Serum and Glucocorticoid-Regulated Kinase Signaling in Breast Cancer

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Serum and Glucocorticoid-Regulated Kinase Signaling in Breast Cancer

A dissertation presented

by

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Serum and Glucocorticoid-Regulated Kinase Signaling in Breast Cancer

Abstract

Oncogenic activating mutations in *PIK3CA*, the gene encoding the catalytic

subunit of phosphoinositide 3-kinase (PI 3-K), are highly prevalent in breast cancer. The

protein kinase Akt is considered to be the primary effector of *PIK3CA*, though the

mechanisms by which PI 3-K mediates tumorigenic signals in an Akt-independent

manner remain obscure. My studies show that the serum and glucocorticoid-regulated

kinases (SGKs) can function as effectors of PI 3-kinase and transduce signals to

phenotypes associated with malignancy. We show that SGK3 is amplified in breast

cancer and identify the mechanism by which SGK3 is activated downstream of *PIK3CA*,

specifically through the catalytic activity of the phosphoinositide phosphatase INPP4B.

Expression of INPP4B promotes SGK3 activation and in turn inhibits Akt

phosphorylation. In breast cancer cell lines with elevated levels of INPP4B, SGK3 is

required for proliferation in 3D and also for invasive migration. SGK3 phenotypes are in

part mediated by phosphorylation of the substrate protein N-myc downstream regulated

1 (NDRG)1, an established metastasis suppressor. The phosphorylation of NDRG1

leads to recruitment by F-box and WD repeat domain-containing 7 (FBW7), the

substrate recognition domain of the Skp, Cullin, F-box containing (SCF) complex. Binding of FBW7 to NDRG1 promotes its polyubiquitination and subsequent degradation by the 26S proteasome. By contrast, our studies also show that the related SGK1 isoform is polyubiquitinated by the functional E3 ubiquitin ligase Rictor-Cullin-1 complex, leading to SGK1 degradation. Proteasomal degradation of SGK1 by Rictor-Cullin-1 is the first identified mTORC2-independent function of the Rictor protein. Moreover, the deregulation of SGK1 ubiquitination highlights a mechanism of SGK1 overexpression in breast cancers.

Taken together, these findings highlight novel post-translational modifications of SGK isoforms converging on the theme of ubiquitination and degradation. Alterations in ubiquitination status lead to the deregulation of cellular phenotypes such as proliferation and migration. The data presented in this thesis also highlight a mechanism by which SGK isoforms transduce oncogenic PI3-kinase signaling in breast cancer. The oncogenic potential of SGK signaling is further emphasized by the aberrant expression levels of SGK1 and SGK3 in breast cancers.

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ABBREVIATIONS

4E-BP	4E-Binding Protein
AAA	ATPases Associated with diverse cellular Activities
AGC	PKA, PKG, PKC
Akt/PKB	Protein Kinase B
AMPK	5′ AMP-activated protein Kinase
ARF	ADP Ribosylation Factor
ATP	Adenosine Triphosphate
BAD	BCL-2 Agonist of cell Death
BMX	Bone Marrow kinase on chromosome X
BTK	Bruton's Tyrosine Kinase
C-terminal	COOH- terminal
C-myc	Cellular v-myc myelocytomatosis viral oncogene homolog
CD44	Cluster of Differentiation 44
CHIP	Carboxyl terminus of Hsc70-Interacting Protein
CK	Cytokeratin
DUB	De-Ubiquitinating enzyme
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial- Mesenchymal Transition
ER	Estrogen Receptor
ERK	Extracellular-Regulated Kinase
FBW7	F-Box and WD repeat domain-containing 7
FBXL	F-box and leucine rich repeats
FBXO	F-box and other domains
FBXW	F-box and WD40 repeats
FGF	Fibroblast-derived Growth Factor
FOXO3A	Forkhead box O 3a
FSH	Follicle Stimulated Hormone
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GPCR	G-protein coupled receptors
GRP1	General Receptor for 3-Phosphoinositides 1
GSK3β	Glycogen Synthase Kinase β
GTP	Guanosine 5'-Triphosphate
HDM2	Human Double Minute 2
HECT	Homologous to E6 –associated protein C-Terminus
HER2/ ERBB2	Human Epidermal growth factor Receptor 2
HIF1	Hypoxia-Inducible Factor 1
HM	Hydrophobic Motif
HSC70	Heat-Shock Cognate Protein 70

IFNγ	Interferon Gamma
IGF	Insulin-like growth factor
IGF1-R	Insulin-like Growth Factor 1-Receptor
ILK	Integrin-Linked Kinase
INPP4A	Inositol Polyphosphate-4-Phosphatase type I
INPP4B	Inositol Polyphosphate-4-Phosphatase type II
ITK	Interleukin-2-inducible T-cell Kinase
JAMM/MPN+	Jab1, Mov34, MPR1 Pad1 N-Terminal
LOH	Loss of Heterozygosity
LRP6	Low-density lipoprotein Receptor-related Protein 6
MAPK	Mitogen-Activated Protein Kinase
mLST8/GβL	G protein β-subunit-like protein
MMP7	Matrix Metalloproteinase 7
mTOR	mammalian Target of Rapamycin
mTORC1	mammalian Target of Rapamycin Complex 1
mTORC2	mammalian Target of Rapamycin Complex 2
N-terminal	NH2-terminal
N-myc	Neuroblastoma-derived Myelocytomatosis
NDRG	N-myc Down-Regulated Gene
NEDD-4	Neural precursor cell Expressed Developmentally Down-regulated protein 4
NFAT	Nuclear Factor of activated-T cells
OTU	Ovarian Tumor-like protease
PDGF	Platelet-derived Growth Factor
PDGF-R	Platelet-Derived Growth Factor- Receptor
PDK-1	Phosphoinositide-Dependent Kinase-1
PH	Pleckstrin homology
PHLPP	PH domain and Leucine rich Protein Phosphatase
PI 3-K	Phosphoinositide 3- Kinase
PIF	PDK-1 Interacting Fragment
PKC	Protein Kinase C
PLCγ	Phospholipase C γ
PR	Progesterone Receptor
PRAS40	Proline-Rich Akt Substrate of 40 kDa
PROTOR	protein observed with Rictor-1
PTEN	Phosphatase and Tensin homolog
PX	Phox Homology
RAC	Ras-related C3 botulinum toxin substrate
RING	Really Interesting New Gene
RTK	Receptor Tyrosine Kinase
S6K	p70 ribosomal S6Kinase
SCF	Skp, Cullin, F-box containing
SGK1	Serum and Glucocorticoid- Regulated Kinase1
SGK2	Serum and Glucocorticoid- Regulated Kinase2

SGK3	Serum and Glucocorticoid- Regulated Kinase3
SH	Src Homology
SHIP	SH2 domain-containing Inositol Phosphatase
SKP2	S phase Kinase-associated Protein 2
STAT	Signal Transducer and Activator of Transcription
T-ALL	T-cell Acute Lymphoblastic Leukemia
TAPP	Tandem PH-domain containing Protein
TCF	T-Cell Factor
TGFβ	Transforming Growth Factor β
TIRF	Total Internal Reflection Fluorescence
TNBC	Triple-Negative Breast Cancer
TSC	Tuberous Sclerosis Complex
UCH	Ubiquitin C-terminal Hydrolase
UPS	Ubiquitin Proteasome System
USP	Ubiquitin-Specific Proteases
VPS34	Vacuolar Protein Sorting 34
WNK1	With No lysine (K) 1
WNT	Wingless-related integration site

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go forth into the world that I have gained a small spec of their comprehensive abilities to build a career in science.

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CHAPTER 1

INTRODUCTION

1. MECHANISMS OF PI 3-KINASE ACTIVATION

Phosphoinositide 3-Kinase (PI 3-K) is a lipid kinase family divided into 3 classes: class I, II, and III. Class IA PI 3-Ks are heterodimeric enzymes composed of regulatory and catalytic subunits that are activated upon growth factor stimulation of Receptor Tyrosine Kinases (RTK) and G-Protein Coupled Receptors (GPCR). The p85α (p85α, p50α, p55α), p85β, and p55γ regulatory subunits are encoded by three distinct genes *PIK3R1*, *PIK3R2*, and *PIK3R3* and are collectively referred to as p85. The regulatory subunits are capable of binding the catalytic subunits p110α, p110β, and p110δ encoded by *PIK3CA*, *PIK3CB*, *PIK3CD*, respectively (Courtney et al., 2010; Hawkins et al., 2006). p85 subunits contain Src Homology 2 (SH)2 and SH3 domains that bind phosphorylated tyrosine residues of RTKs or their adaptor proteins (Manning and Cantley, 2007). RTKs such as Insulin-like Growth Factor 1-Receptor (IGF1-R) and Platelet-Derived Growth Factor-Receptor (PDGF-R) harbor intrinsic tyrosine kinase activity and upon growth factor-induced dimerization of the receptor, the dimers transphosphorylate tyrosine residues in the cytoplasmic tail regions (Hawkins et al., 2006). The p85 regulatory subunits function to inhibit the activity of the p110 catalytic subunit until the SH2 domain of p85 binds phosphotyrosines on the RTK, within the common consensus sequence pYXXM (Y, tyrosine; X, any amino acid; M, methionine) (Hawkins et al., 2006; Okkenhaug, 2013). The binding of p85 to RTKs elicits the release of intermolecular p110 inhibition, thus activating PI 3-K and downstream signaling (Courtney et al., 2010). *In vitro* PI 3-K phosphorylates the 3' position of the inositol ring of the phosphoinositides PI (phosphatidylinositol), PI(3)P

(phosphatidylinositol (3) phosphate) and PI(4,5)P2 (phosphatidylinositol (4,5) bisphosphate) (Toker, 2000). However in cells, class I PI 3-K primarily phosphorylates the substrate PI(4,5)P2 to generate PI(3,4,5)P3 (Miller et al., 2011; Toker, 2000). In turn, PI(3,4,5)P3 recruits downstream effector proteins that harbor lipid binding domains, thus transducing the PI 3-K signal to secondary signal relay pathways (**Figure 1-1**). In addition to RTK regulation, PI 3-K is localized by Ras binding to p110 effectively localizing PI 3-K to the plasma membrane (Courtney et al., 2010; Hawkins et al., 2006). p110β is unique as a Class 1A PI 3-K to be recruited and activated by GPCRs. In response to GPCR ligand stimulation, Class 1B PI 3-K also interconverts PI(3,4,5)P3 from PI(4,5)P2. Unlike Class IA enzymes, Class 1B PI 3-K consists of heterodimers of p110γ encoded by the *PIK3CG* gene and the regulatory subunits p87 or p101. Class 1B PI 3-K are predominantly expressed in immune cells and are known for roles in inflammatory diseases and immunity (Hawkins et al., 2006). Class IA PI 3-K is the class most prominently implicated in cancer etiology and progression by transducing growth factor responses as well mediating oncogenic signaling (Yuan and Cantley, 2008).

Class II PI 3-kinases generate PI(3)P and PI(3,4)P2 from PI and PI(3)P and lack any known regulatory domains. PI 3-K-C2α, PI 3-K-C2β, and PI 3-K-C2δ are class II enzymes and possess a C2 domain (Hawkins et al., 2006; Okkenhaug, 2013). Little is known about the functional roles of Class II PI 3-K, although they are known to be activated by insulin signaling and as such they modulate PI 3-K signaling in insulin-responsive cells and tissues (Okkenhaug, 2013).
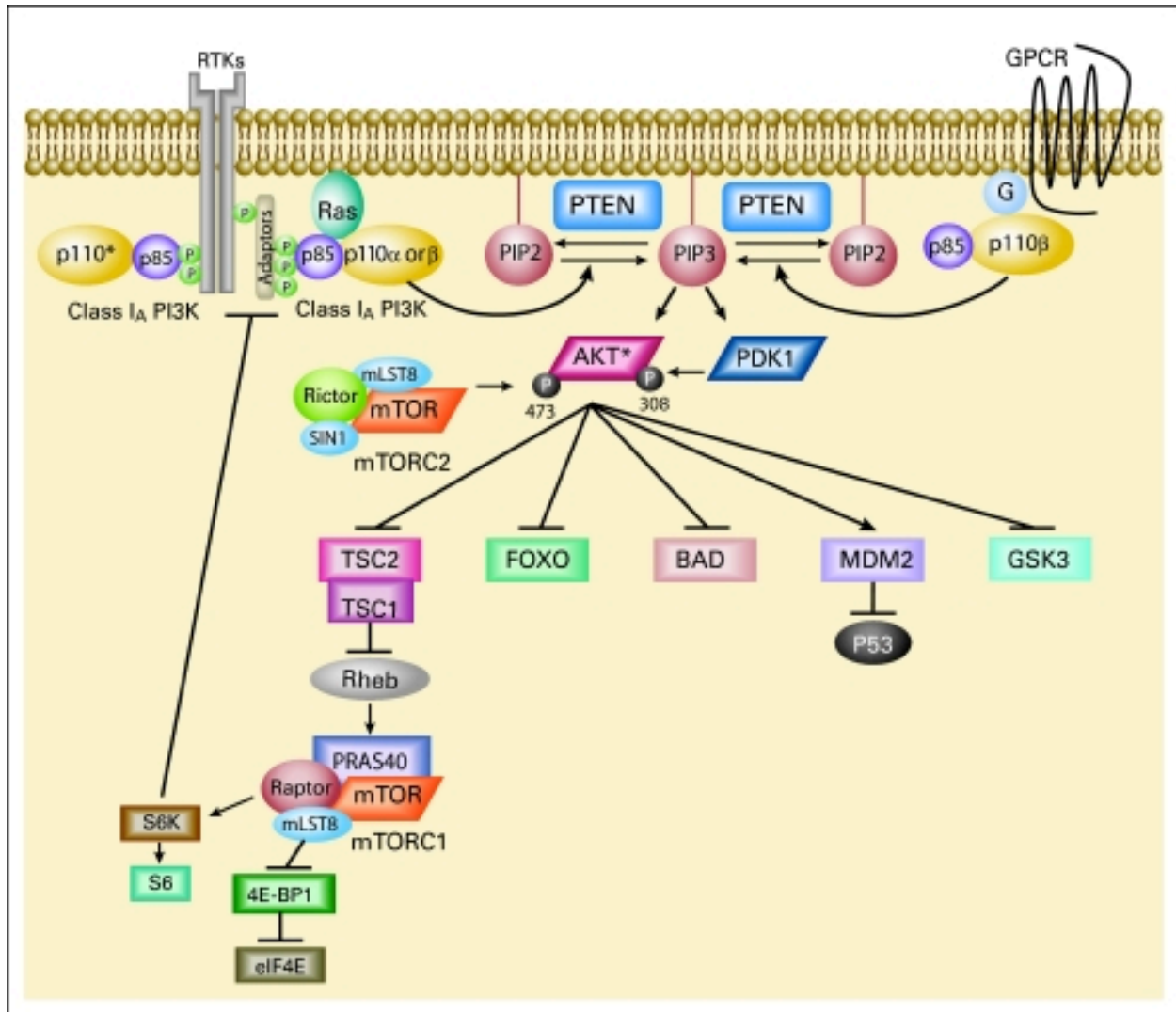
Figure 1-1: The phosphoinositide 3-kinase cascade

The phosphoinositide 3-kinase (PI3-K) signaling cascade. PI3-K signaling impacts on cell growth, survival, and metabolism. Arrows represent activation, while bars reflect inhibition. (*) p110 alpha, beta, or delta. Taken from (Courtney et al., 2010).

Class III PI 3-kinases exclusively produce PI(3)P from PI, and are composed of a

VPS34 catalytic domain and p150 regulatory domain (Backer, 2008). The primary role

of Class III PI 3-kinases and the lipid product PI(3)P is to modulate endosomal protein

sorting and membrane trafficking (Backer, 2008; Okkenhaug, 2013).

The Akt Kinase as a Transducer of PI 3-K Signaling

 A major effector of the PI 3-K signaling axis is the serine/threonine protein kinase

Akt, also known as Protein Kinase B (PKB) (Luo et al., 2003). Under basal serum-

starved conditions Akt is a cytosolic enzyme. Upon activation of PI 3-K, Akt translocates

to the plasma membrane by binding PI(3,4,5)P3 and PI(3,4)P2 through the amino-

terminal Pleckstrin Homology (PH) domain (Luo et al., 2003). As discussed above,

PI(3,4,5)P3 is generated by Class I PI 3-K, whereas PI(3,4)P2 is synthesized by Class II

PI 3-K. An alternate route for PI(3,4)P2 accumulation is dephosphorylation of

PI(3,4,5)P3 by the SH2 domain-containing Inositol 5' Phosphatases (SHIP) 1 and 2,

which are PI(3,4,5)P3 5' inositol phosphatases (Choi et al., 2002; Elong Edimo et al.,

2013; Scheid et al., 2002). Subsequent to the lipid binding-induced conformational

change, Akt is phosphorylated at two critical residues in the catalytic domain, Thr308 in

the activation loop and Ser473 in the Hydrophobic Motif (HM) (Luo et al., 2003; Toker,

2000). Thr308 (in Akt1) is phosphorylated by the Phosphoinositide-Dependent Kinase -

1 (PDK-1), which is also recruited to PI(3,4,5)P3 and PI(3,4)P2 through its carboxyl-

terminal PH domain (Mora et al., 2004). PDK-1 is a constitutively active protein kinase

primarily due to autophosphorylation at the activation loop residue Ser241 (Mora et al.,

2004). The HM of Akt isoforms (Ser473 in Akt1) is phosphorylated by the mammalian

Target Of Rapamycin Complex 2 (mTORC2), although a number of other kinases have

been shown to replace the function of mTORC2 as the Ser473 kinases, including

Protein Kinase C (PKC), Integrin-Linked Kinase (ILK) and Akt itself (Lu et al., 2006;

Miller et al., 2011; Xia et al., 2004). Regardless of the mechanism, phosphorylation of

Akt by mTORC2 promotes a conformational change allowing PDK-1 access to the Akt

activation loop (**Figure 1-2**) (Sarbassov et al., 2005). Both Ser473 and Thr308

phosphorylation are required for maximal Akt catalytic activity (Courtney et al., 2010;

Woodgett, 2005). Upon activation, Akt is locked in the catalytically-active confirmation

and loses the PI(3,4,5)P3 or PI(3,4)P2 binding requirement (Mora et al., 2004;

Sarbassov et al., 2005). Newly activated Akt then translocates to numerous intracellular

compartments to interact and phosphorylate an array of substrates critical for

metabolism, growth, and survival(Luo et al., 2003). Importantly, the levels PI(3,4,5)P3 in

all cells and tissues are negatively regulated by the tumor suppressor Phosphatase and

Tensin homolog deleted on chromosome ten (PTEN). PTEN converts PI(3,4,5)P3 back

to PI(4,5)P2 effectively terminating PI 3-K signaling (**Figure 1-3**) (Li et al., 1997;

Maehama and Dixon, 1998). As discussed below, inactivation of PTEN is a frequent

event in cancer and leads to constitutive PI(3,4,5)P3 levels and hyperactivation of Akt

(Engelman et al., 2006). Alternately, a distinct tumor suppressor Inositol Polyphosphate

4-Phosphatase type II (INPP4B) also negatively regulates Akt by dephosphorylating

PI(3,4)P2 at the 4' position effectively generating PI(3)P (Fedele et al., 2010; Gewinner
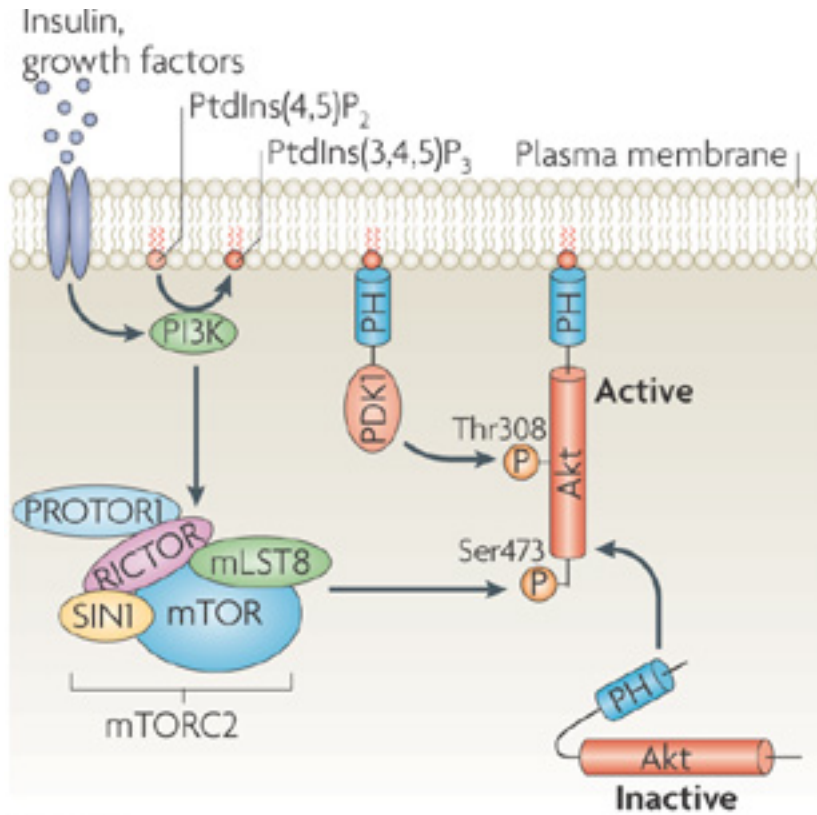
et al., 2009).

