCl and untreated conditions, in siControl and siOSR1 treated cells N.S - Not significant, **P < 0.01, ***P < 0.001.

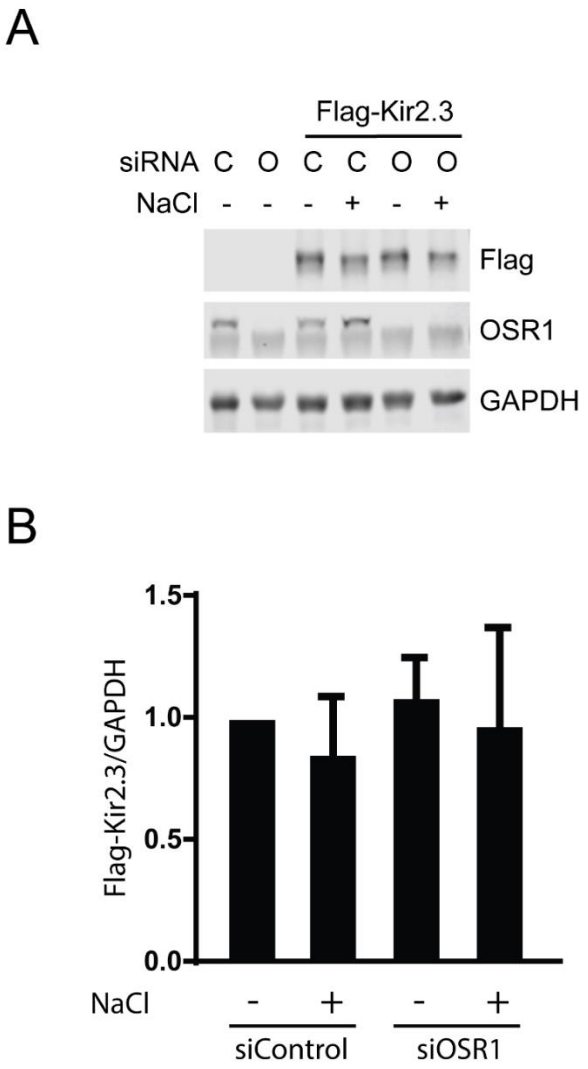

Figure 3.7. OSR1 does not alter the cellular amount of Kir2.3 (A) HeLa cells were plated and treated with 20 nM siControl or siOSR1 oligonucleotides. Then, Flag-Kir2.3 or a control plasmid was expressed, followed by NaCl treatment of cells as described in Figure 3.6.A. Then the cells were lysed, and proteins were analyzed by immunoblotting. (B) Quantitation of relative change of Flag-Kir2.3 in cells in A. N = 2.

**WNK Activity Regulates the Cellular Localization of Kir2.3**

Because the activity assays showed that OSR1 activation is required for its effect on Kir channels, I hypothesized that WNK activity inhibition would recapitulate the effects of OSR1 depletion in the cells. To test this, I expressed a Kir2.3 construct in cells and treated the cells with WNK463, a small molecule pan-WNK inhibitor [200, 335]. Then I treated the cells with NaCl as described earlier and analyzed them by immunofluorescence. Without NaCl or drug treatment, Kir2.3 was perinuclear, and became dispersed and membrane associated following treatment with NaCl, consistent with my previous findings. WNK inhibition alone did not change the localization of Kir2.3; however, it decreased the changes in Kir2.3 localization when cells were exposed to NaCl (Figure 3.8A-D). These results suggest that WNK activity is responsible for the NaCl-induced re-localization of Kir2.3. WNK inhibition also prevented the partial re-localization of OSR1 in the presence of NaCl (Figure 3.8A). OSR1 was colocalized with Kir2.3 in cells. NaCl treatment did not change the amount of colocalization; however, altered the distribution of colocalization between these proteins. WNK inhibition did not prevent this colocalization in either NaCl treated or untreated conditions (Figure 3.8A).

