INTERACTION MAPPING OF THE ATYPICAL PROTEIN KINASE

WITH NO LYSINE 3 (WNK3)

APPROVED BY SUPERVISORY COMMITTEE

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DEDICATION

I dedicate my work to Nephew Zane.

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Thanks first and foremost to Melanie Cobb for honoring me with an invitation to join her lab, for allowing me the good fortune of working with Tony Anselmo during my rotation and for being a supportive mentor. Thanks to my committee members for their time and input. Thanks to my labmates and Dionne Ware for the good atmosphere I've enjoyed the past few years. Thanks especially to Arif Jivan for being a good friend and a real pleasure to share a laboratory bench with, to Colleen Vanderbilt for making our little laboratory corner a happy one, and to Jihan Osborne and Samarpita Sengupta for the working with me during their rotations. Thanks also to Deborah Evalds and Nancy McKinney for their open doors, listening ears and senses of humor. Thanks to Glenn Russell for 11th hour help with illustrations. Lastly, thanks to my parents and siblings for their love and support.

INTERACTION MAPPING OF THE ATYPICAL PROTEIN KINASE WITH NO LYSINE 3 (WNK3)

by

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INTERACTION MAPPING OF THE ATYPICAL PROTEIN KINASE WITH NO LYSINE 3 (WNK3)

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The story of the protein kinase "with no lysine 3" (WNK3) represents a unique chapter in the larger story of protein kinases, the so-called 'molecular switches' of the cell that serve the vital function of phosphorylating myriad proteins. In doing so, these enzymes furnish the cell with one of the primary means by which signals from the external environment are transduced into cellular consequences. At the time our laboratory reported discovery of the first WNK, it was thought that all protein kinases contained an invariant catalytic lysine necessary for phosphoryl

transfer in ß strand 3 (protein kinase subdomain II) of the highly conserved catalytic domain. Analysis of WNK1 uncovered a cysteine in the place of the so-called canonical catalytic lysine--hence the name WNK for "with no lysine". Subsequently, other WNKs came to light, and together with WNK1, they comprise an atypical branch of the kinome--the functions and significance of which are still being elucidated.

Of clinical significance, WNKs 1 and 4 have been implicated in a heritable form of hypertension (pseudohypoaldosteronism type II). WNK3 has been reported to regulate certain members of the SLC12A family of cation/Cl^- cotransporters (KCC1/2; NKCC1; NCC), and also to localize to various Cl^- transporting epithelia and certain brain neurons with GABA-A ionotropic receptors.

My goal with these interaction mapping efforts has been to build a collection of putative WNK3 interactors to serve as a source of information and project leads for the ongoing research program of the Cobb laboratory. The yeast two-hybrid screens described here have yielded hundreds of putative interactors. While this written work deals only with a small number of the most interesting putative interactors, together they point toward a number of unexpected roles for WNK3, including putative interactions with RNA-binding proteins, transcriptional

regulators and proteins implicated in developmental disorders and neurodegenerative disease.

The story of the WNK kinases will go on. With a connection to ion flux diseases well-established, the WNK family will surely continue to attract attention for many years, particularly given their potential as drug targets.

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List of Abbreviations

Akt	protein kinase B
3-AT	3-amino-1,2,4-triazole
A2bp1	ataxin-2 binding protein
Aes	amino terminal enhancer of split
ApoD	apolipoprotein
Atn1	atrophin-1
BLAST	Basic Local Alignment Search Tool
Brunol4	bruno-like 4, RNA binding protein
CALM	clathrin assembly lymphoid myeloid leukemia protein, aka PICALM
CAP	c-Cbl-associated protein
c-Cbl	Cas-Br-M (murine) ecotropic retroviral transforming sequence c
CCD	cortical collecting duct
Celf4	CUG-BP and ETR-3 like factor 4, aka brunol4
CFTR	cystic fibrosis transmembrane conductance regulator
CNT	connecting tubule
DCT	distal convoluted tubule
Drpla	dentatorubral-pallidoluysian atrophy
Eef1a1	eukaryotic translation elongation factor 1 alpha 1
ENaC	epithelial sodium channel
ERK2	extracellular signal-regulated kinase 2
ERK5	extracellular signal-regulated kinase 5
Flot1	flotillin-1
GABA	γ-aminobutyric acid
GLUT4	insulin-responsive glucose transporter type 4
Grg	Gp130-associated protein
IGF-1	insulin-like growth factor 1
KCC1/2	potassium-chloride cotransporter 1/2
Klf10	Krueppel-like factor 10
MAP2K	mitogen-activated protein kinase kinase
MAP4K	mitogen-activated protein kinase kinase kinase kinase
MAPK	mitogen-activated protein kinase, aka Erk
MBP	myelin basic protein
Munc18c	mammalian uncoordinated 18 homolog 3
NCBI	National Center for Biotechnology Information
NCC	sodium-chloride cotransporter

NGF	nerve growth factor
NKCC	sodium-potassium-two-chloride cotransporter
OSR1	oxidative stress response 1
PHAII	pseudohypoaldosteronism II, aka Gordon's syndrome
PKA	protein kinase A
PKB	protein kinase B, aka Akt
Rab14	Rab14, member RAS oncogene family
ROMK	renal outer medullary potassium channel, aka Kir 1.1
RRM	RNA recognition motif
SGK1	serum- and glucocorticoid-regulated protein kinase 1
siRNA	small interfering RNA, aka short interfering RNA or silencing RNA
SLC12A	solute carrier family 12A
Smad	Sma and Mad related protein
Smap2	stromal membrane-associated protein 2
SPAK	ste20-related proline-alanine-rich protein kinase
Syt2	synaptotagmin 2
TGFß	transforming growth factor beta
TRPV	transient receptor potential cation channel, subfamily V
WNK	with no lysine protein kinase

Chapter One: Background

> At heart, science is about telling stories - stories that explain what the world is like, and how the world came to be what it is. –Doyne Farmer

WNKs: An Atypical Family

The story of the protein kinase "with no lysine 3" (WNK3) represents a unique chapter in the larger story of protein kinases, the so-called 'molecular switches' of the cell that serve the vital function of phosphorylating myriad proteins. In doing so, these enzymes furnish the cell with one of the primary means by which signals from the external environment are transduced into cellular consequences. Although phosphoproteins had long been known to exist, it was Edwin Krebs and Edmond Fischer who first elucidated a role for reversible protein phosphorylation in modulation of protein activity in the 1950s; Krebs later made the prescient suggestion that the number of these phosphotransferases would reach into the hundreds, as described in his 1992 Nobel lecture (Krebs, 1993).

At the time, it was thought that all protein kinases contained an invariant catalytic lysine necessary for phosphoryl transfer in ß strand 3 (protein kinase subdomain II) of the highly conserved catalytic domain, as noted by Hanks *et al.* (Hanks *et al.*, 1988). Xu *et al.*, however, challenged this conventional thinking in 2000 with

the discovery of WNK1, analysis of which uncovered a cysteine in the place of the so-called canonical catalytic lysine--hence the name WNK for "with no lysine". Subsequently, other WNKs came to light, and together with WNK1, they comprise an atypical branch of the kinome --the functions and significance of which are still being elucidated (Figure 1, p. 33).

Discovery and early characterization of WNK1

Members of our laboratory uncovered the first WNK protein kinase while conducting a PCR screen in search of new members of the mitogen-activated protein kinase kinase (MAP2K) family. The results of this screen included a protein kinase with 2126 amino acids, which exhibited an unusual N-terminal catalytic domain, 24 PxxP motifs and two putative coiled-coil domains.

Quite distinct from other protein kinases, the catalytic lysine in WNK1 lies at K223 (rat sequence) in ß strand 2 (subdomain I) of the highly conserved protein kinase and replaces a conserved glycine in the phosphate anchor ribbon (Xu *et al.*, 2000). Subsequently identified (human) WNKs share a highly conserved N-terminal protein kinase domain, large proline-rich stretches, putative coiled-coil domains, and--distal to the protein kinase domain--a highly conserved autoinhibitory domain and a short acidic span (Veríssimo and Jordan, 2001; Holden *et al.*, 2004) (Figure 2). Notably, the autoinhibitory domain of WNK1

(residues 485-555), when expressed in isolation, can inhibit the protein kinase activity of the isolated protein kinase domain of the protein--an effect largely obviated by mutation of two phenylalanine residues in the domain (Xu *et al.*, 2002).

In accord with the findings of Xu *et al.*, the crystal structure of WNK1 clearly places catalytic K233 in ß strand 2 (Min *et al.*, 2004). This unusual lysine placement notwithstanding, WNK1 possesses protein kinase activity, which, interestingly, is not altered by a C250A mutation. However, a C250K mutation loses catalytic activity, perhaps due to conformational effects; K233A and K233G mutations are also inactive. (Xu *et al.*, 2000). Attesting to the robustness of the WNK lysine placement, an extracellular signal-regulated protein kinase 2 (ERK2) G35K/K52A mutation that mirrors WNK1 exhibits protein kinase activity (Xu *et al.*, 2000; Xu *et al.*, 2002).

All in the family

WNK1 and subsequently identified members of the WNK family have been shown to appear in a variety of multicellular eukaryotes, including vertebrates. The metazoans thus far shown to carry WNK genes exhibit differing numbers of homologs, and span the gamut from *Arabidopsis thaliana* (9), to *Caenorhabditis*

elegans (1), *Drosophila melanogaster* (1), *Oryza sativa* (rice, 9), and the filamentous fungus *Phycomyces* (1) (Xu *et al.*, 2000; Veríssimo and Jordan, 2001; Wilson, 2001) (Uniprot). Humans carry four WNK paralogs: WNK1 (chromosome 12), WNK2 (chromosome 9), WNK3 (X chromosome) and WNK4 (chromosome 17) (Holden *et al.*, 2004) (Uniprot).

WNK1. The longest splice form of human WNK1 has 2382 amino acids (~250 kD) and exhibits the broadest range of expression among the WNKs (Swissprot) (Figure 2). Early reports identified WNK1 transcripts in spleen, lung, liver, kidneys and testes, in addition to reporting protein in brain lysate, and lysates from 293, COS-1 and Ins-1 cells lines; subsequently, WNK1 transcripts were also reported to express in polarized epithelia in colon, gallbladder, kidney, sweat glands, epididymis, esophagus, skin, and bile ducts of the pancreas and liver (Xu *et al.*, 2000; Choate *et al.*, 2003). In polarized epithelia WNK1 localizes to intercellular junctions lining the lateral membrane, while in other epithelia WNK1 is found in the cytoplasm (Choate *et al.*, 2003). The full-length form of WNK1 appears throughout the body, particularly in the heart, testis, skeletal muscle and kidney (Wilson, 2001). A short kidney-specific WNK1 variant, KS-WNK1, carries a unique N-terminal exon in place of much of the protein kinase domain (Delaloy *et al.*, 2003).

WNK2. WNK2 is 2217 amino acids long (~243 kD) (Figure 2). WNK2 mRNA expression has been detected in human fetal heart and brain, and adult liver and colon (Veríssimo and Jordan, 2001).

WNK3. WNK3 has two isoforms that have been studied, one that is 1800 amino acids (~200 kD) and another that is 1743 amino acids (~192 kD) (Swissprot) (Figure 2 and Figure 3); database searches reveal another isoform, predicted to span 1790 residues (~197 kD), which remains unstudied (XP_914679.2) (UniParc). The yeast two-hybrid screens discussed below made use of baits from the 1743 amino acid splice form of WNK3.

Interestingly, and in contrast to other vertebrate WNKs, *in situ* hybridization has shown that WNK3 mRNA is mostly highly expressed in the brain, although protein, as noted below, is expressed both inside and outside the brain (Veríssimo and Jordan, 2001). Loci of relatively greater brain expression include 1) the dentate gyrus of the hippocampus; 2) the thalamic and hypothalamic nuclei; 3) the reticular formation; 4) the suprachiasmic and supraoptic nuclei; 5) the dorsal raphe; 6) the Purkinje layer of the cerebellum; 7) the locus ceruleus; and 8) the entorhinal cortex. Also, WNK3 transcript expresses highly in several cortical layers and parenchymal areas, while immunofluorescence has shown WNK3

protein in neurons bearing γ-aminobutyric acid A (GABA-A) ionotropic receptors (Figure 4) (Kahle et al., 2005a).

Like WNKs 1 and 4, WNK3 protein has been located at intercellular junctions in diverse epithelia, as shown by immunofluorescence. WNK3 appears in the chloride-secreting parietal cells of the stomach, pancreatic/bile ducts, and crypts of Lieberkühn and epithelium of the small intestine (Kahle et al., 2005a). *In situ* hybridization clearly shows a temporal element in WNK3 expression early in development, because WNK3 in the cerebellum and hippocampus goes from low expression postnatal d10 to high expression d21. Notably, the temporal expression of WNK3 closely tracks that of neuron-specific potassium-chloride cotransporter (KCC) 2, a member of the solute carrier family 12A (SLC12A) (Kahle et al., 2005a).

WNK4. WNK4 is 1243 amino acids long (~135 kD) (Swissprot) (Figure 2). WNK4 expression extends to chloride-transporting epithelia throughout the body, e.g., pancreatic ducts, colonic crypts, bile ducts, sweat glands and epididymis; in these tissues, one sees WNK4 gathered at intercellular junctions (Kahle *et al.*, 2004a). *In situ* hybridization revealed that WNK4 transcripts were found at high levels in the kidney, while immunofluorescence has shown the protein kinase on the lateral membrane and at tight junctions in the distal convoluted tubule (DCT),

the connecting tubule (CNT) and the cortical collecting duct (CCD)--all segments of the aldosterone-sensitive distal nephron. WNK4 permeates the cytoplasm in the CNT and CCD as well (Kahle *et al.*, 2004a).

The presence on the WNKs of multiple interaction motifs suggests they participate in multi-protein complexes (Xu *et al.*, 2005b). These human paralogs and their numerous orthologs are distantly related to the Ste20 branch of the kinome, which contains protein kinases that affect a variety of cellular functions, including cell polarity, cell cycle, ion balance and regulation of mitogen-activated protein kinase (MAPK) cascades (Raman and Cobb, 2003; Lenertz *et al.*, 2005).

WNKs linked to blood pressure

Indeed, early in the story of the WNK family, their role in controlling ion balance garnered a great deal of attention when WNKs 1 and 4 were linked to the pathogenesis of a familial form of hypertension known as pseudohypoaldosteronism II (PHAII), aka Gordon's (hyperkalemia-hypertension) syndrome, or familial hypertensive hyperkalemia (Fhht) (OMIM #145260) (Wilson *et al.* 2001). As such, much of the subsequent research discussed herein relates to the roles of WNKs 1 and 4 in modulation of ion balance vis-à-vis hypertension research.

Dysfunction of the mechanisms orchestrating vasoconstriction, control of blood volume and heart rate can lead to hypertension, a pernicious condition particularly common in industrial societies, in which it contributes to the comorbidity of myocardial infarction, congestive heart failure and stroke. The pathogenesis of hypertension is complex, as it rests upon multiple genetic and environmental factors (Kahle *et al.*, 2008).

A particular form of high blood pressure has come to play an important part in the unfolding story of WNKs. PHAII is a rare autosomal dominant form of hypertension characterized by both increased Na^+ and Cl^- reabsorption and impaired K^+ secretion in the kidney, which leads to hyperkalemia. Additional characteristics include suppressed plasma renin activity, hyperchloremia, reduced plasma bicarbonate; glomerular filtration, however, remains normal (OMIM). PHAII is regularly treated with thiazide diuretics, which inhibit the electroneutral sodium-chloride cotransporter (NCC), an SLC12A family member, thereby demonstrating a Cl^--dependent phenotype (Hadchouel *et al.*, 2006).

PHAII, chromosome 12 and WNK1. Many individuals with PHAII go undiagnosed; thus, its prevalence remains unclear. The condition has, however, attracted greater attention since a gene mapping study implicated WNKs 1 and 4

in the pathogenesis of the condition (Wilson, 2001). Investigation of several affected families with chromosome 12 abnormalities revealed independent, yet overlapping, deletions that both cosegregate with the disease and are not found in control subjects, thus strongly suggesting that these mutations are indeed PHAII-causing. Closer investigation of chromosome 12 mapped the deletions to intron 1 of WNK1 (Wilson, 2001).

This discovery dovetailed nicely with the fact that early in the story of the WNKs, it was noted that NaCl treatment of HEK293 cells lead to increased WNK1 activity--a change that correlates with phosphorylation of myelin basic protein (MBP) substrate, a nearly universal phosphoacceptor (Xu *et al.*, 2000). A variety of hypertonic stressors have been shown to increase WNK1 activity in DCT cells from the mammalian kidney; among these solutes, NaCl at 0.5 M has the greatest effect on WNK1 activity in culture, as it prompts a tenfold increase. By comparison, the lower osmolarity challenge posed by 0.5 M sorbitol, sucrose, mannitol, urea or glucose produces a 3x-5x increase in WNK1 activity. WNK1 responds to hypotonic stress as well (< 40 mOsM), albeit less robustly than it responds to hypertonic stress. There does appear to be specificity in the WNK response, because no increase in WNK1 protein kinase activity was revealed following treatment with dexamethasone, aldosterone, parthyroid hormone,

insulin, transforming growth factor β, forskolin, fetal bovine serum, epidermal growth factor or vasopressin (Lenertz *et al.*, 2005).