Figure 1-2: Activation mechanism of Akt

The generation of PI(3,4,5)P3 from PI(4,5)P2 by PI 3-K recruits Akt to the membrane. Here, it is phosphorylated by PDK-1 and mTORC2 required for maximal Akt activation. Adapted from (Pearce et al., 2010).
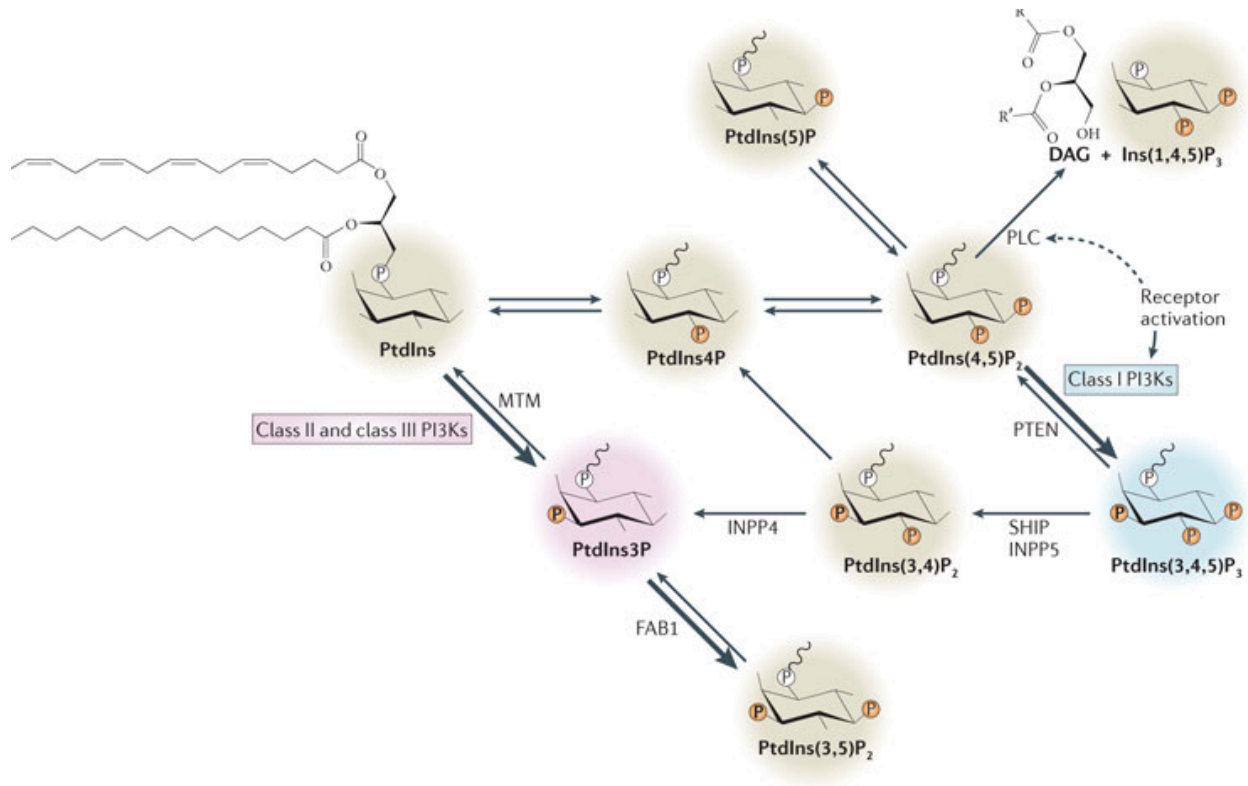
Figure 1-3: PI 3-K catalyzed inositol phospholipid pathways

The main metabolic pathways that interconvert inositol phospholipids in eukaryotic cells are shown. Class I phosphoinositide 3-kinases (PI 3-Ks) are activated by cell surface receptors to make phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P$_3$) and, indirectly, PtdIns(3,4)P$_2$; these two lipids are intracellular signals that carry important information through the receptor signaling network. Class III and probably class II PI3Ks make PI(3)P and play a key part in regulatory networks controlling vesicular trafficking through the endosome–lysosome system. DAG, diacylglycerol; FAB1, PI(3)P 5-kinase; INPP5, inositol polyphosphate 5-phosphatase; MTM, myotubularin; PLC, phospholipase C; PTEN, phosphatase and tensin homologue; SHIP, SH2 domain-containing inositol 5'-phosphatase. Adapted from (Vanhaesebroeck et al., 2012).

Again, inactivation mutations or loss of heterozygosity (LOH) of *INPP4B* leads to

increased accumulation of PI(3,4)P2 and constitutive Akt activation (Gewinner et al.,

2009).

There are three Akt isoforms encoded by three distinct genes: Akt1/PKBα,

Akt2/PKBβ and Akt3/PKBγ. All three isoforms are basophilic-directed serine/threonine

protein kinases that share a conserved consensus substrate phosphorylation motif,

with Arginine at the -3 and -5 position and a preference for hydrophobic residues at +1,

relative to the Ser/Thr phospho-acceptor residue (Murray et al., 2004; Tessier and

Woodgett, 2006b). Akt1, Akt2 and Akt3 are ubiquitously expressed in all tissues and

organs in humans. Akt isoforms are members of the AGC (PKA, PKG, PKC) family of

kinases and share certain common elements in their mechanisms of regulation, since

most AGC kinases are phosphorylated at their activation loop residues by PDK-1 and at

the HM motif by mTORC2. Although all three Akt isoforms are activated by identical

mechanisms through lipid binding and PDK-1- and mTORC2-catalyzed phosphorylation,

Akt1, Akt2 and Akt3 have multiple non-redundant roles in signaling and

pathophysiology. One clear example of Akt isoform-specific signaling was first identified

in breast cancer cells, whereby Akt1 and Akt2 possess opposing roles in the regulation

of cell migration and invasion (Toker and Yoeli-Lerner, 2006). Akt2 functions to promote

invasive migration in part by the induction of β1integrin expression (Arboleda et al.,

2003). Conversely, Akt1 inhibits migration, invasion and even metastatic dissemination

in vivo, in part through the degradation of Nuclear Factor of activated-T cells (NFAT),

modulation of the Extracellular-Regulated Kinase (ERK) pathway, and inhibition of actin

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bundling (Toker and Yoeli-Lerner, 2006; Yoeli-Lerner et al., 2005). Akt isoform signaling specificity is also mediated through inactivation of its catalytic activity by protein phosphatases. Specifically, Akt signaling is terminated by the PH domain and Leucine-rich Protein Phosphatases 1 and 2 (PHLPP)1/2 (Brognard and Newton, 2008; Brognard et al., 2007). PHLPPs are a family of serine/threonine phosphatases that dephosphorylate Akt isoforms at Ser473. Interestingly, PHLPP1 has been shown to dephosphorylate Akt2 and Akt3 at S474 and Ser472, respectively, whereas PHLPP2 dephosphorylates Akt1 and Akt3 at their corresponding HM residue (Brognard and Newton, 2008; Brognard et al., 2007).

Akt transduces the PI 3-K signal in both normal physiological conditions as well as pathophysiological settings by phosphorylating a wide range of substrates that control virtually all aspects of cell biology, including but not limited to cell motility, growth, proliferation and survival (Luo et al., 2003). The first identified function for Akt in both cancer cells and neuronal tissues is induction of cell survival mechanisms under conditions of stress, mediated by the phosphorylation of substrates involved in apoptosis such as BCL-2 Agonist of cell Death (BAD) (Datta et al., 1997), that promotes cell survival subsequent to Akt-mediated phosphorylation. Cell cycle progression is also mediated by Akt signaling, and phosphorylation of the F-box protein S phase Kinase-associated Protein 2 (SKP2) by Akt promotes the formation of the Skp, Cullin, F-box containing (SCF) complex, an E3 ligase leading to p27 degradation, thus promoting cell cycle progression (Gao et al., 2009). Forkhead box O 3A (FOXO3A) phosphorylation by Akt promotes FOXO3A nuclear export and cytoplasmic retention leading to inhibition of

transcription of genes that control cell cycle arrest and apoptosis, effectively promoting survival (Singh et al., 2010). Akt is directly involved in the activation of the mammalian Target of Rapamycin Complex 1 (mTORC1), and does so by phosphorylating the Tuberous Sclerosis Complex (TSC)1/TSC2, leading to TSC inhibition and subsequent downstream activation of mTORC1 (Luo et al., 2003). The activation of mTORC1 in turn integrates critical cellular growth signals including feedback inactivation of the PI 3-K and Akt pathway. mTORC1 activation is further facilitated through Akt-mediated phosphorylation of Proline-Rich Akt Substrate of 40 kDa (PRAS40) leading to binding of 14-3-3 and release of mTORC1 inhibition (Vander Haar et al., 2007).

Although many of the over 200 substrates of Akt are likely to be phosphorylated by all three Akt isoforms, a number of studies have uncovered a number of Akt isoform-specific substrates that explain, in part, the non-redundant function of Akt's in phenotypes associated with malignancy. For example, Akt1 exclusively phosphorylates palladin, an actin bundling protein, and this event mediates the ability of Akt1 to function as a suppressor of breast cancer cell invasive migration. Similarly, SKP2 has been shown to be an exclusive Akt1 substrate. Akt2 specific substrates include AS160 in the regulation of glucose transport and Myosin5a in the regulation of glut4 vesicular translocation (Chin and Toker, 2011; Toker, 2012). To date, no Akt3-specific substrates have been identified, although these are likely to exist.

Alternate PI 3-K Pathway Effectors

Although Akt is generally considered to be the primary effector of PI 3-Kinase, it is by no means the exclusive transducer of PI(3,4)P2 and PI(3,4,5)P3. Since PI 3-K signal relay is initiated by PI(3,4)P2 and PI(3,4,5)P3 accumulation at the plasma membrane, a rate limiting event in PI 3-K signal relay is the recruitment of effectors to the plasma membrane. Other than Akt and PDK-1, other PH domain-containing proteins have been identified and are recruited by binding to PI(3,4,5)P3 and PI(3,4)P2, although it is worth noting that all PH domains bind phosphoinositides selectively or with high affinity. Of the ones that do, the Tec family of non-receptor protein tyrosine kinases, including Tec, Bruton's Tyrosine Kinase (BTK), Interleukin-2-Inducible T-cell Kinase (ITK), and Bone Marrow kinase on chromosome X (BMX), bind to PI(3,4,5)P3 through their PH domains (Vargas et al., 2013).   Moreover, the PH domain of BTK is critical for *in vitro* kinase activity and substrate recognition and binding (Saito et al., 2001). Another class of effectors are Guanine Nucleotide Exchange Factors (GEFs) for small Guanosine 5'-Triphosphate (GTP)ases, whereby recruitment positions the GEF in proximity with its GTPase in addition to relieving PH domain inhibition of the catalytic region (Cherfils and Zeghouf, 2013; Stalder and Antonny, 2013). The GEFs of the small GTPase ADP Ribosylation Factor (ARF) and also Ras-related C3 botulinum toxin substrate (RAC) control ARF and RAC activity thus regulating cytoskeletal changes leading to lamellipodia formation in a manner that is PI 3-K-dependent (DiNitto et al., 2007; Stalder and Antonny, 2013).   Regulation of cytoskeletal dynamics by PI 3-K is also mediated by Tandem PH-domain containing Proteins 1/2 (TAPP)1/2, that contain both amino-

terminal and carboxyl-terminal PH domains (Hogan et al., 2004). In addition, General

Receptor for 3-Phosphoinositides 1 (GRP1) is the GEF that modulates the activation of

ARF6, whereby the PH domain is critical for GRP1 localization in the activation of ARF6,

leading to Glut4 vesicle formation and metabolic homeostasis (Langille et al., 1999).

Therefore, effectors of PI(3,4)P2 and PI(3,4,5)P3 other than Akt and PDK-1 modulate a

large network of downstream secondary signal relay pathways that in turn control

numerous cellular processes from metabolism to growth and cell motility. However, it is

generally considered in the field that many other effectors of PI 3-K exist in cells that

respond to PI(3,4)P2 and PI(3,4,5)P3, and ascribing phenotypes linked to PI 3-K in

pathophysiology to specific effectors remains a challenge in the field.

Oncogenic PI 3-K Pathway Mutations

The functional importance of PI 3-K signaling in cancer is most obviously evident by

the number of oncogenic mutations that exist in genes that encode proteins that both

regulate PI 3-K, PI 3-K itself and effectors of PI 3-K. *PIK3CA* is one of the most

frequently mutated genes in breast cancer, with a mutation rate approximating 35%

especially in hormone receptor positive breast tumors (Yuan and Cantley, 2008). The

majority of *PIK3CA* mutations exist in two hotspot regions of exon 9 and exon 20, within

the helical and catalytic domains, respectively. E542K, E545K, and H1047R mutations

account for 80% of the *PIK3CA* mutations in breast cancer (Courtney et al., 2010; Miller

et al., 2011). These hotspot mutations are gain-of-function mutations that cause

hyperactivation of p110α and downstream signaling by alleviating the inhibitory effects

of the SH2 domain of p85 under basal conditions. *PIK3CA* mutations are also frequent in other solid tumors, including endometrial carcinoma and colon carcinoma. Somatic mutations in Akt genes have also been identified, albeit at lower frequency that *PIK3CA* mutations. The Akt1 E17K mutation is oncogenic and promotes constitutive Akt signaling in the absence of growth factors (Brugge et al., 2007). This mutation at E17 within the PH domain of Akt causing hyperactivation of the kinase, likely due to reduced specificity PI (3,4,5)P3 and PI(3.4)P2 such that the enzyme can interact with the more abundant PI(4,5)P2. In breast cancer Akt1 E17K mutations have been identified at a frequency of 1.5-8%, depending on the study (Yuan and Cantley, 2008). A recent whole cancer genome sequencing study identified an Akt2 E17K mutation in one patient, and an Akt3 E17K mutation has been described in the metastatic setting of a breast cancer patient. Interestingly, E17K mutations in Akt3 appear to occur with high frequency in melanoma (Davies et al., 2008).

   PTEN inactivation is the most common PI 3-K pathway mutation across combined cancer subsets, with an inactivation frequency in breast cancer of approximately 30% (Miller et al., 2011). *PTEN* inactivation leads to hyperactivation of the PI 3-K pathway since the major mechanism of PI 3-K signal termination, PI(3,4,5)P3 dephosphorylation, is absent. However, even in the context of *PTEN* inactivation, PI(3,4,5)P3 must still be synthesized, and generally *PIK3CA* mutations and *PTEN* inactivation are not synonymous, at least in breast cancer. Instead, studies have shown that the primary route of PI(3,4,5)P3 synthesis in PTEN-deficient tumors is the *PIK3CB* isoforms (Wee et al., 2008). Ultimately, elevated levels of PI(3,4,5)P3 lead to constitutive downstream Akt

activation and substrate phosphorylation. *PTEN* inactivation occurs via distinct epigenetic and somatic mechanisms including missense mutations, truncation, homozygous and heterozygous deletion, and epigenetic silencing. Germline *PTEN* mutations lead to hamartoma syndromes of Cowden disease, Bannayan-Riley-Ruvalcaba syndrome and (Hobert and Eng, 2009; Song et al., 2012).

Since p21 Ras binding to p110α controls PI 3-K activation, Ras mutations contribute to pathway activation. Ras mutation across all cancer occur prominently at a frequency of approximately 30% (Adjei, 2001). In breast cancer, Ras mutations are relatively infrequent but nonetheless significant with about 5% hyperactivation across all molecular subtypes (Miller et al., 2011). PI 3-K is critical for tumor initiation in Ras-mutant tumors, however, established tumors show reduced dependence on PI 3-K. Pan-PI 3-K inhibitors alone may not be sufficient to abrogate late stage Ras-mediated tumorigenesis, but may underscore a potential preventative opportunity.

Since Class 1 PI 3-Ks are activated downstream of RTKs, amplification of these receptors also leads to PI 3-K pathway hyperactivation in solid tumors. The Human Epidermal growth factor Receptor 2 (HER2/ ERBB2) is a member of the ErbB family of receptors and overexpression or amplification of HER2 is detected in approximately 20% of all breast cancers (Yuan and Cantley, 2008). HER2 amplification leads to hyperactivation of not only PI 3-K but also PKC, ERK, Phospholipase C γ (PLCγ) and Signal Transducer and Activator of Transcription (STAT) pathways (Masuda et al., 2012). RTK amplification therefore leads to widespread deregulation and uncontrolled cell growth, making them a logical focus for therapeutic intervention. HER2 is the target

for monoclonal antibody drugs such as Herceptin and Pertuzumab (Rexer and Arteaga, 2013).

The Inositol Polyphosphate Phosphatase INPP4B

The importance of INPP4B in PI 3-K signaling was recently appreciated since inactivation of this putative tumor suppressor leads to elevated PI(3,4)P2 levels and consequent Akt hyperactivation, leading to increased cell proliferation and tumor initiation (Gewinner et al., 2009). The same studies also highlighted the fact that INPP4B is lost by genetic inactivation in basal-like/triple negative breast cancer (Fedele et al., 2010; Gewinner et al., 2009). Breast cancer is subdivided into molecular subtypes with distinct profiles: triple-negative breast cancer (TNBC) or basal-like, HER2 positive, Luminal A and Luminal B. TNBC lack estrogen receptor (ER), progesterone receptor (PR), and HER2 (Patani et al., 2013). Basal-like breast cancers are further distinguished from triple negative subtype by immunohistochemical or gene expression analysis establishing basal biomarkers such as Epidermal Growth Factor Receptor (EGFR) and/or basal cytokeratins (CK5/6, CK14, CK17) (Patani et al., 2013). Basal-like breast cancers also typically display a high mitotic index and histological grade, and are associated with more aggressive tumors. HER2+ve breast cancers overexpress HER2, but not ER or PR. Luminal subtype A breast cancers express ER and/or PR but not HER2 and have low Ki67, while Luminal B cancers express ER and/or PR and maintain high Ki67 (Patani et al., 2013). TNBC accounts for 15% of all breast cancer and are associated with poor prognosis. 55% of TNBC and 88% of basal-like carcinomas

display LOH of INPP4B (Fedele et al., 2010; Gewinner et al., 2009). These findings argue that LOH of INPP4B is restricted to ER-ve tumors. Furthermore, INPP4B loss is seen in 49% of *PTEN*-null tumors (Fedele et al., 2010). Therefore, the correlation of INPP4B LOH combined with *PTEN* inactivation status implies that the concurrent loss of both phosphatases leads to hyperactivation of Akt in aggressive basal-like or TNBC, such that these tumors may particularly benefit PI 3-K-directed therapies.

The tumor suppressor activity of INPP4B has been proposed to be due to its ability to dephosphorylate PI(3,4)P2 into PI(3)P, such that *INPP4B* LOH causes constitutive elevated levels of PI(3,4)P2 (Gewinner et al., 2009). Although two isoforms exist, Inositol Polyphosphate-4-Phosphatase type I (INPP4A) and INPP4B. INPP4A is exclusively localized to neuronal tissues with no evidence of functional expression in cancer (Fedele et al., 2010). By contrast, INPP4B is defined as a tumor suppressor in breast, ovarian, prostate carcinomas and melanomas (Agoulnik et al., 2011). INPP4B inactivation correlates with poor prognosis for long-term survival and highly metastatic tumor potential. Interestingly, additional associations for INPP4B are documented in breast cancer, whereby 60% of cancers with *BRCA1* germline mutations also have *INPP4B* LOH (Gewinner et al., 2009). One study has shown that INPP4B knockdown with shRNA in breast cancer cells *in vitro* induces anchorage-independent cell growth (Fedele et al., 2010). Moreover, INPP4B overexpression in xenograft models inhibits the growth of *BRCA1* mutant cells (Gewinner et al., 2009).

Since *INPP4B* is lost by LOH with high frequency TNBC/basal like cancers, INPP4B expression has been proposed as a biomarker for Luminal A and Luminal B

subtypes. Specifically, a statistically significant expression of INPP4B in ER+ve has been described (Fedele et al., 2010). Moreover, ER has been demonstrated to mediate the transcriptional induction of *INPP4B (Fedele et al., 2010).* Importantly, *PIK3CA* oncogenic mutations are mutually exclusive with *INPP4B* LOH, corroborating previous studies identifying 47% of ER+ve breast cancer tumors as *PIK3CA* mutant (Miller et al., 2011). INPP4B expression correlates with 75% of breast cancers that express ER and/or PR (Miller et al., 2011).

2. SERUM AND GLUCOCORTICOID-REGULATED KINASES

Serum and Glucocorticoid-Regulated Kinase 1 (*SGK1*) was initially identified in a rat mammary tumor cell line as a glucocorticoid-induced gene (Tessier and Woodgett, 2006b). SGKs are also in the AGC kinase family and comprise three isoforms each encoded by distinct genes: SGK1, Serum and Glucocorticoid-Regulated Kinase 2 (SGK2) and Serum and Glucocorticoid-Regulated Kinase 3 (SGK3) (**Figure 1-4**). SGK2 and SGK3 were cloned and characterized due to high sequence similarity to SGK1. SGK1 and SGK3 isoforms are ubiquitously expressed, unlike SGK2, which is restricted to the liver, kidney, pancreas, and brain (Tessier and Woodgett, 2006b). SGK isoforms share a combined amino acid sequence identity of ~80% within the catalytic domains and ~50% within the carboxyl-terminal region. SGK isoforms share an overall ~50% homology with the catalytic domains of Akt isoforms. Structurally, three SGK isoforms diverge from one another at the amino-terminus. Specifically, the amino-terminus of SGK1 localizes it to the endoplasmic reticulum for subsequent degradation. SGK2 on the other hand has a blunted amino-terminus with no known functions. SGK3 has a Phox Homology (PX) domain that binds exclusively to PI(3)P (Tessier and Woodgett, 2006a). SGK isoforms share the same substrate consensus phosphorylation motif as Akt, RXRXXS/T (Murray et al., 2004). The only known distinction between the Akt and SGK consensus motifs is the +1 hydrophobic residue that is exclusive to Akt. To date, there is only one SGK substrate that is exclusive to the SGK family and is not targeted by Akt isoforms, N-myc Down-Regulated Gene (NDRG)1 (Tessier and Woodgett, 2006b). NDRG1 was originally identified as an SGK substrate using the KESTREL
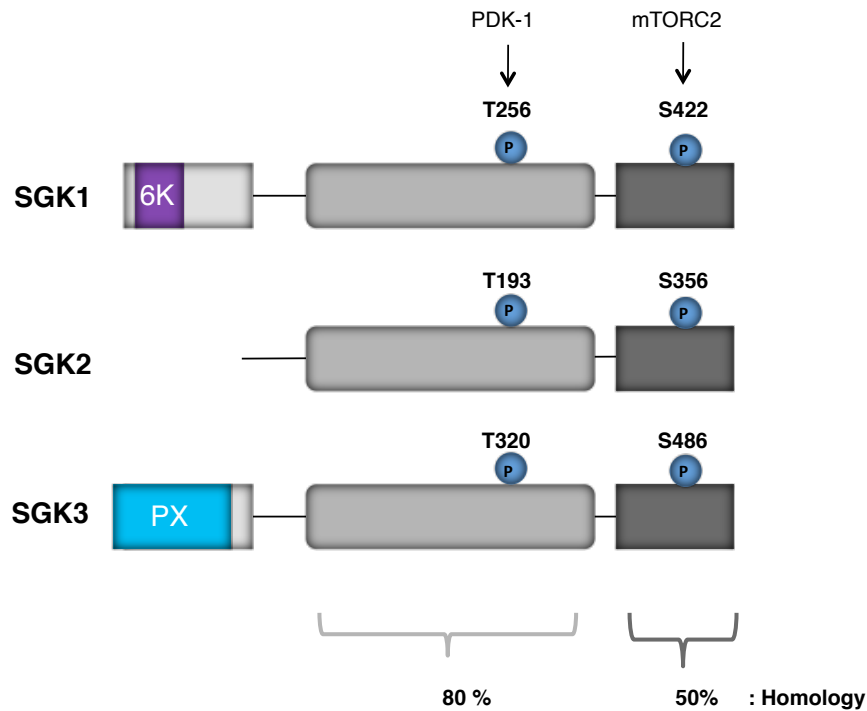
Figure 1-4: Serum and Glucocorticoid-regulated Kinase Isoforms

SGK family isoforms share ~80% sequence homology in their catalytic domains and ~50% sequence homology in their C-terminal region (Tessier and Woodgett, 2006b). They diverge in their N-terminal regions where SGK3 possesses a PX domain and SGK1 has 6 N-terminal Lysines (K) that target it for polyubiquitination and degradation by the 26S proteasome. SGK isoforms have two phosphorylation sites required for maximal activation: PDK-1 regulated catalytic domain site (SGK1, T256; SGK2, T193; SGK3, T320) and Hydrophobic motif site regulated by MTORC2, only formally demonstrated (SGK1, S422).

technology by the Cohen laboratory (Murray et al., 2004). SGK isoforms also phosphorylate known Akt substrates such as FOXO3A, Glycogen Synthase Kinase β (GSK-3β), Raf, Neural precursor cell Expressed Developmentally Down-regulated protein 4-2 (NEDD4-2), and TSC2, highlighting redundancy in Akt and SGK downstream signaling mechanisms (Tessier and Woodgett, 2006b). SGK knockout mice have yielded little information regarding physiological functions, although SGK1 knockout mice have sodium balance intolerances, and SGK3-null mice have defective hair follicle development (Lang et al., 2006; McDonald, 2008). Double knockouts have not yet been made and SGK2 null mice have also not been generated.

