ANA ISABEL VIEIRA LOPES MENDES

SIGNAL TRANSDUCTION PATHWAYS INVOLVING THE HYPERTENSION-RELATED WNK1 AND WNK4 PROTEIN KINASES

Lisboa

2011

n° de arquivo

ANA ISABEL VIEIRA LOPES MENDES

SIGNAL TRANSDUCTION PATHWAYS INVOLVING THE HYPERTENSION-RELATED WNK1 AND WNK4 PROTEIN KINASES

Dissertação apresentada para obtenção do Grau de Doutor em Biologia, na especialidade de Genética Molecular, pela Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia.

Lisboa

2011

A Deus...

Agradecimentos

Ao Doutor Peter Jordan por me ter acolhido no seu laboratório e pela confiança em mim depositada. Agradeço o estímulo, a preciosa orientação e rigor científicos, a amizade e o exemplo constantes, que muito contribuíram para a minha formação e que foram imprescindíveis para a realização deste trabalho.

À Professora Ilda Sanches pela disponibilidade, pelo interesse e supervisão científicos prestados ao longo do decurso de todo o trabalho e pelas sugestões e comentários na preparação deste manuscrito.

Às pessoas anónimas que dando o seu consentimento informado se tornaram o imprescindível objecto de estudo de parte deste trabalho.

Ao Ministério da Ciência e Ensino Superior e à Fundação para a Ciência e a Tecnologia por terem possibilitado a realização deste trabalho através da concessão da bolsa de doutoramento de que fui recipiente de 2006 a 2009 (SFRH/BD/23001/2005), através do Programa de Financiamento Plurianual do Centro de Investigação em Genética Molecular Humana e do projecto POCI/SAU-MMO/56439/2004. À FEBS pelo seu imprescindível apoio para participar num dos mais prestigiados encontros científicos na área da Transdução de Sinal e das Proteínas Cinases.

Ao Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA) pelas condições básicas necessárias para a realização deste projecto. Ao antigo Centro de Genética Humana, agora Departamento de Genética, na pessoa do Doutor João Lavinha, por me ter aceite no seu Departamento proporcionando-me assim a oportunidade de desenvolver este trabalho.

A todos os colegas do Departamento de Genética do INSA que me brindaram ao longo destes anos com o seu conhecimento, interesse, apoio, exemplo ou experiência. Em

particular, ao Zé Manel da Unidade de Citogenética, pela assistência na Cultura de Células e aos colaboradores da Unidade de Tecnologia e Inovação, pelas numerosas sequenciações e análises por dHPLC. Aos colegas do Grupo de Fibrose Quística, a Filipa, o Toby, a Anabela, a Ana Carina, a Marisa, o Carlos e o Simão agradeço a sua singular colaboração e conselhos científicos.

Aos maravilhosos colegas, agora amigos, do Grupo de Oncobiologia pelo acolhimento, pelo excelente ambiente de trabalho e principalmente pela simpatia, camaradagem e cumplicidade que sempre demonstraram e claro, pela amizade e pela infindável paciência concedidas. À Sónia, por tudo o que me ensinou, pela permanente disponibilidade, pelo diálogo científico, e pelas numerosas boleias. Ao Paulo pelo valioso acompanhamento e apoio científicos. À Vânia pelos conselhos úteis e por me demonstrar que é possível (e saudável) ter algum tempo livre durante um doutoramento. À Patrícia pela ajuda e incentivo e pela companhia nas horas indecentes. À Andreia e à Elizabeth que embora já não estejam no laboratório estarão sempre presentes no meu coração.

Aos meus amigos, Inês, Cláudia, Catarina, Tó Zé, Lara, Pedro, Carlos (o meu guru pessoal do LaTeX) e Sara pelo vosso apoio independentemente da distância. Obrigada pela vossa amizade que sobrevive não só à distância que nos separa, bem como à passagem do tempo. E também aos meus amigos mais locais, Cátia, Beta, Zé, Pedro e Ricardo por estarem sempre presentes e disponíveis para me aturarem e me devolverem um pouco à realidade.

Aos meus pais, à minha irmã Sofia e ao meu primo Pedro, pela eterna presença na minha vida e pelo encorajamento, compreensão, apoio, ajuda e paciência que me dedicaram durante mais uma etapa decisiva da minha vida.

Ao Tiago pela compreensão, carinho, amizade e amor, sem os quais nada seria possível.

Resumo

Os genes *WNK1* e *WNK4* pertencem à subfamília WNK de proteínas cinases e a sua mutação causa pseudohipoaldosteronismo tipo II, uma forma rara de hipertensão familiar com hipercalemia e hipercalciuria. Os mecanismos moleculares subjacentes a esta condição envolvem a regulação da homeostase renal de electrólitos e a modulação de diversos canais iónicos e transportadores *via* cinases WNK. Além disso, as WNKs também estão descritas como intervenientes em vias de transdução de sinal relacionadas com sobrevivência e proliferação celulares.

O objectivo desta tese foi identificar novas proteínas que interajam com a WNK1 e a WNK4, e as vias de tradução de sinal subjacentes. Primeiramente, descobriu-se que a WNK1 forma um complexo proteico com a Rab-GAP TBC1D4, fosforilando-a *in vitro*. Mostrou-se ainda que os níveis de expressão da WNK1 regulam a expressão à superfície de células HEK293 do transportador da glucose GLUT1. A WNK1 aumenta a ligação da TBC1D4 às proteínas reguladoras 14-3-3, ao mesmo tempo que reduz a interacção com a pequena GTPase exocítica Rab8A. Estes efeitos são dependentes da sua actividade catalítica. Em conjunto, estes dados sugerem uma via que regula a entrada constitutiva da glucose na célula *via* GLUT1, cujo nível de expressão está relacionado com várias doenças humanas.

Seguidamente, descobriu-se que a WNK4 promove a expressão do canal de cloro CFTR à superfície de células de mamífero. O mecanismo através do qual a WNK4 age sobre a CFTR envolve a interacção com a tirosina cinase Syk, a qual demonstrámos que fosforila o resíduo de tirosina 512 (Tyr512) no primeiro domínio de ligação ao ATP da CFTR. A presença da WNK4 impede esta fosforilação *in vitro* de forma independente da sua actividade de cinase. Em células BHK21 a expressar estavelmente a CFTR, a Syk reduz, enquanto a WNK4 promove, a expressão à superfície deste canal. Mutando a Tyr512

concluiu-se que a sua fosforilação é um novo sinal que regula a prevalência da CFTR à superfície das células e que a WNK4 e a Syk têm papéis antagonistas neste processo.

Por fim, foram detectadas dez variantes da *WNK4* num grupo de doentes e controlos portugueses, que posteriormente foram testadas para associação com a hipertensão e/ou osteoporose. Apesar de nenhuma ter apresentado associação significativa com a hipertensão, uma alteração *missense* rara (rs56116165) num resíduo de arginina altamente conservado apresentou uma associação nominal com a osteoporose. Com esta descoberta defendemos que esta alteração é uma variante alélica rara, num gene candidato implicado numa função biológica na homeostase renal do cálcio, que pode contribuir para uma predisposição genética para a osteoporose.

Abstract

The genes *WNK1* and *WNK4* belong to the subfamily of WNK protein kinases and their mutation causes pseudohypoaldosteronism type II, a rare familial form of hypertension with hyperkalemia and hypercalciuria. The molecular mechanisms underlying this condition involve the regulation of renal electrolyte homeostasis and the modulation of diverse ion channels and transporters *via* WNK kinases. Additionally, WNKs have also been reported to participate in signal transduction pathways related to cell survival and proliferation.

The objective of the present thesis was to identify novel WNK1 and WNK4 interacting proteins and the underlying signal transduction pathways. First, it was found that WNK1 forms a protein complex with the Rab-GAP TBC1D4 and phosphorylates it *in vitro*. It was shown that the expression levels of WNK1 regulate surface expression of the constitutive glucose transporter GLUT1 in HEK293 cells. WNK1 is shown to increase the binding of TBC1D4 to regulatory 14-3-3 proteins while reducing its interaction with the exocytic small GTPase Rab8A. Moreover, these effects were kinase activity-dependent. Together, the data describe a pathway regulating constitutive glucose uptake *via* GLUT1, the expression level of which is related to several human diseases.

Second, WNK4 was found to promote the cell surface expression of the CFTR chloride channel in mammalian cells. The mechanism by which WNK4 acts on CFTR involves interaction with the tyrosine kinase Syk, which we found to phosphorylate tyrosine 512 (Tyr512) in the first nucleotide-binding domain of CFTR. The presence of WNK4 prevents this *in vitro* phosphorylation in a kinase-independent manner. In BHK21 cells stably expressing CFTR, Syk reduces, while WNK4 promotes, the cell surface expression of CFTR. Mutation of Tyr512 revealed that its phosphorylation is a novel signal

regulating the prevalence of CFTR at the cell surface and that WNK4 and Syk play an antagonistic role in this process.

Finally, ten *WNK4* variants were detected in a cohort of Portuguese patients and control individuals, which subsequently were tested for association to hypertension and/or osteoporosis. Despite none of the variants yield any significant association to hypertension, a rare missense alteration (rs56116165) in a highly conserved arginine residue showed a nominal association to osteoporosis. This finding advocates that this polymorphism is a rare allelic variant, in a candidate gene with a biological function in renal calcium homeostasis, that may contribute to a genetic predisposition to osteoporosis.

List of Abbreviations

ARH	autosomal recessive hypercholesterolemia
AS160	Akt substrate of 160 KDa
Asp	aspartic acid residue
ATP	adenosine triphosphate
BHK21	baby hamster kidney
BMD	bone mineral density
cAMP	3′-5′-cyclic adenosine monophosphate
CBP-D28k	calbindin-D28k
CCD	cortical collecting duct
cDNA	mRNA-complementary DNA
CFEX	Cl^-/base exchanger SCL26A6
CFTR	cystic fibrosis transmembrane conductance regulator
Ci	Curie units
C-terminus	carboxy terminus
DAPI	4,6-diamidino-2-phenylindole
DCT	distal convoluted tubule
dHPLC	denaturing high-performance liquid chromatography
DMEM	Dulbecco's modified minimal essential medium
DNA	deoxyribonucleic acid
DSP	dithio-bis-succinimidylpropionate
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ENaC	epithelial sodium channel
ERK	extracellular signal-regulated kinase
F508del	deletion of phenylalanine (F) residue at position 508

GDP	guanosine-5′-diphosphate
GFP	green fluorescent protein
GLUT	glucose transporter
GST	glutathione S-transferase
GTP	guanosine triphosphate
GWAS	genome-wide association study
HEK293	human embryonic kidney
hnRNP	heterogeneous nuclear ribonucleoprotein
IGF-1	insulin-like growth factor 1
IPTG	isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
Kb	Kilo base pairs
KCC2	K^+/Cl^- cotransporter 2
KCC3	K^+/Cl^- cotransporter 3
kd	kinase dead
KDa	Kilodaltons
KS-WNK1	kidney-specific WNK1
L-WNK1	long WNK1
Lys	lysine residue
MAPK	mitogen-activated protein kinase
MAP2K (MEK)	MAPK kinase
MAP3K (MEKK)	MAPK kinase kinase
MAP4K	MAPK kinase kinase kinase
MDCK	Madin-Darby canine kidney II
Myc	epitope tag derived from the *c-myc* gene product
NBD1	nucleotide binding domain 1
NCC	Na^+/Cl^- cotransporter
NHE3	Na^+/H^+ exchanger

NHERF	Na^+/H^+ exchanger regulatory factor
NKCC1	$Na^+/K^+/2Cl^-$ cotransporter 1
NKCC2	$Na^+/K^+/2Cl^-$ cotransporter 2
N-terminus	amino-terminus
OMIM	Online Mendelian Inheritance in Man
OR	odds ratio
OSR1	oxidative stress-responsive kinase 1
PAK	p21-activated kinase
PBS	phosphate-buffered saline
PBS-T	PBS/0.01% Tween
PCR	polymerase chain reaction
PHA-II	pseudohypoaldosteronism type II
Phe	phenylalanine residue
Phe508del	deletion of phenylalanine residue 508
PI3K	phosphatidyl inositol-3-kinase
PKC	protein kinase C
PMSF	phenylmethanesulfonylfluoride
PTH	parathyroid hormone
PVDF	polyvinylidene difluoride
RabGAP	Rab GTPase-activating protein
RIPA	radio-immunoprecipitation assay
RNA	ribonucleic acid
ROMK	renal outer medullary K^+ channel
SDS-PAGE	sodium dodecyl sulfate-polyacrilamide gel electrophoresis
Ser	serine residue
SGK1	serum glucocorticoid-inducible protein kinase 1
SH3	Src-homology 3
siRNA	small interfering RNA oligonucleotide

SNP	single nucleotide polymorphism
SPAK	ste20-related proline alanine-rich kinase
SUMO	small ubiquitin-like modifier
Syk	spleen tyrosine (=Y) kinase
T7	epitope tag from T7 bacteriophage gene10
TAL	thick ascending limb of Henle
TBC1D1	Tre-2/USP6, BUB2, Cdc16 domain family, member 1
TBC1D4	Tre-2/USP6, BUB2, Cdc16 domain family, member 4
TBS	tris-buffered saline
TfR1	transferrin receptor 1
TGFβ	transforming growth factor beta
TGN	*trans*-Golgi network
Thr	threonine residue
TKL	tyrosine kinase-like
Tris	tris(hydroxymethyl)aminomethane
TRPM6	transient receptor potential channel melastatin subtype 6
TRPV4	transient receptor potential channel vanilloid subtype 4
TRPV5	transient receptor potential channel vanilloid subtype 5
TRPV6	transient receptor potential channel vanilloid subtype 6
Tyr	tyrosine residue
WNK	human with no K = lysine
Wnk	mouse with no K = lysine
wt	wild-type
Y512E	substitution of tyrosine (Y) for glutamate (E) at position 512
Y512F	substitution of tyrosine (Y) for phenylalanine (F) at position 512
YFP	yellow fluorescent protein

Table of Contents

List of Figures

List of Tables

I may not have gone

where I intended to go,

but I think I have ended up

where I intended to be.

Douglas Adams (1952-2001)

1

General Introduction

1.1 Cellular signalling and protein kinases

The response of a cell to changes in its environment is initiated by receptors and executed through a diverse array of intracellular signalling pathways. These signal transduction pathways are not linear cascades of events but rather complex networks and circuits of positive and negative feedback loops that serve to relay, amplify, and integrate signals from extracellular stimuli, ultimately resulting in a genomic and physiological response. Protein kinases are central signal transducers involved in control of all facets of cell function, including changes in the cellular program as well as cellular homeostasis. Therefore, it is not surprising that mutations or dysregulation of protein kinases, their regulators and effectors are implicated in several human diseases, making them excellent candidates for the development of agonists and antagonists for therapeutic use (reviewed in Hunter, 2000).

The activity of protein kinases is modulated by the upstream signalling events through a variety of means that often involve alterations in the phosphorylation of key protein kinase residues or changes in the physical association of regulatory proteins with the protein kinase. Signals are typically relayed downstream by the protein kinase through the covalent transfer of the terminal phosphate group from ATP or GTP to serine, threonine or tyrosine residues of substrate proteins. Phosphorylation of the protein substrate then alters its ability to physically interact with other molecules in the cell, or its catalytic activity, or its subcellular localisation (Pearson and Kemp, 1991; Pawson and Wilson, 1997; Cohen, 2000).

Protein kinases share a catalytic domain composed of a contiguous stretch of approximately 250-300 amino acids that can be divided into twelve subdomains. Although no residue in this region is absolutely conserved in all protein kinases, there is a pattern of residue conservation within this core of amino acids that constitutes a family signature (Hanks et al., 1988; Hanks and Hunter, 1995). Protein kinases constitute a large and

diverse evolutionarily conserved gene superfamily composed of 518 genes that can be divided in eight major families (except for 40 atypical protein kinase genes): AGC (containing PK$\underline{A}$, PK$\underline{G}$, PK$\underline{C}$ subfamilies), CAMK (calcium/calmodulin-dependent protein kinases family), CKI (casein kinase 1 family), CMGC (containing $\underline{C}$DK, $\underline{M}$APK, $\underline{G}$SK3, $\underline{C}$LK subfamilies), STE (homologs of the yeast Sterile 7, 11, and 20 families of serine/threonine kinases), TK (tyrosine kinase family), TKL (tyrosine kinase-like family), and RGC (receptor guanylate cyclase family). In addition, there is a group classified as Other Protein Kinases, in which the WNK subfamily of serine/threonine protein kinases is included (Kostich et al., 2002; Manning et al., 2002).

1.2   The WNK subfamily of protein kinases

The WNK protein kinases are found only in multicellular organisms, forming in the human kinome a separated phylogenetical branch (more closely related to the STE and TKL family branches). Their closest human homologues are the protein kinases MEKK (or MAP3K, MAPK kinase kinase), PAK (p21-activated kinase), and Raf, that belong to the mitogen-activated protein kinases (MAPKs) (Veríssimo and Jordan, 2001; Kostich et al., 2002; Manning et al., 2002).

The human WNK kinase subfamily is composed of four members and, despite the fact that all four kinases exhibit similar topology, their overall degree of sequence identity is approximately 40%. The WNKs most conserved region (83-93%) is the unique kinase domain located towards the N-terminus of the protein, containing the expected 12 subdomains (FIG. 1.1). All four WNK kinases have an unusual positioning of the conserved lysine residue essential for catalytic activity on subdomain I (in the majority of protein kinase families it is located on subdomain II) and as a result of their initial sequence analysis they were named WNK, meaning $\underline{w}$ith $\underline{n}$o $\underline{K}$ = lysine. The other conserved regions between the WNK kinases are the autoinhibitory domain, that can
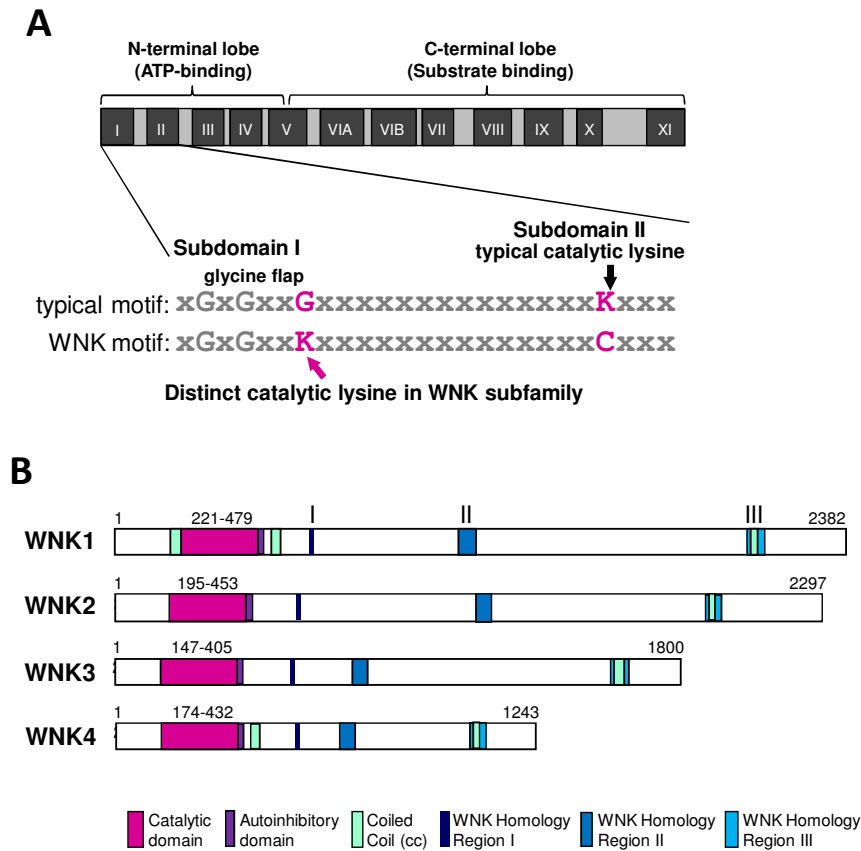
Figure 1.1| **Sequence features of the WNK subfamily of protein kinases. A**| Comparison of the sequence in subdomains I and II of the catalytic domain between a typical protein kinase and the WNK kinases. A conserved lysine in subdomain II, which binds to ATP, is absent in WNK kinases and functionally substituted by another lysine located in subdomain I, as indicated. **B**| Schematic representation of the four human WNK proteins. Catalytic, autoinhibitory, and coiled-coil domains are shown as well as conserved regions of homology between WNK kinases (adapted from Moniz and Jordan, 2010).

suppress protein kinase activity, three short homology regions, and a coiled-coil domain in spite of their considerable size. The homology region closer to the C-terminus contains, besides the coiled-coil domain, multiple PXXP motifs and the typical binding sites for Src-homology 3 (SH3) domains. These features together with their large size suggest that these proteins have a scaffolding function and are involved in multiple cellular signalling pathways (Xu et al., 2000; Veríssimo and Jordan, 2001; Xu et al., 2002a; Xu et al., 2005a).

The four WNK kinases also differ in their tissue specific expression pattern: while WNK1 is almost ubiquitary, the others have a more restrict expression (Xu et al., 2000; Veríssimo and Jordan, 2001). The molecular diversity of the WNK subfamily is further increased by the existence of several alternative splicing variants that have been described for all WNKs (Veríssimo and Jordan, 2001; Delaloy et al., 2003; O'Reilly et al., 2003; Moniz et al., 2007; Holden et al., 2004; Gagnon et al., 2006b; Shekarabi et al., 2008).

Numerous studies in the past few years have shown that WNK kinases play important roles in the regulation of diverse cellular functions including, electrolyte homeostasis, membrane trafficking, cell survival and proliferation, and organ development.

1.3 WNK kinases and pseudohypoaldosteronism type II

Mutations in *WNK1* or *WNK4* cause pseudohypoaldosteronism type II (PHA-II), also known as familial hyperkalemic hypertension (FHH) or Gordon's syndrome [Online Mendelian Inheritance in Man (OMIM) database no. 145260], a rare familial form of hypertension characterised by increased renal salt reabsorption accompanied by hyperkalemia (high serum potassium levels) and metabolic acidosis due to impaired potassium and proton excretion (Wilson et al., 2001).

The *WNK1* gene expression is under the control of three alternative promoters (FIG. 1.2 | A), generating several WNK1 isoforms with a tissue-specific distribution. Two major *WNK1* transcripts have been identified. One, named long WNK1 (L-WNK1), is under the control of 2 proximal promoters and is almost ubiquitous, being mainly expressed in skeletal muscle, heart, and brain. The other, a shorter transcript, has mainly a high-level production in the kidney, predominantly in the distal convoluted tubule (DCT), but also in the cortical collecting duct (CCD). The transcription of this kidney-specific WNK1 (KS-WNK1) isoform is under the control of a kidney-specific

enhancer and of an alternative promoter, rP. The latter is located in intron 4 and is the only *WNK1* variant that contains sequences from an extra exon located between exons 4 and 5, which has been designated as exon 4a (Wilson et al., 2001; Xu et al., 2002b; Delaloy et al., 2003). This isoform lacks the almost entire kinase domain, thus being devoid of serine-threonine kinase activity. However, it contains the autoinhibitory domain (Xu et al., 2002a), and therefore it could act as a dominant negative form of the catalytic L-WNK1 isoform and it could also inhibit other WNKs activity (as it has been shown for WNK3 by Yang et al., 2007).

Reported disease-causing mutations in *WNK1* are large deletions in the first intron that result in the overexpression of L- and KS-WNK1 in the DCT and ubiquitous ectopic KS-WNK1 expression (Wilson et al., 2001; Delaloy et al., 2008). Since PHA-II clinical features are all explained by defects in renal ion transport in the distal nephron, it is highly likely that altering the normal balance between L-WNK1 and KS-WNK1 plays a key role in its pathophysiology.

In contrast, *WNK4* mutations (FIG. 1.2 | B) are all missense alterations, which cluster in short (10 or 16 amino acids) highly conserved segments immediately downstream from the first or second coiled-coil domains of the encoded WNK protein. These mutations result in the substitution of a charged residue in these negatively and positively charged sequences, respectively (Wilson et al., 2001). As coiled-coil domains are generally thought to be involved in protein-protein interactions, these mutations may affect the interaction of WNK4 with its partners. Moreover, WNK4 is expressed predominantly in the kidney, where it is restricted to the DCT and CCD, the distal nephron segments known to be crucial in the homeostasis of electrolytes that is altered in PHA-II (Wilson et al., 2001).

Despite the different nature of *WNK1* and *WNK4* disease-causing mutations, the phenotypes of PHA-II patients harbouring mutations in either gene are not significantly
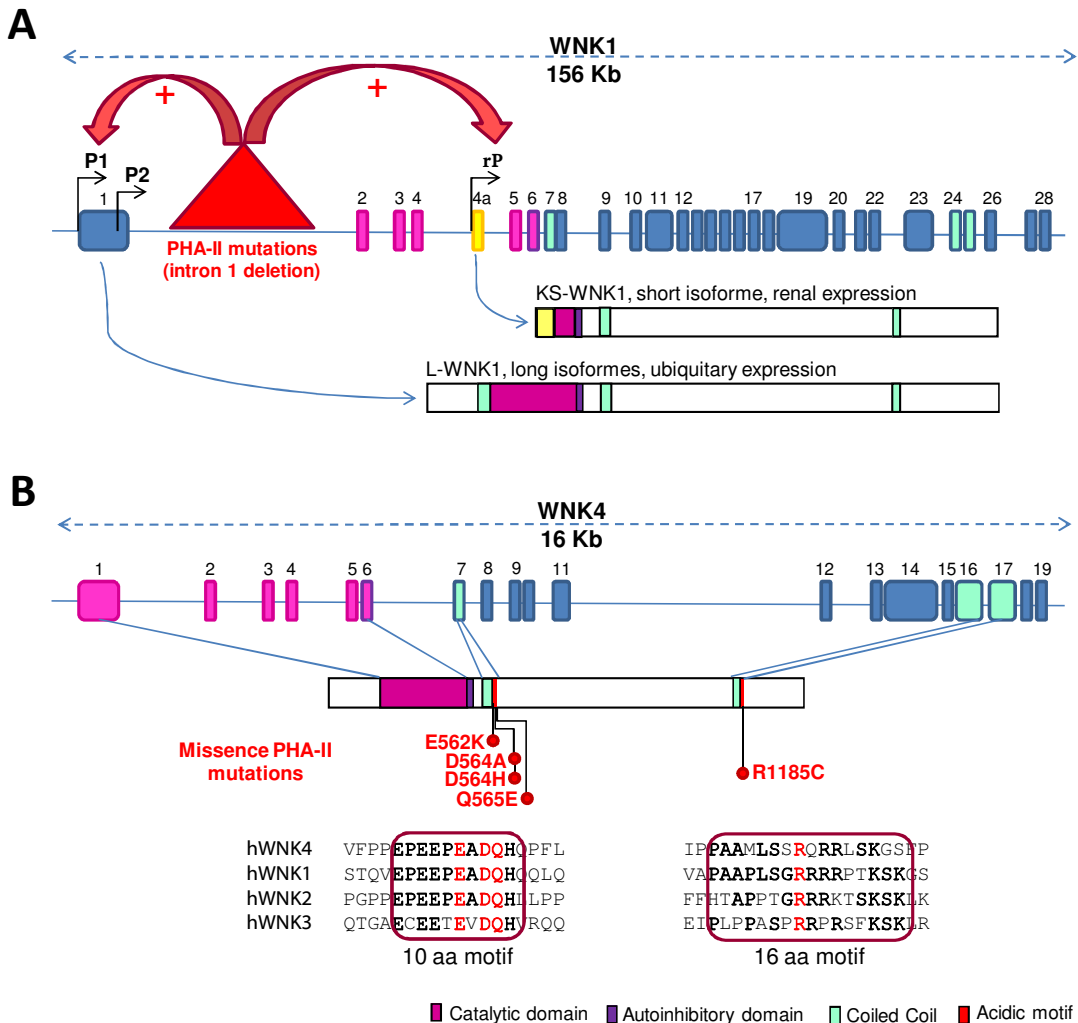
Figure 1.2| Characterisation of *WNK1* and *WNK4* mutations causing PHA-II. A| *WNK1* PHA-II mutations. The genomic segments that are deleted in PHA-II are shown and the two major WNK1 isoforms are represented with their promoters indicated. **B| *WNK4* PHA-II mutations.** Missense mutations causing PHA-II localise to small motifs adjacent to the coiled-coil domains. These 10 and 16 amino acid motifs are conserved among the WNK family. The genomic segment spanning WNK1 and WNK4 are represented by a horizontal line and exons are indicated by numbered rectangles. Catalytic, autoinhibitory and coiled-coil domains are shown (adapted from Hadchouel et al., 2005).

different (Wilson et al., 2001), suggesting that these kinases either act in series in the same pathway or in parallel pathways on similar downstream targets. The most important difference between patients with *WNK1* and *WNK4* mutations is that while the first exhibit normocalciuria (Achard et al., 2003), the latter manifest hypercalciuria and low bone mineral density (BMD) (Mayan et al., 2002).

In agreement with its role in causing this rare monogenic form of hypertension, WNK1 also plays a significant role in blood pressure regulation in the general population. Common *WNK1* variants have been shown to be associated with the regulation of blood pressure (Tobin et al., 2005; Newhouse et al., 2005; Tobin et al., 2008; Osada et al., 2009; Newhouse et al., 2009; Padmanabhan et al., 2010). On the other hand, it remains controversial whether *WNK4* variants may predispose to essential hypertension (Erlich et al., 2003; Speirs and Morris, 2004; Kokubo et al., 2004; Kamide et al., 2004; Tobin et al., 2005; Sun et al., 2009).

Curiously, a nervous system-specific WNK1 splice variant has recently been implicated in another disorder, hereditary sensory and autonomic neuropathy type II (OMIM no. 201300). This is an early-onset autosomal recessive disorder characterised by loss of perception to pain, touch, and heat, attributable to a partial loss of peripheral sensory nerves (Shekarabi et al., 2008).

The discovery that mutations in *WNK1* or *WNK4* are implicated in PHA-II led to a myriad of studies at the physiological level showing effects of WNK1 and WNK4 on several plasma membrane cotransporters and ion channels (both renal and extra-renal), and shedding some light on their mechanisms of action.

1.4 The Role of WNK1 and WNK4 in renal electrolyte homeostasis

An integrated and tightly regulated system of ion channels, exchangers, transporters, and pumps arrayed along the length of the kidney tubule and the paracellular pathway are responsible for maintaining electrolyte and fluid balance through the reabsorption of more than 99% of the salts present in the glomerular filtrate.

The bulk (some 60%) of the filtered salts is reabsorbed in the proximal tubule, mainly by the Na^+/H^+ exchanger (NHE3), chloride/base exchangers or through paracellular

junctions (for calcium and magnesium). A further 20-30% is reabsorbed in the thick ascending limb of Henle (TAL) by the electrically neutral $Na^+/K^+/2Cl^-$ cotransporter 2 (NKCC2), followed by the recycling of potassium through the renal outer medullary K^+ channel (ROMK), which provides the passive driving force for calcium and magnesium reabsortion throught the tight junction. The remainder sodium and cloride (around 10%) are recovered by the electroneutral Na^+/Cl^- cotransporter (NCC) in the DCT and through the epithelial sodium channel (ENaC) in the CCD (reviewed in Reilly and Elisson, 2000). The epithelial calcium channels, transient receptor potential channel vanilloid subtype 4 (TRPV4), 5 (TRPV5) and 6 (TRPV6), and the epithelial magnesium channel, transient receptor potential channel melastatin subtype 6 (TRPM6), are responsible for the calcium and magnesium active reabsortion in both DCT and CCD (5-10%) (Tian et al., 2004; Hoenderop et al., 2005; Schlingmann et al., 2002). All these electrogenic steps provide the electrical driving force for cloride reabsorption *via* the paracellular path and for potassium and proton secretion through the ROMK and the H^+ ATPase, respectively (Reilly and Ellison, 2000).

Despite the minor contribution from the DCT and CCD to salt reabsortion, the distal nephron is the primary target site for the renin-angiotensin-aldosterone system (further detailed in the next subsection), and for the major calciotropic hormones, i.e. parathyroid hormone (PTH), calcitonin, 1,25-dihydroxyvitamin D_3 (active form of vitamin D), and 17β-estradiol, being the final rate of calcium excretion also regulated by calbindin-D28k (CBP-D28k), a cytosolic calcium facilitator (Reilly and Ellison, 2000; Hoenderop et al., 2005). This fine-tuning of electrolyte and fluid homeostasis plays a key role in the control of ion excretion and hence, blood pressure, which also explains the efficacy of anti-hypertensive drugs targeting these sites in the distal nephron. Moreover, PHA-II patients' symptoms can be corrected by thiazide diuretics (Farfel et al., 1978; Semmekrot et al., 1987), which function as antagonists of NCC (Monroy et al., 2000).

Experimental evidence exists showing that WNK1 and WNK4 regulate diverse renal ion transport proteins, including the cation-chloride cotransporters NCC and NKCC2, the ENaC sodium channel, the ROMK potassium channel, the TRPV4, TRPV5 and TRPV6 calcium channels, and the paracellular tight junction proteins claudins (described with more detail in the following).

1.4.1   Upstream regulators of WNKs

At the moment, little is known about potential physiologically important regulators of the WNK kinases role in renal electrolyte homeostasis. The renin-angiotensin-aldosterone system is a hormonal cascade involved in the blood pressure, electrolyte and fluid homeostasis. Its dysregulation plays an important part in the pathogenesis of both renal and cardiovascular diseases, and in hypertension (reviewed in Kappert and Unger, 2008).

Renin is a protease derived from its precursor protein, prorenin, which is released constitutively from juxtaglomerular cells of the kidney. The hydrolysis of angiotensinogen by renin, generating angiotensin I, is the first and rate-limiting step of this system. Subsequently, another enzyme converts angiotensin I into angiotensin II and its binding to type 1 receptors causes most of angiotension II known physiological and pathophysiological effects (Kappert and Unger, 2008).

The activation of this pathway occurs as a result of hypovolemia (low intravascular volume) and/or hypotension, and its immediate effects include vasoconstrition in the arterioles of the glomerulus, thus increasing the peripheral vascular resistance and ultimately increasing blood pressure. In addition, angiotensin II also acts on the adrenal glands cortex causing the secretion of the sodium-retaining hormone aldosterone (Quinn and Williams, 1988; Kappert and Unger, 2008). Upon release, aldosterone binds to its receptor, the mineralocorticoid receptor, in the distal nephron and enhances

sodium reabsorption (*via* NCC and ENaC), being the final outcome an increase in intravascular volume (towards normal) and in blood pressure (Kim et al., 1998; Eaton et al., 2001).

High serum potassium levels (hyperkalemia) can also stimulate aldosterone secretion, and aldosterone can increase the excretion of potassium by the kidney (*via* ROMK), so it is not surprising that there is a continuous relationship between the level of circulating potassium and that of aldosterone (Quinn and Williams, 1988; Beesley et al., 1998).

Recently, it has been shown that aldosterone rapidly induces the expression of KS-WNK1, but not of L-WNK1 (Náray-Fejes-Tóth et al., 2004). L-WNK1 expression is regulated by aldosterone only at the post-transcriptional level, probably *via* miR-192, a micro-RNA expressed only in the kidney and the gastrointestinal tract (whether KS-WNK1 expression is regulated in a similar manner remains to be determined) (Elvira-Matelot et al., 2010). On the other hand, both WNK1 and WNK4 are a target of an aldosterone-responsive gene expressed in the distal nephron, SGK1 (serum glucocorticoid-inducible protein kinase) (Xu et al., 2005b; Ring et al., 2007b; Rozansky et al., 2009). Moreover, WNK4 has been shown to become activated in response to angiotensin II signalling (San-Cristobal et al., 2009), thus providing additional evidence for the renin-angiotensin-aldosterone system importance in WNK kinases regulation.

In addition, it has been suggested that hyperosmotic stress and cell shrinkage (e.g. sorbitol, NaCl, and KCl) induces the phosphorylation (Ser1261), autophosphorylation (Ser382 on the kinase domain) and activation of WNK1, leading to the phosphorylation and activation of its downstream targets. Furthermore, hypertonicity also induces a rapid redistribution of WNK1 from the cytoplasm to vesicular structures (Xu et al., 2000; Lenertz et al., 2005; Zagórska et al., 2007). At the present moment, it is unclear whether WNK4 is regulated in a similar manner and the underlying molecular mechanisms remain to be fully elucidated.

1.4.2   NCC and NKCC2 regulation

The first studies performed in *Xenopus laevis* oocytes strongly suggested that WNK4 is a negative regulator of the thiazide-sensitive sodium transport in the DCT. Therefore, PHA-II could result from unregulated activity of NCC, a member of the *SLC12* gene family (*SLC12A3* gene), as a consequence of *WNK1* and *WNK4* mutations. Specifically, wild-type WNK4 inhibited the activity of NCC through a nearly complete inhibition of the insertion of the channel into the plasma membrane, whereas WNK4 PHA-II-causing mutants seemed to fail to inhibit NCC in *Xenopus* oocytes (Wilson et al., 2003; Yang et al., 2003).

Some years later, two independent studies confirmed that wild-type WNK4 downregulates the surface expression (and thus activity) of the NCC in mammalian cells as well (Yang et al., 2005b; Cai et al., 2006). Furthermore, *Wnk4* Q562E PHA-II mutant expression in mice (transgenic for genomic segments harbouring PHA-II *Wnk4* Q562E mutation homologous to the human WNK4 Q565E mutation) recapitulates the PHA-II phenotype (high blood pressure, hyperkalemia and hypercalciuria). Also, it is entirely dependent upon NCC activity, which shows that the major physiological role of WNK4 is to regulate NCC activity in the DCT (Lalioti et al., 2006).

Unlike WNK4, WNK1 does not seem to directly affect NCC activity but to lie upstream of the WNK4 signalling pathway, modulating the inhibitory effects of WNK4 on NCC (Wilson et al., 2003; Yang et al., 2003). Moreover, KS-WNK1 downregulates NCC activity indirectly, by inhibiting L-WNK1 activity and thereby allowing WNK4 to inhibit NCC (FIG. 1.4 | DCT, on page 27) (Subramanya et al., 2006).

Accordingly, transgenic mice overexpressing KS-WNK1 in the kidney present low effective circulating volume, renal sodium wasting (impaired ability to reabsorb sodium) and low blood pressure in response to dietary sodium restriction, and decreased surface expression of total and phosphorylated NCC and NKCC2 (another member of the

SLC12 gene family, *SLC12A1* gene) in DCT and TAL, respectively. This confirms KS-WNK1 as a negative regulator of both NCC and NKCC2 *in vivo*. These animals also exhibit an increase in the angiotensin II and aldosterone levels, which is likely due to activation of the renin-angiotensin system, consistent with the notion that a low effective circulating volume causes the decrease in blood pressure (Liu et al., 2011).

Conversely, KS-WNK1 knock-out mice display the inverse phenotypes, i.e. sodium retention and elevated blood pressure on a high sodium diet, and increased expression and phosphorylation of NCC and NKCC2 in the respective nephron segments (Hadchouel et al., 2010; Liu et al., 2011).

Recently, a study has shown that SGK1, a mediator of aldosterone signalling, binds to and phosphorylates WNK4 (Ser1190 and Ser1217), relieving the inhibitory effect of WNK4 on NCC activity (FIG. 1.4 on page 27). In addition, *in vitro* kinase assays indicate that L-WNK1 and SGK1 share phosphorylation targets and share similar effects on WNK4 (Rozansky et al., 2009).

Other recent studies have elucidated the mechanism by which WNK4 downregulates NCC surface expression. WNK4 does not stimulate NCC endocytosis (Golbang et al., 2006), but instead attenuates the rate of NCC plasma membrane delivery. In fact, WNK4 acts on newly synthesized NCC in the secretory pathway stimulating interaction with the AP-3 adaptor complex. These proteins mark cargo for transport from endosomes to lysosomes, and thus divert NCC into the lysosomal pathway, where it is ultimately degraded (Subramanya et al., 2009). Furthermore, WNK4 promotes NCC targeting to the lysosome for degradation *via* a mechanism that involves sortilin, a lysosomal targeting receptor expressed in the DCT (Zhou et al., 2010). Interestingly, WNK4 globally stimulates lysosomal activity, therefore it might promote the lysosomal sorting and degradation of cargo other than the NCC (Subramanya et al., 2009).