PHAII, chromosome 17 and WNK4. The WNK4 gene lay at another PHAII locus found on chromosome 17, and indeed, a number of WNK4 mutations were subsequently described in PHAII families. WNK4 PHAII-associated mutations are charge-changing and map to a stretch of only four amino acids located in a conserved, acidic span just distal to the first putative coiled-coiled (Wilson, 2001). Subsequent work with PHAII-mutant mice has confirmed the significance of this cluster of mutations, as the mutants exhibit high blood pressure (Lalioti *et al.*, 2006; Yang *et al.*, 2007c).

The intersection of WNK genes with PHAII loci prompted broader investigation into how deletion or over-expression of WNKs affect activity and cell surface expression of various channels and ion transporters.

WNKs and modulation of electrolyte levels

Much of the work on the role of WNKs in modulating proteins that control electrolyte levels has focused on the functional unit of the kidney, the nephron. The nephron modulates the concentrations of K^+, Na^+, H^+ and Cl^- in both the

vasculature and the extracellular space; in addition, the nephron regulates water volume. This precise regulatory process begins at the closed, web-like end of the nephron known as the glomerulus, or Bowman's capsule, in which water and solutes are filtered by pressure from the blood into the nephron lumen. The nephron then employs an array of transmembrane proteins, including transporters, pumps, exchangers and ion channels, which, alongside the paracellular pathway, determine whether the water and electrolytes that were secreted from the blood are reabsorbed into the blood, or excreted in the urine. When functioning correctly, for instance, the kidney carefully maintains serum K^+ and Na^+ levels, thus allowing for proper function of excitable neurons and normal blood pressure (Guyton, 1991).

Some of the same transmembrane proteins which allow the kidney to maintain electrolyte homeostasis in the blood are also employed by individual cells to modulate electrolyte levels with respect to the extracellular environment (Lang *et al.*, 1998; Kahle *et al.*, 2008). For instance, the SLC12A family of chloride-cation cotransporters effect Cl^- influx (e.g. sodium-potassium-two-chloride cotransporter (NKCC) 1 ubiquitously) and Cl^- efflux (e.g. potassium-chloride cotransporter (KCC2) in neurons) by taking advantage of favorable chemical gradients for K^+ and Na^+ to move Cl^- out of or into the cell--even against its electrochemical potential (see Table 1). The capacity of a cell to control intracellular levels of Cl^-

and its companion ions plays an important role in defense of cell volume and neuronal excitability (Meyer et al., 2002; Gamba, 2005a; Kahle et al., 2005a).

In the particular case of neurons bearing the ionotropic GABA-A receptor (a ligand-gated Cl^--channel), the concentration of intra- versus extracellular Cl^- determines whether the neurotransmitter GABA has an excitatory or inhibitory effect on the neuron, i.e., more or less likely that a neuron will fire an action potential--the rapid, transient, self-propagating electrical signal that travels from the neuronal axon hillock to the tip(s) of the axon, leading to the Ca^{+2}-dependent secretion of neurotransmitters. It is the integration of input from neuronal dendrites that prompts this event when a certain depolarization threshold is reached. Immunofluorescence intracellular Cl^- is higher than extracellular Cl^-, for instance, after GABA binds to the GABA-A receptor and the receptor Cl^--channel opens, the ion would flow down its concentration gradient, exit from the cell, and depolarize the neuron. In this case GABA would be excitatory, as it is early in development (Owens *et al.*, 1996; Kandel, 2000). Levels of intra- versus extracellular electrolytes also play an important role in regulation of defense of cell volume; for instance, under conditions of extracellular hypertonicity, a cell must take action to prevent shrinkage caused by water leaving the cell. In this case, the inhibition of KCC coupled with the activation of NKCC would increase

net Cl^- and ion entry, thereby raising intracellular osmolarity and concomitantly driving reuptake of water (Gamba, 2005a).

It is known that SLC12A cotransporters are regulated by serine/threonine phosphorylation, which activates the N(K)CCs and reciprocally inhibits the KCCs; dephosphorylation has the opposite effects (Gamba, 2005b). Because the phosphorylation state of both the NKCCs and the KCCs changes under different osmotic states, it has been widely suggested that a Cl^-/volume sensitive protein kinase plays a pivotal role in regulatory changes in cell volume. A number of reported characteristics of members of the WNK family suggest that they might be volume/Cl^- sensitive protein kinases. Namely, 1) subcellular localization, autophosphorylation and protein kinase activity of certain family members respond to fluctuations in extracellular osmolarity (Xu *et al.*, 2002; Lenertz *et al.*, 2005; Zagórska *et al.*, 2007); 2) transcript and protein expression patterns of some family members correspond to that of NKCC1 and different KCCs in multiple tissues (Wilson, 2001; Kahle et al., 2004a; Kahle et al., 2005a); 3) WNKs have been reported to regulate activity of SLC12A family member NCC, which shares regulatory motifs with other family members (Wilson et al., 2003; Yang, 2003; Gamba, 2005a), and 4) multiple family members phosphorylate binding partners of NKCC1 and the KCCs: ste20-related proline-alanine-rich

protein kinase (SPAK) and mammalian family member oxidative stress response 1 (OSR1) (Vitari *et al.*, 2005; Anselmo *et al.*, 2006; Gagnon *et al.*, 2006).

WNKs, neurons and the kidney

The fact that WNKs 1 and 4 localize in the kidney to distal nephron segments involved in K^+, Na^+ and Cl^- regulation--in conjunction with the characteristics of PHAII--suggests a role for these phosphotransferases in modulating the action of proteins involved controlling K^+, Na^+ and Cl^- influx/efflux in the nephron. Such targets include (1) regulators of K^+ secretion such as the the renal outer medullary potassium channel (ROMK), the primary modulator of K^+ secretion in the CCD/CNT (Wang *et al.*, 1997); and (2) mediators of Na^+ and Cl^- reabsorption, such as NCC in the DCT, and the epithelial sodium channel (ENaC) in the CCD and CNT (Reilly and Ellison, 2000; Xu *et al.*, 2005c).

For instance, ENaC activity is necessary for normal K^+ secretion, and the activity of this channel is responsible for the lumen-negative potential. It is known that increased ENaC activity often leads to hypokalemia, due to the correspondingly greater lumen-negative potential. In contrast, loss of ENaC activity perturbs the lumen-negative potential and can lead to profound hyperkalemia (Kahle *et al.*, 2008). So a loss of ENaC function might account for the hyperkalemia in PHAII,

but that alone cannot account for hypertension. NCC, for example, is known to be the cause of Gitelman syndrome, the phenotype of which represents the mirror opposite phenotype of PHAII; individuals with Gitelman syndrome have hypokalemia, metabolic alkalosis and low blood pressure (Simon *et al.*, 1996). The involvement of NCC in Gitelman syndrome, conjoined with the fact that NCC-targeting thiazide diuretics are used to treat PHAII, suggest an important role for this SLC12A family member in the pathogenesis of PHAII (Hadchouel *et al.*, 2006).

WNKs and ENaC. WNK1 has been shown to increase sodium influx through ENaC, an amiloride-senstive channel found in areas of the body that control fluid reabsorption such as the colon, lung and kidney. ENaC carries a PPxY motif that binds the E3 ubiquitin ligase developmentally downregulated Nedd4-2, leading to proteasomal degradation; Nedd4-2, in turn, is downregulated following phosphorylation by serum- and glucocorticoid-regulated protein kinase 1 (SGK1), which is itself an indirect WNK1 target (Xu *et al.*, 2005c; Xu *et al.*, 2005a). WNK1 does not directly phosphorylate SGK1, but the two proteins co-immunoprecipitate and expression of WNK1 residues 1-220 leads to higher SGK1 protein kinase activity; thus WNK1, via activation of SGK1, plays a role in modulation of ENaC (Xu *et al.*, 2005c; Xu *et al.*, 2005a). In oocytes, WNK4 inhibited ENaC activity in a protein kinase-independent fashion; this inhibition is

removed by PHAII mutations. It has also been shown that mutation of the PPxY motif necessary for clathrin-mediated endocytosis obviated this effect, suggesting reliance on this method of controlling the amount of ENaC at the plasma membrane (Ring *et al.*, 2007).

WNKs and ROMK. WNK1 has been shown to decrease levels of the renal K^+ channel ROMK1 at the apical membrane by increasing its endocytosis (via clathrin-coated vesicles) and subsequent degradation; residues 1-491 of WNK1 suffice to bring about the same effect (Zeng *et al.*, 2002; Lazrak *et al.*, 2006; He *et al.*, 2007). The activity of this channel, found in the kidney CCD and CNT, changes in tandem with levels of dietary K^+ intake and plays an important role in maintaining homeostatic levels of serum K^+ (Palmer and Frindt, 1999; Hebert *et al.*, 2005); therefore, its misregulation could contribute to the hallmark hyperkalemia seen in WNK1-caused PHAII. The kidney-specific form of WNK1 (KS-WNK1) has been shown to block WNK1's inhibition of ROMK, leading to greater K^+ secretion; accordingly, dietary K^+ restriction leads to higher expression of WNK1 and reduced expression of KS-WNK1--leading to less K^+ secretion, which constitutes an appropriate response to hypokalemia (Lazrak *et al.*, 2006; Wade *et al.*, 2006).

Transgenic mice overexpressing residues 1-253 of KS-WNK1 have more ROMK at the apical membrane, concomitant lower K^+ serum and higher K^+ secretion, thus lending support to the idea that KS-WNK1 plays an important role in maintaining K^+ serum levels (Liu *et al.*, 2009). Similarly, WNK3 and WNK4 inhibit *Xenopus* surface expression and activity of ROMK, even without protein kinase activity; ROMK inhibition, however, appears stronger with WNK4 PHAII mutants or with the homologous PHAII-like form of WNK3 (Kahle *et al.*, 2003; Cope *et al.*, 2006; Lazrak *et al.*, 2006; Leng *et al.*, 2006; Wade *et al.*, 2006; He *et al.*, 2007).

WNKs and NCC. It is well known that NCC expression in oocytes causes vigorous uptake of $^{22}Na^+$, and that this uptake can be blocked by thiazide diuretics, which target NCC (Gamba *et al.*, 1993). WT WNK4 has been shown to curb this robust uptake; however, the WNK4 missense mutations behind PHAII quash the inhibitory effect of WNK4 on NCC (Wilson *et al.*, 2003; Yang, 2003). These results have been confirmed in animal models, as two groups have reported thiazide-sensitive hypertension in mice with WNK4-type PHAII mutations (Lalioti *et al.*, 2006; Yang *et al.*, 2007c).

It has been reported that WNKs modulate levels of NCC at the plasma membrane in a kinase-independent fashion (Yang, 2003; Cai *et al.*, 2006); however, another

group determined this effect to be protein kinase-dependent, and this point remains unresolved (Wilson *et al.*, 2003). It has been shown that WNK1's phosphorylation/activation of OSR1/SPAK--which subsequently leads to OSR1/SPAK-mediated phosphorylation/activation of NCC--results in greater thiazide sensitivity in HEK cells (Richardson *et al.*, 2008). While this result suggests a role for WNK kinase activity in NCC function, it is not known whether this arises from changes in plasma membrane expression or modulation of instrinsic transporter activity (Huang *et al.*, 2008).

WNK1 relieves the inhibitory effect of WNK4, as shown by coexpression in *Xenopus*, thus suggesting that WNK1 mutations contribute to hypertension by removing WNK4's inhibition of NCC in the DCT (Yang, 2003; Yang *et al.*, 2005). Other mechanisms are likely involved in WNK1-related PHAII, as patients with this condition do not respond well to thiazide diuretics (Disse-Nicodeme *et al.*, 2000). One possibility discussed above is the effect of WNK1 on ENaC activity, via WNK1's activation of SGK1. WNK3 is present in the DCT and has recently also been shown to affect the regulation of NCC by WNKS 1 & 4 in oocytes and human kidney cells. Yang *et al.* report that this effect arises from antagonism between WNKs 3 & 4, based on their work in *Xenopus* oocytes injected with both differing ratios of each cRNA, i.e. as WNK3 increases relative

to WNK4, NCC activity progressively increases, but when WNK4 increases relative to WNK3, the opposite result is observed (Yang *et al.*, 2007a).

WNKs, KCCs and NKCCs. *Xenopus* studies have revealed that WNK3 and WNK4 inhibit KCC1/2, and that WNK3 depends upon its protein kinase activity to have this effect (Kahle et al., 2005a; Gagnon et al., 2006). WNK4, expressed by itself, causes inhibition of the kidney KCCs 1, 3 and 4 in a manner dependent on catalytic activity. WNK1, WNK3 and WNK4 all promote NKCC activity in a protein kinase-dependent fashion (Rinehart *et al.*, 2005; Anselmo *et al.*, 2006; Gagnon *et al.*, 2006). WNK1 co-immunoprecipitates with OSR1 and its phosphorylation of OSR1 leads to increased $^{86}Rb^{+}$ uptake by NKCC; in contrast, RNAi of WNK1 or OSR1 in Hela cells leads to lower NKCC activity (Anselmo *et al.*, 2006).

A marked increase in bumetanide-sensitive $^{86}Rb^{+}$ uptake in oocytes occurs following coexpression of WT WNK3 with NKCC1, in comparison to NKCC1 by itself; this occurs in hypertonic medium and even in hypotonic medium--a condition in which NKCC1 activity is normally minimal (Kahle et al., 2005a). On the contrary, a clear inhibition of all four KCCs follows coexpression of WNK3 in oocytes, even under hypotonic conditions, at which time KCCs are

normally maximally active in order to lower the intracellular Cl^- concentration (Kahle et al., 2005a).

Notably, a protein kinase-dead form of WNK3, produced by missense mutations, brings about the exact opposite results, namely, it activates KCCs while inhibiting NKCC1 (Kahle et al., 2005a). Because it had been established that SLC12A cotransporters can be regulated by phosphorylation, the threonine phosphorylation sites of NKCC1 were studied, and it was shown that WNK3 activity led to robust phosphorylation of threonines 212 and 217 (human NKCC1) (Lytle and Forbush, 1992b; Flemmer et al., 2002; Kahle et al., 2005b). In line with the results of rubidium uptake assays discussed above, this effect occurred even under hypotonic conditions, when NKCC1 should be minimally active; similarly, expression of a kinase-inactive mutant of WNK3 protein kinase activity produced no phosphorylation, even in hypertonic conditions, under which NKCC1 is normally found to be maximally active (Kahle et al., 2005a).

These results clearly suggest that WNK3 can reciprocally modulate these important pathways of Cl^- influx and efflux, even in conditions under which the pathways are normally minimally active, and that WNK3 has its effect on KCCs and NKCC1 via protein kinase activity. As for the mirror opposite results obtained with protein kinase-dead WNK3, phosphatase activity may play a role;

namely, KCC activation under hypertonic conditions could follow from involvement of a phosphatase. In accordance with this view, it has been shown that the phosphatase inhibitors calyculin A and cyclosporine A abrogate WNK3-stimulated activity of KCCs under hypertonic conditions, thus connecting protein phosphatases PP1 and 2B with this effect (de Los Heros *et al.*, 2006). In sum, these reports implicate WNK3 as a linchpin protein kinase in the maintenance of intracellular Cl^- vis-à-vis the cellular environment (Table 1).

WNKs, NKCC and OSR1/SPAK. It has been demonstrated that WNKs 1 and 4 affect the function of the KCCs and NKCC1 via activation of the serine/threonine protein kinase SPAK. This Ste20-type protein kinase belongs to the germinal center protein kinase (GCK)-VI subfamily. Both SPAK and mammalian family member OSR1 bind to the N term of NKCC1 at the *([R/K]FX[V/I])* motif (Piechotta *et al.*, 2002). Additionally SPAK and NKCC1 colocalize in salivary glands, the choroid plexus, neurons and other epithelia (Gagnon *et al.*, 2007). The fact that NKCC1 and SPAK both colocalize in certain tissues and bind each other prompted further investigation into their interaction.

Xenopus oocyte studies have revealed that WT WNK4 and SPAK interact, independent of tonicity, to increase NKCC activation; this effect is dependent on

protein kinase activity and an intact SPAK-binding motif on WNK4 (Gagnon *et al.*, 2006). The activation of KCC2/3 under isotonic conditions by WNK3 and WNK4 is greater with SPAK-DN and blocked by phosphatase inhibitors (Garzón-Muvdi *et al.*, 2007).