Interestingly, SGK1 is the only SGK isoform induced by serum and/or glucocorticoids, such that the name 'SGK' is somewhat of a misnomer. A variety of other mechanisms lead to transcriptional induction of SGK1, including p53, Transforming Growth Factor β (TGFβ), Fibroblast-derived Growth Factor (FGF), Platelet-derived Growth Factor (PDGF), Follicle Stimulated Hormone (FSH), hyperosmotic stress, hypoxia, heat shock, UV irradiation and DNA damage (Tessier and Woodgett, 2006b). SGK1 expression is very transient and this underlies its mechanism of regulation. SGK1 is poly-ubiquitinated by NEDD4-2 and Carboxyl terminus of HSC70-Interacting Protein (CHIP) E3 ligases, leading to degradation by the 26S proteasome (Brickley et al., 2002; Zhou and Snyder, 2005). The degradation of SGK1 is controlled through the amino-terminus in which amino acids 18-24 direct SGK1 to the endoplasmic reticulum following poly-ubiquitinatination of six lysine residues within this domain.

In breast cancer, SGK1 expression correlates significantly with pAkt levels, however there is no correlation with tumor grade or ER status. Studies have revealed that 40% of tumors overexpress SGK1 and concurrently elevated pAkt1 at S473 (Sahoo et al., 2005). Immunohistochemical analysis reveals that 48% of breast tumors overexpress SGK1 (Sahoo et al., 2005). Other studies have documented approximately 75% SGK1 overexpression in breast cancer using tissue microarrays (Zhang et al., 2005). However, no studies have to date examined SGK1 activity in human tumors, primarily due to the lack of available tools to accurately determine kinase SGK activity. However, a recent study by the Alessi laboratory identified SGK1 as an alternate pathway for breast cancers treated with Akt inhibitors (Sommer et al., 2013). In this study a significant correlation is detected in cells that overexpress SGK1 and are also resistant to Akt inhibitors. Additionally, these cell lines reveal high phosphorylation levels of NDRG1, a surrogate for high SGK activity (Sommer et al., 2013). Proliferation is also significantly reduced in Akt inhibitor-resistant cells upon SGK1 knockdown, highlighting a novel role for SGK1 as a therapeutic target in breast cancer.

Serum and Glucocorticoid-regulated Kinase Activation Mechanisms

SGK isoforms share the same upstream kinases with Akt, PDK-1 and mTORC2. As with Akt, both PDK-1 and mTORC2 phosphorylation are required to maximally activate SGK (Garcia-Martinez and Alessi, 2008; Kobayashi et al., 1999). mTORC2 phosphorylates the carboxyl-terminal HM of SGK1(Ser422) (Garcia-Martinez and Alessi, 2008). SGK2 and SGK3 are also likely mTORC2 substrates for the HM at Ser356 and

Ser486, respectively, although this has not formally been proven. PDK-1 has been shown to phosphorylate the activation-loop of all SGKs (SGK1, Thr256; SGK2, Thr193; SGK3, Thr320) (Kobayashi et al., 1999).   However, there are some clear distinctions from Akt in the mechanism of regulation of SGK isoforms. For example, the PI 3-K-dependence for SGK1 activation lies with the PDK-1 phosphorylation of the activation-loop phosphorylation. In this context, the With No lysine (K) 1 (WNK1) kinase is required for efficient PDK-1 docking and phosphorylation of SGK1 (Xu et al., 2005). WNK1 Thr58 phosphorylation is required for the correct confirmation of WNK1 to serve as scaffold, whereas its kinase domain is dispensable for SGK1 activation. Interestingly, Akt and SGK both phosphorylate WNK1 at Thr58 indicative of a possible feedback loop to promote SGK1 activity (Xu et al., 2005). Although the precise mechanism by which PI 3-K contributes to SGK2 and SGK3 activation has yet to be defined, PI 3-K inhibitors to block SGK2 and SGK3 activity in cell-based assays (Kobayashi et al., 1999). In the case of SGK3, studies have shown that a functional PX domain and PI 3-K are necessary critical for maximal SGK3 phosphorylation at Thr320 and Ser486, as well as SGK3 catalytic activity (Tessier and Woodgett, 2006a). To this end, a point mutation (Arg90Ala) in the SGK3 PX domain which abolishes PI(3)P binding completely blocks SGK3 phosphorylation, indicating that both PI(3)P binding and PI 3-K signaling converge to activate this SGK isoform (Tessier and Woodgett, 2006a).

Serum and Glucocorticoid-regulated Kinase 3 Regulation

A recent study from the Garraway laboratory demonstrated that SGK3 can function to mediate Akt-independent signaling and cell proliferation in cells harboring oncogenic *PIK3CA* mutations (Vasudevan et al., 2009). This is relevant since studies have shown that a significant proportion of breast cancers are resistant to Akt inhibitors even in the context of oncogenic PI 3-K signaling. In the Garraway study, cell lines that display low levels of Akt phosphorylation have elevated SGK3 activity. Furthermore, breast cancer cell lines that show little or no dependence on Akt for anchorage-independent growth instead require SGK3 activity for proliferation and survival (Vasudevan et al., 2009). However, since SGK3 does not harbor a functional PH domain capable of sensing PI(3,4,5)P3 and PI(3,4)P2, the question remains how *PIK3CA* can utilize SGK3 as a bona fide effector.

As discussed above, the PX domain of SGK3 binds PI(3)P and studies have shown that this event localizes SGK3 primarily to endosomal membranes that are enriched for this lipid. Vacuolar Protein Sorting 34 (VPS34), the only Class III PI 3-Kinase, is thought to be largely if not exclusively responsible for the generating the pool of PI(3)P at the endosome (Backer, 2008). Importantly, as with Akt, SGK3 activity loses the PX domain and PI(3)P dependence after phosphorylation of the HM (Tessier and Woodgett, 2006a). Additionally mutation of the PDK-1 Interacting Fragment (PIF) pocket of SGK3 attenuates Thr320 phosphorylation by PDK-1 (Tessier and Woodgett, 2006a). This indicates that PDK-1 requires the PIF pocket to dock within the HM of SGK3, as with other AGC family kinases such as S6K1/2. Furthermore, PI 3-K inhibitors combined

with PDK-1 overexpression does not inhibit the SGK3 Thr320 phosphorylation, suggesting that SGK1, SGK3 is not PI 3-K-dependent due to stabilization by PDK-1 docking. Instead, it is more likely that SGK3 depends upon PI 3-K for HM phosphorylation mediated by mTORC2, or for its localization and substrate interactions.

Although the *SGK3* gene is not regulated by glucocorticoids, it is transcriptionally upregulated by ER (Wang et al., 2011a). SGK3 protein levels correlate with ER status in both breast cancer cell lines and tumor samples, and SGK3 has been shown to induce estrogen-mediated survival of breast cancer cells (Wang et al., 2011a; Xu et al., 2012). The transcriptional induction by ER is of particular interest in Luminal subtype A and Luminal subtype B breast cancers, since approximately 75% breast cancers are ER+ve and thus likely also display elevated SGK3.

3. N-MYC DOWN-REGULATED GENE 1

N-myc down-regulated gene (*NDRG*) represents a family of four members encoded by distinct genes that share ~50% sequence homology: NDRG1, NDRG2, NDRG3 and NDRG4 (**Figure 1-5**). The NDRG family is categorized within a subgroup of α/β hydrolase enzymes, although NDRG isoforms lack any hydrolytic catalytic activity (Melotte et al., 2010). NDRG isoforms contain an NDR domain and a non-functional α/β hydrolase fold. No enzymatic activity of any kind has been ascribed to NDRG isoforms. NDRG1 is ubiquitously expressed in all human tissues, although elevated levels are detected in epithelial tissues (Sun et al., 2013a). NDRG2 is primarily expressed in the brain, heart, spinal cord, and skeletal muscle. NDRG2 and NDRG4 isoforms display multiple splice variants and expressed exclusively in the brain and heart. Interestingly, NDRG3 is expressed in all tissues except brain and heart (Melotte et al., 2010).

NDRG1 is the only isoform that contains a carboxyl-terminal tandem 3X decapeptide repeat (Melotte et al., 2010). Within each decapeptide are three SGK phosphorylation sites (Thr346, Thr356, Thr366) that are not phosphorylated by Akt. Each SGK site primes NDRG1 for phosphorylation by GSK3β at the -4 residue (Ser342, Ser352 and Ser362) (Murray et al., 2004). Therefore, in order to phosphorylate NDRG1, GSK3β requires prior NDRG1 phosphorylation by SGK3. The overall consequence of these phosphorylation events at the cellular level remains to be determined. The GSK3β and SGK3 sites in NDRG1 are conserved across species and to *Danio rerio.* (**Figure 1-6**) Although NDRG1 is primarily cytoplasmic, some studies show that NDRG1 can be recruited to the plasma membrane, endosomes, mitochondrial membranes, and the
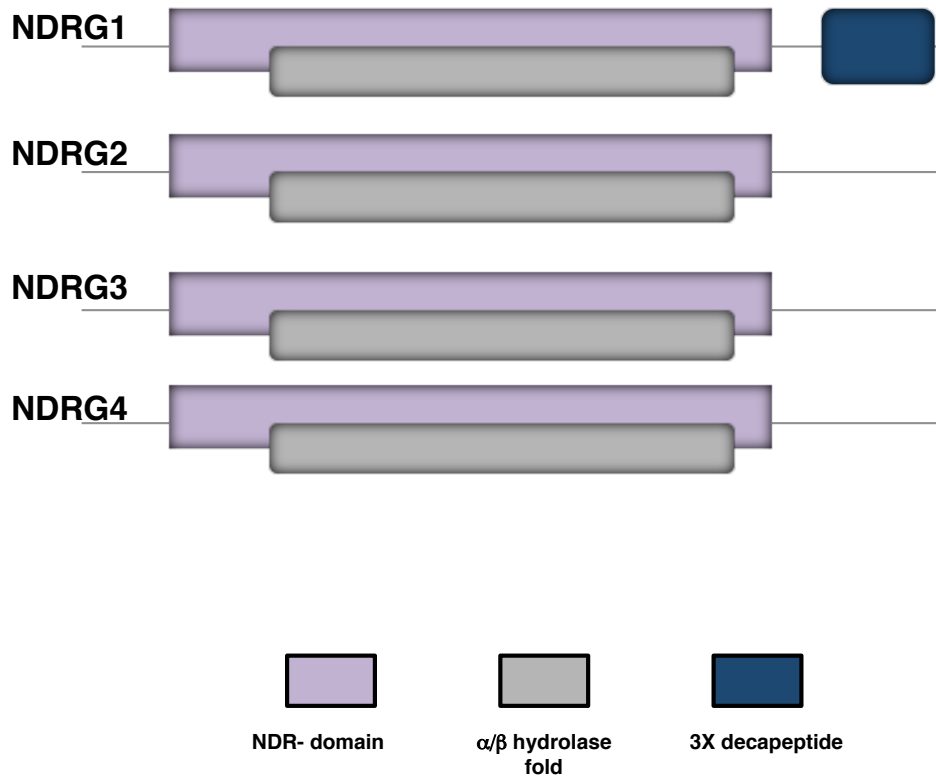
Figure 1-5: NDRG family isoforms

NDRG isoforms 1-4 share common N-myc Down-Regulated (NDR) domains and non-functional a/b hydrolase domains. NDRG1 has a unique C-terminal 3X decapeptide repeat.

```
Homo sapiens           321: trlmrsrtas gssvtsldgt rsrshtsegt rsrshtsegt  rsrshtsega
Mus musculus           321: trlmrsrtas gssvtslegt rsrshtsegp rsrshtsegs  rsrshtseda
Xenopus tropicalus     321: trlmrsrtgs aassssqdgn rsrshtnegs rsrshtgdgn  rsrahtgdgn
Danio rerio            307: trlvrsrtas gssitsfdgn rsrshtnegn rsrshtneg-  rarahtsenq
```

Figure 1-6: NDRG1 3X decapeptide conservation

NDRG1 3X decapeptide repeat is conserved from *Homo sapiens* to *Danio rerio.* The decapeptide repeat is highlighted in grey and SGK substrate phosphorylation motif is written in red.

nucleus (Sun et al., 2013a). Although NDRG1 has no established nuclear localization

sequence, it is known to associate with transport proteins such as Heat-Shock Cognate

Protein 70 (HSC70) (Sun et al., 2013a). Moreover, membrane-associated NDRG1 is

localized to adherens junctions, indicating a possible interaction with the cytoskeletal

components that cooperate with these junctions.

In addition to post-translational modification, NDRG1 is regulated at the

transcriptional level. NDRG isoforms possess a 5' CpG island within their promoter

regions suggesting their expression is regulated by DNA methylation (Melotte et al.,

2010). Oncogenes such as N-neuroblastoma-derived myelocytomatosis (N-myc) and

Cellular V-myc myelocytomatosis viral oncogene homolog (C-myc) down-regulate

NDRG expression, hence their gene nomenclature. In addition, NDRG1 is regulated by

a wide range of other cellular factors and extracellular stimuli and agents, including

Hypoxia-Inducible Factor 1 (HIF1), p53, nickel, cobalt, vitamin A, vitamin D, steroids,

heavy metal chelators, and oxidative stress (Melotte et al., 2010).

NDRG1 Regulation During the Epithelial-Mesenchymal Transition

NDRG1 has been documented to function as a metastasis suppressor (Sun et al.,

2013a). In this context, initial studies identified a role for NDRG1 in the vesicular

recycling of E-cadherin (Kachhap et al., 2007). NDRG1 interacts with membrane-bound

Rab4a GTPase at the recycling endosomes of the Trans Golgi network to shuttle E-

cadherin to the plasma membrane, thereby stabilizing the adherens junction complex

(Kachhap et al., 2007). In turn, stabilization of the actin cytoskeleton leads to decreased

cell motility and reduced invasive migration. The elevated levels of E-cadherin at adherens junctions also reduce the rate of Epithelial- Mesenchymal Transition (EMT), a critical step in the progression to metastasis (**Figure 1-7**).

NDRG1 has been shown to modulate EMT by promoting the down-regulation of the TGFβ signaling pathway (Chen et al., 2012). For example, NDRG1 suppresses the expression of TGFβ targets Snail, Slug and Smad leading to suppression of EMT and metastasis. NDRG1 has also been implicated in inhibiting metastasis through Wingless-related integration site (Wnt) signaling, by binding the Low-density Lipoprotein Receptor-related Protein 6 (LRP6), a coreceptor for the canonical Wnt pathway (Liu et al., 2012). NDRG1 binding blocks Wnt downstream signaling, thus preventing accumulation and nuclear translocation of β-catenin. The nuclear entry of β-catenin upregulates the activity of the T-Cell Factor (TCF) family of transcription factors. TCF family members induce the transcription of oncogenic proteins such as Myc, Cyclin D, Cluster of Differentiation 44 (CD44), and Matrix Metalloproteinase 7 (MMP7) that promote cell cycle progression, tumor growth, and cancer metastasis (Liu et al., 2012).

Clinical Significance of NDRG1 Expression and Function

NDRG1 protein and mRNA levels have been found to be decreased in breast, prostate, colon, and glioma cancers relative to normal tissue (Sun et al., 2013a). Therefore, there is increasing evidence that supports NDRG1 as a tumor suppressor and/or a metastasis suppressor. In breast tumors, an inverse correlation in NDRG1 expression and ERα status has been documented (Sun et al., 2013a). Similarly, ectopic
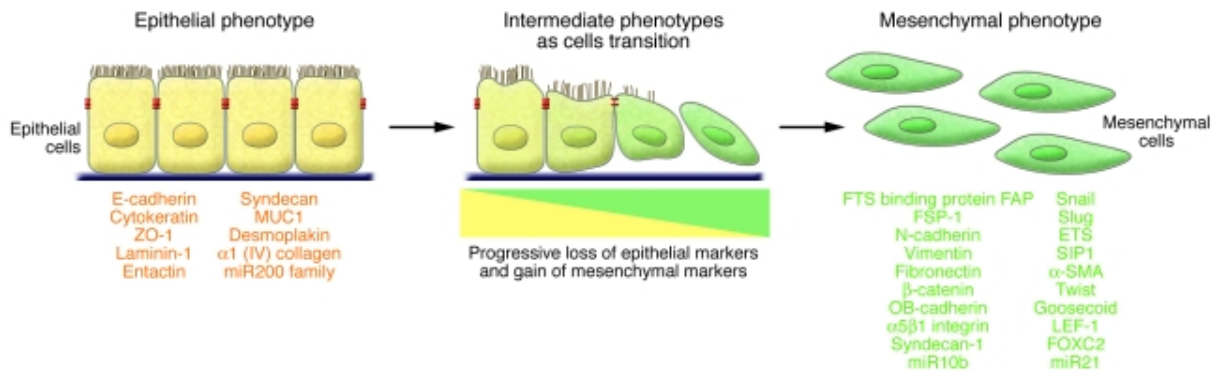
Figure 1-7: EMT

An EMT involves a functional transition of polarized epithelial cells into mobile and ECM component–secreting mesenchymal cells. The epithelial and mesenchymal cell markers commonly used by EMT researchers are listed. Colocalization of these two sets of distinct markers defines an intermediate phenotype of EMT, indicating cells that have passed only partly through an EMT. Detection of cells expressing both sets of markers makes it impossible to identify all mesenchymal cells that originate from the epithelia via EMT, as many mesenchymal cells likely shed all epithelial markers once a transition is completed. For this reason, most studies in mice use irreversible epithelial cell–lineage tagging to address the full range of EMT-induced changes. ZO-1, zona occludens 1; MUC1, mucin 1, cell surface associated; miR200, microRNA 200; SIP1, survival of motor neuron protein interacting protein 1; FOXC2, forkhead box C2. Taken from (Kalluri and Weinberg, 2009).

expression of ERα in ERα negative cells induces down-regulation of NDRG1 and suppression of NDRG1 leads to increased invasive migration that can be rescued by reintroduction of NDRG1.

In a study of 240 colorectal cancers, NDRG1 levels inversely correlated with tumor grade, metastatic lesions, and differentiation status (Mao et al., 2013). Importantly, NDRG1 expression is a positive prognostic marker for long-term survival at 5+ years. Similarly in neuroblastoma patients, decreased NDRG1 mRNA is linked to poor prognosis and metastastatic dissemination (Matsushita et al., 2013). Similarly, in hepatic cancers cells NDRG1 suppresses growth though cell cycle arrest, and loss of NDRG1 in ovarian carcinoma cells leads to enhanced invasive migration (Zhao et al., 2011). In pancreatic cancer NDRG1 is a positive prognostic marker for long-term patient survival, and loss of NDRG1 expression in pancreatic cancer is indicative of invasive tumors with high levels of angiogenesis and overall tumor growth. In this model, rescued expression of NDRG1 leads to suppression of angiogenic phenotypes and reduced tumor growth via suppression of NFκB signaling (Hosoi et al., 2009).

More recently, epigenetic regulation of NDRG1 through a previously undocumented microRNA pathway has been identified. miR 182 promotes prostate cancer proliferation, growth, and invasion through the down-regulation of NDRG1 (Liu et al., 2013). The microRNA binds the 3' untranslated region of NDRG1 leading to its suppression. Thus, the dynamic changes in NDRG1 expression and its potential function as a regulator of EMT and metastasis make it an attractive therapeutic target. In this context, iron chelators have been proposed as a possible chemotherapeutic

strategy due to their ability to induce NDRG1 expression (Sun et al., 2013b). Iron

chelators are a new potential class of anti-metabolites. In colon adenocarcinoma iron

chelators inhibit EMT driven by TGFβ and Wnt pathways (Chen et al., 2012).

It is important to clarify that NDRG1 likely possess tissue-specific functions.

There is evidence of reciprocal function of NDRG1 in certain cancers. For example,

there are conflicting reports addressing the function of NDRG1 in gastric cancer,

whereby one study claims that NDRG1 is a suppressor of invasion while another

demonstrates that NDRG1 promotes angiogenesis and tumor growth (Liu et al., 2011;

Ureshino et al., 2012). Additionally, oral cancers display extensive correlations with

NDRG1 expression using combinatorial microarray analysis and IHC in 103 oral and

oropharyngeal squamous cell carcinomas (Dos Santos et al., 2012). Furthermore, lung

cancers also show positive correlation with NDRG1 expression in tumors and absence

in normal non-neoplastic tissue (Wang et al., 2013). NDRG1 expression in lung cancers

promotes angiogenesis and predicts poor prognosis.

