REGULATION OF CELL MIGRATION BY WNK1

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DEDICATION

This is dedicated to Armando “Mandis” Estrada Jr., his wife Maria Adela “Adelita,” and to their son Alex.

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by

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ROLE OF WNK1 IN CELL MIGRATION

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Cell motility is an immensely complex process that involves reorganization of the cytoskeleton, and consequent membrane deformation, triggered by a variety of motogenic stimuli, including growth and chemotactic factors, hormones, and elements of the extracellular matrix. My graduate research has focused on the regulation of cell motility by a serine/threonine protein kinase, WNK1 (With No lysine (K)1), so named because of the absence of a conserved lysine within the catalytic domain. Although WNK1 has been most thoroughly characterized for its role in controlling ion flux in the kidney, emerging evidence points to its essential participation in both intracellular membrane trafficking and

overall cell movement. Having confirmed a previous observation that depletion of WNK1 in cultured cells interferes with wound closure in an established motility assay, I next sought to identify specific aspects of cytoskeleton remodeling that are impaired by reduction of WNK1 expression. The most dramatic effect of siRNA-mediated WNK1 depletion was an increase in the proportion of polymerized (F) actin. Surprisingly, this redistribution was accompanied by a corresponding increase in the proportion of active, unphosphorylated cofilin, an F-actin depolymerizing factor, most likely reflecting engagement of a feedback homeostatic mechanism. To understand the pathway(s) whereby WNK1 regulates stimulus-dependent actin dynamics, I examined three potential pathways known to impact cell motility: 1. Remodeling of subcellular cytoskeletal structures, including circular dorsal ruffles and stress fibers; 2. Activation of downstream effectors of phosphatidylinositol 3-kinase (PI3K), including Akt and LIMK1; 3. Regulation of proteins that control the cytoskeletal re-organization including the ERM proteins (Ezrin, Moesin, and Radixin), cofilin, and the Rho family of GTPases, including RhoA and Rac1.

TABLE OF CONTENTS

PREVIOUS PUBLICATIONS

A. Estrada, B. Barylko, J.K. Osborne, J. Chen, C.J. Heise**,** M.H. Cobb, and J.P. Albanesi. **With no K/lysine 1 (WNK1) controls cell migration in part through effects on actin polymerization**. In preparation.

C. Byers[†], B. Barylko[†], N. G. James[‡], D. R. Southworth[‖§], L. Wang[†], K. A. Collins[⊥], J. A. Ross[‡], T. C. Tassin[†], **A. Estrada**[†], M. Waung[⊥], K. M. Huber[⊥], D. M. Jameson[‡] and J. P. Albanesi[†*] **Arc Stimulates GTPase Activity and Self-Assembly of Dynamin 2.** Submitted

LIST OF FIGURES

LIST OF ABREVIATIONS

WNK: With No Lysine (K)

WNK OE: WNK1 overexpression

TKL: Tyrosine kinase-like

ATP: Adenosine triphosphate

PKA: Protein Kinase A (cAMP-dependent kinase)

SH3: Src homology 3

KS-WNK1: Kidney-specific WNK1

DCT: Distal convoluted tubule

PHAII: Pseudohypoaldosteronism Type-2

NKCC: Na^+-K^+-Cl^- -co-transporter

KCC: Na^+- Cl^- co-transpcoorter

OSR1: Oxidative stress element type-1 kinase

ROMK1: Renal outer medullary K^+ channel type-1

ITSN1: Intersectin1

MAPK: Mitogen activated protein kinase

EGF: Epidermal growth factor

TGF-B: Transforming growth factor type-beta

HGF: Hepatocyte growth factor

EMT: Epithelial to mesenchymal transition

LPA: Lysophasphatidic acid

SCC-9: Human tongue squamous carcinoma cell

A549: Lung adenocarcinoma cell line

FH1: Formin homology 1 domain

WASP: Wiskott Aldrich Syndrome protein

PIP2: Phosphatidylinositol-4,5-bisphosphate

PIP3: Phosphatidylinositol (3, 4, 5) trisphosphate

PI3K: Phosphatidylinositol 3-kinase

CAT: Collective to ameoboid transition

MAT: Mesenchymal to ameoboid transition

SPAK: Ste 20/sps1 protein kinase

ECM: Extracellular matrix

FL-WNK1: Full-length WNK1

CDR: Circular dorsal ruffles

PMA: Phorbol 13-myristate-13-acetate

PP1: Protein phosphatase type 1

PP2A: Protein phosphatase type 2

LIMK1: LIM kinase type 1

ERM: Ezrin, Radixin, and Moesin

PDK1: Phosphoinositide-dependent kinase type 1

AKT: Protein kinase B type 1

GDP: Guanosine diphosphate

PBD: Protein binding domain

PAK1: P21 activated kinase type 1

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

WNK kinases

Discovery and characterization

WNK1 was the first member of the WNK family of protein kinases to be cloned in the laboratory of Dr. Melanie H. Cobb at UT Southwestern Medical Center. It was identified using a nested PCR strategy aimed at finding novel members of the MEK family. This group of kinases is phylogenetically different from other kinases. Additional WNKs have since been identified and have been determined to be closely related to the sterile 20-like (STE) and tyrosine kinase-like (TKL) family of kinases (Moniz et al. 2010). WNK1 has a kinase domain located within the N-terminal 490 residues, but unlike other members of the MEK family of kinases, WNKs lack the critical lysine within the catalytic domain required for phosphoryl transfer. Instead, the catalytic lysine found in all other protein kinases is located in subdomain II. This catalytic lysine is responsible for binding to ATP; in WNK1, the traditional catalytic lysine is replaced by a cysteine at residue 250 (Xu et al. 2000).

WNKs are expressed in multiple organisms, including model systems such as *Caenorhabditis elegans*, *Oryza phycomyces*, *Drosophila melanogaster*, and *Xenopus sp* (McCormick et al. 2011). To date there are four known members of the WNK family found in humans, as shown on the scheme on figure 1.

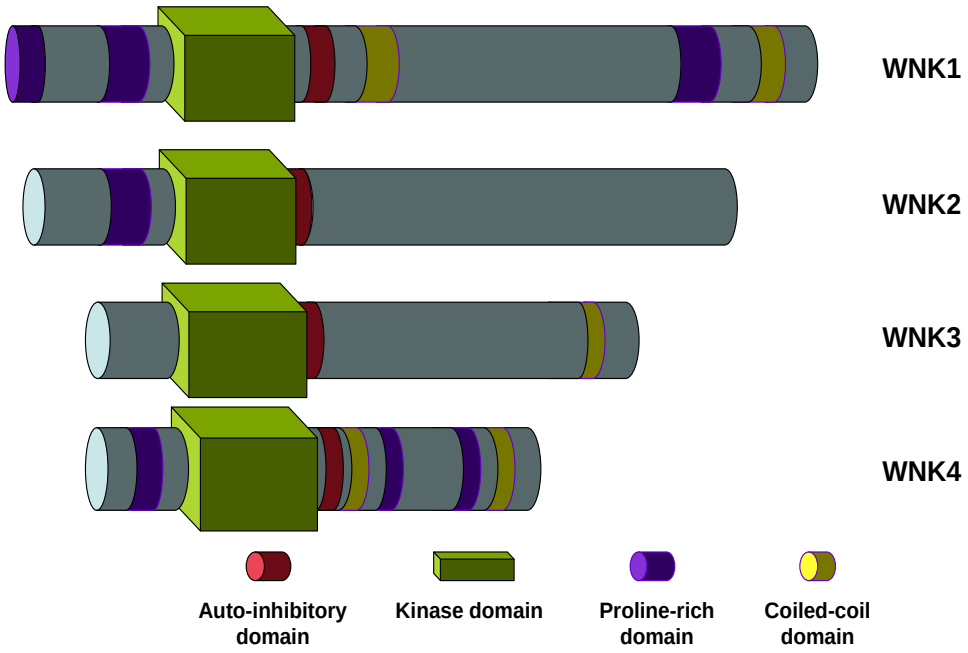

Figure 1. Representative drawing of the four known human WNK family members (Drawn to scale).

Other organisms express from one form of WNK (e.g., *Drosophila melanogaster*) to as many as nine (e.g., *Arabidopsis thaliana*). The importance of the cysteine located at the traditional catalytic position was revealed in studies carried out using a WNK1 C250A clone. The C250A mutant displayed wild-type kinase activity, suggesting that cysteine 250 was not crucial for catalytic activity Xu et al. (2000) subsequently demonstrated that the lysine 17 residues upstream of the classical catalytic lysine position was crucial for kinase activity of WNK1

as was evident in subsequent experiments where mutation of this lysine to methionine (K233M) was shown to abrogate kinase activity.

Much of the information that was first obtained from the Cobb lab regarding the importance of lysine 233 was confirmed when the crystal structure of the kinase domain of WNK1 was solved by Min et al. (2002). The kinase domain localized to residues 218-483 near the amino-terminal end of WNK1, and an auto-inhibitory domain of approximately 60 residues was identified C-terminal to the kinase domain, between residues 490-550 (Xu et al. 2002). Further studies on WNK1 demonstrated the presence of two phenylanaline residues (Phe-524 and Phe-526) which are important in mediating the auto-inhibition of WNK1. The crystal structure of WNK1 demonstrated that, aside from the lack of the classical lysine at the catalytic site, much of the active site appears to be normal when compared to the catalytic site of protein kinase A (PKA). The crystal structure revealed that cysteine 250 occupies a position in strand β3 (subdomain II) and that the lysine residue responsible for catalysis (Lys-233) was found in strand β2 (subdomain I) (Min et al. 2002). The position of this lysine makes WNKs unique within the kinase superfamily.

All four mammalian WNKs have their kinase domain located toward the amino-terminal end of the protein (figure1). This domain displays 85-90% sequence conservation among the four WNKs. WNK1 contains 24 PxxP motifs, which potentially serve as interaction sites for Src homology 3 (SH3) domains in

other proteins. The presence of these motifs have led to the identification of SH3 domain-containing partners, which has provided clues toward unraveling WNK function in the cell (Huang et al. 2008). In addition to these PxxP motifs, WNK family members also contain 1-2 coiled-coil domains, which could be important in mediating protein interactions or in promoting WNK oligomerization. The latter possibility is supported by experiments demonstrating that WNK1 forms a tetramer as seen in gel filtration analysis performed by L. Lenertz (UT Southwestern Dept. of Pharmacology). Aside from these common domains that are present in all four WNKs, there is relatively little sequence identity among the four family members (Huang et al. 2008).

WNK1 is subject to two forms of auto-regulation. The first mechanism is controlled by the aforementioned a auto-inhibitory domain, which suppresses WNK kinase activity (Xu et al. 2002). The second is mediated by auto-phosphorylation of serine residues 382 and 378 (Xu et al. 2000), which is conserved in all members of the MEKK family of kinases. Homologous residues are found in WNKs 2, 3, and 4 (Serines-356, 308, and 332 respectively) (Xu et al. 2002).

WNK proteins are ubiquitously expressed in tissues with specific isoforms being enriched in different tissues. For example, humans express two known WNK1 proteins, a truncated kidney-specific WNK1 (KS-WNK1) and a long form WNK1 of about 23kDa. KS-WNK1 lacks the amino terminal 437 amino acids

which contain the kinase domain; but instead contains 30 amino acids that include a cysteine-rich region. The remainder of the KS-WNK1 sequence is identical to full length WNK1. KS-WNK1 has only been identified within the distal convoluted tubule (DCT) of the kidney and it has been shown to play an important role in renal secretion and blood pressure control (McCormick et al. 2011).

WNK1 is 2382 amino acids in length and is expressed throughout the body, most prominently in the kidney, heart, muscle, and lung (Cope et al. 2005). WNK2, WNK3, and WNK4 are expressed at relatively low levels in the brain, colon, kidney, and pancreas. WNK3 is expressed at higher levels in the liver and the kidney, although its expression is low compared to WNK1, even in this tissue. WNK4 is the least highly expressed of the four but it has been detected in the kidney, colon, brain, and lung (Cope et al. 2005).

Genetics of WNKs

WNKs 1, 2, 3, and 4 are located on chromosomes 12, 9, X, and 17 respectively (Cope et al. 2005). Shortly after the discovery of WNKs; it was reported that mutations in both WNK1 and WNK4 are linked to a rare autosomal dominant disease known as pseudohypoaldosteronism type 2 (PHAII) (Bergaya et al. 2011). Disease-associated missence mutations in WNK4 near the kinase domain lead to a partial loss of function. The disease is also manifested in patients containing deletions within the first intron of WNK1 (Xu et al. 2000), which leads

to an increase in expression of full length WNK1 within the kidney (Huang et al. 2008). PHAII also known as Familial Hyperkalemic Hypertension or Gordon's syndrome has been characterized by hyperkalemia and hyperchloremic metabolic acidosis (Gordon et al. 1986). It has also been observed that heterozygous WNK1-/- mice display an increase in blood pressure with no effect on their kidney function, suggesting that WNK1 may serve other roles outside the kidney (Cope et al. 2005). The link between WNK1 mutations and kidney disease has engendered much work on the potential role(s) of WNKs in renal ion transport.

Roles of WNKs

Studies have demonstrated that WNKs 1, 3, and 4 increase ion transport mediated by Na^+-K^+-Cl^- co-transporters (NKCC1 and NKCC2) (Anselmo et al. 2006 and Rinehart et al. 2005) and that WNKs 3 and 4 regulate K^+-Cl^- co-transporters (KCC1-KCC4) (Vitari et al. 2005). Regulation of ion transport by WNKs is mediated by two members of the Ste20p family protein kinases, SPAK and OSR1 (Piechotta et al. 2002). It is suggested that the two kinases interact and phosphorylate NKCC 1 and 2 at their N-termini (Gimenez and Forbush et al. 2003). WNKs 1 and 4 regulate transporter phosphorylation by forming a tight complex with OSR1 and SPAK, which causes their activation and subsequent phosphorylation of NKCC transporters (Anselmo et al. 2006). In addition to their role in ion transport, WNKs have been implicated in the regulation of endocytosis and exocytosis. For example, WNK1 regulates the membrane-binding properties

of synaptotagmin 2, a protein involved in the membrane fusion process (Lee et al. 2004).

Further putative roles for WNKs have emerged from observations made in WNK knockout mice. Homozygous WNK1 knockout is embryonically lethal in mice, suggesting an important role in development. Heterozygous mice display an increase in blood pressure, perhaps caused by dysfunctional regulation of ion transporters NKCC and KCC, which have been found throughout the body (Huang et al. 2007).

Aside from its role in regulating ion transporters, WNKs have also been implicated in cell survival, signaling, and proliferation. DasGupta et al. (2005) demonstrated that the single *Drosophila* WNK (CG7177) is important in regulating the WNT pathway, a key component in embryonic development. WNK RNAi screen have also revealed that WNKs serve an important role in the survival of cultured *Drosophila* S2 cells (Boutros et al. 2005). WNKs have also been shown to suppress transforming growth factor type beta (TGF-β) signaling by decreasing the amount of phosphorylated Smad2 in the nucleus, while increasing the overall amount of Smad2 in the cell (Lee et al. 2007). However, observations made by Xu et al. (2005) demonstrated that WNK1 activates the ERK5 MAPK signaling pathway, which is important in cell proliferation. These findings may suggest that WNKs have a role in tumor maintenance and development.

WNKs in Cancer

Most physiological studies performed on WNKs have focused on their role in regulating ion transport, in part, because of the known mutations that promote PHAII in humans. However, recent evidence suggests that WNKS may have a role in cancer. A crucial hurdle cells must overcome when undergoing transformation is cell proliferation. Work done by Tu et al. (2010) demonstrated that cells lacking WNK1 displayed defects in mitotic spindles, abscission and cell survival. WNK1 was found to co-localize toward the mitotic spindle in early prophase. Cells lacking WNK1 displayed a range of defects, including misaligned chromosomes and spindles, tripolar spindles, and L-shaped chromosomes (Tu et al. 2010).

Sequence alignment analysis revealed about a 50% similarity between the catalytic domains of WNKs and a subset of MAPK family of members(MEKK, Raf, and Pak) (Verissimo et al. 2001). MAPKs have been linked to pathways that regulate the transition from a quiescent to a proliferative state in epithelial cells (Moniz et al. 2010). MAPK pathways are activated in response to growth factor stimulation and trigger a signaling cascade in which MAPKs are phosphorylated and activated by MAP2Ks, which in turn are activated by MAP3Ks (Moniz et al. 2010). These events then trigger additional signaling pathways that promote cell survival, escape from apoptosis, cell proliferation, and cell migration (Moniz et al. 2010).

The importance of the MAPK signaling cascade arises from the fact that roughly one third of all reported human cancers have a disrupted or deregulated MAPK signaling pathway. Therefore, it is important to understand mechanisms of MAPK regulation (Dhillon et al. 2007). WNKs appear to have a role in regulating several of these processes. WNK1 may be involved in activation of ERK5 after epidermal growth factor (EGF) simulation (Sun et al. 2006). WNK1 is important in activating MEKK3 independently of its kinase activity (Moniz et al. 2010). Regulation of MEKK3 is important for the activation of ERK5. WNK1 has been shown to regulate ERK5 activation in HeLa cells which may be an important function in tumor cell transformation (Xu et al. 2005).

A role for WNKs in cancer is further supported by the fact that WNK2 expression appears to be suppressed in human glioma cells (Haas et al. 2011). Although the underlying mechanism linking suppression of WNK activity to cancer remains to be defined, clues were proved by experiments of Moniz et al. (2008) showing that WNK silencing leads to increases in ERK 1/2 activation, which is triggered by the phosphorylation of MEK1 at a critical activation serine (Ser-298), a known Pak1 phosphorylation site. Moniz et al. (2008) have suggested that MEK1 phosphorylation by Pak may be caused by WNK2 inhibiting Pak via the small GTPase Rac1. These findings demonstrate the potential role WNKs may have in cell proliferation suggesting a possible link between WNKs and cancer.

WNKs may also impact cell signaling pathways by controlling the intracellular trafficking and degradation of growth factor receptors. After ligand binding, many receptors are internalized into endosomes and this endocytic process may influence signaling both positively and negatively. For example, endocytic trafficking of receptors to lysosomes is a well-established mechanism for down-regulation, but there is also strong evidence that some signaling pathways are prolonged or even enhanced by endocytosis (Gonzalez-Gaitan et al. 2003). As previously mentioned, WNKs are key regulators of ion transporters and recent findings suggest that this regulation may be attributed to regulation of transporter internalization. For example, work done by Goucheng et al. (2007) demonstrated that WNKs regulate the endocytosis of renal outer medullar potassium 1 (ROMK1) by clathrin-dependent and -independent mechanisms. The authors further demonstrated that ROMK1 regulation by WNK1 is mediated by the interaction of three amino terminal proline rich motifs (PxxP) of WNK1 with the scaffold protein intersectin1 (ITSN1) (He et al. 2007).

WNK3 has also been implicated in regulating cancer cell proliferation by regulating the calcium transient receptor potential vanilloid (TRPV) channels (Prevarskaya et al. 2007 and Zhang et al. 2008). These channels are important for maintaining cell viability in certain cancers of the ovary, thyroid, breast, and colon. Verissimo et al. (2006) reported that knockdown of WNK3 increased the rate of apoptosis in HeLa cells mediated by caspase-3 suggesting an important

role for the kinase in suppressing cell death via caspase-3, perhaps, by the formation of a protein complex comprising WNK3, pro-caspase-3 and the heat shock protein p70 (Verissimo et al. 2006 and Salveson et al. 2002).