WNK1 and WNK4 phosphorylate SPAK at threonine 233 and serine 373; SPAK then phosphorylates threonine 212 and threonine 217 on NKCC1--threonine sites, the phosphorlation of which corresponds to cotransporter activity (Lytle and Forbush, 1992a; Flemmer *et al.*, 2002; Vitari *et al.*, 2005). WNK1 acts similarly with regard to OSR1 (Anselmo *et al.*, 2006). It has been shown that low levels of chloride inside oocytes--following hypotonic stress or coexpression of KCC2--lead to the phosphorylation and activation of threonines in the N terminus of NKCC2, which are highly conserved across species (e.g., threonines 96 and 101 in rat NKCC2 correspond to threonines 184 and 189 in shark NKCC1) (Gamba *et al.*, 1994; Pacheco-Alvarez *et al.*, 2006; Ponce-Coria *et al.*, 2008). Loss of these residues leaves NKCC2 unresponsive to low intracellular Cl^- as does elimination of the SPAK-binding motif in WNK3. Additionally, WNK3 protein kinase activity is required. These findings suggest that WNK3 plays the role of a chloride sensor under certain circumstances (Ponce-Coria *et al.*, 2008).

How WNKs bring about divergent responses to osmotic stress remains unclear. It could be that WNK1/4 act through SPAK/OSR1 to enhance phosphorylation of regulatory sites on SLC12A family members, while WNK3 could bring about increased phosphorylation of these cotransporters by inhibitory phosphorylation of phosphatases (Kahle *et al.*, 2008). Protein phosphatase 1 could play this part, as it has been shown to interact with NKCC1 via its RVxF binding motif (Darman *et al.*, 2001). Alternatively, sequestration of WNKs could obviate their role in response to osmotic stress.

WNKs and the paracellular pathway

Along the polarized epithelium lining the lumen of the nephron, there is variability in tight junction structure that correlates with paracellular permeability--from the "leaky" tight junctions nearer the glomerulus to the "tight" tight junctions of the epithelium of the CCD. The path of movement of electrolytes through these permeable junctions is known as the paracellular pathway, and it plays a significant role in kidney function, as evidenced by its breakdown, which not only disrupts epithelial polarity but also contributes to the pathogenesis of conditions such as polycystic kidney disease (Brown, 2000). WNK4 has been shown to phosphorylate several claudins, a type of tight junction protein that plays a role in tight junction permeability; also, WNK4 mutants

display greater chloride permeability, which contributes to Na^+ reabsorption as well (Kahle *et al.*, 2004b; Yamauchi *et al.*, 2004). Nonetheless, NCC appears to play a greater role in WNK4-related PHAII, because patients with WNK4-related PHAII are approximately six times more sensitive to thiazide diuretics than patients with essential hypertension (Mayan *et al.*, 2002). Work with mutant mice has borne this hypothesis out, e.g., Yang *et al.* found that WNK4 knockin mice displayed higher plasma membrane levels of NCC but no changes in paracellular permeability (Yang *et al.*, 2007c).

Other parts WNKs play

WNKs and membrane function. Work in our laboratory points to an important role for WNKs in membrane function. WNK1 has been reported to affect exocytosis of insulin granules via its interaction with mammalian uncoordinated 18 homolog c (Munc18c)--an interaction mediated via the kinase domain of WNK1, although Munc18c is not a WNK1 phosphorylation substrate (Oh *et al.*, 2007). Additionally, Lee *et al.* showed that WNK1 selectively binds and phosphorylates synaptotamin 2 (Syt2), an important actor in vesicular fusion and membrane trafficking. The phosphorylation takes place on the calcium binding C2 domains of Syt2, and increases the amount of Ca^{2+} necessary for Syt2 to bind phospholipid vesicles. Thus, WNK1 may play an important role in Ca^{+2}-

dependent Syt2-mediated regulation of membrane events, including those controlling amounts of proteins in the plasma membrane (Lee *et al.*, 2004).

WNK1 has been shown to bind VPS4, which regulates protein sorting at the multivesicular body; WNK1 and VPS4 have also been shown by microscropy to localize on the same vesicles in the cytoplasm (Kieffer et al., 2008; Tu et al., 2009). It has also been demonstrated that WNK1 overexpression or knockdown perturbs both constitutive and ligand-dependent internalization of membrane proteins (Heise et al., 2009). For instance, WNK1 knockdown stabilizes the presence of the epidermal growth factor (EGF) receptor at the plasma membrane, diminishing its ligand-dependent internalization for transport to the lysosomes (Tu et al., 2009).

Cystic fibrosis transmembrane conductance regulator (CFTR). Yang *et al.* have reported both WNK1 and WNK4 negatively affect levels of CFTR at the plasma membrane. The effect of WNK1 is protein kinase-independent, while that of WNK4 is protein kinase-dependent (Yang *et al.*, 2007b). Since both WNKs 1 and 4 are expressed in pulmonary epithelial cells, this finding could be of great relevance to cystic fibrosis research.

Transient receptor potential cation channel, subfamily V (TRPV), member 4 (TRPV4). WNK1 and WNK4 can dampen plasma membrane presentation of overexpressed TRPV4, a nonselective cation channel important in a variety of cellular functions in different tissues, including mechanosensation, osmoregulation and thermosensation (Fu *et al.*, 2006). Conversely, WNK4 augments plasma membrane expression of TRPV5, which plays an essential role in calcium reabsorption in epithelia (Jiang *et al.*, 2007). Zhang *et al.* have provided insight into the relationship of WNK3 and the renal epithelial Ca^{+2} channel TRPV5, which plays a role in Ca^{+2} reabsorption. Coexpression of WNK3 and TRPV5 produced a large increase in Ca^{+2} uptake--and Na^{+} current per voltage clamp--vis-à-vis expression of the channel alone. The group discovered corresponding results with TRPV6, an intestinal paralog of TRPV5. Protein kinase activity was required in both instances and the catalytic domain alone was able to mimic the effect of the whole protein. It was also shown that WNK3 increases both the plasma membrane level of the complexly glycosylated form of TRPV5 and its endocytosis in a microtubule-dependent manner. Thus, WNK3 appears to play a positive role in trafficking of mature TRPV5 (Zhang *et al.*, 2008).

Transforming growth factor beta (TGFß) signaling. WNK1/4 have been implicated in TGFß-Sma- and Mad-related protein (Smad) signaling as well.

When TGFβ binds to its receptor, it initiates a signaling cascade in which the receptor recruits and phosphorylates Smad2 or Smad3, which then dissociates and binds to the common mediator Smad 4. This Smad complex then moves to the nucleus, where it affects regulation of transcription of specific target genes. The Smads often serve antiproliferative roles. WNK1/4 bind several Smads, while WNK1 RNAi perturbs both Smad2's expression and antiproliferative effects (Lee *et al.*, 2007).

Cancer and cell death. Large-scale investigations of protein kinase mutations in a variety of human cancers have uncovered mutations in human WNKs (Greenman *et al.*, 2007). WNK3 has also been shown to aid cells in evading apoptosis by inhibiting caspase-3 (Veríssimo *et al.*, 2006).

ERK5 pathway. WNK1 can function as a MAPK kinase kinase kinase (MAP4K) in the extracellular signal-related protein kinase 5 (ERK5) pathway, which is important in cardiovascular development and neural differentiation (Xu *et al.*, 2004). WNK1 has also been shown to enhance ERK5 activity when overexpressed in 293 cells; this phenomenon is dependent on the presence of MEKK2 and MEKK3, both of which co-immunoprecipitate with, and are phosphorylated by, WNK1 (Xu *et al.*, 2004). Therefore, WNK1 can act upstream

in the ERK5 pathway. EGF-induced activation of ERK5 requires WNK1, which is multiply phosphorylated following EGF binding (Xu *et al.*, 2004).

Development and differentiation. One may infer a vital role for WNK1 in mouse development from the facts that loss of WNK1 causes mouse embryos to die, and that WNK1 mRNA appears preimplantation and at E8.5 in heart/vasculature (Delaloy *et al.*, 2006). Mice generated with one copy of the WNK1 gene exhibit low blood pressure (Zambrowicz *et al.*, 2003) These findings dovetail nicely with the known role of WNK1 in activation of the ERK5 pathway, given its role in cardiac development (Xu *et al.*, 2004). Additionally, WNK1 RNAi dampens migration, differentiation and growth in C17.2 neuronal progenitor cells (Sun *et al.*, 2006). Underscoring the essential nature of WNK family involvement in development, the *Drosophila* WNK ortholog (CG7177; dWNK) exhibits a high degree of conservation, and has been shown by RNAi to be necessary for survival of cells in development (Boutros, 2004). Moreover, it has been demonstrated that dWNK negatively modulates the Wingless/Wnt pathway, which is central cascade during embryonic development (DasGupta *et al.*, 2005).

Developmental disorders. Recent work has also unveiled a potential link between WNK3 and several developmental disorders. Qiao *et al.* screened a population of individuals with autism spectrum disorders and found a deletion of Xp11.22 in

two autistic brothers with intellectual disability and cleft lip/palate; the deletion, confirmed by RT-PCR and fluorescence *in situ* hybridization, encompassed the genes for plant homeodomain finger protein 8 (PHF8), FAM120C and WNK3--the first of which has been linked to both intellectual disability and cleft lip/palate (Qiao *et al.*, 2008). While the loss of PHF8 alone may account for these two linked conditions, the additional presence of autism here implies that deletion of FAM120C and/or WNK3 may play a role in the pathogenesis of this particular disorder (Qiao *et al.*, 2008).

Akt/PKB. It is known that Akt/protein kinase B (PKB), a serine/threonine protein kinase in the insulin signaling pathway, phosphorylates WNK1 at threonine 60 in vitro and in HEK293 cells following insulin-like growth factor 1 (IGF-1) treatment (Jiang *et al.*, 2005; Lenertz *et al.*, 2005). This link, in conjunction with the comorbidity of hypertension and insulin resistance, provides great impetus for further research into WNK1 function.

The least studied family member, WNK2. To date, very little is known about WNK2. Lenertz *et al.* reported that the WNK2 protein kinase domain can be phosphorylated by WNK1 and that its protein kinase activity can similarly be inhibited by the WNK1 autoinhibitory domain (Lenertz *et al.*, 2005). Decreased expression of WNK2 has been linked to the pathogenesis of meningioma, the

most prevalent primary CNS tumor; the researchers hold WNK2 out as a prototypical example of epigenetic silencing, as the WNK2 gene proved to be aberrantly methylated at the 5' CpG island in most Grade II and III meningiomas, but seldom in thirteen other tumor types (Costello *et al.*, 2008). Recently, WNK2 has also been shown to regulate EGFR signaling negatively through inhibition of MEK1 (Moniz *et al.*, 2008).

WNK3: a discovery project

The story of the WNK kinases will go on. With a connection to ion flux diseases well-established, the WNK family will surely continue to attract attention for many years, particularly given their potential as drug targets. As the gamut of WNK functions--and, importantly, dysfunction--comes into sharper focus, it is likely these protein kinases will be shown to play more roles in the cell.

With this in mind, I chose to study WNK3, one of the least characterized WNK family members and the only one to have high levels of transcript expressed in the brain. I have employed the powerful yeast two-hybrid system to develop a picture of the WNK3 interactome,, with the goal of compiling a collection of putative WNK3 interactors to serve as a source of information and project leads for the ongoing research program of the Cobb laboratory. The yeast two-hybrid screens I

will describe below have yielded hundreds of putative interactors that indicate unexpected directions in WNK function.

Chapter One: Figures

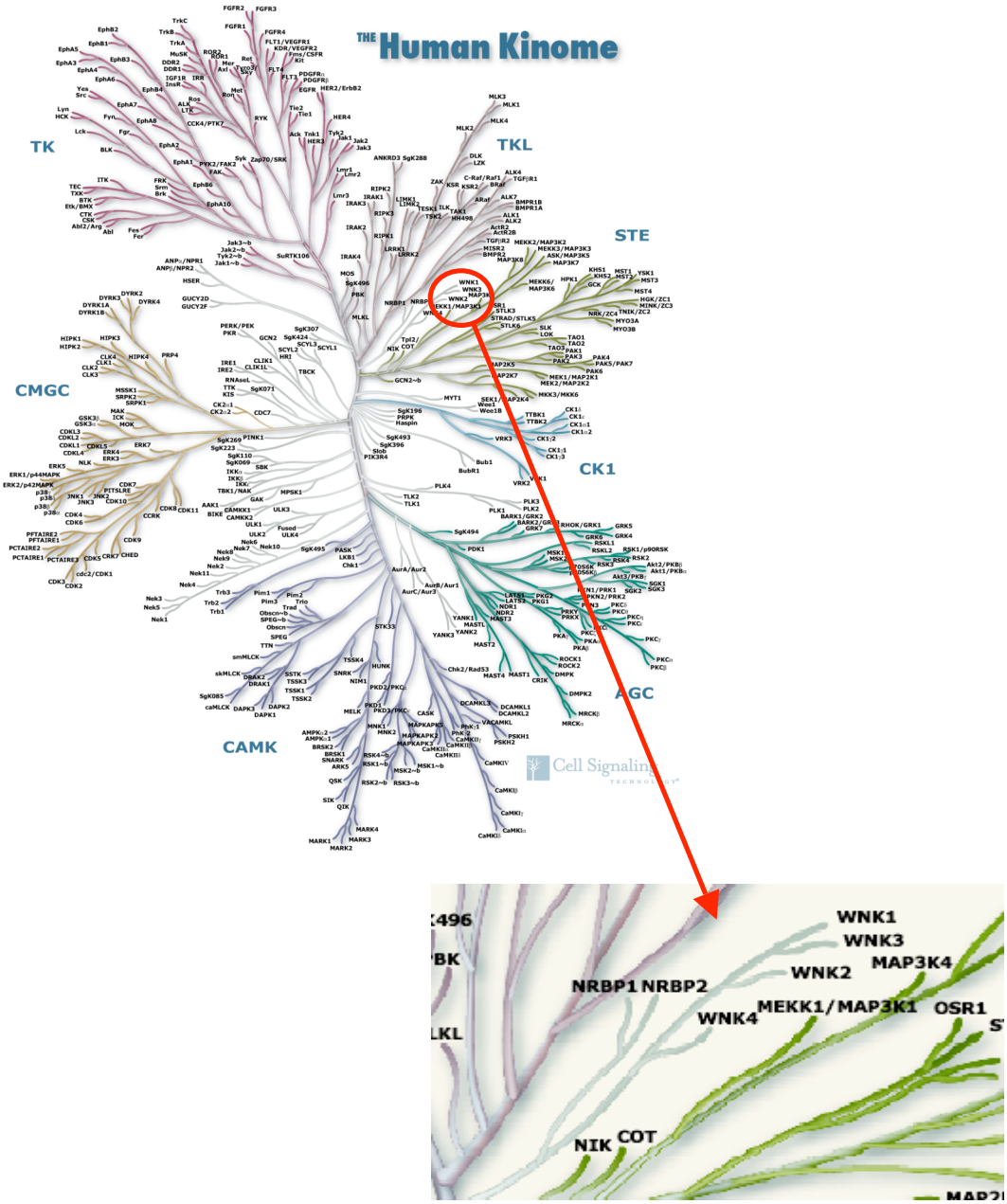


Figure 1. The WNKs occupy a unique position in the human kinome.