In addition to WNK1 and WNK4 effects on NCC and NKCC2 surface abundance, these kinases can also affect NCC and NKCC2 transport activity through its phosphorylation. Both of them can directly bind to their downstream targets, two similar serine/threonine kinases belonging to the Ste20-related kinases family, OSR1 (oxidative stress-responsive kinase 1) and SPAK (ste20-related proline alanine-rich kinase), and phosphorylate their evolutionarily conserved threonine and serine residues (Thr233 and Ser373 in SPAK, or Thr185 and Ser325 in OSR1) *in vitro* (FIG. 1.3) (Moriguchi et al., 2005; Vitari et al., 2005; Vitari et al., 2006; Anselmo et al., 2006). On the other hand, KS-WNK1 antagonizes the effect of WNK1 and WNK4 on NCC and NKCC2 mediated through OSR1 and/or SPAK (FIG. 1.4 | DCT, on page 27) (Liu et al., 2011).
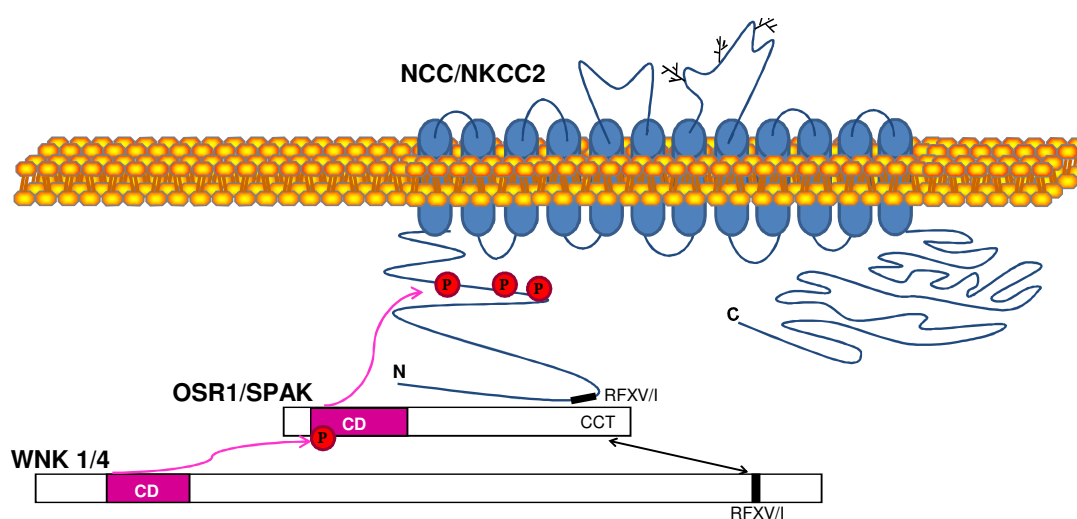
Figure 1.3| Schematic representation of OSR1/SPAK interactions with both WNK 1/4 and NCC/NKCC2. WNK1 and WNK4 interact with OSR1/SPAK, which is mediated by the RFXV/I motif in WNKs and the conserved C-terminal domain (CCT) in OSR1/SPAK, respectively. WNKs activate OSR1/SPAK by phosphorylating its kinase domain, which in turn phosphorylate cation-chloride-coupled cotransporters at conserved threonine or serine residues in the N-terminus. The interaction between OSR1/SPAK and the cotransporters also occurs *via* the CCT domain and RFXV/I motif. CD, catalytic domain.

SPAK and OSR1 possess a conserved C-terminal domain, which interacts with RFXV/I motifs present in both WNK1 and WNK4 C-termini as well as in the N-terminal tail of SLC12 cation/chloride cotransporters, such as NCC and NKCC2. Furthermore,

these Ste20-related kinases have been confirmed to phosphorylate conserved threonine residues in the N-terminal tail of NCC (Thr46, Thr50, Thr60) and NKCC2 (Thr99, Thr104, Thr117) *in vitro* (Moriguchi et al., 2005; Richardson et al., 2008). This phosphorylation of NCC and NKCC2 has been shown to increase their intrinsic transporter activity (Pacheco-Alvarez et al., 2006; Giménez and Forbush, 2005).

These NCC and NKCC2 phosphorylation sites mediate blood pressure control as shown in transgenic knock-in mice. On Spak$^{T243A/+}$ mice, SPAK activation by the WNK kinases is prevented, which leads to significantly reduced blood pressure by insufficient expression and phosphorylation of NCC and NKCC2 (Rafiqi et al., 2010). Hypomorphic *Wnk4* mice (with deletion of *Wnk4* exon 7) that have recently been generated, also display reduced blood pressure and decreased SPAK/OSR1 and NCC phosphorylation, demonstrating that wild-type WNK4 positively regulates NCC function *in vivo* through the WNK-OSR1/SPAK-NCC cascade (Ohta et al., 2009).

Further support for the role of SPAK in blood pressure control comes from a genome-wide association study (GWAS). In this study *STK39*, the human SPAK gene, was identified as a novel hypertension susceptibility gene in which common variants are associated with blood pressure levels, suggesting that SPAK may also regulate blood pressure within the general population. This study showed that higher transcriptional activity of SPAK, due to the presence of the less common G allele in an intronic single nucleotide polymorphism (rs35929607), may increase the activity of downstream NCC and NKCC2, which would promote sodium reabsorption in the kidney, thereby increasing intravascular volume and blood pressure (Wang et al., 2009).

Physiological stimuli such as angiotensin II signal through type I receptors *via* WNK4 and SPAK to increase NCC activity (FIG. 1.4 | DCT, on page 27), since angiotensin II increases WNK4-dependent phosphorylation of both SPAK and NCC in mammalian

cells. NCC activity is also increased by WNK4 PHA-II-causing mutations, and cannot be further augmented by angiotensin II signalling. This is consistent with WNK4 PHA-II mutations providing constitutive activation of the signalling pathway between angiotensin II and NCC (San-Cristobal et al., 2009).

The hypothesis that mutant PHA-II WNK4 enhances this signalling pathway in the renal epithelium and thereby activates NCC (and thus sodium reabsorption) is favoured by the observation that in the distal nephron of the PHA-II mouse model Wnk4$^{D561A/+}$ mice, there is increased phosphorylation of both OSR1/SPAK and NCC (which was concentrated on the apical plasma membranes). Moreover, it was shown that the activation of the OSR1/SPAK-NCC phosphorylation cascade by the mutant WNK4 is the major cause of PHA-II in these mice (Yang et al., 2007c). Conversely, a significant decrease in NKCC2 expression was observed in the TAL of Wnk4$^{D561A/+}$ mice (Yang et al., 2010), however WNK4 protein expression in the TAL is still controversial (Wilson et al., 2001; O'Reilly et al., 2006).

1.4.3 ENaC regulation

Naray-Fejes-Toth and collaborators (2004) reported that aldosterone rapidly induces the expression of KS-WNK1 (but not of L-WNK1) in an unique mouse CCD cell line that expresses functional mineralocorticoid receptors. Moreover, ectopic expression of KS-WNK1 significantly increases transepithelial sodium transport in those cells through the epithelial sodium channel (ENaC), the major mediator of aldosterone-sensitive sodium reabsorption (FIG. 1.4 | CCD, on page 27) . Furthermore, decreased ENaC expression and activity have recently been reported in KS-WNK1$^{-/-}$ mice, although the authors suggest it could be a compensatory mechanism to prevent hypertension as a result of the increased NCC activity following KS-WNK1 inactivation (Hadchouel et al., 2010).

Regardless, other studies suggest that WNK1 N-terminus regulates ENaC through the activation of SGK1 (whose activity is stimulated by mineralocorticoids such as aldosterone), which phosphorylates Nedd4-2, an E3 ubiquitin ligase. This enzyme directly interacts with a PY motif in two subunits of ENaC *via* a WW proline-binding domain, leading to the endocytosis of the channel. Nedd4-2 phosphorylation inhibits this direct interaction, causing the retention of ENaC at the cell surface and, consequently, increased sodium current (FIG. 1.4 | CCD, on page 27) (Xu et al., 2005b).

How WNK1 controls SGK1 activity remains unclear since SGK1 is not a WNK1 substrate, although two sites of activating phosphorylation on SGK1, Thr256 and Ser422, must be present for activation of SGK1 by WNK1. Possibly, as WNK1 binds to SGK1, WNK1 may serve as a scaffold to assemble a protein complex required for efficient SGK1 activation (Xu et al., 2005b; Xu et al., 2005c). Conversely, there is more insight in the upstream pathways involved (FIG. 1.4 | CCD, on page 27), WNK1 contains an N-terminal site (Thr60) for phosphorylation by Akt (also called protein kinase B), and/or SGK1 (WNK1 phosphorylation by SGK1 may reflect positive feedback) (Xu et al., 2005c). Furthermore, Akt phosphorylation of WNK1 increases the activation of SGK1 and ENaC by WNK1, and the activation of Akt requires phosphatidyl inositol-3-kinase (PI3K) catalytic activity. In addition, it has also been shown that WNK1 is required for SGK1 activation by insulin-like growth factor 1 (IGF-1) (Xu et al., 2005c).

A comparable site for Akt/SGK1 phosphorylation is present in WNK4 (Ser47) and its mutation reduces WNK4 capacity to activate SGK1. Furthermore, PI3K activity is also required for SGK1 activation by WNK4. In addition, the N-terminus of all WNKs has been shown to have the capacity to interact with and activate SGK1, increasing ENaC-associated sodium current. Conversely, KS-WNK1, which lacks the WNK1 N-terminus, is unable to activate ENaC, and moreover, inhibits ENaC activation by L-WNK1 and probably by other WNK subfamily members (Heise et al., 2010).

On the other hand, in *Xenopus* oocytes, WNK4 inhibits ENaC activity *via* a mechanism that requires the short cytoplasmic C-terminus of ENaC, which is used by Nedd4-2 to target the channel for degradation. However, it is not known whether WNK4 is acting through Nedd4-2 or in an independent pathway. This inhibition is independent of WNK4 kinase activity and is alleviated by mutations that cause PHA-II, and by mimicking phosphorylation at one SGK1 consensus phosphorylation site (S1190D) in WNK4 (Ring et al., 2007a; Ring et al., 2007b).

The physiological significance of these effects of WNK4 on ENaC *in vivo* is demonstrated by the observation that transepithelial sodium permeability and ENaC protein levels were elevated in the CCD of mutant $Wnk4^{D561A/+}$ mice, even though it was suggested that it could be a secondary mechanism that compensates for reduced luminal sodium availability in CCD and/or hyperkalemia (Yang et al., 2007c). Also the hypomorphic WNK4 mice and the $Spak^{T243A/+}$ animals expressed higher levels of ENaC in the kidney, further suggesting that this is a compensatory mechanism for reduced NCC/NKCC2 activity (Ohta et al., 2009; Rafiqi et al., 2010).

1.4.4 ROMK regulation

As PHA-II is characterised by hyperkalemia due to impaired potassium excretion (Wilson et al., 2001), the involvement of ROMK (primary determinant of potassium ion secretion in the CCD) has been extensively studied.

Work in *Xenopus* oocytes has shown that WNK4 inhibits the activity of ROMK, and this inhibition is due to a significant reduction in membrane expression of ROMK, being this effect independent of WNK4 kinase activity. Furthermore, WNK4 facilitates the retrieval of ROMK from the oocyte membrane through a clathrin-mediated endocytosis pathway (Kahle et al., 2003).

The kinase-independent inhibition of ROMK by WNK4 raised the question of how inhibition is achieved. One possibility is that WNK4 serves as a scaffold to bring together ROMK and other factors that target ROMK for endocytosis (Kahle et al., 2003). Indeed, several years later it was shown in human embryonic kidney (HEK293) cells that WNK4 interacts with intersectin, a multimodular endocytic scaffold protein that recruits endocytic proteins to the clathrin-coated pits, and that this interaction is crucial for stimulation of ROMK endocytosis by WNK4 (FIG. 1.4 | CCD, on page 27). It was also demonstrated that this endocytosis stimulation required only the binding of intersectin to specific proline-rich WNK motifs (PXXP) *via* its SH3 domains and not the WNKs kinase activity (He et al., 2007).

Curiously, the PXXP motifs of WNK4 critical for interaction with intersectin, precede the region of 4 amino acids (EADQ, FIG. 1.2 | B) where most PHA-II-causing mutations are clustered (He et al., 2007). These WNK4 mutations markedly increase inhibition of ROMK-mediated potassium ion flux and surface expression, compared with wild-type WNK4, due to enhanced interactions of WNK4 with intersectin and ROMK (Kahle et al., 2003; He et al., 2007). Therefore, the increased inhibition of ROMK can account for impaired potassium ion secretion and hyperkalemia in patients with PHA-II (Kahle et al., 2003).

Another work in *Xenopus* oocytes has shown that WNK1 can directly affect ROMK-mediated potassium ion current, independent of WNK4, by causing a marked reduction in its surface expression. They have also shown that this effect does not require WNK1 kinase activity (Cope et al., 2006). In HEK293 cells, L-WNK1 (similarly to WNK4) stimulates clathrin-dependent endocytosis of this channel by interacting with the scaffold protein intersectin through its SH3 domains (Cope et al., 2006; Lazrak et al., 2006; He et al., 2007). Moreover, KS-WNK1 acts as a physiological antagonist of L-WNK1, reversing the inhibition of ROMK caused by L-WNK1 (FIG. 1.4 | CCD, on page 27). Accordingly, transgenic mice overexpressing KS-WNK1 display increased surface expression

of ROMK in the renal distal tubules and decreased serum potassium levels (Liu et al., 2009). Thus, hyperkalemia in PHA-II patients with *WNK1* mutations may be caused, at least partially, by increased expression of L-WNK1, which can lead to increased endocytosis of ROMK *via* intersectin (Lazrak et al., 2006; He et al., 2007; Liu et al., 2009).

More recently, the endocytotic signal and the internalisation machinery involved in L-WNK1-stimulated ROMK endocytosis have been further elucidated. ROMK binds directly to the clathrin adaptor molecule autosomal recessive hypercholesterolemia (ARH), and this interaction is mediated by what is believed to be a novel variant of the canonical NPXY endocytotic signal, YxNPxFV. Thus, ARH, which has a scaffolding architecture to couple cargo selection to clathrin-pit localisation, recruits ROMK to clathrin-coated pits, for both constitutive and L-WNK1-stimulated endocytosis, by simultaneously interacting with the internalisation signal, AP-2 and clathrin (Fang et al., 2009).

Nevertheless, transgenic Wnk4 Q562E and knock-in Wnk4$^{D561A/+}$ PHA-II mice models suggest an additional mechanism for hyperkalemia. In both models, hyperkalemia is ameliorated by treatment with thiazide. It was therefore suggested that hyperkalemia developed secondary, because an increase in the activity of NCC decreases sodium reabsorption *via* ENaC, thus diminishing the driving force for potassium secretion *via* ROMK (Lalioti et al., 2006; Yang et al., 2007c). In both studies (also true for D564A Wnk4 transgenic mice), apical abundance of ROMK between wild-type and PHA-II mice were not different (Yamauchi et al., 2005; Lalioti et al., 2006; Yang et al., 2007c). The 'normal' level of apical abundance of ROMK in PHA-II mice, however, may be considered inappropriately low in the setting of hyperkalemia since the abundance of ROMK should be upregulated in these situations, e.g. by increased dietary potassium intake (reviewed in Frindt and Palmer, 2010). The observation that apical abundance of ROMK is unchanged suggests that it is partially inhibited by mutant WNK4. Moreover, hyperkalemia in patients with WNK4 mutations (Mayan et al., 2004), as well as in PHA-II

mice (Yang et al., 2007c), precedes the development of hypertension, suggesting that increased NCC activity is not essential for hyperkalemia. Therefore, inhibition of ROMK by mutant WNK4 likely occurs in PHA-II and contributes to hyperkalemia.

1.4.5 TRPV4, TRPV5, and TRPV6 regulation

The molecular mechanisms underlying hypercalciuria in PHA-II caused by *WNK4* mutations remain unclear. In the distal nephron, calcium channels such as TRPV4 are co-expressed with both WNK1 and WNK4 (Tian et al., 2004; Wilson et al., 2001), thus the osmotically responsive WNK kinases may influence expression, function and/or targeting of TRPV4 in this tissue. Indeed, ectopic expression of either *WNK* downregulates TRPV4 function in HEK293 cells, being the effect mediated *via* decreased cell surface expression of TRPV4 and partially independent of WNK kinase activity, although a direct interaction between the WNK kinases and TRPV4 could not be demonstrated. Regarding WNK4 PHA-II-causing mutations, they abrogate, but do not eliminate, the inhibitory effect of WNK4 on TRPV4 function (Fu et al., 2006).

An effect of WNK4 (but not of WNK1) on TRPV5 function has also been described. However, in contrast to its effect on TRPV4, the study in *Xenopus* oocytes showed that coexpression of *TRPV5* with *WNK4* results in increased TRPV5-mediated calcium uptake due to the increase in TRPV5 surface expression. Another difference is that WNK4-induced increase in TRPV5 plasma membrane expression is abolished by a kinase-dead mutant. Moreover, the positive effect of WNK4 on TRPV5 was greatly reduced when NCC was coexpressed, suggesting that WNK4 may be an important modulator of the inverse relation between calcium and sodium reabsorption in the distal tubule (Jiang et al., 2007).

In *Xenopus* oocytes, WNK4 PHA-II-causing mutants were indistinguishable from wild-type WNK4 in TRPV5 *in vitro* function assays (Jiang et al., 2007), which is inconsistent

with the aberrant calcium balance accompanying familial hyperkalemia and hypertension in WNK4 mutant patients (Mayan et al., 2002). However, in the presence of NCC, the WNK4 PHA-II mutant exhibits an impaired ability to enhance TRPV5 compared with wild-type WNK4, suggesting reduced calcium reabsorption under the disease condition, which could in turn contribute to the hypercalciuria observed in the WNK4 PHA-II patients (Jiang et al., 2007).

Conversely, a more recent study (Cha and Huang, 2010) shows that WNK4 decreases calcium current and cell surface abundance of TRPV5 by enhancing its endocytosis through a caveolae-mediated mechanism, in which intersectin is also important (FIG. 1.4, on page 27). Besides its role in clathrin-coated vesicles, intersectin is also present in caveolae and is believed to play a critical role in fission of caveolar vesicles *via* dynamin, similar to that in clathrin-coated ones (Predescu et al., 2003).

Curiously, parathyroid hormone-activated protein kinase C (PKC) has the opposite effect of WNK4, and increases cell surface abundance of TRPV5 by inhibiting caveolae-mediated endocytosis of the channel (Cha et al., 2008). Therefore, the lower basal TRPV5 level in the presence of WNK4 allows the amplification of the channel stimulation by activated PKC (Cha and Huang, 2010). Surprisingly, the rate of TRPV5 increase stimulated by PKC is also enhanced by WNK4. Nevertheless, further studies are required to determine how WNK4 enhances this rate (Cha and Huang, 2010).

This study also shows that WNK4 PHA-II mutation enhances the ability of WNK4 to inhibit TRPV5 current, raising the possibility that WNK4 regulation of TRPV5 may also contribute to hypercalciuria in PHA-II patients (Cha and Huang, 2010).

On the other hand, WNK4 had direct little effect on TRPV6-mediated calcium uptake (Jiang et al., 2007). However, the analysis of Wnk4$^{D561A/+}$ knock-in transgenic mice has suggested that decreased calcium reabsorption in the upstream nephron, with a secondary adaptive increase in the calcium channel TRPV6, and the cytosolic calcium

facilitator CBP-D28k expression in the distal tubules, is underlying the hypercalciuria of PHA-II patients (Yang et al., 2010).

1.4.6 Paracellular chloride permeability regulation

WNK1 and WNK4, despite being both present in the DCT and CCD, have distinct subcellular distribution patterns. WNK1 is present throughout the cytoplasm whereas WNK4 is present at the subapical plasma membranes and tight junctions in the DCT and in both the cytoplasm and at intercellular junctions in the CCD (Wilson et al., 2001). This pattern suggests that WNK4 might also modulate paracellular ion flux across these epithelia, since it occurs through selective and regulated pores in tight junctions.

Ectopic expression of *WNK4* in Madin-Darby canine kidney (MDCK) II epithelial cells increases paracellular chloride permeability through tight junctions. In addition, the paracellular chloride conductance is greater in cells expressing *WNK4* PHA-II mutants than in cells expressing wild-type proteins (Kahle et al., 2004b; Yamauchi et al., 2004).

WNK4 phosphorylates claudins 1-4, a family of transmembrane tight junction proteins involved in the regulation of paracellular ion permeability (FIG. 1.4 | DCT, on page 27). Furthermore, cells expressing *WNK4* PHA-II mutant contain a higher level of phosphorylated claudins than cells expressing wild-type protein (Yamauchi et al., 2004). Enhanced phosphorylation of claudins by mutant WNK4 is due to the increased claudin-WNK4 association. Possibly the disease-causing mutations in WNK4 somehow affect its interaction with its negative regulators, including its own domains (such as the autoinhibitory one), allowing enhanced binding to claudins (Yamauchi et al., 2004). How this phosphorylation imparts its effect is presently unknown. One possibility is that this post-translational modification alters claudins conductance, as occurs with transcellular ion channels (Marban et al., 1998). One can speculate that the phosphorylation

by WNK4 changes the conformation of claudins, increasing chloride ion pore permeability or affecting their interaction with intracellular regulators, such as cytoskeletal proteins (Heiskala et al., 2001).

Similar to ectopic expression of WNK4, MDCK II cells overexpressing WNK1 display an increase in paracellular permeability, associated with increased phosphorylation of claudin 4 (Ohta et al., 2006). However, an *in vitro* kinase assay (and its cytoplasmic localisation) suggests that WNK1 does not directly phosphorylate claudin 4 (Yang et al., 2007b).

Thus hypertension in PHA-II patients could be caused by increased NaCl reabsorption through both NCC and the paracellular pathway. However, the increase in NaCl reabsorption through NCC may be relatively more critical than that through the paracellular pathway, since hypertension in patients with WNK4 mutations was estimated to be six times more sensitive to thiazide treatment than individuals with essential hypertension (Mayan et al., 2002). Furthermore, recent studies using mice carrying a mutant PHA-II-causing *WNK4* gene (created either by transgene integration or by knock-in) support the hypothesis that increased NCC activity is the primary cause of the hypertension resulting from WNK4 mutations (Lalioti et al., 2006; Yang et al., 2007c).

1.4.7 WNK3 also participates in renal electrolyte homeostasis

Adding to the complexity of ion channel regulation by WNK1 and WNK4 is the finding that WNK3 also participates in renal electrolyte homeostasis (FIG. 1.4 on page 27), although no known mutations link this kinase to PHA-II. Unlike WNK1 and WNK4, WNK3 is expressed throughout the nephron (including the TAL and the DCT) and predominantly at intercellular junctions (Rinehart et al., 2005; Leng et al., 2006).

Wild-type WNK3 activates NCC, NKCC1 and NKCC2 (chloride influx pathways), and inhibits all four KCCs (chloride efflux pathways), even during cell swelling, a condition in which KCCs are maximally active. The catalytically inactive WNK3 has the opposite effects, even in isotonic conditions. Therefore, WNK3 possesses the ability to bypass the tonicity requirements for activation or inhibition of the cotransporters (Kahle et al., 2005; Rinehart et al., 2005; de Los Heros et al., 2006).

The effects of WNK3 are mediated *via* altered phosphorylation and surface expression of its downstream targets, however, WNK3 does not seem to directly phosphorylate these cotransporters. Furthermore, it has been shown that WNK3 modulates KCC1-KCC4 activity *via* a mechanism that requires protein phosphatases, whereas activation of NKCC2 by intracellular chloride depletion requires an interaction between WNK3 and SPAK (in a pathway in which WNK3 lies upstream of SPAK) (Kahle et al., 2005; Rinehart et al., 2005; de Los Heros et al., 2006; Ponce-Coria et al., 2008).

On the other hand, WNK3 also inhibits ROMK activity by decreasing its plasmalemmal surface expression in *Xenopus* oocytes, although in a non-catalytically manner. Conversely, WNK3 has no effect on the activity of ENaC in *Xenopus* oocytes, or on paracellular chloride flux in MDCK II cells, renal NaCl transport processes regulated by WNK1, or by both WNK1 and WNK4, respectively (Leng et al., 2006; Rinehart et al., 2005).

WNK3 positively regulates the epithelial calcium channels TRPV5 and TRPV6 through a kinase-dependent pathway, which involves protein maturation and delivery to the plasma membrane *via* the secretory pathway (Zhang et al., 2008). Similar to the attenuation of the positive effect of WNK4 on TRPV5 when NCC was coexpressed (Jiang et al., 2007), the presence of NCC also partially blocked the positive effect of WNK3 on TRPV5 (Zhang et al., 2008).
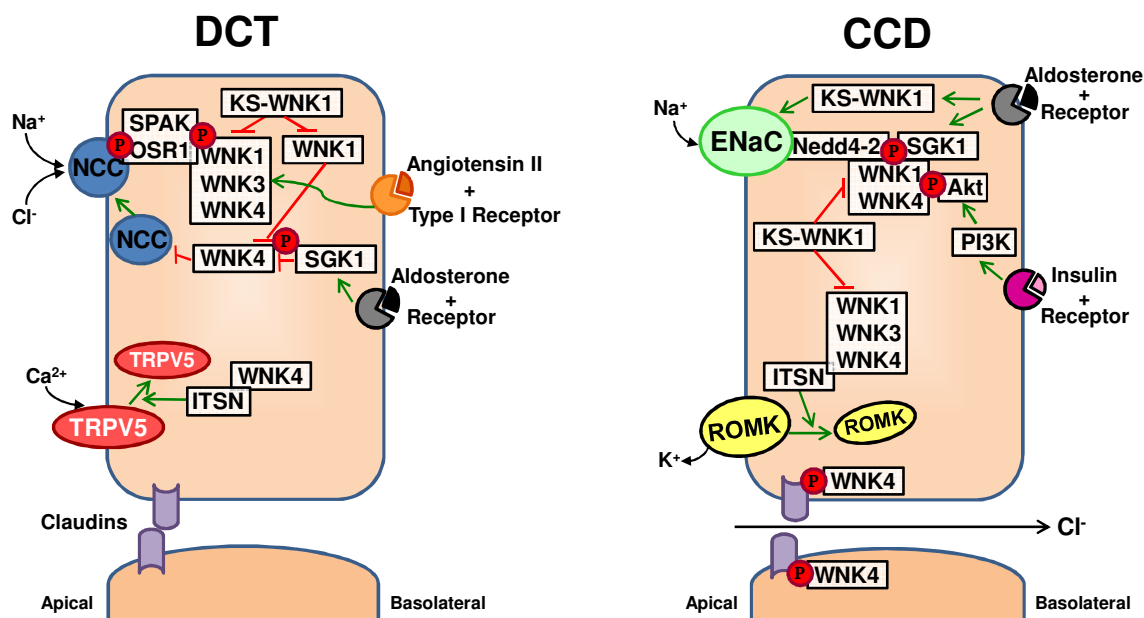
Figure 1.4| Simplified model of WNKs signalling pathways involved in renal electrolyte homeostasis regulation. Distal convoluted tubule (DCT) | WNK4 can interact with NCC, the main channel responsible for NaCl transport in this nephron segment, and reduce its surface expression by stimulating lysosomal degradation. Both SGK1 (whose activity is stimulated by mineralocorticoids such as aldosterone) and WNK1 act upstream of WNK4, inhibiting its function. Moreover, KS-WNK1 antagonises WNK1 activity, thereby allowing WNK4 to inhibit NCC. In addition, there is evidence that WNK1, WNK3 and WNK4 can activate NCC *via* SPAK and/or OSR1, and that KS-WNK1 reverses the activation. Additionally, angiotensin II through type I receptors acts *via* WNK4 and SPAK to increase NCC activity. On the other hand, WNK4 also decreases cell surface abundance of TRPV5 by enhancing its endocytosis through a caveolae-mediated mechanism, in which intersectin (ITSN) is important. **Cortical collecting duct (CCD)** | WNK1 and WNK4 regulate ENaC through the activation of SGK1 (a mediator of aldosterone signalling), which phosphorylates Nedd4-2. This reduces its direct interaction with ENaC and thereby leads to retention of the channel at the cell surface. KS-WNK1 inhibits ENaC activation by WNK1 and probably by other WNK subfamily members. Conversely, there is also evidence that KS-WNK1, in response to aldosterone, signalling enhances ENaC activity. Furthermore, WNK1 and WNK4 (and possibly WNK3) facilitate the retrieval of ROMK from the plasma membrane by a clathrin-mediated process, which also depends on ITSN. Once again, KS-WNK1 acts as a physiological antagonist of WNK1 (and possibly of other WNKs), reversing the inhibition of ROMK. In addition, WNK4 also stimulates paracellular chloride flux by phosphorylating claudins. (For simplicity, TRPV5 regulation is only represented in the DCT. Green arrows – activating pathways, red blunt ends – inhibitory pathways. See text for further details.)

1.4.8 Comprehensive model for WNK renal electrolyte homeostasis (dys)regulation

WNK1, WNK3 and WNK4 have been suggested as multifunctional regulators of diverse renal ion transporters and their different mechanisms of regulation could be a means to achieve independent function modulation of different channels. Specifically, WNK1 and WNK4 kinases can both be seen as a molecular switch that integrates physiologic signals to alter the balance between net renal NaCl reabsorption and potassium secretion.

The ability of WNK1 and WNK4 to differentially regulate NCC and ROMK could explain the mechanism by which the kidney responds differently to aldosterone signalling in the setting of hypovolemia *versus* hyperkalemia (Wilson et al., 2003; Yang et al., 2003; Xu et al., 2005b; Kahle et al., 2003; Cope et al., 2006). Furthermore, both kinases seem to regulate paracellular chloride flux (Yamauchi et al., 2004; Ohta et al., 2006), which plays an important role in NaCl and potassium homeostasis in the CCD, where 70% of chloride reabsorption occurs *via* the paracellular path (Schuster and Stokes, 1987).

In the presence of WNK4 PHA-II mutant, the increase in electroneutral NCC activity in the DCT (Lalioti et al., 2006) could reduce sodium reabsorption *via* ENaC, which would be accompanied by increased paracellular chloride reabsorption and reduced potassium secretion due to the effect of WNK4 to both, reduce the lumen-negative potential (Kahle et al., 2004b; Yamauchi et al., 2004), and to directly inhibit ROMK (Kahle et al., 2003).

Hypertension in PHA-II patients with *WNK1* mutations is likely caused by L-WNK1 mediated-increase of both NCC and ENaC sodium transport (Wilson et al., 2003; Yang et al., 2003; Xu et al., 2005b). More, L-WNK1 might also increase paracellular chloride reabsorption, and the decrease in potassium secretion through ROMK could contribute

to the hyperkalemia phenotype in these patients (Cope et al., 2006). Thus, *WNK1* mutations effect of L-WNK1 expression in the DCT would enable it to counteract KS-WNK1, which is L-WNK1 physiological antagonist (Subramanya et al., 2006).

WNK kinases not only modulate transport pathways directly, but also constitute a multi-gene kinase network responsible for the electrolyte homeostasis in the aldosterone-sensitive distal nephron. In particular, several *in vitro* studies have shown that WNK4, WNK1, KS-WNK1, and recently WNK3, can form heteroligomeric protein complexes and interact functionally amongst each other at the protein-protein level (*via* coiled-coil domains) or by phosphorylation processes (Yang et al., 2003; Yang et al., 2005a; Subramanya et al., 2006; Yang et al., 2007b). These interactions between different WNKs have been suggested to allow the signal transduction pathway to be precisely tuned to meet the different physiological demands of salt balance.

1.5   WNK kinases as regulators of ion flux in extrarenal epithelia

Although PHA-II has a renal phenotype, WNK kinases are widely expressed regulatory proteins. In extrarenal epithelia, WNK1 and WNK4 have been shown to regulate other ion transporters and exchangers, establishing pleiotropic roles for these proteins since ion transport across the plasma membrane is fundamental for many cellular functions, including cell volume and pH regulation, electrochemical gradients, control of neuronal excitability and mucus constitution (Choate et al., 2003; Kahle et al., 2004a; Kahle et al., 2006).

1.5.1 NKCC1 and KCC2 regulation

The WNK1 and WNK4 effectors SPAK and OSR1 are ubiquitously expressed and have multiple functions. For instance, they have been confirmed to phosphorylate other SLC12 cation/chloride cotransporters besides renal NCC and NKCC2, such as the $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1) and the K^+/Cl^- cotransporter 2 (KCC2) *in vitro* in both *Xenopus* oocytes and HEK293 cells (Dowd and Forbush, 2003; Gagnon et al., 2006a; Vitari et al., 2005; Gagnon et al., 2006b). NKCC1 is also ubiquitously expressed and plays a key role in epithelial salt secretion and cell volume regulation, whereas KCC2 is a neuron-specific cotransporter, which functions not only as a significant chloride extrusion mechanism, but also plays an important role in the overall chloride homeostasis of mature neurons (Haas and Forbush, 2000; Kahle et al., 2008b).

NKCCs, which mediate chloride influx, are activated by phosphorylation and inhibited by dephosphorylation (Haas and Forbush, 2000), whereas KCCs, which transport chloride out of cells, show the opposite regulation (Lauf and Adragna, 2000), i.e. these cotransporters are reciprocally regulated by the same stimuli.

Activated WNK1 and WNK4 phosphorylate and activate SPAK and OSR1, which then phosphorylate and stimulate the activity of NKCC1 (Vitari et al., 2005) and phosphorylate and inactivate the function of KCC2 (Gagnon et al., 2006b). In addition to directly activating NKCC1 by phosphorylation, SPAK was suggested to play a scaffolding role in regulating NKCC1 function, perhaps by controlling its phosphorylation by other kinases (Piechotta et al., 2003).

Conversely, another study in *Xenopus* oocytes shows that WNK4 can inhibit NKCC1, which occurs *via* reduced surface expression of the cotransporter (Kahle et al., 2004a). At this stage, there is no explanation why the same cotransporter (NKCC1, although human *versus* mouse), the same kinase (WNK4), and the same expression system (*Xenopus laevis* oocytes) would yield such diametrically opposite results (Gagnon et al., 2006b).

1.5.2 KCC1, KCC3, and KCC4 regulation

KCC1 exhibits ubiquitous expression and its primary role seems to be cell volume regulation. On the other hand, KCC3 and KCC4 are expressed in several tissues, including the central nervous system and the kidney, and may play a role in several physiological processes, such as transepithelial salt absorption, renal potassium secretion and reabsorption, myocardial potassium loss during ischemia, and vascular smooth muscle cell relaxation (Gamba, 2005a; Mount and Gamba, 2001).

In *Xenopus* oocytes, wild-type WNK4 is capable of inhibiting the activity of all three cotransporters under cell swelling (Garzón-Muvdi et al., 2007), a condition in which KCC1, KCC3, and KCC4 are maximally active. The requirement of WNK4 catalytic activity to inhibit KCCs suggests that WNK4 induces their phosphorylation since it is known that KCCs remain inactive when phosphorylated (Lauf and Adragna, 2000). Furthermore, the presence of SPAK does not affect the activity of these cotransporters or change the WNK4 inhibitory effect on non-neuronal KCCs (Garzón-Muvdi et al., 2007).

In contrast, catalytically inactive WNK4 is able to bypass the tonicity requirements for activation of KCC3 (and KCC2) but not for KCC1 and KCC4, and activate this cotransporter in isotonic conditions (Garzón-Muvdi et al., 2007). The activation of KCC3 observed by inactive WNK4 is similar to what was previously observed with the catalytically inactive form of WNK3 (de Los Heros et al., 2006) and it is also similar to what would be expected with activation of the protein phosphatases, suggesting that catalytically inactive WNK4 is able to activate some of the endogenous protein phosphatases in the *Xenopus* oocytes.

The expression of WNK1 (but again not of SPAK or OSR1) also appears to be part of the pathway involved in the inhibitory phosphorylation events of cotransporter KCC3 and it is also suggested that dephosphorylation increases its intrinsic transport activity (Rinehart et al., 2009).

1.5.3   CFTR, SLC26A6 (CFEX), and SLC26A9 regulation

WNK kinases are coexpressed with the cystic fibrosis transmembrane conductance regulator (CFTR) in a variety of tissues, including ciliary epithelial cells in the lung (Choate et al., 2003; Yang et al., 2007a). CFTR is a cAMP-gated chloride channel encoded by the gene that is mutated in cystic fibrosis, being the most frequent mutation the deletion of phenylalanine 508 (Phe508), which causes increased proteolytic degradation of the misfolded mutant CFTR protein. Cystic fibrosis is a recessive genetic disease characterised by progressive lung dysfunction (which is the main cause of morbidity and mortality). This is a consequence of the defect in chloride transport coupled with hyperabsorption of sodium, which leads to the generation of thick and dehydrated mucus, and subsequent colonisation of the conducting airway by bacteria (such as *Pseudomonas aeruginosa*). Other symptoms include pancreatic insufficiency, elevated sweat electrolytes and male infertility (reviewed in Li and Naren, 2010). It has been shown that the disease severity could be significantly reduced if the retention at the plasma membrane of the mutant protein improved (since it could contribute to some residual chloride channel activity) (Amaral, 2005).

WNK1 and WNK4 kinases inhibit CFTR chloride channel activity in *Xenopus* oocytes, but the mechanisms appear to be different and, WNK1 and WNK4 exhibit additive CFTR inhibition. Moreover, the inhibitory effects of WNK1 are kinase dependent and do not affect CFTR abundance at the plasma membrane, whereas the inhibitory effects of WNK4 are kinase independent and associated with a reduction in CFTR cell surface abundance. Furthermore, the Wnk4 Q562E PHA-II-disease mouse model (*Wnk4* mutation homologous to the human Q565E mutation) has enhanced CFTR inhibitor activity when compared to the wild-type Wnk4 (Yang et al., 2007a).

Moreover, WNK4 PHA-II patients have been reported to have changes in epithelial potential difference and conductance that resemble mild cystic fibrosis, which could

be explained by the mutant WNK4 ability to suppress CFTR in nasal epithelia and sweat ducts of PHA-II patients (Farfel et al., 2005; Yang et al., 2007a).

WNK4 has also been reported to reduce the activity of the Cl^-/base exchanger SCL26-A6 (CFEX), in *Xenopus* oocytes (Kahle et al., 2004a). CFEX was first identified in the brush border of renal proximal tubule cells, however it is also expressed in pancreas, liver, and testis (Knauf et al., 2001; Lohi et al., 2000; Waldegger et al., 2001). Intriguingly, in all these tissues epithelial ducts secrete HCO3$^-$ by Cl^-/HCO3$^-$ exchange coupled to CFTR-regulated chloride secretion (Li and Naren, 2010).

SLC26A9 is also a member of the SLC26 family of anion transporters, which is expressed at high levels at the luminal membrane of airway secretory glands and gastric surface epithelial cells, where it functions as a chloride channel. WNK1, WNK3, and WNK4 protein kinases regulate SLC26A9 channel activity and the inhibition does not require the WNKs kinase activity. WNK-mediated inhibition of SLC26A9 activity is caused by reduced SLC26A9 surface expression both in *Xenopus* oocytes and HEK293 cell line (Dorwart et al., 2007).

The effects of WNK4 on CFTR activity and, of WNK1, WNK3, and WNK4 on SLC26A9 activity, are reminiscent of WNK1 and WNK4 effects on ROMK (Kahle et al., 2003; Lazrak et al., 2006; He et al., 2007). Both the effects of WNK4 on CFTR and of WNK1/3/4 on SLC26A9 are kinase activity-independent and associated with a change in protein abundance at the plasma membrane. Therefore, WNKs could act here (as has been shown for ROMK) as scaffolds that recruit other proteins that regulate the activity and/or surface expression of these channels, possibly by an endocytosis mechanism.

1.6 Summary of WNKs regulation of ion transport proteins

Taken together, the published data show that WNK kinases regulate many ion transport proteins by altering membrane trafficking, and/or their activity, and that the mechanisms by which WNK kinases regulate these proteins may vary (TABLE 1.1).

Table 1.1| Ion Transporters regulated by the WNK kinases

Channel	Function	WNK1 activity	WNK4 activity	Effect
NCC	Na^+/Cl^- cotransporter	dependent	dependent	surface and activity
NKCC	$Na^+/K^+/2Cl^-$ cotransporter	dependent	dependent	activity
KCC	K^+/Cl^- cotransporter	dependent	dependent	activity
ENaC	epithelial Na^+ channel	independent	independent	surface
ROMK	K^+ channel	independent	independent	surface
TRPV	Ca^+ channel	dependent	dependent	surface
CFTR	Cl^- channel	dependent	independent	activity or surface
CFEX	Cl^-/base exchanger	unknown	unknown	activity
SLC26A9	Cl^-/base exchanger	independent	independent	surface

In particular, WNK4 decreases cell surface expression of the thiazide-sensitive NaCl cotransporter NCC by inhibiting forward trafficking, whereas L-WNK1 antagonizes WNK4 activity and KS-WNK1 inhibits L-WNK1 action (Wilson et al., 2003; Yang et al., 2003; Yang et al., 2005b; Subramanya et al., 2006). On the other hand, both WNK1 and WNK4 enhance NCC transport activity through the cotransporter phosphorylation *via* their effectors, OSR1 and SPAK (Moriguchi et al., 2005; Vitari et al., 2005; Pacheco-Alvarez et al., 2006; Richardson et al., 2008).

Furthermore, WNK1 activates SGK1 to inhibit Nedd4-2-mediated internalization of the epithelial sodium channel ENaC, and although WNK4 also inhibits ENaC, it is not known whether WNK4 is acting through Nedd4-2 or in an independent pathway (Xu et al., 2005b; Ring et al., 2007a).

Both, WNK1 and WNK4 stimulate clathrin-mediated endocytosis of the potassium channel ROMK (Kahle et al., 2003; Cope et al., 2006; Lazrak et al., 2006; He et al., 2007) and, WNK4 also stimulates caveolae-mediated endocytosis of the epithelial calcium channel TRPV5 (Cha and Huang, 2010). Moreover, WNK4 regulates both clathrin-mediated and caveolae-mediated endocytosis by the same mechanism involving an interaction with intersectin, an endocytic scaffold protein.

Even though both WNK1 and WNK4 kinases inhibit CFTR chloride channel activity, the inhibitory effects of WNK1 are kinase-dependent and do not affect CFTR abundance at the plasma membrane, whereas the inhibitory effects of WNK4 are kinase-independent and associated with a reduction in CFTR cell surface abundance (Yang et al., 2007a).

On the other hand, WNK1 also interacts with, and phosphorylates (within its calcium binding C2 domains) synaptotagmin. This protein is a calcium sensor, which regulates endocytosis and exocytosis and thus may be involved in a general mechanism of WNK1-regulated trafficking of plasma membrane proteins. Phosphorylation by WNK1 increases the amount of calcium required for synaptotagmin binding to phospholipid vesicles. Therefore WNK1 could influence the rate of cycling of synaptotagmins among their calcium-dependent binding states, which would in turn compromise the functionality of a number of transporters that are regulated by their time of residence at the plasma membrane (Lee et al., 2004).

1.7 The Role of WNK kinases in cell survival and proliferation

Besides WNK kinases' role in ion channel regulation, WNKs also participate in signalling pathways involved in cell survival and proliferation. Whether a functional connection between both roles exists has not been established yet.