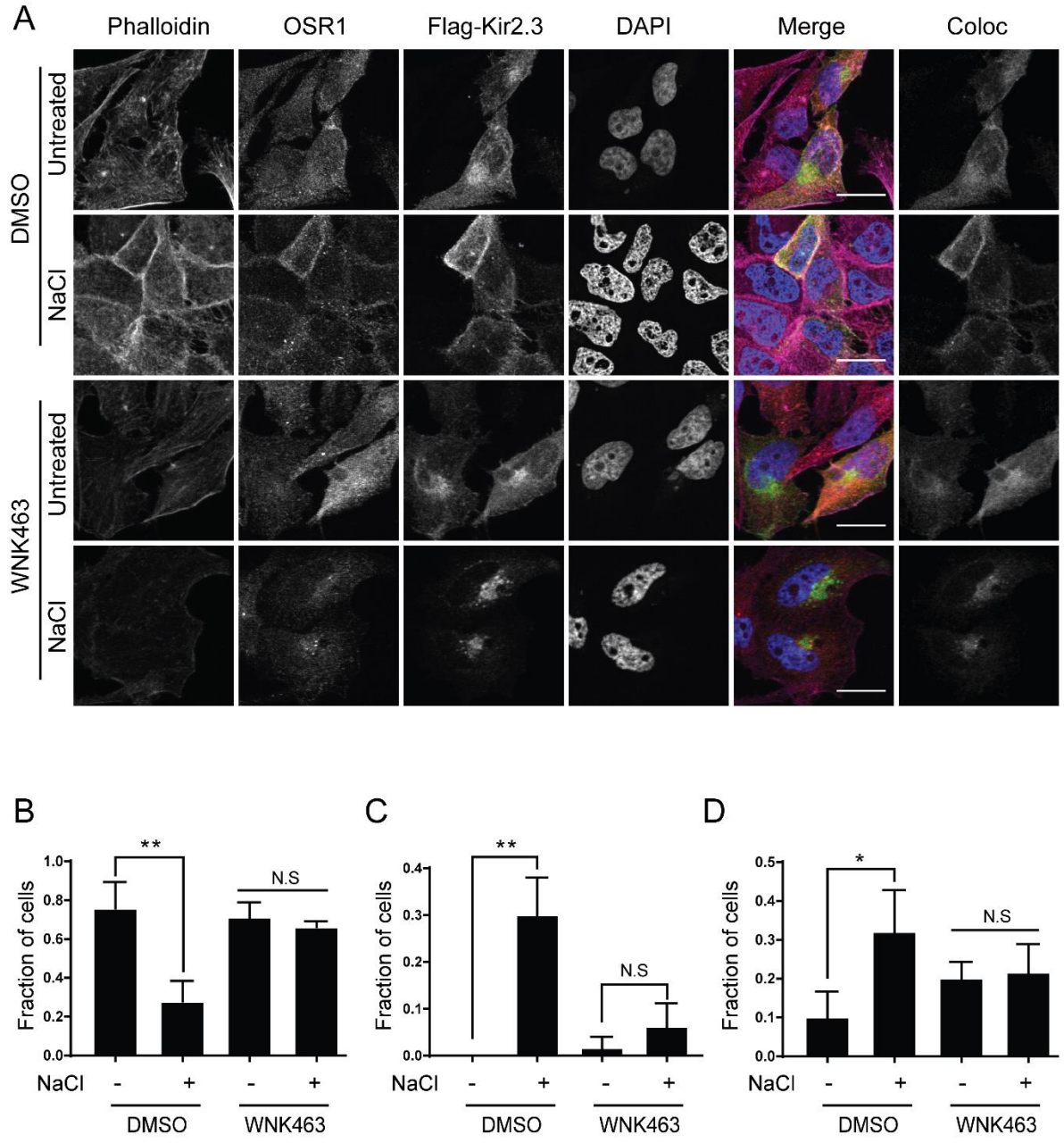


Figure 3.8. WNK activity regulates the cellular localization of Kir2.3 in the presence of NaCl (A) HeLa cells were plated on coverslips. The following day, a plasmid encoding 3x-Flag(A95-A96)-Kir2.3 was transfected into cells. The next day, cells were treated with 1 µM WNK463 or 0.1% DMSO (Control) for 24 hours. The following day, medium was replaced with fresh medium with or without 150 mM NaCl, in the presence or absence of WNK463, for three hours. Cells were then analyzed by immunofluorescence and images were deconvoluted. Colocalization threshold for OSR1 channel was automatically calculated and set at 1000 for Flag-Kir2.3 channel, and a colocalization channel was generated by the Imaris 8 software. (B-D) Quantitation of fractions of cells with distinct Flag-Kir2.3 localization phenotypes in A, as described in Figure 3.6.B-D. The

categories are (B) Having a predominantly perinuclear localization of Flag-Kir2.3 (C) Having a predominantly cell membrane-associated localization of Flag-Kir2.3 and (D) Having Flag-Kir2.3 dispersed throughout the cytoplasm or concentrated away from perinuclear region, based on their Flag-Kir2.3 localization phenotype. The cells with high expressions of Flag-Kir2.3 were omitted from the analysis. (Scale bars, 18 μm). The changes analyzed were between NaCl and untreated conditions, in DMSO and WNK463 treated cells. N.S – Not significant, *P < 0.05, **P < 0.01.