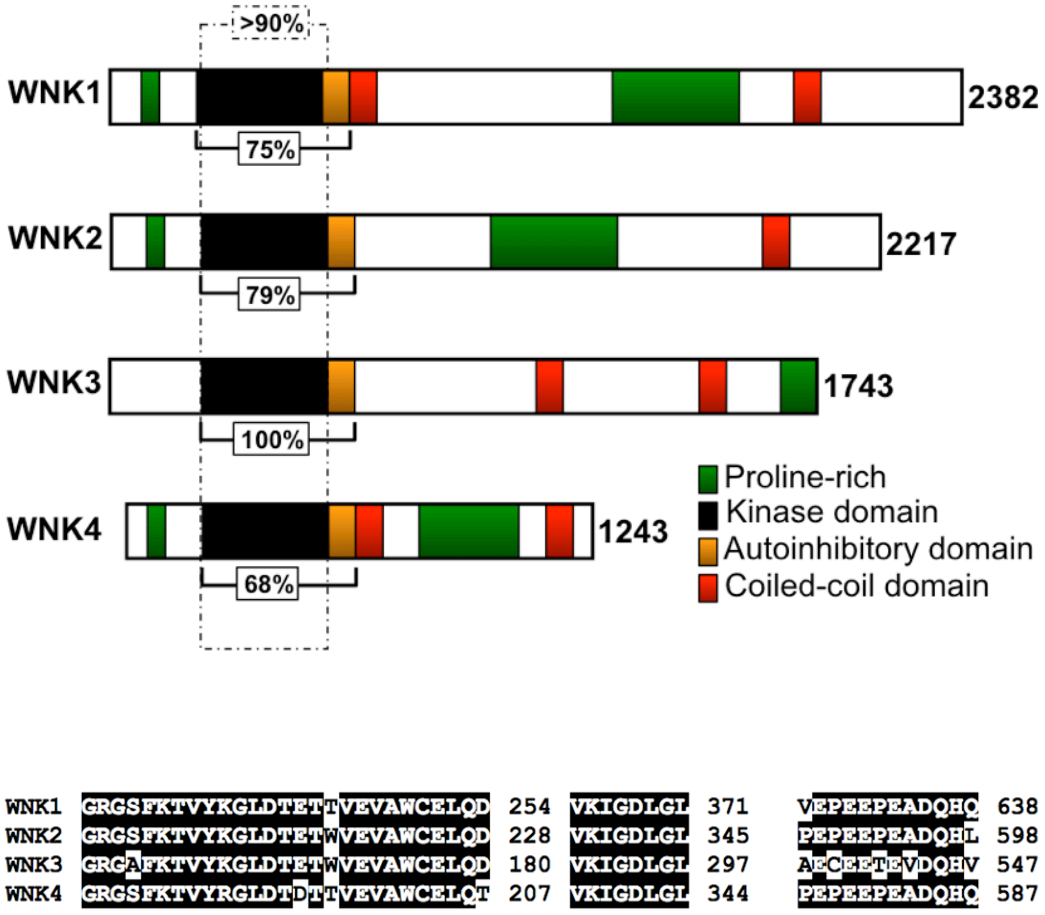


Figure 2. Human WNK Family Architecture.

WNKs possess a highly conserved catalytic domain and adjacent auto-inhibitory domain, and exhibit a high degree of homology among metazoans. The WNK catalytic domain is most similar to human PAK2, MEKK3, and Raf-1. The WNKs differ in tissue-specific expression and in chromosomal location. Note that percentages shown represent identity among the WNK protein kinase domains (dashed lines) and protein kinase+auto-inhibitory domains (solid lines) vis-à-vis WNK3. The specific sequences shown correspond to the portion of the protein kinase domain containing WNK placement of the active lysine and the ten amino acid stretch corresponding to the cluster of PHAII mutations in WNK4.

Figure 3. Alternatively spliced isoforms of human WNK3.

Sequences of human WNK3, variant 1 (1800 residues); human WNK3, variant 2 (1743 residues); and a database variant (1790 residues) that is unreviewed (Uniprot). The longer WNK3 isoform includes a 47 amino acid span and a 10 amino acid span not found in WNK3, variant 2. The C-terminal WNK3 bait (residues 1473-1743) used in the yeast two-hybrid screens discussed come from the shorter isoform. Boxed sequence corresponds to the WNK3 C terminus bait.

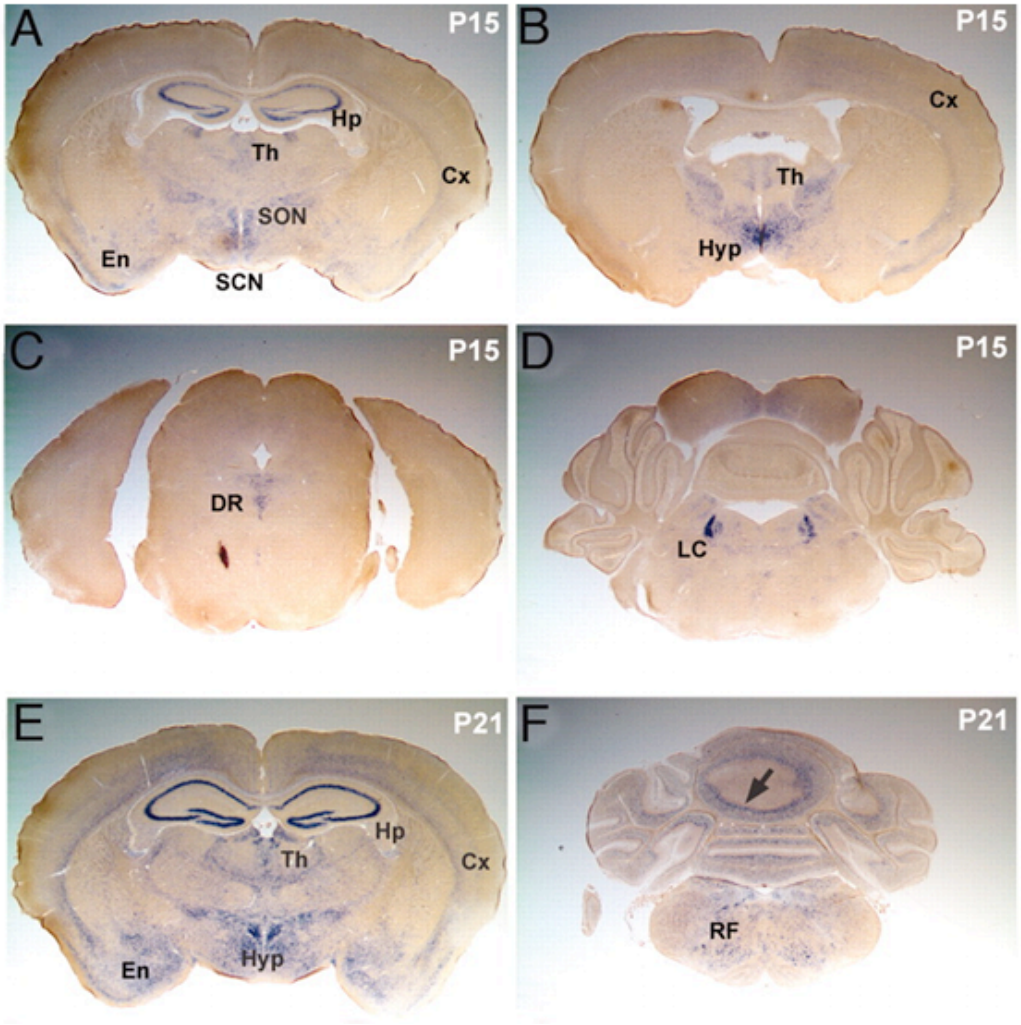


Figure 4. WNK3 expression in brain.

WNK3 expression in brain. (A-F) Antisense riboprobe to WNK3 was hybridized in situ to mouse brain sections. Coronal sections at postnatal day 15 (P15; A-D) and P21 (E and F) are shown. (A) WNK3 expression is high in the hippocampus (Hp), thalamus (Th), layers of the cerebral (Cx), and entorhinal (En) cortices and in the supraoptic (SON) and suprachiasmatic nuclei (SCN). (B) Strong WNK3 expression is seen in hypothalamus (Hyp), Cx, and thalamus (Th). (C) WNK3 is expressed in the dorsal raphe nucleus (DR). (D) Intense WNK3 expression in the locus ceruleus (LC). (E) WNK3 expression is higher at P21, especially in Hp, Cx, and En. WNK3 expression in Hyp is also strong. (F) At P21, WNK3 is expressed in the Purkinje layer (arrow) of the cerebellum (Cb) and the medullary reticular formation (RF). Kahle K. T. et.al. PNAS 2005;102:16783-16788. ©2005 NAS

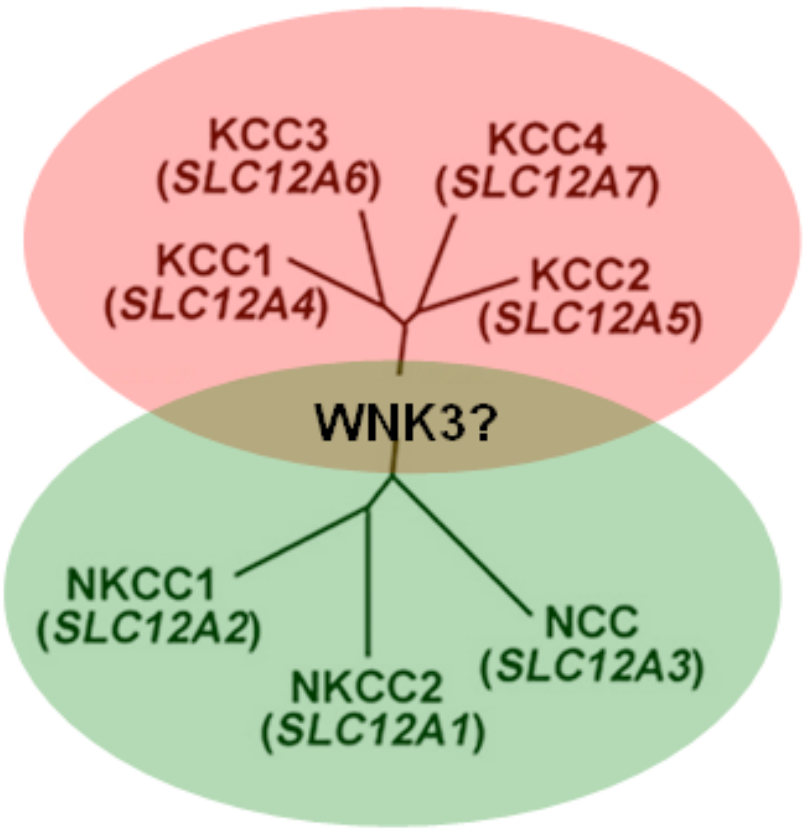


Figure 5. SLC12A family of chloride-cation cotransporters.

Members of the SLC12A family of chloride-cation cotransporters are regulated by serine/threonine phosphorylation, which activates the chloride-importing N(K)CCs and reciprocally inhibits the chloride-exporting KCCs; dephosphorlation has the opposite effects.

Chapter Two: Methodology

Library and contructs. The neonatal mouse brain cDNA library used for yeast two-hybrid screening was a gift of Mark Henkemeyer of UT Southwestern, and all WNK baits were produced by members of the Cobb laboratory (Table 2). The full-length clone of human WNK3, variant 2, containing 1743 residues (Origene, no. SC300456) was kindly provided by the laboratory of Richard Lifton at Yale University. All work with WNK3 described herein made use of this shorter isoform of WNK3. Full-length flotillin-1 was generously made available by the Bickel laboratory at Baylor University.

LexA-Gal4 two-hybrid system. The yeast two-hybrid screens discussed herein were based on the LexA-Gal4 model (Figure 6). The pVJL11 bait plasmid carries a LexA DNA binding domain and a TRP reporter gene, which codes for a protein necessary for tryptophan synthesis; the pGAD-GH prey plasmid carries a Gal4 activation domain and a LEU2 reporter gene, which codes for a protein necessary for leucine synthesis (Vojtek and Hollenberg, 1995). All yeast came from the *S. cerevisiae* two-hybrid tester strain L40, and were grown at 30ºC.

The L40 yeast strain is auxotrophic for tryptophan, leucine and histidine, and carries a LexA binding site followed by a Gal4 activation domain and two reporters genes--the first is HIS3, which produces an enzyme necessary for histidine synthesis, and the second codes for ß-galactosidase. Thus, L40 yeast harboring the bait plasmid grow on complete supplemental medium (CSM) (BIO 101, Inc.) plates lacking tryptophan (CSM-Trp); those containing the prey plasmid grow on plates lacking leucine (CSM-Leu); those hosting both plasmids and an interacting pair of bait and prey fusion proteins grow on triple dropout plates (CSM-Trp-Leu-His) and also express ß-galactosidase.

Autoactivation tests. It is possible for a bait fusion protein to autoactivate, i.e. stimulate transcription of the histidine and ß-galactosidase reporter genes, even without a bait-prey interaction, thereby leading to false positives during screening. To control for this possibility, autoactivation tests were performed by transforming yeast with a given bait plasmid using the Frozen EZ-Yeast Transformation II kit (Zymo Research), and plating them on double dropout CSM-Trp-His plates containing 0-10 mM 3-amino-1,2,4-triazole (3-AT) (Sigma #A8056). 3-AT inhibits an enzyme necessary for histidine biosynthesis, and the lowest concentration of 3-AT at which no yeast grew was subsequently used in screening with that bait. Bait expression was confirmed by immunoblotting with

an anti-lexA antibody (Sigma), following preparation of yeast lysates as described at http://www.protocol-online.org/prot/Model_Organisms/Yeast/Protein.

Library tranformation. For library transformations, L40 yeast were transformed with a given bait as above and grown on CSM-Trp plates; colonies from these plates were introduced into CSM-Trp media and shaken at 30ºC until reaching an optical density of 0.600. Yeast were rocked overnight at room temperature with library DNA and carrier salmon sperm DNA in a PEG/lithium acetate solution, before being spread onto triple dropout CSM-Trp-His-Leu plates with the requisite concentration of 3-AT to prevent autoactivation (Table 2) (Golemis, 2002). Plates were incubated at 30ºC and colonies were collected between three and fourteen days post-transformation. Colonies from transformation plates were streaked onto triple dropout CSM-Trp-His-Leu plates for subsequent assay of ß-galactosidase activity, using the filter assay detailed on the Hershkowitz laboratory homepage, Department of Biochemistry and Biophysics, USCF (http://biochemistry.ucsf.edu/labs/herskowitz/).

Prey identification. Yeast colonies selected for prey plasmid isolation were streaked onto duplicate CSM-Leu and CSM-Trp plates until colonies were found that grew only on CSM-Leu plates. DNA retrieved from such yeast, using the

Zymogen Yeast Plasmid Miniprep Kit, was used to transform bacteria, thus allowing for standard PEG preparation of sufficient quantities of DNA for sequencing using pGAD-GH-specific primers (Table 3).

Pairwise testing of putative interactions. Putative interactions of interest were confirmed by pairwise testing of bait and prey. This involved co-transforming yeast with the interacting bait and isolated prey, followed by plating onto CSM-Trp-His-Leu plates with the amount of 3-AT necessary to prevent bait autoactivation (Table 2).

Two-hybrid bait-prey swap. Swapping inserts between the yeast two-hybrid bait and prey provides one method of confirmation of the protein:protein interaction. For example, the putative interaction of Flot1 and WNK3 was confirmed by this method. Yeast transformed with the swapped pGAD-GH WNK3 C-terminus prey grew on CSM-Leu plates but not on CSM-Trp plates; yeast transformed with the pVJL11 full-length Flot1 bait grew on CSM-Trp plates but not on CSM-Leu plates. Co-transformation of yeast produced growth on CSM-Trp-Leu plates. Autoactivation testing of the pVJL11 full-length Flot1 bait revealed no growth any concentration on CSM-Trp-His plates with 0-10 mM 3-AT, thereby revealing that this bait does not autoactivate. For stringent confirmation, co-transformed colonies from CSM-Trp-Leu plates were re-streaked onto triple dropout CSM-

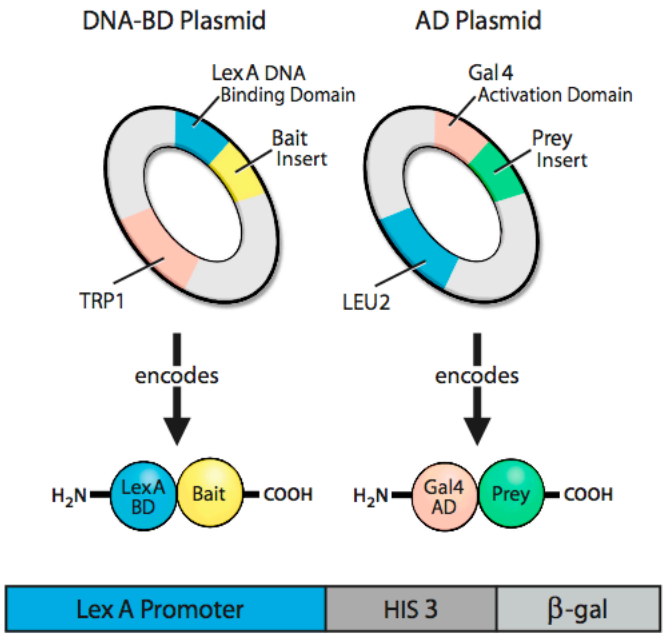
Trp-His-Leu plates with increasing concentrations of 3-AT. Moderate growth was observed at 0.1 mM 3-AT, thus confirming interaction above background and validating this yeast two-hybrid interaction

Bioinformatics. Successfully sequenced prey plasmids were analyzed using the Basic Local Alignment Search Tool (BLAST) suite, available via the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) . Briefly, the nucleotide sequence of prey inserts was subjected to BLASTn analysis, which searches a nucleotide database using a nucleotide query; prey plasmid inserts were also subjected to BLASTx analysis, which searches a protein database using a nucleotide query translated in all six frames. Additionally, the frame of prey plasmid inserts was confirmed by chromatogram analysis using 4Peaks (mekentosj.com/4peaks), and in-frame translations were subjected to BLASTp analysis, which searches a protein database using a protein query. Alignments were performed with ClustalW.