Several studies of somatic mutations in human cancers reported missense mutations in *WNK1* associated with brain, breast, lung, and colon cancers and, also a silent mutation associated with ovary cancer. In contrast, missense *WNK4* mutations were found in melanoma, and ovary carcinoma, and a frameshift deletion was found in gastric cancer. On the other hand, *WNK2* missense mutations have been found in brain and lung cancers, frameshift deletions have been described in gastric and colon cancers, and both types of mutations were found in ovary cancers. Finally, also missense mutations in *WNK3* were reported in lung and kidney cancers and a frameshift deletion was found in brain cancer (Stephens et al., 2005; Davies et al., 2005; Greenman et al., 2007; Parsons et al., 2008).

At the genome-wide level, a recent RNA interference study identified 73 kinases whose suppression increased the level of apoptosis by at least twofold over control, defining them as survival kinases. In this screen, WNK1 and WNK3 scored positive, whereas WNK2 and WNK4 had no effect on cell survival (MacKeigan et al., 2005 and J. Blenis, personal communication, Boston).

Additionally, WNK1 expression was greatly decreased in a tumour cell line, in which ganglioside synthesis was suppressed by genetic manipulation leading to a reduced rate of cell migration and invasiveness, thus indicating a putative role of WNK1 in tumorigenesis (Zeng et al., 2005). Furthermore, WNK3 was found to promote cell survival by inhibiting caspase-3 and thus permitting cells to evade apoptosis (Veríssimo et al., 2006).

All of these findings suggest a relationship between WNK protein kinases and cell survival and proliferation (reviewed in Moniz and Jordan, 2010).

Given that the protein kinases MEKK, PAK, and Raf are the WNK kinases closest homologues, a response to epidermal growth factor (EGF) would be expected. Indeed, WNK1 is involved in the EGF-dependent stimulation of extracellular signal-regulated

kinase 5 (ERK5), a signalling pathway linked to cell proliferation. Specifically, WNK1 activates this pathway in response to low concentrations of EGF in HEK293 cells. It does so by interacting with MEKK2 and MEKK3, which bind to and activate MEK5 kinase, leading in turn to the phosphorylation and activation of ERK5 (Xu et al., 2004; Nakamura and Johnson, 2003). Accordingly, reduction of WNK1 mRNA levels by RNA interference in C17.2 mouse neural progenitor cells suppresses the activation of ERK5 and, greatly reduces cell growth, differentiation and migration (Sun et al., 2006).

In contrast to WNK1, human WNK2 (the only subfamily member not known to be involved in ion channel regulation) has no effect on ERK5 but modulates the activation level of ERK1/2. The molecular mechanism through which a reduction in WNK2 expression can increase ERK1/2 activation involves phosphorylation of MEK1 at serine residue 298, a modification that increases its affinity towards ERK1/2. The increase in ERK1/2 activation promotes cell cycle progression through G1/S and sensitises cells to respond to lower concentrations of EGF (Moniz et al., 2007; Moniz et al., 2008).

Ectopic expression of WNK4 has also been shown to increase the phosphorylation of ERK1/2 and p38 MAPKs following EGF stimulation or hyperosmotic stress in HEK293 cells. However, the mechanism of WNK4 action remains unclear and the corresponding effect of endogenous WNK4 has not been addressed yet (Shaharabany et al., 2008).

WNKs can also affect other signalling pathways related to cell proliferation, including the inhibition of Smad2/transforming growth factor beta (TGFβ) signalling since both WNK1 and WNK4 directly bind to and phosphorylate Smad2. There are two effects of WNK1 on Smad2/TGFβ. One is to increase the cellular Smad2 level in HeLa cells, probably through an increase in its transcription. The other is to decrease the accumulation of phosphorylated Smad2 in the nucleus, causing inhibition of Smad2-mediated transcription (Lee et al., 2007). The dual actions of WNK1 may be relevant to the complex feedback regulatory mechanism for Smad2/TGFβ signalling, which can

lead to both tumour suppression and progression depending on the cell context and the microenvironment (Derynck et al., 2001; Massagué and Gomis, 2006).

As already mentioned (see 1.4.3 ENaC regulation), WNK1 also serves as a substrate for the protein kinase Akt (Vitari et al., 2004; Jiang et al., 2005). This kinase is an effector of the PI3K pathway, which is involved in the metabolic and mitogenic functions of insulin, including the regulation of gene expression, cell survival, cell growth, glucose transport, protein and glycogen synthesis, amongst many others (Lawlor and Alessi, 2001; Whiteman et al., 2002; Franke, 2008).

Moreover, WNK1 associates with syntaxin 4-regulatory protein Munc18c (Oh et al., 2007), which is known to participate in insulin-stimulated GLUT4 vesicle translocation and fusion in 3T3L1 adipocytes (Thurmond et al., 1998; Thurmond et al., 2000; Thurmond and Pessin, 2000). Although neither ectopic WNK1 expression alone nor small interfering RNA oligonucleotide (siRNA)-mediated depletion of WNK1 affects insulin secretion, endogenous WNK1-Munc18c complexes are important for glucose-stimulated insulin secretion *via* a syntaxin 4-dependent mechanism. This finding identifies a new role for WNK1 in vesicle/granule exocytosis and it is independent of its role as a kinase. Thus WNK1 could function as a scaffold, recruiting Munc18c as a substrate for another kinase or phosphatase (Jiang et al., 2005; Oh et al., 2007).

On the other hand, siRNA-induced depletion of WNK1 enhances insulin-stimulated DNA synthesis and cell growth in 3T3-L1 adipocytes suggesting a negative regulatory role for WNK1 in insulin-mediated proliferation control (Jiang et al., 2005).

As also previously mentioned, SGK1, which is activated by PI3K (and WNK1), regulates sodium transport regulation in the kidney, however, in other cell types it also mediates cyclin-dependent kinase inhibitor protein p27 phosphorylation, causing its cytoplasmic mislocalisation. This impairs the inhibition of cyclin E-Cdk2 (leading to

G1 progression) and contributes to increased cell motility, thus dysregulating cell cycle, cell proliferation and cell migration (Hong et al., 2008; McAllister et al., 2003).

1.8 The Role of WNK1 in organ development

The *WNK1* gene appears to be essential for normal embryonic development in mice, since homozygous knock-out is lethal *in utero*; heterozygotes, however, are viable despite presenting low blood pressure (Zambrowicz et al., 2003). Through the analysis of embryos of time-mated WNK1-null mice, it was found that WNK1 is indeed essential for cardiovascular development. Since *WNK1* is expressed both in the developing and adult heart there could be effects of *WNK1* overexpression on the cardiovascular system of PHA-II patients which might contribute to hypertension (Delaloy et al., 2006; Xie et al., 2009).

1.9 Objectives

Since the molecular mechanisms and interactions involved in WNK function are largely unknown, the elucidation of the molecular details involving human WNK1 and WNK4 protein kinases was the main objective of this thesis. Specifically, the identification of novel interacting cellular protein partners and the underlying signal transduction pathways was pursued. A proteomic or an antibody array approach was used to identify partners for PHA-II-causing family members WNK1 (Chapter 2) and WNK4 (Chapter 3), respectively. The results were expected to deepen the understanding on the role of WNK1 and WNK4 in normal cell physiology. This knowledge could contribute to clarify their role in hypertension, but may have implications for other ion channel-related disorders as well.

In addition, it is known that *WNK4* mutations detected so far in PHA-II patients are all missense alterations clustered in exons 7 or 17 (Wilson et al., 2001) and WNK4 PHA-II patients present hypercalciuria and low BMD besides hypertension. Accordingly, another objective was established, to conduct a mutational screen for *WNK4* exons 7 and 17 in a cohort of Portuguese patients and control individuals, and to evaluate if any of the alterations encountered in the population under study may confer genetic predisposition to hypertension and/or osteoporosis, through a case-control study (Chapter 4).

If you don't know where you are going,

any road will get you there.

Lewis Carroll (1832-1898)

2

Protein kinase WNK1 promotes cell surface expression of glucose transporter GLUT1 by regulating a TBC1D4/Rab8A complex

Mendes AI, Matos P, Moniz S and Jordan P

2.1 Summary

One mechanism by which mammalian cells regulate the uptake of glucose is the number of glucose transporter (GLUT) proteins present at the plasma membrane. In insulin-responsive cells types, GLUT4 is released from intracellular stores through inactivation of the Rab GTPase activating protein Tre-2/USP6, BUB2, Cdc16 domain family, member 4 (TBC1D4) (also known as AS160). Here we describe that TBC1D4 forms a protein complex with protein kinase WNK1 in HEK293 cells. We show that WNK1 phosphorylates TBC1D4 *in vitro* and that the expression levels of WNK1 in cells regulate surface expression of the constitutive glucose transporter GLUT1. WNK1 was found to increase the binding of TBC1D4 to regulatory 14-3-3 proteins while reducing its interaction with the exocytic small GTPase Rab8A. These effects were dependent on the catalytic activity because expression of a kinase-dead WNK1 mutant had no effect on binding of 14-3-3 and Rab8A, or on surface GLUT1 levels. Together, the data describe a pathway regulating constitutive glucose uptake *via* GLUT1, the expression level of which is related to several human diseases.

Keywords: AS160, Cell surface protein, Glucose transport, Glut1, Intracellular trafficking, Protein phosphorylation, Signal transduction, TBC1D4, WNK1

2.2 Background

WNK1 is one of four human members of the WNK subfamily of serine/threonine protein kinases and is the only family member with a ubiquitous expression pattern (Veríssimo and Jordan, 2001; Wilson et al., 2001; Choate et al., 2003; Delaloy et al., 2006). The WNK1 protein contains 2382 amino acids with a predicted molecular weight of 251 KDa and has its catalytic domain near the N-terminus, including the unique sequence variation around a catalytically-important lysine residue that characterises the WNK subfamily (Xu et al., 2000; Min et al., 2004). WNK1 contains coiled-coil domains but the bulk sequence reveals no homology to other known protein domains.

Currently, the knowledge on WNK1-regulated cellular pathways is still limited (Moniz and Jordan, 2010). On one hand, WNK1 can affect multiple signalling pathways related to cell proliferation, including the epidermal growth factor-dependent stimulation of ERK5 in HEK293 cells (Xu et al., 2004) and the inhibition of Smad2/TGFβ signalling (Lee et al., 2007) in HeLa cells. Additionally, IGF-1 treatment of HEK293 cells or adipocytes stimulates protein kinase Akt to phosphorylate WNK1 at threonine residue 60 (Thr60). However, the physiological relevance of this event is unclear because phosphorylation did not affect WNK1 kinase activity, or its subcellular localisation (Vitari et al., 2004; Jiang et al., 2005; Xu et al., 2005c). On the other hand, WNK1 is involved in restoring osmotic homeostasis. It directly phosphorylates and activates SPAK and OSR1 protein kinases, which interact with and stimulate the activity of the cation-chloride co-transporters NKCC1 (Vitari et al., 2005; Moriguchi et al., 2005; Anselmo et al., 2006; Vitari et al., 2006) or NCC (Moriguchi et al., 2005; Richardson et al., 2008) in HEK293 or HeLa cells. The expression of WNK1 also appears to be part of a pathway involved in the inhibitory phosphorylation events of cotransporter KCC3 (Rinehart et al., 2009).

Mutations in the *WNK1* and *WNK4* genes have been linked to PHA-II, a rare familial form of hypertension (Wilson et al., 2001) which is characterised by increased renal salt reabsorption and decreased potassium secretion. The identified *WNK1* mutations increase *WNK1* expression levels (Wilson et al., 2001; Delaloy et al., 2008) and *in vitro* data indicate that WNK1 inhibits WNK4, which itself regulates the renal sodium cotransporter NCC and the potassium transporter ROMK, in particular the amount of these channel proteins present at the surface of CCD cells (Gamba, 2005b; Kahle et al., 2008a; Richardson and Alessi, 2008). In addition, WNK1 was shown to have WNK4-independent effects on ion channels: it stimulates clathrin-dependent endocytosis of ROMK by interaction with the scaffold protein intersectin and this does not require WNK1 kinase activity (Cope et al., 2006; He et al., 2007). Moreover, WNK1 activates the SGK1, which phosphorylates the ubiquitin-protein isopeptide ligase Nedd4-2 so that less epithelial sodium channel (ENaC) is endocytosed from the plasma membrane of HEK293 cells (Xu et al., 2005b).

In addition to its role in ion channel regulation, WNK1 has also been implicated in protein secretion. In pancreatic beta cells WNK1 binds to synaptotagmin 2 (Lee et al., 2004) and Munc18c (Oh et al., 2007), two proteins involved in the fusion of secretory membrane vesicles.

Altogether, these data suggest that WNK1 regulates the retention or insertion of various transmembrane channel proteins and that different downstream effector mechanisms are involved. Here we describe a role for WNK1 in regulating glucose transporter GLUT1 (*SLC2A1* gene) in HEK293 cells. The constitutive expression of this transmembrane protein at the cell surface ensures glucose uptake in most tissues. We found that WNK1 through interaction with TBC1D4, a Rab GTPase-activating protein (RabGAP) involved in regulated exocytosis of glucose transporters, promotes GLUT1 surface expression.

2.3 Experimental Procedures

2.3.1 Cell culture and transfections

HEK293 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10 units/mL Penicillin, 10 μg/mL Streptomycin and 10% fetal calf serum (Invitrogen S.A., Barcelona, Spain), and regularly checked for absence of mycoplasm infection.

For ectopic expression of plasmid cDNAs, HEK293 cells were transfected at 80-90% confluence using Metafectene (Biontex, Martinsried/Planegg, Germany) according to the manufacturer's instructions. Transfection efficiencies were found to be 90%, as determined microscopically using a GFP expression vector. Total amounts of transfected plasmid DNA were kept constant at 6 μg per 60 mm dish or 2 μg per 35 mm dish and adjusted with empty vector if required. Cells were analysed after 20 hours for biochemical assays or after 16 hours for immunofluorescence experiments.

For gene silencing HEK293 at 30% confluence were transfected with 200 pmol small interfering RNA oligonucleotides (siRNAs) per 35 mm dish or 400 pmol per 60 mm dish using LipofectAMINE 2000 (Invitrogen). Cells transfected with siRNAs were analysed after 24 or 48 hours, and the achieved reduction in target gene expression was determined in each experiment by removing a 40 μL aliquot from the cell lysate for Western blot analysis. All results were confirmed in at least three independent experiments.

2.3.2 Expression constructs and small interfering RNA oligonucleotides

The previously described WNK1 full length cDNA encoding amino acids 1-2382 (Veríssimo and Jordan, 2001) was subcloned as an *Eco*RI fragment into expression plasmids pcDNA3-Myc or pEGFP-C2 (Invitrogen or BD Biosciences Europe, Erembodegem, Belgium). The kinase-dead WNK1 mutant K233M was made by site-directed mutagenesis of

codon AAG to ATG using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA, USA). WNK1 fragments were generated as follows: WNK1(1-538) was amplified with primer pair PK12-5-FL1 (5'-TCT GGC GGC GCC GCA GAG AA) and PK12-R2 (5'-CTT GTG CAA CAT CTT CTG GGA CA), cloned into pCR2.1-Topo (Invitrogen) and then transferred with *Eco*RI into pEGFP; WNK1(1-1340) was obtained by digestion of pEGFP-C2-WNK1 with *Afl*II to delete an internal fragment and religation of the remaining vector; for WNK1(1307-2382) pEGFP-C2-WNK1 was digested with *Stu*I and *Sal*I, the fragment cloned into pBluescript and transferred as an *Eco*RI fragment into pEGFP-C2; for WNK1(2030-2382) pEGFP-C2-WNK1 was digested with *Sac*I and *Eco*RI and the fragment recloned into pEGFP. Previously published constructs used in this study were Myc-tagged hnRNP A1 (Matter et al., 2000) and pCR3.1/AS160-2Myc (Peck et al., 2006). All constructs were verified by automated DNA sequencing.

Pools of three siRNA oligonucleotides were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) to deplete expression of endogenous TBC1D4 (sc-61654), or Rab8A (sc-41828), or Rab8B (sc-106474). The siRNA targeting WNK1 was the previously validated siWNK1-a (5'-GCA GGA GUG UCU AGU UAU ATT) (Moniz et al., 2007). As controls, pre-designed luciferase GL2 siRNA (5'-CGU ACG CGG AAU ACU UCG ATT) or a GFP-specific siRNA (5'-GGC UAC GUC CAG GAG CGC ACC TT) were used (all obtained from MWG-Biotech AG, Ebersberg, Germany).

2.3.3 Immunoprecipitation and Western blot procedures

For co-immunoprecipitation experiments, cells were grown in either 60 mm (transfected cells) or 100 mm dishes (endogenous proteins), lysed on ice in 250 µL non-denaturing lysis buffer (50 mM Tris-HCl pH 7.5, 1% NP-40, 100 mM NaCl, 10% glycerol, 10 mM $MgCl_2$) supplemented with a protease inhibitor cocktail composed of 1 mM PMSF, 1 mM 1,10-phenanthroline, 1 mM EGTA, 10 µM E64, and 10 µg/mL

of each aprotinin, leupeptin, and pepstatin A (all from Sigma-Aldrich Quimica, Madrid, Spain). The cell lysates were incubated for 2 hours at 4ºC with the specified antibodies (2.5 µg/mL anti-GFP ab1218 or anti-TBC1D4 ab24469 (Abcam, Cambridge, UK), anti-Myc clone 9E10 (M5546, Sigma-Aldrich), rabbit anti-Myc A14 (sc-789 from Santa Cruz Biotechnologies), or affinity-purified rabbit anti-WNK1 serum 308), then further incubated for 1 hour with protein G-Agarose beads (Roche Applied Science, Indianapolis, IN, USA), and finally washed three times in cold lysis buffer containing 300 mM NaCl. Proteins were solubilised from the beads in 2x SDS sample buffer, boiled, and separated in a 10% SDS-PAGE Protean III mini-gel (BioRad, Hercules, CA, USA).

For proteomic identification of proteins, the gel was stained in standard Coomassie Brillant Blue R250, destained and a digital image taken over a light box. Candidate bands were excised from the gel using a stereomicroscope, placed into sterile water and shipped for mass spectrometry as indicated by the service provider Alphalyse (Odense, Denmark, www.alphalyse.com).

For detection of specific proteins, the polyacrylamide gel was transferred onto a PVDF membrane (BioRad) using a Mini Trans-Blot cell (BioRad; 100V for 1 hour) followed by Coomassie-staining to check for equal transfer. Western blot membranes were blocked in TBS, 0.1% Triton X-100, 5% milk powder, probed using the indicated antibodies, and then incubated with a secondary peroxidase-conjugated antibody (BioRad) followed by chemiluminescence detection. Primary antibodies used for Western blots were rabbit anti-WNK1 serum 308 (affinity-purified polyclonal antibody raised and purified by Eurogentec (Ougree, Belgium) against peptide TSKDRPVSQPSLVGSKEC) (Veríssimo and Jordan, 2001), rabbit anti-Myc A14 (sc-789), rabbit anti-pan 14-3-3 K19 (sc-629) and anti-GLUT1 H43 (sc-7903) from Santa Cruz Biotechnologies, rabbit anti-GFP ab290 and rabbit anti-TBC1D4 ab24469 from Abcam, mouse anti-Rab8 (clone 4/Rab4) and mouse anti-Rab4 (clone 7/Rab4) from BD Biosciences Europe, mouse anti-T7-tag and mouse

anti-PCNA ab-1 (clone PC10) from Merck4Biosciences (Nottingham, UK), mouse anti-α-tubulin (clone B5-1-2) from Sigma-Aldrich and mouse anti-human transferrin receptor (clone H68.4, Invitrogen).

2.3.4 Production of recombinant OSR1 and *in vitro* protein kinase assays

For the production of recombinant OSR1, a reported WNK1 substrate (Zagórska et al., 2007), its coding sequence was amplified from cDNA clone IRAUp969A0428D (ima-Genes, Berlin, Germany) by PCR with primers Bam-Osr1-F (5'-GGA TCC ATG TCC GAG GAC TCG AG) and Hind-Osr1-R (5'-TTC GAA TTA GCT GAT GCT GAG CTG), cloned into pCR2.1 Topo, and then subcloned into the *Bam*HI and *Hin*dIII sites of T7/His-tagged pET28 vector (Merck4Biosciences). Subsequently, codon 164 of the cDNA was mutated from GAC to GCC using the Quick Change mutagenesis kit (Stratagene) to generate the kinase-dead OSR1-D164A mutant. The protein was expressed in the *E. coli* BL21 strain under IPTG induction and the bacterial pellets harvested at $1,400 \times g$, 20 min and frozen. For protein extraction pellets were resuspended in 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT in the presence of the protease inhibitor cocktail described above and then sonicated on ice in 10 cycles of 30 seconds with 10 seconds intervals (Sonics Vibra Cell sonicator, set at 40% amplitude). Following centrifugation of the extract at $16,000 \times g$, the supernatant was incubated with Ni-NTA agarose beads (Qiagen, Hilden, Germany) for 1 hour at 4°C. Beads were washed twice with cold lysis buffer containing 20 mM imidazole and protease inhibitors. Recombinant OSR1 was eluted in cold lysis buffer containing 250 mM imidazole, quantified, and stored in aliquots at -80°C.

For *in vitro* protein kinase assays, cells were lysed under strigent conditions in 250 μL RIPA buffer (50 mM Tris-HCl pH 7.5, 1% NP-40, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with protease inhibitors (see above). To reduce extract

viscosity 500 units/mL benzonase (Sigma-Aldrich) and 5 mM $MgCl_2$ were added. Following immunoprecipitation of WNK1 as described above, the resulting beads were washed three times in cold lysis buffer containing 300 mM NaCl, and then resuspended in 20 μL kinase reaction buffer (30 mM Tris/HCl pH 7.5, 10% glycerol, 1 mM DTT, 1 mM Na_3VO_4, 10 mM $MgCl_2$, 100 μM ATP), mixed with their substrate (either beads containing immunoprecipitated Myc-TBC1D4 or 500 ng recombinant kinase-dead OSR1), and incubated in the presence of 5 μCi γ-[^{32}P] ATP at 30°C for 30 min. Finally, 2x SDS sample buffer was added, samples boiled and separated by SDS-PAGE followed by Western blot. Membranes were exposed to X-ray films, and subsequently incubated with the indicated antibodies in order to document protein quantities.

2.3.5 Biotinylation of cell surface proteins

HEK293 cells were transfected as described above, washed twice with warm culture medium to remove dead cells, and placed on ice in a cold room. Cells were incubated for 5 min with ice-cold PBS-CM (PBS pH 8.0 containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) to ensure arrest of endocytic traffic. Then, cells were incubated for 30 min with 0.5 mg/mL EZlink sulfo-NHS-SS-biotin (Pierce Biotechnology, Rockford, IL, USA) in PBS-CM, before being rinsed twice and left for 10 min on ice with ice-cold Tris/Glycine (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glycine, 1% BSA) to terminate the biotinylation reaction. Cells were again washed 3x with cold PBS-CM and lysed in 250 μL pull-down buffer (50 mM Tris/HCl pH 7.5, 100 mM NaCl, 10% glycerol, 1% NP-40) in the presence of the aforementioned protease inhibitor cocktail. The cell lysates were harvested by scraping and cleared by centrifugation at 16,000 × g at 4°C for 5 min. An aliquot of 40 μL representing the total GLUT1 level was removed and added to 5x SDS sample buffer, while 200 μL lysate were added to 50 μL Streptavidin-agarose beads (Sigma-Aldrich), previously incubated for 1 hour in 1 mL cold pull-down buffer containing 2% non-fat milk powder, and washed 3x in

pull-down buffer. For purification of biotinylated proteins, lysate and beads were incubated under rotation for 1 hour at 4ºC, the beads collected by centrifugation (1 min at 3,000 × g), and washed 3x in cold wash buffer (100 mM Tris/HCl pH 7.5, 300 mM NaCl, 1% Triton X-100). Captured proteins were recovered by boiling the beads in 25 µL 2x SDS sample buffer plus 2 µL of 1 M DTT. The biotinylated protein fraction and one twentieth of the corresponding whole cell lysates were analysed alongside by SDS-PAGE followed by Western blot.

2.3.6 Immunoprecipitation from cell lysates stabilised by cross-linking

For experiments using a cleavable chemical cross-linker, HEK293 cells were seeded in 60 mm dishes, transfected as described, rinsed 3x in PBS-CM at room temperature and incubated for 30 min on ice with 0.5 mM DSP (Sigma-Aldrich). Subsequently, cells were rinsed twice and left for 15 min on ice with ice-cold Tris/Glycine buffer (see above) to terminate the cross-linking reaction. Cells were again washed 3x with cold PBS-CM and lysed in 500 µL RIPA buffer supplemented with protease inhibitors and treated with benzonase (see above). An aliquot of 80 µL representing the total protein levels was removed and added to 5x SDS sample buffer, while 400 µL lysate were used for immunoprecipitation with anti-Myc antibodies, as described above. Proteins were solubilised from the beads in 2x SDS sample buffer supplemented with 5% β-mercaptoethanol to reverse the introduced cross-links, and analysed by SDS-PAGE followed by Western blot.

2.3.7   Immunofluorescence microscopy

HEK293 cells were grown on coverslips, transfected as indicated, fixed after 16 hours with 4% (v/v) formaldehyde (Merck Chemicals, Darmstadt, Germany) in PBS and permeabilised with 0.2% Triton X-100 in PBS. Cells were then immunolabeled for 1 hour with rabbit anti-GLUT1 (USBiological, Massachusetts, MA, USA, catalog G3900-03D), washed 3x 5 min in PBS/0.01% Tween (PBS-T), and incubated with a secondary Alexa532-conjugated antibody (Invitrogen-Molecular Probes). Next, cells were washed, stained briefly in DAPI solution (Sigma; 1:1000 in PBS-T), washed again, and post-fixed for 15 min in 4% formaldehyde. Coverslips were mounted on microscope slides with Vectashield (Vector Laboratories, Burlingame, CA, USA), images recorded on a Leica TCS-SPE confocal microscope and processed with Adobe Photoshop software.

2.4   Results

2.4.1   WNK1 exists in a complex with TBC1D4

In order to identify proteins interacting under physiological conditions, endogenous WNK1 was immunoprecipitated under non-denaturing conditions from HEK293 cells using a previously described anti-WNK1 serum (Veríssimo and Jordan, 2001). The obtained protein fraction was then separated by gel electrophoresis and stained for the presence of co-immunoprecipitated proteins (FIG. 2.1 | A), which were excised from the gel and identified by mass spectrometry. Besides WNK1 itself, we identified a major co-purifying protein migrating above 150 KDa as TBC1 (Tre-2/USP6, BUB2, Cdc16) domain family, member 4, also known as AS160 (Akt substrate of 160 KDa), a protein involved in insulin-stimulated glucose transport in adipocytes (Watson and Pessin, 2006; Sakamoto and Holman, 2008).
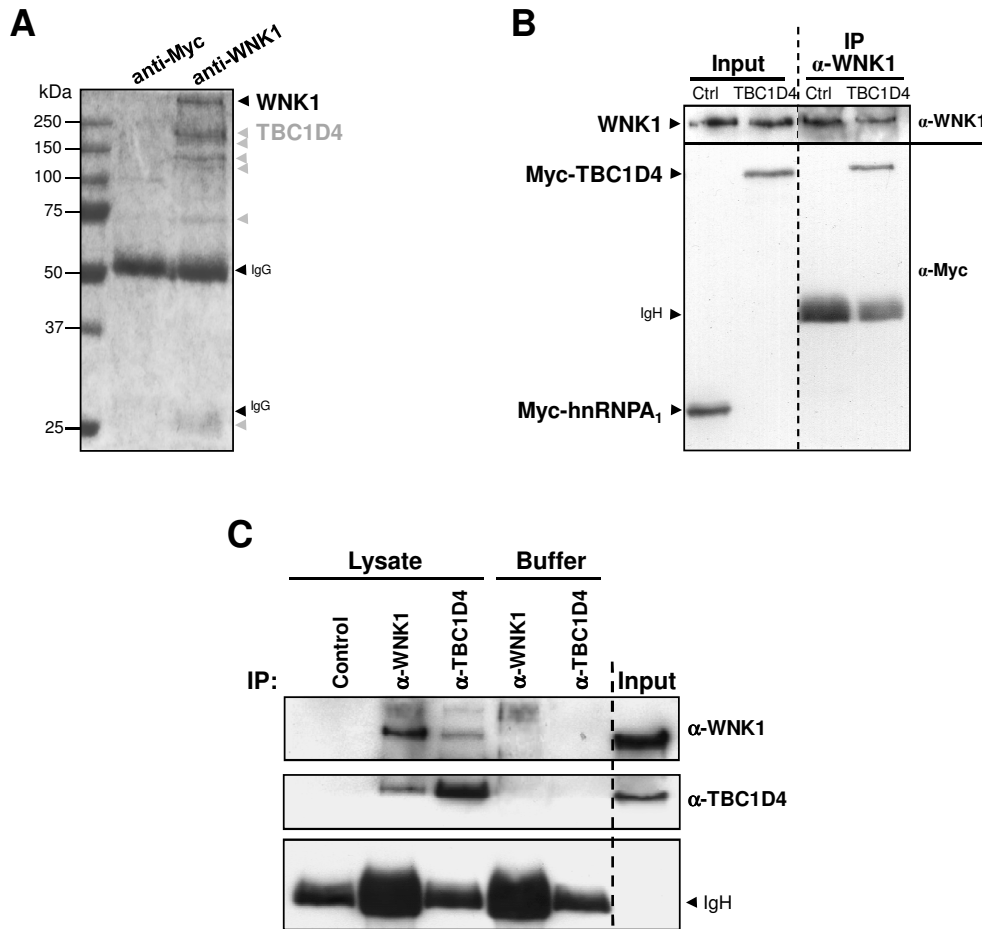
Figure 2.1| WNK1 forms a protein complex with TBC1D4 in HEK293 cells. A| Cells were lysed under non-denaturing conditions and incubated with affinity-purified anti-peptide antibodies against either human WNK1 or the Myc-epitope (negative control). Shown is the digital image of a Coomassie-stained gel and gray arrowheads indicate the bands that specifically co-precipitated with the anti-WNK1 antibodies. These bands were excised from the gel for mass spectrometric identification, and those identified as WNK1 and TBC1D4 are indicated. Note that endogenous WNK1 protein has been described to migrate at an apparent molecular weight above 251 KDa (Veríssimo and Jordan, 2001; Vitari et al., 2004). **B|** HEK293 cells were transfected with Myc-TBC1D4 or Myc-hnRNP A1 (negative control), then lysed under non-denaturing conditions and incubated with anti-WNK1 to immunoprecipitate endogenous WNK1. The total cell lysate (Input) and the precipitate (IP) were analysed by Western blot with the indicated antibodies. Note that Myc-TBC1D4 but not Myc-hnRNP A1 co-immunoprecipitates with endogenous WNK1. **C|** Co-immunoprecipitation of endogenous TBC1D4 and WNK1. HEK293 cells were lysed under non-denaturing conditions, incubated for immunoprecipitation with a control rabbit antibody, or anti-WNK1 or anti-TBC1D4 (IP), and the resulting fraction analysed by Western blot. In addition, anti-WNK1 and anti-TBC1D4 antibodies were incubated in the absence of cell lysate (buffer) to exclude that non-specific antiserum-derived bands would confound the results. Note the presence of TBC1D4 in the sample precipitated with anti-WNK1 and the detection of WNK1 in the sample precipitated with anti-TBC1D4. The lane marked as 'Input' shows the expression levels observed in whole cell lysates, and immunoglobulin heavy chains (IgH) are indicated to document success and comparability of the immunoprecipitation reactions.

For confirmation of the observed interaction, Myc-TBC1D4 was transfected into HEK293 cells and endogenous WNK1 immunoprecipitated as above. Anti-Myc antibodies clearly detected the presence of TBC1D4 in the precipitate. As a control, the unrelated nuclear protein Myc-hnRNP A1 was expressed but did not co-immunoprecipitate with WNK1 (FIG. 2.1 | B). Moreover, both endogenous proteins co-immunoprecipitated, i.e. using specific anti-TBC1D4 or anti-WNK1 antibodies we could demonstrate by Western blot that endogenous TBC1D4 co-immunoprecipitated with endogenous WNK1, or *vice versa* (FIG. 2.1 | C).

In order to determine which WNK1 domain mediates the interaction with TBC1D4, several GFP-tagged WNK1 deletion mutants were expressed and tested for their ability to co-immunoprecipitate with Myc-TBC1D4 (FIG. 2.2 | A). The results showed that, besides full-length WNK1(1-2382), the N-terminal WNK1(1-538) fragment had the strongest affinity to TBC1D4 (FIG. 2.2 | B). The weak affinity for intermediate fragment WNK1(1-1340) suggests that intra-molecular interactions exist that can modulate access to the N-terminus. Together, these data validate the existence of a protein complex containing WNK1 and TBC1D4 in HEK293 cells.

2.4.2 WNK1 phosphorylates TBC1D4 *in vitro*

Given the preferential interaction with the N-terminal WNK1 fragment, which contains the catalytic domain, we next tested whether TBC1D4 served as a substrate for WNK1 kinase activity. For this, WNK1 was immunoprecipitated from HEK293 cells transfected with either GFP-WNK1 or the N-terminal GFP-WNK1(1-538) fragment. Cells were lysed in RIPA buffer and the immunoprecipitated WNK1 beads washed in high salt buffer so that contamination with other kinases from the cell lysate was minimised. The immunoprecipitated kinases were analysed in an *in vitro* kinase assay followed by electrophoretic protein separation and transfer to PVDF membranes. As a

A

B
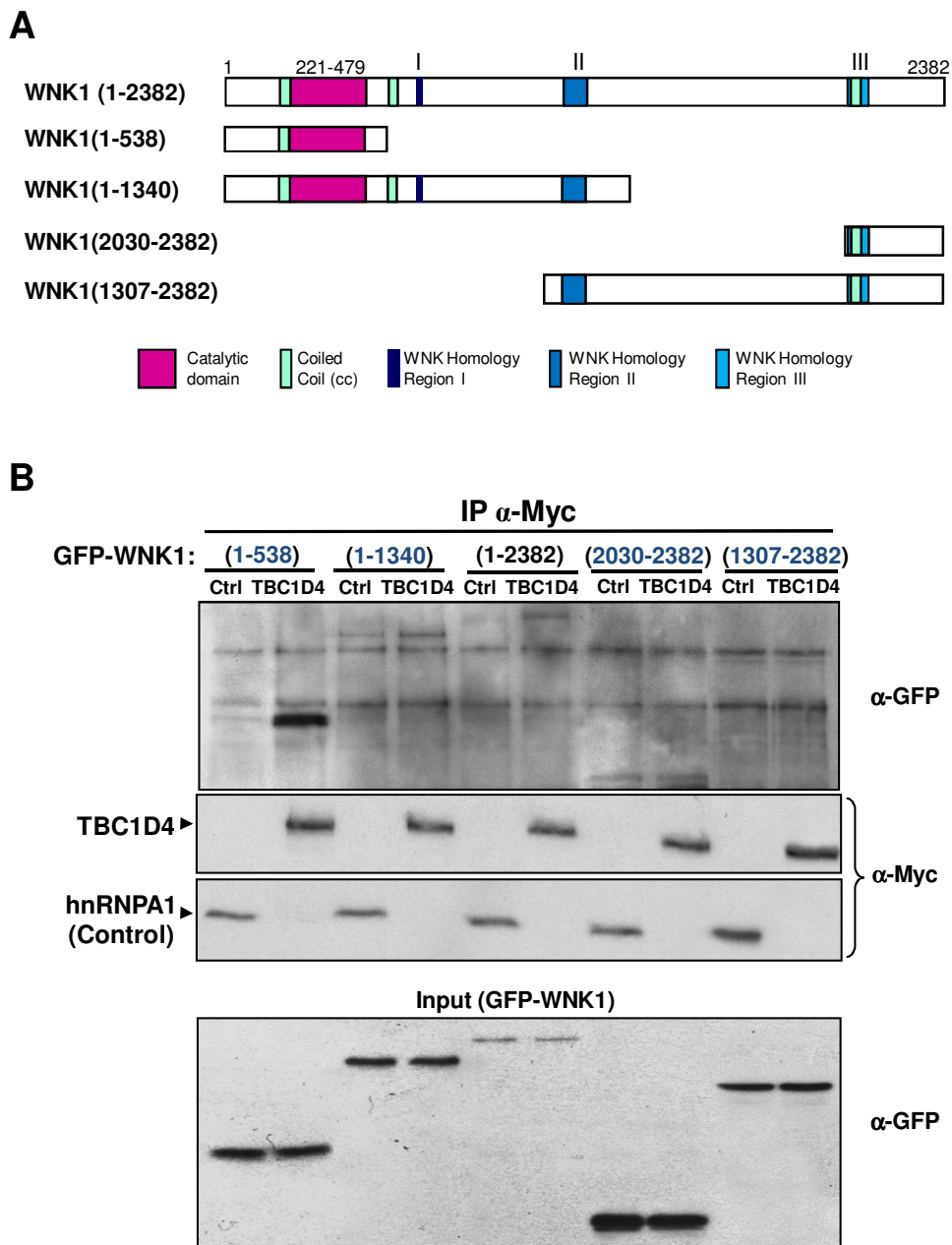
Figure 2.2| The N-terminal WNK1 region is required for protein complex formation with TBC1D4.
A| Schematic representation of full-length human WNK1 and the four fragments tested with the respec-
tive amino acid numbers in parentheses. **B|** HEK293 cells were co-transfected with one of the indicated
GFP-WNK1 constructs and either Myc-TBC1D4 or Myc-hnRNP A1 (negative control). Following cell ly-
sis under non-denaturing conditions, anti-Myc antibodies were added and immunoprecipitated proteins
analysed by Western blot. The lower panel shows the expression levels of GFP-WNK1 constructs in the
whole cell lysate (Input), the middle panels the quantities of precipitated Myc-tagged TBC1D4 and hn-
RNP A1, and the upper panel the amounts of co-precipitated GFP-WNK1 constructs. Note the specific
co-immunoprecipitation of full-length WNK1 and its N-terminal fragment (1-538).

positive control we added recombinant OSR1 to the assay, a recently described WNK1 substrate (Zagórska et al., 2007). As shown in FIG. 2.3, both WNK1 and its N-terminal fragment autophosphorylated and phosphorylated OSR1. In order to test TBC1D4 as substrate, Myc-TBC1D4 was immunoprecipitated from transfected cells in RIPA buffer, the beads washed in high-salt buffer and added as substrate to the kinase assay. We observed that both WNK1 and its N-terminal fragment phosphorylated TBC1D4. In contrast, the respective kinase-dead WNK1 mutants did not phosphorylate it, confirming the absence of contaminant kinase activities (FIG. 2.3).

2.4.3 WNK1 catalytic activity increases the association of endogenous TBC1D4 with 14-3-3 proteins

Previously, it has been described that phosphorylated TBC1D4 is a phosphoprotein that interacts with 14-3-3 (Jin et al., 2004; Ramm et al., 2006; Geraghty et al., 2007), a family of proteins that bind phosphoproteins and modulate the corresponding signalling activities (Mackintosh, 2004). We thus tested the effect of WNK1 expression on the interaction of TBC1D4 with 14-3-3 proteins. Endogenous TBC1D4 was immunoprecipitated from HEK293 cells and the precipitate analysed by Western blot. It was found that the protein complex formed between the endogenous TBC1D4 and WNK1 contained considerably increased amounts of 14-3-3 proteins. In contrast, cells expressing the kinase-dead WNK1 mutant showed only co-immunoprecipitation between WNK1 and TBC1D4, and no change in the association with 14-3-3, which remained at background level comparable to that observed in GFP-expressing control cells (FIG. 2.4).