4. THE UBIQUITIN PROTEASOME SYSTEM

The Ubiquitin Proteasome System (UPS) is a central node of regulation monitoring protein levels of virtually all signaling networks in all cells and tissues. The UPS controls the abundance of proteins that are critical mediators of metabolism, cell cycle progression, cytoskeletal changes, motility, survival and proliferation. Intracellular protein levels cell are regulated by the concerted action of UPS components: ubiquitin-activating enzymes (E1); ubiquitin-conjugating enzymes (E2); and ubiquitin-protein ligases (E3) (Lau et al., 2012). E1, E2, and E3 enzymes cooperate to poly-ubiquitinate proteins leading to their degradation by the 26S proteasome (**Figure 1-8**). E3 ligases are the enzymes responsible for protein target selection and thus the diversity of the UPS. E3 ligases function as the substrate recognition domain of the UPS system to recognize and bind substrates intended for degradation. There are approximately 1000 E3 ligases in the human proteome allowing sufficient specificity to regulate the entire network of intracellular proteins (Lau et al., 2012). Additionally, phosphorylation or hydroxylation is often required to promote E3 ligase binding. A post-translational modification prerequisite facilitates precise modulation of the spatiotemporal expression of proteins throughout the cell cycle. E1 enzymes activate ubiquitin through an ATP-dependent process where the E1 enzyme forms a thiol ester bond with an ubiquitin molecule. This activated form of ubiquitin is next transferred to the E2 molecule, which facilitates transfer of the ubiquitin to the substrate through E2 interaction with E3 ligases. The carboxyl-terminus of ubiquitin is bound to the epsilon-amino group of a lysine on the substrate (Lau et al., 2012).
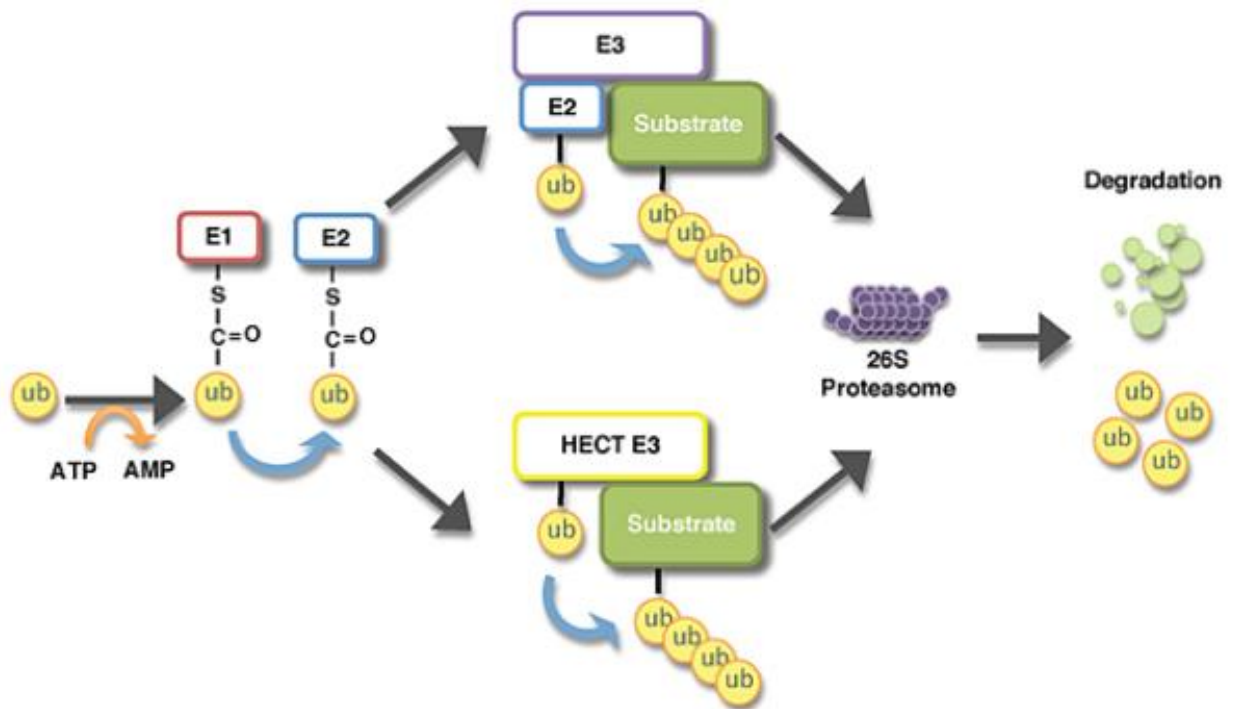
Figure 1-8: Ubiquitin Reaction Pathways

Initially, the E1 enzyme activates, in an ATP-dependent manner, the 76 amino acid ubiquitin molecule by forming a high-energy thiol ester bond with ubiquitin. This activated molecule is then transferred to the E2 conjugating enzyme. The E3 ligase then positions the target substrate near the E2 enzyme allowing for the transfer of ubiquitin. In the case of HECT E3 ligases however, the E3 ligase directly transfers the ubiquitin molecule onto the target substrate. Once a chain of four or more ubiquitin molecules is placed onto the substrate protein, the molecule is then targeted for proteolysis by the 26 proteasome. Taken from (Lau et al., 2012).

A single ubiquitin bound to a substrate is characterized as monoubiquitination.   A monoubiquitination can either lead to polyubquitination or it can instead label a protein for vesicular trafficking, DNA repair, or endocytosis. There are 7 lysines in the 76 amino acid ubiquitin sequence that can participate in ubiquitin chain elongation. Lys48 is the most well studied acceptor site leading to polyubiquitination. A chain of 4 or more Lys48 linked ubiquitins targets the substrate protein for proteasomal degradation by the 26S proteasome (Fasanaro et al., 2010). Alternately, Lys63-linked polyubiquitination targets the substrate for non-proteolytic cellular processes such as DNA repair, endocytosis, inflammation, and translation. Lys6, Lys11, Lys27, Lys29, and Lys33 polyubiquitination are less well studied and are not comprehensively understood (Zhang and Sidhu, 2013). Likewise, branched ubiquitination chains with multiple forms of linkage are known to exist, but their cellular functions are unclear.

De-Ubiquitinating Enzymes

Ubiquitination is a bi-directional system in which De-Ubiquitinating enzymes (DUBs) can reverse the E1-3 coordinate ubiquitin tagging by cleaving the ubiquitin moiety from the protein substrate (Fasanaro et al., 2010). DUB activity may terminate non-proteolytic functions and rescue polyubiquitinated substrates from proteolysis through the removal of the ubiquitin chain. Additionally, DUBS may recycle ubiquitin from substrates undergoing proteolysis through the removal of ubiquitin moieties as the substrate enters the 26S proteasome. DUB functions are completed by 5 subgroups of enzymes that include Jab1, Mov34, MPR1 Pad1 N-Terminal (JAMM)/(MPN+)

metalloproteases, Josephins, Ovarian tumor-like proteases (OTUs), ubiquitin C-terminal hydrolases (UCHs), and ubiquitin-specific proteases (USPs) (Zhang and Sidhu, 2013). Josephins, OTUs, UCHs, and USPs are cysteine protease DUB groups, whereas JAMM is the only identified metalloprotease subgroup.

The 26S proteasome

The 26S proteasome is the most common pathway for protein degradation in eukaryotes. Each 26S proteasome consists of one 20S core subunit and two additional 19S subunits. The 20S core is assembled using 7α subunits (α1-7) and 7 β subunits (β1-7) (Jung and Grune, 2013). In the 'modern' or eukaryotic form, the 20S core is composed of 4 stacked rings. The two outer rings each consist of 7α subunits. The α subunits serve structural roles and allow regulatory proteins to dock in addition to performing gating functions with their amino-terminal region to restrict inappropriate access to the core center. The two heptameric inner rings are composed of β subunits. The β1-3 subunits possess active protease sites and provide the catalytic activity of the 20S core (Jung and Grune, 2013). Alternatively, in mammals there are a set of β subunits inducible by TGFα and Interferon Gamma (IFNγ) for *de novo* synthesis of the 20S core (Jung and Grune, 2013). This 20S core can bind the 11S regulatory subunit. Together the 20S and 11S subunits can degrade short peptides but not entire proteins. The lack of ATP-binding and hydrolysis likely leads to the inability to unfold complete proteins.

The 20S core is only capable of independently degrading unfolded proteins. Damaged and misfolded proteins can be proteolytically degraded by the β subunits within the 20S core. In order to degrade folded proteins, the 20S core must bind the 19S subunits. The 19S regulatory subunits consist of a 9 subunit base that binds to the α heptameric ring and a 10 subunit lid. 6 of the 9 base subunits are ATPases Associated with diverse cellular Activities (AAA) ATPase units, which allow the binding of the 19S regulator to the 20S core(Bar-Nun and Glickman, 2012). ATP binding to the hexameric AAA ATPase subunits is required for 19S and 20S binding. ATP hydrolysis is only required for the unfolding of the substrate protein, but not for subunit interaction.

E3 LIGASES

The mechanism of E3 ligase ubiquitin-substrate conjugation is unique to E3 ligases. There are 3 primary families of E3 ligases, categorized as follows: Homologous to E6–associated protein C-Terminus (HECT); U-box; and Really Interesting New Gene (RING) domains (Fasanaro et al., 2010). HECT E3 ligases directly conjugate the ubiquitin moiety to the substrate. HECT E3s contain an active Cysteine residue that functions as an intermediary binding site to transfer the ubiquitin molecule to the substrate (Lau et al., 2012). U-box and RING E3 ligases are scaffolding proteins that facilitate the co-localization of the E2 conjugating enzyme and the substrate.

The SCF family of E3 ubiquitin ligases

SCF complex family of ligases is composed of the Cul1 scaffolding subunit, the Rbx RING subunit, the Skp1adaptor subunit, and an F-box protein that functions as the substrate recognition domain for the complex (Lau et al., 2012). The F-box protein varies in accordance with the substrate. The F-box subunit is the primary means to induce diversity in the recognition of substrates for proteolytic degradation. The SCF complex contributes to the regulation of cellular processes such as cell cycle progression, apoptosis, and lipid metabolism. The deregulation of the SCF complex can lead to carcinogenesis due to the aberrant signaling that stems from uncontrolled levels of pathway regulators.

F-box proteins interact with substrates through a carboxyl-terminal protein binding sequence (**Figure 1-9**). There are three categories of F-box proteins as defined by their substrate-binding sequence. F-box categories include WD40 repeats (FBXW), leucine rich repeats (FBXL) or other domains (FBXO) (Lau et al., 2012).   Most F-box proteins require post-translational modification of the substrate within a degron (phospho-degron) to promote substrate and F-box protein interaction.

FBW7

The protein structure of F-Box and WD repeat domain-containing 7 (FBW7) contains 8 C-terminal WD-40 repeats in addition to its WD-40 F-box motif and a D domain required for FBW7 dimerization (Cheng and Li, 2012). Within the substrate binding motif there
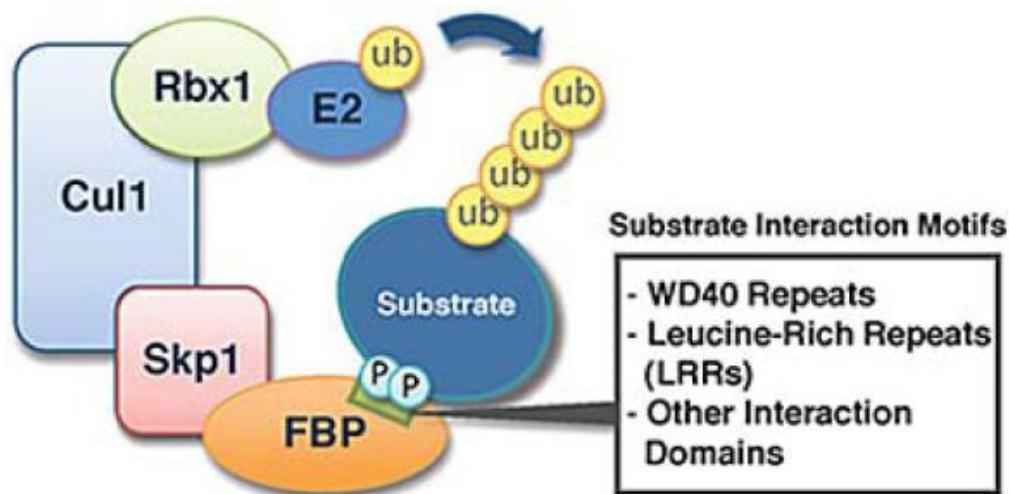
Figure 1-9: SCF Family of E3 ligases

An SCF-type E3-ligase is a multi-subunit complex consisting of three invariable subunits and one variable subunit. The three static subunits include a catalytic RING subunit (Rbx1) that interacts with the E2, a scaffolding subunit (Cul1), and an adaptor subunit (Skp1). The variable molecule is known as the F-box protein (FBP). The major function of the F-box protein is to recruit specific substrates to the E3 complex via substrate interaction domains. The two largest classes of interaction domains found on FBPs are WD40 repeats and leucine-rich repeats (LRRs). A third type of FBP also exists which contains neither WD40 repeats nor LRRs. This third class of F-box proteins contains other types of interaction domains or no recognizable domain at all. To date, there have been approximately 69 FBPs identified in the human genome. Furthermore, unlike HECT E3 ligases that can directly conjugate ubiquitin onto the target, SCF complexes bridge the interaction between the E2 enzyme and the substrate. Taken from (Lau et al., 2012).

are three sequential Arg residues that interact with phosphorylated target substrates.

The typical FBW7 phospho-degron is phosphorylated by GSK3β at a (L)-X-pT/pS-P-(P)-

X-pS/pT/E/D sequence (Lau et al., 2012). There are three amino-terminal splice

variants of FBW7 (α, β, γ) that display distinct tissue and cellular localization (Cheng and

Li, 2012). At the cellular level FBW7α is nucleoplasmic, FBW7β is cytoplasmic, and

FBW7γ is nucleolar. FBW7 has been characterized as a tumor suppressor in T-cell

Acute Lymphoblastic Leukemia (T-ALL) largely through its degradation of a large panel

of oncogenes. FBW7 regulation includes oncogenic substrates such as Cyclin E, C-

Myc, and Notch-1 (Wang et al., 2012). In 6% of all human primary tumors *FBXW7* is

mutated, 43% of those cases occur within a hotspot sequence existing in the RRR

phospho-degron binding region (Wang et al., 2012).   *FBWX7* mutations affect

numerous cellular processes and thus highlight the oncogenic potential of deregulation

in the UPS.

5. CONTRIBUTION OF THIS THESIS

This thesis focuses on Akt-independent PI 3-K signaling mechanisms. Chapter 2 identifies a previously unknown mechanism of PI 3-K-dependent activation of SGK3. My studies show that INPP4B promotes SGK3 activation via its conversion of PI(3,4)P2 to PI(3)P. This same event inhibits Akt signaling due to suppression of PI(3,4)P2. I thus propose that INPP4B is a signaling switch between Akt and SGK3 signaling downstream of PI 3-K. These findings indicate an oncogenic capacity for INPP4B, which as up until now been proposed to function exclusively as a tumor suppressor.

Chapter 2 also characterizes SGK3 and INPP4B tumorigenic phenotypes such as promotion of anchorage-independent growth and invasive migration under conditions of suppressed Akt activation. SGK3 regulates these phenotypes in part through phosphorylation of NDRG1, a known metastasis suppressor and SGK-specific substrate. SGK3 phosphorylation primes NDRG1 for GSK3β phosphorylation leading binding by FBW7. FBW7 is the substrate recognition domain of the E3 ligase SCF complex, which signals NDRG1 for proteolytic degradation by the proteasome. SGK3-induced NDRG1 degradation is a novel mechanism of PI 3-K-driven oncogenic signaling.

Chapter 3 examines SGK1 and the regulation of its stability in the context of PI 3-K. The data reveal that Rictor, a component of the mTORC2 complex, forms a complex with Cullin1 to function as an E3 ligase directing SGK1 polyubiquitination and proteolytic degradation. This is the first mTORC2-independent function of Rictor that has been identified. Additionally, an AGC phosphorylation site on Rictor1 is identified, and

phosphorylation of this site leads to the disassembly of this E3 complex that functions

as a feedback loop.   Chapter 3 elucidates a mechanism by which PI 3-K

hyperactivation can drive SGK1 overexpression. This SGK1 overexpression will then

lead to hyperactivation of its substrates perpetuating the aberrant pathway signaling.

CHAPTER II

SGK3 MEDIATES INPP4B-DEPENDENT PI 3-KINASE SIGNALING IN BREAST

CANCER

1. ABSTRACT

Oncogenic mutations in *PIK3CA*, the gene encoding the catalytic subunit of PI 3-K, occur with high frequency in breast cancer. The protein kinase Akt is considered to be the primary effector of *PIK3CA*, though the mechanisms by which PI 3-K mediates tumorigenic signals in an Akt-independent manner remain obscure. We show that the SGK3 is amplified in breast cancer and activated downstream of *PIK3CA* in a manner dependent on the phosphoinositide phosphatase INPP4B. Expression of INPP4B leads to enhanced SGK3 activation and suppression of Akt phosphorylation. Activation of SGK3 downstream of *PIK3CA* and INPP4B is required for 3D proliferation and invasive migration. We further show that SGK3 targets the metastasis suppressor NDRG1 for degradation by FBW7. We propose a model in which breast cancers harboring oncogenic *PIK3CA* and INPP4B expression activate SGK3 signaling while suppressing Akt, indicative of an oncogenic function for both INPP4B and SGK3 in these tumors.

2. INTRODUCTION

Somatic mutations, amplifications and other genetic lesions in genes that encode

proteins in the PI 3-K pathway play a critical role in breast cancer etiology and

progression by regulating phenotypes such as cell proliferation, survival and metastasis.

The importance of PI 3-K signaling is highlighted by identification of activating

oncogenic mutations of *PIK3CA*, the gene that encodes the p110α catalytic subunit of

class I PI 3-K. Oncogenic *PIK3CA* mutations are frequent in breast cancers, particularly

in estrogen receptor positive disease where approximately 40% of cases harbor one of

the two most frequent mutations, H1047R and E545K (Cancer Genome Atlas, 2012;

Engelman et al., 2006; Lee et al., 2005; Samuels et al., 2004). Class I PI 3-K activate

signaling cascades by generating the phosphoinositides PI(3,4)P$_2$ and PI(3,4,5)P3

(Manning and Cantley, 2007). Arguably the most studied and best understood effector

of PI 3-K is the serine/ threonine protein kinase Akt/protein kinase B (PKB). Activation of

Akt is initiated though interaction of the Pleckstrin homology (PH) domain with either

PtdIns-3,4-P$_2$ or PtdIns-3,4,5-P$_3$ (Chin and Toker, 2009; Franke et al., 1997; Woodgett,

2005). This is followed by phosphorylation of Akt by the PDK-1 and mTORC2 locking

the enzyme in the catalytically competent conformation (Mora et al., 2004; Sarbassov et

al., 2005).

Signal termination of PI 3-K and Akt signaling is mediated by PTEN, a tumor

suppressor protein that dephosphorylates PI(3,4,5)P3 converting it back to PI(4,5)P2 (Li

et al., 1997; Maehama and Dixon, 1998). LOH, inactivating mutations or deletions in

PTEN are frequent in many cancers, and lead to excessive PI(3,4,5)P3 accumulation

and hyperactivation of downstream effectors, including Akt (Engelman et al., 2006). An

alternative mechanism of negative regulation of the Akt pathway is through the SHIP

family of proteins that dephosphorylate PI(3,4,5)P3 and generate PI(3,4)P2, (Choi et al.,

2002; Scheid et al., 2002). In turn, PI(3,4)P2 signaling is terminated by

dephosphorylation, mediated by INPP4A and INPP4B, resulting in PI(3)P generation

(Gewinner et al., 2009; Norris et al., 1997; Norris and Majerus, 1994). INPP4A and

INPP4B both function as suppressors of Akt activity (Ivetac et al., 2009), however,

INPP4A expression is primarily restricted to the brain while INPP4B is expressed most

tissues, including breast (Fedele et al., 2010).

Despite numerous studies pointing to Akt as a primary transducer of the PI 3-K

signal, *PIK3CA* mutant tumors have strikingly low levels of phosphorylated (hence

activated) Akt, indicating that other PI(3,4)P2 and PI(3,4,5)P3 effectors link PI 3-K to

tumorigenesis (Stemke-Hale et al., 2008; Vasudevan et al., 2009). Such effectors

include the Tec family kinases Btk and Itk (Luo et al., 2003; Miao et al., 2010).

Moreover, GTPase activating proteins for Rho family GTPases also transduce PI 3-K

signaling, such as GRP1 (Shiota et al., 2006). A more recent study showed that

PIK3CA-mediated breast cancer cell growth and survival is dependent on SGK3, in

cases where Akt was dispensable (Vasudevan et al., 2009). SGK3 is an AGC protein

kinase family member along with two other isoforms, SGK1 and SGK2. SGK isoforms

share ~55 % sequence identity with the Akt1-3 catalytic domains (Firestone et al., 2003;

Kobayashi et al., 1999; Tessier and Woodgett, 2006b). SGK and Akt isoforms also

phosphorylate the same consensus substrate motif, RXRXXS/T, and thus possess a

large number of shared substrates (Murray et al., 2004). SGK isoforms are also activated by the same upstream kinases as Akt, PDK-1 at the activation loop residue and TORC2 at the hydrophobic motif (Garcia-Martinez and Alessi, 2008; Kobayashi and Cohen, 1999; Kobayashi et al., 1999; Liu et al., 2000). As with Akt, SGK phosphorylation at these residues is necessary for catalytic activity. However, unlike Akt, SGKs have unique regulatory regions. In the case of SGK3, this includes an amino-terminal PX domain (Xu et al., 2001). The SGK3 PX domain binds to PI(3)P, thereby localizing a pool of the kinase to endosomal membranes (Tessier and Woodgett, 2006a; Xu et al., 2001).

Despite the nomenclature, SGK3 expression is not regulated by glucocorticoids, instead ER signaling has been shown to induce SGK3 transcription (Wang et al., 2011a; Xu et al., 2012). Interestingly, *INPP4B* is also an ER-induced gene (Fedele et al., 2010). Luminal breast cancers are defined by their expression of estrogen and progesterone receptors, distinguishing them from HER2 and basal-like (triple-negative) subtypes (Fedele et al., 2010; Sorlie et al., 2001). *INPP4B* inactivation by LOH is a frequent event in basal-like cancers, and its loss leads to Akt hyperactivation (Cancer Genome Atlas, 2012; Fedele et al., 2010; Gewinner et al., 2009). Conversely, INPP4B has been proposed to be a novel biomarker for luminal-type breast cancers, which also harbor frequent *PIK3CA* oncogenic mutations.

The mechanisms linking *PIK3CA* to SGK3 signaling and downstream phenotypes have not been defined. Here we show that INPP4B mediates *PIK3CA*-dependent SGK3 activation in breast cancer cells. We also show that SGK3 regulates N-Myc downstream

regulated 1 (NDRG1) leading to ubiquitination and degradation mediated by the E3

ligase F-box and WD repeat domain-containing 7 (FBW7). Finally, we show that SGK3

functions as a PI 3-K effector in the control of oncogenic signals promoting cell growth

and migration of breast cancer.

3. MATERIALS AND METHODS

Cell culture and transfection

BT-20, DLD-1, HEK293T, HCC1937, HeLa, MDA-MB-435, MDA-MB-231, NIH/3T3 and

MCF7 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Cellgro)

supplemented with 10% Fetal Bovine Serum (FBS; Gibco). ZR-75-1 cells were grown in

RPMI 1640 medium supplemented with 10% FBS. MCF10A cells were maintained in

DMEM/Ham's F12 medium supplemented with 5% equine serum (Cellgro), 10 μg/ml

insulin, 500 ng/ml hydrocortisone (Sigma-Aldrich), 20 ng/ml epidermal growth factor

(EGF) (R&D systems) and 100 ng/ml cholera toxin (List Biological Labs). T47D and BT-

549 were cultured in RPMI 1640 supplemented with 10% FBS and 10 μg/ml insulin.

SUM159PT were grown in HAM's F12 supplemented with 5% FBS and 5 μg/ml insulin

and 1μg/ml hydrocortisone. Hs578T were growing in DMEM supplemented with 10%

FBS and 0.01 mg/ml bovine insulin. *VPS34* fl/fl MEFs were provided by Wei-Xing Zong

(Stony Brook University) and cultured as previously described (Guertin and Sabatini,

2007). Transient transfections were performed using X-tremeGENE HP (Roche).