There were several preys that corresponded to different coding regions of the same gene, but that were not all in frame. The nucleotide sequences of these preys were translated in different 5'-3' frames (http://ca.expasy.org/tools/dna.html), and the resulting protein sequences were subjected to PRATT analysis (http://www.expasy.ch/tools/pratt/) to reveal any conserved protein patterns.

PRATT was then used to scan the UniProtKB/Swiss-Prot database for matching patterns (Table 4).

Chapter Two: Figures



	L40 yeast	Bait	Prey
Plasmid	-----	pVJL11	pGAD-GH
Promoter	LexA	ADH	ADH
Fusion	-----	LexA DBD	Gal4 AD
Reporter	LacZ His	Trp	Leu
Phenotype	Trp- Leu- His-	Trp+	Leu+

Figure 6. LexA-Gal4 two-hybrid system.

Chapter Three: Interaction mapping of WNK3

The following discussion will focus upon a number of the more interesting finds from a series of yeast two-hybrid screens with baits made from WNK3 (Table 3), including membrane-, DNA- and RNA-associated proteins, and putative interactions that have not been grouped by possible function. These putative interactions were all confirmed by pairwise testing.

Membrane-associated proteins

Flotillin-1 (Flot1). Eight preys isolated from the fourth large-scale two-hybrid screen with the C terminus of human WNK3 proved to be Flot1. Interestingly, the eight clustered into two groups: six prey plasmid inserts corresponded to the in-frame nucleotide sequence coding for residues 1-263 of Flot1, while the other two putative interactors corresponded to the out-of-frame sequence (+2) coding for the last 399-428 residues of Flot1 (Figure 7) (Figure 8). The significance of such noncoding preys is not clear.

This 428 amino acid, membrane-associated protein and its paralog, flotillin-2 (~44% identical), were first described in 1997 by two groups; one named them flotillins for their flotation in certain density gradients, and the other dubbed them

reggies for their regenerative role in goldfish retinal ganglion cells (Bickel *et al.*, 1997; Schulte *et al.*, 1997). The flotillins occur across kingdoms and have been found conserved in bacteria, fungi, plants, and animals; indeed, Flot1 is 61% identical between Drosophila and mouse, and 99% identical between man and rodents (Rivera-Milla *et al.*, 2006; Babuke and Tikkanen, 2007) (Figure 8).

Flotillins are expressed most highly in neuronal tissues and have been described as localizing to the plasma membrane, the Golgi, endosomal compartments, exosomes and phagosomes (Dermine *et al.*, 2001; Babuke and Tikkanen, 2007). The flotillins are not transmembrane proteins, but instead associate with the plasma membrane via fatty acid side chains (flotillin-2) or, as is the case with Flot1, two stretches of hydrophobic residues (Liu *et al.*, 2005). The flotillins have been shown to associate constitutively with caveolae, membrane rafts, and non-caveolar rafts even in cells that bear caveolae (Bickel *et al.*, 1997; Stuermer *et al.*, 2004).

Notably, Flot1 has been shown to play a vital role in ERK-mediated neurite outgrowth signaling in PC12 cells, in response to the binding of nerve growth factor (NGF) to its receptor, TrkA. This binding event both activates the receptor and prompts its translocation to lipid rafts, where it is recruited and retained by Flot1 via the adaptor protein c-Cbl associated protein (CAP); once tethered to a

lipid raft, TrkA, is able to participate in the formation of signaling complexes, which leads to efficient activation of ERK1/2 (Limpert *et al.*, 2007).

The putative interaction between WNK3 and Flot1 was validated using a bait-prey swap, as described in Chapter Two. Flot1 remains a dimly elucidated protein that appears in a variety of membrane milieu; thus, its putative interaction with the C terminus of WNK3 would allow for any number of roles for WNK3 in membrane function, e.g., membrane trafficking, or as intimated by the recent work with Flot1 and TrkA, a part in raft-mediated signaling.

Stromal membrane-associated protein 2 (Smap2). The two-hybrid screen with the C terminus of WNK3 uncovered two Smap2 fragments, one corresponding to Smap2 residues 75-357 of 428 and another to residues 258-428. The region common to the inserts, residues 258-357, includes most of a unique, highly conserved region Smap2 shares with the only other member of the Smap family, Smap1 (Uniprot) (Figure 9). No function has been identified for this conserved region, which corresponds to residues 231-339 in Smap2; in each Smap, this region lies between a clathrin-interacting domain and a clathrin assembly lymphoid myeloid leukemia protein (CALM)-interacting domain (Tanabe *et al.*, 2008) (Figure 9).

Together Smaps 1 and 2 constitute a unique pair of small GTPase-activating proteins (GAPs) that specifically target ADP ribosylation factors (Arfs), thus they are called ArfGAPs (Natsume *et al.*, 2006). ArfGAPs function, in essence, to speed the inactivation of Arfs (which belong to the Ras superfamily of GTP-binding proteins) by enhancing their hydrolysis of GTP. The Smaps localize quite distinctly from one another, with Smap1 diffuse throughout the cytoplasm and Smap2 concentrated on the Golgi and early endosomes, as shown by immunofluorescence with overexpressed proteins. It has been reported that Smap2 preferentially regulates Arf1, while Smap1 targets Arf6 (Tanabe *et al.*, 2005; Tanabe *et al.*, 2008).

Arf1 recruits coat proteins required for both membrane budding and membrane fission (Nie *et al.*, 2003), which suggests Smap2 may indirectly have a role in Golgi maintenance (Natsume *et al.*, 2006). However, its full role in the life-cycle of clathrin-coated vesicles remains obscure.

RNA-associated proteins

Ataxin-2 binding protein 1 (A2bp1). Three putative interactions from the two-hybrid screen with the C terminus of human WNK3 were identified as A2bp1

(mouse sequence). Two of these corresponded to the in-frame nucleotide sequence coding for residues 168-313 of 396. The third A2bp1 insert was a longer in-frame nucleotide sequence, which included all of the sequence in the other two preys, and coded for residues 111-396. Thus, all three preys have residues 168-313 in common (Figure 10).

A2bp1 was first identified through a yeast two-hybrid screen with the C terminus of ataxin-2, which causes the neurodegenerative disorder, spinocerebellar ataxia type 2, when it carries an expanded polyglutamine tract (Shibata *et al.*, 2000). Mutations in the A2bp1 gene have since been linked by deletion studies, in both individuals and groups, to autism, epilepsy, global developmental delay and, quite recently, bipolar disorder (Bhalla *et al.*, 2004; Martin *et al.*, 2007; Le-Niculescu *et al.*, 2009). A clear understanding of the role of mutations in A2bp1 in the pathogenesis of such disorders is lacking.

Mouse A2bp1 is approximately 43 kD and has highly conserved orthologs among a number of vertebrates, including zebrafish and humans; between mice and humans, the level of identity exceeds 98% (Uniprot) (Figure 10). A2bp1 transcript appears highly expressed in heart, muscle and brain; protein expression data, however, is more limited, but A2bp1 has been shown by immunofluorescence to localize to the Golgi (along with ataxin-2) (Shibata *et al.*, 2000; Jin *et al.*, 2003).

A2bp1 contains an RNA recognition motif that bears exceptional affinity for the sequence 5'-UGCAUGU-3' (Auweter *et al.*, 2006) and, as such, has been shown to play roles in tissue-specific splicing, and also in repression of pre-spliceosome formation (Zhou and Lou, 2008).

Wei Chen in our laboratory demonstrated in 293 cells that A2bp1 co-immunoprecipitates with both full-length WNK3 and the C terminus of WNK3, but not with the N terminus of WNK3 or a negative control (Myc-ERK2) (Figure 11). These findings provide validation of the yeast two-hybrid results and demonstrate the interaction of WNK3 with A2bp1 in mammalian cells.

CUG-BP and ETR-3 like factor 4 (Celf4). Celf4 (aka bruno-like 4, RNA binding protein (Brunol4)) came to light as a potential WNK3 interactor following the two-hybrid screen with WNK3's C terminus. Two preys encoded Celf4 residues 153-479, and 344-445, of 486. Thus, the smaller prey insert is entirely included in the larger prey insert (Figure 12).

Celf4 came to light in 2001 as a founding member of the CELF family: a group of novel RNA-binding proteins determined to be important actors in the developmentally-regulated pre-mRNA alternative splicing of cardiac troponin T

(Ladd *et al.*, 2001). The existence of CELF orthologs extends from the animal into the plant kingdom, and vertebrate family members bear strong resemblance to one another, i.e., they each harbor a highly conserved region of ambiguous function set amid three RNA recognition motifs (RRMs) (Uniprot).
Celf4 transcript finds highest expression in the nervous system; nonetheless its protein levels appear greatest in muscle (Ladd *et al.*, 2001; Brimacombe and Ladd, 2007).

Wei Chen in our laboratory demonstrated in 293 cells that Celf4 co-immunoprecipitates with both full-length WNK3 and the C terminus of WNK3, but not with the N terminus of WNK3 or a negative control (Myc-ERK2) (Figure 11). These findings provide validation of the yeast two-hybrid results and demonstrate the interaction of WNK3 with Celf4 in mammalian cells.

DNA-associated proteins

Kruppel-like factor 10 (Klf10). Klf10 came to our attention as a result of the two-hybrid screen with the C terminus of WNK3. Its prey plasmid coded for residues 126-425 of 479, which harbor a protein pattern conserved with another prey from the same screen, Flot1 (Figure 13) (Table 4).

Klf10 is a repressor of transcription with three zinc fingers near its C-terminus, which bind the consensus sequence 5'-GGTGTG-3' (Uniprot). Its expression and nuclear localization both increase within two hours of TGFß treatment, and immunoblotting has revealed Klf10 in tissues throughout the body--except the kidney (Subramaniam *et al.*, 1998). Strikingly, loss of Klf10 produces a number of divergent phenotypes between the sexes: male knockouts experience cardiac hypertrophy, while female $Klf10^{-/-}$ mice exhibit severely reduced bone density, suggesting a role in mediation of estrogen signaling in bone. Additionally, knockout mice, of both sexes, exhibited delayed expression of TGFß following tendon laceration (Subramaniam *et al.*, 2007).

Of potential interest, PRATT analysis (http://www.expasy.ch/tools/pratt/) has revealed a protein pattern shared between Klf10 and another prey, Flot1 (Table 4).

Amino-terminal enhancer of split (Aes). Preys corresponding to various portions of the Aes coding in-frame sequence came out of the screen with the C terminus of WNK3 more than thirty times, with many of these including the full-length coding sequence (Figure 14). Aes was the most frequently isolated prey plasmid, although the significance of this is unclear. It may corresponded to the number of copies of Aes in the neonatal mouse library used for the two-hybrid screens.

Aes belongs to the Gp130-associated protein (Grg) family of transcriptional corepressors, which is highly conserved among vertebrates (Uniprot) (Brinkmeier *et al.*, 2003). Of the five Grg family members, Aes alone lacks the WD-40 domain responsible for interaction with DNA binding proteins that contain an engrailed repressor homology domain (the signature target of Grg family members); however, it does retain the amino-terminal glutamine-rich domain necessary for interaction among family members (Pickles *et al.*, 2002). Aes functions as a repressor of the other members of the Grg family. It also bears strong resemblance to its *Drosophila* cousin (~50% identity), a protein implicated in neuronal development via the Notch pathway (Miyasaka *et al.*, 1993). With this in mind, a deficiency in Aes itself has been linked to anomalous pituitary development in mice (Brinkmeier *et al.*, 2003).

Other putative interactions of interest

Apolipoprotein D (ApoD). This protein implicated in neurodegenerative disorders was pulled out of the WNK3 C-terminal screen three times (Figure 15). Two of the inserts encode residues 50-189 of 189, while the third encodes residues 69-189; thus the two longest inserts include the full sequence of the third ApoD prey sequence.

Primarily neuronal in expression, ApoD has been found to function in transport of cholesterol and other hydrophobic molecules, and is reportedly overexpressed in bipolar disorder, schizophrenia and Alzheimer's disease (Thomas *et al.*, 2001; Navarro *et al.*, 2004; Carter, 2007). This ~21 kD protein is 72% identical between mouse and human, and its orthologs appear across the vertebrate class throughout development (Uniprot) (Figure 15). Overexpression of its ortholog in *Drosophila* lengthened lifespan almost 30%, in addition to improving resistance to both hyperoxia and starvation (Walker *et al.*, 2006). ApoD knockout mice display altered locomotion and diminished learning capacity, and a less robust protective response to oxidative insult; taken together, these results indicate an important, likely neuroprotective, role for ApoD in brain function (Ganfornina *et al.*, 2008).

This interaction has been confirmed using immunoprecipitation in 293 cells. Wei Chen in our laboratory successfully co-immunoprecipitated an HA-tagged WNK3 C terminus, and a Myc-tagged ApoD (corresponding to the fragments pulled out of the screen); importantly, Myc-ERK2 was not co-immunoprecipitated (Figure 16).

Atrophin-1 (Atn1). Two Atn1 preys came out of the two-hybrid screen with the C terminus of WNK3. They corresponded to residues 1051-1166 of 1175 of Atn1.

Like ApoD, Atn1 has also been implicated in neurodegeneration. The condition linked to Atn1 is dentatorubral-pallidoluysian atrophy (Drpla), which arises following expansion of a CAG repeat in the Atn1 gene (Burke *et al.*, 1994; Koide *et al.*, 1994). Along with Huntington's disease, another poly-glutamine disorder, Drpla is characterized, in part, by ataxia and dementia (OMIM). Although the transcript appears throughout the body, Atn1 protein resides primarily in the cytosol of neurons, as shown by immunohistochemical analyses of monkey brain (Knight et al., 1997). Atn1 carries several motifs conserved among proteins that function in transcriptional repression, and overexpression of a fusion Atn1-GAL4 protein has been shown in flies to repress transcription of a GAL4-dependent reporter gene (Zhang et al., 2002)

This interaction has been confirmed using immunoprecipitation in 293 cells. Wei Chen in our laboratory successfully co-immunoprecipitated an HA-tagged WNK3 C terminus, and a Myc-tagged Atn1 (corresponding to the fragments pulled out of the screen); importantly, Myc-ERK2 was not co-immunoprecipitated (Figure 16).

Pairwise two-hybrid tests using other WNK baits

As an inroad to a deeper understanding of the human WNK family, I conducted a series of pairwise yeast two-hybrid tests using prey from my WNK3 yeast two-hybrid screens and a number of bait constructs made from WNKs 1, 2 and 4 (Table 2) (Figure 17).

Briefly, two preys from my screen with the WNK3 N-terminal bait were selected: Rab14 and Eef1a1. These were both tested against a two-hybrid bait made from WNK1 N-terminal residues 1-222. Neither Rab14 nor Eef1a1 interacted with this WNK1 bait (Table 5).

Eight preys from the screen with the WNK3 C-terminal bait were also selected: A2bp1, Aes, Atn1, Celf4, Flot1, Flot1 (out-of-frame), Klf10 and Smap2. Each of these preys was tested in a pairwise fashion against C-terminal baits from WNKs 1, 2 and 4, which corresponded to the following residues: WNK1 2021-2382, WNK1 2031-2126; WNK2 1967-2217; and WNK4 1093-1222 (Table 5).

The overlapping WNK1 C-terminal baits produced different results. WNK1 2021-2382 bound A2bp1, Aes, Atn1, Celf4, Flot1, Flot1 (out-of-frame) and Klf10. WNK1 2031-2126, which contains a short region highly conserved in WNK3, bound only one of the eight preys tested, Celf4. Like WNK1 2021-2382,

WNK2 1967-2217 interacted with Flot1 and Klf10, but it was the only C-terminal bait to interact with Smap2 (except WNK3). Lastly, WNK4 1093-1222 interacted only with Flot1 (out-of-frame) (Table 5).

Thus, each of the WNK1/2/4 C-terminal baits interacted with one or more of the eight preys from the WNK3 C-terminal screen (at or above the concentration of 3-AT necessary to control background). None of these baits, however, interacted above background with all eight preys from the WNK3 C-terminal screen (Table 5).

These results provide a useful source of information for further comparative study of the WNK family.