Controls

rOSR1	+	+	+	−	−	−	−	−
TBC1D4	−	−	−	+	+	+	+	+
GFP	+	−	−	+	−	−	−	−
WNK1 wt	−	−	+	−	+	−	−	−
WNK1 kd	−	−	−	−	−	+	−	−
1-538 wt	−	+	−	−	−	−	+	−
1-538 kd	−	−	−	−	−	−	−	+
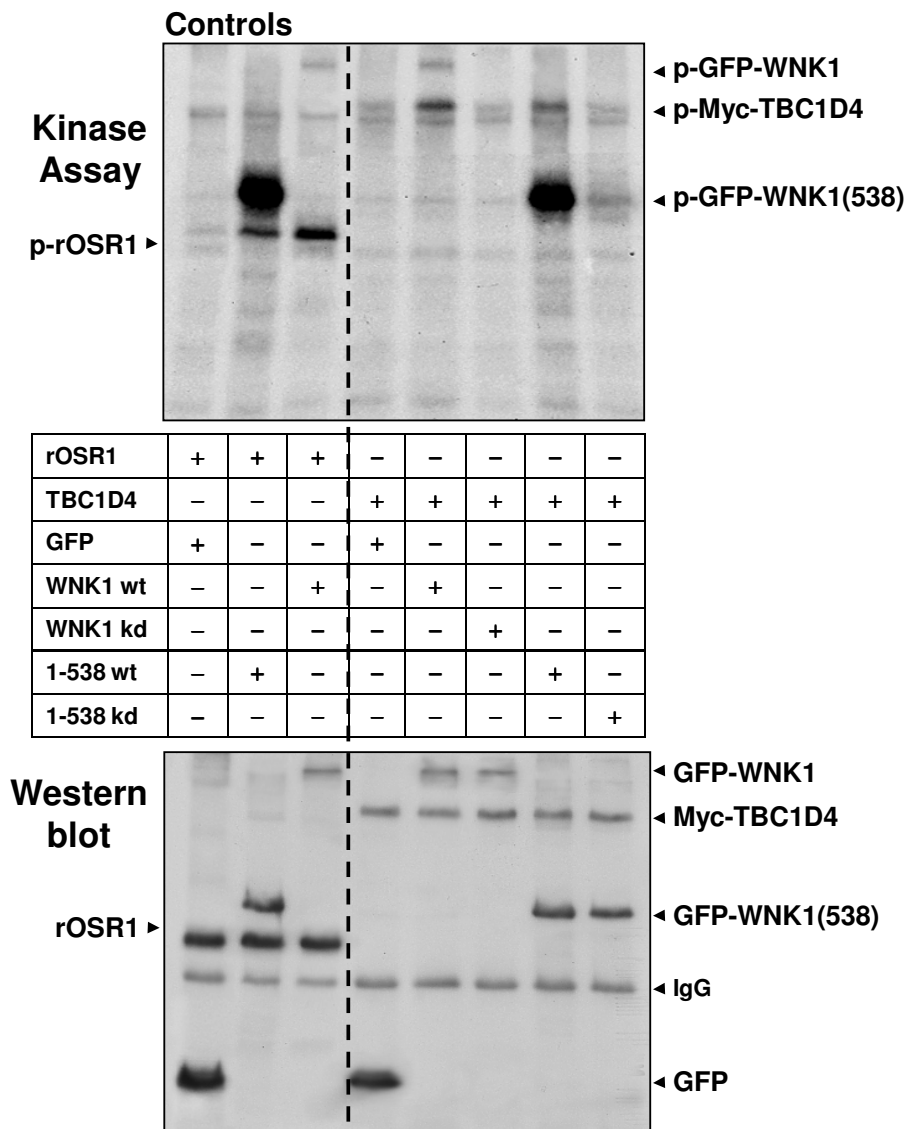
Figure 2.3| WNK1 phosphorylates TBC1D4 *in vitro*. HEK293 cells were transfected with either GFP empty vector, wild-type (wt) GFP-WNK1, wt fragment GFP-WNK1(1-538), or the respective kinase-dead (kd) WNK1-K233M mutants. WNK1 was immunoprecipitated using anti-GFP antibodies and tested in an *in vitro* protein kinase assay. As substrates served either recombinant OSR1 (rOSR1), as a previously described physiological substrate (Zagórska et al., 2007)(Controls), or beads containing immunoprecipitated Myc-TBC1D4. After 30 min of incubation the samples were denatured by adding SDS sample buffer and boiling, then separated by SDS-PAGE and transferred to PVDF blotting membranes. Incorporated radioactive [^{32}P]-phosphate was detected by exposing the membranes for 24 hours to X-ray films (Kinase assay). Subsequently, the amounts of recombinant T7-OSR1 or of immunoprecipitated proteins were documented by Western blot using anti-GFP, and then reprobed with either anti-T7 or anti-Myc antibodies. Note that both wt full-length WNK1 and its fragment (1-538) autophosphorylate, and also phosphorylate the physiological substrates OSR1 and TBC1D4. Whereas fragment (1-538) shows stronger autophosphorylation activity, possibly due to partial deletion of the inhibitory domain (Xu et al., 2002a), wt full-length WNK1 has higher activity towards TBC1D4. Beads containing the respective kd mutants revealed no phosphorylation activity.
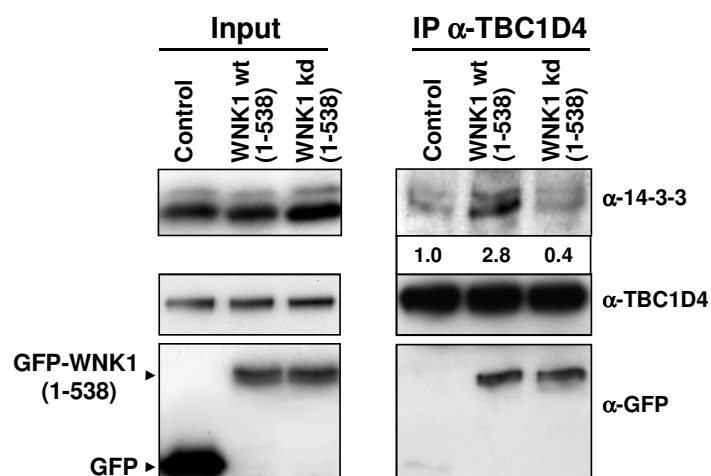
Figure 2.4| Expression of WNK1 increases the association of endogenous TBC1D4 with 14-3-3 proteins. HEK293 cells were transfected with either GFP empty vector (control), or the wild-type (wt) fragment GFP-WNK1(1-538), or its respective kinase-dead (kd) mutant. Endogenous TBC1D4 was immunoprecipitated from cell lysates prepared under non-denaturing conditions and probed by Western blot. Shown are the levels of GFP-WNK1 and endogenous TBC1D4 and 14-3-3 proteins in total cell lysates (Input; left panel) and in the immunoprecipitate (IP; right panel). The quantification of the 14-3-3 band intensities is given below the blot as fold-over-control values. Note that the amount of 14-3-3 co-precipitating with TBC1D4 increased considerably in the presence of wt WNK1, not however of mutant kd WNK1.

2.4.4 WNK1 leads to increased expression of GLUT1 at the cell surface

In insulin-sensitive adipocytes and skeletal muscle cells TBC1D4 was shown to control the translocation of the insulin-responsive glucose transporter GLUT4 from intracellular storage vesicles to the plasma membrane (Sakamoto and Holman, 2008; Koumanov and Holman, 2007). Most other cell types, however, express the transporter GLUT1 for their constitutive uptake of glucose (Zhao et al., 1993). We thus tested whether the observed phosphorylation of TBC1D4 by WNK1 affected the expression of endogenous GLUT1 at the cell surface. HEK293 cells were transfected with either full-length WNK1, or WNK1(1-538), or their kinase-dead mutants K233M, and after 20 hours of expression transferred to 4°C for biotinylation of cell surface proteins. Following cell lysis, biotinylated proteins were captured using Streptavidin-agarose beads and analysed by Western blot using specific antibodies. As positive and negative controls, we confirmed that the biotinylated protein fraction contained the surface protein E-cadherin

but not cytosolic tubulin or the nuclear protein PCNA (data not shown). Under these experimental conditions we found increased GLUT1 cell surface expression in the presence of catalytically active WNK1 (either full-length or fragment (1-538)) (FIG. 2.5). In contrast, expression of the kinase-dead mutants inhibited the level of surface GLUT1 compared to control cells. As further support for the role of WNK1, we depleted endogenous WNK1 from HEK293 cells by RNA interference and analysed endogenous
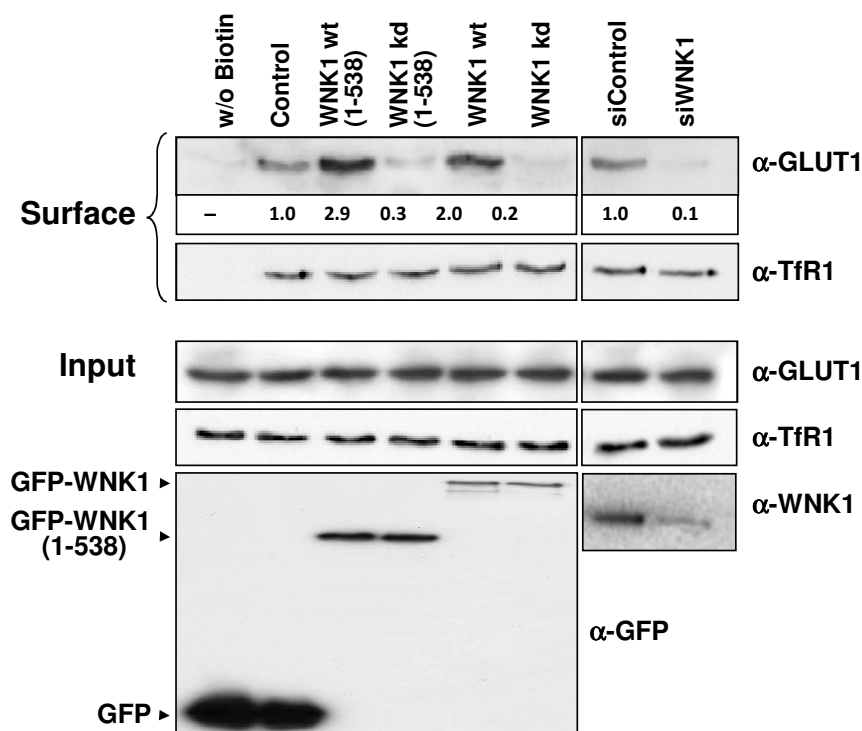
Figure 2.5| Expression of *WNK1* affects the amount of GLUT1 expressed at the cell surface. HEK293 cells were transfected with either GFP empty vector (Control), or wild-type (wt) full-length GFP-WNK1, or fragment GFP-WNK1(1-538), or the respective kinase-dead (kd) mutants, as indicated. In separate experiments, HEK293 cells were transfected with either control (siControl) or WNK1-specific (siWNK1) small interfering oligonucleotides. After 20 hours cell surface proteins were biotinylated and captured from the cell lysate using Streptavidin-beads. Shown are Western blots detecting GLUT1 or TfR1 in the biotinylated protein fraction (top panels, Surface). The corresponding quantification of GLUT1 band intensities is given as fold-over-control values below the blot. Shown are also the expression levels in whole cell lysates (Input) of endogenous GLUT1 and TfR1 (middle panels), and of GFP-tagged proteins or endogenous WNK1 (bottom panels). Note that GLUT1 surface levels increase upon expression of catalytically active WNK1 but decrease in WNK1-depleted cells, whereas surface levels of TfR1 remained unaffected.

GLUT1 surface expression. In WNK1-depleted cells of a significant reduction in biotinylated GLUT1 was detected (FIG. 2.5). Under these experimental conditions the cell surface levels of the endogenous transferrin receptor 1 (TfR1) did not change.

The cell surface expression of endogenous GLUT1 was also determined by immunofluorescence microscopy. In HEK293 cells expressing catalytically active WNK1 as above, we observed increased surface staining with anti-GLUT1 antibodies compared to non-transfected cells (FIG. 2.6). On the contrary, expression of kinase-dead WNK1 reduced GLUT1 staining at the cell surface, while increasing the intracellular GLUT1 signal (FIG. 2.6).
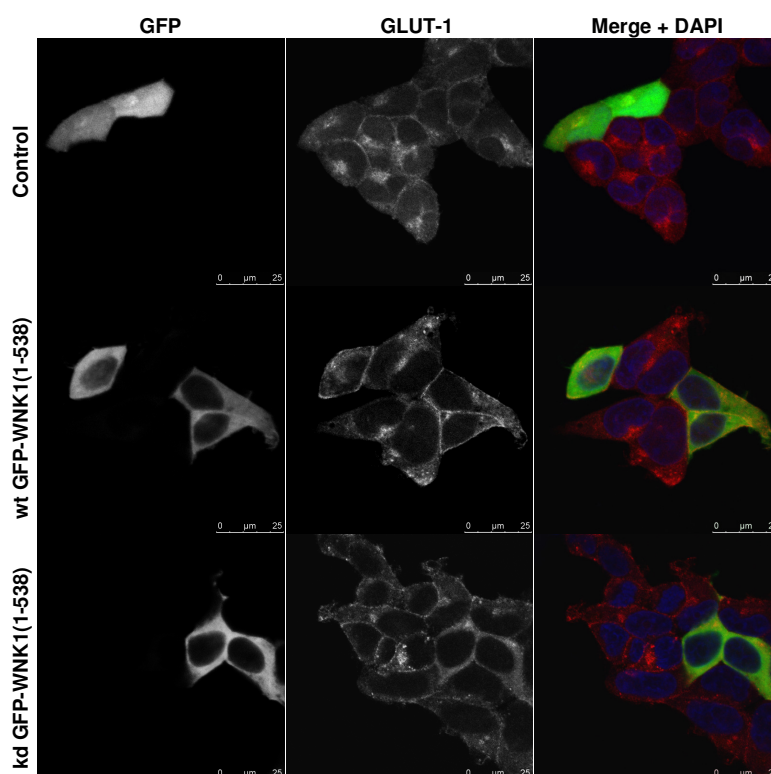
Figure 2.6| Visualisation of HEK293 endogenous GLUT1 protein by immunofluorescence microscopy. HEK293 cells were transfected as indicated with either GFP empty vector (control), or wild-type (wt) GFP-WNK1(1-538), or its respective kinase-dead (kd) mutant. Cells were fixed after 16 hours of expression, stained with anti-GLUT1 followed by a secondary Alexa532-conjugated antibody, and images recorded on a Leica SPE confocal microscope. Transfected cells are marked in green, DAPI in blue and GLUT1 in red. Note the increased surface staining of GLUT1 in cells expressing wt WNK1 in contrast to increased intracellular GLUT1 staining upon expression of kd WNK1.

2.4.5 Rab8A is required for GLUT1 surface expression downstream of WNK1

The above data are compatible with the idea that phosphorylation of TBC1D4 by WNK1 increases its association with 14-3-3 proteins and inhibits TBC1D4 as a negative regulator of GLUT1 surface expression. TBC1D4 was described to act as a GTPase-activating protein (GAP) for Rab2A, Rab8A, Rab10 and Rab14 (Mîinea et al., 2005; Ishikura et al., 2007). Since Rab8 is involved in constitutive biosynthetic trafficking from the *trans*-Golgi network (TGN) to the plasma membrane (Zerial and Mcbride, 2001; Stenmark, 2009), we tested its involvement in GLUT1 surface expression. We co-expressed wild-type GFP-WNK1(1-538) or its kinase-dead mutant, and Myc-TBC1D4 in HEK293 cells. Due to the transient nature of GAP-GTPase interactions, cells were briefly treated with the mild cross-linking reagent DSP before cell lysis. Then Myc-TBC1D4 was immunoprecipitated, and the precipitated fraction analysed for the presence of either endogenous Rab8 or Rab4 (as negative control). Whereas no Rab4 was detected, the endogenous Rab8 clearly co-immunoprecipitated. In addition, the amount of Rab8 in the complex with TBC1D4 was reduced in the presence of wild-type WNK1, but increased when the kinase-dead WNK1 mutant was expressed (FIG. 2.7 | A). These data indicate that the Rab-GAP TBC1D4 interacts transiently with Rab8 in HEK293 cells, and that phosphorylation of TBC1D4 by WNK1 affects the amount of Rab8 engaged.

To substantiate that GLUT1 traffic is regulated by WNK1/TBC1D4 through Rab8, endogenous Rab8A and Rab8B were depleted by specific small interfering oligonucleotides. These experiments (data not shown) identified the slower migrating band as Rab8A and revealed it to be the predominantly expressed form in HEK293 cells. In order to provide evidence that Rab8A operates downstream of WNK1, HEK293 cells were first transfected with siRab8A and 24 hours later transfected with Myc-WNK1(1-538). Under these conditions of Rab8A depletion, the transfection of WNK1 no longer promoted GLUT1 surface expression (FIG. 2.7 | B). Also, the depletion of endogenous

A

B
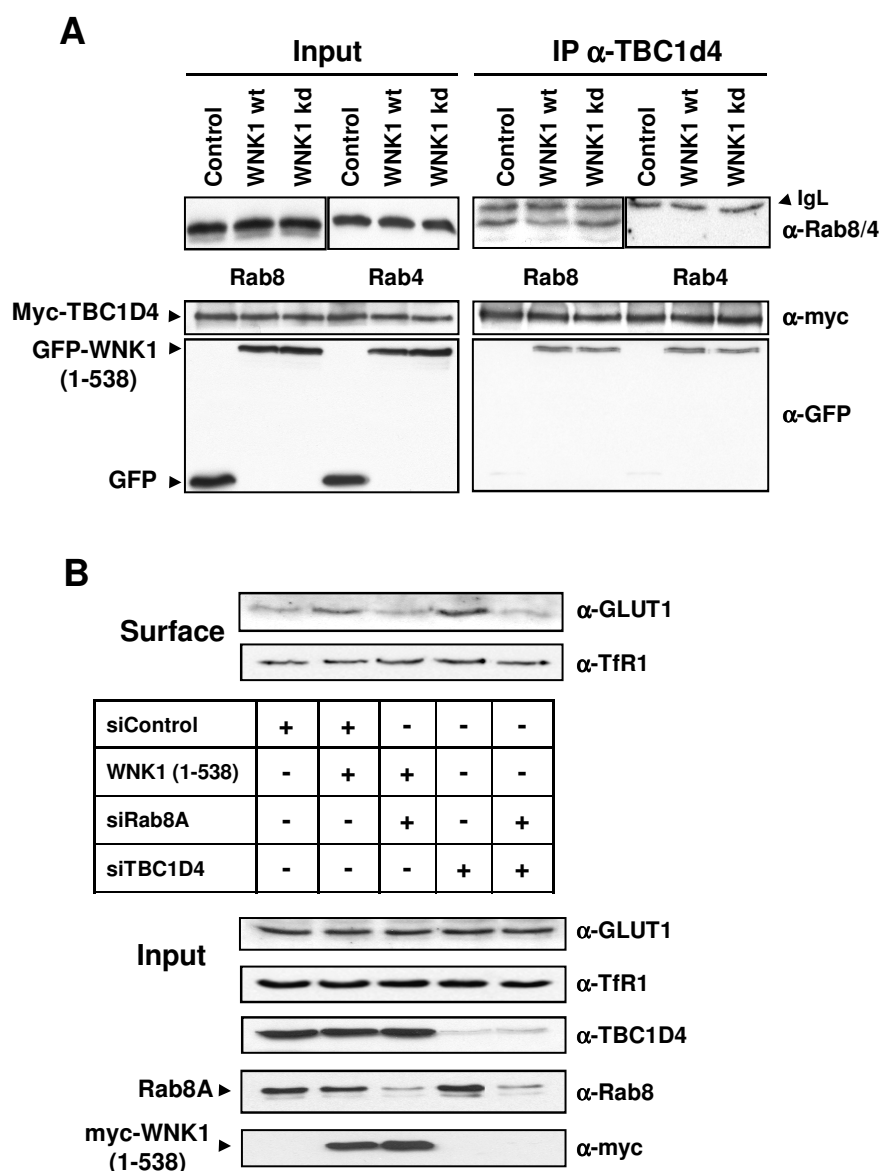
Figure 2.7| Rab8A is required for GLUT1 surface expression downstream of WNK1. A| HEK293 cells were transfected as indicated and treated briefly with the mild reversible cross-linking reagent DSP before lysis. Myc-TBC1D4 was immunoprecipitated and analysed by Western blot. Shown are the expression levels of GFP-WNK1(1-538), Myc-TBC1D4 and of the endogenous Rab8 and Rab4 proteins in total cell lysates (left side panels, Input) as well as the immunoprecipitated fractions (right side panels, IP). Note that Rab8A, but not Rab4, co-precipitated with TBC1D4, and that the amount decreased in the presence of wt WNK1, whereas increased in the presence of kinase-dead WNK1. **B|** HEK293 cells were transfected with control (siGFP) or Rab8A-specific (siRab8A) small interfering RNAs, either alone or in the presence of Myc-WNK1(1-538). In parallel, cells were transfected with TBC1D4-specific siRNAs (siTBC1D4), either alone or in combination with siRab8A. As described in caption of Figure 2.5, the biotinylated cell surface proteins (Surface) and whole cell lysates (Input) were analysed by Western blot to document the amounts of endogenous GLUT1, TfR1, TBC1D4 or Rab8A, as well as of transfected Myc-WNK1(1-538). Note the successful depletion of TBC1D4 (lanes 4 and 5) and of Rab8A (lanes 3 and 5). GLUT1 surface expression was promoted by either the expression of WNK1, or the depletion of TBC1D4, and the simultaneous knockdown of Rab8A impaired both effects. Under these conditions the surface levels of endogenous TfR1 remained unchanged.

TBC1D4 by siRNAs clearly increased GLUT1 levels at the surface, but the simultaneous transfection with siRab8A completely impaired the increase in GLUT1 observed upon depletion of TBC1D4 alone. Again, under these conditions the cell surface levels of endogenous TfR1 remained unaffected (FIG. 2.7 | B). Together, these experiments clearly revealed that Rab8A is involved downstream of WNK1 and TBC1D4 in the control of GLUT1 expression at the surface of HEK293 cells.

2.5 Discussion

The main conclusion from the present work is that protein kinase WNK1 forms a complex with the Rab-GAP TBC1D4, which is involved in regulating the amount of glucose transporter GLUT1 expressed at the cell surface. The complex formation between TBC1D4 and WNK1 was initially identified using an unbiased proteomic approach but then confirmed by immunoprecipitation using various antibodies. In particular, anti-Myc antibodies detected co-immunoprecipitation of epitope-tagged Myc-TBC1D4 together with GFP-WNK1 (FIG. 2.2), as well as with endogenous WNK1 (FIG. 2.1 | B). Furthermore, GFP-tagged WNK1 was pulled down when antibodies against endogenous TBC1D4 were used (FIG. 2.4), and the co-immunoprecipitation of both the endogenous proteins could also be demonstrated in HEK293 cells (FIG. 2.1 | C). Together, these experiments provide solid support for the specificity of the observed interaction between WNK1 and TBC1D4.

TBC1D4 was previously identified to regulate GLUT4 trafficking to the cell surface following insulin stimulation of 3T3L1 adipocytes or skeletal muscle cells. This pathway involves phosphorylation of TBC1D4 by protein kinase Akt and has led to its alternative designation as Akt substrate of 160 KDa (AS160) (Watson and Pessin, 2006; Sakamoto and Holman, 2008; Koumanov and Holman, 2007; Mîinea et al., 2005; Sano et al., 2003). Experimental suppression of TBC1D4 leads to leaky and insulin-independent

release of GLUT4 into the plasma membrane (Eguez et al., 2005). There is considerable evidence that in the absence of insulin the GAP domain of TBC1D4 keeps a critical Rab protein in its inactive GDP-bound state to prevent GLUT4 vesicle release (Stöckli et al., 2008). Upon insulin treatment of adipocytes, Akt-phosphorylated TBC1D4 associates with 14-3-3 proteins (Ramm et al., 2006; Ishikura et al., 2007; Sano et al., 2003) and becomes inhibited. In agreement with a previous report, TBC1D4 can act also as a negative regulator of ubiquitously expressed GLUT1 and not only of GLUT4 in adipocytes (Ngo et al., 2009). Our results show that protein kinase WNK1 is involved in the regulation of GLUT1, which secures glucose transport in non-insulin target cells. WNK1 forms a complex with TBC1D4, and its phosphorylation by WNK1 leads to increased interaction of TBC1D4 with 14-3-3 proteins (FIG. 2.4). Interestingly, a previous proteomic analysis in HEK293 cells used mass spectrometry to identify proteins that associate with Flag-tagged 14-3-3 proteins, and identified both TBC1D4 and WNK1 among a list of 170 unique proteins (Jin et al., 2004).

We found that TBC1D4 interacted preferentially with an N-terminal WNK1 fragment containing the catalytic domain (FIG. 2.2). Consistent with this observation, we detected the phosphorylation of TBC1D4 by WNK1 *in vitro* (FIG. 2.3). For these experiments WNK1 was precipitated under stringent conditions including SDS-containing RIPA lysis buffer and 300 mM NaCl containing washing steps. Because no phosphorylation was obtained when a kinase-dead WNK1 mutant was prepared under these conditions, our data provide strong evidence that TBC1D4 is a specific WNK1 substrate. The observed phosphorylation activity was comparable to that of the described physiological model substrate OSR1 (Zagórska et al., 2007).

TBC1D4 is known to be a phosphoprotein and eight sites (Ser318, Ser341, Thr568, Ser570, Ser588, Thr642, Ser666 and Ser751) have been identified that can be phosphorylated *in vivo* (Geraghty et al., 2007; Sano et al., 2003). Further *in vitro* studies have revealed that at least four kinases (Akt, SGK1, RSK1 and AMPK) can phosphorylate TBC1D4

in distinct stimulus-dependent patterns (Geraghty et al., 2007). However, the functional difference between the individual phosphorylation sites is unclear and requires more detailed studies.

The WNK1-mediated phosphorylation of TBC1D4 resulted in increased amounts of GLUT1 at the surface of HEK293 cells. Under these experimental conditions we also observed a slight increase in cell number (data not shown), indicative of stimulated metabolic activity and cell growth. In contrast, expression of kinase-dead WNK1 mutants inhibited the level of surface GLUT1 compared to control cells. Moreover, the suppression of endogenous WNK1 by small interfering RNA oligonucleotides also decreased surface amounts of GLUT1. These effects were demonstrated by biotinylation of cell surface proteins (FIG. 2.5) and by immunofluorescence microscopy (FIG. 2.6). Under these experimental conditions no changes in the surface levels of the endogenous transferrin receptor 1 were observed (FIG. 2.5), demonstrating the specificity of WNK1/TBC1D4 activity on GLUT1 surface expression.

The WNK1 substrate TBC1D4 has been found to exhibit selective GAP activity towards Rab2A, -8A, -8B, -10, and -14 *in vitro*. In adipocytes, specific depletion of Rab10 was identified to reduce GLUT4 translocation (Mîinea et al., 2005; Sano et al., 2007; Sano et al., 2008), whereas in L6 muscle cell lines Rab8A and Rab14 appeared to be more important for regulating GLUT4 (Ishikura et al., 2007; Ishikura and Klip, 2008). Thus, depending on the cell type, TBC1D4 may control distinct Rab GTPases.

In HEK293 cells we provide evidence that Rab8A is involved in the WNK1-regulated GLUT1 traffic. Rab8 is known to participate in exocytic events (Zerial and Mcbride, 2001; Stenmark, 2009) and exists in two isoforms: Rab8A and Rab8B. Our studies show that Rab8A is the predominantly expressed form in HEK293 cells and that endogenous Rab8A associates with TBC1D4 in a complex, the amount of which depends on the catalytic activity of WNK1 (FIG. 2.7 | A). When HEK293 cells were transfected

with Rab8A-specific siRNAs, the WNK1-induced surface expression of GLUT1 was significantly reduced (FIG. 2.7 | B). Similarly, depletion of Rab8A specifically prevented the increase in GLUT1 surface expression observed following depletion of TBC1D4 by siRNA treatment. Under the same experimental conditions the surface levels of TfR1 did not change (FIG. 2.7 | B). These results demonstrate the requirement of Rab8A for GLUT1 regulation in HEK293 cells, and that it operates downstream of WNK1 and TBC1D4.

These data are in agreement with the current view in the literature that TBC1D4 maintains an intracellular storage compartment for glucose transporter proteins under basal conditions (Pessin et al., 1999; Dugani and Klip, 2005; Leney and Tavaré, 2009). This compartment can be mobilised upon phosphorylation of TBC1D4, by promoting its binding to 14-3-3 proteins and subsequent inactivation of its Rab-GAP activity. This permits the forward trafficking of the transporters to the apical membrane. Based on this view, the pathway involving WNK1 in the control of GLUT1 levels at the surface of HEK293 cells is schematically depicted in FIG. 2.8 (see figure caption for details).

Taken together, our data describe a pathway regulating surface expression of GLUT1 that is of considerable biomedical interest. First, malignant cells have accelerated metabolism and satisfy their increased requirements for ATP production by aerobic glycolysis (Gatenby and Gillies, 2004). Especially during the initiation of tumour formation, cells experience suboptimal supply of oxygen and nutrients due to diffusion limits. One mechanism that tumour cells use to adapt to these conditions is GLUT1 overexpression (Macheda et al., 2005), and our data suggest that changes in expression or regulation of WNK1 may be an alternative route for adaptation. The WNK catalytic domain represents a unique target site for the development of small molecule kinase inhibitors, since it is characterised by a typical sequence variation in which a lysine from subdomain I substitutes for a highly conserved catalytic lysine in subdomain II (hence their name <u>w</u>ith <u>n</u>o [<u>K</u>] = lysine) (Xu et al., 2000; Min et al., 2004). Second,
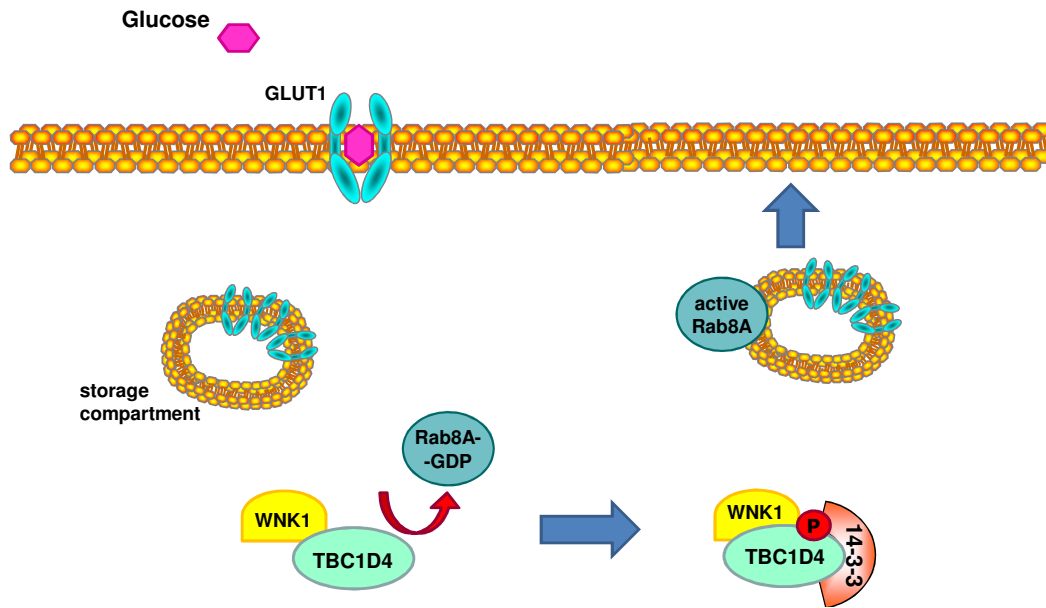
Figure 2.8| Proposed model for the role of the WNK1/TBC1D4 complex in regulating GLUT1 traffic in HEK293 cells. A fraction of GLUT1 is kept in an endosomal storage compartment in the cytosol. There, TBC1D4 keeps Rab8A in the inactive GDP-bound conformation, and prevents vesicle trafficking to the plasma membrane. Upon activation of WNK1, TBC1D4 becomes phosphorylated, and associates with the phosphoprotein-binding 14-3-3 adaptor molecules, so that the GAP activity of TBC1D4 is inhibited. Following Rab8A activation by GDP/GTP exchange, delivery of GLUT1-containing vesicles to the plasma membrane can occur, promoting increased cellular glucose uptake. The upstream stimuli regulating WNK1 activation remain to be determined.

the described pathway may be an interesting drug target in certain diseases linked to epilepsy and mental retardation that are characterised by insufficient GLUT1 activity (Klepper and Leiendecker, 2007; Brockmann, 2009). Finally, hypertensive glomerular injury has been linked to pathological overexpression of GLUT1 in renal cells (Gnudi et al., 2003). This is intriguing because some patients with PHA-II carry germline *WNK1* mutations that lead to *WNK1* overexpression. Our data clearly suggest that increased expression of *WNK1* promotes GLUT1 surface expression in a human kidney-derived cell line. The clinical features of *WNK1* mutant families have been suggested to reflect a form of hypertension that is not exclusively salt dependent, as opposed to families with mutant *WNK4* (Xie et al., 2006; Hadchouel et al., 2006). Thus, WNK1 may in part affect renal salt balance by glucose overload causing glomerular insufficiency.

In the future it will be important to elucidate the upstream mechanisms involved in the WNK1-mediated activation of GLUT1 trafficking. Previous studies reported that hypertonic stress, including glucose, can increase WNK1 activity. Treatment of distal CCD cells with 0.5 M glucose, for instance, promoted a significant increase in WNK1 activity (Lenertz et al., 2005).

Additionally, very recent evidence revealed that TBC1D4 regulates other transporter proteins. Following aldosterone stimulation of CCD cells, TBC1D4 was shown to regulate trafficking of the epithelial sodium channel ENaC to the apical membrane, leading to increased sodium absorption (Liang et al., 2010). Similar to our results, this involved phosphorylation of TBC1D4 and increased association with 14-3-3 proteins, but was mediated by another kinase, SGK1. Intriguingly, WNK1 has been proposed to serve as a scaffold required for efficient SGK1 activation and there is evidence that SGK1 may in turn phosphorylate WNK1 on Thr60 (Xu et al., 2005c).

Taken together, these data indicate TBC1D4 as a central player in the surface expression levels of GLUT4, GLUT1 and ENaC, and it will be interesting to determine whether the phosphorylation by WNK1, SGK1, or Akt represent cell type specific pathways or a common regulatory kinase cascade.

When shall we three meet again

in thunder, lightning, or in rain?

When the hurlyburly's done,

when the battle's lost and won.

W. Shakespeare (1564-1616)

3

Antagonistic regulation of CFTR cell surface expression by protein kinases WNK4 and Syk

Mendes AI, Matos P, Moniz S, Luz S, Amaral M, Farinha C and Jordan P

3.1 Summary

Members of the WNK subfamily of protein kinases regulate various ion channels involved in sodium, potassium, and chloride homeostasis by either inducing their phosphorylation or regulating the number of channel proteins expressed at the cell surface. Here we describe that WNK4 promotes the cell surface expression of the cystic fibrosis transmembrane conductance regulator (CFTR) in mammalian cells. The mechanism by which WNK4 acts on CFTR involves interaction with spleen tyrosine kinase (Syk), which we find to phosphorylate Tyr512 in the first nucleotide-binding domain (NBD1) of CFTR. The presence of WNK4 prevents the phosphorylation of NBD1 by Syk *in vitro* in a kinase-independent manner. In baby hamster kidney (BHK21) cells stably expressing CFTR, catalytically active Syk reduces, while WNK4 promotes, the cell surface expression of CFTR. This is shown by biotinylation of surface proteins, immunofluorescence microscopy, and functional efflux assays. Mutation of Tyr512 to either glutamic acid or phenylalanine is sufficient to alter CFTR surface levels. Together, our results identify that Tyr512 phosphorylation is a novel signal regulating the prevalence of CFTR at the cell surface, and describe an antagonistic role of WNK4 and Syk in this process.

Keywords: Cell surface protein, CFTR, Cystic fibrosis, Intracellular trafficking, Ion transport, Protein phosphorylation, Signal transduction, Syk, WNK4

3.2 Background

Ion transport across the plasma membrane responds to sudden changes in the extracellular environment by post-translational mechanisms that rapidly modify either the amount of ion channel proteins present at the plasma membrane, and/or their transport activity. This process requires tight regulation by signalling mechanisms, however current knowledge on the underlying molecular pathways is still incomplete.

One novel signalling pathway involving members of the recently characterised WNK protein kinase subfamily of serine/threonine protein kinases (Veríssimo and Jordan, 2001) has been identified in the kidney. There, a complex interplay between different ion channels monitors excretion, fluid balance and salt reabsorption. It was found that mutations in the *WNK1* and *WNK4* genes result in increased renal chloride reabsorption and impaired potassium secretion, manifesting as the inherited syndrome of hypertension and hyperkalemia, also known as PHA-II or Gordon's syndrome (Wilson et al., 2001). Affected patients carry missense mutations in *WNK4*, which when expressed in cell models lead to dysregulation of the renal sodium chloride cotransporter NCC and the potassium channel ROMK (Wilson et al., 2003; Gamba, 2005b; Kahle et al., 2008a; Richardson and Alessi, 2008). The analysis of transgenic mouse models of the disease suggests that changes in both the surface expression and the activity of NCC are the main pathological mechanism underlying PHA-II (Lalioti et al., 2006; Yang et al., 2007c; Ohta et al., 2009).

The ion transport activity of NCC, as well as of the related NKCC1 and NKCC2 channels, is regulated by phosphorylation on N-terminal threonine residues by one of two Ste20-family protein kinases, SPAK or OSR1 (Pacheco-Alvarez et al., 2006; Kahle et al., 2006; Richardson et al., 2008). Both kinases require activation by WNK4, which phosphorylates residue Thr233 in SPAK, or Thr185 in OSR1 (Moriguchi et al., 2005; Vitari et al., 2005; Gagnon et al., 2006b). Besides its role as upstream activator of SPAK and OSR1,

WNK4 was shown to inhibit the amount of NCC expressed at the cell surface (Wilson et al., 2003; Yang et al., 2003; Yang et al., 2005a; Cai et al., 2006; Golbang et al., 2006; Subramanya et al., 2009; Zhou et al., 2010), an effect lost in the presence of WNK4 PHA-II-missense mutations.

Furthermore, WNK4 can regulate the surface amount of other renal or extra-renal ion channels in a kinase-independent manner when expressed in *Xenopus* oocytes or mammalian cells. These include the $Cl^-/HCO3^-$ exchangers SLC26A6 (Kahle et al., 2004a) and SLC16A9 (Dorwart et al., 2007), the ROMK potassium channel (Kahle et al., 2003; Ring et al., 2007b; Murthy et al., 2008), the ENaC sodium channel (Ring et al., 2007a; Heise et al., 2010), the TRPV4 (Fu et al., 2006) and TRPV5 (Jiang et al., 2007; Cha and Huang, 2010) calcium channels, and the CFTR chloride channel (Yang et al., 2007a), the mutation of which is the cause for cystic fibrosis.

In case of ROMK and TRPV5, mutant WNK4 was shown to interact with the scaffold protein intersectin and promote channel endocytosis (Kahle et al., 2003; He et al., 2007; Cha and Huang, 2010), but whether other mechanisms exist through which WNK4 regulates ion channel surface expression remains unknown. The elucidation of such mechanisms may help to identify drug targets to correct insufficient channel activity that underlies several diseases (Jentsch et al., 2004; Camerino et al., 2008). For instance, in cystic fibrosis the lack of CFTR activity leads to dehydrated, thick mucus in the lungs with subsequent chronic bacterial infections. However, disease symptoms would be significantly reduced if only about 10% of CFTR activity could be restored in patients (Amaral, 2005).

Here we identified a novel signalling mechanism linking the cell surface expression of CFTR to protein kinases WNK4 and Syk. Syk is a cytoplasmic non-receptor tyrosine kinase best known for its pro-inflammatory role in immunoreceptor signalling in leukocytes (Mócsai et al., 2010). We show in this work that Syk can phosphorylate

CFTR and decrease its surface expression, whereas WNK4 interaction with Syk prevents CFTR phosphorylation, and thus promotes an increase in CFTR-mediated ion transport.

3.3 Experimental Procedures

3.3.1 Cell culture and transfections

HEK293 cells were maintained in DMEM supplemented with 10 units/mL Penicillin, 10 μg/mL Streptomycin and 10% fetal calf serum (Invitrogen). BHK21 cells stably expressing human wild-type CFTR (Tabcharani et al., 1991; Mendes et al., 2003) were maintained in DMEM/F12 medium supplemented with L-glutamine, 15 mM HEPES, 10 units/mL Penicillin, 10 μg/mL Streptomycin, 0.2% methotrexate and 5% fetal calf serum (Invitrogen). Both cells lines were regularly checked for the absence of mycoplasm infection.

For ectopic expression of plasmid cDNAs, HEK293 cells were transfected at 80-90% confluence using Metafectene (Biontex) according to the manufacturer's instructions. BHK21 cells were transfected at 80-90% confluence using LipofectAMINE 2000 (Invitrogen). Transfection efficiencies were found to be around 90%, as determined microscopically using a GFP expression vector. The amount of transfected plasmid DNA was kept constant at 4 μg per 60 mm dish, or 2 μg per 35 mm dish, and constructs supplemented with empty vector if required. Cells were analysed after 20 hours for biochemical assays or after 16 hours for immunofluorescence experiments. All results were confirmed in at least three independent transfection experiments.

3.3.2 Expression constructs

Partial cDNA clones encoding human WNK4 and its kinase-dead mutant K186A (kindly provided by X. Jeunemaitre, Paris) were completed by substituting a PCR-amplified 2 Kb fragment to generate the complete WNK4 sequence identical to Genbank accession number NM_032387. The WNK4 cDNAs were then subcloned into either pcDNA3-Myc or pEGFP expression vectors (Invitrogen or BD Biosciences Europe). The YFP-Syk vector was purchased from RZPD-imaGenes, and its kinase-dead mutant YFP-Syk-K402R generated by mutating codon 402 from AAA to AGA using the Quick Change mutagenesis kit (Stratagene). pET-SUMO-NBD1 (kindly provided by P. J. Thomas, Dallas) was mutated at CFTR codon 512 from TAT to TTT to obtain pET-SUMO-NBD1-Y512F. For subcloning of Myc-tagged NBD1 and NBD1-Y512F, their cD-NAs were PCR-amplified from pET-SUMO-NBD1 with primers NBD1-F (5'-ACG ACT ACA GAA GTA GTG ATG) and NBD1-R (5'-TTA GAC AGG AGC ATC TCC TTC), cloned into pCR2.1 TOPO-TA vector (Invitrogen), and transferred into a pcDNA3-Myc expression vector. CFTR-Y512F and CFTR-Y512E mutants were generated by changing codon 512 of human pNUT-CFTR (Mendes et al., 2003) from TAT to TTT, or GAG, respectively. Protein kinase OSR1 was amplified from cDNA clone IRAUp969A0428D (RZPD-imaGenes) by PCR with primers Bam-OSR1-F (5'-GGA TCC ATG TCC GAG GAC TCG AG) and Hind-OSR1-R (5'-TTC GAA TTA GCT GAT GCT GAG CTG), cloned into pCR2.1 TOPO-TA vector, and then subcloned into the *Bam* HI and *Hin*d III sites of T7/His-tagged pET28 vector (Merck4Biosciences). Subsequently, codon 164 of the cDNA was mutated from GAC to GCC to generate the kinase-dead OSR1-D164A mutant, which was used as a substrate for *in vitro* protein kinase assays. Myc-tagged hnRNP A1 (gift from H. König, Karlsruhe) was as described (Matter et al., 2000). All constructs were verified by automated DNA sequencing.