**Discussion**

In this study, I show that WNKs and OSR1 regulate the localization of Kir2.3 in the presence of NaCl, which activates the WNK pathway. The unpublished data from the Huang lab show that activated OSR1 increases the activation of two Kir channels that contain the OSR1/SPAK recognition motif. It seems likely that this channel activation is caused by an increased membrane localization of Kir channels by the WNK pathway (Figure 3.9). It is also possible that WNKs increase the activation of Kir channels by other mechanisms as well, such as through post-translational modifications that alter channel structure.

Depletion of OSR1 prevents Kir2.3 from migrating towards the cell membrane in response to increased extracellular NaCl, remaining in the perinuclear region of the cells. It is possible that in the absence of OSR1-mediated regulation, Kir2.3 is localized to a subcellular compartment close to nucleus. The trafficking of the Kir channels to cell membranes has been studied before. They contain an endoplasmic reticulum trafficking signal in their C-terminal intracellular region and a Golgi trafficking signal that contains parts of their N- and C-terminal intracellular regions [334, 336-338]. Mutation of the Golgi trafficking sequence prevents the cell membrane localization of Kir2.1, Kir2.3 and Kir4.1, and confine their localization to Golgi [334, 338]. Therefore, it is possible that Kir2.3 is in either endoplasmic reticulum or Golgi near the nucleus in the absence of OSR1 activity.

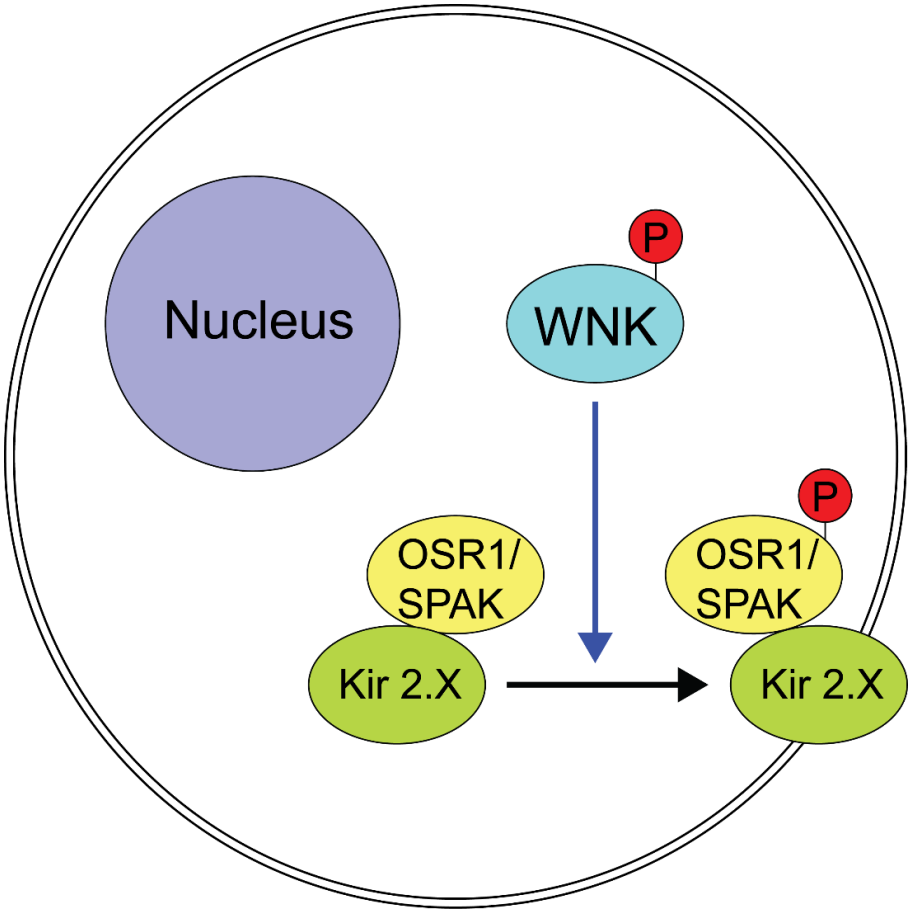


Figure 3.9. A model of the WNK pathway regulation of Kir channel activity.