Hong et al. (2007) demonstrated that many human glioma cells display high levels of methylation of the 5'-end of the WNK2 gene, suggesting that WNK2 may serve as a tumor suppressor within the brain, perhaps by inhibiting pathways downstream of EGF, a major component in maintaining brain tumor pathogenesis (Hong et al. 2007). Although large scale screens of the cancer genome have revealed point mutations in all four WNKs, currently it is not clear whether these mutations contribute to tumor pathogenesis (Moniz et al. 2010). However, these findings suggest that WNKs have much broader roles within cells then regulation of ion transport and may contribute to pathways involved in tumor pathogenesis, including cell proliferation, cell survival and possibly tumor cell migration.

WNK1 and cell migration

WNKs were implicated in the control of cell motility in studies showing their involvement in TGF-β signaling, which is important in the epithelial-mesenhymal transition (Lee et al. 2007). Xutong et al. (2006) reported that WNKs are important in development due to WNK1 homozygous mice being embryonically lethal. The authors further reported that suppression of WNK1 resulted in a decrease in EGF signaling, specifically in ERK5 phosphorylation,

which is critical for inducing C17.2 cell migration. Indeed, cells lacking WNK1 displayed a reduction in C17.2 cell migration in response to EGF stimulation (Xutong et al. 2006).

Mausbacher et al. (2010) observed increased WNK1 phosphorylation in response to lysophosphatidic acid (LPA) in a glycoproteome phosphoproteomic screen of SCC-9 tong squamous carcinoma cells. WNK1 phosphorylation was identified at serine 382 with a 2-fold induction upon LPA treatment. Because LPA was reported to induce SCC-9 cell migration, the authors performed migration assays in SCC-9 cells depleted of WNK1, and showed that LPA-mediated wound closure was impaired compared to controls.

Haas et al. (2011) reported that WNK3 regulates glioma cell migration by regulating the overall cell volume. The authors suggested that cell volume is critical in that invading cells need to alter their cell volume in order to pass across tight cellular barriers. Once across the barriers, cells will resume their normal volumes (Hass et al. 2011).

Although observations demonstrate WNK1 depletion impairs cell migration, the mechanistic basis for this effect is not understood, and represents the major effort of my studies.

Cell migration

Eukaryotic cells have the ability to undergo organized, directed migration under a variety of circumstances, including for example growth factor simulation,

nutrient deficiency, and development (Pantaloni et al. 2001). This cellular locomotion is driven by molecular motors which transport cellular components along microtubules and actin filaments in one direction. This unilateral movement is driven by ATP hydrolysis and is considered an integral physiological, but a potentially pathogenic processm, found in nature (Locascio et al. 2001; Pantaloni et al. 2001). During embryonic development, cell migration is crucial for gastrulation, in that, neural crest cells require cell migration in order to colonize tissues during vertebrate embryogenesis (Locascio et al. 2001).

Cell migration is possible due to an abundance of the 43-kD globular protein, actin (Wegner et al. 1976). *In vitro*, actin has the ability to polymerize into helical, polar filaments characterized by a 167^{o} rotation and a 2.7-nm axial rise; this phenomena of actin polymerization is the crucial mechanism that drives cell migration (Wegner et al. 1976). Beckerle et al. (1998) reported that actin filaments have a quick turnover within cellular protrusions, termed lamellipodia, which extend outward toward a stimulus; At the leading edges of these lamellipodia, polymerization occurs at the barbed ends of actin filaments, whereas depolymerization occurs at the opposite, pointed ends in a process described as "treadmilling" (Bugyi et al. 2010). This mechanism was first described in cells infected with *Listeria*, where barbed actin ends were located facing toward the bacterium. It was also noticed that the rate of which the barbed end of the actin filament grew was directly associated with the rate of which the cell was moving

(Dabiri et al. 1990). Treadmilling is a very rapid process, with a turnover rate of about 3-μm filament per min (Pantaloni et al. 2001).

(Bugyi et al. 2010). G-actin monomers polymerize to form actin filaments with assembly beginning after nucleation of three actin monomers resulting in a trimer followed by polymerization at both ends resulting in extended filaments called F-actin (Bugyi et al. 2010). As the F-actin filament grows, polymerization at both ends is reversible and actin monomers associate and dissociate from the filament (Bugyi et al. 2010). At high concentrations of free actin subunits, the filament grows at each end but polymerization at the barbed ends is considerably quicker. However, as concentrations of free actin monomers fall below a certain threshold, the barbed ends continue to polymerize and elongate in length, at the same time, polymerization at the pointed end will stop and the pointed end begins to decrease in length (Bugyi et al. 2001; Clainche et al. 2008).

Treadmilling is a cyclical process in which there is an overall net gain in subunits at the barbed end of the filament and an equal net loss of subunits at the pointed end. Although experiments demonstrate that actin alone does polymerize *in vitro*, several factors promote actin polymerization at a much higher rate in cells. Although the overall process of actin driven cell migration is a very complicated process which involves a vast amount of different proteins, here are several of the key components that promote actin-based cell migration.

Factors the regulate cell migration

Actin polymerization depends on both ATP binding and ATP hydrolysis. ATP is found bound to G-actin, which has a preference of polymerizing toward the leading edge of filaments at the barbed ends (Bugyi et al. 2010). As soon as ATP-actin is polymerized at the barbed end, the bound ATP is hydrolyzed to ADP. This hydrolysis creates a difference between the critical concentration (Cc) of the ATP-bound barbed end (Cc=0.06 μM) and the ADP-bound pointed end (Cc=0.6 μM). At a steady state, the value of the concentration of unbound monomeric actin is about 0.1 μM, suggesting that elongation at the barbed end is equal to the depolymerizing of the pointed end. Therefore, filaments keep the same length as they move forward (Clainche et al. 2008). Actin bound with ADP are therefore found near the pointed ends of F-actin filaments.

Two important protein groups of proteins that control actin polymerization states are the actin depolymerizing factor (ADF/cofilin) family of proteins and the capping proteins (Pollard et al. 2003). ADF proteins, specifically cofilin1, is crucial in cell migration and are negatively regulated by phosphorylation in response to stimulus, such as growth factors. Regulation of cofilin1 will be discussed later (chapter 3). Cofilin1 accelerates the de-polymerization of actin at the pointed end due in part that de-polymerization are the rate limiting step in treadmilling. The acceleration of actin depoylmerization leads to a higher steady-state concentration of monomeric ATP-actin *in vitro* (Pollard et al. 2003). Cofilin1 accelerates treadmilling by increasing the reverse rate constant (K) so

that the critical concentration at steady state (Css) has a higher value so that barbed-end growth, $r = K_{+}{}^{b}(C_{ss}-C_{c})$ (where C_c is critical concentration) balances faster pointed end de-polymerization (Pantaloni et al. 2001). Cofilin1 therefore enhances actin-based motility by enhancing treadmilling (Aizawa et al. 1996; Pantaloni et al. 2001). However, recent evidence suggests that cofilin1 enhances treadmilling by severing actin filaments although this remains controversial due to the creation of new barbed ends creates an equal amount of pointed ends which will increase the overall turnover of the filaments but will not change the overall net speed of treadmilling (Carlier et al. 1999).

Profilin is a member of the actobindin family, which similar to its *Drosophila* homologue ciboulot, functions as an actin binding protein that preferentially binds to ATP-G actin at barbed ends of filaments forming a complex with actin (Pollard et al. 1984 and Boquet et al. 2000). This complex is converted into an ATP-bound profilin-actin complex which polymerizes at the barbed ends only (Pantaloni et al. 2001). *In vitro* experiments demonstrate that profilin enhances treadmilling by 125-fold, which is similar to known physiological rates of treadmilling (Didrey et al. 1998). Binding of profilin to actin results in a lowered critical concentration of free ATP-G-actin which requires ATP hydrolysis, which suggests that polymerization of these filaments interact with the actin-motor protein Formin (Bugyi et al. 2010; Perelroizen et al. 1999; Romero et al. 2007).

Formins function as motors that assemble at barbed-ends of filaments in cell migration. Formins contain two forming homology 1 and 2 domains (FH1 and FH2) which are responsible for binding profilin and actin, respectively (Bugyi et al. 2010; Goode et al. 2007). Formins bind to monomeric G-actin and facilitates the nucleation process in which three monomers are polymerized and form a trimer. After forming-mediated nucleation of the actin trimer, polymerization of actin proceeds at a quick pace (Bugyi et al. 2010). Other proteins important in actin-based migration are the capping proteins.

Capping proteins prevent the formation of large amounts of barbed ends formation, which in turn, promote depolymerizing actin monomers toward the growth of a few uncapped filaments. These uncapped filaments grow at a much faster rate when compared to growth in filaments which are not capped (Hug et al. 1995). During treadmilling, capped proteins are recycled which is crucial for maintaining cell motility in response to stimulation; this process of maintaining cell motility is regulated by the Arp2/3 complex (Pantaloni et al. 2001).

The Arp2/3 complex is ubiquitously expressed protein complex that stimulates actin polymerization composed of two actin-binding proteins: Arp2 and Arp3 (Welch et al. 1997). Arp2/3 was first described in actin based studies performed on *Listeria* where Arp 2 and 3 enhanced actin polymerization at the surface of the bacteria activated by members of the Wiskott-Aldrich syndrome protein (WASP) family (Machesky et al. 1999: Amann et al. 2002). WASP

proteins are scaffolding proteins that connect signaling pathways that are downstream of receptor tyrosine kinases, small GTPases Cdc42 and Rac, and heterotrimeric GTP-binding proteins. WASP proteins contain a COOH- terminal domain that constitutively activates the Arp2/3 complex which is homologous to the COOH-domain of the *Listeria* protein ActA. WASP also interact with Cdc42-GTP, phosphatidylinositol-4,5-bisphosphate (PIP_2), profilin, and proteins containing SH3 domains (Rohatgi et al. 2000). The interaction of WASP with these proteins promotes actin polymerization locally within the cell and thus drives stimulated- dependent cell migration.

The Arp2/3 complex multiplies the number of filaments by branching off filaments at the leading edges of lamellipodium in a "Y" shape like phenotype in which daughter branches were at 70^o angles from the mother branch; the Arp 2/3 complex is found within the barbed ends of the two daughter branches facing the lamelipodium (Svitkina et al. 1999). Arp2/3 mediated "daughter" filaments do not originate by nucleation but instead contain the Arp2/3 complex within the new filament. It has long been debated on the mechanism of how the Arp2/3 complex causes the filaments branch out, even in solution. Two models have been proposed: side branching and barbed-end branching (Higgs et al. 1999 and Amano et al. 2002). In the barbed-end branching model, it is suggested that the mother and daughter filaments be of equal length. In contrast, the side branching

model suggests that the daughter branches will always be smaller then the mother branches (Amano et al. 1996 and 2001).

Cell migration continues being a topic of great importance as yet many questions remain unanswered as to the mechanisms underlying the regulation and control by which actin driven migration occurs. One aspect of cell migration that is still controversial is the transformation of a quiescent cell into a highly motile cell which is of great importance in tumor pathogenesis.

Cell migration and cancer

About half a million people will die from cancer this year most likely due to tumor cells escaping the primary tumor and entering the vasculature followed by invasion of cells into the surrounding tissue, nearby lymph nodes and colonization of organs (Zigl et al. 2011). Understanding cancer cell migration and secondary tumor formation is currently a major goal of cancer therapy due in fact that about 90% of cancer deaths are related to metastasis (Sporn et al. 1996).

Dating as far back as 1889, in Stephan Paget's theory of "seed and soil," metastasis was suggested as being an integral mechanism of tumor pathogenesis (Zijl et al. 2011). Although a tumor roughly the size of 1 cm promotes the metastasis of one million cells per day to enter the circulatory systems, colonization of these cells is very rare with less then 0.1% of these cells developing into a metastasis (Fidler et al. 2005). This is due to the fact that there are hurdles motile cells must overcome to properly colonize distant sites making it

an inefficient process (Chambers et al. 2002). Today the origin of metastasis is not well understood but two separate models have been proposed. The first model suggests that after the primary tumor reaches a critical size, cells disseminate from the primary tumor and spread. The second school of thought argues that during early tumor development, tumor cells disseminate early in tumor formation in tumors ranging from 1 to 4 mm in size (Olivier et al. 2006).

Tumors arsing from different tissues tend to invade different areas of the body. For example, metastasis of breast cancer cells tends to favor metastasis into the bone, the brain, and in the lung; whereas tumor cells originating within the colon and pancreas prefer to colonize the liver and lung. However, gliomas are generally found within close proximity of the primary tumor, although it is believed that distant metastasis is not observed due to the early lethality of the disease (Mourad et al. 2005).

Migration of cancer into surrounding tissues occurs in several different ways; either as a collective epithelial sheets, detached cluster of cells, or as a single cell in either a mesenchymal or an amoeboid cell type (Friedl et al. 2003). Cells typically undergo several phenotypical and morphological changes which lead to their ability to migrate into surrounding tissues which are characterized as: (1) epithelial to mesenchymal transition (EMT); (2) collective to amoeboid transition (CAT); (3) mesenchymal to amoeboid transition (MAT) (Zijl et al. 2011).

Mechanisms of cancer cell migration

EMT is a crucial event in cancer metastasis and progression, which was first demonstrated in experiments done by Hay et al. (1995), who described an "epithelial-mesenchymal transformation;" since then, the word "transformation" has been replaced with "transition" due to evidence suggesting that this process is reversible. EMT is a highly conserved process which occurs during inflammation, fibrosis, and embryogenesis; EMTs are described as a loss of epithelial phenotypes and genotypes in which the characteristics of a typical epithelial cell are lost (Kalluri et al. 2009). Cells undergoing EMT demonstrate enhanced cell motility potential and an increase in apoptosis resistance (Kalluri et al. 2009). In order to achieve successful EMT, cells must complete several molecular hurdles: expression of specific cell-surface proteins, transcription factor activation, expression of ECM-degrading enzymes, and reorganization of the cytoskeletal network and proteins (Kalluri et al. 2009). EMT is a common morphological change observed in normal development. During embryogenesis and organ development, different cells differentiate into a phenotype that appears to display a plasticity which allows them to morph back and forth from a mesenchymal state to an epithelial state via EMT and meschymal to epithelial transition (MET) (Lee et al. 2006). Cells can also undergo EMT in response to tissue repair, inflammatory response, and pathological stresses (Kalluri et al. 2009).

EMTs are classified into three different subclasses; type I, type II, and type III (Zeisberg et al. 2009). The first subclass, type I, is assigned to EMTs in which no observed fibrosis is detected. For example, type I EMT occurs during embryogenesis in the formation of the placenta. During development, cytotrophoblast precursor cells known as trophoectoderm cells undergo EMT in order to invade the endometrium and anchor the placenta; this anchoring is critical in order to achieve nutrient and gas exchange (Aplin et al. 1998; Kalluri et al. 200).

Type II EMTs are classified as EMTs which occur in wound repair, the fibrosis of organs, and in tissue regeneration (Kalluri et al. 2009). This was first observed by Zavadil et al. (2005), who performed experiments on mice utilizing epithelial, cell-specific promoters by bone marrow transplant studies demonstrate that during kidney fibrosis, about 12% of fibroblasts originate from the bone marrow and about 30% derive from tubular epithelial cells of the kidney via EMT. A key difference between type I and type II is that type II EMTs are limited by the inflammatory response; once inflammation is attenuated, type II EMTs cease. However, chronic inflammation leads to sustained type II EMTs which leads to fibrosis and eventual organ destruction (Kalluri et al. 2009).

Type III EMTs occur in cells that have undergone genetic and epigenetic changes within neoplastic cells which favor clonal outgrowth and tumor development. Cells express or inhibit both oncogenes and tumor suppressor

genes which “highjack” the EMT machinery to promote a highly metastatic and invasive phenotype commonly observed in late stage and lethal forms of cancer (Kalluri et al. 2009). Although all EMTs share common molecular mechanisms, types III EMTs demonstrate unique molecular events that contribute to tumor invasiveness. Recent evidence demonstrates that cells undergoing type III EMTs depend on TGF-β. TGF- β induces EMT by via the activation of Smad proteins via the ALK-5-receptor (TGF- β receptor type I) (Kalluri et al. 2009). Signaling via TGF- β is thought to activate p38 MAPK leading to the increase expression of Fiulin-5; Fibulin-5 promotes metalloproteinase expression, which in turn help breakdown the ECM and promote cell migration (Lee et al. 2008).

Although types III EMTs are of critical importance in understanding tumor pathogenesis, all three EMTs share common signaling mechanisms. One hallmark of EMT is the loss of epithelial (E-) cadherin, which is promoted by various proteins that lead to the breakdown of adherence junctions (Thiery et al. 2009). The reduction of E-cadherin expression is driven by transcription factors Snail and Slug, which are zinc-finger transcription factors that bind E-box motifs and are believed to repress the expression of E-cadherin; many types of cancer cells demonstrate increase in Snail and Slug gene expression as well as a loss of E-cadherin (Cavallro et al. 2004). The loss of E-cadherin is accompanied by the production of N-cadherin (Neuronal) known as the “cadherin switch,” a hallmark of EMT development, which in turn leads to the formation of lamellopodia

(Cavallaro et al. 2004; Kalluri et al. 2009). During tumorigenesis, there is a high amount of growth factors released in the surrounding tissue or by the tumor cells themselves. These factors, which include hypatocyte growth factor (HGF), platelet derived growth factor (PDGF), and transforming growth factor (TGF)-β; these growth factors, in turn, promote the progression of tumor growth as well as promoting cell migration in both in vitro and in vivo systems (Zavadil et al. 2005).

Collective to amoeboid (CAT) transition is described as cell detachment from other cells and display an amoeboid form of migration in which may of the cell to cell interactions are lost, allowing these cells to squeeze through tight junctions between the ECM (Friedl et al. 2010). CAT mediated migration is thought to occur after the loss of beta-1-integrins; a process that is commonly associated with melanomas (Hegerfeldt et al. 2002). Currently, it is unknown if CAT mediated cell migration is dependent or independent of an EMT transitional period.

The third known mechanism of tumor cell migration, described as the transition from a mesenchymal to an amoeboid cell, is known as MAT, and been detected in breast cancer and melanomas (Friedl et al. 2005). Molecular aspects of MAT include dependence on Rac and Rho signaling in that tumor cells undergoing MAT can change from an EMT phenotype to an amoeboid state depending on the expression of regulatory proteins like EphA2 kinase (Zijl et al.

2011). Interestingly, cells undergoing MAT demonstrate a change in phenotype in response to chemotherapy. It is believed that under chemotherapeutic conditions, cells demonstrating mesenchymal phenotypes will undergo a morphological change which in turn, enhances their aggressiveness by switching their mechanism of cell migration (Zijl Et al. 2011).

Therefore it is important in understanding the underlying mechanisms that control cell migration. Roles which are regulated by WNKs is an emerging field since their discovery over a decade ago; since then, WNKs have been implicated in ion transport, cell proliferation and regulating growth factor signaling.