3.3.3   Immunoprecipation and Western blot procedures

For co-immunoprecipitation experiments, cells were grown in 60 mm (transfected cells), lysed on ice in 250 μL non-denaturing lysis buffer (see previous Chapter's Experimental Procedures) supplemented with the aforementioned inhibitor cocktail. The cell lysates were incubated for 2 hours at 4°C with the specified antibodies (2.5 μg/mL anti-GFP ab1218 (Abcam) or anti-Myc clone 9E10 (M5546, Sigma-Aldrich)), then further incubated for 1 hour with protein G-Agarose beads (Roche Applied Science), and finally washed three times in cold lysis buffer containing 200 mM NaCl. Proteins were solubilized from the beads in 2x SDS sample buffer, boiled, and separated in a 10% SDS-PAGE Protean III mini-gel (BioRad). Gels destined to assess CFTR expression contained 1% glycerol and were run at 4°C.

For detection of specific proteins, the polyacrylamide gel was transferred onto a PVDF membrane (BioRad) using a Mini Trans-Blot cell (BioRad; 100V for 1 hour) followed by Coomassie-staining to check for equal transfer. Western blot membranes were blocked in TBS, 0.1% Triton X-100, 5% milk powder, probed using the indicated antibodies, and then incubated with a secondary peroxidase-conjugated antibody (BioRad) followed by chemiluminescence detection. Primary antibodies used for Western blots were rabbit anti-Myc A14 (sc-789) from Santa Cruz Biotecnologies, rabbit anti-GFP ab290 from Abcam, mouse anti-T7-tag from Merck4Biosciences, mouse anti-CFTR clone L12B4 (mAb3484) from Chemicon International (Billerica, MA, USA), mouse anti-CFTR clone 596 (obtained through the UNC CFTR antibody distribution program sponsored by CFFT) and affinity-purified rabbit anti-CFTR (Lis1) (Farinha et al., 2004).

For protein-protein interaction screening, a signal transduction antibody array from Hypromatrix (Worcester, MA, USA) containing antibodies against 400 cellular proteins involved in signal transduction was used. The array membranes were incubated for 2 hours at room temperature with cell lysates (prepared in non-denaturing lysis buffer)

from four 60 mm dishes of HEK293 cells transfected with either Myc-WNK4 or empty vector, then washed like Western blots before staining the complexes containing Myc-WNK4 with an anti-Myc peroxidase conjugate (Hypromatrix).

3.3.4 Biotinylation of cell surface proteins

BHK21 cells were transfected as described above, washed twice with warm culture medium to remove dead cells, and placed on ice in a cold room. Cells were washed three times with ice-cold PBS-CM (see previous Chapter) and left 5 min in cold PBS-CM to ensure arrest of endocytic traffic. Cells were then incubated for 30 min with 0.5 mg/mL EZlink sulfo-NHS-SS-biotin (Pierce Biotechnology) in PBS-CM before being rinsed twice and left for 15 min on ice with ice-cold Tris/Glycine (see Chapter Two) to quench the biotinylation reaction. Cells were again washed 3x with cold PBS-CM and lysed in 250 μL pull-down buffer (see previous Chapter) in the presence of the afore-mentioned protease inhibitor cocktail. The cell lysates were harvested by scraping and cleared by centrifugation at 16,000 × g at 4°C for 5 min. An aliquot of 40 μL representing the total CFTR level was removed and added to 2x SDS CFTR sample buffer (62.5 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 0.02% Bromophenol Blue, 160 mM DTT), while 200 μL lysate were added to 25 μL Streptavidin-agarose beads (Sigma-Aldrich), previously incubated for 1 hour in 1 mL cold pull-down buffer containing 2% non-fat milk powder, and washed 3x in pull-down buffer. For purification of biotiny-lated proteins, lysate and beads were incubated under rotation for 1 hour at 4°C, the beads collected by centrifugation (1 min at 3,000 × g), and washed 3x in cold wash buffer (see Chapter Two). Captured proteins were recovered from the beads in 25 μL 2x SDS CFTR sample buffer. The biotinylated protein fraction and one twentieth of the corresponding whole cell lysates were analysed alongside by SDS-PAGE followed by Western blot.

3.3.5 Production of recombinant OSR1 or SUMO-NBD1 and *in vitro* protein kinase assays

For the production of recombinant human OSR1, a reported WNK4 substrate (Vitari et al., 2005), or of recombinant human NBD1, pET-OSR1-D164A, pET-SUMO-NBD1 or pET-SUMO-NBD1-Y512F were expressed in the *E. coli* BL21 strain under IPTG induction and the bacterial pellets harvested at $1,400 \times g$, 20 min and frozen. For protein extraction pellets were resuspended in either conventional lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) for OSR1, or CFTR lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM L-Arginine, 50 mM NaCl, 5 mM $MgCl_2$, 12.5% glycerol, 0.25% NP-40, 0.1% SDS, 1 mM DTT, 2 mM ATP) in the presence of the protease inhibitor cocktail described above, and then sonicated on ice in 10 cycles of 30 seconds with 10 seconds intervals (Sonics Vibra Cell sonicator, set at 40% amplitude). Following centrifugation of the extracts at $16,000 \times g$, the supernatant was incubated with Ni-NTA agarose beads (Qiagen) for 1 hour at 4ºC. Beads were washed twice with cold lysis buffer containing 20 mM imidazole and protease inhibitors. Recombinant OSR1 was eluted and stored in cold lysis buffer containing 250 mM imidazole, whereas recombinant SUMO-NBD1 proteins were dialysed against an imidazole-free lysis buffer, and then concentrated. Proteins were quantified and stored in aliquots at -80ºC.

For *in vitro* protein kinase assays, cells were lysed under stringent conditions in 250 µL RIPA buffer supplemented with protease inhibitors (see previous Chapter). To reduce extract viscosity 500 units/mL benzonase (Sigma-Aldrich) and 5 mM $MgCl_2$ were added. Following immunoprecipitation of WNK4 or Syk as described above, the resulting beads were washed three times in cold RIPA buffer followed by three washes with non-denaturing lysis buffer and then resuspended in 20 µL kinase reaction buffer (30 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM DTT, 1 mM Na_3VO_4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 100 µM ATP), mixed with their substrate (either beads containing immunoprecipitated Myc-tagged protein, or 1000 ng recombinant kinase-dead OSR1, or

recombinant NBD1), and incubated in the presence of 5 μCi γ-[^{32}P] ATP at 30°C for 30 min. Finally, 2x SDS sample buffer was added, samples boiled and separated by SDS-PAGE followed by Western blot. Membranes were exposed to X-ray films, and subsequently incubated with the indicated antibodies in order to document protein quantities.

3.3.6 Immunofluorescence microscopy

BHK21 cells were grown on coverslips, transfected as indicated, fixed after 16 hours with 4% (v/v) formaldehyde (Merck Chemicals) in PBS and permeabilised with 0.2% Triton X-100 in PBS. Cells were then immunolabeled for 1 hour with mouse anti-CFTR (clone 570; obtained through the UNC CFTR antibody distribution program sponsored by CFFT), washed 3x 5 min in PBS-T, incubated with a secondary Alexa 568-conjugated antibody (Invitrogen-Molecular Probes). Cells were then washed, stained briefly in DAPI solution (Sigma; 1:1000 in PBS-T), washed again and post-fixed for 15 min in 4% formaldehyde. Coverslips were mounted on microscope slides with Vectashield (Vector Laboratories), images recorded on a Leica TCS-SPE confocal microscope and processed with Adobe Photoshop software.

3.3.7 Iodide efflux assay

CFTR-mediated iodide efflux assay was adapted from previously described methods (Long and Walsh, 1997; Tang and Wildey, 2004; Zheng et al., 2009). BHK21 cells were grown in 6-well dishes, transfected in duplicates as indicated, and 20 hours later washed twice and incubated for 30 min at 37°C in prewarmed Iodide Loading Buffer (136 mM NaI, 3 mM KNO$_3$, 2 mM Ca(NO$_3$)$_2$, 0.5 mM MgSO$_4$, 10 mM glucose, 20 mM HEPES pH 7.5). Cells were then washed 4x with Efflux Buffer (EB) (136 mM NaNO$_3$, 3 mM KNO$_3$, 2 mM Ca(NO$_3$)$_2$, 0.5 mM MgSO$_4$, 10 mM glucose, 20 mM HEPES pH 7.5) to

completely remove extracellular iodide. Next, cells were left for equilibration during 10 min at 37ºC in EB (first set of replicates), or EB containing 10 μM of cell-permeable CFTR-specific inhibitor-172 (Merck4 Biosciences) (second set of replicates), before 10 μM Forskolin and 50 μM IBMX (both from Sigma) were added for 5 min to allow for PKA-dependent CFTR-stimulation. The presence of 10 μM of CFTR inhibitor-172 (second set of replicates) allowed correcting for endogenous non-CFTR-mediated iodide efflux activities. Cells from all replicates were then quickly washed with fresh EB and the dishes completely drained. Cells were lysed for 5 min in 1 mL EB plus 0.5% Triton X-100, scraped and centrifuged at 16,000 $\times$ g for 5 min. Two sample aliquots were removed to quantify CFTR by Western blot and total protein using Precision Red Protein Assay Reagent (Cytoskeleton, Denver, CO, USA). The iodide content of the lysates was then determined using an Iodide-Selective Electrode (Consort) connected to an MP225 General Purpose GLP pH/mV/°C Meter (Mettler Toledo). Sample voltages generated by the iodide in solution were interpolated from the standard curve generated by measuring 1, 10, 50 and 100 μM solutions and normalised to the amount of protein in the sample. The iodide effluxed from transfected cells was calculated as the difference between the amounts of iodide remaining in inhibitor-172-treated *versus* non-treated replicates, and expressed in nmol.

3.4 Results

3.4.1 WNK4 modulates the cell surface abundance of CFTR in mammalian cells

WNK4 can regulate various ion channels, and one previous report identified a role in the expression of CFTR at the cell surface of *Xenopus* oocytes (Yang et al., 2007a). In order to confirm this effect in mammalian cells, we used BHK21 cells engineered to stably express the human CFTR protein (Tabcharani et al., 1991; Mendes et al., 2003). These cells are a well characterised model system in the field and were transfected with either GFP

empty vector, or wild-type GFP-WNK4, or its GFP-WNK4-K186A kinase-dead mutant. The consequences on the CFTR protein were subsequently analysed by three different techniques. First, cell surface proteins were labeled by biotinylation and isolated from cell lysates using Streptavidin-beads. As shown in FIG. 3.1 | A, expression of both wild-type and kinase-dead WNK4 increased the amount of CFTR detected at the cell surface. We confirmed that the biotinylation reaction did not label cytosolic proteins such as tubulin (data not shown). Second, confocal immunofluorescence microscopy analysis confirmed an increase in the CFTR staining at the cell surface in both wild-type and kinase-dead WNK4 transfected cells (FIG. 3.1 | B). Third, CFTR-mediated ion transport activity in transfected cells was determined by an iodide efflux assay, and revealed a roughly two-fold increase in iodide release upon wild-type WNK4 or kinase-dead WNK4 mutant expression (FIG. 3.1 | C).

3.4.2 WNK4 exists in a complex with protein kinase Syk

In order to identify the molecular mechanism underlying this effect of WNK4 on CFTR surface expression, we screened proteins interacting with WNK4 using an antibody array. To this end, HEK293 cells were transfected with Myc-tagged WNK4, lysed under non-denaturing conditions to preserve protein-protein interactions, and the extract was incubated with an antibody array. Briefly, the array contained antibodies against 400 cellular proteins involved in signal transduction, which are able to bind to their cognate proteins from the cell lysate. Those proteins forming a complex with Myc-WNK4 in the lysate were revealed by chemiluminescence staining of the array membrane with an anti-Myc peroxidase conjugate. After subtraction of unspecific signals generated by the anti-Myc antibody alone, one clearly positive hit for WNK4 was obtained at the position of anti-Syk (FIG. 3.2 | A).
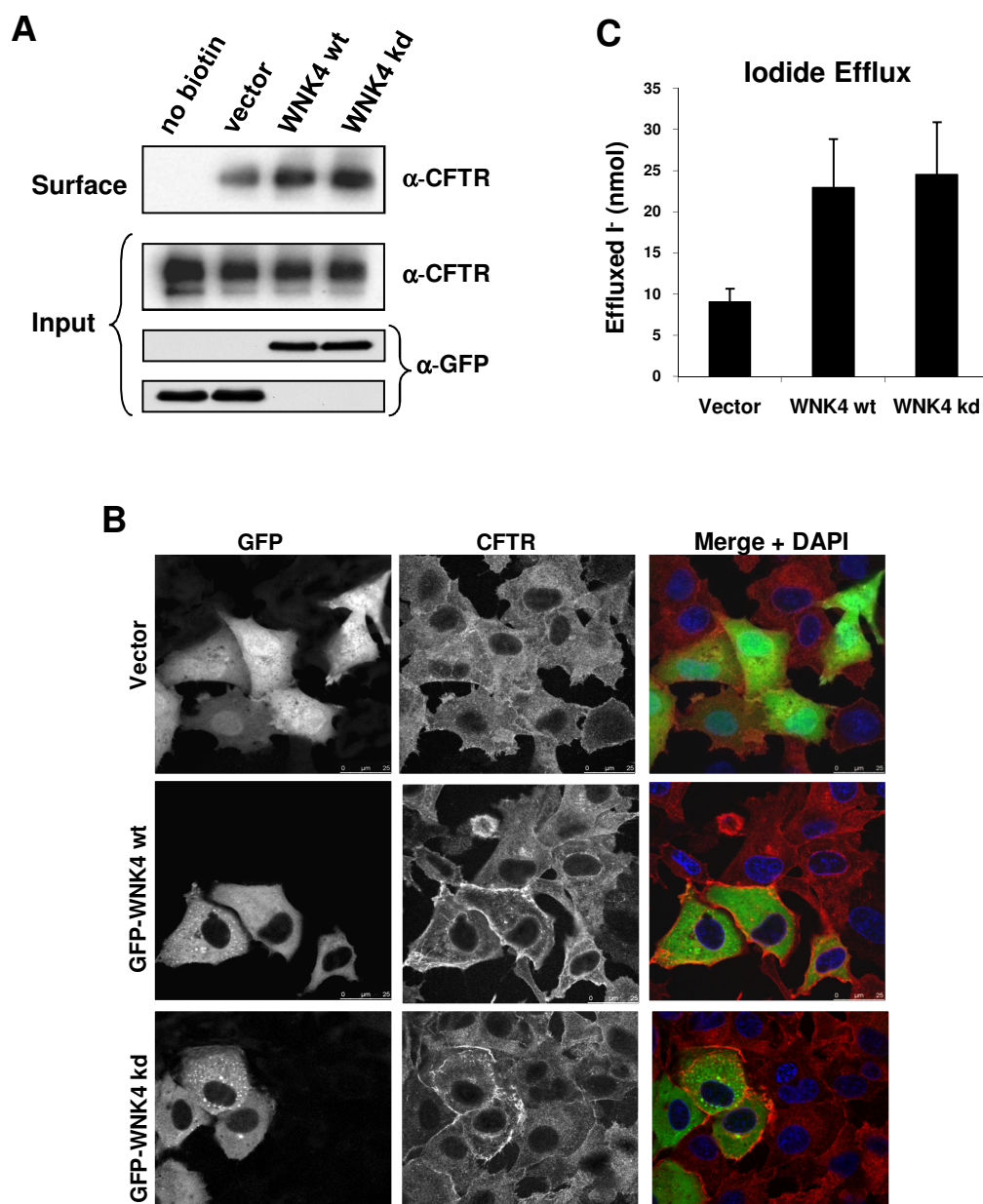
Figure 3.1| Expression of *WNK4* in mammalian cells increases cell surface expression of CFTR. BHK21 cells stably expressing the human CFTR protein were transfected with GFP-tagged expression vectors encoding either GFP alone (vector), or wild-type (wt) WNK4 or its WNK4-K186A kinase-dead (kd) mutant, and then analysed through different techniques for changes in the cell surface expression of CFTR. **A| Biotinylation of cell surface proteins.** Western blots of the CFTR protein, in either the fraction of biotinylated cell surface proteins pulled-down with streptavidin-beads (Surface), or in whole cell lysates (Input) are shown. The latter were further stained to document the expression levels of transfected GFP, wt GFP-WNK4 or its kd mutant. **B| Confocal immunofluorescence microscopy.** Transfected cells were fixed and stained with anti-CFTR followed by anti-mouse Alexa 568-conjugate. Images were recorded sequentially for DAPI, GFP and Alexa stainings. **C| CFTR-mediated ion transport activity by iodide efflux.** For details see EXPERIMENTAL PROCEDURES.
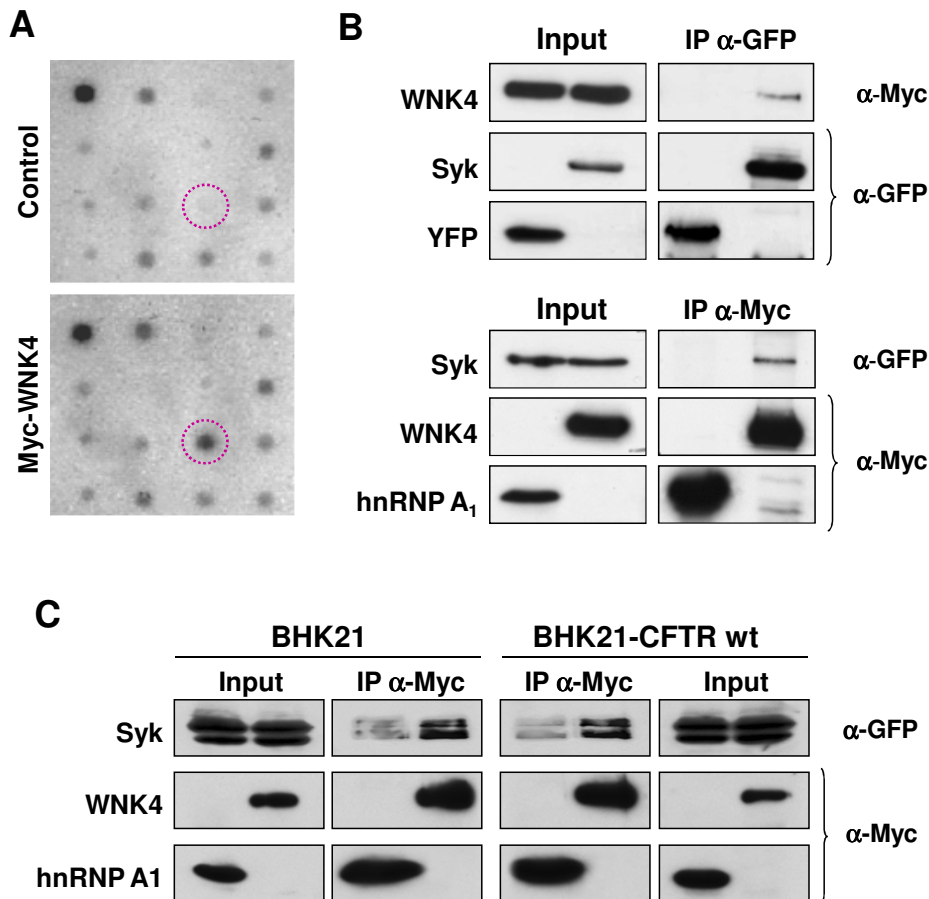
Figure 3.2| WNK4 forms a protein complex with protein kinase Syk. A| Identification of Syk as a WNK4 interacting protein using an antibody array. Lysates of HEK293 cells transfected with either Myc-WNK4 or empty vector (Control) were incubated with an antibody array. WNK4-protein interactions were then revealed by chemiluminescence using an anti-Myc peroxidase-conjugated antibody. The relevant array areas with one WNK4-specific signal that corresponded to the position of Syk are shown. **B| Co-immunoprecipitation experiments to confirm the observed WNK4-Syk interaction.** HEK293 cells were co-transfected with Myc-WNK4 and YFP-Syk, as well as YFP empty vector and Myc-hnRNP A1 as controls, lysed under non-denaturing conditions, and incubated with either anti-GFP antibodies to precipitate Syk (top panels) or with anti-Myc antibodies to precipitate WNK4 (bottom panels). The Western blot analyses of the expression levels in whole cell lysates (Input) and the immunoprecipitated fractions (IP) are shown. As respective controls, the YFP protein or Myc-tagged-hnRNP A1 were used. Note that immunoprecipitated YFP-Syk pulls down WNK4 and, *vice-versa*, that immunoprecipitates of Myc-WNK4 revealed the presence of Syk. **C| Validation of the observed interaction in CFTR-expressing BHK21 cells.** Parental and CFTR-expressing BHK21 cells were co-transfected with YFP-Syk and either Myc-WNK4 or Myc-hnRNP A1 (Control). Myc-tagged proteins were immunoprecipitated and analysed by Western blot as described above.

For confirmation of the observed interaction, Myc-WNK4 and YFP-Syk were co-transfected into HEK293 cells (as well as YFP empty vector or Myc-hnRNP A1 as respective controls) and immunoprecipitated. Anti-GFP antibodies immunoprecipitated Syk, and the presence of WNK4 was clearly detected in the YFP-Syk precipitate, but not with YFP alone. *Vice-versa*, anti-Myc antibodies precipitated WNK4 and the presence of Syk was detected with anti-GFP antibodies. Also as a control, the unrelated nuclear protein Myc-hnRNP A1 was expressed but did not co-immunoprecipitate with Syk (FIG. 3.2 | B). The endogenous WNK4 protein could not be immunoprecipitated due to a lack of suitable commercial antibodies.

Since HEK293 cells do not express endogenous CFTR protein, the co-immunoprecipitation studies were repeated in parental and CFTR-expressing BHK21 cells. This confirmed that Syk precipitated specifically with Myc-WNK4 but not with Myc-hnRNP A1, and that this interaction occurred independently of the presence of the CFTR protein (FIG. 3.2 | C).

3.4.3   Syk forms a complex with CFTR and phosphorylates the NBD1 *in vitro*

Given that so far no functional interaction between protein kinase Syk and CFTR has been described, we first tested whether both proteins were able to co-immunoprecipitate. BHK21-CFTR cells were transfected to express either YFP protein or YFP-Syk, and both were immunoprecipitated from cell lysates using the anti-GFP antibody. The precipitates only revealed the presence of CFTR protein in case of YFP-Syk (FIG. 3.3 | A).

We then analysed the CFTR peptide sequence and found a single Syk recognition motif (Y-D/E-D/E-X) at Tyr512 in NBD1 (FIG. 3.3 | B). We thus tested next whether Syk could phosphorylate CFTR *in vitro*. Wild-type Syk or a K402R kinase-dead Syk mutant were immunoprecipitated from cell lysates in SDS-containing RIPA buffer so that contamination with other kinases from the cell lysate was minimised. The beads were then
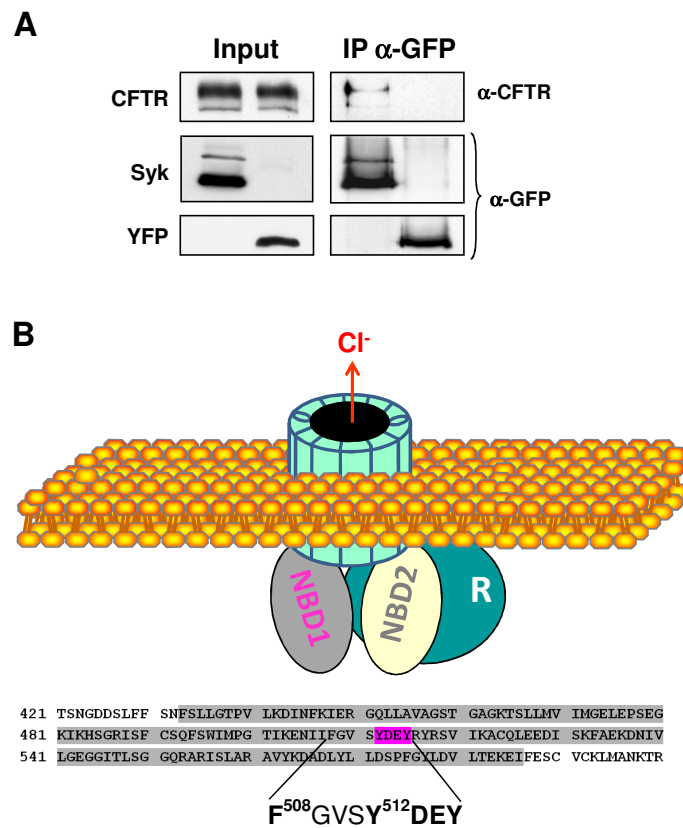
Figure 3.3| CFTR co-immunoprecipitates with Syk and harbours a Syk substrate motif in NBD1. A| Syk forms a protein complex with CFTR. YFP empty vector or YFP-Syk were transfected into CFTR-expressing BHK21 cells, and immunoprecipitated with anti-GFP antibodies. Whole cell lysates and precipitates were then separated in acrylamide gels optimized for best resolution of CFTR bands B and C. Shown are Western blots with the expression levels of YFP, YFP-Syk (anti-GFP) and CFTR (rabbit anti-CFTR Lis1) in whole cell lysates (Input) and the immunoprecipitated fractions (IP). Note that only YFP-Syk co-precipitated the CFTR protein. The second visible Syk protein band occurred as an effect of the CFTR-specific gel conditions. **B| Model of the CFTR ion channel structure with part of its protein sequence.** The sequence of the cytoplasmic nucleotide binding-domain 1 (NBD1) is highlighted in grey, the relevant residues containing the Syk substrate motif in pink and zoomed in below. Note that Tyr512 is close to Phe508, the most frequently mutated residue in cystic fibrosis patients.

washed in protein kinase buffer and incubated with radioactive ATP in the presence of isolated NBD1 as substrate.

In a first set of experiments, recombinant NBD1 (kind gift of P. J. Thomas) was found to be phosphorylated by Syk but not by its kinase-dead mutant (FIG. 3.4 - left panels). In order to demonstrate that Tyr512 was indeed the target residue of this activity, the

	YFP	YFP-Syk wt	YFP-Syk kd		YFP	YFP-Syk wt		YFP	YFP-Syk wt
rNBD1 wt	+	−	+	+	−	−	−	−	−	−
rSUMO-NBD1 wt	−	−	−	−	+	+	−	−	−	−
rSUMO-NBD1 Y512F	−	−	−	−	−	−	+	−	−	−
Myc-NBD1 wt	−	−	−	−	−	−	−	+	+	−
Myc-NBD1 Y512F	−	−	−	−	−	−	−	−	−	+

Kinase Assay (Syk) — Input: α-GFP, α-CFTR — rNBD1 | transfected
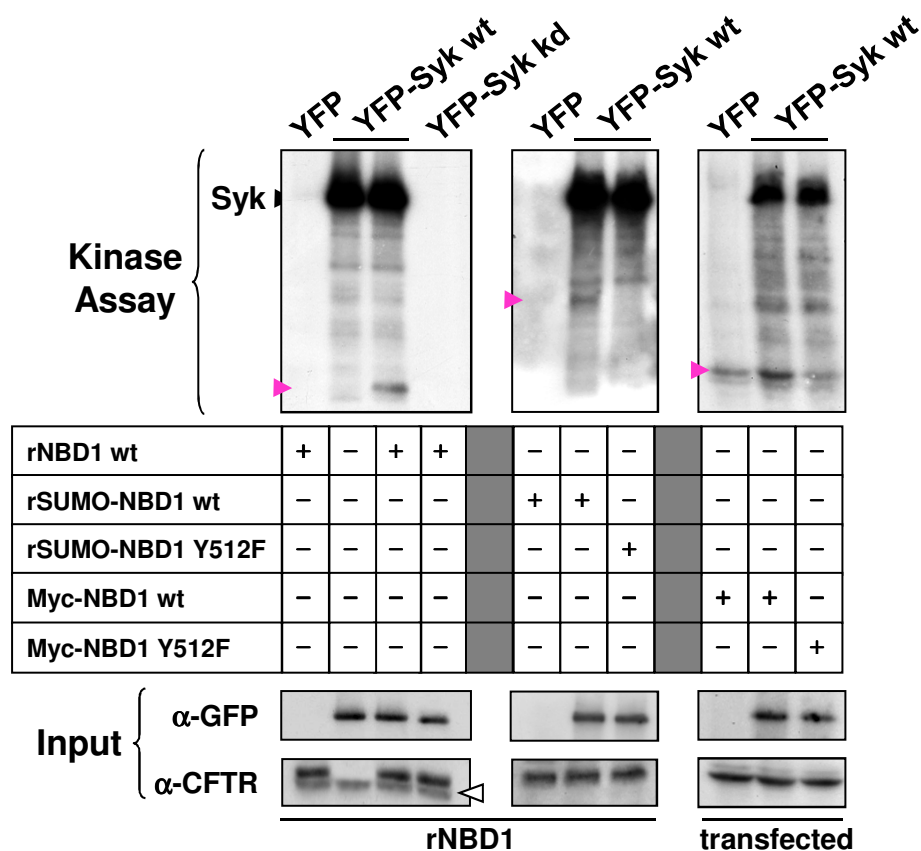
Figure 3.4| Syk phosphorylates CFTR NBD1 *in vitro*. YFP empty vector, or wild-type (wt) YFP-Syk, or K402R kinase-dead (kd) YFP-Syk mutant were transfected into HEK293 cells, immunoprecipitated and tested in *in vitro* protein kinase assays for their ability to phosphorylate CFTR. The added substrates were either recombinant NBD1 (left panel), SUMO-tagged recombinant NBD1, or its Y512F derivative (middle panel), or transfected Myc-tagged NBD1 or Myc-NBD1-Y512F immunoprecipitated from HEK293 cells (right panel). Samples were separated by gel electrophoresis and transferred to PVDF blotting membranes. Shown are the X-ray films (Kinase Assay) detecting incorporated radioactive [^{32}P]-phosphate and pink arrowheads indicate the position of NBD1. Note that wt Syk (but not its kd mutant) autophosphorylates and phosphorylates NBD1 at Tyr512. In order to document equal quantities of immunoprecipitated Syk or NBD1 substrate protein, membranes were subsequently immunostained with anti-GFP and anti-CFTR L12B40 antibodies (Input). White arrowhead indicates the position of immunoglobulin light chains which retained some radioactive phosphate.

NBD1 coding sequence was point mutated to a phenylalanine at position 512 and then expressed as SUMO-tagged recombinant protein. While Syk clearly phosphorylated wild-type SUMO-NBD1 it no longer phosphorylated the Y512F NBD1 mutant (FIG. 3.4 - middle panels). Since the phosphorylation of recombinant proteins *in vitro* can

sometimes result from an incorrect conformation, we confirmed these data using Myc-tagged CFTR immunoprecipitated from HEK293 used as substrate for the Syk kinase assay. Given that the high phosphorylation of full-length CFTR in the regulatory domain hampered analysis of the activity of Syk, we expressed only Myc-NBD1 and the corresponding Y512F NBD1 mutant in these cells. The results confirmed that Syk can only phosphorylate wild-type but not Y512F NBD1 (FIG. 3.4 - right panels).

3.4.4 WNK4 inhibits NBD1 phosphorylation by Syk

Since we found above that Syk interacts with WNK4, we tested the effect of WNK4 on the NBD1 phosphorylation by Syk. For this purpose, wild-type Myc-WNK4 or its kinase-dead mutant were expressed, immunoprecipitated and added to Syk and recombinant NBD1 in the *in vitro* kinase assay. It was found that the presence of either wild-type WNK4 or its kinase-dead mutant completely prevented Syk from phosphorylating NBD1 (FIG. 3.5 | A).

In order to further study how WNK4 inhibits the NBD1 phosphorylation by Syk, we analysed whether WNK4 could directly phosphorylate Syk *in vitro*. Myc-WNK4 was expressed in cells, immunoprecipitated and mixed in *in vitro* kinase assays with either wild-type or kinase-dead Syk. As a positive control, recombinant protein OSR1 was added as known WNK substrate (Vitari et al., 2005), and found to become phosphorylated by WNK4 (FIG. 3.5 | B). In contrast, Syk kinase-dead mutant was not phosphorylated by WNK4, and wild-type Syk revealed only its own auto-phosphorylation level without any evidence for additional phosphorylation by WNK4.

Figure 3.5 (see next page)| WNK4 inhibits the Syk-mediated NBD1 phosphorylation. A| WNK4 inhibits NBD1 phosphorylation by Syk. Wild-type (wt) YFP-Syk, K402R kinase-dead (kd) YFP-Syk, wt GFP-WNK4 or K186A kd GFP-WNK4 were transfected into HEK293 cells, immunoprecipitated and pooled as indicated to test their ability to phosphorylate recombinant NBD1 (rNBD1) in *in vitro* protein kinase assays. Samples were separated and analysed as described in the caption of FIG. 3.4. (continues on the next page)

A

Kinase Assay   Input (α-GFP + α-CFTR)

WNK4 ►   ◄ GFP-WNK4
Syk ►   ◄ YFP-Syk
    ◄ IgG-hc
NBD1 ►   ◄ rNBD1
    ► IgG-lc

YFP-Syk kd	+	−	−	−		+	−	−	−
YFP-Syk wt	−	+	+	+		−	+	+	+
GFP-WNK4 wt	−	−	+	−		−	−	+	−
GFP-WNK4 kd	−	−	−	+		−	−	−	+
rNBD1	+	+	+	+		+	+	+	+

B

Kinase Assay

WNK4 ►
Syk ►
OSR1 ►

Myc-WNK4 wt	-	+	-	+	-	+
GFP-vector	+	-	+	-	-	-
YFP-Syk kd	+	+	-	-	-	-
YFP-Syk wt	-	-	+	+	-	-
T7-rOSR1 kd	-	-	-	-	+	+

Input

α-Myc

α-GFP

α-T7
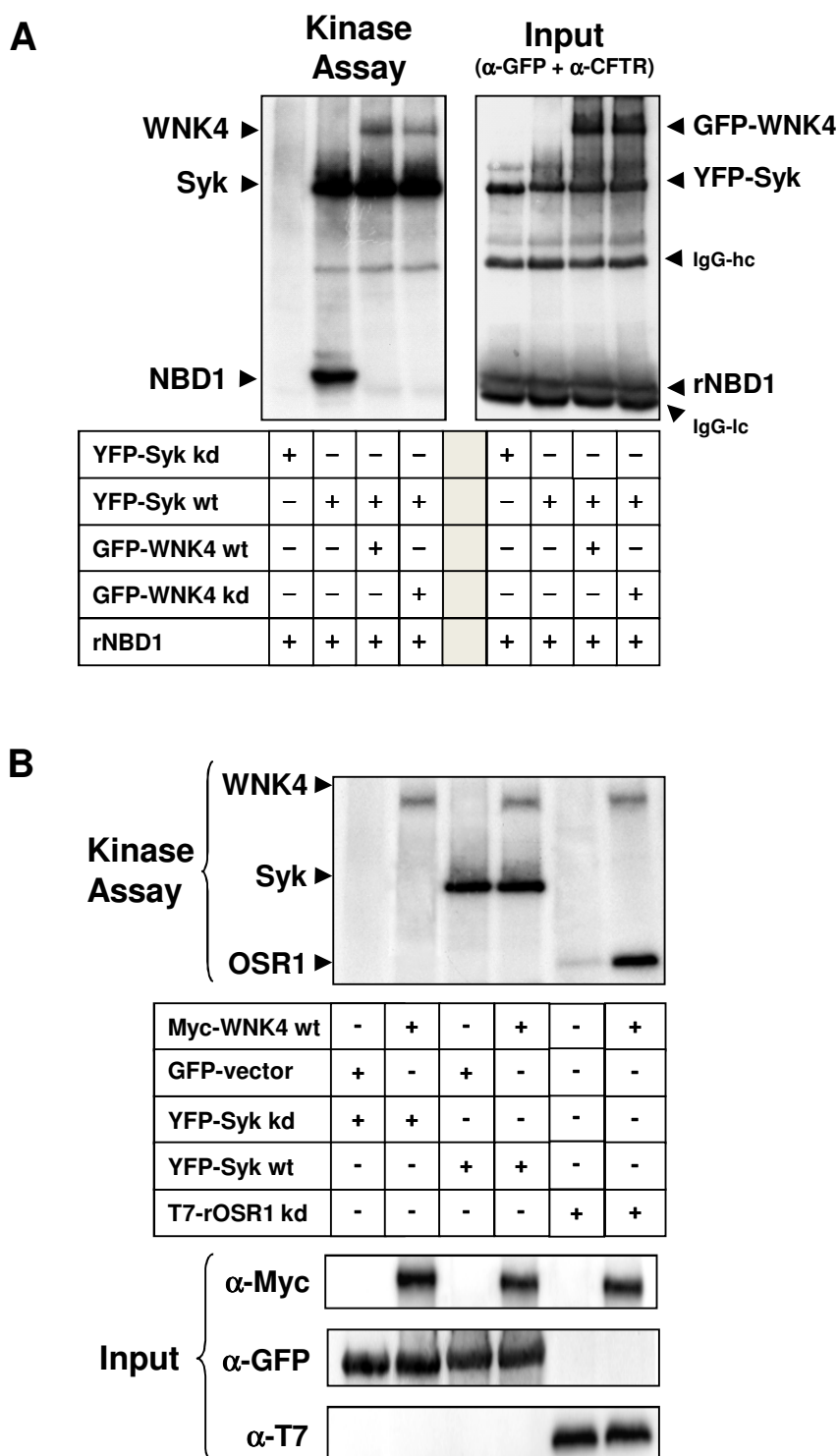
Figure 3.5 (cont.)| A| Note that Syk and WNK4 autophosphorylate and that the presence of WNK4 prevents Syk from phosphorylating NBD1. **B| WNK4 does not phosphorylate Syk *in vitro*.** wt YFP-Syk, or its kd mutant, or wt Myc-WNK4 were transfected into HEK293 cells, immunoprecipitated and pooled as indicated for *in vitro* protein kinase assays. As a positive control, Myc-WNK4 was incubated with rOSR1, a previously described substrate. Samples were separated and analysed as described in the caption of FIG. 3.4. Note that WNK4 phosphorylates OSR1 but not the Syk protein.

3.4.5 Syk activity modulates the expression of CFTR at the cell surface

Since Syk phosphorylated NBD1 of CFTR *in vitro*, we asked whether Syk could modulate the expression of CFTR at the cell surface. Thus, BHK21-CFTR cells were transfected with YFP-tagged expression vectors encoding either YFP alone (empty vector), wild-type Syk, or its kinase-dead mutant, and then analysed for the expression of CFTR at the cell surface by biotinylation of cell surface proteins, immunofluorescence microscopy and iodide efflux assays, all in comparison to the described effect of wild-type WNK4. The expression of wild-type Syk led to a decrease in the amount of CFTR at the cell surface, both as biotinylated protein (FIG. 3.6 | A), or immunofluorescent staining (FIG. 3.6 | B). In contrast, expression of Syk kinase-dead increased the amounts of plasma membrane-associated CFTR in both assays to levels comparable to those obtained upon expression of WNK4 (see also FIG. 3.1 | B for WNK4 immunofluorescences). These data were confirmed by iodide efflux measurements (FIG. 3.6 | C) demonstrating that the observed changes in surface amounts of CFTR reflected different capacities of cells to efflux iodide upon Forskolin/IBMX stimulation.