Growth factors and inhibitors

Cells were stimulated for 20 min with 100 ng/ml recombinant human IGF-1 (R&D

systems). MG132 (Cayman Chemicals) was used at 10 μM for 16 hr. Cycloheximide

was used at 10 μg/ml. Inhibitors were as follows: BKM120, 1μM; A66, 1μM; rapamycin,

100nM; GSK650394, 10μM; and SB415286, 25 μM.

Plasmids

SGK3-GST was provided by Sandra Marmiroli (University of Modena and Reggio Emilia). FLAG- p110α/ pCMV2 (#16643) FLAG-p110α H1047R/ pCMV2 (#16639) and FLAG-p110α E545K/ pCMV2 (#16642) plasmids were obtained from Addgene. HA-FBW7 and HA-GSK3 were described previously (Wei et al., 2005). NDRG1-FLAG was provided by Sushant Kachhap (Johns Hopkins Medical Institute) (Kachhap et al., 2007). pEAK-FLAG/INPP4B was provided by Lewis Cantley (Weill Cornell Medical College). FLAG-VPS34 was provided by Junying Yuan (Harvard Medical School). pHAGE-N-eGFP was provided by Wade Harper (Harvard Medical School). His-Ub was previously published (Reiling and Sabatini, 2006). FLAG-INPP4B.C842A was generated by site-directed mutagenesis with the following primers: sense, 5'-AATGGTATTCGTTTCACCTGTGCTAAAAGTGCCAAAGACAGGAC -3'; anti-sense: 5'-GTCCTGTCTTTGGCACTTTTAGCACAGGTGAAACGAATACCATT-3'. FLAG-1A-NDRG1 was constructed by site- directed mutagenesis with the following primers: 346A-sense: 5'-ACCCGCAGCCGCTCCCACGCCAGCGAGGGCA-3'; 346A-anti-sense, 5'-TGCCCTCGCTGGCGTGGGAGCGGCTGCGGGT-3'. FLAG-8A-NDRG1 was generated using site- directed mutagenesis and the following primers: 328A/ 330A-sense, 5' TGCGGTCCCGCGCAGCCGCTGGTTCCAGC 3'; 328A/ 330A-anti-sense, 5' GCTGGAACCAGCGGCTGCGCGGGACCGCA 3'; 342A –sense, 5' GATGGCACCCGCGCCCGCTCCCACGC 3'; 342A-anti-sense, 5' GCGTGGGAGCGGGCGCGGGTGCCATC 3'; 352A/362A-sense, 5' CGAAGCCGCTCCCACGCCAGCGAGGGCACCCGCAGCCGCTC 3'; 352A/362A-anti-

sense, 5' CTCGCTGGCGTGCGAGCGGCTGCGGGTGCCCTCGCTGGCGTGGGAGC

3'; 346A-sense: 5' ACCCGCAGCCGCTCCCACGCCAGCGAGGGCA 3'; 346A-anti-

sense, 5' TGCCCTCGCTGGCGTGGGAGCGGCTGCGGGT 3'; 356A/366A-sense,

5'AAGCCGCTCCCACGCCAGCGAGGGCACCCGCAGCCGCTCGCAC 3'; 356A/ 366A-

antisense, 5'

CTCGCTGGCGTGCGAGCGGCTGCGGGTGCCCTCGCTGGCGTGGGAGCGGCTT 3'.

shRNA-resistant SGK3 and INPP4B were made by Gateway Cloning (Life

Technologies) into lentiviral pHAGE-N-eGFP vector followed by site-directed

mutagenesis. SGK3.shRNA2-resistant SGK3-GFP (SGK3*) was generated by site-

directed mutagenesis with the following primers: sense, 5'

AGATTTTATTAAACAAAGACGAGCAGGACTGAATGAGTTTATTCAGAACCTAGTTAG

GTATCCAGAACTT 3': anti-sense, 5'

AAGTTCTGGATACCTAACTAGGTTCTGAATAAACTCATTCAGTCCTGCTCGTCTTTG

TTTAATAAAATCT 3'. INPP4B.shRNA2-resistant INPP4B-GFP (INPP4B*) was

generated using site-directed mutagenesis with the following primers: sense, 5'-

TTCATCCTTGCATGCAAGGATCTCGTGGCACCAGTGAGGGATCGTAAACTGAATAC

ACTGGTGC-3': anti-sense, 5'-

GCACCAGTGTATTCAGTTTACGATCCCTCACTGGTGCCACGAGATCCTTGCATGCA

AGGATGAA-3'. All point mutations were verified by DNA sequencing.

shRNA-containing vectors: shRNA-mediated silencing of Fbw7, INPP4B, SGK3, and

Akt1 each required the synthesis of a set of oligonucleotides composed of a target

shRNA sequence and its complement against each respective gene. Two hairpins were generated against each gene as follows: Fbw7.shRNA1-sense, 5'-AACCTTCTCTGGAGAGAGAAA-3'; Fbw7.shRNA1-anti-sense, 5'-TTTCTCTCTCCAGAGAAGGTT-3'; Fbw7.shRNA6-sense, 5'-CCAGAGACTGAAACCTGTCTA-3'; Fbw7.shRNA6-anti-sense, 5'-TAGACAGGTTTCAGTCTCTGG-3'; INPP4B.shRNA2-sense, 5'-CCGGCCATCTGAGTATCCCATCTATCTCGAGATAGATGGGATACTCAGATGGTTTTTG-3'; INPP4B.shRNA2-anti- sense, 5'-AATTCAAAAACCATCTGAGTATCCCATCTATCTCGAGATAGATGGGATACTCAGATGG-3'; INPP4B.shRNA6-sense, 5'-CCGGAGATACTCCAGCACCGAAATTCTCGAGAATTTCGGTGCTGGAGTATTTTTG-3'; INPP4B.shRNA6-anti-sense, 5'-AATTCAAAAAGATACTCCAGCACCGAAATTCTCGAGAATTTCGGTGCTGGAGTATCT-3'; SGK3.shRNA1-sense, 5'-CCGGGCGAGACCCTAGTTAAGAGAACTCGAGTTCTCTTAACTAGGGTCTCGCTTTTT-3'; SGK3.shRNA1-anti-sense, 5'-AATTCAAAAAGCGAGACCCTAGTTAAGAGAACTCGAGTTCTCTTAACTAGGGTCTCGC-3'; SGK3.shRNA2-sense, 5'-CCGGGCAGGACTAAACGAATTCATTCTCGAGAATGAATTCGTTTAGTCCTGCTTTTT-3'; SGK3.shRNA2-anti-sense, 5'-AATTCAAAAAGCAGGACTAAACGAATTCATTCTCGAGAATGAATTCGTTTAGTCCTGC-3'; Akt1.shRNA, sense, 5'-

CCGGGAGTTTGAGTACCTGAAGCTGCTCGAGCAGCTTCAGGTACTCAAACTCTTTT

TG-3′; Akt1.shRNA, anti-sense, 5′-

AATTCAAAAAGAGTTTGAGTACCTGAAGCTGCTCGAGCAGCTTCAGGTACTCAAAC

TC-3′

 The primer sets were each annealed and ligated into pLKO.   Lentiviral supernatants

were produced by transfection of HEK293T cells with VSVG, Δ8.2, and control-PLKO,

Fbw7, INPP4B, or SGK3 shRNA-containing vectors for 48 hr.

Antibodies

Anti-Akt (#4685), anti-phospho-Akt Ser473 (#4060), anti-phospho-Akt Thr308 (#9275),

anti-Cyclin E (#4129), anti-GSK3β (#9315), anti-phospho-GSK3β (#9336), anti-NDRG1

(# 5196), anti-phospho-NDRG1 Thr346 (#3217), anti-p110α (#4249), anti-S6K (#2705),

anti-phospho-S6K Thr389 (#9205), anti-SGK3 (#8156), anti-Vps34 (#4263) and anti-β-

actin (#4970) were purchased from Cell Signaling Technologies. Anti-FLAG M2

(#F3165) was from Sigma-Aldrich. Anti-phospho-SGK3 Thr320 (# S1010-85W8) was

from US Biologicals. Anti-GFP (# sc-9996), anti-GST (# sc-459) and anti-INPP4B (#sc-

12318) were from Santa Cruz. Anti-FBW7 (#A301-720A) was from Bethyl Laboratories.

Anti-SGK3 (#LS-C132061) for immunoprecipitation was from LifeSpan BioSciences.

Anti-HA was generated and purified from the 12CA5 hybridoma. Anti-p85 was

generated in house and has been previously described (Kapeller et al., 1995).

Horseradish peroxidase-conjugated anti-goat was from Millipore. Horseradish

peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulin G (IgG) antibodies were from Chemicon.

In vitro protein kinase assays

MCF7 cells infected with pLKO-control or INPP4B.shRNA-pLKO were transfected with SGK3-GST. 24 hr post-transfection cells were serum-starved for 16 hr followed by 20 min stimulation with IGF-1. SGK3 was isolated with glutathione beads and incubated with 75ng GST-GSK3β substrate peptide and 250 μM cold ATP in kinase assay buffer (Cell Signaling Technologies) for 35 min at 30°C. The kinase reaction was terminated by the addition of 1x SDS-PAGE loading buffer. Alternatively, 293T cells were co-transfected with SGK3-GST and wild type, E545K, or H1047R *PIK3CA*. SGK3 was precipitated and used for *in vitro* kinase assays as described in the above protocol.

Transwell migration and invasion assays

Transwell assays were carried out essentially as previously described (Chin and Toker, 2010). Briefly, NIH 3T3 conditioned media was added to the lower chamber of 8 μm pore Transwells (Corning). For invasion assays, filters were coated with 10 mg Matrigel (BD Biosciences). 10×10^5 cells were added to the upper chamber, in triplicate and migration allowed to proceed for 2-24 hr at 37°C, depending on the cell line. Cells that migrated or invaded to the lower chamber were fixed, stained using Hema-3® stain kit (Protocol; Fisher Scientific; Pittsburgh), and counted.

Immunoblotting

Immunoblotting was carried out as previously described (Chin and Toker, 2010). Briefly, cell lysates were separated on SDS-PAGE followed electrophoretic transfer to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk/TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.6) for 30 min. Blots were rinsed in TBST, and incubated at 4°C in primary antibody dilutions in 5% nonfat dry milk/ TBST for 16 hr. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr at 25°C. Membranes were rinsed in TBST and developed with chemiluminescence HRP substrate (Millipore).

Quantitative RT-PCR analysis

RNA was extracted using the Qiagen RNeasy. Reverse transcription reaction was carried out with ABI Taqman Reverse Transcriptional Reagent (N808-0234). After mixing the resulting cDNA template with *NDRG1* (sense: 5′-CCTGAGATGGTAGAGGGTCTC-3′ and antisense: 5′-CCAATTTAGAATTGCATTCCACC-3′) or glyceraldehyde-3-phosphate dehydrogenase (sense: 5′-GCAAATTCCATGGCACCGT-3′ and antisense: 5′-TCGCCCCACTTGATTTTGG-3′) primers quantitative RT-PCR was performed using SYBR Green PCR master mix in an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA).

Copy number analysis

The algorithm GISTIC (Genomic Identification of Significant Targets in Cancer) was used to analyze chromosome 8 gene copy numbers of the Tumorscape database. Tumorscape data analysis was performed as described (Beroukhim et al., 2010). SGK3 GISTIC profile was generated using 3131 cell lines and tumors. GISTIC analysis for breast cancer was performed using 243 breast samples and a limit of Q-value < 0.250 for focal peak amplification. Significance values were determined using the FDR (false discovery rate) test.

3D Culture Assay

3D cultures were performed as described (Debnath et al., 2003). Chamber slides were coated with growth factor-reduced Matrigel (BD Biosciences) and allowed to solidify at 37°C for 30 min. Subsequently, 3000 cells were seeded to each chamber slide in assay media (10% FBS/RPMI, 2 μg/ml puromycin, and 2% Matrigel). The assay media was aspirated and replaced 4 days after seeding. Quantitation was conducted using ImageJ quantitation of 2D surface area on Day 8(NIH).

Soft Agar Colony Formation Assay

Soft agar assays were performed by coating 6 cm^3 tissue culture plates with 5 mL of 0.8% Noble agar / growth media (10% FBS/DMEM and 2 μg/ml puromycin) and allowed to solidify at 20°C. 5×10^4 MCF7 breast cancer cells were plated in 1 mL top layer 0.4% Noble agar/ growth media (10% FBS/DMEM and 2 μg/ml puromycin). Soft agar was solidified at 20°C for 4 hr then transferred to 37°C incubator for the duration of the

assay. Growth media (10% FBS/DMEM and 2 μg/ml puromycin) was added every 4 days and cells were counted and measured 28 days after seeding. Quantitation was performed using MatLab software (MathWorks).

Ubiquitination Assay

Cell based *in vivo* ubiquitination assays was performed as described (Chan et al., 2012). 293T cells were transfected with indicated plasmids for 48 hr followed by lysis in denatured buffer (6M guanidine-HCl, 0.1M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole). The cell lysates were incubated with nickel beads for 3 hr, washed, and subjected to immunoblotting analysis.

4. RESULTS

**SGK3 is amplified and hyperactivated in breast cancer**

A recent study demonstrated that SGK3 is required for the survival of certain breast cancer cell lines with oncogenic *PIK3CA* mutations. These same cells showed minimal Akt activity and furthermore, Akt was dispensable for survival (Vasudevan et al., 2009). The Akt PH domain binds the PI 3-K lipids PI(3,4)P2 and PI(3,4,5)P3, however the SGK3 regulatory region lacks a functional PH domain. Instead, SGK3 regulation is in part mediated by the PX domain that primarily binds PI(3)P (Tessier and Woodgett, 2006a). Since PI(3)P is not a product of class I PI 3-kinases, the mechanism by which SGK3 functions as an effector of *PIK3CA* remains undefined.

Somatic activating mutations in the *SGK3* gene have not been identified with any appreciable frequency. We examined whether amplifications or deletions of *SGK3* exist in human cancers and cancer cell lines in a published database of 3131 cancers (Beroukhim et al., 2010). Amplifications were present in 30% of tumors. In 4.8% of cases, these were focal events encompassing less than half of chromosome 8q, a rate significantly above the genome-wide average (q=0.00168) (**Figure 2-1**). Among the 243 breast cancers in the study, 54% exhibited amplifications of *SGK3*, including 12% with focal alterations (q=0.186), and indeed SGK3 is one of 64 genes within the "peak region" where these amplifications most overlap (**Figure 2-2**). Chromosome 8q also includes *MYC*, which is the most commonly amplified gene among these cancers. However, the SGK3 peak was significant even after discounting amplifications which encompassed MYC.
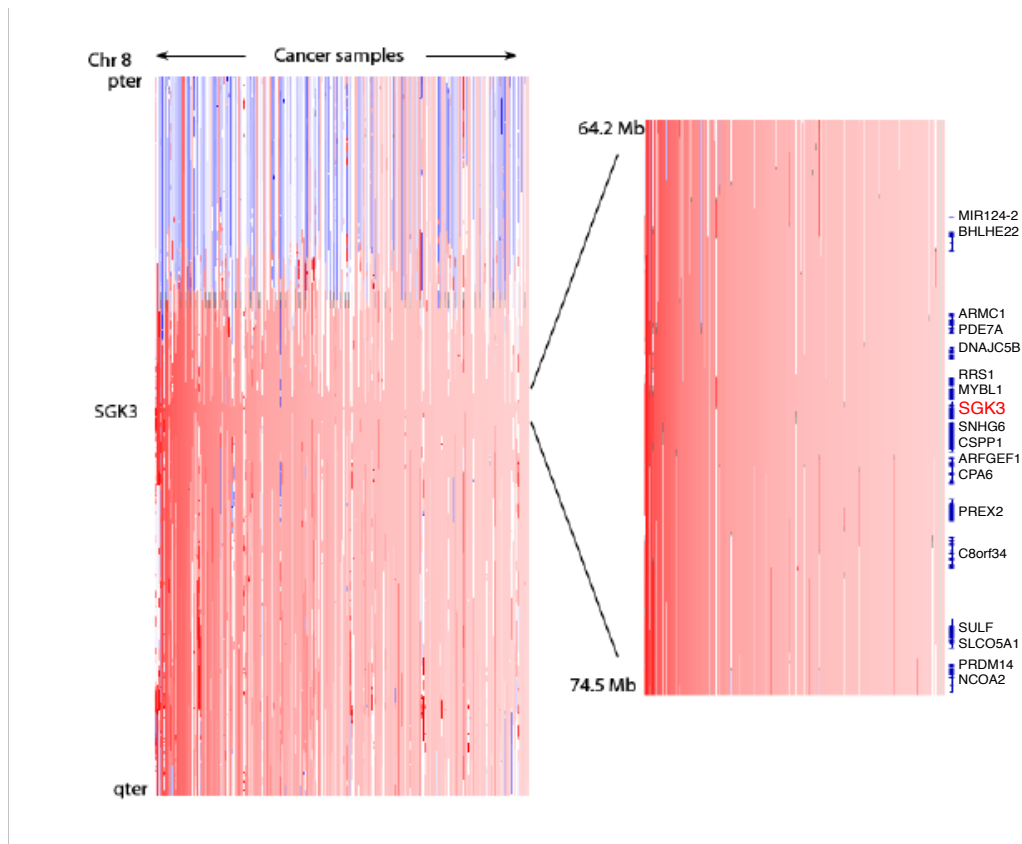
Figure 2-1: SGK3 is amplified in cancer

Copy-number profiles across chromosome 8 (y-axis) for 3,131 cancer samples (x-axis). A magnified view of the region including the *SGK3* gene locus is shown in the inset. Analysis was performed from a data set obtained from a study of 3,131 cancers of over 25 subtypes. Samples are sorted by frequency of amplification at the *SGK3* locus.
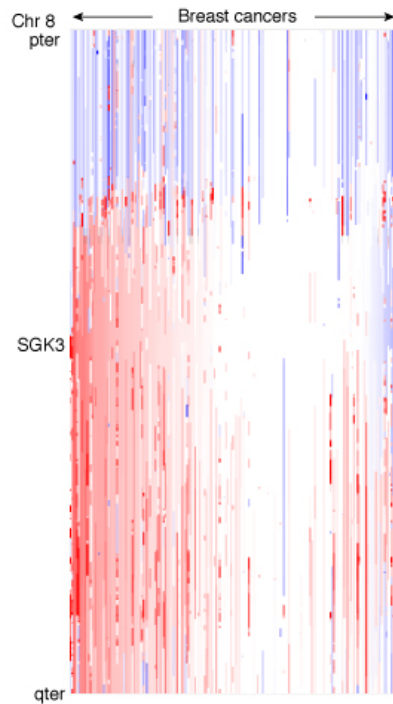
Figure 2-2: SGK3 is focally amplified in breast cancer

Copy-number profiles from 243 breast cancers selected from the data set described in (Figure2-1).

We next analyzed SGK3 protein levels in a panel of breast cancer cell lines informed by the Tumorscape analysis to have amplified SGK3, along with *PIK3CA* and *PTEN* mutation status (**Table 2-1**). Cells were serum-starved and stimulated with insulin-like growth factor-1 (IGF-1) to activate PI 3-K and Akt signaling (**Figure 2-3**). We used an antibody against the SGK3 activation loop site Thr320, targeted by PDK-1, as a surrogate for SGK3 activation. Concomitantly, we evaluated Akt phosphorylation at the activation loop residue (Thr308), also targeted by PDK-1, and hydrophobic motif (Ser473), phosphorylated by the TORC2 complex. An antibody that recognizes the endogenous SGK3 hydrophobic motif site at Ser486 is not available. We also used total and phospho-NDRG1 antibodies as surrogates for the activity of SGK3, noting that NDRG1 is a pan-SGK substrate (Murray et al., 2004; Najafov et al., 2011). We find that in cells that harbor oncogenic *PIK3CA* mutations, SGK3 is basally phosphorylated and further stimulated by IGF-1 (ZR-75-1, MCF7, T47D, Figure 1C). Interestingly, in these same cells total levels of NDRG1 are significantly lower, and since NDRG1 is an SGK substrate, this suggests that phosphorylation by SGK3 may lead to NDRG1 degradation.

Not all breast cancer cell lines that harbor *PIK3CA* mutations or *PTEN* inactivation display elevated SGK3 phosphorylation (e.g., SUM159PT, BT-549) (**Figure 2-3**). We therefore considered additional PI 3-K pathway alterations that would account for SGK3 phosphorylation. INPP4B is 4' phosphoinositide phosphatase that exclusively dephosphorylates the PI 3-K lipid PI(3,4)P2, converting it to PI(3)P.
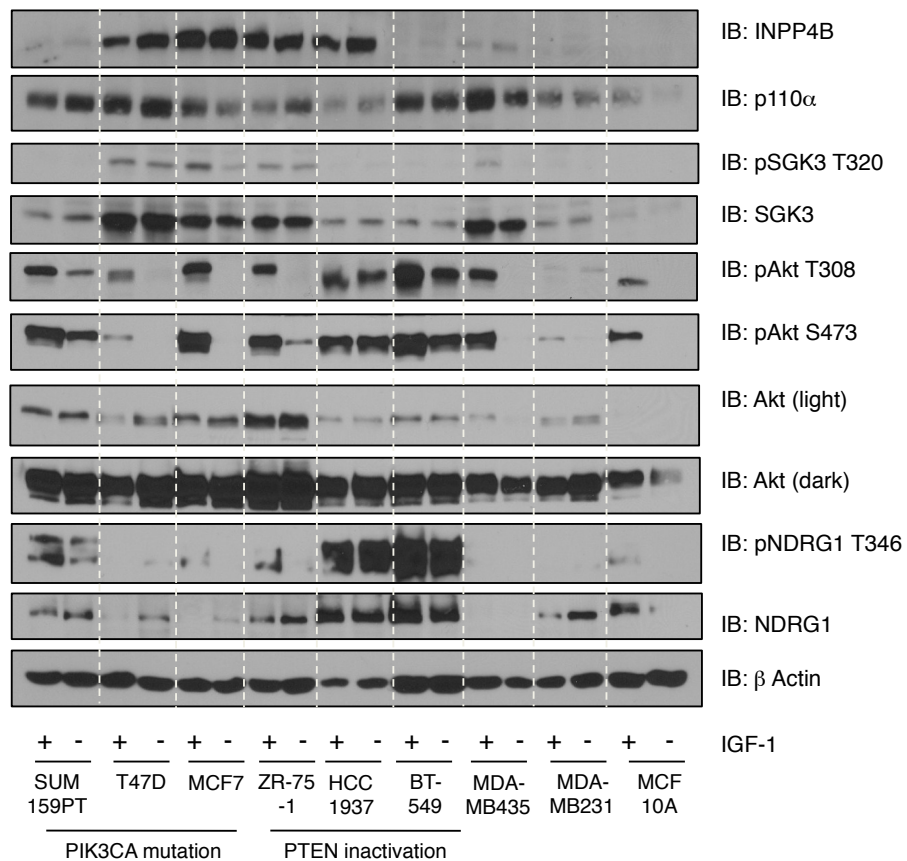
Figure 2-3: SGK3 expression analysis in cell lines

Cell lysates derived from the indicated breast epithelial and cancer cell lines, serum-starved (-) or stimulated with IGF-1 for 20 min (+) were immunoblotted (IB) with the indicated antibodies.