General Hypothesis

In the years since the discovery of WNK1, emphasis has been placed on its role in regulation of ionic balance, particularly in the kidney. However, novel roles for WNKs in other process are becoming increasingly evident. While cell migration is a very well understood process, new factors, which contribute to regulating these factors, are still being identified either mechanistically (like the phosphorylational state of actin-binding proteins like cofilin) or at the physiologically level (the induction of stress fibers). Given previous observations that link WNK1 to cell migration, the hypothesis of this dissertation is that WNK1 regulates cell motility by controlling the polymerization state of actin, and the organization of actin into supramolecular structures, including stress fibers and circular dorsal ruffles. The following chapters presented here will be the basis for

my thesis dissertation defense and represent my attempt to address this hypothesis.

CHAPTER TWO

Effects of manipulating WNK1 expression on cell motility

Introduction

Cell motility is a complex process that involves the coordinated activities of dozens of interacting cytoskeletal elements. Not surprisingly, it is subject to precise spatio-temporal regulation mediated by a variety of kinases, phosphatases, and GTP-binding proteins. Several studies have implicated WNKs in motility; although there is some disagreement as to whether they act as positive or negative regulators. For example, migration of C17.2 neuronal progenitor cells was inhibited by antisense RNA- mediated depletion of WNK1 (Sun et al. 2006). Likewise, lysophasphatidic acid (LPA)-dependent migration of SCC-9 squamous cell carcinoma cells was suppressed by RNAi-mediated depletion of WNK1 (Mausbacher et al. 2010). Finally, glioma cell motility was found to promote WNK3-mediated activation of the NKCC1 trnasporter, which facilitates focal adhesion turnaround (Haas et al. 2011). In contrast to the findings, it was very recently reported that reduction of WNK2 expression by promoter hypermethylation resulted in increased invasiveness of glioma cells, possibly triggered by Rac1 activation (Moniz et al. 2010).

The principle goal of my research has been to define the mechanisms whereby WNK1 regulates the actin cytoskeleton. To this end, my first objective

was to establish the role of WNK1 in overall motility of the cell types used in my investigation: HeLa and A549.

Materials and Methods

Cell Culture- HeLa and A549 cells were maintained 10cm plastic cell culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g glucose, and L-glutamine. Cells were incubated at 37^{o}C and maintained in 5% CO_2.

Immunoblotting- Cells were washed with cold 1X phosphate buffered saline (PBS) and lysed with RIPA buffer (150mM NaCl, 1% NP40, 50mM TRIS ph 8.0, 0.5% DOC, and 0.05% SDS. Lysate was then centrifuged for 15 min at max speed on a table centrifuge at 4^{o}C. Cleared lysate was then treated with 5X SDS sample buffer (1:4 stacking buffer (0.15M TRIS pH 8.0), 4% SDS, 20% Glycerol, 10% Betamercaptoethanol (BME), and 0.02g bromophenol blue) and boiled for 2 minutes. SDS-PAGE gels were loaded with 20 µl of lysate and transferred onto a 2 µm nitrocellulose membrane (BIO-RAD). Membrane was then blocked with 5% Milk for one hour and antibodies were added as suggested by the manufacture.

Antibodies and reagents- antibodies to the following proteins were as indicated: WNK1 immunofluoresence and immunoblotting (no. 4979; Cell Signaling). Secondary antibodies for immunofluoresence were goat anti-mouse Alexa 488 or 546, goat anti rabbit-Alexa 488 or 546. DAPI was purchased from Invitrogen. Phosphorylated Akt (T308; 2965S), total Akt (2977S), phosphorylated cofilin

(3311S), phosphorylate ERM (3149S), and phosphorylate LIMK (3841S) were all purchased from Cell Signaling. Secondary antibodies donkey anti-mouse (IRDye 800; 926-32212) and rabbit (IRDye680; 926-32223) were purchased from LiCOR.

Scratch Wound Cell Migration Assay- Cells were plated in a 6 well plate and allowed to reach 80% confluence in DMEM with 10% FBS. After 72 hours, a 10 µl pipette tip was used to form a cross-shaped scratch wound within each well. Cells were washed with DMEM to remove floating debris from the well and replaced with fresh medium and treated the cells with either 10% serum DMEM or DMEM without serum. Photographs were taken after one hour, and then 24 hours, using an inverted light microscope (Zeiss #00593) at 20X magnification.

Boyden Chamber Assay- Cells were seeded until confluency in 10 cm plates. Confluent cells were then treated with 0.25% trypsin-EDTA and then counted. Suspended cells were then incubated in separate 1.5 ml Eppendorf tube. 400 µl of DMEM with 10% FBS was placed in the bottom wells of the Boyden chamber. An 8 µm pore polycarbonate membrane filter (Neuro Probe) was placed above the bottom chamber and the top half of the chamber was filled with 285 µl of DMEM and 5,000 cells/each chamber. The "sandwiched" chamber was then placed between two 6 well plate lids (to cover the open chamber) and incubated at 37^{o}C for 24 hours. The top chamber was then aspirated and the filter was removed and washed with 1X PBS by placing the membrane over the buffer without allowing it

to submerge on the side where cells were originally incubated, i.e., the "reverse side." Migrating cells on the reverse side or the "cell side," were not washed. The "reverse side," was then dragged carefully across a razor blade with forceps to remove any non migrating cells and washed three times. Cells were then fixed in ice-cold methanol for 5 min, placed on a glass slide and allowed to air dry. Two drops of Vectashield with DAPI mounting medium was placed on the filter and covered with a cover slip; the membrane was then sealed with nail polish and allowed to dry overnight at room temperature in the dark. Migrating cells were visualized by DAPI fluorescence and taking cell counts of separate fields.

RNAi knockdown transfections- Knockdown experiments were performed utilizing lipofectamine RNAiMAX according to the manufacture's recommendations (Invitrogen). The following oligonucleotides were used: WNK1: sense, GGAUCAAGUGCGAGAAAUUUTT, and antisense, AAUUUCUCGCAUUGAUCCTT. RNAi oligo-lipofectamin complex were created by incubating either WNK1 oligo or scrambled oligo with a final concentration of 10 nM in 250 μl of optimem and incubated for 15 min at room temprature. RNAiMax complexes were created by incubating 6 pmol of RNAiMAX duplexes with 250 μl of optimem and incubated for 15 min at room temperature. After incubation the RNAiMAX duplexes were mixed with the oligo complexes and incubated at room temperature for 45 min. During incubation, HeLa Cells were trypsinized and counted as previously mentioned. After

incubation, the oligo/RNAiMAx complexes were placed in a 6 well plate with 1.5 ml of DMEM supplemented with 10% FBS. Cells were resuspended to 0.5 ml and a final concentration of 300,000 cells/ml then added to the RNAi/medium mixture for a final volume of 2 ml. Cells were then incubated at 37^{o}C for 48 h.

Viability assay-Cells were counted and seeded at 50,000 cells/well on a 96 well plate. After transfection, cells were kept at 37^{o}C in 5% CO_2 for 48 h in 100 µl of DMEM supplemented with 10% FBS. Cell titer-blue reagent was added as suggested by the manufacturer (Promega). 20 µl of reagent was added to each 100 µl of medium (thus, 20µl/ well) and incubated at 37^{o}C in 5% CO_2 for 3 h. Cell viability was then measured using a Bio-Tek 96 well plate reader using excitation wave length of 530 nm and an emission wave length of 580 nm.

Results

We first confirmed previous results (mentioned in the introduction to this chapter) that silencing of WNK1 interferes with overall cell motility. As shown in figure 2, HeLa cells depleted by WNK1 were severely impaired in their ability to migrate into a furrow or “wound” made by scratching confluent cells with a pipette tip.

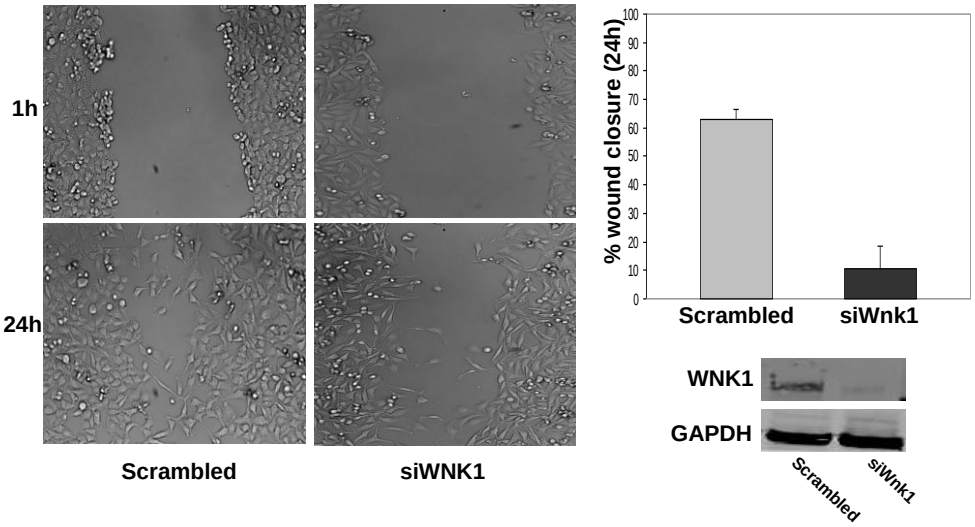

Figure 2. WNK1 knockdown inhibits migration of HeLa cells. HeLa cells were transfected with either scrambled or WNK1 siRNA oligonucleotides, grew for 48 hours, then a wound was made by scratching confluent cells with a pipette tip. Left panel - Representative micrographs taken 1 hour and 24 hours after wounds were made. Right upper panel - The distance between wound edges in the scrambled- or WNK1 siRNA- treated cells is presented as a percent of wound closure after 24 hours. Bar graphs represent the results of three independent experiments. Right lower panel- immunoblot showing efficiency of WNK1 knockdown (upper part); GAPDH (lower part) – loading control.

As shown in figure 3, a similar effect was observed in A549 cells depleted of WNK1.

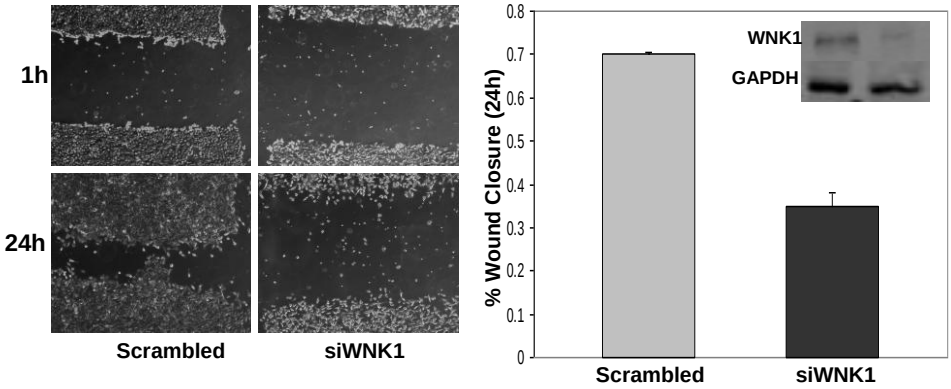

Figure 3. WNK1 knockdown inhibits migration of A549 cells. A549 cells were transfected with either scrambled or WNK1 siRNA oligonucleotides, grew for 48 hours, then an furrow was made by scratching confluent cells with a pipette tip. Left panel - Representative micrographs taken 1 hour and 24 hours after wounds were made. Right upper panel - The distance between furrow edges in the scrambled- or WNK1 siRNA-treated cells is presented as a percent of wound closure after 24 hours. Bar graphs represent the results of three independent experiments. Right lower panel- immunoblot showing efficiency of WNK1 knockdown (upper part); GAP-DH (lower part) – loading control.

This defect in motility was also observed using boyden chamber assays, which score cells which have migrated from one side of a filter to the to the other (figure 4).

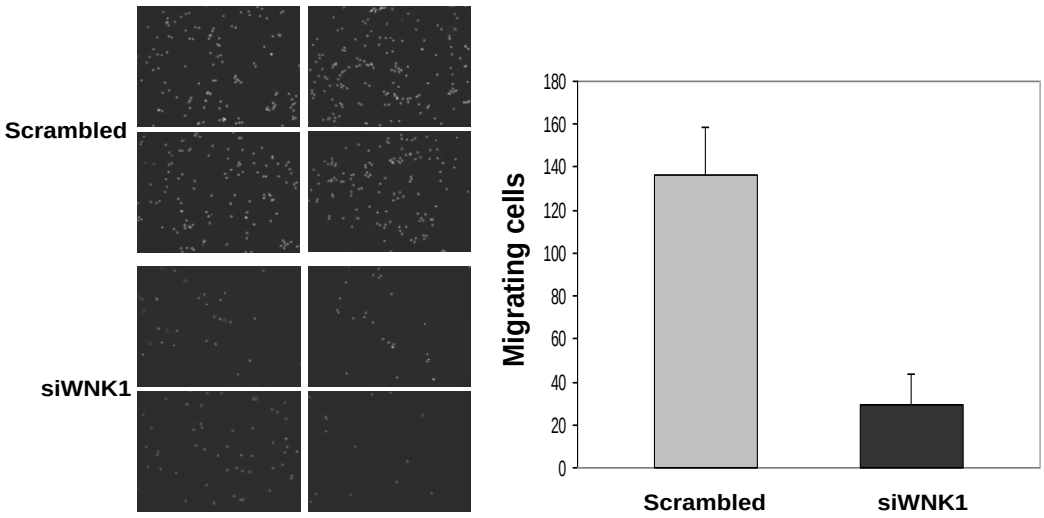

Figure 4. WNK1 knockdown inhibits Hela cell migration. HeLa cells were transfected with either scrambled or WNK1 siRNA oligonucleotides, grew for 24 hours then placed in a boyden chamber and incubated for 24 hours. Migrating cells on the filter were quantified using DAPI stain and observed under 10X magnification using a light microscope. Graph is indicative of 4 panals.

Because floating cells were somewhat more evident in the two motility assays in WNK-depleted samples than in controls, it was important to rule out the possibility that the loss of migration was due simply to reduction of cell viability. This consideration was particularly important given past studies showing that : (a) knockdown of WNK3 induces increased expression of the pro-apoptotic factor, caspase 3 (Verissimo et al. 2006); (b) WNK1 and WNK4 were identified in a genome-wide RNAi screen for apoptosis-inducing proteins (Boutros et al. 2004 and Choe et al. 2007); and (c) impaired ability to cope with osmotic stress, as might occur upon WNK depletion, has been linked to cell death (Delpire et al. 2008 and Richardson et al. 2008). Despite these concerns, our results

demonstrated that reduction of cell viability was similar in control and WNK-depleted HeLa cells (figure 5).

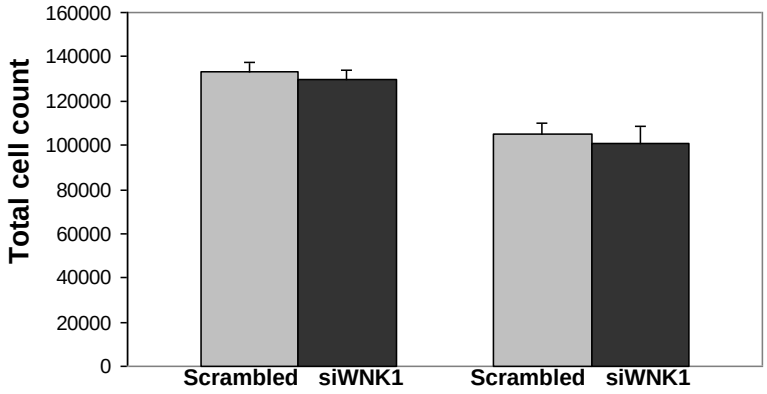

Figure 5. WNK1 knockout does not affect cell viability. HeLa cells transfected with either scrambled or WNK1 siRNA oligonucleotides were grown for 48 hours in a 96 well plate. Cells were then incubated with titer blue assay reagent for 4 hours. Fluorescence was measured using a 96 well plate reader at 560 EX and 590 EM. Right and left bars indicate different concentrations of cells utilized in experiments. Bars are indicative of 3 independent experiments.

Effect of over-expression of WNK1 and WNK1 fragments on cell motility

In view of our finding that WNK1 depletion interferes with cell migration, it would be reasonable to expect that WNK1 over-expression would have no effect, or might even enhance motility. Moreover, unpublished data of L. Lenertz (Cobb laboratory, UT Southwestern Dept. of Pharmacology) indicated that WNK1 phosphorylates Pak1 at threonine 146 and 213. Although subsequent work did not reveal the importance of phosphorylation at these residues, L. Lenertz

demonstrated that hyperosmotic stress did promote Pak1 activity. Therefore it is possible that WNK1 phosphorylation of Pak1 may promote its activation.

To our surprise, over-expression of WNK1 yielded the same phenotype observed in cells with depleted WNK1. i.e., disruption of cell motility as measured by scratch-wound assays. Potential implications of this observation are outlined in this chapter's discussion.

The extent of WNK1 over-expression for a given amount of cDNA was estimated by immunoblotting with anti-WNK1 antibodies. Figure 6 shows that WNK1 levels increase approximately 6-fold in transfected cells (taking transfection efficiency into account) using 4 µg of cDNA per 2 ml.

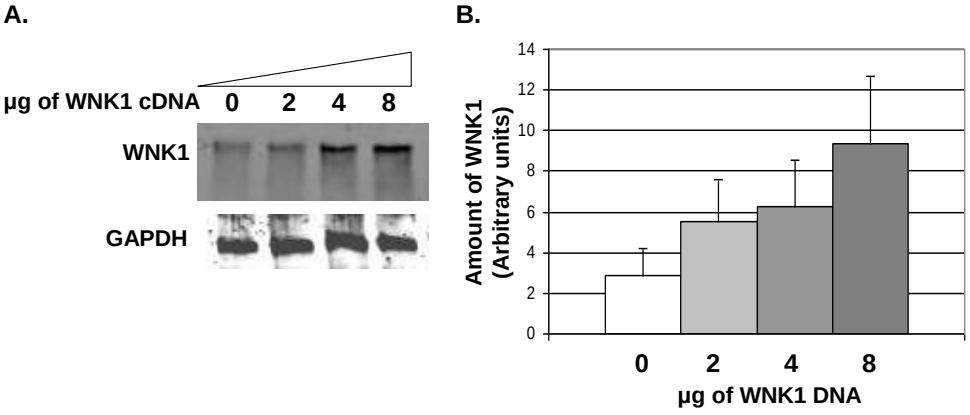

Figure 6. Correlation between WNK1 overexpression and cDNA used for transfection in A549 cells. A. Representative western blot showing increased amounts of expressed WNK1 due to increased amounts of cDNA. Immunoblot with anti-GAP DH was done as control for equal loading of samples. B. Quantification of WNK1 expression. Bar graphs show the average of… experiments.

As shown in figure 7 and 8 respectively, this concentration of cDNA diminished motility of A549 and HeLa cells to comparable extents as observed upon WNK1 depletion.