I observed that the Kir2.3 localization in cells adopt four major localization morphologies: largely perinuclear, largely cell membrane-associated, localized in both perinuclear and membrane regions, and dispersed throughout the cytoplasm of cells. With NaCl treatment, the number of cells with both cell membrane-associated and dispersed morphologies increased. The appearance of these distinct phenotypes could be a result of the dynamic nature of Kir2.3 transport within the cells. Kir2.3 moves from the perinuclear region to the plasma membrane, and the dispersed localization could be an intermediate step between those two phenotypes.

I also demonstrated that OSR1 depletion almost completely prevented Kir2.3 from acquiring a membrane-associated localization phenotype in the presence of NaCl, while WNK463 was less

effective. This slight increase of membrane-associated Kir2.3 phenotype even in the absence of WNK activity could be due to the presence of basal activity of OSR1. In addition, WNK463 treatment showed a pattern of increasing number of cells with the dispersed phenotype in the absence of NaCl. This could be likely due to activation-independent and OSR1-independent effects of WNKs on Kir2.3.

OSR1 itself was localized more to the cell membrane in the presence of NaCl, and this effect was inhibited by WNK463. Therefore, it seems that WNK-mediated activation drives OSR1 to cell membranes. At the plasma membrane OSR1/SPAK may be more able to activate cation chloride cotransporters as shown before [6, 8].

## Materials and Methods

### Materials

The following antibodies were used: OSR1 and AKT1 (Cell Signaling Technology); GAPDH (Santa Cruz); Flag M2 (Sigma-Aldrich). The siOSR1 (Silencer select validated s19303) was obtained from Life Technologies/Ambion. The plasmid 3xFlag (A95-A96) Kir2.3 was generated by Steve Stippec to insert the Flag tag within the indicated position in the coding sequence. The chemical WNK463 was obtained from Selleckchem.

### Cell Culture and Transfection

HeLa cells were grown in DMEM with 10% (vol/vol) FBS, 1% (vol/vol) L-glutamine at 37 °C, 5% $CO_2$. siRNA-mediated knockdown and plasmid expression were performed as stated in Chapter 2.

**Immunofluorescence and Image Representation, SDS-PAGE and Immunoblotting Analysis, and Statistical Analyses**

All of these procedures were performed as stated in Chapter 2. Image deconvolution was also performed as described in Chapter 2.

# Chapter 4

# Future Directions

## Regulation of Autophagy, Intracellular Trafficking and Associated Components by the WNK Pathway

I demonstrated that WNK1 is an inhibitor of autophagy. Two previous reports suggested that WNK2 is a regulator of autophagy [148, 149]. However, it is still not known how and whether WNK3 and WNK4 play any role in regulating autophagy. In addition, my work focused on analyzing the effect of WNK1 on macroautophagy [207, 217]. Whether WNK1 regulates other forms of autophagy such as chaperone-mediated autophagy [339] and microautophagy [340] is not known. Moreover, it is not clear whether WNK1 regulates selective forms of autophagy such as mitophagy and pexophagy, which clear specific organelles or molecules from cells [341, 342]. Future studies should address whether all four WNKs are involved in controlling autophagy, either positively or negatively, and if they influence multiple types of autophagy.

WNKs regulate endocytosis and surface expression of ion channels and cotransporters, and it seems a mechanism of regulation of ion channel/cotransporter activity by WNK pathway [20, 75-77, 79, 80, 84, 343, 344]. Binding to ITSN is one such mechanism for WNK control of endocytosis [19]. The inhibitory role of WNK1 on the PI3KC3 complex and production of PI3P suggest that there could be multiple mechanisms WNKs employ to regulate endocytosis, because PI3KC3 is a major mediator of this process [152]. PI3KC3 complex regulates not only endocytosis and autophagy, but also other forms of intracellular trafficking, such as phagocytosis and endosome to Golgi retrograde trafficking [152]. Therefore, it is likely that WNKs regulate other cellular trafficking events apart from autophagy and endocytosis. Future studies should identify these WNK-regulated processes and the underlying mechanisms.

I identified that WNK1 inhibited the cellular expression of ULK1. It is not clear whether this is an effect on ULK1 transcription, translation or protein stability. Therefore, it is important to identify the regulatory mechanism(s). Actions of WNK1 on other components of the ULK1 complex, ATG13, ATG101 and FIP200, such as controlling their expression or post translational modifications, are also unknown. It is not clear how WNK1 inhibits the ULK1 upstream kinase AMPK. AMPK is generally activated by low cellular energy or by phosphorylation by LKB1 or CaMKK2 [345, 346]. However, my findings suggest that there is no significant change in the ATP/AMP ratio in cells following depletion of WNK1. It will be important for future studies to address the precise regulation of the AMPK-ULK1 axis by WNKs.