Table 2-1: Table of breast cancer cell lines

Cell line name	Tissue of origin	Tumorigenicity	Mutation	ER	PR	P53	HER2	Others
BT20	Breast	+	*PIK3CA* (H1047R)	-	-	mutant	-	*EGFR* amplified
BT-549	Breast	+	*PTEN* truncation	-	-	mutant	-	
HCC1937	Breast	+	*PTEN* deletion	-	-	mutant	-	
Hs578T	Breast	+	*PIK3R1* *HRas* (G12D)	-	-	mutant	-	
MCF10A	Breast	-		-	-	WT	-	
MCF7	Breast	+	*PIK3CA* (E545K)	+	+	WT	-	
MDA-MB231	Breast	+	*KRAS* (G13D)	-	-	mutant	-	
MDA-MB435	Breast	+	*BRaf* (V600E)	-	-	mutant	-	
SUM159PT	Breast	+	*PIK3CA* (H1047L)	-	-	mutant	-	
T47D	Breast	+	*PIK3CA* (H1047R)	+	+	mutant	-	
ZR-75-1	Breast	+	*PTEN* missense	+	-	WT	-	

Loss of *INPP4B* by LOH is frequently observed in basal-like cancers and leads to elevated PI(3,4)P2 levels and in turn, Akt hyperactivation (Fedele et al., 2010; Gewinner et al., 2009). However, *INPP4B* loss is not observed in estrogen receptor-positive breast cancers, where *PIK3CA* mutations occur with the highest frequency (Cancer Genome Atlas, 2012). In our comparative analysis, we find that elevated SGK3 phosphorylation correlates directly with both elevated INPP4B expression and *PIK3CA* mutation status (**Figure 2-3**).

To provide cause-and-effect evidence for SGK3 as a PI 3-K effector, we used pathway inhibitors in cells with *PIK3CA* mutation and high INPP4B, as well as robust SGK3 phosphorylation. Since phosphorylation-state antibodies such as pThr320 (for SGK3) or pThr308 and pSer473 (for Akt) do not directly measure protein kinase activity, we developed a kinase assay to measure SGK3 activity. Endogenous SGK3 was immunoprecipitated and used in *in vitro* kinase assays with a peptide substrate derived from GSK-3β that is a defined optimal Akt and SGK substrate, since they share a similar consensus phosphorylation motif (Murray et al., 2004). Using this assay, we show that SGK3 activity is stimulated by IGF-1, concomitant with increased Thr320 phosphorylation and increased NDRG1 phosphorylation (**Figure 2-4**). SGK3 activation is attenuated by both BKM-120 and A66, pan class I and p110α specific inhibitors, respectively (Buonamici et al., 2010; Jamieson et al., 2011). Conversely, inhibition of TORC1 using Rapamycin does not block SGK3 activation, consistent with the notion that SGK isoforms are TORC2 targets (Garcia-Martinez and Alessi, 2008). A
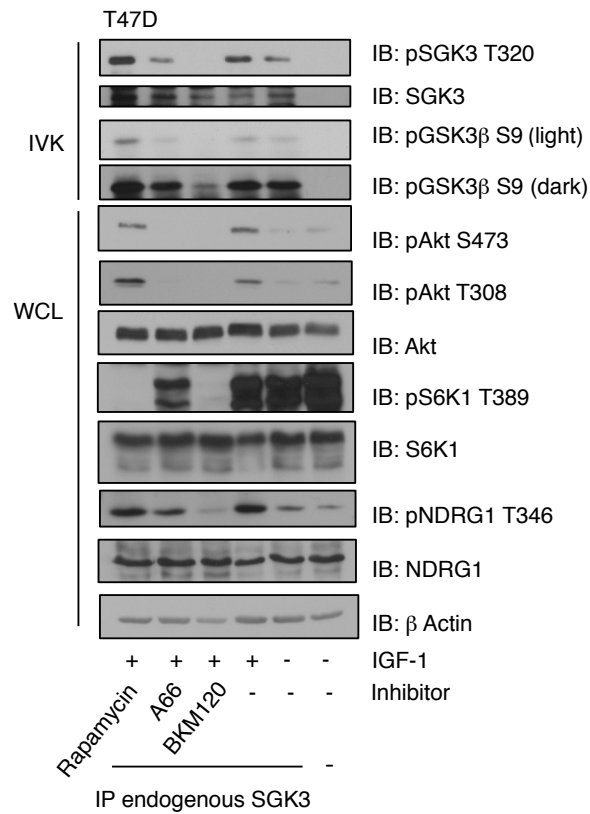
Figure 2-4: inhibitor analysis of SGK3 signaling

T47D cells were serum-starved for 24 hr, then pretreated with the invocated inhibitors, rapamycin (100nM), A66 (1μM) or BKM120 (1μM) for 20 min, then stimulated with IGF-1 for a further 20 min. SGK3 was immunoprecipitated with anti-SGK3 and subjected to an *in vitro* kinase assay (IVK) using GSK-3β peptide substrate. Peptide substrate phosphorylation was detected using anti-GSK-3β phospho-antibody. SGK3 phosphorylation was detected using anti-SGK3 pT320. Total SGK3 was evaluated as control. Whole cell lysates (WCL) were immunoblotted with the indicated antibodies. All results are representative of at least 3 independent experiments.

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requirement for *PIK3CA* in SGK3 activation is further substantiated by the fact that both

basal and IGF-1-stimulated SGK3 activity is enhanced in cells ectopically expressing

wild-type and oncogenic *PIK3CA* mutants (**Figure 2-5**). Collectively, these data

demonstrate that SGK3 is amplified in breast cancer and its activity is dependent on

oncogenic PI 3-K signaling.

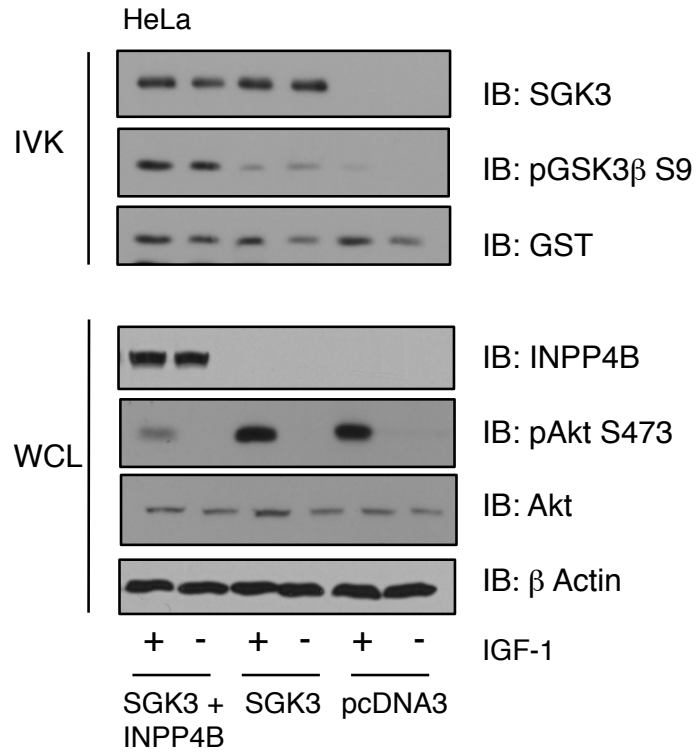**INPP4B regulates SGK3 activation**

PtdIns-3-P directly binds to the SGK3 PX domain (Tessier and Woodgett, 2006a).

Since high INPP4B levels correlate with the ability of IGF-1 to stimulate SGK3 in

PIK3CA mutant cells (**Figure 2-3**), and INPP4B generates PI(3)P, we reasoned that

INPP4B levels are rate-limiting for SGK3 activation downstream of PI 3-K. SGK3 activity

measured by *in vitro* kinase assay is significantly enhanced in cells co-expressing

INPP4B, whereas Akt phosphorylation is attenuated, as expected (**Figure 2-6**).

Moreover, endogenous SGK3 phosphorylation is also stimulated by expression of wild-

type INPP4B, but not by a catalytically- inactive phosphatase mutant (**Figure 2-7**).

Although the primary route of PI(3)P synthesis is through the class III PI 3-K VPS34,

expression of an active VPS34 allele does not promote SGK3 phosphorylation (**Figure

2-7**). Moreover, *VPS34* null MEFs (fl/fl) display the same level of IGF-1-stimulated

SGK3 and NDRG1 phosphorylation as control cells (**Figure 2-8**).

Silencing INPP4B using lentiviral shRNA completely attenuates SGK3

phosphorylation in response to IGF-1, with a concomitant decrease in phospho-NDRG1
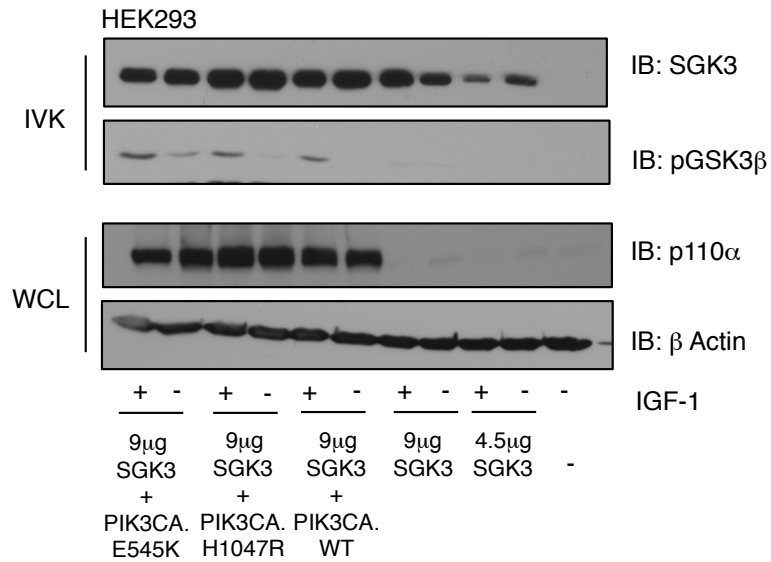
Figure 2-5: Oncogenic PIK3CA mutants hyperactivate SGK3

HEK293 cells were transfected with SGK3-GST alone or in combination with PIK3CA.WT, PIK3CA.H1047R, or PIK3CA.E545K followed by 24 hr serum- starvation before stimulation with IGF-1. Glutathione agarose was used to isolate SGK3 prior to incubation with GST-GSK3β peptide in an *in vitro* kinase (IVK) assay. Whole cell lysates (WCL) and *in vitro* kinase assay were immunoblotted as indicated.

Figure 2-6: INPP4B positively regulates SGK3 activity

HeLa cells were transfected with SGK3-GST alone or with INPP4B and serum-starved for 24 hr, prior to stimulation with IGF-1 for 20 min. SGK3 was isolated from cell lysates with glutathione beads, and incubated with GST-GSK3β peptide in an in vitro kinase assays (IVK). Peptide substrate phosphorylation was detected using anti-GSK-3β phospho-antibody. Total SGK3 was evaluated as control. Whole cell lysates were immunoblotted with the indicated antibodies.

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HEK293

IB: VPS34

IB: INPP4B

IB: pSGK3 T320

IB: SGK3

IB: β Actin

| + | - | + | - | + | - | + | - | IGF-1 |

VPS34   INPP4B.   INPP4B   pcDNA3
         C842A
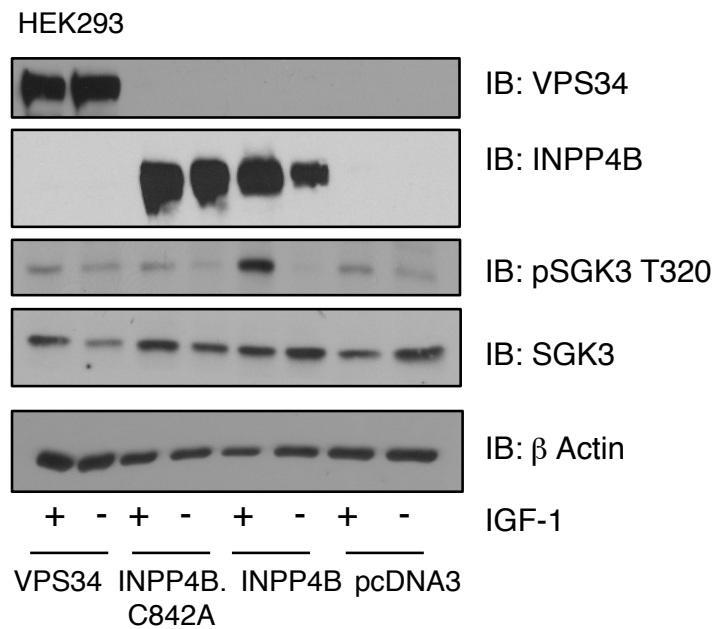
Figure 2-7: INPP4B catalytic activity specifically promotes SGK3 phosphorylation

HEK293T cells were transfected with wild-type INPP4B, catalytically-inactive INPP4B.C842A or VPS34, then serum-starved for 24 hr and stimulated with IGF-1 for 20 min. Whole cell lysates were immunoblotted with the indicated antibodies.

VPS34 fl/fl MEFs

IB: VPS34

IB: pSGK3 T320

IB: SGK3

IB: pNDRG1 T346

IB: β Actin
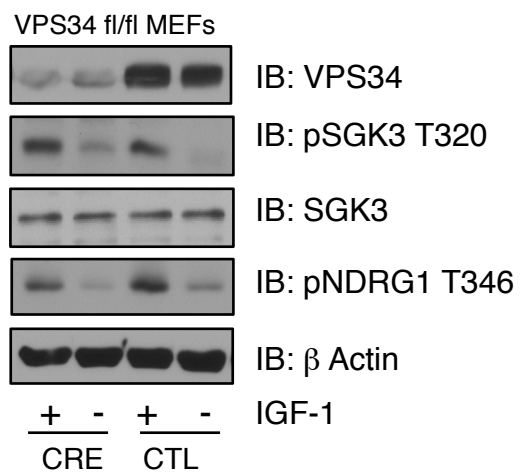
+	-	+	-	IGF-1
CRE		CTL		

Figure 2-8: VPS34 loss does not affect SGK3 phosphorylation

VPS34 fl/fl MEFs were infected with control (CTL) or Cre adenovirus. Control and VPS34-null MEFs were starved for 18 hr and stimulated for 20 min with IGF-1. Whole cell lysates were immunoblotted with the indicated antibodies.

(**Figure 2-9**). Similarly, reduced SGK3 phosphorylation induced by INPP4B silencing is rescued by expression of an shRNA-resistant INPP4B allele (**Figure 2-10**). Inhibition of both exogenous and endogenous SGK3 protein kinase activity is also observed in cells transduced with INPP4B shRNA (**Figure 2-11**). We conclude that INPP4B is required for the ability of PI 3-K to activate SGK3, and that the phosphatase activity of INPP4B is essential. Moreover, we propose that VPS34 is dispensable for *PIK3CA*-mediated SGK3 activation.

SGK3 promotes proliferation in 3D and anchorage independence of growth

To explore the functional significance of SGK3 downstream of *PIK3CA*, we investigated the ability of SGK3 and INPP4B to drive anchorage independent growth, since *PIK3CA* has been shown to promote this phenotype (Isakoff et al., 2005). MCF7 cells (*PIK3CA* E545K, high SGK3 and high INPP4B, **Figure 2-3**) were transduced with vector control, or two distinct SGK3 and INPP4B shRNA's, and colonies allowed to form (**Figure 2-12A and 2-12C**). Whereas control cells form robust colonies in soft agar, silencing either SGK3 or INPP4B significantly inhibits the number of colonies growing in an anchorage-independent manner (**Figure 2-12B**). Additionally, introduction of shRNA-resistant SGK3 and INPP4B cDNAs partially rescues the defect in anchorage-independent growth (**Figure 2-12**). Representative images of each condition are shown in Figure 2-12A.

Growth of cancer cells in 3 dimensional (3D) culture more faithfully recapitulates phenotypes that control tumor growth *in vivo* (Debnath and Brugge, 2005).
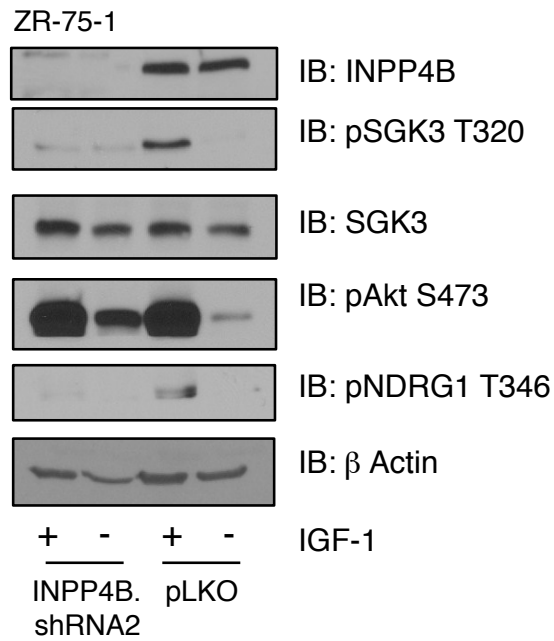
Figure 2-9: INPP4B silencing attenuate SGK3 phosphorylation

ZR-75-1 cells were infected with shRNA lentivirus targeting INPP4B, or control pLKO, serum-starved and stimulated with IGF-1 for 20 min. Cell lysates were immunoblotted with the indicated antibodies.
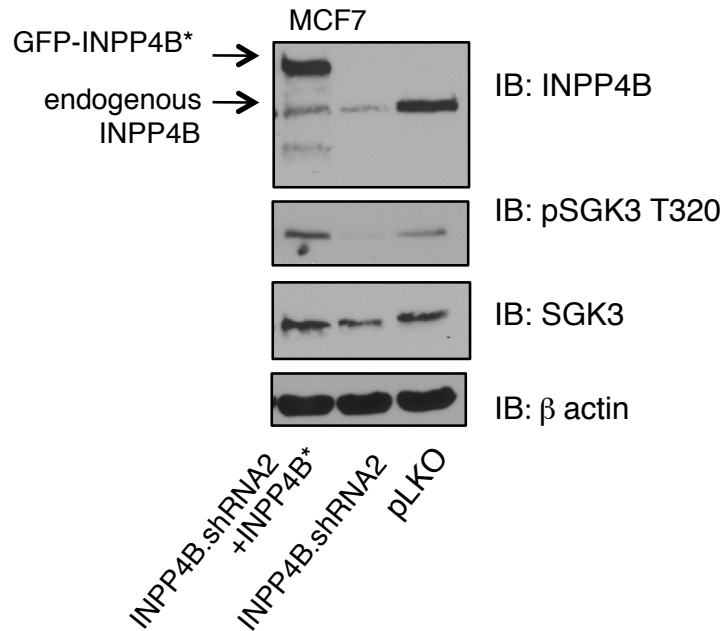
Figure 2-10: shRNA-resistant INPP4B allele rescues SGK3 phosphorylation

MCF7 cells were infected shRNA lentivirus targeting INPP4B, followed by retroviral infection with INPP4B cDNA rescue virus . Cell lysates were immunoblotted with the indicated antibodies. All results are representative of at least 3 independent experiments.
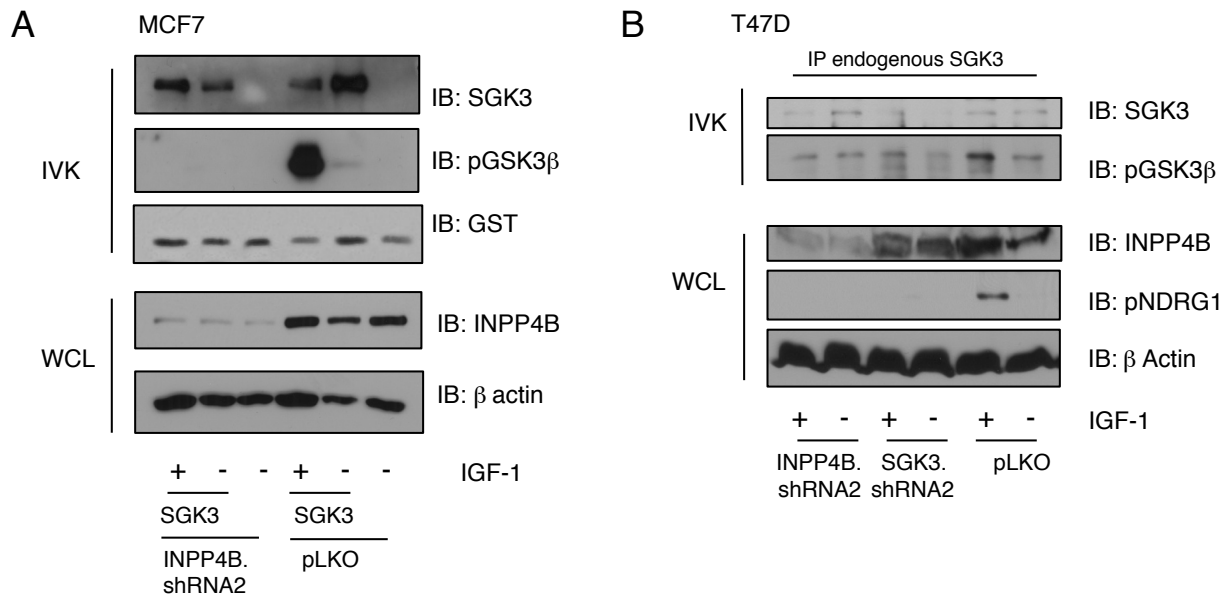
Figure 2-11: INPP4B silencing inhbits SGK3 activity

(A) MCF7 cells were infected with INPP4B.shRNA2 prior to transfection with SGK3-GST. Cells were serum-starved for 18 hr before stimulation with IGF-1. Glutathione agarose beads were used to isolate SGK3 from cell lysates, and incubated with GST-GSK3β in an *in vitro* kinase (IVK) assay.