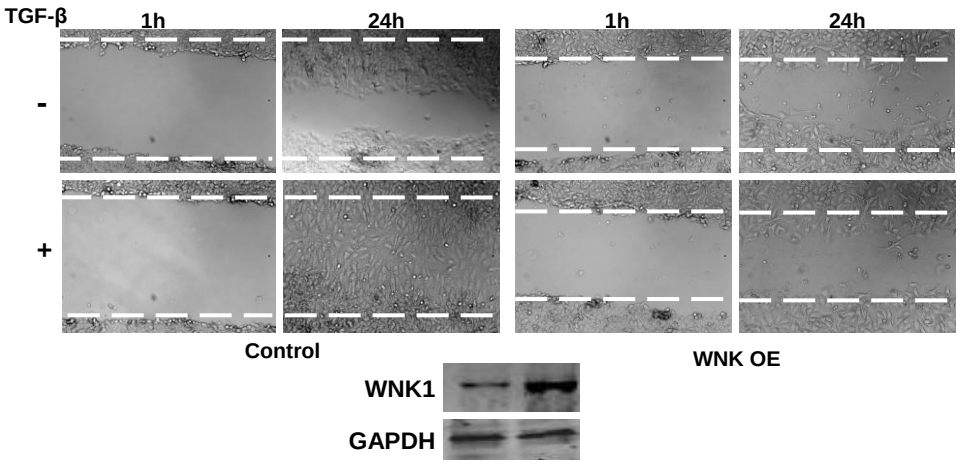

Figure 7. WNK1 overexpression impairs A549 cell migration. A549 cells were transfected with either PCMV5 alone or WNK1 cDNA and grown for 48 hours. At 48 hours, a wound was made by scratching confluent cells with a pipette tip. Left panel - Representative micrographs taken 1 hour and 24 hours after wounds were made. Right lower panel- immunoblot showing efficiency of WNK1 knockdown (upper part); GAP-DH (lower part) – loading control.

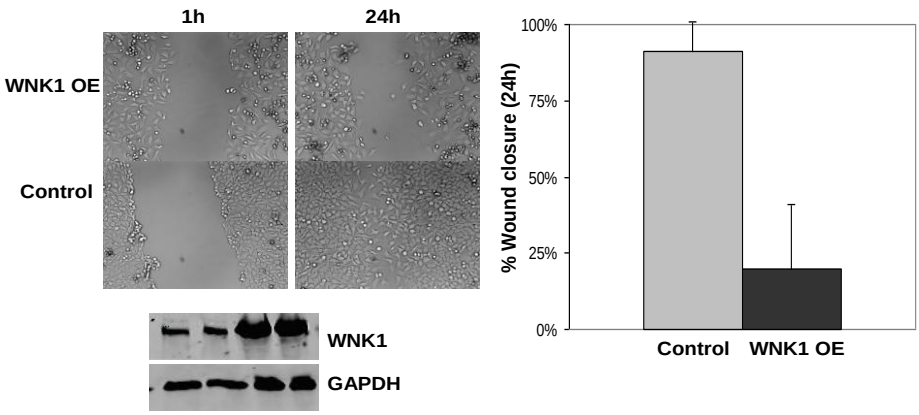

Figure 8. WNK1 overexpression impairs Hela cell migration. HeLa cells were transfected with either PCMV5 alone or WNK1 cDNA and grown for 48 hours. At 48 hours, a wound was made by scratching confluent cells with a pipette tip. Left panel - Representative micrographs taken 1 hour and 24 hours after wounds were made. Right upper panel - The distance between wound edges in the control or WNK1 overexpressed treated cells is presented as a percent of wound closure after 24 hours. Bar graphs represent the results of three independent experiments. Right lower panel- immunoblot showing efficiency of WNK1 knockdown (upper part); GAP-DH (lower part) – loading control.

Due to this finding, I wanted to verify this and subsequent experiments verified that over-expression of WNK1 inhibits A549 cell migration as shown in figure 9 and 10.

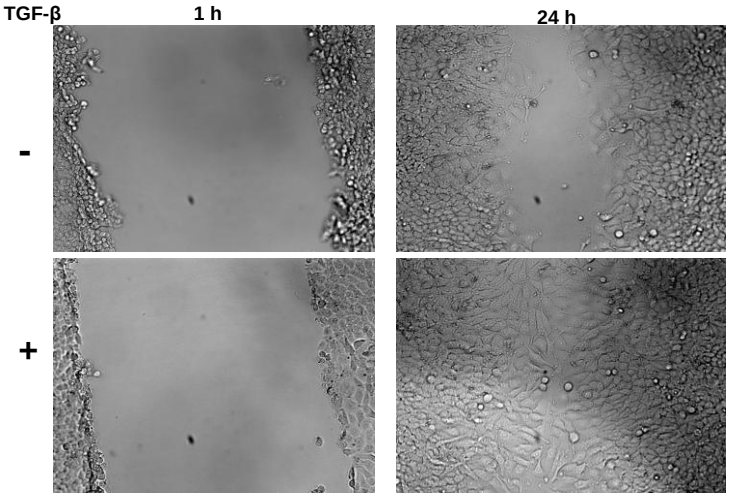

Figure 9. Non-transfected cell wound closure. HeLa cells were transfected with either PCMV5 alone or WNK1 cDNA (or fragments) and grown for 48 hours. At 48 hours, a wound was made by scratching confluent cells with a pipette tip. Top panal indicates cells without TGF-β and the bottom panal indicates cells treated with 5ng/ml of TGF-β. Left side panals indicate time point of 1 hour, right sided panals indicate a 24 hour time point. Immunoblots indicate expression of WNK1 fragments and bottom panal indicate GAPDH loading control.

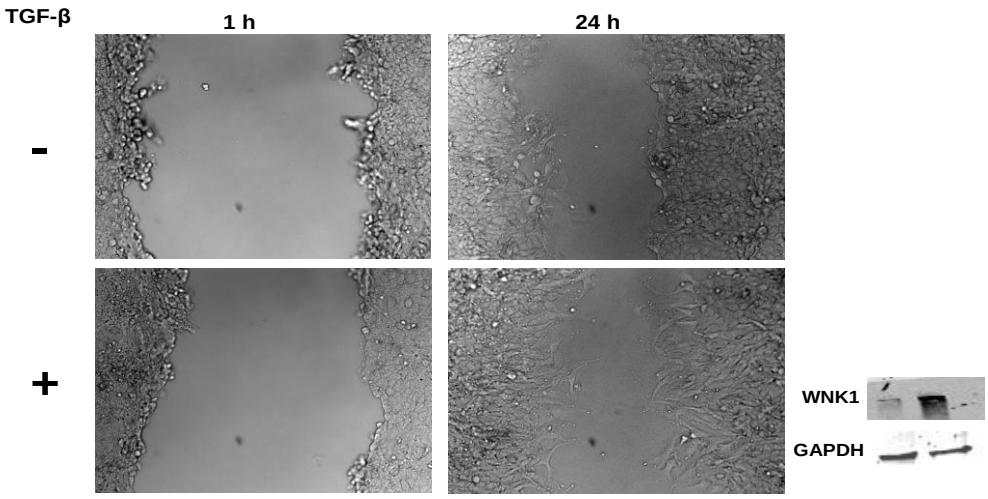

Figure 10. Overexpession of FL WNK1 (1-2126) impares A549 motility.
Immunoblot indicates WNK1 fragment expression and loading control (GAPDH).

I next sought to identify the region(s) in WNK1 responsible for suppression of motility. Figure 10 depicts the constructs used in these experiments, which monitored migration of A549 cells in the absence or presence of TGF-β.

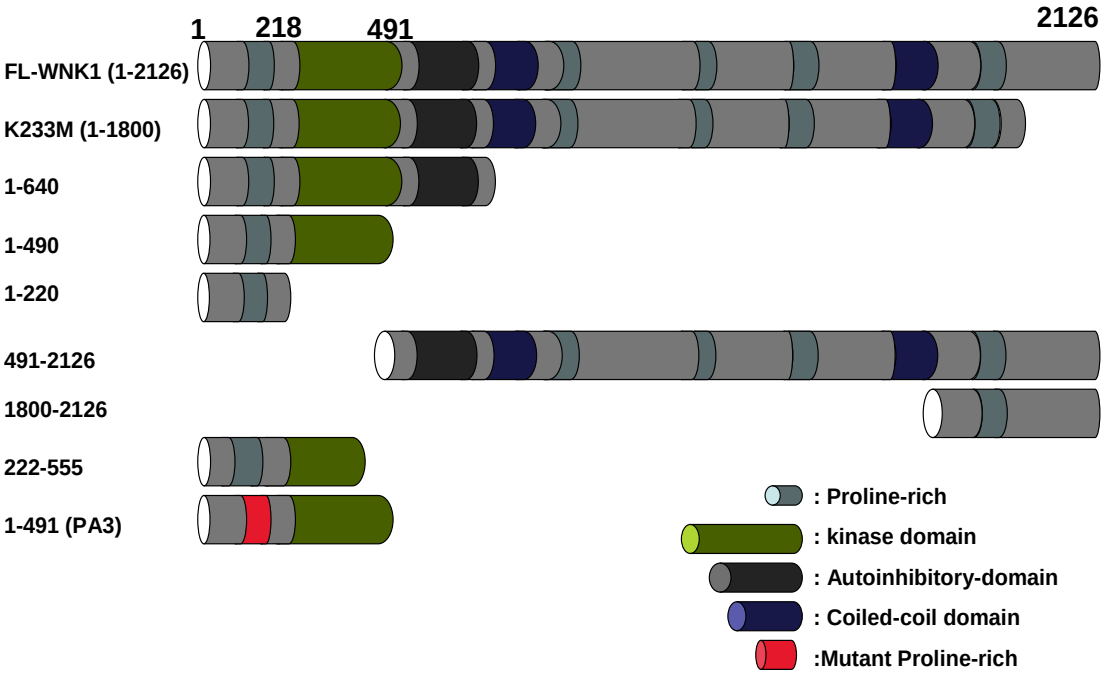

Figure 11. **Scheme of WNK1 fragments utilized in cell migration experiments.** The source of full length WNK1 (FL-WNK) is from rat. All of the fragments were generated from FL-WNK1 and verified sequence analysis.

Expression of fragments 222-555 and 1800-2126, did not affect motility (Figure 13 and 14) suggesting that the auto-inhibitory and c-terminal domain may not be relevant to the observed phenotype.

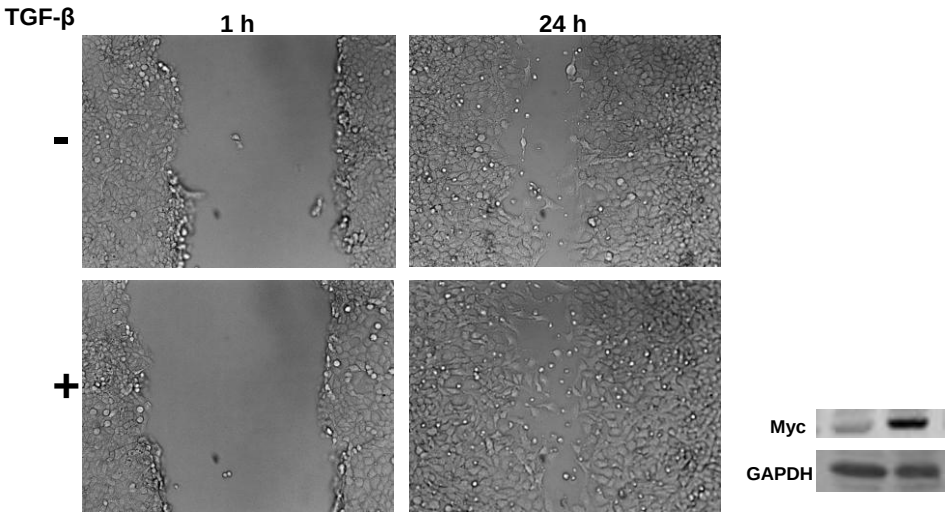

Figure 12. Over-expession of FL WNK1 (222-555) has no affect on A549 motility. Immunoblot indicates WNK1 fragment expression and loading control (GAPDH).

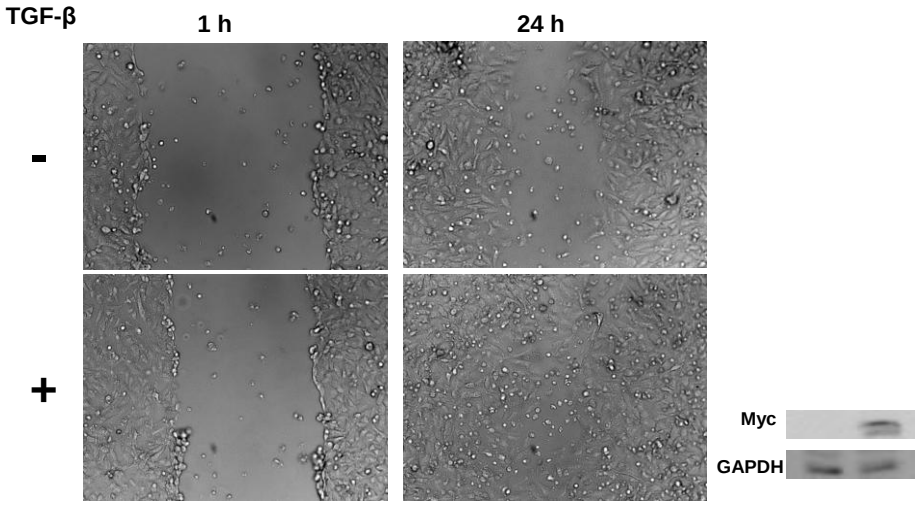

Figure 13. Over-expession of FL WNK1 (1800-2126) has no affect on A549 motility. Immunoblot indicates WNK1 fragment expression and loading control (GAPDH).

In contrast, motility was most strongly impaired by all constructs containing the N-terminal 220 residues, including 1-220, 1-640, and 1-490 (Figure 14, 15, 16) suggesting that catalytic activity may not be a major determinant of motility suppression.

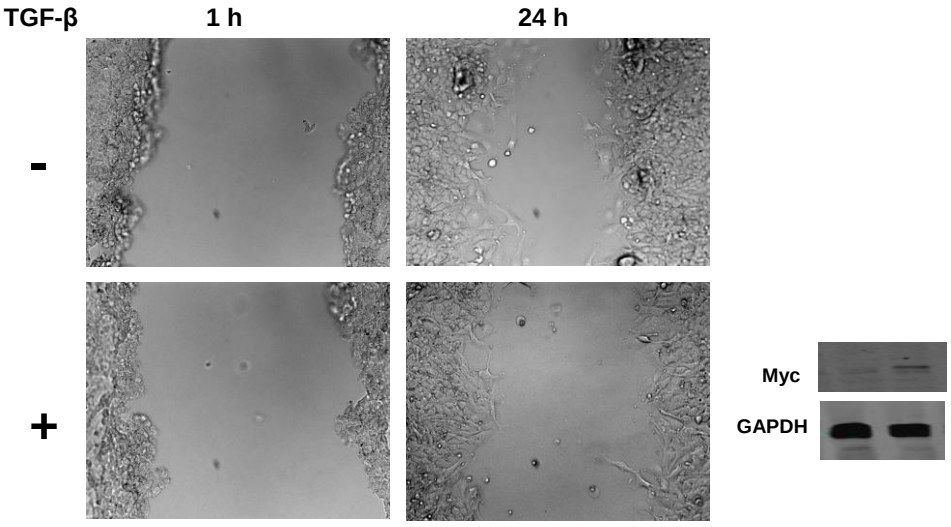

Figure 14. Over-expession of FL WNK1 (1-220) impares A549 motility.
Immunoblot indicates WNK1 fragment expression and loading control (GAPDH).

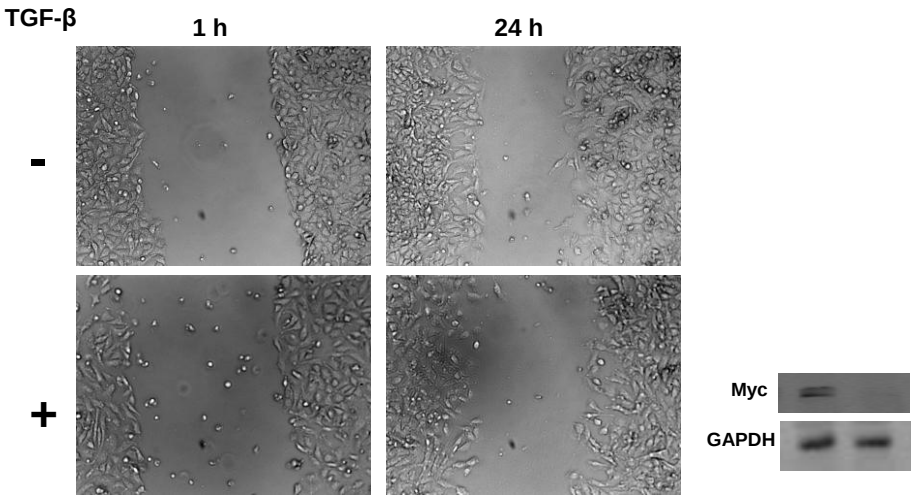

Figure 15. Over-expession of FL WNK1 (1-640) impares A549 motility. Immunoblot indicates WNK1 fragment expression and loading control (GAPDH).

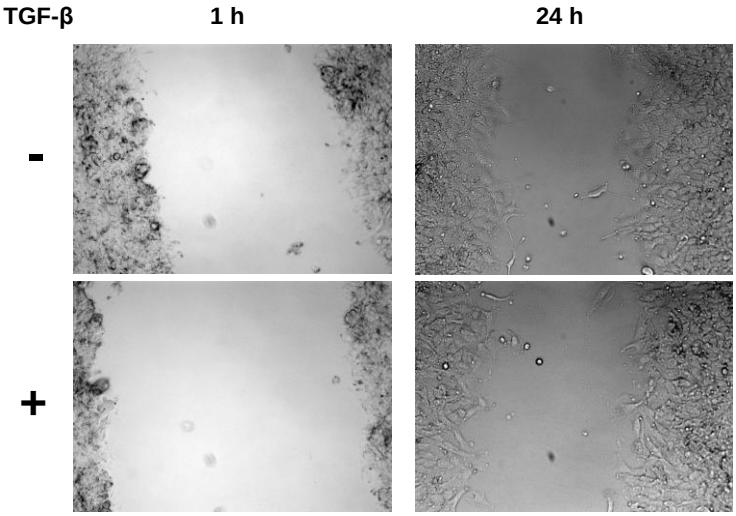

Figure 16. WNK1 (1-490) over-expression has no effect on cell migration. Immunoblot for WNK1 1-491 is on next figure.

Our finding that over-expression of a kinase-dead WNK1 mutant (K233M-1-1800) was as affective at inhibiting motility as its catalytically active full-length WNK1 (figure 17).