3.4.6 Mutation of Tyr512 is sufficient to promote changes in the expression of CFTR at the cell surface

In order to demonstrate that the observed phosphorylation in the NBD1 is functionally relevant *in vivo*, Tyr512 was changed by site-directed mutagenesis into either a non-phosphorylatable phenilalanine residue (Y512F) or a phospho-mimetic glutamic acid residue (Y512E). These CFTR mutants were stably transfected into parental BHK21 cells, and CFTR activity measured by the iodide efflux technique (FIG. 3.7 | A). CFTR-Y512E-expressing cells showed a significant decrease in CFTR-mediated ion transport, while cells expressing CFTR-Y512F released almost twice the amount of iodide as wild-type CFTR-expressing cells. These data were found to correlate to the increased
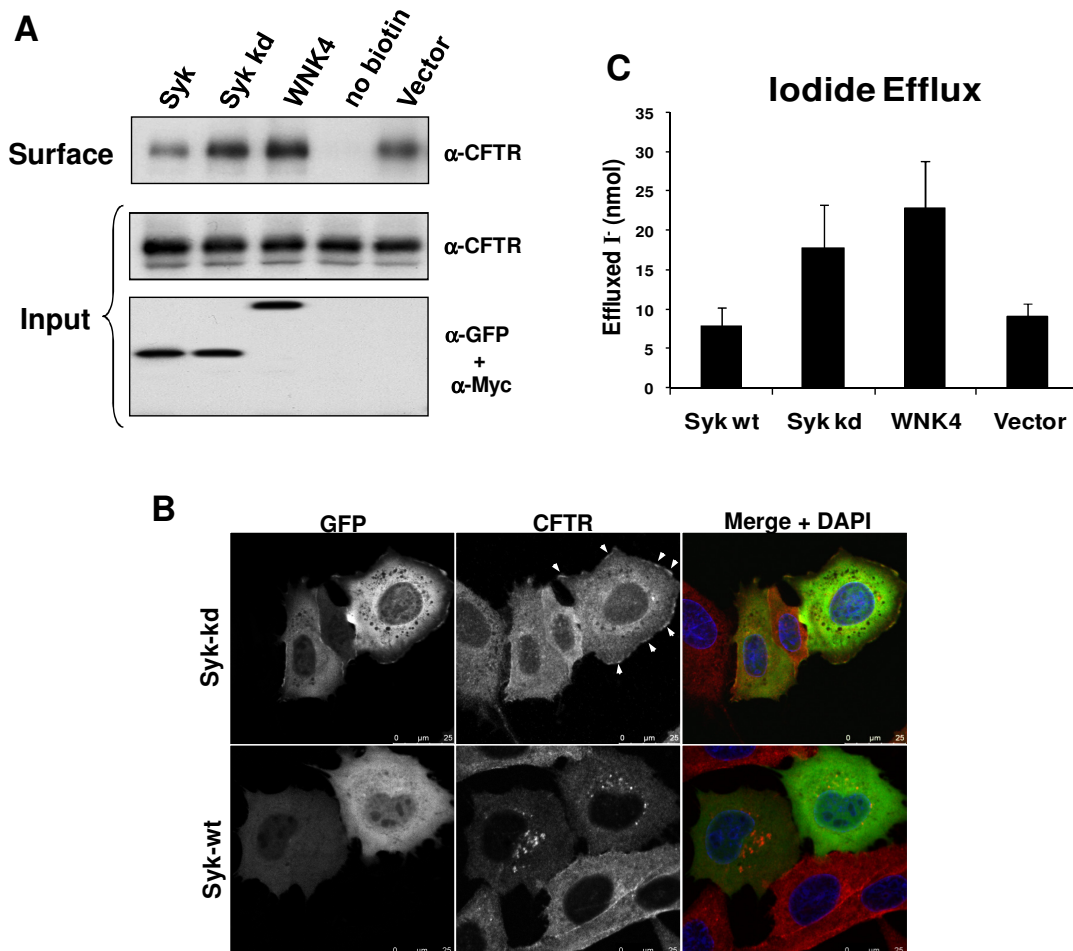
Figure 3.6| WNK4 and Syk have antagonistic effects on the cell surface expression of CFTR. BHK21-CFTR cells were transfected with GFP-tagged expression vectors encoding either GFP alone (vector), wild-type WNK4, wild-type Syk, or its kinase-dead mutant, and then analysed by different techniques for changes in the expression of CFTR at the cell surface, as described in the caption of FIG. 3.1. **A| Biotinylation of cell surface proteins. B| Confocal immunofluorescence microscopy. C| CFTR-mediated ion transport by iodide efflux.** Note that expression of Syk decreased, whereas kinase-dead Syk or WNK4 increased (see FIG. 3.1 for WNK4 immunofluorescences), cell surface CFTR compared to control cells.

amount of biotinylated CFTR protein at the cell surface compared to BHK21-CFTR cells (FIG. 3.7 | B). These mutants thus mimic the effects on CFTR of transfecting either Syk or its kinase-dead mutant. To further confirm that Tyr512 mediates the effects of Syk and WNK4 *in vivo*, we first transfected the non-phosphorylatable CFTR-Y512F-expressing cells with YFP-Syk. Surface biotinylation revealed that wild-type Syk could no longer reduce CFTR-Y512F surface expression (FIG. 3.7 | B - two middle lanes). Second, results showed that in cells expressing the phospho-mimetic CFTR-Y512E mutant and transfected with Myc-WNK4, expression of WNK4 was no longer able to rescue surface levels of the CFTR-Y512E mutant protein (FIG. 3.7 | B - two right lanes) to those of wild-type CFTR (second lane from the left).
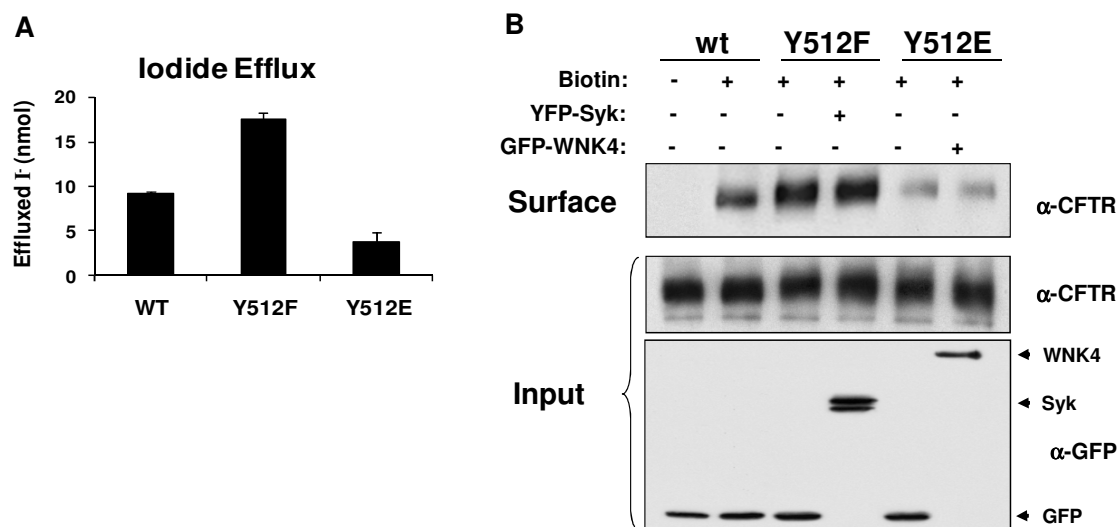
Figure 3.7| Phospho-mimetic mutants of CFTR Tyr512 are sufficient to change CFTR cell surface expression. BHK21 cells were stably transfected with CFTR mutant constructs carrying point mutations in Tyr512, so that either a phospho-mimetic glutamic acid residue (Y512E) or an unphosphorylatable phenylalanine residue (Y512F) were present. Cells were analysed by different techniques for changes in the expression of CFTR at the cell surface, as described in the caption of FIG. 3.1. **A| CFTR-mediated ion transport by iodide efflux. B| Biotinylation of cell surface proteins.** Note that CFTR-Y512E is less, but CFTR-Y512F is more abundant at the cell surface than wild-type CFTR. In addition, surface abundance of CFTR-Y512F can no longer be decreased by transfection of YFP-Syk, and abundance of CFTR-Y512E is no longer increased by expression of GFP-WNK4.

3.5 Discussion

The data presented in this chapter provide two novel insights into the regulation of cell surface expression of CFTR. First, we show that the CFTR protein is a substrate for phosphorylation by protein kinase Syk at NBD1 residue Tyr512, leading to a reduction in the cell surface expression of CFTR. Second, we describe an antagonistic role for WNK4 and Syk in regulating the cell surface expression of CFTR.

In our experiments, Syk was initially identified as a WNK4 partner protein in HEK293 cells, when proteins involved in signal transduction were screened for formation of complexes with Myc-WNK4 under non-denaturing conditions. The interaction was clearly confirmed by co-immunoprecipitation when either WNK4 or Syk were pulled-down (FIG. 3.2). The observed interaction occurred in both human HEK293 and in rodent BHK21 cells, and is independent of the presence of CFTR.

Since expression of WNK4 enhanced the amount of CFTR at the plasma membrane (FIG. 3.1) we searched for a possible role of Syk in this process and found a Syk phosphorylation motif at Tyr512 of the CFTR sequence, i.e. in its NBD1. In protein kinase assays performed *in vitro*, this CFTR domain was indeed found to be phosphorylated by Syk at Tyr512. Furthermore, when Syk is overexpressed in cells it associates with, and co-immunoprecipitates CFTR, an interaction resulting in a slight reduction in the amount of cell surface biotinylated CFTR. Even more significant was the outcome of expressing a kinase-dead (dominant negative) Syk mutant, which can interact with CFTR but not phosphorylate it, causing a significant increase in cell surface CFTR. This indicates that Syk activity normally restricts expression of CFTR at the plasma membrane. This increase in surface CFTR was confirmed by fluorescence microscopy and by measuring CFTR-mediated iodide efflux in living cells (FIG. 3.6). Finally, CFTR mutations mimicking either a constitutive phosphorylated or a non-phosphorylated

Tyr512 were sufficient to provoke the corresponding changes in the CFTR cell surface abundance and iodide efflux (FIG. 3.7).

Together, these data make a convincing case that the phosphorylation of CFTR at Tyr512 by Syk is a novel regulatory mechanism to modulate the amount of CFTR present at the plasma membrane. Although phosphorylation of CFTR is known to occur in the regulatory domain of CFTR leading to channel opening and activation of transport activity (Gadsby and Nairn, 1999), our data on Syk revealed a yet unknown role for phosphorylation in NBD1 for the regulation of CFTR expression at the cell surface. The physiological relevance of these findings is supported by the fact that Syk expression was detected in airway epithelial cells (Ulanova et al., 2005b), and confirmed by us in Western blot analyses of the lung cell lines CFBE and Calu-3 (data not shown).

Our data further revealed that WNK4 acts in an antagonistic way to Syk and promotes cell surface expression of CFTR. A previous analysis of WNK4 in extra-renal tissues found expression in lung and other chloride-transporting epithelia, including sweat ducts, colonic crypts, pancreatic ducts, and epididymis (Kahle et al., 2004a). Thus, the tissue-specific WNK4 expression pattern largely overlaps with that of CFTR. Given that WNK kinases can be activated by changes in extracellular osmolarity (Kahle et al., 2008a), it is tempting to postulate that WNK4 responds to fluctuations of the airway surface fluid and adjusts the amount of CFTR activity accordingly.

The *in vitro* phosphorylation of CFTR NBD1 at Tyr512 by Syk was inhibited by WNK4 as well as by its kinase-dead K186A mutant. Thus, the inhibition is most likely mediated by the protein-protein interaction observed between Syk and WNK4. Other WNK4-mediated effects on ion channels were also previously described to be kinase-independent, and include the inhibition of ROMK (Kahle et al., 2003), and of ENaC (Ring et al., 2007a) surface expression in *Xenopus* oocytes. In both cases, changes in

endocytosis of the respective channels are involved, albeit through different molecular pathways. While the inhibitory effect of WNK4 on ROMK involves binding to the linker protein intersectin to facilitate clathrin-dependent endocytosis (He et al., 2007), the downregulation of ENaC is most likely mediated by the E3 ubiquitin ligase Nedd4-2 to direct ENaC into the lysosomal pathway for degradation (Ring et al., 2007a). Whether the antagonistic effects of WNK4 and Syk on CFTR also involve endocytosis or rather changes in membrane traffic and CFTR delivery remains to be determined. Intriguingly, when we expressed a kinase-dead Syk mutant, which leads to an increase in surface CFTR, our immunofluorescence analysis revealed that this mutant co-localised at the plasma membrane with sites of increased CFTR staining (FIG. 3.6 | B). This is suggestive that under normal conditions Syk is recruited to the plasma membrane CFTR, and then phosphorylates it triggering CFTR endocytosis. The presence of WNK4 would then sequester Syk and increase retention of CFTR at the cell surface. This hypothesis is schematically depicted in FIG. 3.8.
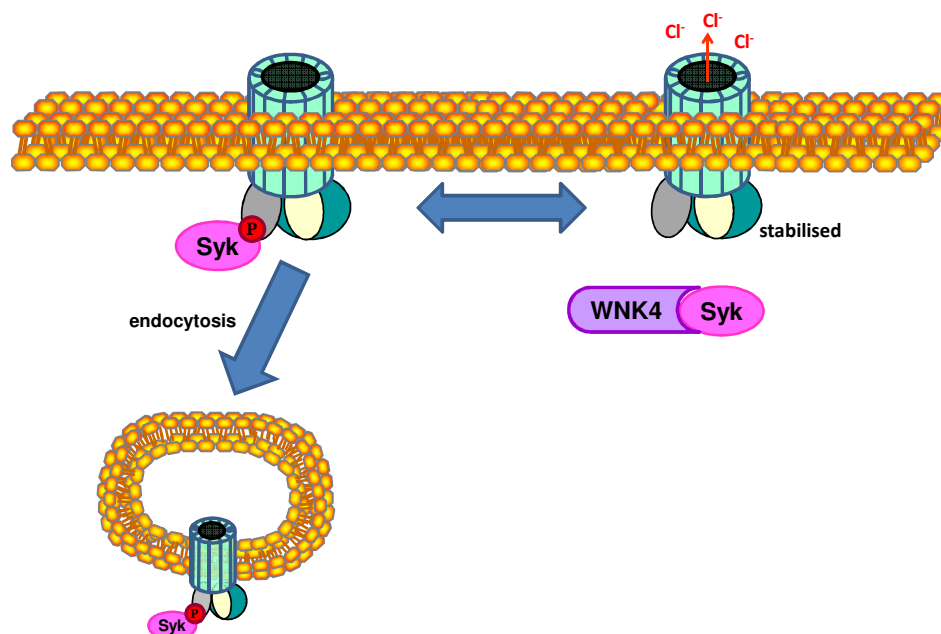
Figure 3.8| Proposed model for the role of WNK4 and Syk in regulating cell surface levels of CFTR. Syk-mediated phosphorylation of CFTR at the plasma membrane triggers its endocytosis. WNK4 can sequester Syk and thus increase the prevalence of CFTR at the cell surface. Upstream signals regulating activation or expression of Syk and WNK4 remain to be determined.

Our findings have several remarkable implications. First, the WNK4/Syk interplay identified for CFTR may also operate in the regulation of other chloride cotransporters. Given that an increase in apical CFTR activity would require concomitant activation of basolateral ion channels to coordinate a transepithelial chloride flux (Kahle et al., 2006), it is appealing to speculate that WNK4 and/or Syk control this coordination. Second, the WNK4/Syk pathway may be of therapeutic interest in cystic fibrosis. The most frequent mutation in patients is the deletion of phenylalanine 508 (Phe508del), which causes increased proteolytic degradation of the misfolded mutant CFTR protein. Interestingly, however, the mutant protein could contribute to some residual chloride channel activity and significantly reduce disease severity if its retention at the plasma membrane is improved (Amaral, 2005). Our data show that Syk normally inhibits CFTR surface expression and suggest that Syk inhibitors may also benefit the prevalence of mutant CFTR Phe508del protein at the plasma membrane. Third, Syk inhibitors are currently used in the clinic to inhibit inflammation and treat allergic rhinitis and rheumatoid arthritis (Ulanova et al., 2005a; Sanderson et al., 2009). This is due to the role of Syk in immunoreceptor signalling in leukocytes (Sanderson et al., 2009; Mócsai et al., 2010). Interestingly, recent studies have suggested that Syk also functions in the lung inflammatory response, being involved in tumour necrosis factor-induced nitric oxide production by airway epithelial cells (Ulanova et al., 2006). Thus, Syk inhibition may further be beneficial in cystic fibrosis therapy because the disorder is characterised by chronic lung inflammation due to impaired clearance of the thick mucus that forms in the absence of CFTR-mediated chloride transport (Belcher and Vij, 2010).

3.6 Acknowledgements

WNK4-K174A cDNAs, and P. J. Thomas (University of Texas Southwestern Medical Center, Dallas) and the CFTR Folding Consortium (CFF, Bethesda, MD, USA) for pET-SUMO-NBD1. This work was supported by the Portuguese Fundação para a Ciência e Tecnologia through grant POCI_PPCDT/SAU-MMO/56439/04, Programa de Financiamento Plurianual do BioFIG, and fellowship BD 23001/05 to AIM.

The important thing is

not to stop questioning;

curiosity has its own reason

for existing.

Albert Einstein (1879 -1955)

4

A *WNK4* gene variant relates to osteoporosis and not to hypertension in the Portuguese population

Mendes AI, Mascarenhas MR, Matos S, Sousa I, Ferreira J, Barbosa AP, Bicho M, and Jordan P

4.1 Summary

Germline mutations in the *WNK4* gene originate Gordon's syndrome or pseudohypoaldosteronism type II (PHA-II), a familial form of hypertension with hyperkalemia and hypercalciuria. In order to elucidate the contribution of *WNK4* genetic variants to hypertension and/or osteoporosis, we analysed 271 control individuals and a cohort of 448 hypertensive and 372 osteoporosis patients from the Portuguese population. Ten genetic variants were detected in 4.3% of the population under study, none of which revealed any significant association to the hypertension phenotype. In contrast, a rare missense alteration (R1204C) within exon 17 in a highly conserved arginine residue showed a possible tendency for association to the osteoporosis group. Our data suggest that *WNK4* polymorphism rs56116165 is a rare allelic variant in a candidate gene with a biological function in renal calcium homeostasis that may contribute to a genetic predisposition to osteoporosis.

Keywords: Gene variant, Hypertension, Low bone mineral density, Osteoporosis, Predisposition, WNK4 protein kinase

4.2 Background

Mutations in the *WNK1* and *WNK4* genes have been identified to cause PHA-II or Gordon's syndrome (Wilson et al., 2001), a rare familial form of hypertension accompanied by hyperkalemia and metabolic acidosis. *WNKs* encode a small family of protein kinases with a sequence variation in the otherwise conserved catalytic domain (Xu et al., 2000; Veríssimo and Jordan, 2001; Min et al., 2004). WNK kinases control the activity of various ion channels and their mutation leads to dysregulated renal sodium absorption and potassium secretion (reviewed in Gamba, 2005; Kahle et al., 2008a).

The mutations identified thus far in the *WNK4* gene are missense changes outside the protein kinase domain, in particular mutations E562K, D564A, Q565E, and R1185C, that cluster in exons 7 or 17 (Wilson et al., 2001). Besides hypertension and hyperkalemia, patients with *WNK4* mutations also present hypercalciuria and low BMD (Mayan et al., 2002).

Transgenic mouse models expressing the disease-causing D564A or Q565E *Wnk4* mutations have demonstrated the typical hypertension, hyperkalemia and hypercalciuria phenotypes (Lalioti et al., 2006; Yang et al., 2007c). They further allowed concluding that increased transport activity of the sodium chloride cotransporter (NCC) is the primary cause for the hypertension syndrome. With regard to hypercalciuria, these mouse models revealed an underlying reduced renal calcium reabsorption since normal values were observed for glomerular filtration rate, serum calcium, and PTH or $1,25(OH)_2D_3$ hormone levels. This is consistent with the notion that calcium reabsorption is inversely related to sodium reabsorption in the distal tubule. Besides NCC, other candidate ion channels have been reported to be affected by *WNK4* mutations, including the transient receptor potential channel vanilloid subtype calcium channels (TRPV4, 5 and 6) (Jiang et al., 2007; Fu et al., 2006) and the renal potassium channel ROMK

(Kahle et al., 2003; Gamba, 2005b). These could contribute to the hypercalciuria and hyperkalemia phenotypes, respectively.

The objective of the work described in the following was to screen a cohort from the Portuguese population for the presence of genetic *WNK4* variants, which might confer an increased risk for either hypertension and/or decreased BMD.

4.3 Experimental Procedures

4.3.1 Subjects

The following DNA samples were studied: the initial hypertension samples included 82 normal control individuals, 125 essential hypertension patients, 63 pregnancy-induced hypertensive women; 10 pre-eclampsia and 9 post-menopause hypertensive women. The study was then extended to include hypertension and/or osteoporosis samples containing 189 normal control individuals, 110 essential hypertension patients, 69 combined low BMD and hypertension, 153 low BMD, 88 osteoporosis and 62 combined osteoporosis and hypertension patients. Patients with alterations in sodium or potassium levels due to primary hyperaldosteronism were excluded from the study. The total numbers were 271 normal control individuals, 448 hypertensive patients, 512 normotensive individuals, 372 patients with decreased BMD (low BMD or osteoporosis) and 381 individuals with documented normal BMD. The genetic screening of exons 7 and 17 was performed in 601 and 960 samples, respectively.

Individuals were accessed for BMD (g/cm^2) by dual X-ray absortiometry at the lumbar spine (L1-L4), hip and distal forearm, using the QDR Discovery W densitometer (Hologic Inc.). The lowest T-score at those skeletal regions was used to qualify BMD, according to the World Health Organization's operational definition of osteoporosis: T-score $\leq$-2.5 SD is classified as osteoporosis, -2.5 SD$\leq$T-score$\leq$-1 SD as low BMD and

T-score $\geq$-1 SD as normal BMD. Blood samples were taken following the primary BMD screening scan before any pharmacological patient treatment.

All patients signed an informed consent declaration.

4.3.2 PCR-dHPLC screening and DNA sequencing

Exons 7 and 17 of the *WNK4* gene (GeneID: 65266, GenBank NC_000017; transcript NM_032387 or ENST00000246914) were amplified from DNA (100 ng) using primer pairs WNK4 ex7-F (5'-GGC GTC CTG ATG GAT CTT TG) and WNK4ex7-R (5'-AGG CCA AGG TGT TCT GGG A) or WNK4ex17-F (5'-CCT GGG AGA GCA AGG TGT GT) and WNK4ex17-R2 (5'-CCT GGA GAA AAA CAG TCG ACA GAA) under standard PCR conditions. Fragments were screened for mutations by dHPLC elution (WAVE; Transgenomic, www.transgenomic.com), in a gradient of 57%-65% dimethylformamide solution B during 4 min at 62°C. Exons showing abnormal dHPLC patterns were subsequently sequenced from an independently amplified PCR product using fluorescent dye terminators and an automated DNA sequencer (ABI Prism 3100, Applied Biosystems).

4.3.3 Statistical Analysis

A case-control association study was conducted comparing individuals with and without our variant of interest (rs56116165) in *WNK4* exon 17, for each of the traits under study. First, we compared the variant's frequency between the individuals with low BMD (N=372) against healthy unselected controls (N=271), as well as between the group of individuals with hypertension (N=448) against the same control group (N=271). Since the variant in question was more frequent in the low BMD group, we did one further test comparing the group of individuals with low BMD (N=372) against the group of all individuals with documented normal BMD (N=381). Logistic regression as well as Fisher's exact test were applied to test association between

rs56116165 and each of the phenotypes under study, thus results were confirmed between both methods. These were implemented in the R software, using the genetics library (http://cran.r-project.org). For each comparison group, the standard error and the respective 95% confidence intervals were also estimated. Both logistic regression and Fisher's exact test are commonly used when dealing with dichotomous traits, especially, the latter test when sample sizes are small such as the populations under investigation in this study. Although performing multiple statistical tests can lead to inflation in the occurrence of false positives (requiring adjustment of the statistical significance (*P*-value) threshold in order to account for the number of independent tests), our study only investigated one variant, so no additional correction was required. Further confounding effects were not considered in the regression analysis.

4.4   Results

Given that *WNK4* mutations cause PHA-II with hypertension and hypercalciuria, genetic variants in this gene may exist in the population that predispose to essential hypertension and/or low BMD. Thus, we genotyped *WNK4* exons 7 and 17, to which all known mutations cluster, in DNA samples from normal individuals and from hypertensive or osteoporosis patients.

Altogether, we analysed 960 individual DNA samples and detected ten (2 intronic and 8 exonic) different genetic variants. These were present in 41 individuals (4.3%). Some individuals carried two alterations so that altogether 52 variant alleles were detected. The corresponding variants and their allele frequencies in the Portuguese population studied are shown in TABLE 4.1. Interestingly, we identified one variant in exon 7 (c.1598T>C, L533P) that has not been previously described.

Table 4.1| Allele frequencies of the genetic *WNK4* variants identified in the Portuguese population. The number of amplified DNA samples (N) was 601 for exon 7 and 960 for exon 17. Sequence numbering was in agreement with public database entries NM_032387 or ENST00000246914. Note that variant c.1598T>C, L533P has not been previously described (n.a. – not applicable).

Exon/Intron	Nucleotide	Protein	SNP	Frequency
IVS6	c.1477(-13)A>G	n.a.	rs61754357	6/601 = 1.0%
7	c.1523G>A	R508H	rs5599715	1/601 = 0.2%
7	c.1524T>C	R508R	rs55879206	2/601 = 0.3%
7	c.1598T>C	L533P	not assigned	1/601 = 0.2%
7	c.1641C>T	A547A	rs9916754	15/601 = 2.5%
7	c.1653C>T	P551P	rs55751736	8/601 = 1.3%
7	c.1664C>G	P555R	rs57737815	4/601 = 0.7%
7	c.1719C>T	H573H	rs56243382	2/601 = 0.3%
IVS16	c.3448(-20)T>C	n.a.	rs61755630	4/960 = 0.4%
17	c.3610C>T	R1204C	rs56116165	9/960 = 0.9%

When the identified variants were matched to the subjects' clinical phenotypes, we observed that most variants occurred in both control and patient populations, without revealing any significant correlation to essential hypertension (TABLE 4.2). Although the exon 7 variants R508R, R508H and L533P were found only in hypertensive pregnant women, they occurred only in one sample each, thus precluding a statistically significant interpretation.

In contrast, we observed that R1204C (rs56116165), a variant already described in exon 17, was almost exclusively found in patients with decreased BMD or osteoporosis (TABLE 4.2). This variant was rare in the general population (0.9%) but found in 8 of the 372 patients with decreased BMD (2.2%). The clinical characterisation of the eight corresponding osteoporosis patients revealed that they were unrelated, mostly female (7/8) and had different pathologies with underlying osteoporosis, including type 2 diabetes

Table 4.2| Correlation between clinical phenotypes and *WNK4* sequence variants. 601 Portuguese individuals were screened for the exon 7, and 960 for the exon 17 region. When divided into phenotypic groups the analysed number of samples covered 448 hypertensive and 512 normotensive individuals, 372 individuals with decreased BMD, and 381 with normal BMD, as well as, 271 normal control individuals. Ten different genetic variations were detected in 41 individuals (4.3%), summing up to a total of 52 variant alleles. (n.a. – not applicable)

Sample	Patient group	Exon/Intron	Nucleotide	Protein	Frequency
D2	Normal Control				
D109	Normal Control				
201	Normal Control	IVS 6	c.1477(-13)A>G	n.a.	6/601
5MF	Essential Hypertension				
7MF	Essential Hypertension				
47MF	Essential Hypertension				
32G	Pregnancy-induced hypertension	Ex 7	c.1523G>A	R508H	1/601
9BN	Essential Hypertension	Ex 7	c.1524 T>C	R508R	2/601
29G	Pregnancy-induced hypertension				
PC2	Preeclampsia	Ex 7	c.1598T>C	L533P	1/601
D4	Normal Control				
D45	Normal Control				
D109	Normal Control				
201	Normal Control				
261	Normal Control				
1MF	Essential Hypertension				
7MF	Essential Hypertension				
47MF	Essential Hypertension	Ex 7	c.1641C>T	A547A	15/601
66MF	Essential Hypertension				
273	Essential Hypertension				
67G	Pregnancy-induced hypertension				
92G	Pregnancy-induced hypertension				
172	Low BMD + Hypertension				
182	Low BMD + Hypertension				
25	Osteoporosis				
D102	Normal Control				
D111	Normal Control				
34MF	Essential Hypertension				
284	Essential Hypertension				
2G	Pregnancy-induced hypertension	Ex 7	c.1653C>T	P551P	8/601
166	Low BMD + Hypertension				
79	Low BMD				
23	Osteoporosis				
261	Normal Control				
1MF	Essential Hypertension	Ex 7	c.1664C>G	P555R	4/601
273	Essential Hypertension				
182	Low BMD + Hypertension				
D45	Normal Control	Ex 7	c.1719 C>T	H573H	2/601
92G	Pregnancy-induced hypertension				
173	Low BMD + Hypertension				
362	Low BMD + Hypertension	IVS 16	c.3448(-20)T>C	n.a.	4/960
123	Low BMD				
133	Low BMD				
420	Normal Control				
70	Osteoporosis + Hypertension				
150	Osteoporosis + Hypertension				
317	Osteoporosis + Hypertension				
699	Osteoporosis+ Hypertension	Ex 17	c.3610C>T	R1204C	9/960
104	Low BMD				
2	Osteoporosis				
514	Osteoporosis				
606	Osteoporosis				

mellitus (2/8), hypercholesterolemia and goiter (3/8), or hyperthyroidism (2/8) (TA-BLE 4.3). All patients had normal serum calcium levels (9.2-10.3) and showed no alterations in sodium or potassium values (data not shown). Only 1 in 381 individuals with normal BMD had the same variant, but presented hypothyroidism.

Table 4.3| Clinical description of the nine individuals carrying the *WNK4* exon 17 variant rs56116165 (R1204C). (F – female, M – male, n.d. – not determined)

ID	Age	Sex	HT	Low BMD	Osteo-porosis	Serum Ca^{2+}	Additional Diagnosis
70	58	F	+	-	+	10.0	Type 2 Diabetes Mellitus
317	74	F	+	-	+	9.6	Type 2 Diabetes Mellitus, Hepatic Steatosis, Esophagitis
150	65	F	+	-	+	9.3	Goiter, Hypercholesterolemia, Depression, Parkinsonism
2	68	F	-	-	+	9.3	Goiter, Hypercholesterolemia, Ischemic Cardiopathy, Chronic Adrenal Insufficiency
606	52	F	-	-	+	n.d.	Goiter, Obesity
514	45	F	-	-	+	10.3	Hyperparathyroidism
699	61	M	+	-	+	9.6	Hyperthyroidism, Hypercholesterolemia, Kidney Cyst Right
104	46	F	-	+	-	n.d.	Iatrogenic Hyperthyroidism, Asthma, Depression
420	44	F	-	-	-	9.2	Hypothyroidism, Chronic lymphocytic thyroiditis, Depression

No significant association between variant rs56116165 and the hypertension phenotype was detected (*P*>0.05 - TABLE 4.4). Moreover, a non significant association value was obtained when the low BMD group was compared to the healthy control individuals (*P*>0.05 - TABLE 4.4). However, when all individuals with clinically documented normal BMD were considered as the control group, the tendency for association with the low BMD phenotype was highlighted (*P*>0.05 - TABLE 4.4), indicating an increased risk (OR>1) for low BMD in carriers of this variant.

Table 4.4| Case-control association results for the variant R1204C in the Portuguese population.
(BMD – bone mineral density, CI – confidence interval, logOR – odds ratio logarithm. Significant results are shown in bold.)

Comparison Groups	Fisher's exact test			Logistic regression model		
	logOR	CI	*P*-value	logOR	CI	*P*-value
Hypertensive *versus* Controls	0.38	(-0.70; 2.10)	0.650	0.89	(-1.30; 3,08)	0.428
Low BMD *versus* Controls	0.72	(-0.09; 2.40)	0.086	1.78	(-0.30; 3.86)	0.094
Low BMD *versus* Normal BMD	0.92	(0.04; 2.60)	**0.020**	2.12	(0.04; 4.20)	**0.046**

In order to evaluate the functional consequence of the observed variation for the *WNK4* gene product, we analysed the evolutionary conservation of arginine 1204. A sequence alignment of 12 different mammalian species revealed that arginine 1204 and its surrounding amino acid residues form a highly conserved sequence (FIG. 4.1), indicating that the amino acid exchange in variant R1204C has possibly functional relevance.

Man (*Homo sapiens*)	–SKGSFPTSRRNSLQRSEPPGP
Chimp (*Pan troglodytes*)	–SKGSFPTSRRNSLQRSEPPGP
Gorilla (*Gorilla gorilla*)	–SKGSFPTSRRNSLQRSEPPGP
Orangutan (*Pongo pygmaeus*)	–SKGSFPTSRRNSLQRSEPPGP
Horse (*Equus caballus*)	–SKGSFPTSRRNSLQRSEPPGL
Hedgehog (*Erinaceus europaeus*)	–SKGSFPTSRRNSLQRSEPLGP
Cow (*Bos taurus*)	–SKGSFPTSRRNSLQRSEPLGP
Pig (*Sus scrofa*)	–SKGSFPTSRRNSLQRSEPLGP
Dog (*Canis familiaris*)	–SKGSFPTSRRNSLQRSEPLGP
Rabbit (*Oryctolagus cuniculus*)	–SKGSFPTSRRNSLQRSEPLGP
Mouse (*Mus musculus*)	–SKGSFPTSRRNSLQRSDLPGP
Rat (*Rattus norvegicus*)	–SKGSFPTSRRNSLQRSDLPGP

Figure 4.1| R1204 is highly conserved among mammalians. A WNK4 protein sequence alignment is shown corresponding to the 3' end of exon 17 in the twelve eutherian mammals indicated. R1204 is marked by a box and sequence differences highlighted by black shading. Note the high conservation of the whole region.

4.5 Discussion

The main conclusions from the present work are that *WNK4* polymorphic variants are rare in the Portuguese population studied and revealed no significant association to essential hypertension. In contrast, the variant rs56116165 was identified as being potentially associated to low BMD.

In this work we describe single nucleotide polymorphisms (SNPs) in exons 7 and 17 of the *WNK4* gene found in the Portuguese population. Ten different rare SNPs were detected, including the identification of one novel mutation/variant found in a preeclampsic pregnant woman (exon 7, c.1598T>C, L533P). Among these polymorphic variants, only one was found to be disease-related, namely exon 17 variant rs56116165, which changes an arginine residue highly conserved among mammalian species into a cysteine (R1204C). Data deposited in the current public NCBI SNP database (http://www.ncbi.nlm.nih.gov/) indicated an allele frequency of 0.6%. In the Portuguese population studied, this variant is similarly rare (0.9%) but was preferentially encountered in low BMD patients (2.2%). The *P*-values obtained with the association tests indicate a clear tendency for an association of this variant (rs56116165) with low BMD. However, as we are dealing with a rare variant, association tests are not the most adequate approach, as they possibly lack statistical power to find an unbiased association.

There is no doubt that genetic variants of an individual can confer susceptibility to certain diseases. It is at present unclear, however, to what extent this susceptibility is contributed by either common variants with low penetrance or rare variants with high penetrance (Schork et al., 2009; McClellan and King, 2010). Following the complete sequencing of the human genome, GWAS have been reported for the unbiased identification of common susceptibility variants involved in complex diseases including hypertension (Levy et al., 2009; Ehret, 2010) and osteoporosis (Rivadeneira et al., 2010; Li

et al., 2010; Cheung et al., 2010). One important lesson from these studies has been that no major common disease-causing variants were found, but rather that many 'low risk variants' exist, each conferring only a small degree of risk. In hypertension, for instance, a *WNK1* variant (SNP rs2301880) was identified which exhibited a reduction in systolic blood pressure of 1.78 mm Hg in the general population, thus contributing a proportion of 1.4% to the additive polygenic variance in mean blood pressure (Tobin et al., 2005). Therefore, development of complex diseases is postulated as the result of the combined effects of many such alleles (McClellan and King, 2010). The GWAS in osteroporosis identified candidate genes with a role in biological pathways of osteoblastogenesis and osteoclastogenesis (Li et al., 2010; Cheung et al., 2010).

This does not exclude, however, that rare gene variants exist in key genes which contribute a large effect in predisposition to complex diseases. The current GWAS approach is likely to exclude them from the initial association analysis because of their low allele frequency (McClellan and King, 2010). This is a major limitation of the GWAS approach. The contribution of rare variants is likely to be important because the common variants identified in GWAS have been estimated to cover less than 3% of the estimated genetic susceptibility to diseases (McClellan and King, 2010). Their clinical applicability is further unclear because many of the identified variants have no known associated function or pathogenetic importance and are merely assumed to influence gene function or expression.

In the study described herein we have shown that the rare variant rs56116165 in *WNK4* exon 17 has clear tendency for association to low BMD. We propose to consider R1204C as a rare susceptibility variant in a gene with an established biological function related to renal calcium homeostasis. In particular, the analysis of *Wnk4* transgenic mice has suggested that decreased calcium reabsorption in the upstream nephron, with a secondary adaptive increase in calcium channel (TRPV6 and CBP-D28k) expression in the

distal tubules is underlying the hypercalciuria of PHA-II (Yang et al., 2010). Interestingly, one PHA-II kindred was described with a close-by *WNK4* mutation in exon 17 (R1185C) that confers hyperkalemia without hypertension (Baz et al., 1990; Mayan et al., 2002). This may indicate that exon 17 mutations differ in biological function from exon 7 mutations and explain our observation that R1204C was found in low BMD patients but not in patients with hypertension alone.

In conclusion, we identified rs56116165 as a rare *WNK4* variant that may predispose to osteoporosis. In order to statistically consolidate its contribution towards this predisposition, future independent studies from different populations should include larger numbers of both cases and controls, inquiring not only the R1204C variant but its surrounding gene region using deep-sequencing strategies. With next-generation sequencing technologies it is hoped to obtain a relevant list of potential susceptibility variants, providing hope for a truly personalised preventive medicine, as well as explaining the missing heritability of the current GWAS studies performed for complex disorders.

4.6 Acknowledgements

Not all those who wander

are lost.

J. R. R. Tolkien (1892-1973)

5

General Discussion

Similarly to the thesis objectives, the general discussion and conclusions are divided into two different parts. In the first part the identification of novel WNK interacting cellular protein partners is discussed in light of the current paradigms on signalling transduction mechanisms, and their importance for cellular homeostasis. The second part of this chapter discusses the value to the broad context of hypertension and/or osteoporosis polygenic inheritance that was added by a *WNK4* mutational screen in a Portuguese cohort followed by the analysis of genetic predisposition to these disorders.

5.1 Novel signalling mechanisms involving human WNK1 and WNK4 protein kinases

The flow of ions and other molecules across the membrane of cells and organelles is crucial to many of life's processes, and so are ion channels and transporters. An important aspect of transporters and ion channel biology in several disorders is the actual amount of protein expressed at the cell surface. Among the multiple factors that regulate the functioning of these transporters and channels in cells, their redistribution between intracellular vesicular pools and the plasma membrane is one such key factor in which the WNK kinases are involved.

WNKs are unique and very large protein kinases that have been shown to participate in a variety of signalling processes and to interact with different proteins *via* several motifs or domains, as summarized in FIG. 5.1. The unusual position of their catalytic lysine may be related to some yet to be identified regulatory mechanism. Moreover, due to their large size it has been speculated that they can act as scaffolds that recruit other proteins which regulate the activity and/or surface expression of ion channels and transporters. Furthermore, in some cases, their kinase activity may not be relevant but rather the conformation of the kinase domain.
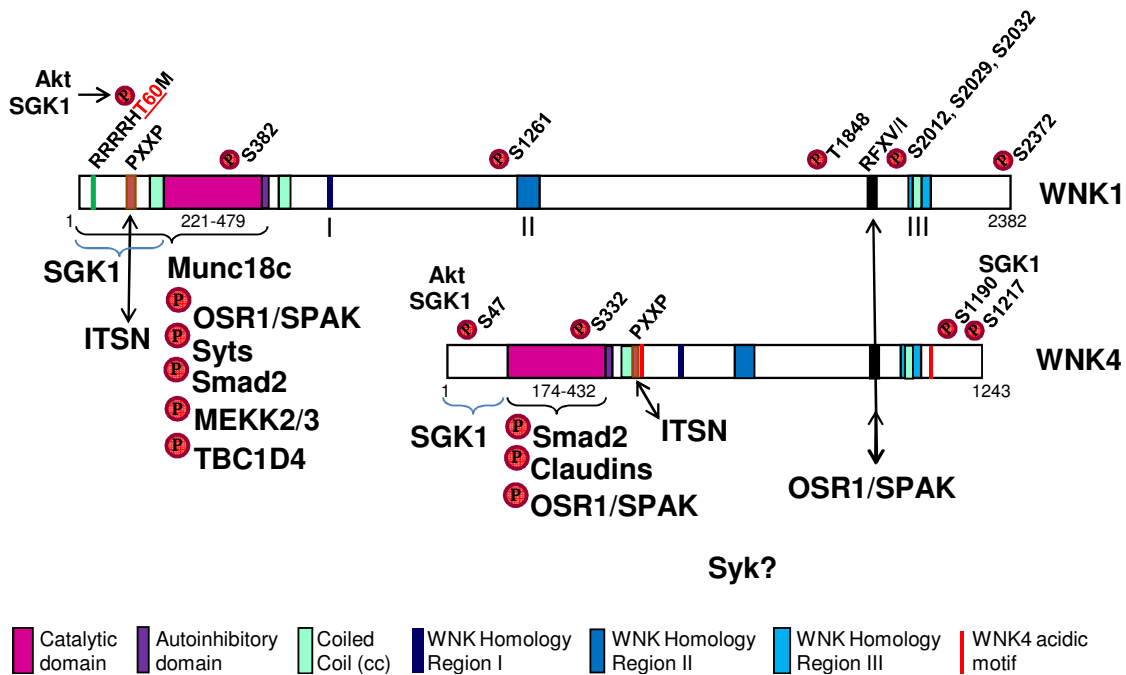
Figure 5.1│ Schematic representation of WNK1 and WNK4 phosphorylation sites, interacting protein partners and respective interaction domains. WNK1 and WNK4 known phosphorylation sites are shown, except for WNK1 Ser15 and Ser167 for simplicity reasons. WNK1 Ser382 and WNK4 Ser332 are autophosphorylation sites (Xu et al., 2000; Lenertz et al., 2005), WNK1 Ser2012, Ser2029, Ser2032 are constitutive sites of phosphorylation, and the other WNK1 sites are sorbitol-induced ones (Zagórska et al., 2007). Both WNK1 and WNK4 have an Akt/SGK1 substrate motif (RXRXX(p)S/T) in their N-terminal (Thr60/Ser47) (Jiang et al., 2005; Heise et al., 2010), and WNK4 has 2 additional motifs on its C-terminus (Ring et al., 2007b; Rozansky et al., 2009). WNK4 Ser1217 is phosphorylated both by SGK1 and WNK1 (Rozansky et al., 2009). WNK1 and WNK4 interact with intersectin (ITSN) *via* a PXXP motif (He et al., 2007) and WNK1 also interacts with Munc18c through its N-terminus (Oh et al., 2007). Interactions with substrates involving phosphorylation are also shown. Both WNKs phosphorylate Smad2 (Lee et al., 2007) and OSR1/SPAK (Vitari et al., 2005), the later bind to WNKs RFXV/I motifs. WNK1 also phosphorylates synaptotagmins (Syts) (Lee et al., 2004), MEKK2/3 (Xu et al., 2004) and TBC1D4 (see Chapter 2), while WNK4 directly phosphorylates claudins (Yamauchi et al., 2004). Syk interaction region/motif within WNK4 is still unknown.

This has been shown for ROMK regulation, in which the mutation of Lys233 or Asp368, two residues conserved in most protein kinases and commonly mutated to generate kinase-dead variants, disrupts an ion pair interaction between them, that is critical for the kinase domain conformation. Thus, mutation of these residues may rather affect

the overall structure of the kinase domain, which is required for PXXP motifs in the N-terminus of WNK1 to interact with the SH3 domain of intersectin, which in turn regulates the endocytosis of the channel (He et al., 2007). This raises the question of whether other WNK regulated mechanisms have been misdeclared as kinase-dependent.