Chapter Three: Figures

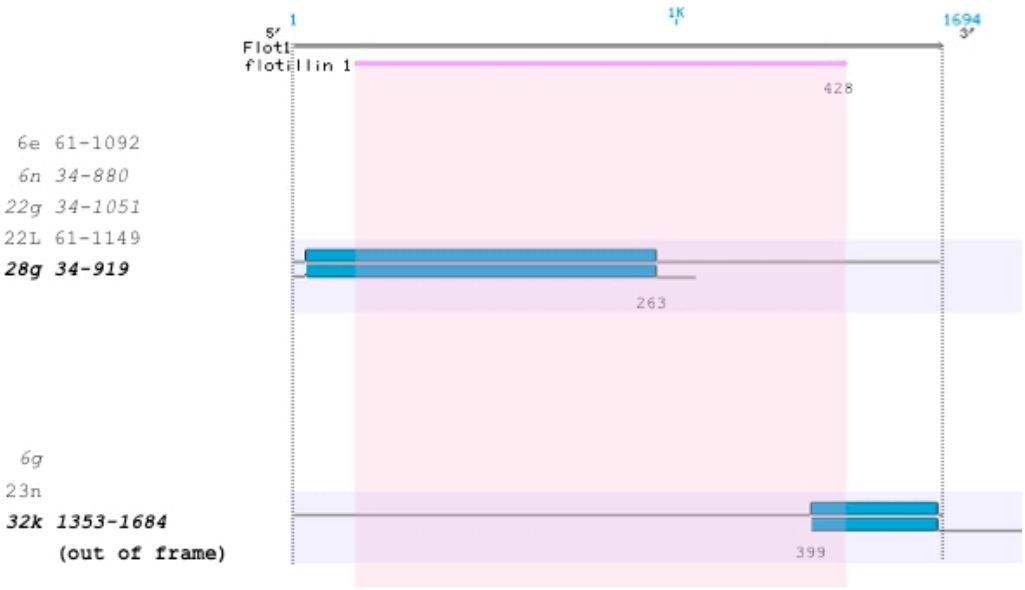


Figure 7. The WNK3 C-terminal screen yielded 8 inserts from Flot1.

Six preys encoded residues 1-263 of 428 (mouse); two preys corresponded to out-of-frame sequence for residues 399-428 of 428. (Pink, CDS; blue, insert).

```
CLUSTAL 2.0.10 multiple sequence alignment


O75955          MFFTCGPNEAMVVSGFCRSPPVMVAGGRVFVLPCIQQIQRISLNTLTLNVKSEKVYTRHG 60 FLOT1_HUMAN
O08917          MFFTCGPNEAMVVSGFCRSPPVMVAGGRVFVLPCIQQIQRISLNTLTLNVKSEKVYTRHG 60 FLOT1_MOUSE
                ************************************************************

O75955          VPISVTGIAQVKIQGQNKEMLAAACQMFLGKTEAEIAHIALETLEGHQRAIMAHMTVEEI 120
O08917          VPISVTGIAQVKIQGQNKEMLAAACQMFLGKTEAEIAHIALETLEGHQRAIMAHMTVEEI 120
                ************************************************************

O75955          YKDRQKFSEQVFKVASSDLVNMGISVVSYTLKDIHDDQDYLHSLGKARTAQVQKDARIGE 180
O08917          YKDRQKFSEQVFKVASSDLVNMGISVVSYTLKDIHDDQDYLHSLGKARTAQVQKDARIGE 180
                ************************************************************

O75955          AEAKRDAGIREAKAKQEKVSAQYLSEIEMAKAQRDYELKKAAYDIEVNTRRAQADLAYQL 240
O08917          AEAKRDAGIREAKAKQEKVSAQCLSEIEMAKAQRDYELKKATYDIEVNTRRAQADLAYQL 240
                ********************* *****************:******************

O75955          QVAKTKQQIEEQRVQVQVVERAQQVAVQEQEIARREKELEARVRKPAEAERYKLERLAEA 300
O08917          QVAKTKQQIEEQRVQVQVVERAQQVAVQEQEIARREKELEARVRKPAEAERYRLERLAEA 300
                ************************************************:*******

O75955          EKSQLIMQAEAEAASVRMRGEAEAFAIGARARAEAEQMAKKAEAFQLYQEAAQLDMLLEK 360
O08917          EKAQLIMQAEAEAESVRMRGEAEAFAIGARARAEAEQMAKKAEAFQMYQEAAQLDMLLEK 360
                **:********** ********************************:*************

O75955          LPQVAEEISGPLTSANKITLVSSGSGTMGAAKVTGEVLDILTRLPESVERLTGVSISQVN 420
O08917          LPQVAEEISGPLTSANKITLVSSGSGTMGAAKVTGEVLDILSRLPESVERLTGVSISQVN 420
                ****************************************:*******************

O75955          H-KPLRTA 427
O08917          HNKPLRTA 428
                *  ******

22L yellow
32K blue (insert out of frame)
```

Figure 8. Flotillin-1 is 98% identical between mice and humans.

The shaded areas represent the sequences that were subjected to PRATT analysis (Table 4).

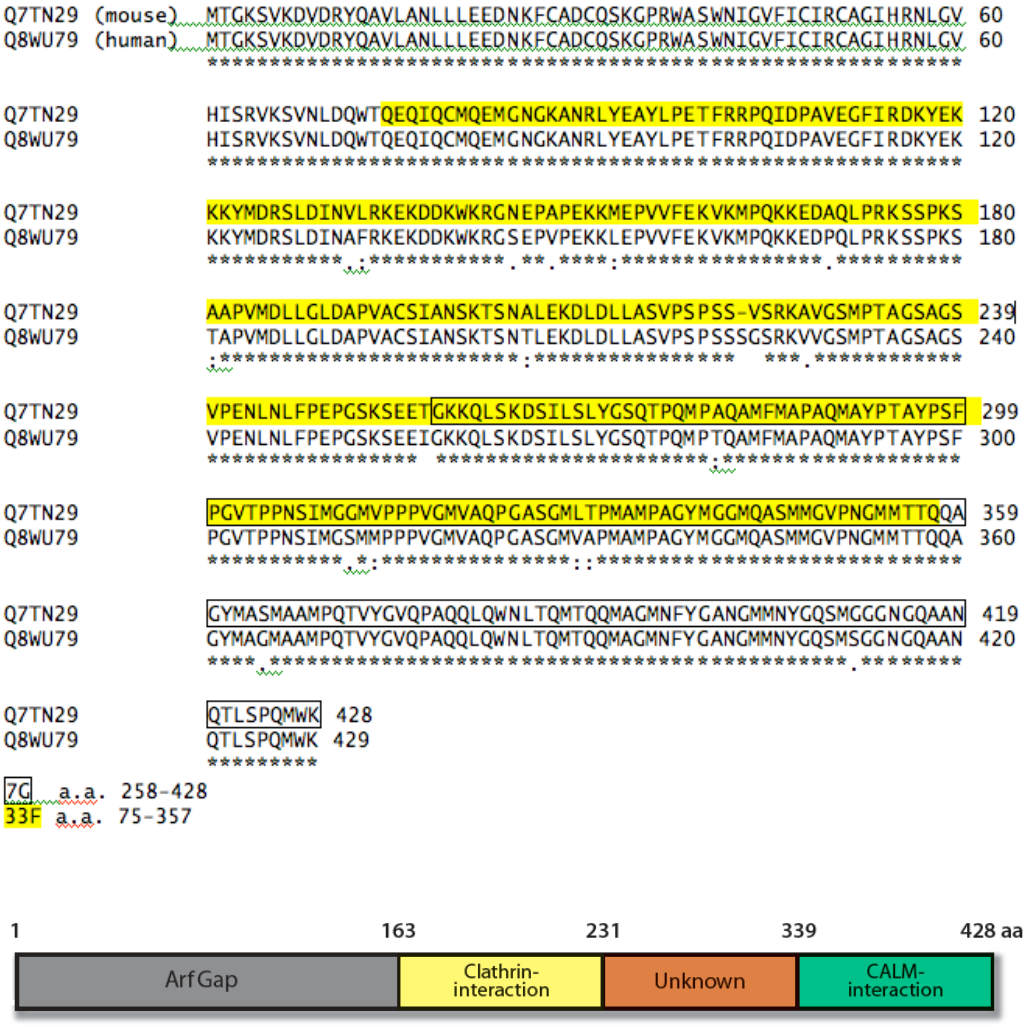


Figure 9. Smap2 is 95% identical between humans and mice.

Figure 10. A2bp1/Fox1a is 98% identical between humans and mice.

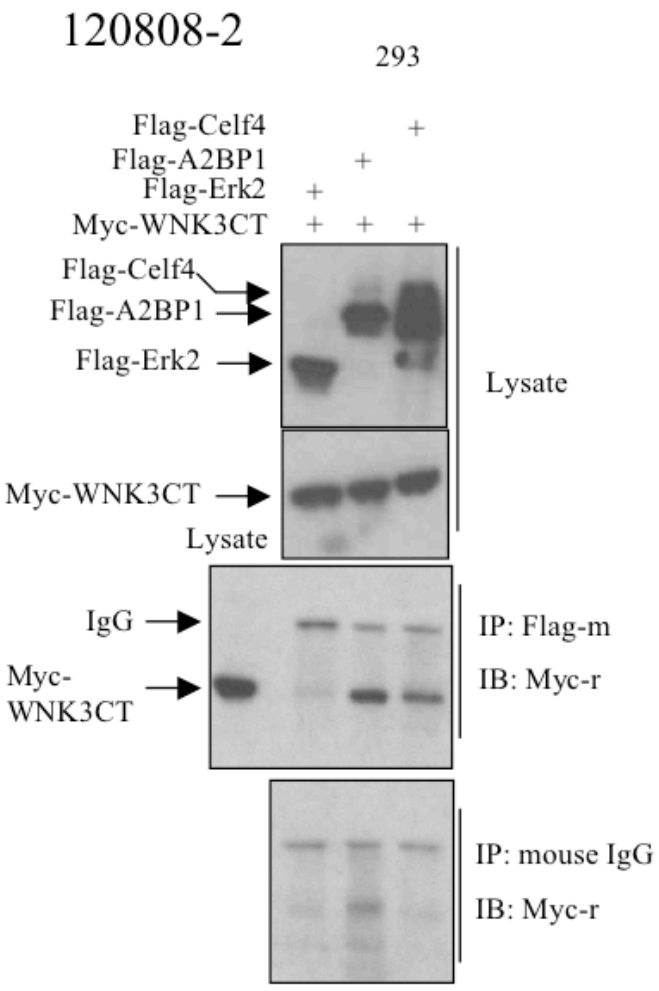


Figure 11. A2bp1 & Celf4 co-immunoprecipitate with the WNK3 C terminus. Courtesy of Wei Chen.

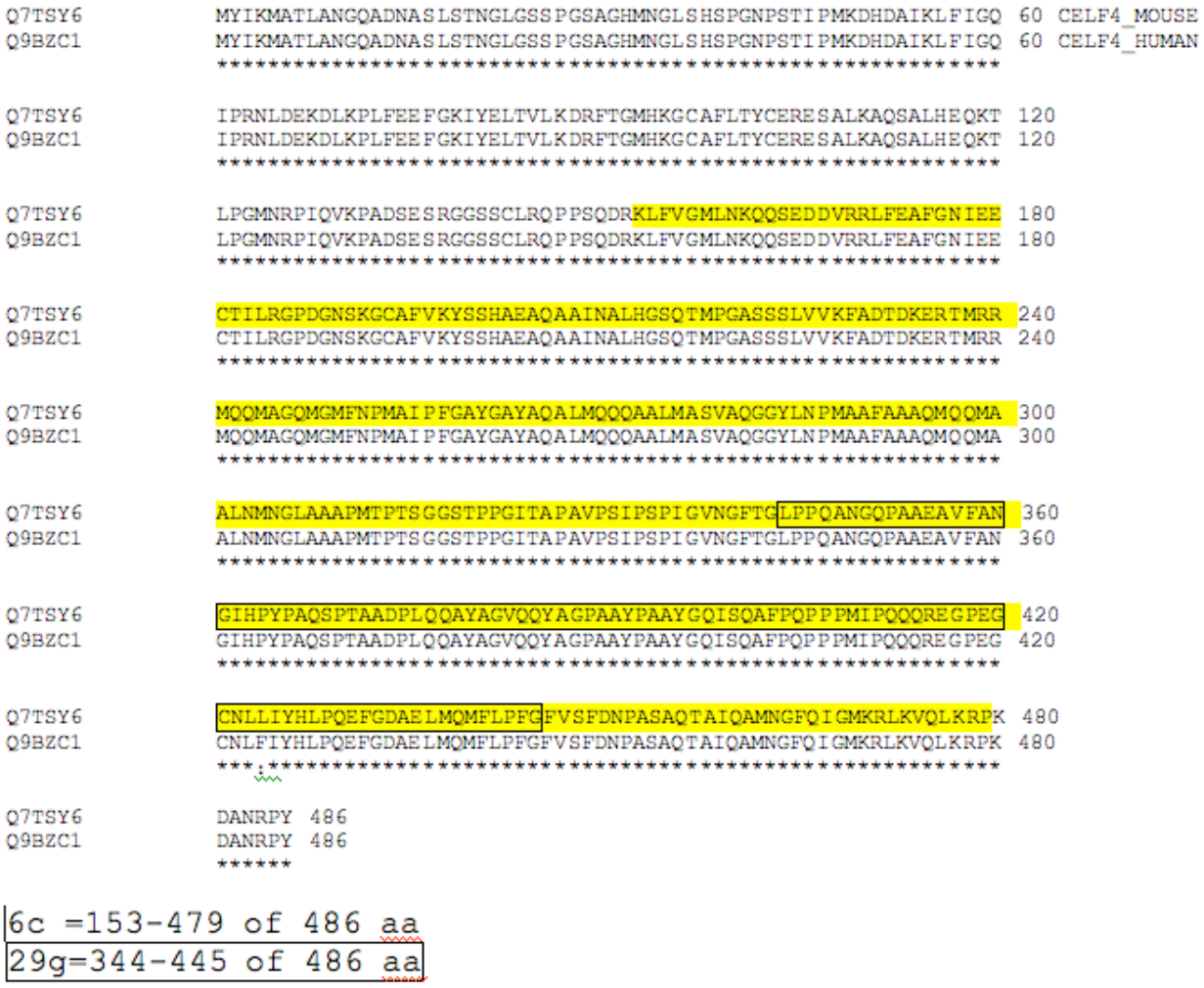


Figure 12. Celf4 came out of the WNK3 C-terminal screen two times.

Figure 13. Klf10 contains a protein pattern found in other preys.

Klf10 emerged from the WNK3 C-terminal screen and has been shown by PRATT analysis (Table 4) to contain a protein pattern similar to two in Flot1, which came out of the same screen.

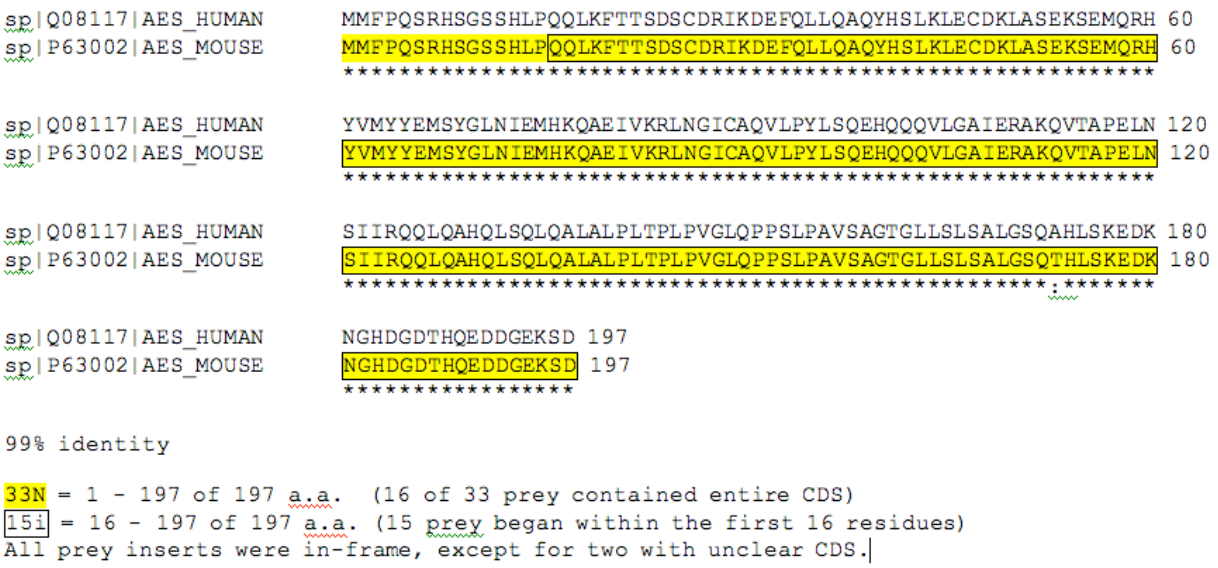


Figure 14. Aes came out of the WNK3 C-terminal screen over thirty times.