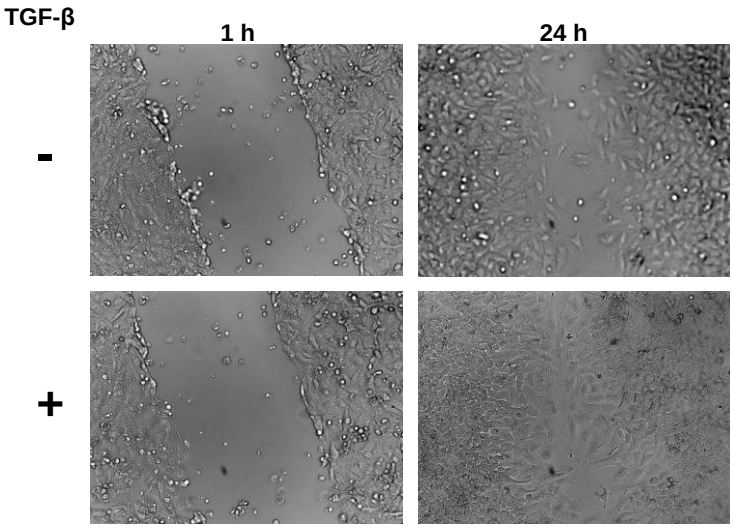

Figure 17. Overexpession of FL WNK1 (K233M-1-1800) has no affect on A549 motility.

The importance of the N-terminal portion of WNK1 in motility was further highlighted by the finding that expression of fragment 491-2126 did not disrupt wound closure (figure 18). Based on these results, we decided to focus our analysis on the effects of the 1-220 N-terminal fragment of WNK1.

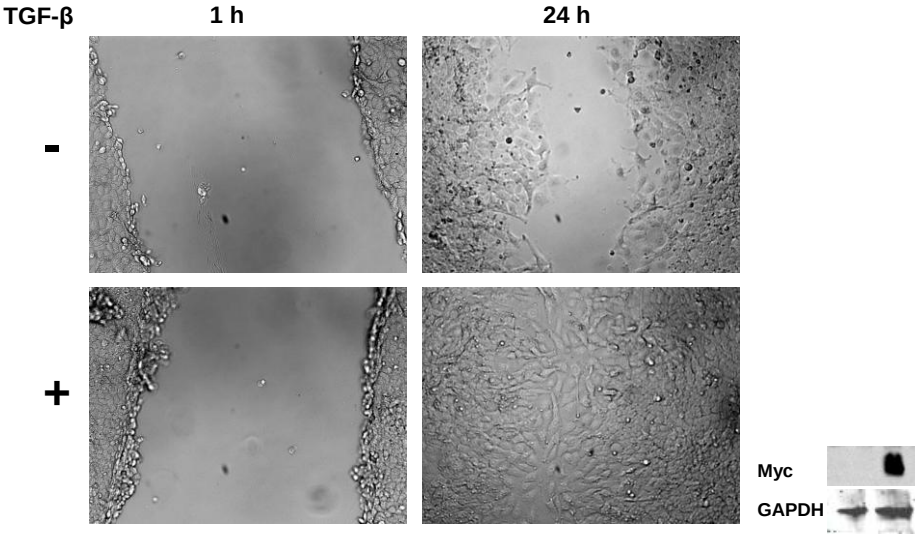

Figure 18.. **WNK1 (491-2126) over-expression has no effect on cell migration**
Immunoblot indicates WNK1 fragment expression and loading control (GAPDH).

I obtained a WNK 1-220 mutant in which three proline residues were mutated to alanine at positions 94, 103, and 114. These proline residues proved crucial in the mediating the interaction between WNK1 and intersectin. I therefore was interested in understanding if a mutant WNK1 (1-220-PA3) would have a similar effect on cell migration when compared to WNK1 (1-220) wild-type. Interestingly, (figure 19) A549 cells over-expressing WNK1 (1-220-PA3) mutant demonstrated no inhibition of cell migration thus further establishing the importance of the WNK1 N-terminal domain.

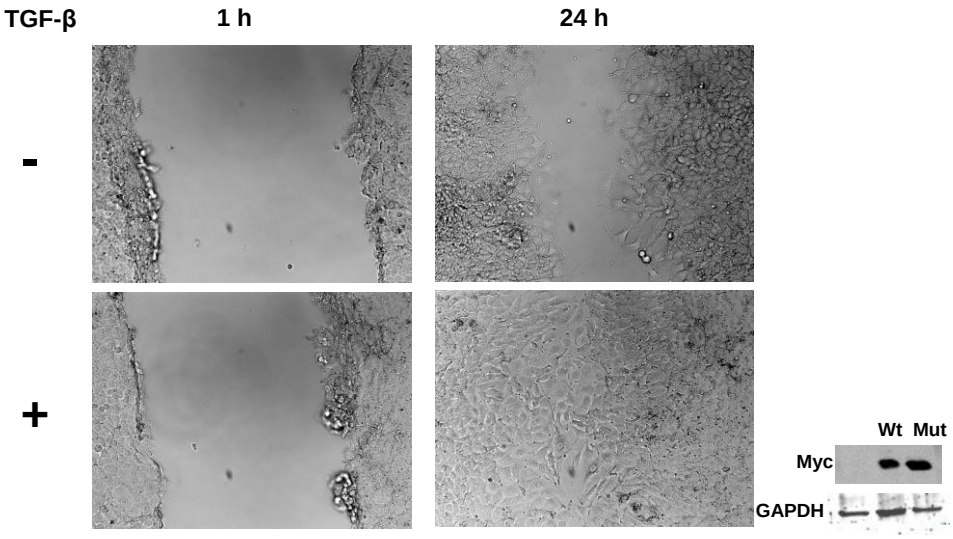

Figure 19. Over-expression of WNK1 (1-491) PA3 has no effect on cell migration. Immunoblot indicates WNK1 fragment expression (WNK1 (1-491) (**figure 15)** and WNK1 (1-491) PA3) and loading control (GAPDH).

A summary of the results from over-expression studies can be seen in figure 20; figure 21 summarizes the effect of TGF-β has on cells over-expressing the WNK1 fragments.

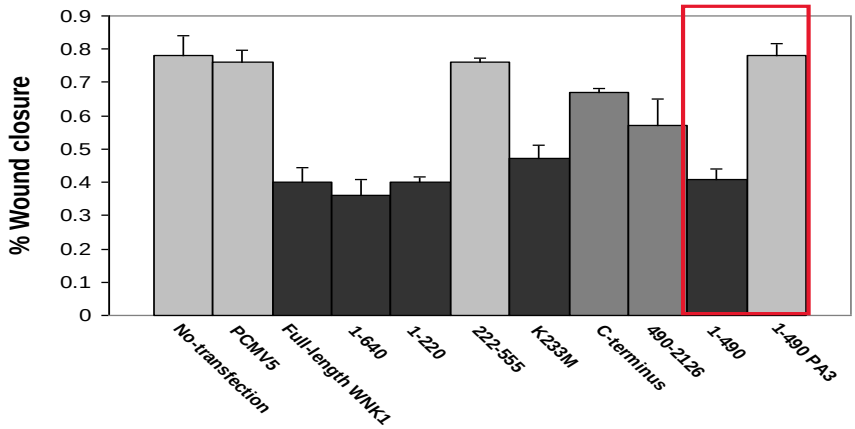

Figure 20. Summary of overexpression effects on wound healing assay. Light grey bars indicate no affect on cell migration; dark grey bars indicate a partial affect and dark bars indicate inhibition of cell migration. Error bars on bars are representative of three experiments. Bars in highlighted in red indicate the difference between the Wild-type 1-491 and WNK1 (1-491) PA3 mutant.

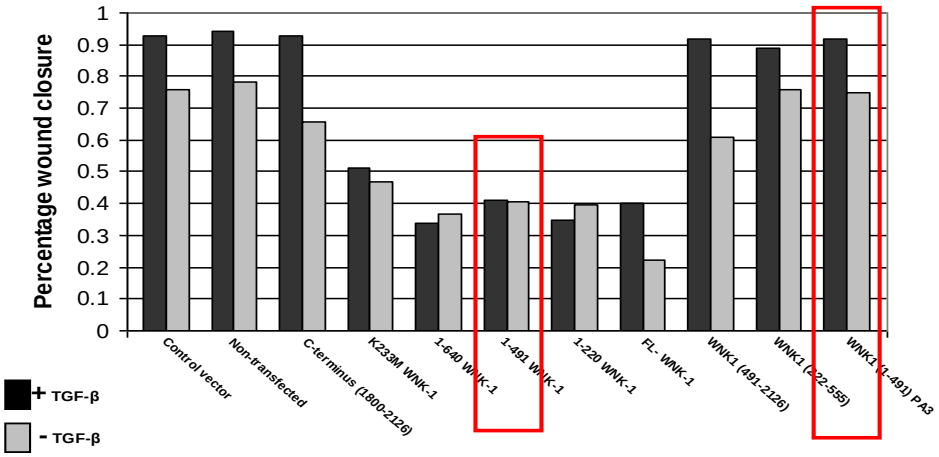

Figure 21. WNK1 overexpression inhibits TGF-β induced migration of HeLa cells. HeLa cells were transfected with either control or WNK1 fragments, grown for 48 hours, then a furrow was made by scratching confluent cells with a pipette tip. Cells were then treated with 5ng/ml TGF-β. The distance betweefn furrow edges in the control or WNK1 cDNA treated cells is presented as a percent of wound closure after 24 hours. Black bars indicate TGF-β treated cells, grey bars indicate non-treated cells. Bars indicate the difference between WNK1 (1-491) and mutant WNK1 (1-491).

As previously mentioned, floating cells were somewhat more evident in the motility assays in WNK-depleted samples than in controls, similar observations were noticed in cells over-expressing WNK1, and therefore it was important to rule out the possibility that the loss of migration was due simply to reduction of cell viability. Figure 22 demonstrates that cells over-expressing WNK1 had similar viability compared to control cells.

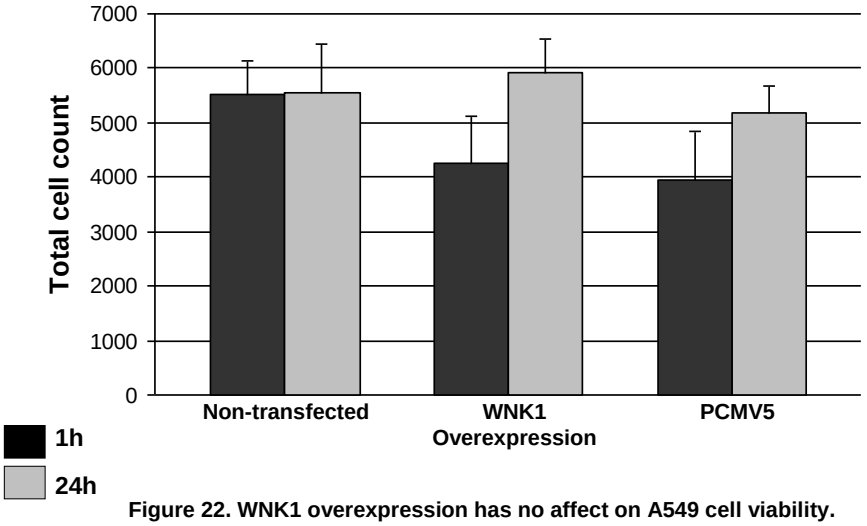

Figure 22. WNK1 overexpression has no affect on A549 cell viability. A549 cells transfected with either PCMV5 or WNK1 cDNA and were grown for 48 hours in a 96 well plate. Cells were then incubated with titer blue assay reagent for 4 hours. Fluorescence was measured using a 96 well plate reader at 560 EX and 590 EM. Right and left bars indicate different concentrations of cells utilized in experiments. Bars are indicative of 11 experiments.

Discussion

The experiments described in this chapter were originally motivated by my interest in confirming previous reports indicating that WNK depletion interferes with cell motility. However, my findings that over-expression of WNK1 also impairs motility, and that WNK kinase activity is not the sole determinant of these effects, demonstrate that a great deal of work remains to be performed in order to understand these events; a summary of the effects of WNK1 fragment over-expression are summarized on table 1.

WNK-1 Fragment	Cell invasion	Inhibition
Full length WNK-1		X
1-1800 (K233M)		X
1-640		X
1-490		X
1-220		X
1800-2126	X	
222-555	X	
Empty vector	X	
491-2126	X	
PA3 WNK1 (1-491)	X	
No transfection	X	

Table 1. Summary of the effects of overexpression of WNK1 fragments in A549 cell migration. (**x**) Indicates outcome of over-expression ofeach fragment.

One mechanism that could explain the observation-that both under- and over-expression of WNK1 inhibits cell motility is the action of WNK1 as a scaffold that facilitates macromolecular interactions important for cytoskeletal regulation. This is supported by figures 20 and 21 in which mutant WNK1 (1-490-PA3) failed to inhibit cell migration when compared to wild-type WNK1 (1-490) (Red boxes). Furthermore, it was suggested by Guocheng et al. (2007) that the N-terminal PxxP motifs of WNK1 recruit proteins, such as intersectin, which in turn recruits factors such as WASP. Another potentially interesting interaction is with Nck1 (Heise dissertation, unpublished observation), an SH3 domain-

containing protein involved in growth factor-mediated changes in actin assembly, in part by recruiting Pak1 to activated receptor (reviewed in Buday 2002). Thus, depletion of WNK1 may hinder motility by preventing its essential scaffolding function. Contrariwise, over-expression of WNK1 may inhibit critical interactions by dispersing the interactors amount the now-increased number of WNK1 molecules. Although in this project I mad some initial attempts to identify novel WNK1-(1-220) binding proteins, my focus has been on characterizing effects of WNK1 depletion on actin cytoskeletal dynamics, and these effects are described in subsequent chapters.

CHAPTER THREE
WNK1 regulation of the actin-cytoskeleton

Introduction

The actin cytoskeleton controls a vast array of cellular process, inclding vesicle transport, maintenance of cell rigidity, signaling cascades, cell division, and cellular migration. Currently there is little information concerning the role of WNK1 in regulation of the actin cytoskeleton. However, evidence reported by Lee et al. (2007) suggests that WNK1 functions downstream of receptors, such as TGF-β, influence cytoskeletal organization suggests that this work is worth pursuing. One way in which WNKs may influence the actin cytoskeleton is by regulation of the Rho family GTP-binding proteins (Ridley et al. 2007). For example, RhoA activity was found to be reduced, whereas Rac1 activity was elevated, in cells depleted of WNK1 (Hong et al. 2007). Moreover, WNK1 was isolated in a complex with Rho-GDI and this interaction was suggested to play a role in mediating neurite out growth (Zhang et al. 2009). Finally, the rate of tumor cell invasiveness directly correlates with the amount of WNK1 expression in F11 neural tumorgenic cells (Zen et al. 2005 and Ridley et al. 2003). In light of these observations, I wanted to determine the effects of WNK1 depletion on total F-actin in cells. To this end, I collaborated with Dr. Barbara Barylko (UT Southwestern) in measuring F-actin content by quantification of bound phalloidin, as described in materials and methods.

Materials and methods

RNAi knockdown transfections- as previously described in chapter 2

Measurements of cellular F-actin- The procedure to determine F-actin in cells was based on the protocol described by Gevrey J. et al. (Immunology 2005, 175,3737-3745). Briefly, HeLa cells were seeded in 96-well plates at a density of 10 $x10^5$ cells per well in 200 ul and incubated for 48 – 60 hours in phenol red free medium with 10% FBS. Cells were fixed for 20 min by the addition of 25 ul 37% formaldehyde solution to each well followed by three washes with PBS. Cells were permeabilized with 0.1% Triton X100 in PBS for 15 min and washed three times with PBS. Cells were then incubated for 20 min with PBS containing 0.30 uM rhodamine phalloidin to stain F-actin and 5uM YO-PRO1 to stain nucleic acids. After several washes with PBS, fluorescence was measured in Synergy 2 (BioTek) plate reader at 485 nm and 528 nm (excitations) and 508 nm and 590 nm (emissions) for YO-PRO1 and rhodamine phalloidin, respectively. F-actin content was normalized as the ratio of rhodamine to YO-PRO1 fluorescence.

Immunofluorescence- Immunofluorescence experiments were carried as previously described (Tu et al. 2010). Cells were cultured in DMEM with 10% FBS and 1% L-glutamine at 37^oC under 5 % CO_2. Cells were plated on a 6 well plates. For immunofluorescence, cells were washed with PBS at 37^oC; fixed with 4% paraformaldehyde in 60 mM Pipes, 25mM Hepes (pH 6.9), 10 mM EGTA,

and 2mM MgCL2 for 10 min; cells were then washed twice for 5 minutes each time with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at 4^{o}C then washed as above. Cells were then incubated in 10% normal goat serum at room temperature for 30 minutes and then incubated with primary antibody at 4^{o}C overnight. Cells were then washed with PBS, incubated with Alexa fluor-conjugated secondary antibody at room temperature for 1 hour, washed twice with PBS, and imaged by fluorescent microscopy.

Cell Culture- as preciously described in chapter 2

Transfections- as previously described in chapter 2

RNAi knockdown transfections- as previously described in chapter 2

Results

As shown in Figure 23, depletion of WNK1 from HeLa cells increased the amount of F-actin by 1.5-fold. I wanted to determine the effects of Oxidative stress-responsive kinase 1 (OSR1) depletion on F-actin due to reported evidence that OSR1 regulates the cytoskeleton and is a known WNK1 substrate (Dan et al. 2001; Ki et al. 2004).

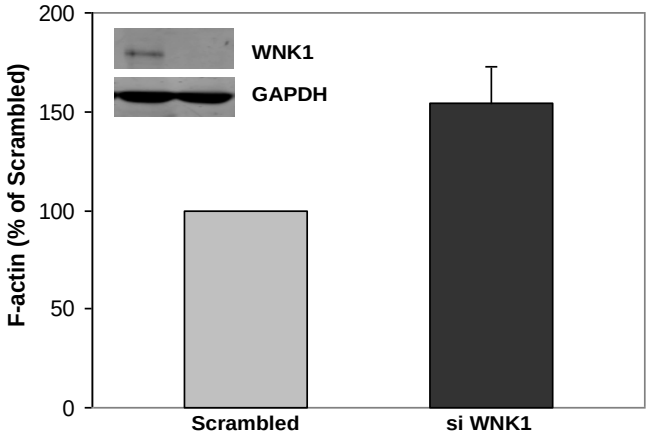

Figure 23. Knockdown of WNK1 increases cellular F-actin content. HeLa cells transfected with either scrambled or WNK1 siRNA oligonucleotides were seeded in 96-well plates at a density of 10 x105 cells per well and incubated for 48 – 60 hours in phenol red free medium with 10% FBS, then fixed be adding formaldehyde for final concentration of 4%. After washes with PBS cells were permeabilized with 0.1% Triton X100. Finally cells were incubated with 300 nM rhodamine phalloidin and 5μM YO-PRO to stain nucleic acids as control. Fluorescence was measured at 485nm and 528 nm (excitations) and 508 and 590 nm (emissions) for YO-PRO and rhodamine phalloidin, respectively. Bar graph represents average of measurements in nine wells in two experiments. Inset: immunoblot showing efficiency of WNK1 knockdown.

As shown in figure 24, depletion of OSR1 from HeLa cells demonstrated no change in the amount of F-actin when compared to control. To identify the structures that might contain this excess polymerized actin, I analyzed the distribution of fluorescently-tagged phalloidin in cultured cells, focusing on the effect of WNK1 depletion on stress fibers and ruffles.