5.1.1 WNK1

The work presented in Chapter 2 of this thesis is the first example of a non-ion channel regulated by a WNK kinase. The molecular details through which WNK1 regulates the surface expression of GLUT1, the glucose transporter in non-insulin target cells, could be elucidated. WNK1 forms a complex with TBC1D4, a Rab-GAP involved in regulated exocytosis of glucose transporters, and with Rab8A-GDP. Upon activation of WNK1, TBC1D4 is phosphorylated and associates with the phosphoprotein-binding 14-3-3 adaptor molecules so that, its GAP activity becomes inhibited. Following Rab8A activation by GDP/GTP exchange, delivery of GLUT1-containing vesicles to the plasma membrane can occur promoting increased cellular glucose uptake.

Curiously, a recent study revealed that another Tre-2/USP6, BUB2, Cdc16 domain family member, TBC1D1 (also an Akt substrate), seems to be a negative regulatory element in the insulin signalling pathway of mTOR that regulates GLUT1 protein expression. It was shown that a siRNA-mediated gene silencing of *TBC1D1* induced the expression of *GLUT1* and increased basal glucose transport in adipocytes, through a mechanism dependent on mTOR (Zhou et al., 2008). It was suggested that the phosphorylation of TBC1D1 could result in the inactivation of its GAP domain, which is similar to what we observed as a consequence of TBC1D4 phosphorylation by WNK1. This raises the question of whether WNK1 could also be mediating TBC1D1 effects on GLUT1 or if these regulatory pathways could occur completely independent in different cell types.

Additionally, it should also be investigated if the WNK1-TBC1D4 interaction is WNK1-specific or if other WNK kinases can also interact and phosphorylate TBC1D4.

Moreover, translocation of the insulin-regulated glucose transporter GLUT4 to adipocytes or muscle cells surface is also known to be regulated by TBC1D4 (Sano et al., 2003; Bruss et al., 2005), and its interaction with 14-3-3 proteins is insulin- and Akt-dependent, *via* an Akt phosphorylation site, Thr642 (Ramm et al., 2006). In addition, WNK1 has been shown to associate with Munc18c (as aforementioned in Chapter 1) (Oh et al., 2007). This syntaxin 4-regulatory protein also participates in insulin-stimulated GLUT4 vesicle translocation in adipocytes, namely at the stage of fusion between the vesicle and plasma membranes (Thurmond et al., 1998; Thurmond et al., 2000; Thurmond and Pessin, 2000). Thus, WNK1 could be involved in a more general mechanism regulating plasma membrane transporter traffic than originally considered.

More recently, TBC1D4 was also shown to regulate ENaC trafficking. Specifically, it stabilises ENaC within an intracellular compartment under nonstimulated conditions, and aldosterone(SGK1)-dependent TBC1D4 phosphorylation permits accumulated ENaC forward trafficking to the apical membrane of cortical collecting duct (CCD) cells (Liang et al., 2010). Therefore, phosphorylation of TBC1D4 appears to be a mechanism shared by regulated exocytosis of various membrane transport proteins.

Taking all these studies into consideration, it is reasonable to speculate that TBC1D4 phosphorylation by Akt and/or SGK1 may also be playing a part in GLUT1 regulation by WNK1. *Vice-versa*, it should be explored whether WNK1 is involved in the regulation of GLUT4 in insulin-responsive cell types. However, since WNK1 not only also directly phosphorylates TBC1D4, but is itself an Akt/SGK1 substrate (as previously mentioned in Chapter 1), the mechanisms underlying GLUT1 and GLUT4 cell surface expression could be more intricate. Specifically, it is unknown whether TBC1D4 phosphorylation by WNK1 is antagonistic or cooperative to its phosphorylation by

Akt/SGK1. Also, the upstream stimuli regulating WNK1 activation should be clarified, and in particular, the effect of insulin or IGF-1 stimulation should be addressed.

Interestingly, insulin-induced phosphorylation of Akt and TBC1D4 is reduced in adipocytes and skeletal muscle cells of patients with type 2 diabetes mellitus, which is accompanied by impaired GLUT4 translocation (Karlsson et al., 2005). Type 2 diabetes is a complex disorder with diminished insulin secretion and insulin action, that contribute to the hyperglycemia phenotype (reviewed in DeFronzo, 1992). In muscle cells, glucose transport is the rate-limiting step for glucose metabolism in both normal glucose-tolerant (Yki-Järvinen et al., 1987; Fink et al., 1992) and diabetic (Butler et al., 1990; Yki-Jarvinen et al., 1992) persons. Defects in glucose transport into muscle cells have also been implicated in the reduced insulin sensitivity observed in type 2 diabetes (Garvey et al., 1998).

The results presented in this thesis raise the possibility that pharmacologic induction of WNK1 activity could increase concentrations of glucose transporters which would provide a therapeutic opportunity in insulin-resistant type 2 diabetic patients.

5.1.2 WNK4

In Chapter 3, a novel signalling mechanism linking the protein kinases WNK4 and Syk to the modulation of CFTR cell surface expression was unravelled. It was shown that Syk can phosphorylate CFTR, leading to a decrease in CFTR expression in the surface of mammalian cells. In contrast, WNK4 interaction with Syk prevents CFTR phosphorylation, and consequently increases CFTR surface expression, thus promoting an increase in CFTR-mediated ion transport.

The exact nature of Syk inhibition by WNK4 remains to be determined. WNK4 could shield the NBD1 domain and prevent access of Syk; however, it seems more likely that

Syk is inhibited by the observed protein-protein interaction with WNK4, since this interaction also occurred in the absence of CFTR in parental BHK21 cells. Nevertheless, whether the antagonistic effect of WNK4 and Syk on CFTR involves endocytosis (similarly to ROMK; Kahle et al., 2003) or rather changes in membrane traffic and CFTR delivery (as for NCC; Subramanya et al.,2009) remains unknown. Therefore, it will be important not only to map the interacting regions involved, but also to investigate possible partners recruited by WNK4 and mediating its effects on Syk, and subsequently on CFTR surface expression.

Given that any increase in apical CFTR activity requires concomitant activation of basolateral ion channels, such as NKCC1, to coordinate a transepithelial chloride flux (Kahle et al., 2006) and, knowing that WNK4 regulates both apical and basolateral chloride flux in extrarenal epithelia (Kahle et al., 2004a), it is tempting to hypothesise that WNK4 (possibly *via* Syk) could be involved in this coordination. This way WNK4 may play a general role in epithelial chloride homeostasis. These observations can afford the speculation that pharmacologic modulation of the WNK4/Syk pathway could be explored to partially restore chloride flux in the airway epithelium of patients with cystic fibrosis.

On the other hand, in addition to functioning as a secretory chloride channel in epithelial cells, CFTR can also regulate several transport proteins, including ENaC (Stutts et al., 1995), ROMK (Yoo et al., 2004; Lu et al., 2006), and chloride/base exchangers (Lee et al., 1999; Ko et al., 2004). These transport proteins are known to be regulated by the WNK kinases (Ring et al., 2007a; Ring et al., 2007b; Kahle et al., 2003; Kahle et al., 2004a; Dorwart et al., 2007). Moreover, it has been shown that the tissue-specific WNK4 expression pattern largely overlaps with that of CFTR (Yang et al., 2007a). Consequently, WNK4 and CFTR may be involved in either cooperative or parallel physiologic pathways that regulate ion flux in these epithelia.

Furthermore, a sequence analysis of a variety of ion channels for the presence of Syk phosphorylation motifs revealed such motifs in the sequence of the renal NKCC2 (Tyr45) and the ubiquitously expressed KCC3 (Tyr63), whose activities are known to be regulated by the WNK kinases (de Los Heros et al., 2006; Garzón-Muvdi et al., 2007; Rinehart et al., 2009). Thus, an interesting subject for future investigations is to examine if a WNK4/Syk regulatory mechanism is also responsible for the modulation of these channels' cell surface abundance. Such a mechanism could also be contributing to the phenotypes observed in carriers of *WNK4* mutations.

5.2   *WNK4* mutational screen and analysis of hypertension/osteoporosis susceptibility

Essential hypertension afflicts nowadays industrialised populations (and continues to increase) leading to augmented morbidity and mortality. This may be partly due to increased salt consumption of a population that has been overtime put at risk by genes that once aided survival, by facilitating salt and water conservation when these resources were scarce (Meneton et al., 2005; O'Shaughnessy and Karet, 2006; Ritz, 2010).

On the other hand, osteoporosis is also a relevant disorder due to its high prevalence worldwide. This disorder poses a major challenge to modern medicine since not only it is a 'silent' condition, predisposing people to fractures at the hip, spine, forearm, or other skeletal sites, but also its treatment requires high costs (Lau and Cooper, 1996; Pongchaiyakul et al., 2008).

Both are age-related, complex and heterogeneous disorders, in which the extent of contributions from genetic polymorphisms to their heterogeneity is still not entirely understood. A small percentage of all cases are due to highly penetrant mutations in single genes, whereas low penetrance susceptibility genes are estimated to account

for a high proportion of cases, with most of the genes yet to be identified (Rosskopf et al., 2007; Ferrari, 2008; Li et al., 2010). Therefore, the search for additional hypertensive/osteoporosis predisposition genes is a main goal towards the improvement of their early diagnosis and to a better understanding of the biological mechanisms underlying these disorders.

The use of molecular markers and gene expression profiling provides a promising approach for improving the predictive accuracy of current disease prognostic indices. Molecular tests for diagnosis, disease prognosis and selection of treatment options, particularly in these disorders, are gaining increased acceptance by physicians and patients. Clinicians who are aware of the progress of the diagnosis tools and, in particular, of how this effort may be effectively facilitated through a comprehensive family history in concert with molecular genetic studies, are in a better position for designing highly targeted screening and management programs for the affected families. This knowledge may have impact upon the progress in the earlier diagnosis of the disorders and provide an impetus for better diagnostic methods. One example of a prognosis/diagnosis tool could be the use of a gene chip with different genetic variants known to be associated with the disorder. In this sense, a better understanding of *WNK4* variants and their contribution to hypertension and/or osteoporosis may have prognostic value and potential therapeutic applications in personalised medicine.

In the work presented in this thesis (Chapter 4) we analysed 271 control individuals, 448 hypertensive and 372 osteoporosis patients from the Portuguese population. Ten genetic variants were detected in 4.3% of the population under study, none of which revealed any significant association to the hypertension phenotype. In contrast, a rare missense alteration (R1204C) in a highly conserved arginine residue in exon 17 was identified as being potentially associated to low bone mineral density. Taken together, these data provide new insights into the genetic basis of osteoporosis, and support the hypothesis that *WNK4* variants can underlie the pathogenesis of this disorder. This

result has a statistical significance towards positive association considered nominal, as it is close to the 5% threshold established for this analysis. Nevertheless, the confidence interval is narrow and the trend is towards increased risk, as the respective OR is above one (TABLE 4.4). This result warrants further validation that could be achieved for example by genotyping a larger group of both cases and controls, through an independent method (like the Taqman platform) in order to gain statistical power. This way, we could confirm whether the initial signal was real or not. It would also be interesting to go back to the clinical data, and find additional relevant factors (such as diet, medication and physical exercise) that may have an impact in the disorder's manifestation. This new data could then be included in the logistic regression analysis performed.

Additionally, a replication study using independent cohorts should be pursued to confirm the positive association signal of this SNP (rs56116165) towards increasing osteoporosis susceptibility. This replication study could also include an improved sample size, once again to empower the study in question.

In a long-term future, it would be thrilling to comprehend the relation between this *WNK4* variant and its contribution for the development of osteoporosis. Overall, there is still a lot of work to do in order to master all the mechanisms involved, since the exact WNK4 biological function in renal calcium homeostasis remains unclear. As described in Chapter 1, there is some evidence that WNK4 could be directly regulating calcium channels (Fu et al., 2006; Jiang et al., 2007; Cha et al., 2008; Cha and Huang, 2010), or may be an important modulator of the inverse relation between sodium and calcium reabsorption in the distal tubule (Jiang et al., 2007; Yang et al., 2010).

Future functional studies could be performed to analyse the effect of the WNK4 variant protein on the regulation of ion channels such as NCC or TRPVs. It is also possible that

the variant acts through differences in *WNK4* expression levels of carriers of this variant. For this, cells lines generated both from carriers and control individuals should be compared. In addition, luciferase reporter vectors (if possible using the endogenous *WNK4* promoter) with each allele of rs56116165 could also be constructed, and subsequently tested for expression levels in an appropriate cell line (e.g. HEK293). If a trend for increased or decreased expression is observed this should be further pursued using mRNA and microRNA chip expression arrays to investigate other possible differences in gene expression/regulation.

Another idea would be to create genetically manipulated animal models for *WNK4* alleles and study the physical (e.g. bone mineral density) and physiological consequences, especially the ones related to ion channels regulated by WNK4.

In the wider field of complex disorders the strategies developed so far only cover either common variants of small effect or rare variants of major effect. Consequently, the systematic identification of rare variants with small effect is mostly still beyond the power of current genetics. Nevertheless, a new era in human genetic research is upon us. The systematical usage of Hapmap common variants reference panels and of high-throughput genotyping systems, will soon be overtaken by next generation sequencing approaches of whole exome or even the entire genome, in increasingly larger numbers of individuals and families. Next generation sequencing is a new increasingly affordable technology which will lead to the creation of a comprehensive list of potential susceptibility variants, providing hope for a truly personalised preventive and curative medicine. Therefore, as we are dealing with a rare variant (rs56116165), the strategy chosen might have been a lucky approach, and should definitely move towards deep-sequencing approaches.

All in all, this work highlights the importance of the evaluation of *WNK4* genetic polymorphisms in the general population and the need for a biological monitoring of the

patients in order to better understand the aetiology of osteoporosis. Similarly, polymorphisms in the *WNK1* gene have been described to cause small but measurable changes in blood pressure in the general population (as mentioned in Chapter 1). Both WNK1 and WNK4 were initially identified as the cause of a rare hypertensive genetic disorder, PHAII, but in addition they also seem to contribute with rare gene variants to the genetic predisposition to hypertension and osteoporosis in the general population. Unravelling the genetic basis of complex disorders is not only a crucial step towards finding answers to reduce these disease prevalences, but also to improve the respective therapies, and consequently ameliorate the patients' quality of life.

References

Achard, J., Warnock, D. G., Disse-Nicodeme, S., Fiquet-Kempf, B., Corvol, P., Fournier, A., and Jeunemaitre, X. (2003). Familial hyperkalemic hypertension: phenotypic analysis in a large family with the WNK1 deletion mutation. *The American Journal of Medicine*, 114(6):495–498.

Amaral, M. D. (2005). Processing of CFTR: traversing the cellular maze – how much CFTR needs to go through to avoid cystic fibrosis? *Pediatric Pulmonology*, 39(6):479–491.

Anselmo, A. N., Earnest, S., Chen, W., Juang, Y.-C., Kim, S. C., Zhao, Y., and Cobb, M. H. (2006). WNK1 and OSR1 regulate the Na+, K+, 2Cl- cotransporter in HeLa cells. *Proceedings of the National Academy of Sciences of the United States of America*, 103(29):10883–10888.

Baz, M., Berland, Y., Dussol, B., Jaber, K., and Boobes, Y. (1990). Syndrome d'hyperkaliémie familiale (syndrome de Gordon). *La Presse Médicale*, 19(43):1981–1984.

Beesley, A. H., Hornby, D., and White, S. J. (1998). Regulation of distal nephron K+ channels (ROMK) mRNA expression by aldosterone in rat kidney. *The Journal of Physiology*, 509(3):629–634.

Belcher, C. N. and Vij, N. (2010). Protein processing and inflammatory signaling in Cystic Fibrosis: challenges and therapeutic strategies. *Current Molecular Medicine*, 10(1):82–94.

Brockmann, K. (2009). The expanding phenotype of GLUT1-deficiency syndrome. *Brain & Development*, 31:545–552.

Bruss, M. D., Arias, E. B., Lienhard, G. E., and Cartee, G. D. (2005). Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes*, 54(1):41–50.

Butler, P. C., Kryshak, E. J., Marsh, M., and Rizza, R. A. (1990). Effect of insulin on oxidation of intracellularly and extracellularly derived glucose in patients with NIDDM. Evidence for primary defect in glucose transport and/or phosphorylation but not oxidation. *Diabetes*, 39(11):1373–1380.

Cai, H., Cebotaru, V., Wang, Y.-H., Zhang, X.-M., Cebotaru, L., Guggino, S. E., and Guggino, W. B. (2006). WNK4 kinase regulates surface expression of the human sodium chloride cotransporter in mammalian cells. *Kidney International*, 69(12):2162–2170.

Camerino, D. C., Desaphy, J.-F., Tricarico, D., Pierno, S., and Liantonio, A. (2008). Therapeutic approaches to ion channel diseases. *Advances in Genetics*, 64:81–145.

References

Cha, S.-K. and Huang, C.-L. (2010). WNK4 kinase stimulates caveola-mediated endo-cytosis of TRPV5 amplifying the dynamic range of regulation of the channel by protein kinase C. *The Journal of Biological Chemistry*, 285(9):6604–6611.

Cha, S.-K., Wu, T., and Huang, C.-L. (2008). Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5. *American Journal of Physiology. Renal Physiology*, 294(5):F1212–1221.

Cheung, C.-L., Xiao, S.-M., and Kung, A. W. C. (2010). Genetic epidemiology of age-related osteoporosis and its clinical applications. *Nature Reviews Rheumatology*, 6:507–517.

Choate, K. A., Kahle, K. T., Wilson, F. H., Nelson-Williams, C., and Lifton, R. P. (2003). WNK1, a kinase mutated in inherited hypertension with hyperkalemia, localizes to diverse Cl- -transporting epithelia. *Proceedings of the National Academy of Sciences of the United States of America*, 100(2):663–668.

Cohen, P. (2000). The regulation of protein function by multisite phosphorylation - a 25 year update. *Trends in Biochemical Sciences*, 25(12):596–601.

Cope, G., Murthy, M., Golbang, A. P., Hamad, A., Liu, C.-H., Cuthbert, A. W., and O'Shaughnessy, K. M. (2006). WNK1 affects surface expression of the ROMK potassium channel independent of WNK4. *Journal of the American Society of Nephrology*, 17(7):1867–1874.

Davies, H., Hunter, C., Smith, R., Stephens, P., Greenman, C., Bignell, G., Teague, J., Butler, A., Edkins, S., Stevens, C., Parker, A., O'Meara, S., Avis, T., Barthorpe, S., Brackenbury, L., Buck, G., Clements, J., Cole, J., Dicks, E., Edwards, K., Forbes, S., Gorton, M., Gray, K., Halliday, K., Harrison, R., Hills, K., Hinton, J., Jones, D., Kosmidou, V., Laman, R., Lugg, R., Menzies, A., Perry, J., Petty, R., Raine, K., Shepherd, R., Small, A., Solomon, H., Stephens, Y., Tofts, C., Varian, J., Webb, A., West, S., Widaa, S., Yates, A., Brasseur, F., Cooper, C. S., Flanagan, A. M., Green, A., Knowles, M., Leung, S. Y., Looijenga, L. H. J., Malkowicz, B., Pierotti, M. A., Teh, B. T., Yuen, S. T., Lakhani, S. R., Easton, D. F., Weber, B. L., Goldstraw, P., Nicholson, A. G., Wooster, R., Stratton, M. R., and Futreal, P. A. (2005). Somatic mutations of the protein kinase gene family in human lung cancer. *Cancer Research*, 65(17):7591–7595.

de Los Heros, P., Kahle, K. T., Rinehart, J., Bobadilla, N. A., Vázquez, N., San Cristobal, P., Mount, D. B., Lifton, R. P., Hebert, S. C., and Gamba, G. (2006). WNK3 bypasses the tonicity requirement for K-Cl cotransporter activation via a phosphatase-dependent pathway. *Proceedings of the National Academy of Sciences of the United States of America*, 103(6):1976–1981.

DeFronzo, R. (1992). Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: a balanced overview. *Diabetologia*, 35(4):389–397.

Delaloy, C., Elvira-Matelot, E., Clemessy, M., Zhou, X.-o., Imbert-Teboul, M., Houot, A.-M., Jeunemaitre, X., and Hadchouel, J. (2008). Deletion of WNK1 first intron

results in misregulation of both isoforms in renal and extrarenal tissues. *Hypertension*, 52(6):1149–1154.

Delaloy, C., Hadchouel, J., Imbert-Teboul, M., Clemessy, M., Houot, A.-M., and Jeunemaitre, X. (2006). Cardiovascular expression of the mouse Wnk1 Gene during development and adulthood revealed by a BAC reporter assay. *The American Journal of Pathology*, 169(1):105–118.

Delaloy, C., Lu, J., Houot, A.-M., Disse-Nicodeme, S., Gase, J.-M., Corvol, P., and Jeunemaitre, X. (2003). Multiple promoters in the WNK1 Gene: one controls expression of a kidney-specific kinase-defective isoform. *Molecular and Cellular Biology*, 23(24):9208–9221.

Derynck, R., Akhurst, R. J., and Balmain, A. (2001). TGF-beta signaling in tumor suppression and cancer progression. *Nature Genetics*, 29(2):117–129.

Dorwart, M. R., Shcheynikov, N., Wang, Y., Stippec, S., and Muallem, S. (2007). SLC26A9 is a Cl(-) channel regulated by the WNK kinases. *The Journal of Physiology*, 584(Pt 1):333–345.

Dowd, B. F. X. and Forbush, B. (2003). PASK (proline-alanine-rich STE20-related kinase), a regulatory kinase of the Na-K-Cl cotransporter (NKCC1). *The Journal of Biological Chemistry*, 278(30):27347–27353.

Dugani, C. B. and Klip, A. (2005). Glucose transporter 4: cycling, compartments and controversies. *EMBO reports*, 6(12):1137–1142.

Eaton, D. C., Malik, B., Saxena, N. C., Al-Khalili, O. K., and Yue, G. (2001). Mechanisms of aldosterone's action on epithelial Na + transport. *The Journal of Membrane Biology*, 184(3):313–319.

Eguez, L., Lee, A., Chavez, J. A., Miinea, C. P., Kane, S., Lienhard, G. E., and McGraw, T. E. (2005). Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. *Cell Metabolism*, 2(4):263–272.

Ehret, G. B. (2010). Genome-wide association studies: contribution of genomics to understanding blood pressure and essential hypertension. *Current Hypertension Reports*, 12:17–25.

Elvira-Matelot, E., Zhou, X.-o., Farman, N., Beaurain, G., Henrion-Caude, A., Hadchouel, J., and Jeunemaitre, X. (2010). Regulation of WNK1 expression by miR-192 and aldosterone. *Journal of the American Society of Nephrology*, 21(10):1724–1731.

Erlich, P. M., Cui, J., Chazaro, I., Farrer, L. A., Baldwin, C. T., Gavras, H., and Destefano, A. L. (2003). Genetic variants of WNK4 in whites and African Americans with hypertension. *Hypertension*, 41(6):1191–1195.

Fang, L., Garuti, R., Kim, B.-Y., Wade, J. B., and Welling, P. A. (2009). The ARH adaptor protein regulates endocytosis of the ROMK potassium secretory channel in mouse kidney. *The Journal of Clinical Investigation*, 119(11):3278–3289.

References

Farfel, Z., Iaina, A., Rosenthal, T., Waks, U., Shibolet, S., and Gafni, J. (1978). Familial hyperpotassemia and hypertension accompanied by normal plasma aldosterone levels: possible hereditary cell membrane defect. *Archives of Internal Medicine*, 138(12):1828–1832.

Farfel, Z., Mayan, H., Yaacov, Y., Mouallem, M., Shaharabany, M., Pauzner, R., Kerem, E., and Wilschanski, M. (2005). WNK4 regulates airway Na+ transport: study of familial hyperkalaemia and hypertension. *European Journal of Clinical Investigation*, 35(6):410–415.

Farinha, C. M., Mendes, F., Roxo-Rosa, M., Penque, D., and Amaral, M. D. (2004). A comparison of 14 antibodies for the biochemical detection of the cystic fibrosis transmembrane conductance regulator protein. *Molecular and Cellular Probes*, 18(4):235–242.

Ferrari, S. (2008). Human genetics of osteoporosis. *Best Practice & Research. Clinical Endocrinology & Metabolism*, 22(5):723–735.

Fink, R. I., Wallace, P., Brechtel, G., and Olefsky, J. M. (1992). Evidence that glucose transport is rate-limiting for in vivo glucose uptake. *Metabolism: Clinical and Experimental*, 41(8):897–902.

Franke, T. F. (2008). PI3K/Akt: getting it right matters. *Oncogene*, 27(50):6473–6488.

Frindt, G. and Palmer, L. G. (2010). Effects of dietary K on cell-surface expression of renal ion channels and transporters. *American Journal of Physiology. Renal Physiology*, 299(4):F890–F897.

Fu, Y., Subramanya, A., Rozansky, D., and Cohen, D. M. (2006). WNK kinases influence TRPV4 channel function and localization. *American Journal of Physiology. Renal Physiology*, 290(6):F1305–F1314.

Gadsby, D. C. and Nairn, A. C. (1999). Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiological reviews*, 79(1 (Suppl)):S77–S107.

Gagnon, K. B. E., England, R., and Delpire, E. (2006a). Characterization of SPAK and OSR1, Regulatory Kinases of the Na-K-2Cl Cotransporter. *Molecular and Cellular Biology*, 26(2):689–698.

Gagnon, K. B. E., England, R., and Delpire, E. (2006b). Volume sensitivity of cation-Cl- cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. *American Journal of Physiology. Cell Physiology*, 290(1):C134–C142.

Gamba, G. (2005a). Molecular physiology and pathophysiology of electroneutral cation-chloride cotransporters. *Physiological reviews*, 85:423– 493.

Gamba, G. (2005b). Role of WNK kinases in regulating tubular salt and potassium transport and in the development of hypertension. *American Journal of Physiology. Renal Physiology*, 288:F245–F252.

Garvey, W. T., Maianu, L., Zhu, J. H., Brechtel-Hook, G., Wallace, P., and Baron, A. D. (1998). Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *The Journal of Clinical Investigation*, 101(11):2377–2386.

Garzón-Muvdi, T., Pacheco-Alvarez, D., Gagnon, K. B. E., Vázquez, N., Ponce-Coria, J., Moreno, E., Delpire, E., and Gamba, G. (2007). WNK4 kinase is a negative regulator of K+-Cl- cotransporters. *American Journal of Physiology. Renal Physiology*, 292(4):F1197–F1207.

Gatenby, R. A. and Gillies, R. J. (2004). Why do cancers have high aerobic glycolysis? *Nature reviews. Cancer*, 4(11):891–899.

Geraghty, K. M., Chen, S., Harthill, J. E., Ibrahim, A. F., Morrice, N. A., Toth, R., Vandermoere, F., Moorhead, G. B., Hardie, D. G., and MacKintosh, C. (2007). Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to IGF-1, EGF, PMA and AICAR. *The Biochemical Journal*, 407(2):231–241.

Giménez, I. and Forbush, B. (2005). Regulatory phosphorylation sites in the NH2 terminus of the renal Na-K-Cl cotransporter (NKCC2). *American Journal of Physiology. Renal Physiology*, 289(6):F1341–F1345.

Gnudi, L., Viberti, G., Raij, L., Rodriguez, V., Burt, D., Cortes, P., Hartley, B., Thomas, S., Maestrini, S., and Gruden, G. (2003). GLUT-1 overexpression: Link between hemodynamic and metabolic factors in glomerular injury? *Hypertension*, 42(1):19–24.

Golbang, A. P., Cope, G., Hamad, A., Murthy, M., Liu, C.-H., Cuthbert, A. W., and O'Shaughnessy, K. M. (2006). Regulation of the expression of the Na/Cl cotransporter by WNK4 and WNK1: evidence that accelerated dynamin-dependent endocytosis is not involved. *American Journal of Physiology. Renal Physiology*, 291(6):F1369–F1376.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Edkins, S., Meara, S. O., Vastrik, I., Schmidt, E. E., Avis, T., Bhamra, G., Buck, G., Choudhury, B., Clements, J., Cole, J., Dicks, E., Forbes, S., Gray, K., Halliday, K., Harrison, R., Hills, K., Hinton, J., Jenkinson, A., Jones, D., Menzies, A., Perry, J., Raine, K., Richardson, D., Shepherd, R., Small, A., Tofts, C., Varian, J., Webb, T., West, S., Widaa, S., Yates, A., Cahill, D. P., Louis, D. N., Goldstraw, P., Nicholson, A. G., Brasseur, F., Looijenga, L., Weber, B. L., Chiew, Y.-E., Greaves, M. F., Green, A. R., Campbell, P., Birney, E., Easton, F., Chenevixtrench, G., Tan, M.-H., Khoo, S. K., Tean, B., Yuen, S. T., Leung, S. Y., Wooster, R., and Futreal, P. A. (2007). Patterns of somatic mutation in human cancer genomes. *Nature*, 446(7132):153–158.

Haas, M. and Forbush, B. (2000). The Na-K-Cl cotransporter of secretory epithelia. *Annual Review of Physiology*, 62:515–534.

References

Hadchouel, J., Delaloy, C., Fauré, S., Achard, J.-M., and Jeunemaitre, X. (2006). Familial Hyperkalemic Hypertension. *Journal of the American Society of Nephrology*, 17:208–217.

Hadchouel, J., Delaloy, C., and Jeunemaitre, X. (2005). WNK1 et WNK4 nouveaux acteurs de l'homéostasie hydrosodée. *Medicine Sciences*, 21:55–60.

Hadchouel, J., Soukaseum, C., Büsst, C., Zhou, X.-o., Baudrie, V., Zürrer, T., Cambillau, M., Elghozi, J.-L., Lifton, R. P., Loffing, J., and Jeunemaitre, X. (2010). Decreased ENaC expression compensates the increased NCC activity following inactivation of the kidney-specific isoform of WNK1 and prevents hypertension. *Proceedings of the National Academy of Sciences of the United States of America*, 107(42):18109–18114.

Hanks, S. K. and Hunter, T. (1995). Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *The FASEB Journal*, 9(8):576–596.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, 241(4861):42–52.

He, G., Wang, H.-r., Huang, S.-k., and Huang, C.-l. (2007). Intersectin links WNK kinases to endocytosis of ROMK1. *The Journal of Clinical Investigation*, 117(4):1078–1087.

Heise, C. J., Xu, B.-E., Deaton, S. L., Cha, S.-K., Cheng, C.-J., Sengupta, S., Juang, Y. C., Stippec, S., Xu, Y., Zhao, Y., Huang, C.-L., and Cobb, M. H. (2010). Serum and glucocorticoid-induced kinase (SGK) 1 and the epithelial sodium channel are regulated by multiple with no lysine (WNK) family members. *The Journal of Biological Chemistry*, 285(33):25161–25167.

Heiskala, M., Peterson, P., and Yang, Y. (2001). The roles of claudin superfamily proteins in paracellular transport. *Traffic*, 2(18):92–98.

Hoenderop, J. G. J., Nilius, B., and Bindels, R. J. M. (2005). Calcium absorption across epithelia. *Physiological Reviews*, 85:373–422.

Holden, S., Cox, J., and Raymond, F. L. (2004). Cloning, genomic organization, alternative splicing and expression analysis of the human gene WNK3 (PRKWNK3). *Gene*, 335:109–119.

Hong, F., Larrea, M. D., Doughty, C., Kwiatkowski, D. J., Squillace, R., and Slingerland, J. M. (2008). mTOR-raptor binds and activates SGK1 to regulate p27 phosphorylation. *Molecular Cell*, 30(6):701–711.

Hunter, T. (2000). Signaling - 2000 and Beyond. *Cell*, 100:113–127.

Ishikura, S., Bilan, P. J., and Klip, A. (2007). Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells. *Biochemical and Biophysical Research Communications*, 353(4):1074–1079.

136

Ishikura, S. and Klip, A. (2008). Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. *American Journal of Physiology. Cell Physiology*, 295(4):C1016–C1025.

Jentsch, T. J., Hübner, C. A., and Fuhrmann, J. C. (2004). Ion channels: function unravelled by dysfunction. *Nature Cell Biology*, 6(11):1039–1047.

Jiang, Y., Ferguson, W. B., and Peng, J.-B. (2007). WNK4 enhances TRPV5-mediated calcium transport: potential role in hypercalciuria of familial hyperkalemic hypertension caused by gene mutation of WNK4. *American Journal of Physiology. Renal Physiology*, 292(2):F545–F554.

Jiang, Z. Y., Zhou, Q. L., Holik, J., Patel, S., Leszyk, J., Coleman, K., Chouinard, M., and Czech, M. P. (2005). Identification of WNK1 as a substrate of Akt/protein kinase B and a negative regulator of insulin-stimulated mitogenesis in 3T3-L1 cells. *The Journal of Biological Chemistry*, 280(22):21622–21628.

Jin, J., Smith, F. D., Stark, C., Wells, C. D., Fawcett, J. P., Kulkarni, S., Metalnikov, P., Donnell, P. O., Taylor, L., Taylor, P., Zougman, A., Woodgett, J. R., Langeberg, L. K., Scott, J. D., and Pawson, T. (2004). Proteomic, functional, and domain-based analysis of in vivo 14-3-3 binding proteins involved in cytoskeletal regulation and cellular organization. *Current Biology*, 14:1436–1450.

Kahle, K. T., Gimenez, I., Hassan, H., Wilson, F. H., Wong, R. D., Forbush, B., Aronson, P. S., and Lifton, R. P. (2004a). WNK4 regulates apical and basolateral Cl- flux in extrarenal epithelia. *Proceedings of the National Academy of Sciences of the United States of America*, 101(7):2064–2069.

Kahle, K. T., MacGregor, G. G., Wilson, F. H., Van Hoek, A. N., Brown, D., Ardito, T., Kashgarian, M., Giebisch, G., Hebert, S. C., Boulpaep, E. L., and Lifton, R. P. (2004b). Paracellular Cl- permeability is regulated by WNK4 kinase: insight into normal physiology and hypertension. *Proceedings of the National Academy of Sciences of the United States of America*, 101(41):14877–14882.

Kahle, K. T., Rinehart, J., de Los Heros, P., Louvi, A., Meade, P., Vazquez, N., Hebert, S. C., Gamba, G., Gimenez, I., and Lifton, R. P. (2005). WNK3 modulates transport of Cl- in and out of cells: implications for control of cell volume and neuronal excitability. *Proceedings of the National Academy of Sciences of the United States of America*, 102(46):16783–16788.

Kahle, K. T., Rinehart, J., Ring, A., Gimenez, I., Gamba, G., Hebert, S. C., and Lifton, R. P. (2006). WNK protein kinases modulate cellular Cl- flux by altering the phosphorylation state of the Na-K-Cl and K-Cl cotransporters. *Physiology*, 21:326–235.

Kahle, K. T., Ring, A. M., and Lifton, R. P. (2008a). Molecular physiology of the WNK kinases. *Annual Review of Physiology*, 70:329–355.

Kahle, K. T., Staley, K. J., Nahed, B. V., Gamba, G., Hebert, S. C., Lifton, R. P., and Mount, D. B. (2008b). Roles of the cation-chloride cotransporters in neurological disease. *Nature Clinical Practice. Neurology*, 4(9):490–503.

References

Kahle, K. T., Wilson, F. H., Leng, Q., Lalioti, M. D., O'Connell, A. D., Dong, K., Kapson, A. K., MacGregor, G. G., Giebisch, G., Hebert, S. C., and Lifton, R. P. (2003). WNK4 regulates the balance between renal NaCl reabsorption and K+ secretion. *Nature Genetics*, 35(4):372–376.

Kamide, K., Takiuchi, S., Tanaka, C., Miwa, Y., Yoshii, M., Horio, T., Mannami, T., Kokubo, Y., Tomoike, H., Kawano, Y., and Miyata, T. (2004). Three novel missense mutations of WNK4, a kinase mutated in inherited hypertension, in Japanese hypertensives: implication of clinical phenotypes. *American Journal of Hypertension*, 17(5 Pt 1):446–449.

Kappert, K. and Unger, T. (2008). Role of the renin-angiotensin system in hypertension. *Hot Topics in Hypertension*, 4:7–23.

Karlsson, H., Zierath, J., Kane, S., Krook, A., Lienhard, G., and Wallberg-Henriksson, H. (2005). Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects. *Diabetes*, 54(6):1692–1697.

Kim, G. H., Masilamani, S., Turner, R., Mitchell, C., Wade, J. B., and Knepper, M. A. (1998). The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proceedings of the National Academy of Sciences of the United States of America*, 95(24):14552–14557.

Klepper, J. and Leiendecker, B. (2007). GLUT1 deficiency syndrome - 2007 update. *Developmental Medicine & Child Neurology*, 49:707–716.

Knauf, F., Yang, C. L., Thomson, R. B., Mentone, S. A., Giebisch, G., and Aronson, P. S. (2001). Identification of a chloride-formate exchanger expressed on the brush border membrane of renal proximal tubule cells. *Proceedings of the National Academy of Sciences of the United States of America*, 98(16):9425–9430.

Ko, S. B. H., Zeng, W., Dorwart, M. R., Luo, X., Kim, K. H., Millen, L., Goto, H., Naruse, S., Soyombo, A., Thomas, P. J., and Muallem, S. (2004). Gating of CFTR by the STAS domain of SLC26 transporters. *Nature Cell Biology*, 6(4):343–350.

Kokubo, Y., Kamide, K., Inamoto, N., Tanaka, C., Banno, M., Takiuchi, S., Kawano, Y., Tomoike, H., and Miyata, T. (2004). Identification of 108 SNPs in TSC, WNK1, and WNK4 and their association with hypertension in a Japanese general population. *Journal of Human Genetics*, 49(9):507–515.

Kostich, M., English, J., Madison, V., Gheyas, F., Wang, L., Qiu, P., Greene, J., and Laz, T. M. (2002). Human members of the eukaryotic protein kinase family. *Genome Biology*, 3(9):1–12.

Koumanov, F. and Holman, G. D. (2007). Thrifty Tbc1d1 and Tbc1d4 proteins link signalling and membrane trafficking pathways. *The Biochemical Journal*, 403(2):e9–e11.

Lalioti, M. D., Zhang, J., Volkman, H. M., Kahle, K. T., Hoffmann, K. E., Toka, H. R., Nelson-Williams, C., Ellison, D. H., Flavell, R., Booth, C. J., Lu, Y., Geller, D. S., and Lifton, R. P. (2006). Wnk4 controls blood pressure and potassium homeostasis via regulation of mass and activity of the distal convoluted tubule. *Nature Genetics*, 38(10):1124–1132.

Lau, E. M. and Cooper, C. (1996). The epidemiology of osteoporosis. The oriental perspective in a world context. *Clinical Orthopaedics and Related Research*, (323):65–74.

Lauf, P. K. and Adragna, N. C. (2000). K-Cl cotransport: properties and molecular mechanism. *Cellular Physiology and Biochemistry*, 10(5-6):341–354.

Lawlor, M. A. and Alessi, D. R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *Journal of Cell Science*, 114(Pt 16):2903–2910.

Lazrak, A., Liu, Z., and Huang, C.-L. (2006). Antagonistic regulation of ROMK by long and kidney-specific WNK1 isoforms. *Proceedings of the National Academy of Sciences of the United States of America*, 103(5):1615–1620.

Lee, B.-H., Chen, W., Stippec, S., and Cobb, M. H. (2007). Biological cross-talk between WNK1 and the transforming growth factor beta-Smad signaling pathway. *The Journal of Biological Chemistry*, 282(25):17985–17996.

Lee, B.-H., Min, X., Heise, C. J., Xu, B.-e., Chen, S., Shu, H., Luby-Phelps, K., Goldsmith, E. J., and Cobb, M. H. (2004). WNK1 phosphorylates synaptotagmin 2 and modulates its membrane binding. *Molecular Cell*, 15(5):741–751.

Lee, M. G., Wigley, W. C., Zeng, W., Noel, L. E., Marino, C. R., Thomas, P. J., and Muallem, S. (1999). Regulation of Cl-/ HCO3- exchange by cystic fibrosis transmembrane conductance regulator expressed in NIH 3T3 and HEK 293 cells. *The Journal of Biological Chemistry*, 274(6):3414–3421.

Lenertz, L. Y., Lee, B. H., Min, X., Xu, B. E., Wedin, K., Earnest, S., Goldsmith, E. J., and Cobb, M. H. (2005). Properties of WNK1 and implications for other family members. *The Journal of Biological Chemistry*, 280(29):26653–26658.

Leney, S. E. and Tavaré, J. M. (2009). The molecular basis of insulin-stimulated glucose uptake: signalling, trafficking and potential drug targets. *The Journal of Endocrinology*, 203(1):1–18.

Leng, Q., Kahle, K. T., Rinehart, J., MacGregor, G. G., Wilson, F. H., Canessa, C. M., Lifton, R. P., and Hebert, S. C. (2006). WNK3, a kinase related to genes mutated in hereditary hypertension with hyperkalaemia, regulates the K+ channel ROMK1 (Kir1.1). *The Journal of Physiology*, 571(Pt 2):275–286.

Levy, D., Ehret, G. B., Rice, K., Verwoert, G. C., Launer, L. J., Dehghan, A., Glazer, N. L., Morrison, A. C., Johnson, A. D., Aspelund, T., Aulchenko, Y., Lumley, T., Köttgen, A., Vasan, R. S., Rivadeneira, F., Eiriksdottir, G., Guo, X., Arking, D. E., Mitchell,

References

G. F., Mattace-Raso, F. U. S., Smith, A. V., Taylor, K., Scharpf, R. B., Hwang, S.-J., Sijbrands, E. J. G., Bis, J., Harris, T. B., Ganesh, S. K., O'Donnell, C. J., Hofman, A., Rotter, J. I., Coresh, J., Benjamin, E. J., Uitterlinden, A. G., Heiss, G., Fox, C. S., Witteman, J. C. M., Boerwinkle, E., Wang, T. J., Gudnason, V., Larson, M. G., Chakravarti, A., Psaty, B. M., and van Duijn, C. M. (2009). Genome-wide association study of blood pressure and hypertension. *Nature Genetics*, 41(6):677–687.