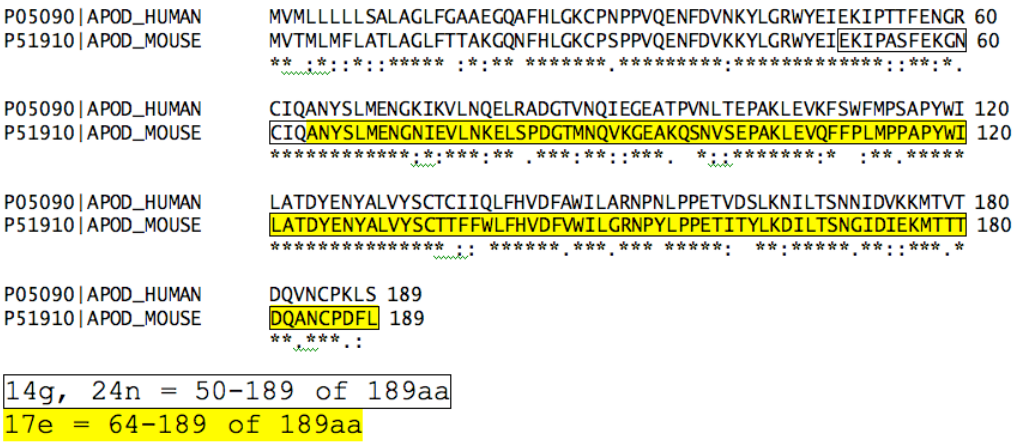


Figure 15. ApoD is 72% identical between mice and humans.

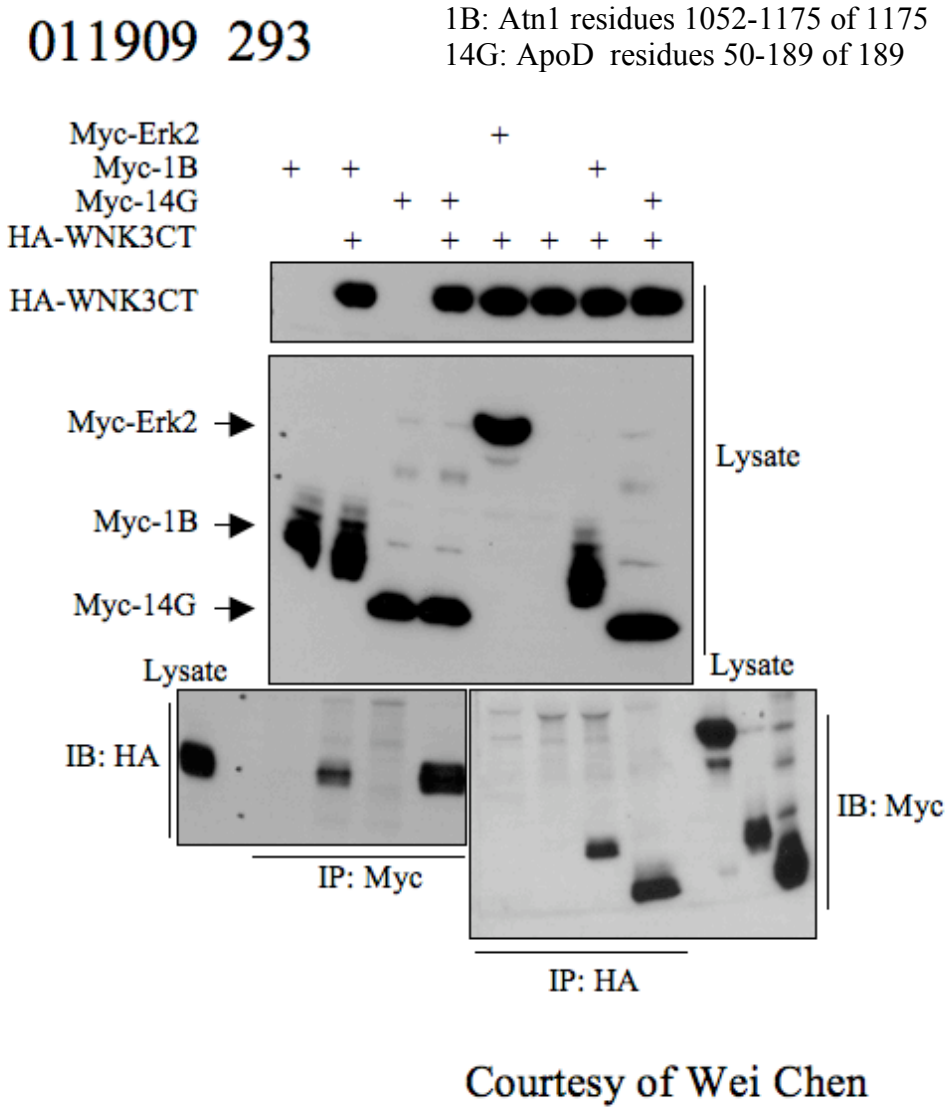


Figure 16. ApoD (14G) & Atn1 (1B) co-immunoprecipate with the WNK3 C terminus.

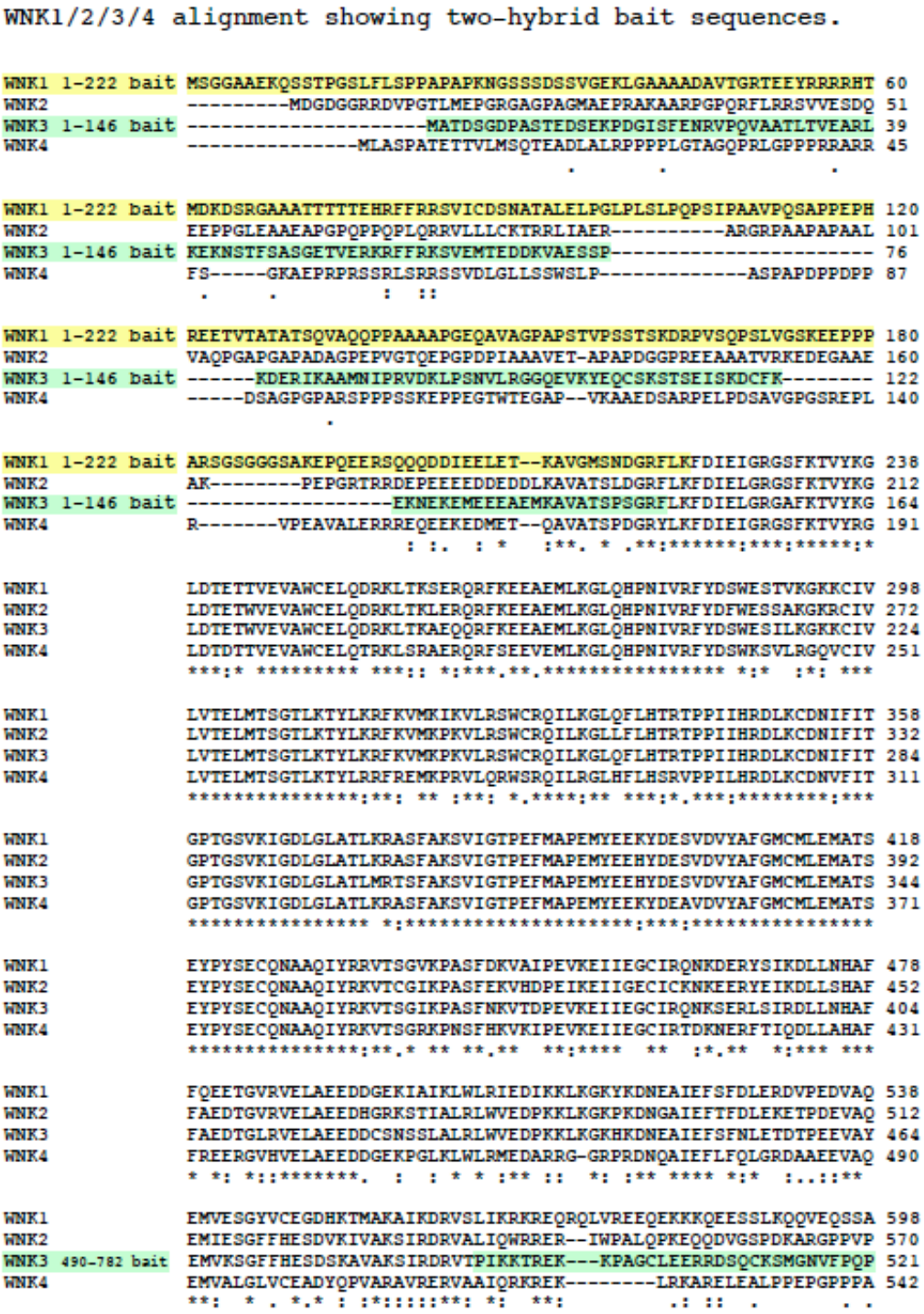


Figure 17. WNK1-4 alignment showing two-hybrid baits.

WNK1/2/3/4 alignment showing two-hybrid bait sequences (cont.).

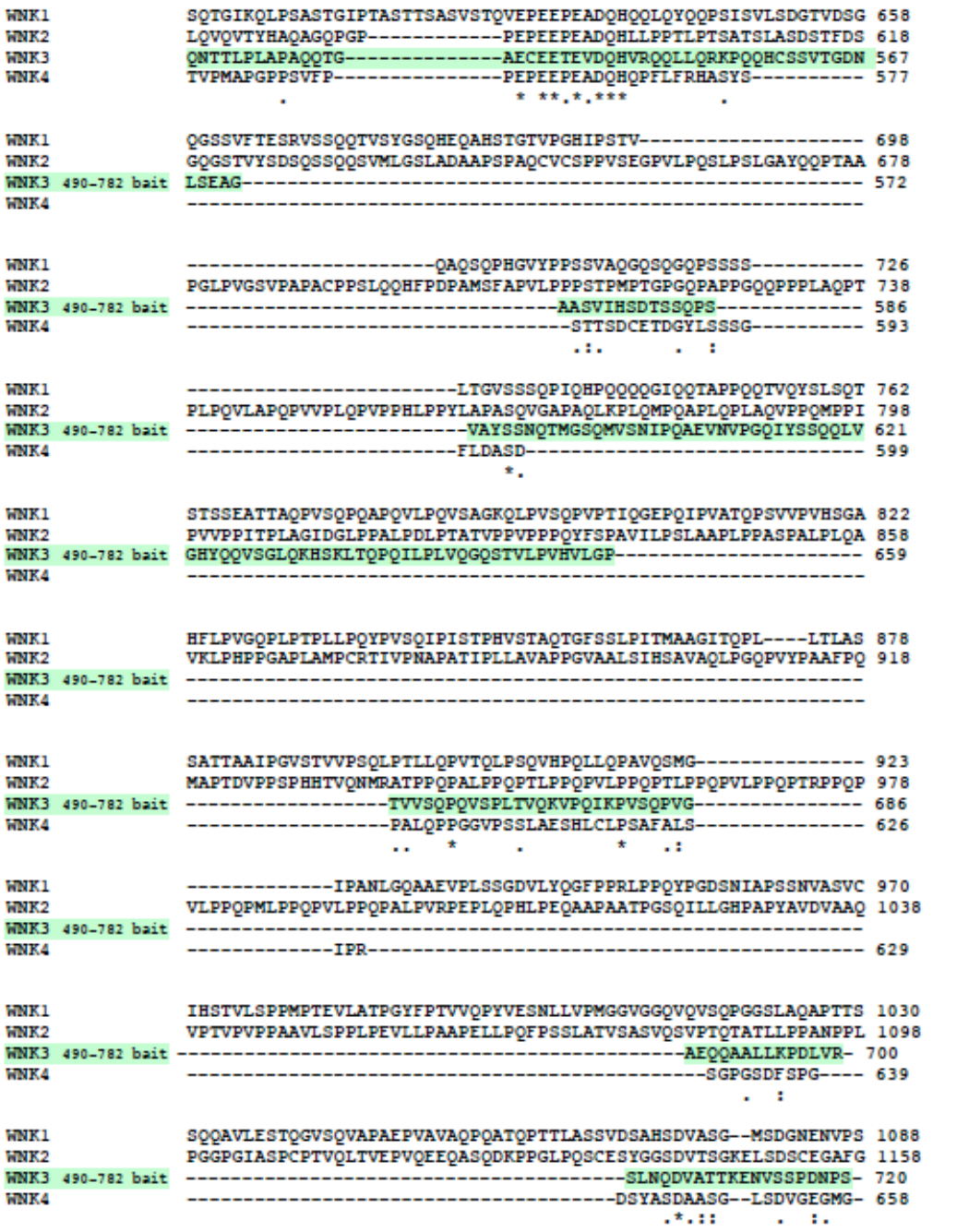


Figure 17 (pg 2 of 5)

WNK1/2/3/4 alignment showing two-hybrid bait sequences (cont.).

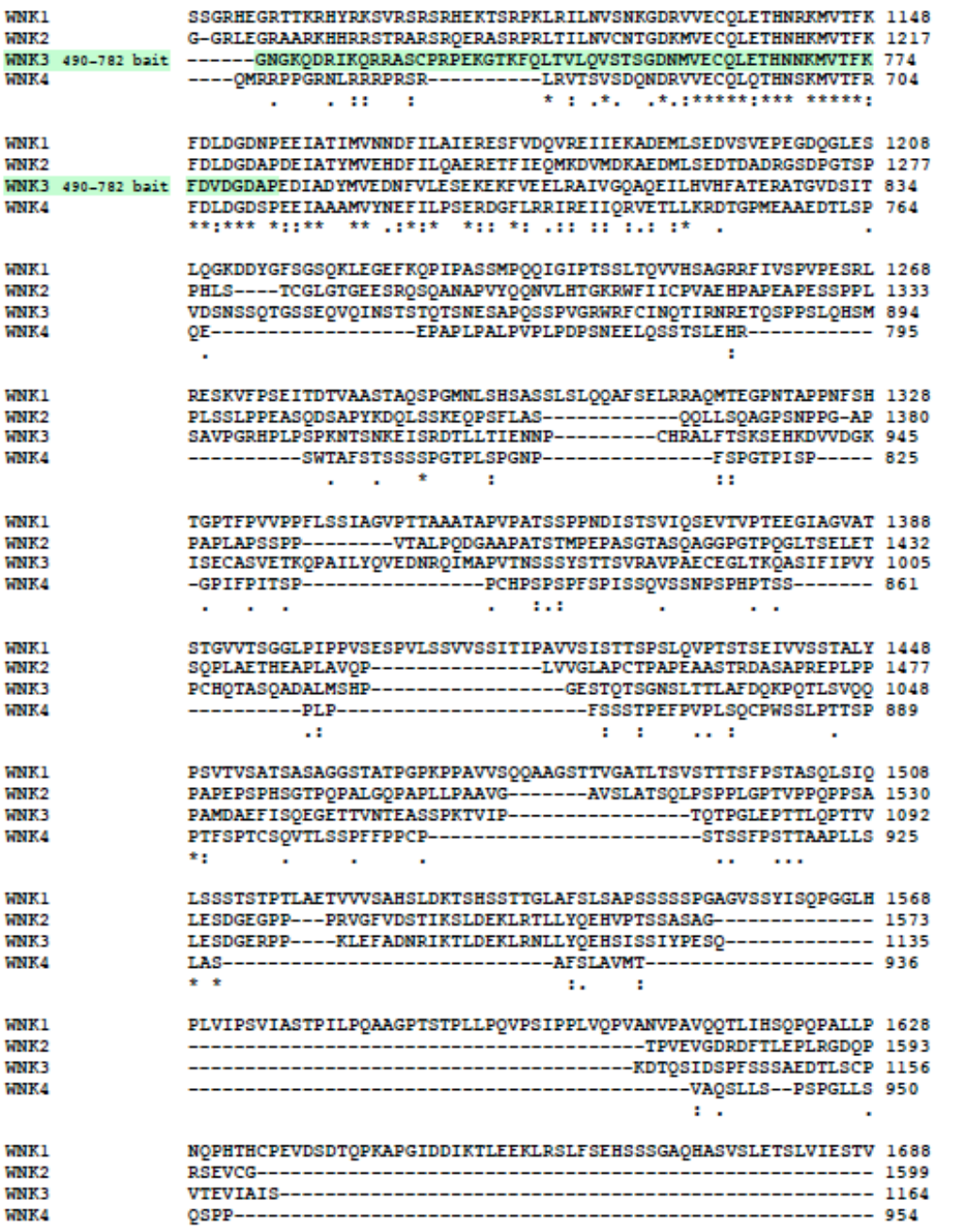


Figure 17 (pg 3 of 5)