(B) T47D cells were infected with SGK3.shRNA2 and INPP4B.shRNA2 prior to serum-starvation for 18 hr prior to IGF-1 stimulation. SGK3 antibody was used to immunoprecipitate SGK3 for 2 hr. In vitro kinase (IVK) assay was then performed with isolated SGK3 and GST-GSK3β peptide. Whole cell lysates (WCL) were immunoblotted with the indicated antibodies.
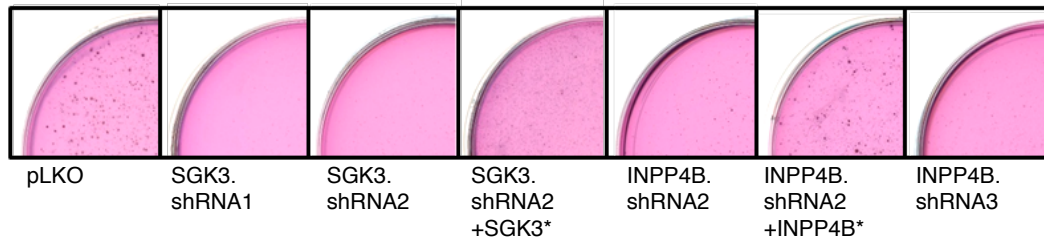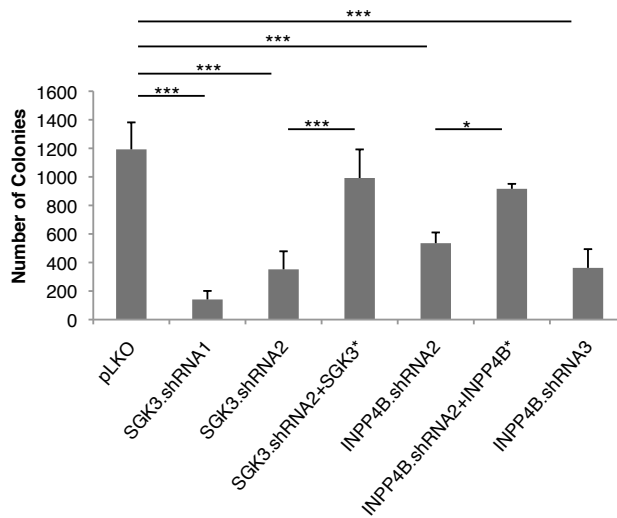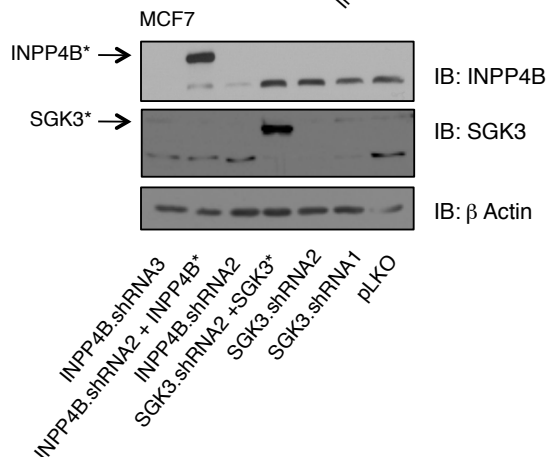
Figure 2-12: SGK3 promotes anchorage independent growth

(A) MCF7 cells infected with pLKO vector control, or two distinct INPP4B and SGK3 shRNA lentiviral vectors and grown in agar/growth media for 28 days. Additional conditions included retroviral infection of INPP4B (INPP4B*) and SGK3 (SGK3*) cDNA rescue virus. Representative images of culture dishes used for the analysis in (B and C) Results are representative of at least 3 independent experiments.
(B) Number of colonies in soft agar obtained in (A) was quantitated using MatLab.
(C) Western blot analysis of MCF7 cells with indicated antibodies.

3D Matrigel growth of ZR-75-1 breast cancer cells (*PTEN* missense, high SGK3, high INPP4B) is also significantly attenuated upon silencing either SGK3 or INPP4B with 2 distinct shRNA sequences each (**Figure 2-13A, 2-13B, and 2-13C**). Inhibition of 3D Matrigel growth upon SGK3 or INPP4B silencing is also observed in MCF7 cells (**Figure 2-14**).

SGK3 promotes breast cancer cell invasive migration

Signaling through *PIK3CA* also modulates breast cancer cell migration and invasion, pre-requisite phenotypes for metastatic dissemination of tumor cells (Pang et al., 2009). We therefore examined if SGK3 is required for breast cancer cell invasive migration. Using *in vitro* Transwell migration assays, we first evaluated SUM159PT cells that express low levels of endogenous SGK3 (**Figure 2-3**). Ectopic expression of SGK3 robustly enhances SUM159PT cell migration (**Figure 2-15A**). Conversely, silencing SGK3 with shRNA significantly attenuates MCF7 cell migration (**Figure 2-15B**). A catalytic small molecule pan-SGK inhibitor, GSK650394 (Sherk et al., 2008) also significantly blocks MCF7 cell migration (**Figure 2-15C**). Finally, ectopic expression of SGK3 also promotes invasive migration through Matrigel (**Figure 2-15D**). Therefore, SGK3 kinase activity is both necessary and sufficient to promote invasive migration of breast cancer cells.

SGK3 mediates NDRG1 proteolytic processing

Finally, since NDRG1 is an established metastasis suppressor and is also an SGK substrate (Kachhap et al., 2007; Murray et al., 2004; Najafov et al., 2011), we evaluated

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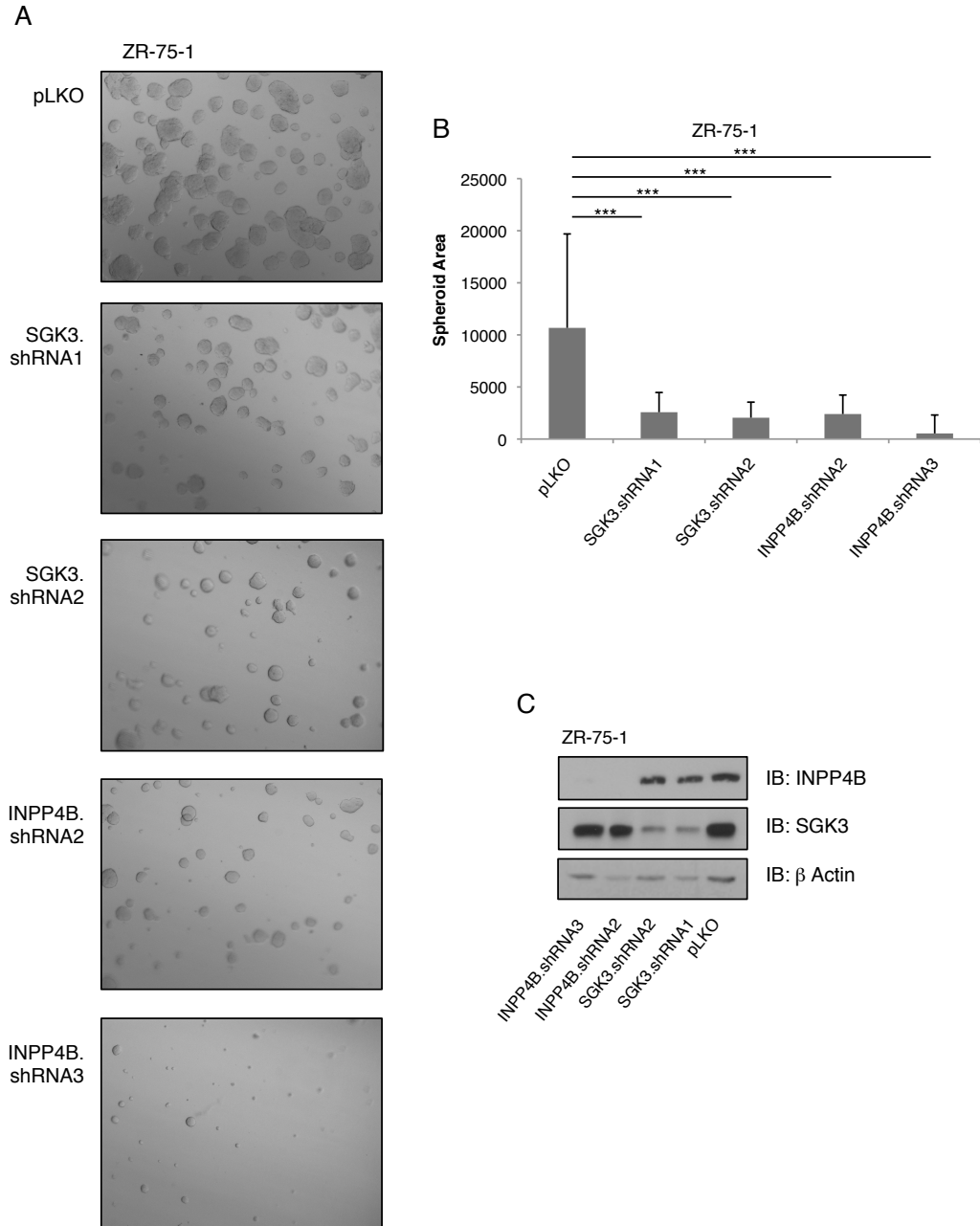
Figure 2-13: SGK3 regulates 3D growth of breast cancer cells

(A) ZR-75-1 cells were infected with control pLKO or two distinct SGK3 shRNA or INPP4B shRNA's and grown for 8 days in 3D Matrigel/cell culture media. Representative images spheroids at 4x magnification are shown.
(B) Quantitation of 2D spheroid size from (A) was performed using ImageJ software. Error bars represent mean ± SEM. *P < 0.05 ; **P < 0.01; ***P < 0.001. Results are representative of at least 3 independent experiments.
(C) Western blot analysis of 3D culture ZR-75-1cell lysates with indicated antibodies.
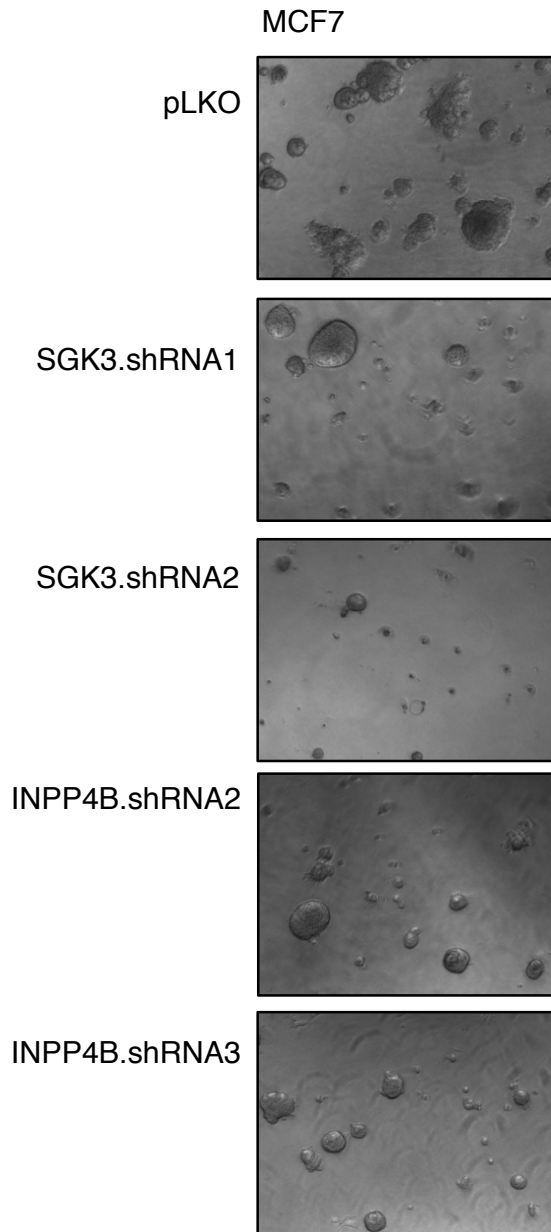
Figure 2-14: SGK3 regulates 3D growth of PIK3CA mutant breast cancer cells

MCF7 cells were infected with control pLKO or one of two distinct SGK3 or INPP4B shRNA lentivirus and grown for 8 days in 3D Matrigel/culture media. Representative spheroid images for each condition are shown at 4X magnification.
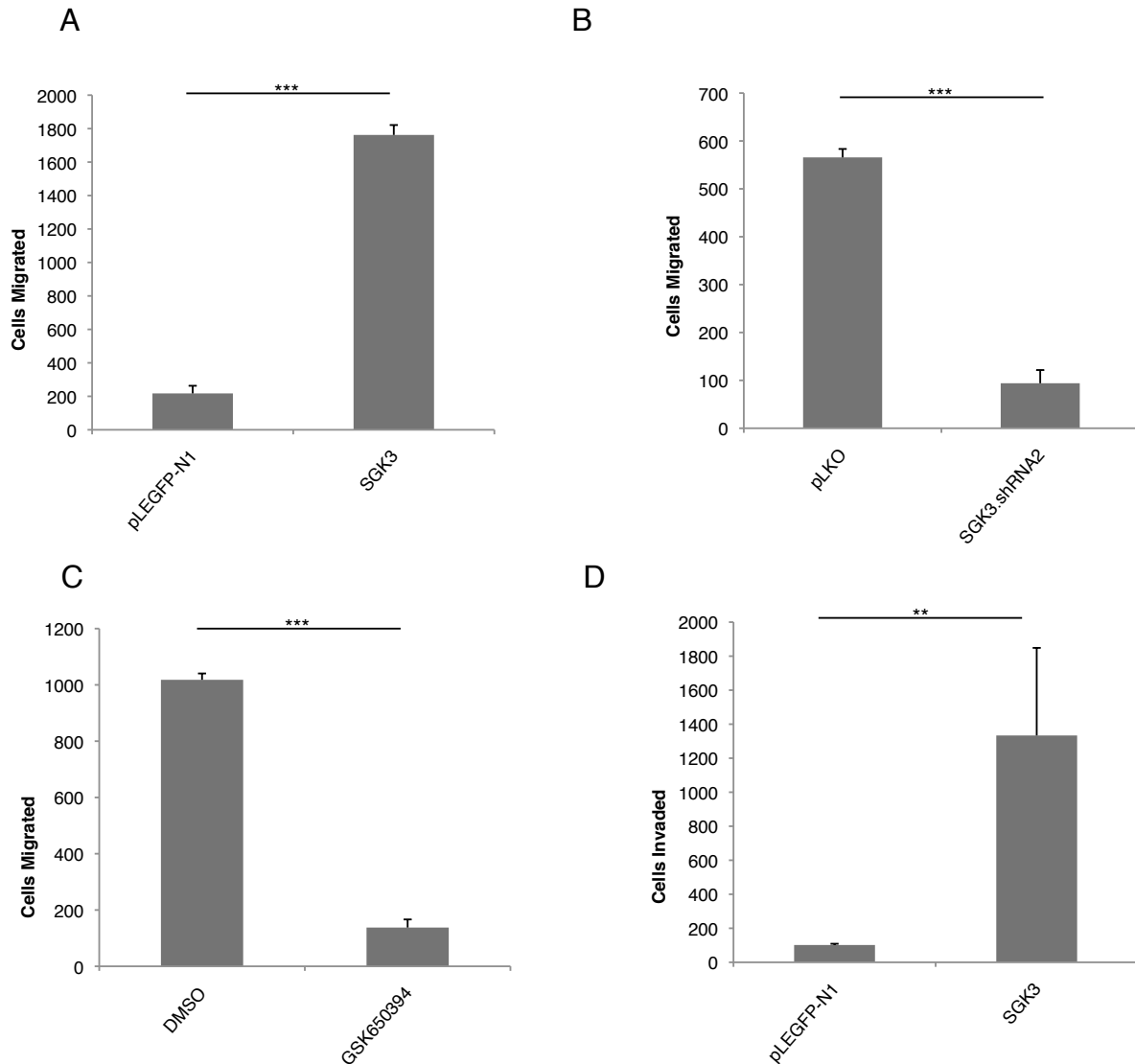
Figure 2-15: SGK3 promotes breast cancer cell invasive migration

(A) SUM159PT breast cancer cells were infected with SGK3 or control pLEGFP-N1 and followed by 2 hr migration through Transwell assays. Relative migration is measured along the Y-axis as the ratio of migrated cells in the test condition versus the control condition.

(B) MCF7 breast cancer cells were infected with SGK3 shRNA lentiviral vector or control empty vector for 48 hr followed by Transwell migration assays.

(C) MCF7 cells were inhibited with SGK inhibitor, GSK650394 (10uM), or control DMSO for 20 min prior to Transwell migration assays.

(D) HS578T breast cancer cells were infected with SGK3 of control pLEGFP-N1 followed by Transwell Matrigel invasion assays.

the mechanism by which SGK3 controls NDRG1 stability. We initially noted a correlation

of NDRG1 protein levels and SGK3 phosphorylation (**Figure 2-3**). Consistent with this,

silencing SGK3, but not Akt1, results in a decrease in phosphorylated NDRG1

(pThr346), with a concomitant increase in total NDRG1 (**Figure 2-16A**). A

cycloheximide chase experiment reveals that the half-life of NDRG1 in serum-containing

media is approximately 1 hour (**Figure 2-16B**). We also examined NDRG1 protein

stability in cells treated with a proteasome inhibitor, and find that NDRG1 levels are

dramatically enhanced upon inhibition of the 26S proteasome with MG132 (**Figure 2-16C**). Curiously, in T47D cells, NDRG1 levels are not affected by MG132. T47D cells

are PIK3CA H1047R mutant, have high SGK3 and INPP4B, and moreover are *FBXW7*

null (Mao et al., 2008). FBW7 is an F-box E3 ubiquitin ligase and substrate recognition

domain of the SKP1-cullin-F-box (SCF) ubiquitin ligase complex. To determine whether

FBW7 mediates SGK3-dependent NDRG1 degradation, we first used wild-type and

FBW7 null DLD-1 colon carcinoma cells (Rajagopalan et al., 2004). FBW7 deletion

leads to a dramatic increase in NDRG1 protein levels, compared to wild-type cells,

whereas *NDRG1* message levels remain unchanged (**Figure 2-17A and Figure 2-17B**).

Moreover, silencing of FBW7 using two distinct shRNA sequences leads to significantly

increased NDRG1 protein levels compared to control cells (**Figure 2-18A**). Increased

NDRG1 levels upon FBW7 silencing are also observed in a distinct breast cancer cell

line, BT-20 (**Figure 2-18B**). Importantly, the fold increase in NDRG1 is comparable to

that observed with Cyclin E, a known FBW7 substrate (**Figure 2-18A**).

A

MCF7

IB: Akt

IB: SGK3

IB: pNDRG1 T346

IB: NDRG1

IB: β actin

| + | - | + | - | + | - | IGF-1 |

Akt1.   SGK3. pLKO
shRNA shRNA2

B

MCF10A

IB: p85

IB: NDRG1

6   3   2 1 0.5 - CHX hr

C

IB: NDRG1

IB: p85

+ -   + -   + -   + -   MG132

T47D MCF10A MCF7 ZR-75-1
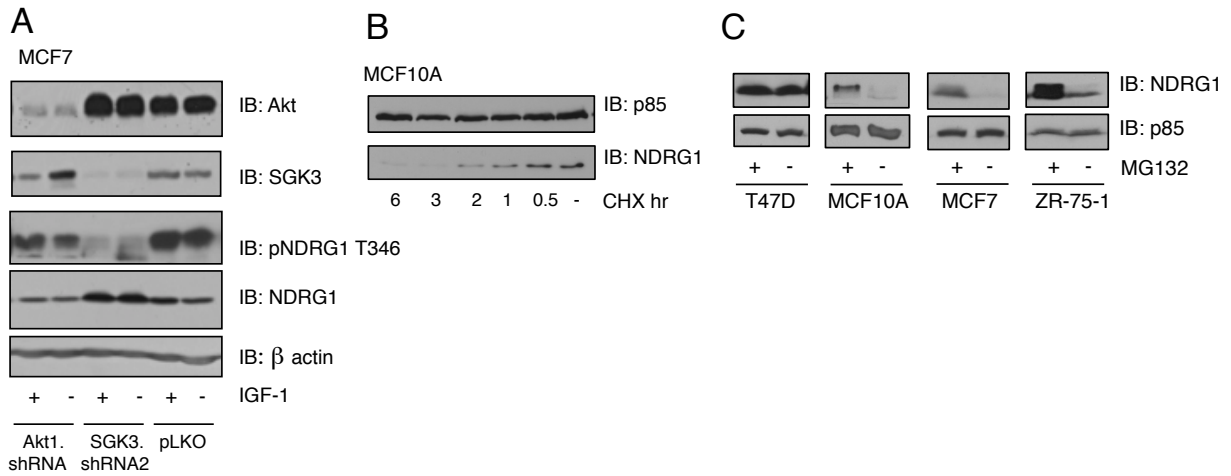
Figure 2-16: NDRG1 is degraded by the 26S proteasome

(A) MCF7 cells infected with SGK3. shRNA, Akt1. shRNA or control pLKO, serum-starved then stimulated with IGF-1 for 20 min. Cell lysates were immunoblotted with the indicated antibodies.

(B) MCF10A cells were treated with cycloheximide or control DMSO for the indicated times and cell lysates immunoblotted with anti-NDRG1 and anti-p85.