The results from this study suggested that the actions of WNK1 on autophagy were mediated to only a small extent through the AMPK-ULK1 axis. Therefore, it is possible that the regulation of ULK1 by WNK1 is important for other cellular processes controlled by ULK1. The ULK1 complex participates in other cellular functions apart from autophagy such as interferon signaling and lipid metabolism [347, 348]. Future studies should analyze whether WNKs affect those cellular functions through ULK1. UVRAG is also involved in regulating non-autophagic and non-endocytic cellular processes, such as DNA double-strand-break repair and T cell function [349, 350]. It would be interesting to assess whether WNK kinases regulate these UVRAG-mediated cellular functions, because WNK1 interacts with UVRAG.

Another approach to understand WNK functions would be to analyze whether WNKs or their downstream target kinases interact with other autophagy proteins apart from UVRAG. This would shed light on other mechanisms of WNK-mediated autophagy regulation. For example, our mass spectrometry assays of immunoprecipitated WNK1 or OSR1/SPAK have yielded several interacting proteins that have roles in autophagy and cellular trafficking (Data not shown).

Therefore, it is likely that WNK1 also regulates autophagy through currently unidentified mechanisms.

Because WNK1 is a kinase, it will be important to determine whether its kinase activity is required for autophagy control. Preliminary data suggest that some WNK1 effects on autophagy are mediated by its kinase function. This is consistent with my observation that SPAK depletion only displayed a fraction of the effect that WNK1 depletion had on autophagy. Therefore, it is possible that WNK1 kinase function-mediated effects on autophagy are relayed through SPAK, while its scaffold function-mediated effects on autophagy are exerted by direct binding to PI3KC3. It is important that future studies also characterize this hypothesis.

**Regulation of Inward-rectifier Potassium Channels by the WNK Pathway**

I discovered that WNK/OSR1 regulates the cellular localization Kir2.3. Several unanswered questions need to be explored. Activation of the WNK pathway resulted in activation of Kir2.1 and Kir2.3, which suggests that WNKs are a common mechanism to regulate Kir channels. Less clear is whether membrane translocation is a common mechanism for regulation of Kir channels. Therefore, future experiments should determine the modes of activation for Kir2.1 and other Kir channels with OSR1 binding motifs by WNKs.

In the presence of NaCl, the electrophoretic mobility of Flag-Kir2.3 is altered, suggesting differential post-translational modifications (PTM) of these channels (Figure 3.7A). It is important to characterize these PTMs, their contribution to activation and localization of Kir2.3, the PTMs dependent on activation of the WNK pathway, and the PTMs conserved in other related Kir channels. This information will help us understand the mechanisms of regulation of Kir channels and their associated physiology better.

It is still not known how OSR1 is able to drive Kir2.3 towards the cell membrane, although it seems that binding between OSR1 and Kir2.3 is important for this effect. It is possible that direct phosphorylation of Kir2.3 by OSR1 is necessary. Alternatively, it is possible that OSR1 functions as a scaffold to bring together Kir2.3 with its effector proteins, and phosphorylate the effector proteins to promote cell membrane localization of Kir2.3.

In addition, it is not yet clear whether the increased Kir2.3 localization at the cell membrane is a result of increased membrane targeting of the channels, or a decrease of channel endocytosis. Therefore, it is important to assess the nature of this Kir2.3 cellular localization to better understand the mechanism of its regulation by the WNK pathway.

My experiments relied on using a pan-WNK inhibitor, WNK463 to inhibit WNK activity [200, 335]. Therefore, it is not known what the contributions of individual WNK proteins are on the regulation of Kir channels. WNKs may have similar or opposite effects on Kir channels and therefore, it is important to understand these effects. The attempts to reduce WNK1 activity in cells by depleting it by siRNA failed, because NaCl treatment killed siWNK1 treated and Flag-Kir2.3 expressed cells (Data not shown). The most feasible method seems to be the use of specific WNK inhibitors for individual kinases. It will also be of value to explore whether the OSR1-related kinase SPAK has similar effects on Kir channels as OSR1. This seems likely as the binding motif interacted with fragments of both OSR1 and SPAK (Clinton A. Taylor, Ph.D. Unpublished data).

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