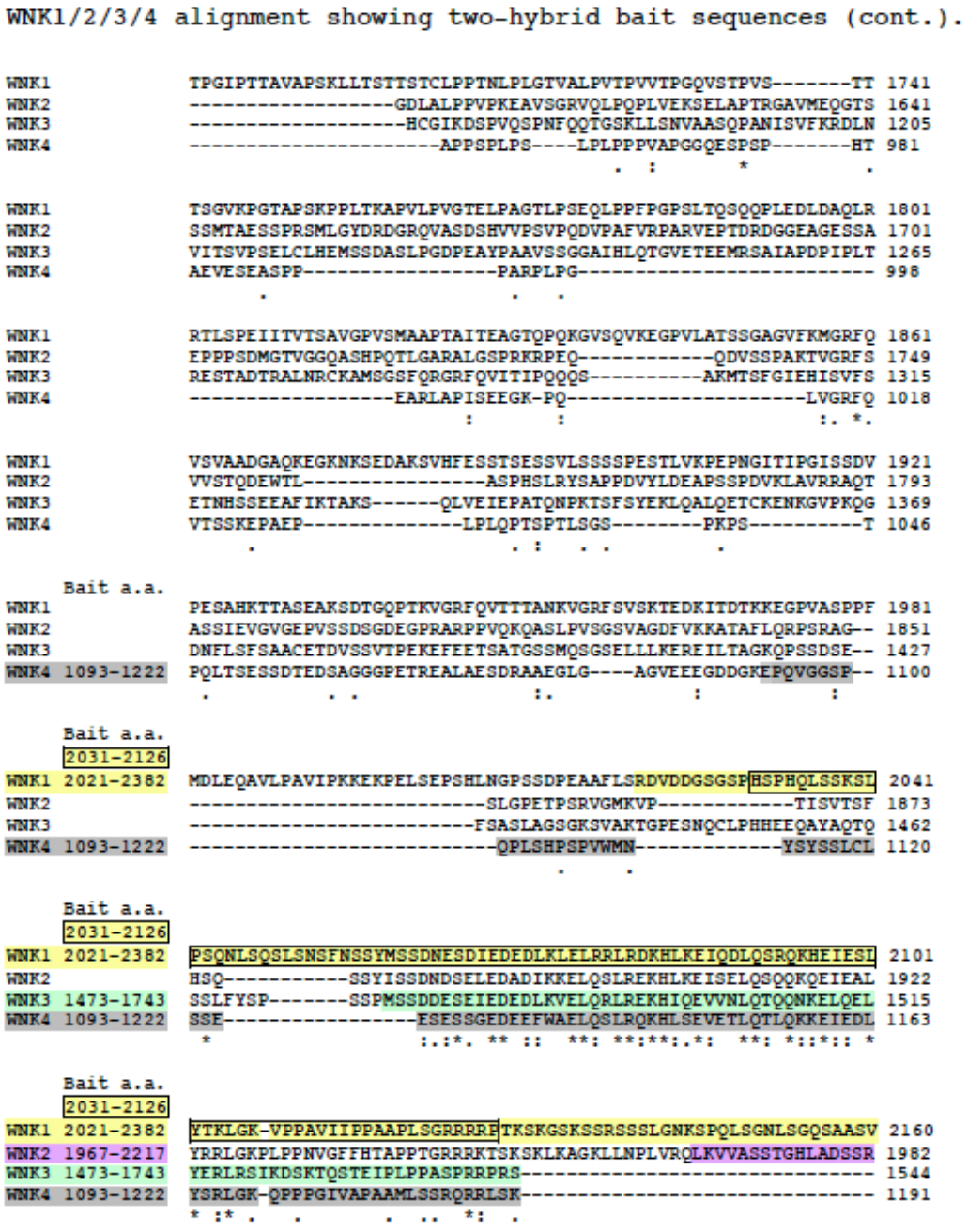


Figure 17 (pg 4 of 5)

WNK1/2/3/4 alignment showing two-hybrid bait sequences (cont.).

```
     Bait a.a.
WNK1 2021-2382  LHPQQTLHPPGNIPESGQNQLL----QPLKPSPSSDNLYSAFTSDGAISVPSLSAPGQGT 2216
WNK2 1967-2217  GPPAKDPAQASVGLTADSTGLSGKAVQTQQPCSVR----------ASLSSDICSGLASDG 2032
WNK3 1473-1743  ---------FKSKLRSRPQSLT-------------------HVDNGIVATDPLCVESNAA 1576
WNK4 1093-1222  ----------GSFPTSRRNSLQ----RSEPPGP------------GIMRRNSLSGSSTGS 1225
                              :    *                        . :     .  .

     Bait a.a.
WNK1 2021-2382  SSTNTVGATVNSQAAQAQPPAMTSSRKGTFTDDLHKLVDNWARDAMNLSGRRGSKGHMNY 2276
WNK2 1967-2217  GGARGQGWTVYHPTSERVTYKSSSKPRARFLSGPVSVSIWSALKRLCLGKEHSSRSSTSS 2092
WNK3 1473-1743  SCQQ-----------------SPASKKGMFTDDLHKLVDDWTKEAVGNSLIKPSLNQLKQ 1619
WNK4            QEQ-------------------RASKGVTFAGDVGRM----------------------- 1243
                                       :.    * ..   :

     Bait a.a.
WNK1 2021-2382  EGPGMARKFSAPGQLCISMTSNLGGSAPISAASATSLGHFTKSMCPPQQYG--------- 2327
WNK2 1967-2217  LAPGPEPGPQPALHVQAQVNNSNNKKGTFTDDLHKLVDEWTSKTVGAAQLKPTLNQLKQT 2152
WNK3 1473-1743  SQHKLETENWN--KVSENTPSTMGYTSTWISSLSQIRGAVPTSLPQGLSLPSFPGPLSSY 1677
WNK4            ------------------------------------------------------------

     Bait a.a.
WNK1 2021-2382  --FPATPFGAQWSGTGGPAPQPLG------QFQPVGT-ASLQNFNISNLQKSISNPPGSN 2378
WNK2 1967-2217  QKLQDMEAQAGWAAPGEARAMTAPRAGVGMPRLPPAP-GPLSTTVIPGAAPTLSVPTPDP 2211
WNK3 1473-1743  GMPHVCQYNAVAGAGYPVQWVGISGTTQQSVVIPAQSGGPFQPGMNMQAFPTSSVQNPAT 1737
WNK4            ------------------------------------------------------------

     Bait a.a.
WNK1 2021-2382  LRTT-- 2382
WNK2 1967-2217  ESEKPD 2217
WNK3 1473-1743  IPPGPK 1743
WNK4            ------
```

Figure 17 (pg 5 of 5)

Chapter Four: Conclusions and future directions

My goal with these interaction mapping efforts has been to build a collection of putative WNK3 interactors to serve as a source of information and project leads for the ongoing research program of the Cobb laboratory. The yeast two-hybrid screens described here have yielded hundreds of putative interactors. While this written work has only dealt with a small number of the most interesting putative interactors, together they point toward a number of unexpected roles for WNK3, including putative interactions with RNA-binding proteins, transcriptional regulators and proteins implicated in developmental disorders and neurodegenerative disease.

Bearing in mind the effects of WNK1 on vesicular fusion, exocytosis and membrane trafficking--in conjunction with its primary localization patterns--it is plausible to infer a role for WNK3 in, e.g., the trafficking of ApoD, or the dynamics of vesicular budding via interaction with Smap2. Additionally, WNK3 has a number of binding domains that, e.g., could allow it to participate in signaling events at lipid rafts via Flot1. This platform function could also account for the interaction of WNK3 with A2bp1 and Celf4, giving it a role in staging

RNA-binding events. Because WNK3 (like WNK1) resides in both the cytoplasm and the nucleus, its interaction with these two RNA-binding proteins could be nuclear in scope and involve control of their localization.

Putative interactors chosen for follow-up should undergo a two-hybrid bait-prey swap, because it makes use of readily available materials and provides further validation of a given protein:protein interaction. Pairwise two-hybrid testing with various truncations of the WNK3 bait and chosen prey would also define the minimum regions necessary for interaction. Similarly, pairwise two-hybrid testing of preys with other WNK family two-hybrid baits may yield useful information, as discussed herein.

A battery of tools will be needed to evaluate interactions. Antibodies will be needed for immunofluorescence and immunoprecipitation. Binding-deficient mutants would also be of value, because binding-deficiency is less disruptive than protein elimination. Such mutants would make it possible to use small interfering RNA (siRNA) to knockdown endogenous protein, followed by introduction of binding-deficient, siRNA-resistant mutants. Choosing cell lines and establishing a system amenable to testing will depend on the interaction being tested. For instance, the choice of a putative interactor based on the hypothesis that WNK3 plays a role in exocytosis might ultimately be most easily demonstrated in bovine

adrenal chromaffin cells, but this would not necessary be the appropriate type of cell for evaluating all interactions. Eventually, the development of an inducible knockout animal could produce the most biologically relevant insight.

The story of the WNK kinases will go on. With a connection to ion flux diseases well-established, the WNK family will surely continue to attract attention for many years, particularly given their potential as drug targets. As the gamut of WNK functions--and, importantly, dysfunction--comes into sharper focus, it is likely these protein kinases will be shown to play more roles in the cell. I hope that the work I will leave behind may provide some foundation for clearer insight into these peculiar proteins.

Tables Section

	Concentration (mM)	
	Intracellular	Extracellular
Na^{+}	5-15	145
K^{+}	140	5
Cl^{-}	4-30	110
Ca^{+2}	0.0001	1-2

Table 1. Electrolyte concentrations inside and outside mammalian cells.

pVJL11 bait	Residues	Made by	3-AT (mM)
WNK1 N terminus	1-222 of 2382	Byung-Hoon Lee	0
WNK1 C terminus	2021-2382 of 2382	Tony Anselmo	0.5
WNK1 C terminus	2031-2126 of 2382	Byung-Hoon Lee	3
WNK2 C terminus	1967-2217 of 2217	Staci Cummings	0
WNK3 N terminus	1-146 of 1743	Jon Self	2.5
WNK3 midsection	490-782 of 1743	Jon Self	6
WNK3 C terminus	1473 - 1743 of 1743	Steve Stippec	2.5
WNK4 C terminus	1093-1222 of 1243	Svetlana Earnest	0.5
Flot1 full-length	1-428 of 428	Steve Stippec	0

Table 2. Two-hybrid screen pVJL11 bait constructs.

The amount of 3-AT necessary to control background is shown.

Yeast two-hybrid screens with WNK3				
Bait	Y2H#	Strong interactors	Identified	# replicates
C terminus	Y2H4	267	201	35
Midsection	Y2H15	180	11	0
N terminus	Y2H12	224	101	9

Table 3. Results of two-hybrid screens with WNK3 baits.

Following library transformation, colonies exhibiting robust growth and/or strong beta-gal activity were selected for prey plasmid isolation and sequencing. Some prey plasmids appeared multiple times and in different frames.

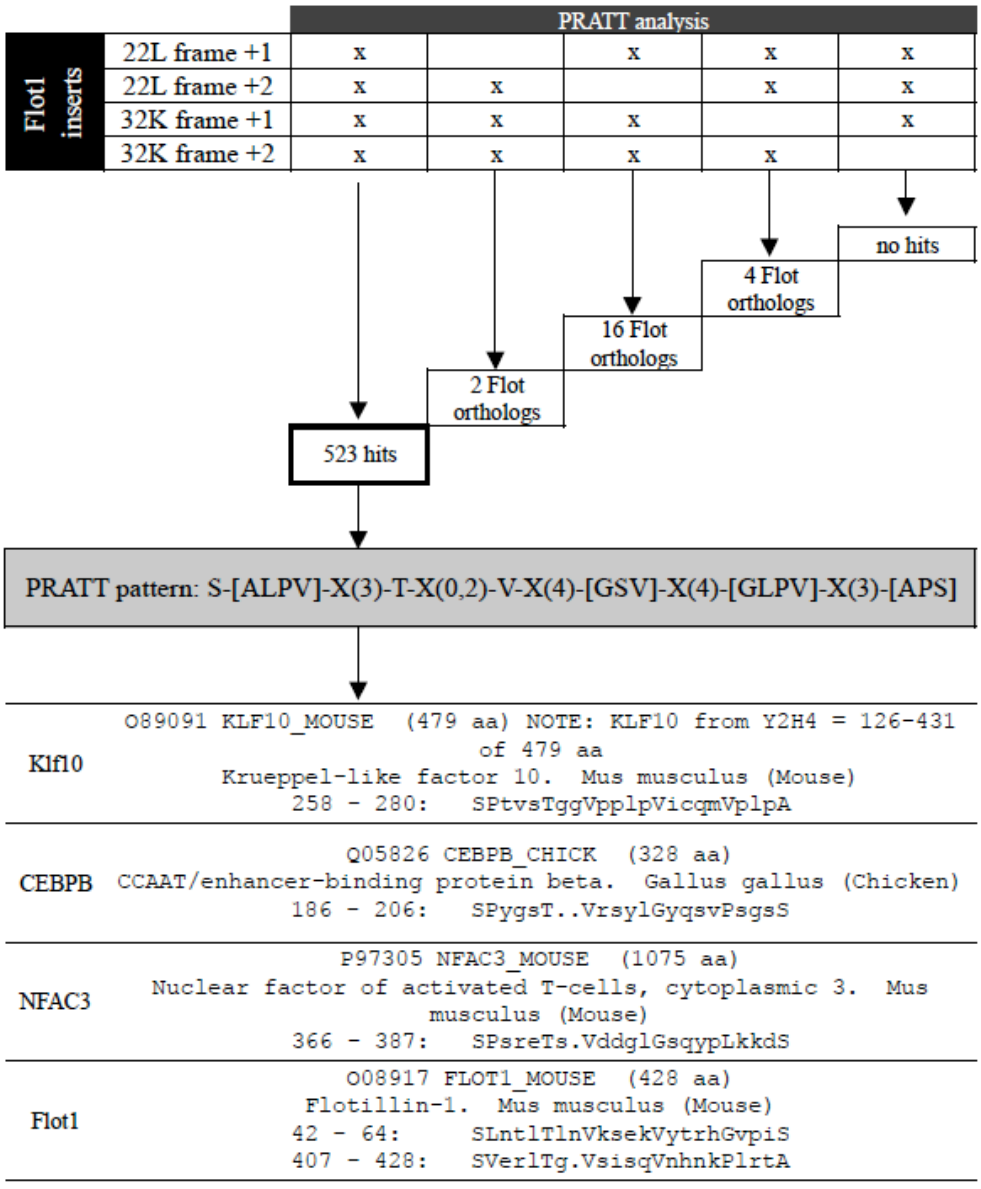


Table 4. PRATT analysis of Flot1 prey inserts.

Two Flot1 inserts from the WNK C-terminal screen (22L and 32K) (Figure 7 and Figure 8) were subjected to PRATT analysis. Each insert was analyzed in frames +1 and +2. Prey insert sequences follow on next page.

(continued from previous page)

>22L frame +1 (encodes 1-263 of 428 residues) Flot1
AVPTPFWGSAGLRASQDPGGVAYGDVPEAAAST **MFFTCGPNEAMVVSGFCRSPPVMVAGG**
RVFVLPCIQQIQRISLNTLTLNVKSEKVYTRHGVPISVTGIAQVKIQGQNKEMLAAACQM
FLGKTEAEIAHIALETLEGHQRAIMAHMTVEEIYKDRQKFSEQVFKVASSDLVNMGISVV
SYTLKDIHDDQDYLHSLGKARTAQVQKDARIGEAEAKRDAGIREAKAKQEKVSAQCLSEI
EMAKAQRDYELKKATYDIEVNTRRAQADLAYQLQVAKTKQQIEEQRVQVQVVERAQ QWQC
RSRRSPGARRSWRLACASQQRLSATAGALRXEXPADHAAEPKLSLCGCEGKLRPLL-
356 residues total

>22L frame +2 (encodes 49-63 of 145 residues) predicted Flot1 fragment; B0V2I9_HUMAN; unreviewed
QSPPRSGVLRASALLKTPVGLLTGTFRKLQLQ **PCFSLVAQMRPWWSP** GSAGAPQSWWPEA
ECLSYPAFSKSRGSLSTH-
78 residues total

>32K frame 1
GHLEPPAGECGEAHRRQHLPGKSQQAFKDSVSPQSWTVWPERSHWGVVSSHAPHLASLSM
APDRGAHLSLPNSPCLALEGLPFLAHCQCPAPRLIIFSPSQLFNFLIKLD
(110 residues)

>32K frame +2 (encodes 399-428 of 428 residues) Flot1
DILSRLPESVERLTGVSISQVNHNKPLRTA-
30 residues total

	WNK1 C-term bait residues 2021-2382	WNK1 C-term bait residues 2031-2126	WNK2 C-term bait residues 1967-2217	WNK4 C-term bait residues 1093-1222	WNK1 N-term bait residues 1-222
A2bp1	+++	--	--	--	
Aes	+++	--	--	--	
Atn1	++++	--	--	--	
Celf4	++	++++	--	--	
Flot1	++++	--	++++	--	
Flot1*	++++	--	--	+	
Klf10	+	--	+++	--	
Smap2	--	--	+++	--	
Rab14					--
Eef1a1					--

*out of frame

Table 5. WNK3 two-hybrid preys tested against other WNK baits.

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