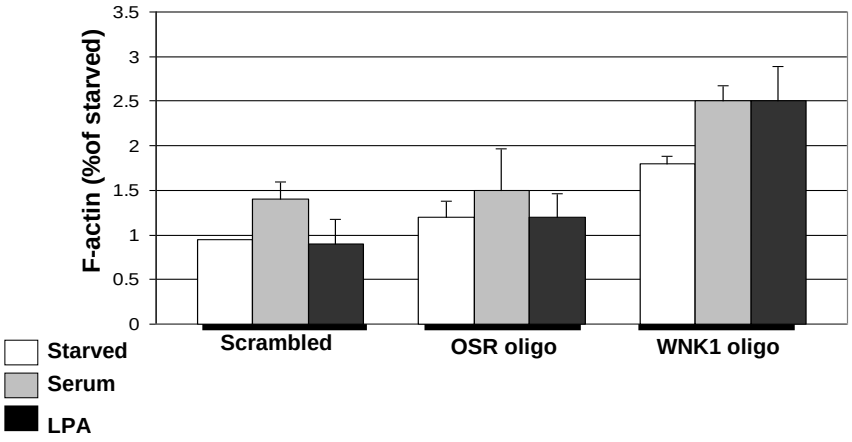

Figure 24. OSR1 knockdown has no affect on F-actin. HeLa cells transfected with either scrambled, OSR1 oligonucleotide, or with WNK1 siRNA oligonucleotides and were seeded in 96-well plates at a density of 10 x10^5 cells per well and incubated for 48 – 60 hours in phenol red free medium with 10% FBS, then fixed be adding formaldehyde for final concentration of 4%. After washes with PBS cells were permeabilized with 0.1% Triton X100. Finally cells were incubated with 300 nM rhodamine phalloidin and 5µM YO-PRO to stain nucleic acids as control. Fluorescence was measured at 485nm and 528 nm (excitations) and 508 and 590 nm (emissions) for YO-PRO and rhodamine phalloidin, respectively. Bar graph represents average of measurements in nine wells in two experiments.

Stress fibers consist of approximately 30 actin filaments bundled together by α-actinin. (Cramer et al. 1997; Lazarides et al. 1975). They are aligned in anti-parallel fashion and are anchored to the substratum by attachment to integrin complexes at focal adhesions (Svitkina et al. 1997; Pellegrin et al. 2007)). Although myosin-dependent contraction of stress fibers is a key step in motility, inordinately large or stable stress fibers may also interfere with motility (Couchman and Rees et al. 1979; Burridge et al. 1981). Figure 25 shows that WNK1 silencing promoted stress fiber formation in HeLa cells.

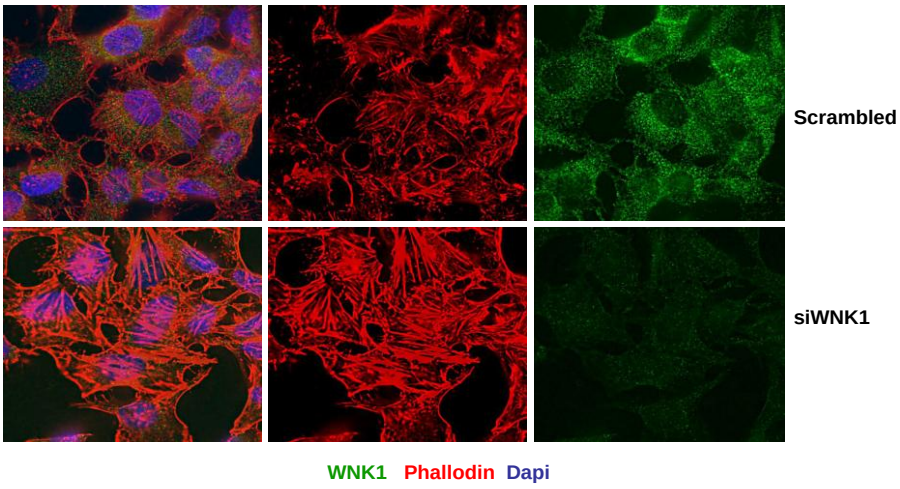

Figure 25. WNK1 Knockdown promotes stress fiber formation. Hela cells were transfected with either scrambled oligo or WNK1 siRNA for 48 hours. Cells were placed on cover slips and immunostained with anti WNK, phallodin, and DAPI. Cells were visualized by confocal microscopy.

In contrast, ruffle formation was impaired in WNK1-depleted cells. Growth factor treatment of many cell types induces the formation of two types of lateral ruffles (LRs), located near the leading edges of moving cells, and circular dorsal ruffles (CDRs), which emerge from the dorsal surfaces and occasionally form waves that move toward the rear of the cell (Abercrombie et al. 1970; Sueetsugu et al. 2003). There is evidence that CDR formation is stimulated by Rac but inhibited by Rho (Zeng et al. 2002; Hoon et al. 2012). In this study I analyzed the effect of manipulating WNK1 expression on CDR formation, which was more easily quantifiable than LR formation. A549 cells were used in the majority of experiments as CDRs formed more readily, and were more evident, in these cells than in HeLa cells. As show in Figures 26, CDRs were evident in A549 cells

grown in serum for 48 h, and increased in frequency by more than 3-fold upon TGF-β treatment.

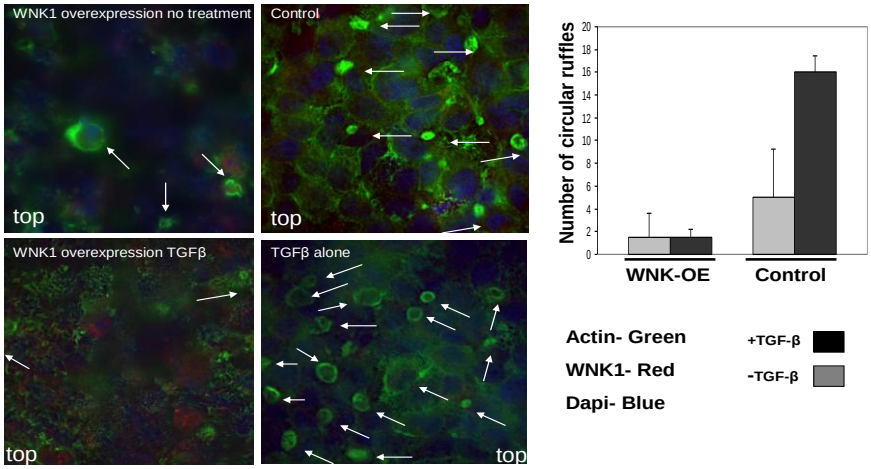

Figure 26. WNK1 overexpression diminishes dorsal ruffle formation in A549 cells. A549 cells were transfected with either PCMV5 or WNK1 cDNA for 48 hours. Cells were placed on cover slips and immunostained with anti -WNK, phallodin, and DAPI. Bar graphs indicate number of dorsal ruffles observed in 3 independent experiments. Cells were visualized by confocal microscopy. (**White arrows**) Indicate the presence of circular dorsal ruffle.

WNK1 over-expression reduced CDR formation both in the absence or presence of TGF-β. Similar results were obtained in both, A549 and HeLa cells depleted of WNK1 (Figure 27 and 28), though in this case TGF-β treatment was not attempted. WNK1-depleted cells also had a lower propensity to form CDRs in response to stimulation by phorbol 12-myristate 13-acetate (PMA), which was previously shown to strongly induce CDRs in A549 cells (Caino et al. 2012).

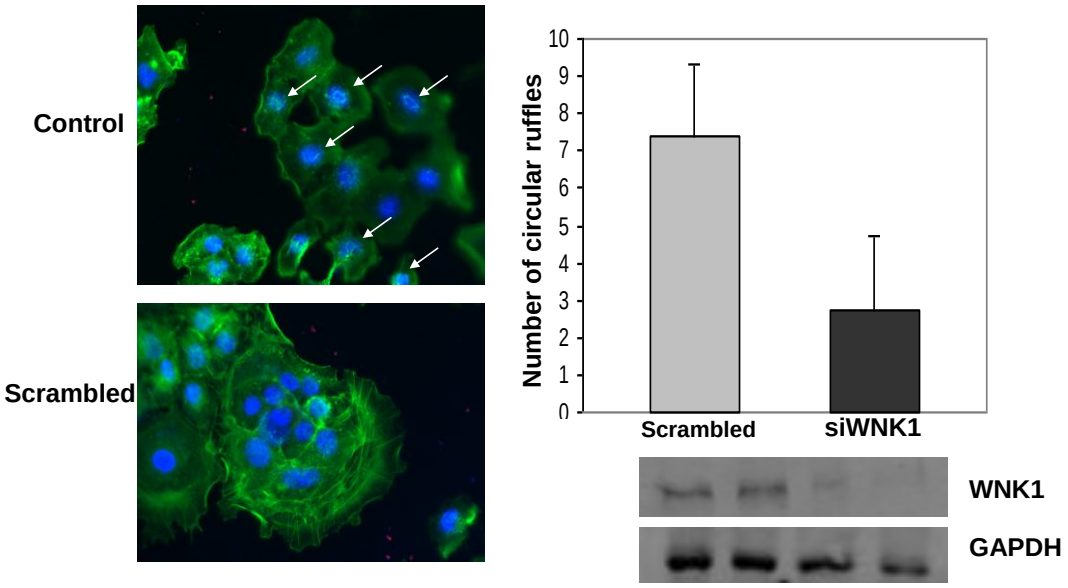

Figure 27. WNK1 depleated cells reduce dorsal ruffle formationin A549 cells. A549 cells were transfected with either scrambled oligo or WNK1 siRNA for 48 hours. Cells were placed on cover slips and immunostained with anti -WNK, phallodin, and DAPI. Bar graphs indicate number of dorsal ruffles observed in 11 independent fields. Cells were visualized by confocal microscopy. Immunoblot demonstrates WNK1 knockdown take from same population of cells of two independent experiments. GAPDH was utilized for loading control. (**White arrows**) Indicate the presence of circular dorsal ruffle.

Finally, to establish that the effect of WNK1 knockdown is not specific for A549 cells, I showed that CDR formation was also suppressed in PMA-treated HeLa cells (Figure 29).

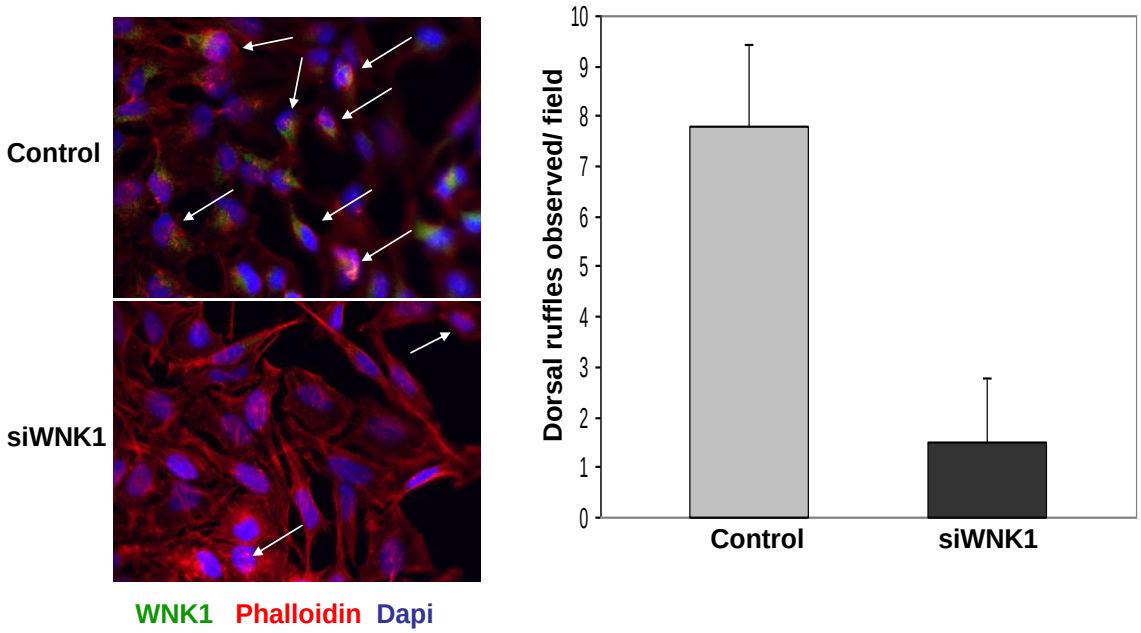

Figure 28. WNK1 depleated cells reduce dorsal ruffle formationin A549 cells. Hela cells were transfected with either scrambled oligo or WNK1 siRNA for 48 hours. Cells were placed on cover slips and immunostained with anti WNK, phallodin (Green), and DAPI. Bar graphs indicate number of dorsal ruffles observed in 4 independent fields. Cells were visualized by confocal microscopy. Immunoblot demonstrates WNK1 knockdown take from same population of cell. GAPDH was utilized for loading control. (**White arrows**) Indicate the presence of circular dorsal ruffle.

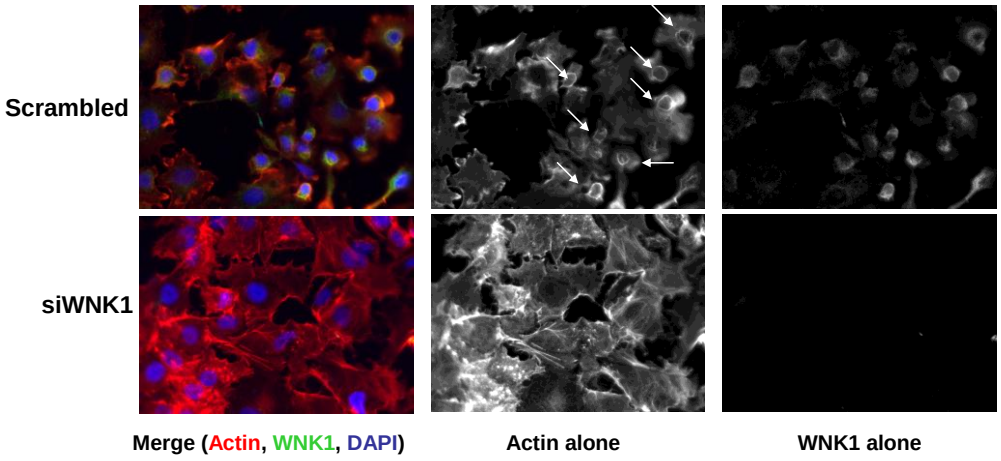

Figure 29. Cells lacking WNK1 fail to form Circular Dorsal ruffles after PMA stimulation. A549 cells were transfected with either scrambled oligo or WNK1 siRNA for 48 hours. Cells were placed on cover slips and immunostained with anti WNK, phallodin, and DAPI. Right column demonstrates merger of three fields; Middle column displays actin alone; Right column demonstrates WNK1 alone. White arrows indicate a dorsal ruffle. Both rows were stimulated with PMA. Un-stimulated cells demonstrated no noticeable dorsal ruffles.

Discussion

In this Chapter we demonstrate that WNK1 deletion increases the proportion of polymerized actin in cells, and induces the formation of prominent stress fibers. In contrast, dorsal ruffle formation is reduced both by WNK1 depletion and over-expression. These results are consistent with reduction in overall cell motility observed upon depletion or over-expression of WNK1 (Chapter 2).

At present, we have insufficient information to ascribe these actin phenotypes on a specific WNK1-dependent regulatory mechanism. For example, our results

could be explained by increased activation of RhoA or reduced activation of Rac1, which are known to have reciprocal effects on actin organization. Rac1 is activated by GDP-GTP exchange catalyzed by guanine nucleotide exchange factors (GEFs), which in turn are activated by recruitment to membranes by phosphatidylinositol (3,4,5) trisphosphate (PIP_3) (Giry et al. 1995; ishizaki et al. 1996). In addition, growth factor-dependent Rac1 activation has been shown to occur, at least in part, on Rab5-positive early endosomes (Zech et al. 2008). Rac1 activation is a potential mechanism whereby WNK1 contributes to membrane ruffling, and this process could be influenced by WNK1 at multiple steps, including: 1.) increasing PIP_3 synthesis by activating PI 3-kinase(s); 2.) promoting early stages of the endocytic pathway; 3. direct binding to Nck, thereby recruiting Pak1, a downstream Rac1 effector that promotes membrane ruffling; and/or 4.) controlling intracellular ionic conditions and osmotic strength via regulation of ion transporters. As shown above, the latter possibility was not supported in preliminary experiments involving OSR1 knockdown or inhibition of NKCC1 by bumetanide, although more work needs to be done to confirm these initial observations

CHAPTER FOUR

WNK1 REGULATION OF THE ACTIN CYTOSKELETON

Introduction

The actin cytoskeleton is controlled by a vast number of proteins which promote actin-polymerization and de-polymerization. Crucial in this process are members of the ADF/cofilin family of proteins. For example, cofilin1 severs actin filaments and increases the amount of monomeric G-actin (Maciver et al. 2002). Cofilin1 activation is suppressed by phosphorylation on serine 3 mediated by LIM kinase (LIMK) (Toshima et al. 2001; Scott et al. 2006). Here we show that depletion of WNK1 increase the proportion of unphosphorylated (i.e., active) cofilin 1, and that both depletion and overexpression of WNK1 impairs the activity of LIMK. We further show that WNK1 depletion enhances the phosphorylation of ERM (ezrin, radixin, moesin) proteins, which serve as linkers between membranes and actin stress fibers.

Dr. Charles Heise (Formerly of the Cobb laboratory, UT Southwestern) reported that WNK1 interacts with Nck1, a known activator of LIMK (Figure 29) (Heise, PhD Dissertation, data). Dr. Lisa Lenertz (also a former member of the Cobb laboratory) found that WNK1 phosphorylates Pak1, which in turn activates LIMK (Lenertz, PhD Dissertation, data). At present, the relevance of these observation to the findings reported here remain to be determined.

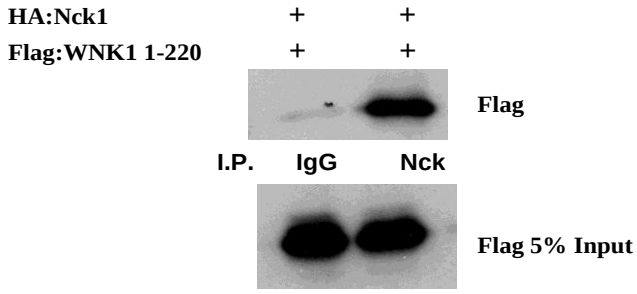

Figure 30. WNK1 interacts with Nck1. Overexpression experiments demonstrate that a Flag-WNK1 (1-220) interacts with HA tagged Nck1 as demonstrated by Charles Heise (2006).

Results

As shown in figure 31, depletion of WNK1 from HeLa cells caused a decrease in the amount of phosphorylated cofilin1 by 4 fold as quantitative immunoblotting using a phospho-specific serine 3 antibody.