Li, C. and Naren, A. P. (2010). CFTR chloride channel in the apical compartments: spatiotemporal coupling to its interacting partners. *Integrative Biology*, 2(4):161–177.

Li, W.-F., Hou, S.-X., Yu, B., Li, M.-M., Férec, C., and Chen, J.-M. (2010). Genetics of osteoporosis: accelerating pace in gene identification and validation. *Human Genetics*, 127:249–285.

Liang, X., Butterworth, M. B., Peters, K. W., and Frizzell, R. (2010). AS160 modulates aldosterone-stimulated epithelial sodium channel forward trafficking. *Molecular Biology of the Cell*, 21:2024 –2033.

Liu, Z., Wang, H.-R., and Huang, C.-L. (2009). Regulation of ROMK channel and K+ homeostasis by kidney-specific WNK1 kinase. *The Journal of Biological Chemistry*, 284(18):12198–12206.

Liu, Z., Xie, J., Wu, T., Truong, T., Auchus, R., and Huang, C. (2011). Downregulation of NCC and NKCC2 cotransporters by kidney-specific WNK1 revealed by gene disruption and transgenic mouse models. *Human Molecular Genetics*, 20(5):855–866.

Lohi, H., Kujala, M., Kerkelä, E., Saarialho-Kere, U., Kestilä, M., and Kere, J. (2000). Mapping of five new putative anion transporter genes in human and characterization of SLC26A6, a candidate gene for pancreatic anion exchanger. *Genomics*, 70(1):102–112.

Long, K. and Walsh, K. (1997). Iodide efflux measurements with an iodide-selective electrode: A non-radioactive procedure for monitoring cellular chloride transport. *Methods in Cell Science*, 19(3):207–212.

Lu, M., Leng, Q., Egan, M., Caplan, M., Boulpaep, E., Giebisch, G., and Hebert, S. (2006). CFTR is required for PKA-regulated ATP sensitivity of Kir1. 1 potassium channels in mouse kidney. *Journal of Clinical Investigation*, 116(3):797–807.

Macheda, M. L., Rogers, S., and Best, J. D. (2005). Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *Journal of Cellular Physiology*, 202(3):654–662.

MacKeigan, J. P., Murphy, L. O., and Blenis, J. (2005). Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. *Nature Cell Biology*, 7(6):591–600.

140

Mackintosh, C. (2004). Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *The Biochemical Journal*, 381(Pt 2):329–342.

Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The protein kinase complement of the human genome. *Science*, 298:1912–1934.

Marban, E., Yamagishi, T., and Tomaselli, G. F. (1998). Structure and function of voltage-gated sodium channels. *Journal of Physiology*, 508:647–657.

Massagué, J. and Gomis, R. R. (2006). The logic of TGFbeta signaling. *FEBS letters*, 580(12):2811–2820.

Matter, N., Marx, M., Weg-Remers, S., Ponta, H., Herrlich, P., and König, H. (2000). Heterogeneous ribonucleoprotein A1 is part of an exon-specific splice-silencing complex controlled by oncogenic signaling pathways. *The Journal of Biological Chemistry*, 275(45):35353–35360.

Mayan, H., Munter, G., Shaharabany, M., Mouallem, M., Pauzner, R., Holtzman, E. J., and Farfel, Z. (2004). Hypercalciuria in familial hyperkalemia and hypertension accompanies hyperkalemia and precedes hypertension: description of a large family with the Q565E WNK4 mutation. *The Journal of Clinical Endocrinology and Metabolism*, 89(8):4025–4030.

Mayan, H., Vered, I., Mouallem, M., Tzadok-Witkon, M., Pauzner, R., and Farfel, Z. (2002). Pseudohypoaldosteronism type II: marked sensitivity to thiazides, hypercalciuria, normomagnesemia, and low bone mineral density. *The Journal of Clinical Endocrinology and Metabolism*, 87(7):3248–3254.

McAllister, S., Becker-Hapak, M., Pintucci, G., Pagano, M., and Dowdy, S. (2003). Novel p27kip1 C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions. *Molecular and Cellular Biology*, 23(1):216–228.

McClellan, J. and King, M.-C. (2010). Genetic heterogeneity in human disease. *Cell*, 141:210–217.

Mendes, F., Roxo Rosa, M., Dragomir, A., Farinha, C. M., Roomans, G. M., Amaral, M. D., and Penque, D. (2003). Unusually common cystic fibrosis mutation in Portugal encodes a misprocessed protein. *Biochemical and Biophysical Research Communications*, 311(3):665–671.

Meneton, P., Jeunemaitre, X., de Wardener, H., and Macgregor, G. (2005). Links between dietary salt intake, renal salt handling, blood pressure, and cardiovascular diseases. *Physiological Reviews*, 85(2):679–715.

Mîinea, C. P., Sano, H., Kane, S., Sano, E., Fukuda, M., Peränen, J., Lane, W. S., and Lienhard, G. E. (2005). AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *The Biochemical Journal*, 391(Pt 1):87–93.

References

Min, X., Lee, B.-H., Cobb, M. H., and Goldsmith, E. J. (2004). Crystal structure of the kinase domain of WNK1, a kinase that causes a hereditary form of hypertension. *Structure*, 12(7):1303–1311.

Mócsai, A., Ruland, J., and Tybulewicz, V. L. J. (2010). The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nature Reviews Immunology*, 10(6):387–402.

Moniz, S. and Jordan, P. (2010). Emerging roles for WNK kinases in cancer. *Cellular and Molecular Life Sciences*, 67(8):1265–1276.

Moniz, S., Matos, P., and Jordan, P. (2008). WNK2 modulates MEK1 activity through the Rho GTPase pathway. *Cellular Signalling*, 20(10):1762–1768.

Moniz, S., Veríssimo, F., Matos, P., Braza, R., Silva, E., Kotevelets, L., Chastre, E., Gespach, C., and Jordan, P. (2007). Protein kinase WNK2 inhibits cell proliferation by negatively modulating the activation of MEK1/ERK1/2. *Oncogene*, 26:6071–6081.

Monroy, A., Plata, C., Hebert, S. C., and Gamba, G. (2000). Characterization of the thiazide-sensitive Na(+)-Cl(-) cotransporter: a new model for ions and diuretics interaction. *American Journal of Physiology. Renal Physiology*, 279(1):F161–F169.

Moriguchi, T., Urushiyama, S., Hisamoto, N., Iemura, S.-i., Uchida, S., Natsume, T., Matsumoto, K., and Shibuya, H. (2005). WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. *The Journal of Biological Chemistry*, 280(52):42685–42693.

Mount, D. B. and Gamba, G. (2001). Renal potassium-chloride cotransporters. *Current Opinion in Nephrology and Hypertension*, 10(5):685–691.

Murthy, M., Cope, G., and O'Shaughnessy, K. M. (2008). The acidic motif of WNK4 is crucial for its interaction with the K channel ROMK. *Biochemical and Biophysical Research Communications*, 375(4):651–654.

Nakamura, K. and Johnson, G. L. (2003). PB1 domains of MEKK2 and MEKK3 interact with the MEK5 PB1 domain for activation of the ERK5 pathway. *The Journal of Biological Chemistry*, 278(39):36989–36992.

Náray-Fejes-Tóth, A., Snyder, P. M., and Fejes-Tóth, G. (2004). The kidney-specific WNK1 isoform is induced by aldosterone and stimulates epithelial sodium channel-mediated Na+ transport. *Proceedings of the National Academy of Sciences of the United States of America*, 101(50):17434–17439.

Newhouse, S., Farrall, M., Wallace, C., Hoti, M., Burke, B., Howard, P., Onipinla, A., Lee, K., Shaw-Hawkins, S., Dobson, R., Brown, M., Samani, N. J., Dominiczak, A. F., Connell, J. M., Lathrop, G. M., Kooner, J., Chambers, J., Elliott, P., Clarke, R., Collins, R., Laan, M., Org, E., Juhanson, P., Veldre, G., Viigimaa, M., Eyheramendy, S., Cappuccio, F. P., Ji, C., Iacone, R., Strazzullo, P., Kumari, M., Marmot, M., Brunner, E., Caulfield, M., and Munroe, P. B. (2009). Polymorphisms in the

WNK1 gene are associated with blood pressure variation and urinary potassium excretion. *PLoS One*, 4(4):e5003.

Newhouse, S. J., Wallace, C., Dobson, R., Mein, C., Pembroke, J., Farrall, M., Clayton, D., Brown, M., Samani, N., Dominiczak, A., Connell, J. M., Webster, J., Lathrop, G. M., Caulfield, M., and Munroe, P. B. (2005). Haplotypes of the WNK1 gene associate with blood pressure variation in a severely hypertensive population from the British Genetics of Hypertension study. *Human Molecular Genetics*, 14(13):1805–1814.

Ngo, S., Barry, J. B., Nisbet, J. C., Prins, J. B., and Whitehead, J. P. (2009). Reduced phosphorylation of AS160 contributes to glucocorticoid-mediated inhibition of glucose uptake in human and murine adipocytes. *Molecular and Cellular Endocrinology*, 302(1):33–40.

Oh, E., Heise, C. J., English, J. M., Cobb, M. H., and Thurmond, D. C. (2007). WNK1 is a novel regulator of Munc18c-syntaxin 4 complex formation in soluble NSF attachment protein receptor (SNARE)-mediated vesicle exocytosis. *The Journal of Biological Chemistry*, 282(45):32613–32622.

Ohta, A., Rai, T., Yui, N., Chiga, M., Yang, S.-S., Lin, S.-H., Sohara, E., Sasaki, S., and Uchida, S. (2009). Targeted disruption of the Wnk4 gene decreases phosphorylation of Na-Cl cotransporter, increases Na excretion and lowers blood pressure. *Human Molecular Genetics*, 18(20):3978–3986.

Ohta, A., Yang, S.-S., Rai, T., Chiga, M., Sasaki, S., and Uchida, S. (2006). Overexpression of human WNK1 increases paracellular chloride permeability and phosphorylation of claudin-4 in MDCKII cells. *Biochemical and Biophysical Research Communications*, 349(2):804–808.

O'Reilly, M., Marshall, E., Macgillivray, T., Mittal, M., Xue, W., Kenyon, C. J., and Brown, R. W. (2006). Dietary electrolyte-driven responses in the renal WNK kinase pathway in vivo. *Journal of the American Society of Nephrology*, 17(9):2402–2413.

O'Reilly, M., Marshall, E., Speirs, H. J. L., and Brown, R. (2003). WNK1, a Gene within a Novel Blood Pressure Control Pathway, Tissue-Specifically Generates Radically Different Isoforms with and without a Kinase Domain. *Journal of the American Society of Nephrology*, 14(10):2447–2456.

Osada, Y., Miyauchi, R., Goda, T., Kasezawa, N., Horiike, H., Iida, M., Sasaki, S., and Yamakawa-Kobayashi, K. (2009). Variations in the WNK1 gene modulates the effect of dietary intake of sodium and potassium on blood pressure determination. *Journal of Human Genetics*, 54(8):474–478.

O'Shaughnessy, K. M. and Karet, F. E. (2006). Salt handling and hypertension. *Annual Review of Nutrition*, 26:343–365.

References

Pacheco-Alvarez, D., Cristóbal, P. S., Meade, P., Moreno, E., Vazquez, N., Muñoz, E., Díaz, A., Juárez, M. E., Giménez, I., and Gamba, G. (2006). The Na+Cl- cotransporter is activated and phosphorylated at the amino-terminal domain upon intracellular chloride depletion. *The Journal of Biological Chemistry*, 281(39):28755–28763.

Padmanabhan, S., Menni, C., Lee, W. K., Laing, S., Brambilla, P., Sega, R., Perego, R., Grassi, G., Cesana, G., Delles, C., Mancia, G., and Dominiczak, A. F. (2010). The effects of sex and method of blood pressure measurement on genetic associations with blood pressure in the PAMELA study. *Journal of Hypertension*, 28(3):465–477.

Parsons, D., Jones, S., Zhang, X., Lin, J., and Leary, R. (2008). An integrated genomic analysis of human glioblastoma multiforme. *Science*, 321(5897):1807–1812.

Pawson, T. and Wilson, S. G. (1997). Signaling through scaffold, anchoring, and adaptor proteins. *Science*, 278(5346):2075–2080.

Pearson, R. and Kemp, B. (1991). Protein kinase phosphorylation site sequences and consensus specificity motifs: Tabulations. *Methods in Enzymology*, 200:62–81.

Peck, G. R., Ye, S., Pham, V., Fernando, R. N., Macaulay, S. L., Chai, S. Y., and Albiston, A. L. (2006). Interaction of the Akt substrate, AS160, with the glucose transporter 4 vesicle marker protein, insulin-regulated aminopeptidase. *Molecular Endocrinology*, 20(10):2576–2583.

Pessin, J. E., Thurmond, D. C., Elmendorf, J. S., Coker, K. J., and Okada, S. (1999). Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location! *The Journal of Biological Chemistry*, 274(5):2593–2596.

Piechotta, K., Garbarini, N., England, R., and Delpire, E. (2003). Characterization of the interaction of the stress kinase SPAK with the Na+-K+-2Cl- cotransporter in the nervous system: evidence for a scaffolding role of the kinase. *The Journal of Biological chemistry*, 278(52):52848–52856.

Ponce-Coria, J., San-Cristobal, P., Kahle, K. T., Vazquez, N., Pacheco-Alvarez, D., de Los Heros, P., Juárez, P., Muñoz, E., Michel, G., Bobadilla, N. A., Gimenez, I., Lifton, R. P., Hebert, S. C., and Gamba, G. (2008). Regulation of NKCC2 by a chloride-sensing mechanism involving the WNK3 and SPAK kinases. *Proceedings of the National Academy of Sciences of the United States of America*, 105(24):8458–8463.

Pongchaiyakul, C., Songpattanasilp, T., and Taechakraichana, N. (2008). Osteoporosis: overview in disease, epidemiology, treatment and health economy. *Journal of the Medical Association of Thailand*, 91(4):581–594.

Predescu, S., Predescu, D., Timblin, B., Stan, R., and Malik, A. (2003). Intersectin regulates fission and internalization of caveolae in endothelial cells. *Molecular Biology of the Cell*, 14:4997–5010.

Quinn, S. J. and Williams, G. H. (1988). Regulation of aldosterone secretion. *Annual Review of Physiology*, 50:409–426.

Rafiqi, F. H., Zuber, A. M., Glover, M., Richardson, C., Fleming, S., Jovanović, S., Jovanović, A., O'Shaughnessy, K. M., and Alessi, D. R. (2010). Role of the WNK-activated SPAK kinase in regulating blood pressure. *EMBO Molecular Medicine*, 2(2):63–75.

Ramm, G., Larance, M., Guilhaus, M., and James, D. E. (2006). A role for 14-3-3 in insulin-stimulated GLUT4 translocation through its interaction with the RabGAP AS160. *The Journal of Biological Chemistry*, 281(39):29174–29180.

Reilly, R. F. and Ellison, D. H. (2000). Mammalian distal tubule: physiology, pathophysiology, and molecular anatomy. *Physiological Reviews*, 80(1):277–314.

Richardson, C. and Alessi, D. R. (2008). The regulation of salt transport and blood pressure by the WNK-SPAK/OSR1 signalling pathway. *Journal of Cell Science*, 121(Pt 20):3293–3304.

Richardson, C., Rafiqi, F. H., Karlsson, H. K. R., Moleleki, N., Vandewalle, A., Campbell, D. G., Morrice, N. A., and Alessi, D. R. (2008). Activation of the thiazide-sensitive Na+-Cl- cotransporter by the WNK-regulated kinases SPAK and OSR1. *Journal of Cell Science*, 121(Pt 5):675–684.

Rinehart, J., Kahle, K. T., de Los Heros, P., Vazquez, N., Meade, P., Wilson, F. H., Hebert, S. C., Gimenez, I., Gamba, G., and Lifton, R. P. (2005). WNK3 kinase is a positive regulator of NKCC2 and NCC, renal cation-Cl- cotransporters required for normal blood pressure homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, 102(46):16777–16782.

Rinehart, J., Maksimova, Y. D., Tanis, J. E., Stone, K. L., Hodson, C. A., Zhang, J., Risinger, M., Pan, W., Wu, D., Colangelo, C. M., Forbush, B., Joiner, C. H., Gulcicek, E. E., Gallagher, P. G., and Lifton, R. P. (2009). Sites of regulated phosphorylation that control K-Cl cotransporter activity. *Cell*, 138(3):525–536.

Ring, A. M., Cheng, S. X., Leng, Q., Kahle, K. T., Rinehart, J., Lalioti, M. D., Volkman, H. M., Wilson, F. H., Hebert, S. C., and Lifton, R. P. (2007a). WNK4 regulates activity of the epithelial Na+ channel in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 104(10):4020–4024.

Ring, A. M., Leng, Q., Rinehart, J., Wilson, F. H., Kahle, K. T., Hebert, S. C., and Lifton, R. P. (2007b). An SGK1 site in WNK4 regulates Na+ channel and K+ channel activity and has implications for aldosterone signaling and K+ homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, 104(10):4025–4029.

Ritz, E. (2010). Salt and hypertension. *Nephrology*, 15 Suppl 2:49–52.

Rivadeneira, F., Styrkársdottir, U., Estrada, K., Halldórsson, B. V., Hsu, Y.-H., Richards, J. B., Zillikens, M. C., Kavvoura, F. K., Amin, N., Aulchenko, Y. S., Cup11es, L. A., Deloukas, P., Demissie, S., Grundberg, E., Hofman, A., Kong, A., Karasik, D., van Meurs, J. B., Oostra, B., Pastinen, T., Pols, H. A., Sigurdsson, G., Soranzo, N., Thorleifsson, G., Thorsteinsdottir, U., Williams, F. M., Wilson, S. G., Zhou, Y., Ralston,

References

S. H., van Duijn, C. M., Spector, T., Kiel, D. P., Stefansson, K., Ioannidis, J. P., Uitterlinden, A. G., and Consortium, T. G. F. F. O. G. (2010). Twenty bone mineral density loci identified by large-scale meta-analysis of genome-wide association studies. *Nature Genetics*, 41(11):1199–1206.

Rosskopf, D., Schürks, M., Rimmbach, C., and Schäfers, R. (2007). Genetics of arterial hypertension and hypotension. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 374(5-6):429–469.

Rozansky, D. J., Cornwall, T., Subramanya, A. R., Roger, S., Yang, Y.-F., David, L. L., Zhu, X., Yang, C.-L., and Ellison, D. H. (2009). Aldosterone mediates activation of the thiazide-sensitive Na-Cl cotransporter through an SGK1 and WNK4 signaling pathway. *The Journal of Clinical Investigation*, 119(9):2601–2612.

Sakamoto, K. and Holman, G. D. (2008). Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. *American Journal of Physiology. Endocrinology and Metabolism*, 295(1):E29–E37.

San-Cristobal, P., Pacheco-Alvarez, D., Richardson, C., Ring, A. M., Vazquez, N., Rafiqi, F. H., Chari, D., Kahle, K. T., Leng, Q., Bobadilla, N. A., Hebert, S. C., Alessi, D. R., Lifton, R. P., and Gamba, G. (2009). Angiotensin II signaling increases activity of the renal Na-Cl cotransporter through a WNK4-SPAK-dependent pathway. *Proceedings of the National Academy of Sciences of the United States of America*, 106(11):4384–4389.

Sanderson, M. P., Lau, C. W., Schnapp, A., and Chow, C.-W. (2009). Syk: a novel target for treatment of inflammation in lung disease. *Inflammation & Allergy Drug Targets*, 8(2):87–95.

Sano, H., Eguez, L., Teruel, M. N., Fukuda, M., Chavez, J. A., Chuang, T. D., Lienhard, G. E., and McGraw, T. E. (2007). Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. *Cell Metabolism*, 5(4):293–303.

Sano, H., Kane, S., Sano, E., Mîinea, C. P., Asara, J. M., Lane, W. S., Garner, C. W., and Lienhard, G. E. (2003). Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *The Journal of Biological Chemistry*, 278(17):14599–14602.

Sano, H., Roach, W. G., Peck, G. R., Fukuda, M., and Lienhard, G. E. (2008). Rab10 in insulin-stimulated GLUT4 translocation. *The Biochemical Journal*, 411(1):89–95.

Schlingmann, K. P., Weber, S., Peters, M., Niemann Nejsum, L., Vitzthum, H., Klingel, K., Kratz, M., Haddad, E., Ristoff, E., Dinour, D., Syrrou, M., Nielsen, S., Sassen, M., Waldegger, S., Seyberth, H. W., and Konrad, M. (2002). Hypomagnesemia with secondary hypocalcemia is caused by mutations in TRPM6, a new member of the TRPM gene family. *Nature Genetics*, 31(2):166–170.

Schork, N. J., Murray, S. S., Frazer, K. A., and Topol, E. J. (2009). Common vs. rare allele hypotheses for complex diseases. *Current Opinion in Genetics & Development*, 19:212–219.

Schuster, V. L. and Stokes, J. B. (1987). Chloride transport by the cortical and outer medullary collecting duct. *American Journal of Physiology. Renal Physiology*, 253(2 Pt 2):F203–F212.

Semmekrot, B., Monnens, B., Theelen, B. G. A., Rascher, W., Gabreels, F., and Willems, J. (1987). The syndrome of hypertension and hyperkalaemia with normal glomerular function (Gordon's syndrome). *Pediatric Nephrology*, 1(6):473–478.

Shaharabany, M., Holtzman, E. J., Mayan, H., Hirschberg, K., Seger, R., and Farfel, Z. (2008). Distinct pathways for the involvement of WNK4 in the signaling of hypertonicity and EGF. *The FEBS Journal*, 275(8):1631–1642.

Shekarabi, M., Girard, N., Rivière, J.-b., Dion, P., Houle, M., Toulouse, A., Lafrenière, R. G., Vercauteren, F., Hince, P., Laganiere, J., Rochefort, D., Faivre, L., Samuels, M., and Rouleau, G. A. (2008). Mutations in the nervous system - specific HSN2 exon of WNK1 cause hereditary sensory neuropathy type II. *The Journal of Clinical Investigation*, 118(7):2496–2505.

Speirs, H. J. L. and Morris, B. J. (2004). WNK4 intron 10 polymorphism is not associated with hypertension. *Hypertension*, 43(4):766–768.

Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. *Nature reviews. Molecular Cell Biology*, 10(8):513–525.

Stephens, P., Edkins, S., Davies, H., Greenman, C., Cox, C., Hunter, C., Bignell, G., Teague, J., Smith, R., Stevens, C., O'Meara, S., Parker, A., Tarpey, P., Avis, T., Barthorpe, A., Brackenbury, L., Buck, G., Butler, A., Clements, J., Cole, J., Dicks, E., Edwards, K., Forbes, S., Gorton, M., Gray, K., Halliday, K., Harrison, R., Hills, K., Hinton, J., Jones, D., Kosmidou, V., Laman, R., Lugg, R., Menzies, A., Perry, J., Petty, R., Raine, K., Shepherd, R., Small, A., Solomon, H., Stephens, Y., Tofts, C., Varian, J., Webb, A., West, S., Widaa, S., Yates, A., Brasseur, F., Cooper, C. S., Flanagan, A. M., Green, A., Knowles, M., Leung, S. Y., Looijenga, L. H. J., Malkowicz, B., Pierotti, M. A., Teh, B., Yuen, S. T., Nicholson, A. G., Lakhani, S., Easton, D. F., Weber, B. L., Stratton, M. R., Futreal, P. A., and Wooster, R. (2005). A screen of the complete protein kinase gene family identifies diverse patterns of somatic mutations in human breast cancer. *Nature Genetics*, 37(6):590–592.

Stöckli, J., Davey, J. R., Hohnen-Behrens, C., Xu, A., James, D. E., and Ramm, G. (2008). Regulation of glucose transporter 4 translocation by the Rab guanosine triphosphatase-activating protein AS160/TBC1D4: role of phosphorylation and membrane association. *Molecular Endocrinology*, 22(12):2703–2715.

Stutts, M., Canessa, C., Olsen, J., Hamrick, M., Cohn, J., Rossier, B., and Boucher, R. (1995). CFTR as a cAMP-dependent regulator of sodium channels. *Science*, 269(5225):847–850.

References

Subramanya, A. R., Liu, J., Ellison, D. H., Wade, J. B., and Welling, P. A. (2009). WNK4 diverts the thiazide-sensitive NaCl cotransporter to the lysosome and stimulates AP-3 interaction. *The Journal of Biological Chemistry*, 284(27):18471–18480.

Subramanya, A. R., Yang, C. L., Zhu, X., and Elisson, D. H. (2006). Dominant-negative regulation of WNK1 by its kidney-specific kinase-defective isoform. *American Journal of Renal Physiology. Renal Physiology.*, 290:619–624.

Sun, X., Gao, L., Yu, R. K., and Zeng, G. (2006). Down-regulation of WNK1 protein kinase in neural progenitor cells suppresses cell proliferation and migration. *Journal of Neurochemistry*, 99(4):1114–1121.

Sun, Z.-J., Li, Y., Lu, J.-Y., Ding, Q., Liang, Y., Shi, J.-P., Li-Ling, J., and Zhao, Y.-Y. (2009). Association of Ala589Ser polymorphism of WNK4 gene with essential hypertension in a high-risk Chinese population. *The Journal of Physiological Sciences*, 59(2):81–86.

Tabcharani, J. A., Chang, X. B., Riordan, J. R., and Hanrahan, J. W. (1991). Phosphorylation-regulated Cl- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature*, 352(6336):628–631.

Tang, W. and Wildey, M. J. (2004). Development of a colorimetric method for functional chloride channel assay. *Journal of Biomolecular Screening*, 9(7):607–613.

Thurmond, D. C., Ceresa, B. P., Okada, S., Elmendorf, J. S., Coker, K., and Pessin, J. E. (1998). Regulation of insulin-stimulated GLUT4 translocation by Munc18c in 3T3L1 adipocytes. *The Journal of Biological Chemistry*, 273(50):33876–33883.

Thurmond, D. C., Kanzaki, M., Khan, A. H., and Pessin, J. E. (2000). Munc18c function is required for insulin-stimulated plasma membrane fusion of GLUT4 and insulin-responsive amino peptidase storage vesicles. *Molecular and Cellular Biology*, 20(1):379–388.

Thurmond, D. C. and Pessin, J. E. (2000). Discrimination of GLUT4 vesicle trafficking from fusion using a temperature-sensitive Munc18c mutant. *The EMBO Journal*, 19(14):3565–3575.

Tian, W., Salanova, M., Xu, H., Lindsley, J. N., Oyama, T. T., Anderson, S., Bachmann, S., and Cohen, D. M. (2004). Renal expression of osmotically responsive cation channel TRPV4 is restricted to water-impermeant nephron segments. *American Journal of Physiology. Renal Physiology*, 287(1):F17–F24.

Tobin, M. D., Raleigh, S. M., Newhouse, S., Braund, P., Bodycote, C., Ogleby, J., Cross, D., Gracey, J., Hayes, S., Smith, T., Ridge, C., Caulfield, M., Sheehan, N. A., Munroe, P. B., Burton, P. R., and Samani, N. J. (2005). Association of WNK1 gene polymorphisms and haplotypes with ambulatory blood pressure in the general population. *Circulation*, 112(22):3423–3429.

Tobin, M. D., Timpson, N. J., Wain, L. V., Ring, S., Jones, L. R., Emmett, P. M., Palmer, T. M., Ness, A. R., Samani, N. J., Smith, G. D., and Burton, P. R. (2008). Common variation in the WNK1 gene and blood pressure in childhood: the Avon Longitudinal Study of Parents and Children. *Hypertension*, 52(5):974–979.

Ulanova, M., Duta, F., Puttagunta, L., D Schreiber, A., and Befus, A. D. (2005a). Spleen tyrosine kinase (Syk) as a novel target for allergic asthma and rhinitis. *Expert Opinion on Therapeutic Targets*, 9(5):901–921.

Ulanova, M., Marcet-Palacios, M., Muñoz, S., Asfaha, S., Kim, M.-K., Schreiber, A. D., and Befus, A. D. (2006). Involvement of Syk kinase in TNF-induced nitric oxide production by airway epithelial cells. *Biochemical and Biophysical Research Communications*, 351(2):431–437.

Ulanova, M., Puttagunta, L., Marcet-Palacios, M., Duszyk, M., Steinhoff, U., Duta, F., Kim, M.-K., Indik, Z. K., Schreiber, A. D., and Befus, A. D. (2005b). Syk tyrosine kinase participates in beta1-integrin signaling and inflammatory responses in airway epithelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 288(3):L497–L507.

Veríssimo, F. and Jordan, P. (2001). WNK kinases, a novel protein kinase subfamily in multi-cellular organisms. *Oncogene*, 20(39):5562–5569.

Veríssimo, F., Silva, E., Morris, J. D., Pepperkok, R., and Jordan, P. (2006). Protein kinase WNK3 increases cell survival in a caspase-3-dependent pathway. *Oncogene*, 25(30):4172–4182.

Vitari, A. C., Deak, M., Collins, B. J., Morrice, N., Prescott, A. R., Phelan, A., Humphreys, S., and Alessi, D. R. (2004). WNK1, the kinase mutated in an inherited high-blood-pressure syndrome, is a novel PKB (protein kinase B)/Akt substrate. *Biochemistry Journal*, 378:257–268.

Vitari, A. C., Deak, M., Morrice, N. A., and Alessi, D. R. (2005). The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. *The Biochemical Journal*, 391(Pt 1):17–24.

Vitari, A. C., Thastrup, J., Rafiqi, F. H., Deak, M., Morrice, N. A., Karlsson, H. K. R., and Alessi, D. R. (2006). Functional interactions of the SPAK/OSR1 kinases with their upstream activator WNK1 and downstream substrate NKCC1. *The Biochemical Journal*, 397(1):223–231.

Waldegger, S., Moschen, I., Ramirez, A., Smith, R. J., Ayadi, H., Lang, F., and Kubisch, C. (2001). Cloning and characterization of SLC26A6, a novel member of the solute carrier 26 gene family. *Genomics*, 72(1):43–50.

Wang, Y., Connell, J. R. O., Mcardle, P. F., Wade, J. B., Dorff, S. E., Shah, S. J., Shi, X., Pan, L., Rampersaud, E., Shen, H., Kim, J. D., Subramanya, A. R., Steinle, N. I., Parsa, A., Ober, C. C., Welling, P. A., Chakravarti, A., Weder, A. B., Cooper, R. S., Mitchell,

References

B. D., Shuldiner, A. R., and Chang, Y.-p. C. (2009). From the cover: Whole-genome association study identifies STK39 as a hypertension susceptibility gene. *Proceedings of the National Academy of Sciences of the United States of America*, 106(1):226–231.

Watson, R. T. and Pessin, J. E. (2006). Bridging the GAP between insulin signaling and GLUT4 translocation. *Trends in Biochemical Sciences*, 31(4):215–222.

Whiteman, E., Cho, H., and Birnbaum, M. (2002). Role of Akt/protein kinase B in metabolism. *Trends in Endocrinology and Metabolism*, 13(10):444–451.

Wilson, F. H., Disse-Nicodeme, S., Choate, K. A., Ishikawa, K., Nelson-Williams, C., Desitter, I., Gunel, M., Milford, D. V., Lipkin, G. W., Achard, J. M., Feely, M. P., Dussol, B., Berland, Y., Unwind, R. J., Mayan, H., Simon, D. B., Farfel, Z., Jeune-maitre, X., and Lifton, R. P. (2001). Human hypertension caused by mutations in WNK kinases. *Science*, 293:1107–1112.

Wilson, F. H., Kahle, K. T., Sabath, E., Lalioti, M. D., Rapson, A. K., Hoover, R. S., Hebert, S. C., Gamba, G., and Lifton, R. P. (2003). Molecular pathogenesis of inherited hypertension with hyperkalemia: the Na-Cl cotransporter is inhibited by wild-type but not mutant WNK4. *Proceedings of the National Academy of Sciences of the United States of America*, 100(2):680–684.

Xie, J., Craig, L., Cobb, M. H., and Huang, C.-L. (2006). Role of with-no-lysine [K] kinases in the pathogenesis of Gordon's syndrome. *Pediatric Nephrology*, 21:1231–1236.

Xie, J., Wu, T., Xu, K., Huang, I. K., Cleaver, O., and Huang, C.-L. (2009). Endothelial-specific expression of WNK1 kinase is essential for angiogenesis and heart development in mice. *The American Journal of Pathology*, 175(3):1315–1327.

Xu, B.-e., English, J. M., Wilsbacher, J. L., Stippec, S., Goldsmith, E. J., and Cobb, M. H. (2000). WNK1, a Novel Mammalian Serine/Threonine Protein Kinase Lacking the Catalytic Lysine in Subdomain II. *The Journal of Biological Chemistry*, 275(22):16795–16801.

Xu, B.-e., Lee, B.-h., Min, X., Lenertz, L., Heise, C. J., Stippec, S., Goldsmith, E. J., and Cobb, M. H. (2005a). WNK1: analysis of protein kinase structure, downstream targets, and potential roles in hypertension. *Cell Research*, 15(1):6–10.

Xu, B. E., Min, X., Stippec, S., Lee, B. H., Goldsmith, E. J., and Cobb, M. H. (2002a). Regulation of WNK1 by an autoinhibitory domain and autophosphorylation. *The Journal Biological Chemistry*, 277(50):48456–48462.

Xu, B.-e., Stippec, S., Chu, P.-Y., Lazrak, A., Li, X.-J., Lee, B.-H., English, J. M., Ortega, B., Huang, C.-L., and Cobb, M. H. (2005b). WNK1 activates SGK1 to regulate the epithelial sodium channel. *Proceedings of the National Academy of Sciences of the United States of America*, 102(29):10315–10320.

Xu, B.-E., Stippec, S., Lazrak, A., Huang, C.-L., and Cobb, M. H. (2005c). WNK1 activates SGK1 by a phosphatidylinositol 3-kinase-dependent and non-catalytic mechanism. *The Journal of Biological Chemistry*, 280(40):34218–34223.

Xu, B.-E., Stippec, S., Lenertz, L., Lee, B.-H., Zhang, W., Lee, Y.-K., and Cobb, M. H. (2004). WNK1 activates ERK5 by an MEKK2/3-dependent mechanism. *The Journal of Biological Chemistry*, 279(9):7826–7831.

Xu, Q., Modrek, B., and Lee, C. (2002b). Genome-wide detection of tissue-specific alternative splicing in the human transcriptome. *Nucleic Acids Research*, 30(17):3754–3766.

Yamauchi, K., Rai, T., Kobayashi, K., Sohara, E., Suzuki, T., Itoh, T., Suda, S., Hayama, A., Sasaki, S., and Uchida, S. (2004). Disease-causing mutant WNK4 increases paracellular chloride permeability and phosphorylates claudins. *Proceedings of the National Academy of Sciences of the United States of America*, 101(13):4690–4694.

Yamauchi, K., Yang, S.-S., Ohta, A., Sohara, E., Rai, T., Sasaki, S., and Uchida, S. (2005). Apical localization of renal K channel was not altered in mutant WNK4 transgenic mice. *Biochemical and Biophysical Research Communications*, 332(3):750–755.

Yang, C.-L., Angell, J., Mitchell, R., and Ellison, D. H. (2003). WNK kinases regulate thiazide- sensitive Na-Cl cotransport. *The Journal of Clinical Investigation*, 111(7):1039–1045.

Yang, C.-L., Liu, X., Paliege, A., Zhu, X., Bachmann, S., Dawson, D. C., and Ellison, D. H. (2007a). WNK1 and WNK4 modulate CFTR activity. *Biochemical and Biophysical Research Communications*, 353(3):535–540.

Yang, C.-L., Zhu, X., and Ellison, D. (2007b). The thiazide-sensitive Na-Cl cotransporter is regulated by a WNK kinase signaling complex. *Journal of Clinical Investigation*, 117(11):3403–3411.

Yang, C.-L., Zhu, X., Wang, Z., Subramanya, A. R., and Ellison, D. H. (2005a). Mechanisms of WNK1 and WNK4 interaction in the regulation of thiazide-sensitive NaCl cotransport. *The Journal of Clinical Investigation*, 115(5):1379–1387.

Yang, S.-S., Hsu, Y.-J., Chiga, M., Rai, T., Sasaki, S., Uchida, S., and Lin, S.-H. (2010). Mechanisms for hypercalciuria in pseudohypoaldosteronism type II-causing WNK4 knock-in mice. *Endocrinology*, 151(4):1829–1836.

Yang, S.-S., Morimoto, T., Rai, T., Chiga, M., Sohara, E., Ohno, M., Uchida, K., Lin, S.-H., Moriguchi, T., Shibuya, H., Kondo, Y., Sasaki, S., and Uchida, S. (2007c). Molecular pathogenesis of pseudohypoaldosteronism type II: generation and analysis of a Wnk4(D561A/+) knockin mouse model. *Cell Metabolism*, 5(5):331–344.

Yang, S.-S., Yamauchi, K., Rai, T., Hiyama, A., Sohara, E., Suzuki, T., Itoh, T., Suda, S., Sasaki, S., and Uchida, S. (2005b). Regulation of apical localization of the thiazide-sensitive NaCl cotransporter by WNK4 in polarized epithelial cells. *Biochemical and Biophysical Research Communications*, 330(2):410–414.

References

Yki-Jarvinen, H., Vuorinen-Markkola, H., Koranyi, L., Bourey, R., Tordjman, K., Mueckler, M., Permutt, A., and Koivisto, V. (1992). Defect in insulin action on expression of the muscle/adipose tissue glucose transporter gene in skeletal muscle of type 1 diabetic patients. *Journal of Clinical Endocrinology & Metabolism*, 75(3):795–799.

Yki-Järvinen, H., Young, A. A., Lamkin, C., and Foley, J. E. (1987). Kinetics of glucose disposal in whole body and across the forearm in man. *The Journal of Clinical Investigation*, 79(6):1713–1719.

Yoo, D., Flagg, T. P., Olsen, O., Raghuram, V., Foskett, J. K., and Welling, P. A. (2004). Assembly and trafficking of a multiprotein ROMK (Kir 1.1) channel complex by PDZ interactions. *The Journal of Biological Chemistry*, 279(8):6863–6873.

Zagórska, A., Pozo-Guisado, E., Boudeau, J., Vitari, A. C., Rafiqi, F. H., Thastrup, J., Deak, M., Campbell, D. G., Morrice, N. A., Prescott, A. R., and Alessi, D. R. (2007). Regulation of activity and localization of the WNK1 protein kinase by hyperosmotic stress. *The Journal of Cell Biology*, 176(1):89–100.

Zambrowicz, B. P., Abuin, A., Ramirez-Solis, R., Richter, L. J., Piggott, J., Beltrandel-Rio, H., Buxton, E. C., Finch, R. A., Edwards, J., Friddle, C. J., Gupta, A., Hansen, G., Hu, Y., Huang, W., Jaing, C., Key, B. W., Kipp, P., Kohlhauff, B., Ma, Z.-Q., Markesich, D., Payne, R., Potter, D. G., Qian, N., Shaw, J., Schrick, J., Shi, Z.-Z., Sparks, M. J., Van Sligtenhorst, I., Vogel, P., Walke, W., Xu, N., Zhu, Q., Person, C., and Sands, A. T. (2003). Wnk1 kinase deficiency lowers blood pressure in mice: a gene-trap screen to identify potential targets for therapeutic intervention. *Proceedings of the National Academy of Sciences of the United States of America*, 100(24):14109–14114.

Zeng, G., Gao, L., Xia, T., Gu, Y., and Yu, R. K. (2005). Expression of the mouse WNK1 gene in correlation with ganglioside GD3 and functional analysis of the mouse WNK1 promoter. *Gene*, 344:233–239.

Zerial, M. and Mcbride, H. (2001). Rab Proteins as Membrane Organizers. *Nature Reviews. Molecular Cell Biology*, 2(February):107–117.

Zhang, W., Na, T., and Peng, J.-B. (2008). WNK3 positively regulates epithelial calcium channels TRPV5 and TRPV6 via a kinase-dependent pathway. *American Journal of Physiology. Renal Physiology*, 295(5):F1472–F1484.

Zhao, F. Q., Glimm, D. R., and Kennelly, J. J. (1993). Distribution of mammalian facilitative glucose transporter messenger RNA in bovine tissues. *The International Journal of Biochemistry*, 25(12):1897–1903.

Zheng, W., Kuhlicke, J., Jäckel, K., Eltzschig, H. K., Singh, A., Sjöblom, M., Riederer, B., Weinhold, C., Seidler, U., Colgan, S. P., and Karhausen, J. (2009). Hypoxia inducible factor-1 (HIF-1)-mediated repression of cystic fibrosis transmembrane conductance regulator (CFTR) in the intestinal epithelium. *The FASEB Journal*, 23(1):204–213.

Zhou, B., Zhuang, J., Gu, D., Wang, H., Cebotaru, L., Guggino, W. B., and Cai, H. (2010). WNK4 enhances the degradation of NCC through a sortilin-mediated lysosomal pathway. *Journal of the American Society of Nephrology*, 21(1):82–92.

Zhou, Q. L., Jiang, Z. Y., Holik, J., Chawla, A., Hagan, G. N., Leszyk, J., and Czech, M. P. (2008). Akt substrate TBC1D1 regulates GLUT1 expression through the mTOR pathway in 3T3-L1 adipocytes. *The Biochemical Journal*, 411(3):647–655.