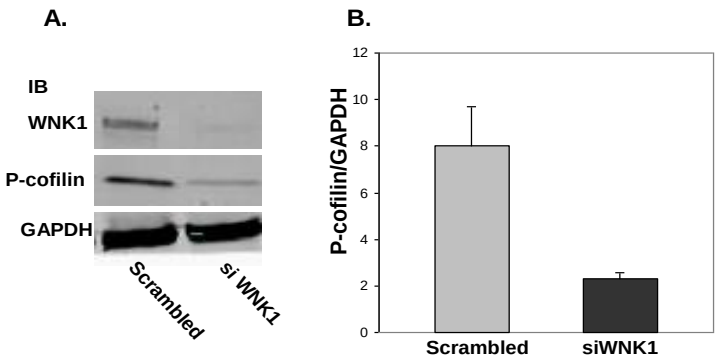

Figure 31. Knockdown of WNK1 promotes dephosphorylation of cofilin. Hela cells transfected with either scrambled or WNK1 siRNA oligonucleotides were grown for 48 hours, lysed in RIPA solution, and the extracts were analyzed by immunoblotting with anti-phosphocofilin antibodies for the presence of P-cofilin. **A**. Immunoblot showing the efficiency of WNK1 knockdown and decrease of P-cofilin. GAPDH – control for loading. **B**. Bar graphs represent the average of three experiments.

Interestingly, WNK1 depleted cells demonstrated a decrease in cofilin after stimulation with various stimulants as shown in figure 32. To better understand the loss of phosphorylation of cofilin1 in cells depleted of WNK1, I analyzed the phosphorylational state of LIMK1.

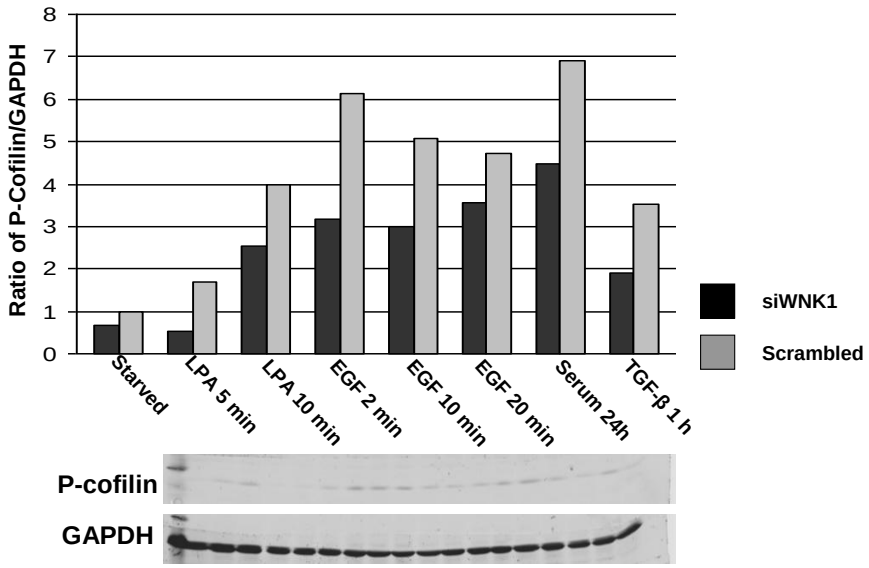

Figure 32.Stimulus-dependent phosphorylation of cofilin is reduced in heLa cells depleted of WNK1. Hela cells transfected with either scrambled or WNK1 siRNA oligonucleotides were grown for 48 hours followed by stimulation with various stimuli, lysed, and the extracts were analyzed by immunoblotting for the presence of P-cofilin with a phospho-cofilin specific antibody to ser-3. **A.** Bar graphs represent the average of two experiments. **B**. Immunoblot showing the efficiency of WNK1 knockdown, p-ERM, and GAPDH – control for loading

As shown in figure 33(A), depletion of WNK1 from HeLa cells decreased the amount of LIMK1 phosphorylation at Threonine 508 which is crucial for LIMK1 activity and the subsequent phosphorylation of cofilin1.

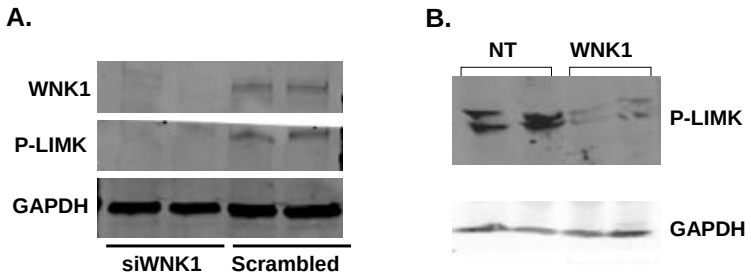

Figure 33. Overexpression and knockdown of WNK1 impairs LIMK activity. A. Hela cells were transfected with either scrambled oligo or WNK1 siRNA and incubated for 48 hours. Cells were then lysed and run on SDS-PAGE gradient from 4-20% and immunoblotted for phospho-specific LIMK, and WNK1. GAPDH was utilized as a loading control in each experiment. **B.** A549 cells were transfected with either control cDNA or WNK1 for 48 hours. Cells were lysed after 48 hours and immunobloted for phosphorylated LIMK.

Over-expression of WNK1 in A549 cells demonstrated a similar effect in that LIMK1 phosphorylation is decreased as shown in figure 33 (B). To further gain insight into how WNK1 may regulate the actin cytoskeleton, I decided to monitor the phosphorylational state of the three members of the ERM family of proteins; Ezrin, Radixin, and Moesin of cells depleted of WNK1.

ERM proteins are a family of proteins which are 82, 80, and 75kDa in size and are critical for mediating the anchoring of the actin-cytoskeleton to the plasma membrane via its C-terminal actin-binding domain (Bretscher et al. 1983; Louvet-Valee et al. 2000). Moreover, ERM protein activation is mediated by phosphorylation at conserved residues threonine 567, 564, and 558, respectively (Pietromonaco et al. 1998). In light of the importance ERM proteins have in

promoting the linkage of the plasma membrane with the cytoskeleton, I sought to determine the activity of ERM proteins in WNK1 depleted HeLa cells. As shown in Figure 34, WNK1 depleted cells exhibited a 3 fold increase in phosphorylated ERM proteins at the threonine residues critical for activation.

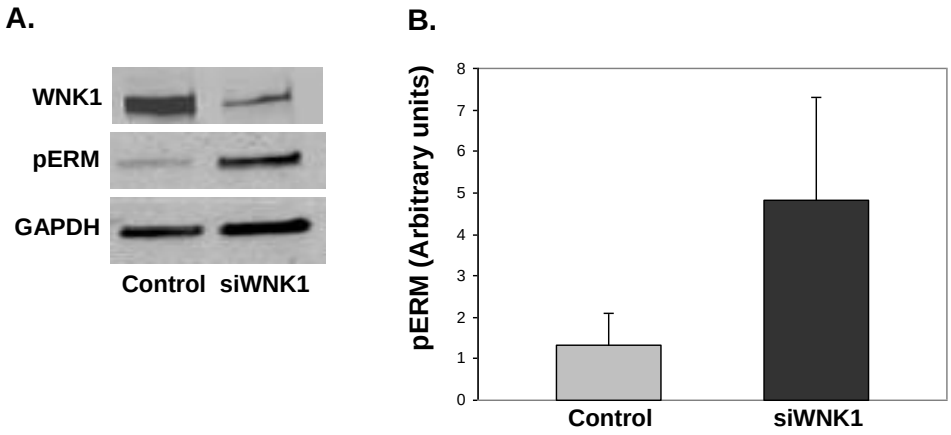

Figure 34. Activated ERM (ezrin, moesin, and radixin) is elevated in the absence of WNK1. Hela cells transfected with either scrambled or WNK1 siRNA oligonucleotides were grown for 48 hours, lysed in RIPA solution, and the exctracts were analyzed by immunoblotting for the presence of P-ERM with a phospho-ERM specific antibody. **A**. Immunoblot showing the efficiency of WNK1 knockdown, p-ERM, and GAPDH – control for loading. **B.** Bar graphs represent the average of three experiments.

It was reported by Pearson et al. (2000) that ERM protein phosphorylation was induced by growth factors, including TGF-β, and EGF. I therefore attempted to determine if WNK1 depletion impaired stimulus-dependent ERM phosphorylation. Figure 35 demonstrates that regardless of the mode of

stimulation, cells depleted of WNK1 demonstrate an increase in ERM phosphorylation.

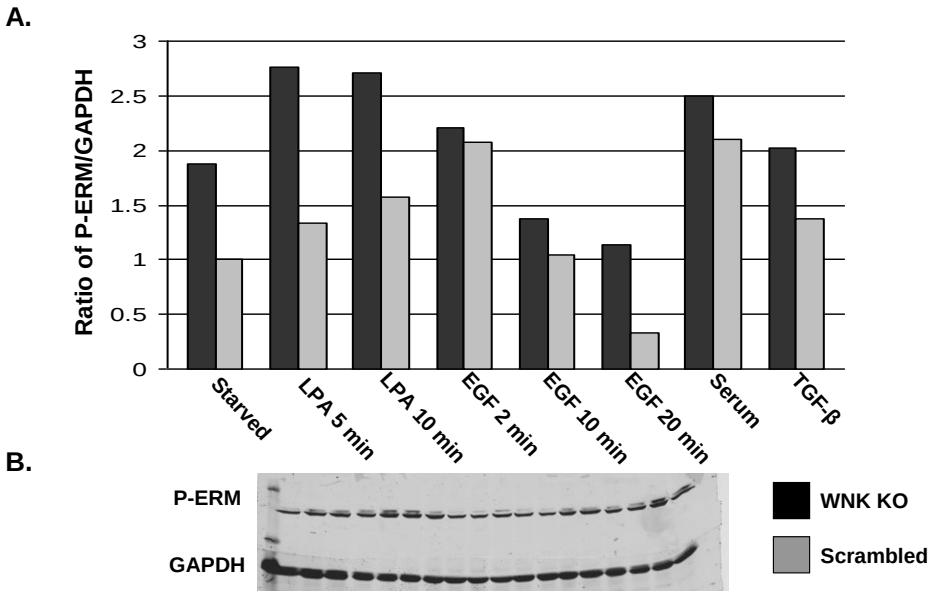

Figure 35. Phosphorylated ERM increases in WNK1 knockout Hela Cells independently of stimulation. Hela cells transfected with either scrambled or WNK1 siRNA oligonucleotides were grown for 48 hours followed by stimulation with various stimuli, lysed, and the exctracts were analyzed by immunoblotting for the presence of P-ERM with a phospho-ERM specific antibody. **A.** Bar graphs represent the average of three experiments. **B**. Immunoblot showing the efficiency of WNK1 knockdown, p-ERM, and GAPDH – control for loading.

A key protein important in cell migration, which has also been reported as a WNK1 substrate is protein kinase B (Akt) (Jiang et al. 2004). Akt is a ubiquitously expressed, serine/threonine protein kinase that belongs to the AGC subfamily of protein kinases (Fayard et al. 2005; Hanada et al. 2004). Activation of Akt is mediated by phosphorylation at two critical residues: threonine 308 and serine 473; phosphorylation of these residues is mediated by phosphophoinositide-dependent kinase 1 (PDK1) and other kinases like the

mTOR complex TORC2 (Fayard et al. 2005; Sarboasov et al. 2005). Akt is crucial for cell migration by phosphorylating Girdin, an actin binding protein important for leading edge stress fiber formation (Enomoto et al. 2008) and cells depleted of Akt1 have impaired cell migration (Jiang et al. 2005). In light of these findings, I wanted to measure Akt1 activity in cells depleted with WNK1. As shown in figure 35, HeLa cells depleted of WNK1 demonstrates a reduction in Akt1 phosphorylation at T308. Interestingly figure 37 shows that cells over-expressing WNK1 display no increase in Akt 1 phosphorylation in response to TGF-β, a known Akt1 activator.

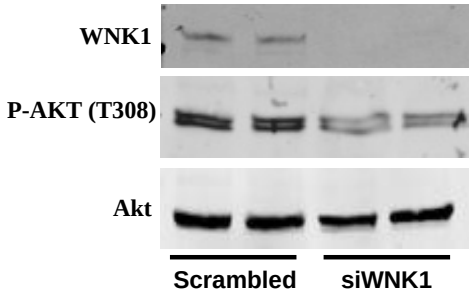

Figure 36. Knockdown of WNK1 impairs Akt activity. A549 cells were transfected with either control cDNA or WNK1 oligo for 48 hours. Cells were lysed after 48 hours and immunbloted for phosphorylated Akt (T308). WNK1 and GAPDH immunoblotting was performed to estimate knockdown and loading control; immunoblot results from duplicate experiments are shown.

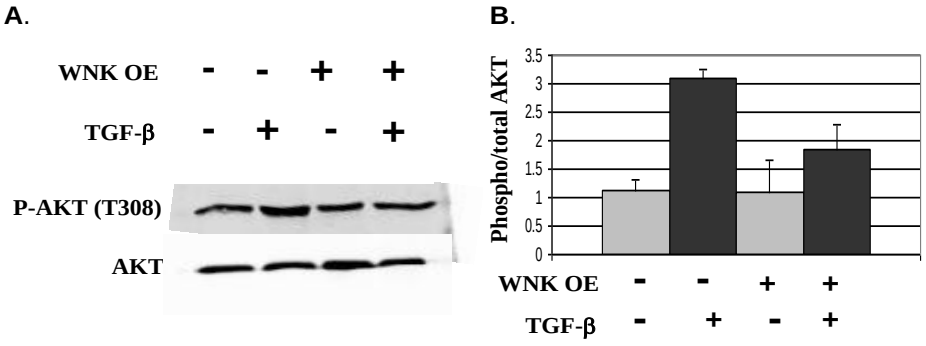

Figure 37. WNK1 overexpession results in suppression of TGF-β dependent AKT activation. cells transfected with either scrambled or WNK1 siRNA oligonucleotides were treated with TGF-β **(**5ng/ml) for 1hr. **A**.**Upper panel** shows western blot of phosphorylated (P-AKT) and total amount (T) of ATK in **lower panel. B**. Bar graphs show the average of 3 experiments.

RhoA and Rac1, two members of the Ras superfamily of small GTPases, are critical for the regulation of the actin cytoskeleton (Parrie et al. 2010). Rac and Rho switch between an "off" GDP bound form and an "on" GTP bound form (Ridley et al. 2006; Bosco et al. 2009; Parrie et al. 2010). Rho GTPases have been demonstrated to regulate cell migration; for example, Rac1 is important for promoting the extension of lamellipodium and the formation of new focal adhesions at the leading edge, whereas RhoA is important for tail detachment (Parrie et al. 2010). Given the importance in cell motility, I sought to monitor Rac1 and RhoA activity in WNK1 depleted cells. In collaboration with Dr. Barbara Barylko (UT Southwester) we performed Rac1 and Rho experiments where we exploited several known Rho GTPase protein binding sites; the PBD-

domain of Pak1 and the protein Rhotekin to isolate active Rac1 and RhoA respectively. Unfortunately we were unable to detect any Rac1 activity in cells depleted of WNK1 as shown in figure 38.

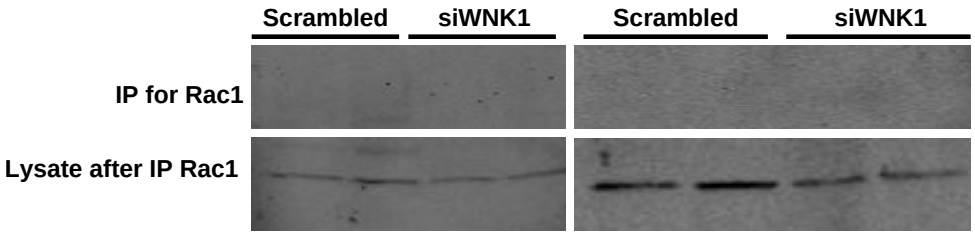

Figure 38.Effect of WNK1 knockdown on Rac1 activation. Hela cells were transfected with either scrambled oligo or WNK1 oligo for 48 hours. Cells were then lysed in GTP lysis buffer and IP was performed using the PBD domain of Pak1. **Right and Left panels** indicate results of two independent experiments. **Top two panals** indicate IP for active Rac1, **bottom two panels** indicate total Rac1.

However, cells over-expressing WNK1 demonstrated a decrease in Rac1 activity even after stimulation with TGF-β as shown in figure 39.

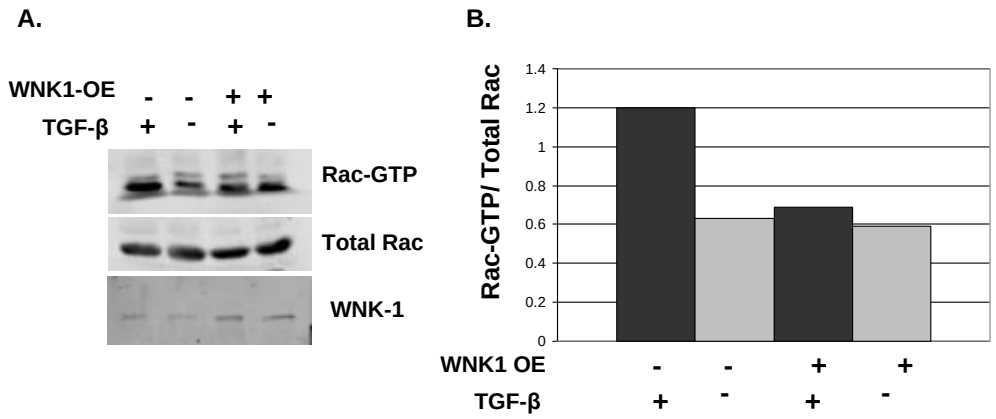

Figure 39. Overexpression of WNK1 impairs Rac1 activity. Figure A. A549 cells were transfected with either control cDNA or WNK1 oligos for 48 hours. Cells were lysed after 48 hours and bloted for Rac1 and WNK1. Top immunoblot is post immunoprecipitation and therefore demonstrates active GTP-bound Rac1. **Panel B** depicts the results of two independent experiments.

Much to my frustration, we were unable to detect any active RhoA in cells depleted of WNK1, as shown in figure 40.

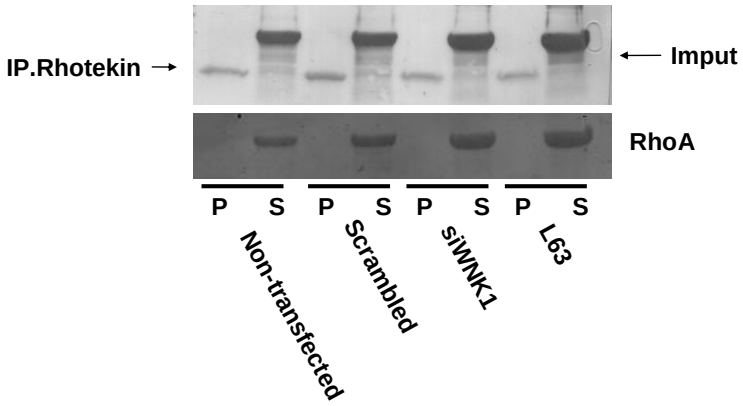

Figure 40. Pulldown of activated RhoA. HeLa cells were transfected with either scrambled or WNK1 oligo or transfected with a constitiutively active Rho (L63) for 48 hours. **Top panel**, indicates imput and purified rhotekin GST-beads (**lower band**). **Lower panel**, indicates anti-RhoA. (**P**) pelleted GST-beads after imunoprecipitation, (**S**) supernatant after pulldown.

Based on these findings I wished to gain a better understanding of how WNK1 may regulate cofilin and LIMK1. Interestingly C. Heise (UT Southwestern Dept. of Pharmacology, unpublished data) demonstrated that WNK1 interacts with the adaptor protein Nck1 *in vitro*. Nck1 has been reported to recruit Pak1 to re-organize the actin cytoskeletal in response to growth-factor stimulation (Buday et al. 2002). Further observations made by L. Lenertz (UT Southwestern Dept. of Pharmacology) indicated that WNK1 phosphorylates Pak1, although, does not interact directly. Therefore it is possible that Nck1 interacts with WNK1 and recruits Pak1 for phosphorylation. Although the effect of Pak1 phosphorylation by WNK1 is poorly understood, it is important to verify the

interaction between WNK1 and Nck1 with endogenous proteins. I therefore collaborated with Dr. Barbara Barylko to identify a potential Nck1/WNK1 complex. Unfortunately, we were unable to identify any interaction between endogenous Nck1 and WNK1 (Figure 41).

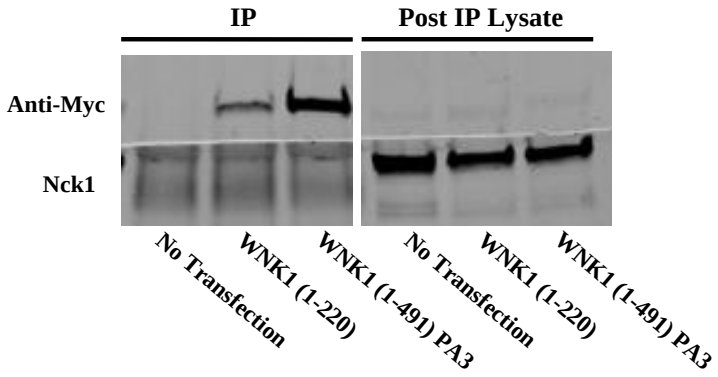

Figure 41. Immunoprecipitation of WNK1 and Nck1. Hela cells were grown to confluency and transfected with either WNK1 wild-type or WNK1 (1-220) PA3 mutant for 48 hours. Cells were then lysed and immunoprecipitation was carried out by utilizing anti-myc antibody and immunoblot was performed against anti-myc and anti-nck1.

Discussion

In this chapter, we demonstrate that depletion of WNK1 increases cofilin1 activity as measured by a decrease in phosphorylation of cofilin on serine 3. WNK1 depletion also elicits a decrease in the activity of the cofilin1 kinase, LIMK1. Moreover, LIMK1 phosphorylation was reduced in cells that both under-

and over-expressed WNK1. Although an increase in cofilin1 activity may seem conter-intuitive, in that actin filaments were stabilized by WNK1 depletion (see previous chapter), a similar finding was reported by another group. Oleinik et al. (2010) reported an increase in cofilin1 activity, together with an increase in F-actin, which resulted in impaired cell migration. This may be due to our current understanding of how cell migration occurs. Cell migration is described as a cyclic process in which polymerization and de-polymerization of actin occurs simultaneously parts of the cell. Furthermore, established data indicates that cofilin1 is crucial for regulating both, actin polymerization and de-polymerization in a cyclic cycle manner (Oleinik et al. 2010). Moreover, cofilin1 is believed to function by translocating between the plasma membrane, the cytosol and actin-filaments in response to its phosphorylated/un-phosphorylated state suggesting that any disruption which shifts cofilin1 toward a phosphorylated (i.e. inactive) or a de-phosphorylated (i.e. active) state in excess will disrupt actin-cytoskeletal dynamics and downstream processes, including cell migration. However, the decrease in cofilin1 de-phosphorylation in cells depleted with WNK1 may be due to the inhibition of its activator, LIMK1, which was impaired as observed in cells both under-and over-expressing WNK1. Although we currently have insufficient evidence as to how WNK1 inhibits LIMK1 activity, the data reported here demonstrate that WNK1 regulates the actin cytoskeleton by regulating known upstream effectors like LIMK1 and cofilin1.

WNK1 knockdown and over-expression resulted in the inhibition of Akt phosphorylation at T308. Interestingly, Akt phosphorylates WNK1 at threonine 60, which is important in regulating blood pressure (Vitari et al. 2004). Moreover, a yeast-two hybrid screen demonstrated that WNK1 may interact with the p85 subunit of phosphatidyliinositol-3-kinase, which produces the second messenger PIP_3 and activates PDK1 and hence Akt. Therefore it is possible that WNK1 may regulate Akt by a feed-forward mechanism in which WNK1 enhances Akt activity by regulating upstream effectors like the p85 subunit of PI3K. Currently, work is being performed to establish a potential WNK1 and p85 interaction.

Understanding the effects of WNK1 depletion on the Rho GTPases will provide crucial insight into how WNK1 regulates cell migration. Although initial observations suggest that WNK1 over-expression caused a reduction of active Rac1, further work is needed to monitor the active state of Rac1 and RhoA in cells depleted with WNK1.

Materials and Methods

Immunoblotting-as previously described (see chapter 2).

Antibodies and reagents- as previously described (See chapter 2)

Transfections- as previously described (see chapter 2)

Rac/Rho-GTP Pulldown Assay- Rac and Rho activity was measured using a glutathione S-transferase (GST) fusion protein containing the PBD domain of human p21 activated kinase 1 (PAK) protein containing the CRIB domain which was purchased from Cytoskeleton Inc. Rho activity was determined utilizing the rhotekin kit also purchased from Cytoskeleton and utilized according to the manufacturer recommendations. Harvested cells were first washed with ice-cold PBS and lysed with ice-cold lysis buffer (1M HEPES, 5M NaCl, 4.9M, MgCl2, .5M EDTA, 50% Glycerol, phosphatase inhibitor cocktail 2 and 3 (SIGMA), 50 µl PMSF, and 10µl protease inhibitors). Cleared lysate was then incubated with 20 µl of GST fusion protein and incubated at 4^{o}C overnight on a rocker. The following day, lysate-GST protein mix was incubated with GST-beads after washed with lysis buffer. GST beads were incubated with the lysate for 4 hours at 4^{o}C. Beads were then centrifuged and treated with 5X SDS sample buffer and run on SDS –PAGE and immunoblotted for Rac1 at 1:1000 (Cell Signaling).

Co-immunoprecipitation assay- HeLa cells were grown to confluency on 10cm plates and transfected with either WNK1 (1-491) or WNK1 (1-491) PA3 as previously mentioned, for 48 hours. A separate plate was prepared without any

transfection for control. Cells were washed in PBS and lysed in ice-cold 400 µl of lysis buffer (50mM HEPES, ph7.5, 50mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.1% Triton X100, and protease and phosphatese inhibitor cocktail). Lysate was centrifuged and lysate was separated to three separate tubes. 800 µl of lysate was added to each tube for IP and was incubated with 16 µl of anti-myc antibodies and incubated overnight. The following day, lysate was incubated with pre-washed G beads for 4 hours followed by centrifugation and separation of lysate from beads. Beads were then washed three times in lysis buffer. Both, lysate and beads were prepared in 5X loading buffer and ran on a Bio-RAD gradient gel 4-20%. Protein was transferred to nitrocellulose and immunoblotted for anti-myc and anti-nck1.

Chapter five

SYNOPSIS

Introduction

The evidence obtained in this study demonstrates that WNK1 has a role in the regulation of cell migration. Three independent groups, including our own, have found that cell migration is inhibited in cells depleted of WNK1. My goal was to extend the work of the other two groups by providing information regarding mechanisms whereby WNK1 influences cytoskeletal dynamics. The majority of studies performed to date on WNK1 have focused on its role in regulating ion transport, and this process clearly contributes to cell motility, as recently reviewed by Schwab et al. (2012). However, my working assumption as been that WNK1 also affects the cytoskeleton by interacting with, and in some cases phosphorylating multiple elements of the actin polymerization machinery. As stated below, future efforts should be directed at identifying these elements and defining the effects of WNK1 on their cellular activities.

Materials and methods

Overlay Experiment- Cells were washed with cold 1X phosphate buffered saline (PBS) and lysed with RIPA buffer (150mM NaCl, 1% NP40, 50mM TRIS ph 8.0, 0.5% DOC, and 0.05% SDS. Lysate was then centrifuged for 15 min at max speed on a table centrifuge at 4^{o}C. Cleared lysate was then treated with 5X SDS sample buffer (1:4 stacking buffer (0.15M TRIS pH 8.0), 4% SDS, 20% Glycerol, 10% Betamercaptoethanol (BME), and 0.02g bromophenal blue) and boiled for 2 minutes. Gradient SDS-PAGE gels (5 to 20%) were loaded with 40 µl of lysate and transferred onto a 2 µm nitrocellulose membrane (BIO-RAD). Membrane was then blocked with 5% Milk for one hour. GST-WNK1 (1-220) or GST proteins were then incubated with membrane overnight. The next day, membrane was washed and anti-GST antibody was incubated overnight at 4^{o}C. The following day, anti-GST antibody was washed out and secondary antibody was incubated for one hour and image was processed by LiCOR.

Protein purification- E.coli BL21 cells were transformed with GST-WNK1 (1-220) plasmid and plated on a 100µg/ml amp-LB plate overnight. The following day, colonies were plated onto a 50ml LB media with 100 µg/ml ampicillin and grown at 37^{o}C on a shaker, overnight. The next day, about 5 mls was added to 1 liter 100 µg/ml ampicillin media and grown for several hours at 28oC. OD600 readings were performed at varies time points to measure concentration of bacteria until OD was at .600. Bacteria was then incoculated with 0.1 IPTG to

0.1mM to induce protein expression and incubated for 3 hours. Cells were then place in two 500 ml spin tubes and centrifuged at 2500 rpm for 10 min at 4^{o}C. Pellet was resuspended in 10ml GST binding buffer (25mM Tris pH 7.5, 150mM NaCl, 1 mM EDTA (Protease inhibitors) on ice. Cells were then treated with 1% triton X100 to a final concentration of .5% and cells were lysed for 5 min on ice. Cells were then sonicated at 15% for 10 seconds to shear DNA, repeated three more times. Lysate was then centrifuged for 10 minutes at 10,000 rpm at 4^{o}C by ultra-centrifugation. Lysate was transferred to 50 ml tubes. Tubes were then incubated with 500 µl of pre-washed Glutathione beads (prewashed with GST buffer and resuspended in 50% slurry). The lysate/beads were then incubated at 4^{o}C for 4 hours on a rocker. Beads were then spun down for 1 minute at 1,000 rpm and beads were resuspended with 20ml GST binding buffer; and spun down, soup was removed and repeated twice. Beads were then incubaded with 500 µl of elution buffer (10mM reduced glutathione/50mM Tris-HCL pH 7.5 and 5% glycerol) for 15 minutes. Beads were spun down and sup was removed to a separate tube labeled 1. For every tube of lysate, 10 µl was utilized to measure protein concentration. And tubes were collected as need be. Lysate was dialyzed overnight at 4^{o}C in dialysis buffer (5mM Hepes pH 7.6, 1mM DTT, .2 mM PMSF, 1mM EDTA, 10% glycerol and protease inhibitors). Protein fractions were then frozen at -20^{o}C. Protein fractions were then quantified by SDS-PAGE for purity.

Results

Interference of cell migration both by WNK1 depletion and over-expression.

My interest in this project arose from the lack of mechanistic information regarding how cells depleted in WNK1 interfere with cell migration. Although this observation had been reported twice before by independent groups, my initial experiments were aimed at confirming their results, and extending them to different cell types. But the questions remained as to "how" the absence of WNK1 disrupts cell migration and whether, as appeared logical at the time, over-expression of WNK1 could enhance cell migration. To my surprise, depletion and over-expression of WNK1 yielded virtually identical cytoskeletal phenotypes, and these were not due to effects of these interventions on cell viability. A potential explanation for this outcome is that WNK1 acts as a scaffold, facilitating interactions among cytoskeletal components and their upstream or downstream regulators. As well-established from analyses of cell signaling pathways (e.g., Bray and Lay (1997); Ferrell (2000); Levchenko et al. (2000)),an important feature of scaffolding proteins is their biphasic concentration dependence. Above an optimal concentration, non-functional complexes containing only one interacting (i.e., "scaffolded") partner become prevalent. The kinase domain represents only 10% of the total WNK1 sequence, suggesting that at least a subset of its functions are mediated by kinase-independent protein interactions. Indeed, Tu et al. (2010) have shown that mitotic spindle defects caused by WNK1

depletion can be rescued by expression of catalytically inactive WNK1 fragments (Tu et al. 2010). In this context it should be noted that WNK1 contains 24 proline-rich motifs, which commonly serve as interaction sites for several binding modules, including SH3 and WW domains (Kay et al. 2000). Previous studies have established the importance of protein interactions with three PXXP motifs within the N-terminal 220 residues of WNK1 in the regulation of the ROMK1 channel (He et al. 2007). Experiments described above demonstrate that the ame region of WNK1 is also likely to play a role in regulation of cell motility.

Regulates of the actin cytoskeleton by WNK1.

To better understand how WNK1 regulates cell migration, I first sought to define the effects of manipulating WNK1 expression on actin polymerization and on the formation of actin-based subcellular structures, specifically stress fibers and dorsal ruffles. In HeLa cells, the proportion of actin in the polymerized state (i.e., F-actin) increased by approximately 1.5-fold upon siRNA-mediated depletion of WNK1. This treatment also induced the formation of more pronounced actin stress fibers, a phenotype characteristic of elevated RhoA activity, or of an imbalance between RhoA and Rac1 activities (Sander et al. 1999), both of which can lead to impaired cell movement. WNK1 depletion inhibited the formation of circular dorsal ruffles, which were recently shown to play a critical role in growth factor-stimulated integrin trafficking during cell

migration (Gu et al. 2011). Dorsal ruffle formation is a Rac1 dependent process, further supporting the possibility that WNK1 participates in activation of Rac1, or suppression of its antagonist, RhoA, or both.

Having found that WNK1 knockdown (and over-expression) disrupts actin-rich structures, I attempted to identify upstream regulators responsible for the observed phenotypes. Novel findings presented here indicate that WNK1 regulates actin re-organization through regulation of the actin-severing protein cofilin1. Cofilin phosphorylation was greatly reduced in the absence of WNK1, suggesting that WNK1 may serve to maintain cofilin in its phosphorylated, inactive state. Indeed, WNK1 depletion and over-expression resulted in a decrease in the phosphorylation state of the known cofilin activator, LIMK1. Current models suggest that cell motility requires a balance between cofilin activation and inactivation Oleinik et al. 2011), and that therefore the phosphorylation state of cofilin is subject to precise spatio-temporal regulation. Further work will be needed to understand how WNK1 contributes to this regulation.

Further clues toward understanding the mechanisms whereby WNK1 regulates cell migration will be provided by the identification of binding partners which have already been implicated in cytoskeletal assembly. Although time constraints prevented me from pursuing this line of investigation, previous studies have yielded a list of interesting candidate interactors, including Nck1 (C. Heise,

unpublished results) and Pak1 (L. Lenertz, unpublished results). Nck1 binds directly to Pak1 and recruits it to activated receptors (Buday et al. 2002). Pak1, a downstream effector of Rac1, phosphorylates and activates LIMK. Based on these interactions, I propose the following model depicting a potential pathway linking WNK1 to the regulation of the actin cytoskeleton (Figure 42).

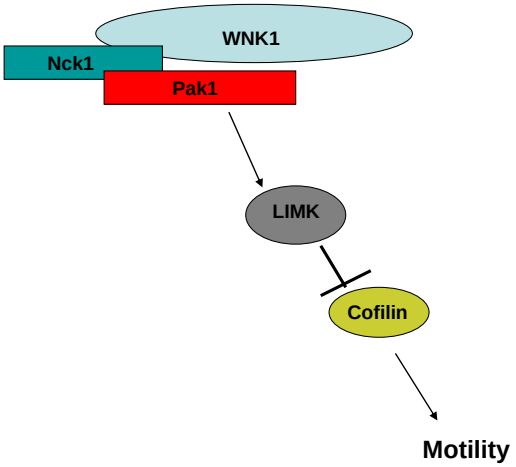

Figure 42. Proposed mechanism by which WNK1 may regulate cell mgiration. It is possible that WNK1 will interact with Nck1 to recruit Pak1 and thus promoting its phosphorylation in turn, activates LIMK, which phosphorylates cofilin1 and thus regulates cell migration.

Future Directions

Future experiments should be directed at cataloguing WNK1 interactors that regulate the cytoskeleton, identifying mutations that inhibit the interactions but are otherwise minimally disruptive, and testing the effects of these mutations

on cell motility and cytoskeletal assembly. As stated, time constraints prevented me from carrying out a thorough search for relevant WNK1 binding partners, although gel overlay experiments with GST-WNK1 (1-220) reproducibly revealed an electrophoretic band corresponding to a protein of approximately 90kDa (Figure 43). Efforts are underway to identify his protein by mass spectrometry.

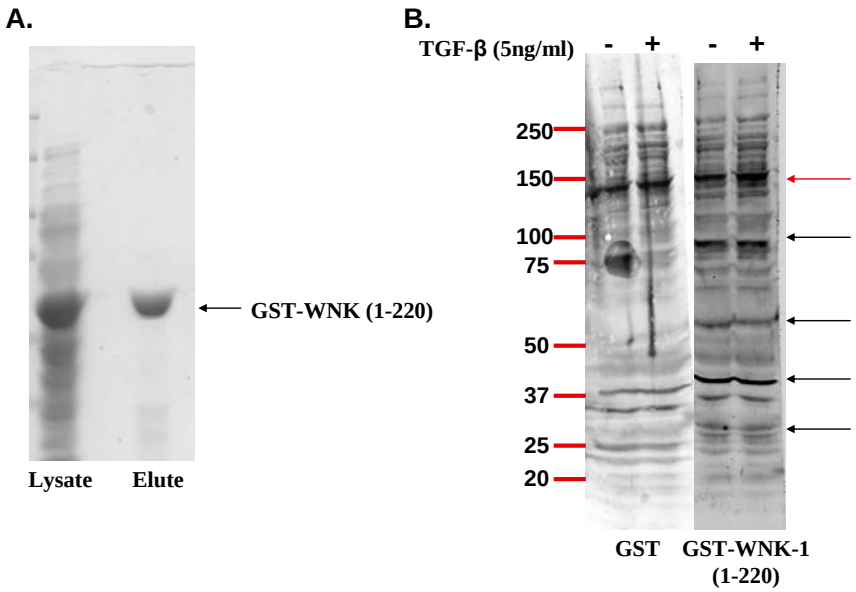

Figure 43. Identification of new potential interacting partners with WNK1. Hela cells were grown to confluence. Confluent cells were treated with or without TGF-β for 1 hour then lysed and run on a Gradient gel between 5 to 15%, transferred to nitrocellulose, and then incubated with either GST alone or purified **(A)** GST-WNK1 (1-220). Panel **(B) (Black Arrows)** demonstrates potential interaction with proteins at around 150, 90, 60, 40, and 30 as indicated by the arrows. **Red arrow** demonstrates a shift in band at 150 after TGF- β.

More work will also be required to reliably monitor the activities of Rho family GTPases in cells depleted of WNK1 and to understand how WNK1-dependent phosphorylation of Pak1 (observed by L. Lenertz) influences these

activities. Finally, it will be important to determine how the regulation of ion flux by WNK1 contributes to its role in regulating cell motility.

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