(C) T47D, MCF10A, MCF7, and ZR-75-1 cells were treated with MG132 for 16 hr and immunoblotted with anti-NDRG1 and anti-p85.
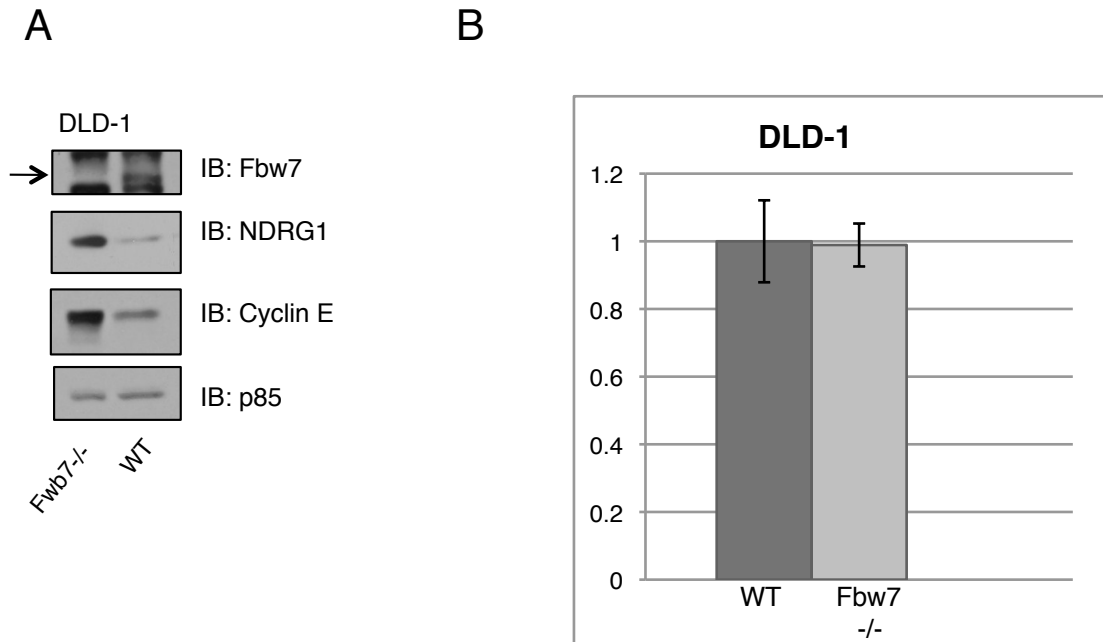
Figure 2-17: FBW7 regulates NDRG1 expression at the protein level

(A) Cell lysates of wild-type (FBW7 WT) or DLD-1 colon cancer cells null for *Fbw7* (*Fbw7* -/-) were immunoblotted with the indicated antibodies.
(B) Quantitative RT-PCR analysis to determine the relative mRNA expression of *NDRG1* in wild-type or *Fbw7*-/- DLD-1 cell lines.
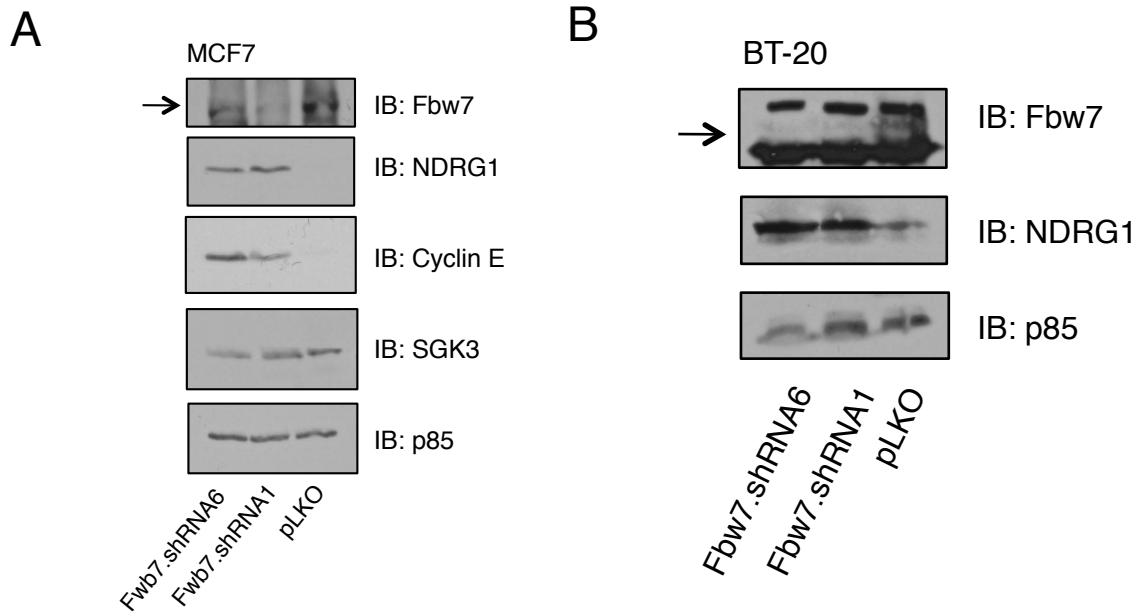
Figure 2-18: Fbw7 regulates NDRG1 protein levels in breast cancer cells

(A) MCF7 cells were infected with two distinct Fbw7 shRNA's or control pLKO, and lysates immunoblotted with the indicated antibodies
(B) BT-20 cells were infected with two distinct Fbw7 shRNA or control pLKO, and cell lysates were immunoblotted with the indicated antibodies

Studies have shown NDRG1 phosphorylation by SGK3 at Thr346/Thr356/Thr366 primes for phosphorylation by GSK-3β at Ser342/Ser352/Ser462 (Murray et al., 2004). GSK-3β phosphorylation is a common priming event for FBW7 substrate binding (Wang et al., 2011b). To assess whether NDRG1 stability is dependent on the concerted action of SGK3, GSK-3β and FBW7, we co-expressed each component in combination and measured NDRG1 stability. Maximal NDRG1 degradation is only observed when both SGK3 and GSK-3β are simultaneously co-expressed with FBW7 (**Figure 2-19A**). Treatment of cells with SGK inhibitor, GSK650394, or GSK3 inhibitor, SB415286, conversely causes an increase in NDRG1 levels, while dual treatment with both GSK650394 and SB415286 heightens NDRG1 stability even further (**Figure 2-19B**). Finally, we used an established, cell-based ubiquitination assay to evaluate NDRG1 ubiquitination. NDRG1 ubiquitination is increased when FBW7 is co-expressed, and ubiquitination is maximal when SGK3 and GSK-3β are co-expressed. In the absence of MG132, NDRG1 ubiquitination is completely eliminated. Furthermore, NDRG1 ubiquitination is significantly attenuated in a mutant that lacks SGK3 and GSK-3β consensus phosphorylation sites. Therefore, NDRG1 stability is regulated by SGK3 activity and subsequent FBW7-mediated degradation. To address FBW7 binding to NDRG1 a GST-binding assay was performed. NDRG1.T346A has reduced ability to bind FBW7-GST and the 8A-NDRG1 eliminates binding to FBW7, demonstrating SGK3 and GSK3 phosphorylation is necessary for FBW7 binding (**Figure 2-20B**). In order to determine the phenotypic significance of SGK3 regulation of NDRG1 we investigated the ability of
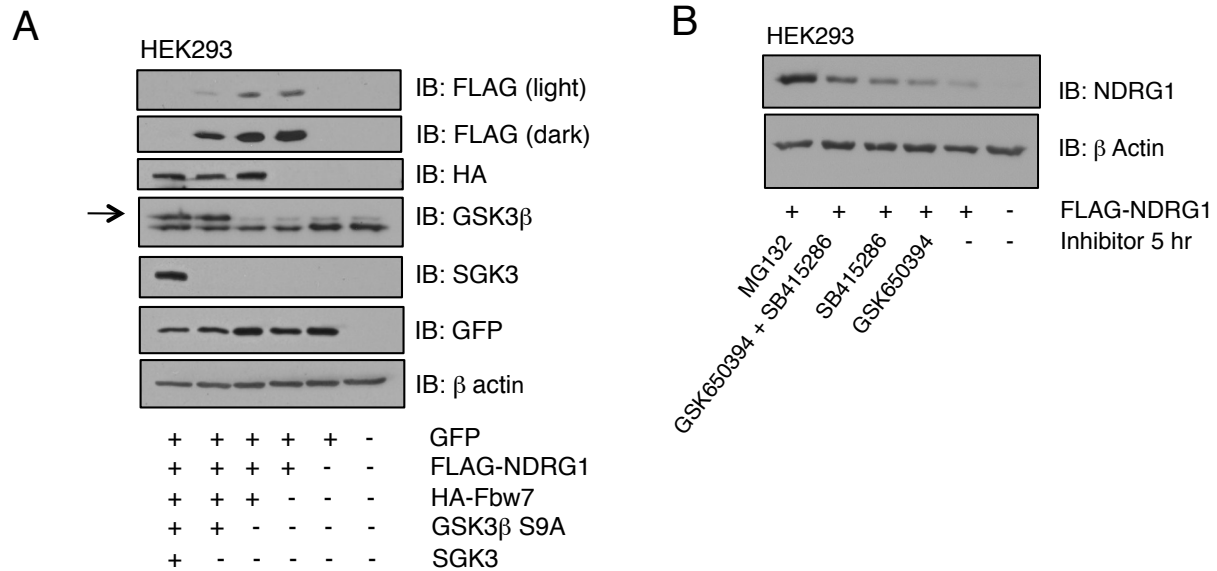
Figure 2-19: GSK3 and SGK3 have additive effects on NDRG1 degradation

(A) HEK293T cells were transfected with control GFP vector, alone or with the indicated combinations of NDRG1-FLAG, HA-Fbw7, HA-GSK3β.S9A and SGK3-GST. NDRG1 stability was evaluated by immunoblotting whole cell lysates with anti-FLAG, and indicated antibodies.
(B) HEK293T cells were transfected for 48 hr with NDRG1-FLAG prior to 5 hr treatment with GSK650394, SB415286, GSK650394 and SB415286, or MG132 in order to examine NDRG1 protein stability changes.
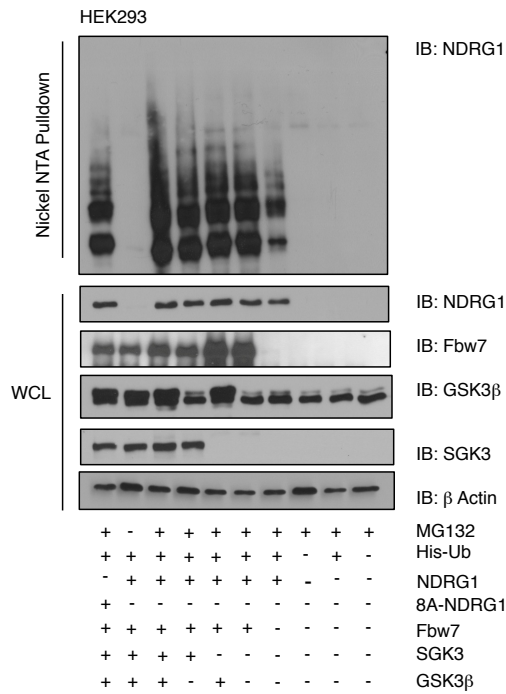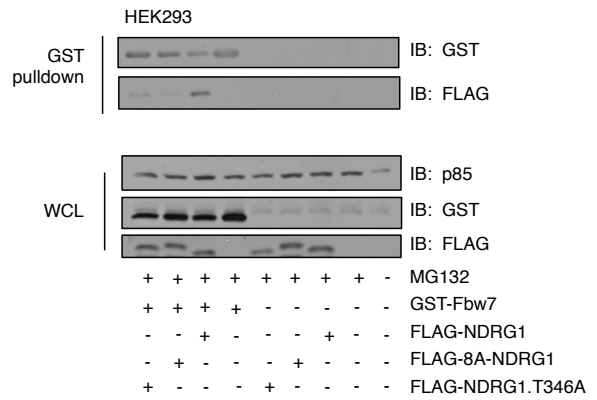
Figure 2-20: NDRG1 is an FBW7 substrate

(A) A His-Ubiquitin assay was performed after transfection of HEK293T cells with FLAG-NDRG1, GST-Fbw7, HA-GSK3β.S9A, SGK3-GFP, and His-Ub, followed by treatment with MG132 for 12 hr. Nickel NTA beads were used to isolate His-ubiquitin complexes, followed by immunoblotting with the indicated antibodies.
(B) HEK293 were transfected with GST-Fbw7 and NDRG1, 8A-NDRG1, or NDRG1.T346A and treated with MG132 for 18 hr. Glutathione agarose was used to isolate Fbw7 and associated proteins. Pulldown and whole cell lysates (WCL) were immunoblotted with the indicated antibodies.

NDRG1 to mediate migration. In ZR-75-1 cells the expression of NDRG1 dramatically inhibits Transwell cellular migration, while co-expression with SGK3, GSK3β, and FBW7 causes a dramatic increase in migration rescuing the NDRG1 suppression of motility (**Figure 2-21**). We therefore argue that NDRG1 degradation provides a significant contribution to the SGK3 downstream signaling.
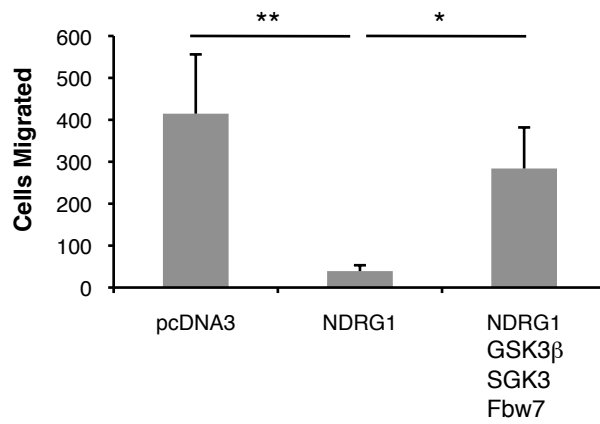
Figure 2-21: NDRG1 inhibits breast cancer cell migration

ZR-75-1 cells were transfected for 24 hr with pcDNA3, NDRG1, or NDRG1, Fbw7, GSK3β, and SGK3 followed by an 18 hr Transwell migration assay.

5. DISCUSSION

The frequency of PI 3-K pathway mutations in many human solid tumors, including breast cancer, has led to the development of numerous small molecule inhibitors currently in phase I and II clinical trials (Baselga, 2011). These include inhibitors to *PIK3CA*, such as the pan class I PI 3-K inhibitor BKM-120, as well as p110α-specific inhibitors such as BYL-719 and A66. Various Akt catalytic inhibitors are also undergoing pre-clinical validation for many tumor types (Cleary and Shapiro, 2010; Courtney et al., 2010). Arguably, most studies that have evaluated the mechanism by which PI 3-kinases transduce signals to phenotypes associated with malignancy have focused on Akt as the effector molecule. However, other PI 3-K targets have been shown to signal downstream of PI(3,4,5)P3, for example the Tec family kinases such as Btk in hematological malignancies (Davids and Brown, 2012). A more recent study pointed to SGK3 as a critical effector of oncogenic *PIK3CA*-mutant breast cancer cells in which Akt is dispensable (Vasudevan et al., 2009). This finding provides insight into an additional target for the development of new drugs for combination therapy in PI 3-K pathway mutant tumors.

Since SGK3 does not possess a PH domain in the regulatory region that would bind PI(3,4,5)P3, the mechanism by which *PIK3CA* can activate SGK3 has remained obscure (Tessier and Woodgett, 2006b). We reasoned that such a mechanism would have to take into account the lipid-binding property of the SGK3 PX domain, which bind PI(3)P (Tessier and Woodgett, 2006a). Although PI 3-K has been shown to activate SGK isoforms through PDK-1 and TORC2 (Garcia-Martinez and Alessi, 2008), it has

also been shown that a PX domain lipid-binding mutant of SGK3 is catalytically inactive (Tessier and Woodgett, 2006a). This same PX domain mutant is not phosphorylated at the corresponding Thr320 and Ser486 sites, indicating PI(3)P binding as a pre-requisite for phosphorylation and activation. Since class I PI 3-kinases do not synthesize PI(3)P directly, an intermediate step(s) is required for SGK3 activity. Our data show that INPP4B is both necessary and sufficient to transduce the *PIK3CA* signal to SGK3 activation. INPP4B is a PI(3,4)P2 phosphatase, such that inactivation of INPP4B by LOH in human tumors leads to Akt hyperactivation (Fedele et al., 2010; Gewinner et al., 2009). Conversely, one would predict that in tumors with normal or elevated expression of INPP4B, the synthesis of PI(3)P would promote the activation of an alternative effector(s). We propose that SGK3 is one such effector, whose activation by *PIK3CA* is mediated by INPP4B in breast cancer cells. This model is supported by several lines of evidence. First, breast cancer cell lines with high INPP4B levels show elevated SGK3 phosphorylation and activity, whereas lines with low or undetectable INPP4B do not (Figure 1C). Expression of catalytically active, but not inactive INPP4B leads to activation of SGK3 with a concomitant inhibition of IGF-1-stimulated Akt phosphorylation (Figure 2A). Moreover, silencing INPP4B with shRNA attenuates SGK3 activation, and also leads to inhibition of anchorage-independent growth and proliferation in 3D (**Figure 2-9, Figure 2-12 and Figure 2-13**). In a previous study, silencing INPP4B with shRNA was shown to result in increased anchorage-independent growth, also in MCF7 cells (Fedele et al., 2010). The reason for the discrepancies between the two studies is unclear, but could be due to differences in shRNA sequences used. In our studies, two

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distinct shRNA sequences were used, and introduction of INPP4B cDNA rescued the phenotype. Regardless, our data support a model in which INPP4B functions as both an oncogene and a tumor suppressor. A tumor suppressor function is evident in basal-like tumors, which show a high frequency of INPP4B LOH, and a concomitant increase in Akt activity (Fedele et al., 2010). Conversely, an oncogenic role for INPP4B is supported by breast cancer cell lines derived from luminal tumors that show elevated SGK3 activation and high INPP4B expression.

INPP4B dephosphorylates PI(3,4)P2 to generate PI(3)P, and our results show that INPP4B catalytic activity is required for SGK3 activation. Since the SGK3 PX domain binds PI(3)P, this would argue that SGK3 activation would occur at sites of PtdIns-3-P accumulation. PI(3)P is also be generated by class I and II PI 3-kinases. Specifically, a significant pool of PI(3)P is generated by the class III enzyme VPS34 at endosomal membranes (Backer, 2008). Previous studies have shown that SGK3 is localized at the endosome in a manner that depends on an intact PX domain (Tessier and Woodgett, 2006a). Since SGK3 phosphorylation and subsequent activation are dependent on a functional PX domain, this would argue that SGK3 activation occurs primarily at the endosome. However, in our studies we find that *PIK3CA*-mediated activation of SGK3 does not require VPS34 (**Figure 2-8**). The products of *PIK3CA*, PI(3,4,5)P3 and PI(3,4)P2 are synthesized primarily at the plasma membrane, indicating that the pool of PI(3)P generated by INPP4B would also reside at the plasma membrane. In turn, a plasma membrane pool of PI(3)P would recruit SGK3 leading to its activation. Although we cannot formally rule out that the PI(3)P generated by INPP4B recruits SGK3 at the

endosome, this is unlikely because studies have demonstrated that the endosomal

recruitment of SGK3 is PI 3-K-independent (Tessier and Woodgett, 2006a). Tools for

detecting endogenous SGK3 localization, such as activation-state antibodies, are not of

sufficient sensitivity for use in immunofluorescence or immunohistochemistry, and to

date our results aimed at localizing SGK3 in response PI 3-K and INPP4B signaling

have been inconclusive. Future studies using more specific reagents will be required to

address the relevant localization of SGK3 downstream of *PIK3CA*.

Oncogenic *PIK3CA* mutations are particularly prevalent in ER-positive breast

cancers (Cancer Genome Atlas, 2012). Interestingly, INPP4B has been identified as a

potential biomarker for luminal-type breast cancers (Fedele et al., 2010). Moreover, both

INPP4B and SGK3 are transcriptionally regulated by estrogen (Harvell et al., 2006;

Wang et al., 2011a; Xu et al., 2012). This suggests that the *PIK3CA*/INPP4B/SGK3

signaling axis is particularly important at promoting tumorigenesis in hormone receptor-

positive cancers. It is also interesting to note that the original characterization of SGK3

as a required target for *PIK3CA*-mediated growth and survival was conducted MCF7

cells, which express estrogen receptor (Vasudevan et al., 2009). Whether ER

expression is necessary or rate- limiting for the *PIK3CA*/INPP4B/SGK3 pathway

remains to be determined, since other mechanisms contribute to the transcriptional

induction of both INPP4B and SGK3.

The majority of SGK3 substrates identified to date have also been shown to be

phosphorylated by Akt, and thus likely share overlapping phenotypes during tumor

progression (Tessier and Woodgett, 2006b). However, NDRG1 is the only known

specific SGK substrate, although the functional consequence of SGK-mediated phosphorylation of NDRG1 is not known. We explored NDRG1 as a target of SGK3 in breast cancer cells, since NDRG1 is a known metastasis suppressor and its expression inversely correlates with breast cancer grade (Kovacevic and Richardson, 2006). Our results show that SGK3 is required for invasive migration, a pre-requisite for metastatic dissemination (**Figure 2-15**). We also find that NDRG1 protein levels are inversely correlated with SGK3 activity, suggesting that SGK3 might control NDRG1 stability (**Figure 2-3**). It has been shown that SGK3 phosphorylation of NDRG1 primes for subsequent phosphorylation by GSK3β (Murray et al., 2004). Our studies provide additional insight into NDRG1 regulation, as we show that SGK3 and GSK-3β control the interaction of NDRG1 with the substrate recognition domain of the E3 ligase SCF complex, FBW7 (**Figure 2-19 and Figure 2-20**). FBW7 targets NDRG1 for degradation by the 26S- proteasome. In turn, loss of NDRG1 expression is known to reduce the recycling of E-cadherin to the plasma membrane, increase cell migration and eventual metastatic dissemination (Kachhap et al., 2007). Consistent with this, we find that silencing SGK3 inhibits cell migration, an effect that is phenocopied by expression of NDRG1 (**Figure 2-15 and Figure 2-21**). In this model, *PIK3CA*-mutant tumor cells with elevated SGK3 activity would maintain low NDRG1 levels, resulting in enhanced invasive migration.

6. CONCLUSION

In summary, we have identified a new mechanism by which *PIK3CA* transduces tumorigenic phenotypes in breast cancer, specifically though SGK3 activation. INPP4B catalytic activity is required for the ability of breast cancer cells with oncogenic *PIK3CA* to activate SGK3. This provides an alternative mechanism for *PIK3CA* to drive cancer progression under conditions in which Akt is inactive. These findings advocate for the development of small molecule inhibitors targeting SGK3, for *PIK3CA*-addicted tumors resistant to Akt inhibition.

Acknowledgements

CHAPTER III

RICTOR FORMS A COMPLEX WITH CULLIN-1 TO PROMOTE SGK1

UBIQUITINATION AND DESTRUCTION

1. ABSTRACT

The Rictor/mTOR complex (also known as mTORC2) plays a critical role in cellular homeostasis by phosphorylating AGC kinases such as Akt and SGK at their hydrophobic motifs to activate downstream signaling. However, the regulation of mTORC2 and whether it has additional function(s) remains largely unknown. Here we report that Rictor associates with Cullin-1 to form a functional E3 ubiquitin ligase. Rictor, but not Raptor or mTOR alone promotes SGK1 ubiquitination. Loss of Rictor/Cullin-1-mediated ubiquitination leads to increased SGK1 protein levels as detected in Rictor null cells. Moreover, as part of a feedback mechanism, phosphorylation of Rictor at T1135 by multiple AGC kinases disrupts the interaction between Rictor and Cullin-1 to impair SGK1 ubiquitination. These findings indicate that the Rictor/Cullin-1 E3 ligase activity is regulated by a specific signal relay cascade and that misregulation of this mechanism may contribute to the frequent overexpression of SGK1 in various human cancers.

(Gao et al., 2010)

2. INTRODUCTION

The mammalian target of Rapamycin (mTOR) plays a critical role in regulation of cellular homeostasis, cell growth and survival pathways by acting as a sensor for upstream inputs from multiple growth-promoting signals which are then transduced to downstream effectors (Guertin and Sabatini, 2007; Reiling and Sabatini, 2006). In order to fulfill this complex regulatory function, the mTOR kinase assembles into at least two distinct complexes termed mTORC1 and mTORC2 (Guertin and Sabatini, 2007; Reiling and Sabatini, 2006). These two multi-component subcomplexes differ both structurally and functionally and signal to distinct downstream substrates. mTORC1 is composed of mTOR, Raptor, PRAS40 and mLST8/GβL (G protein β-subunit-like protein). The best characterized mTORC1 kinase substrates include S6K (p70 S6 ribosomal kinase) and 4E-BP1 (phosphorylated 4E-binding protein). The mTORC2 complex includes mTOR, Rictor, mLST8/GβL, PROTOR (protein observed with Rictor-1)/PRR5 and Sin1 (Jacinto et al., 2006; Shiota et al., 2006). mTORC2 phosphorylates the hydrophobic motif of Akt at Ser473 (Sarbassov et al., 2005)and SGK1 at Ser422 (Garcia-Martinez and Alessi, 2008), leading to full kinase activation. Since aberrant activation of Akt is a hallmark of many types of cancers (Manning and Cantley, 2007), hyperactivation of mTORC2 activity has been implicated in cancer progression (Guertin and Sabatini, 2007).

The activity of the mTORC1 complex is highly regulated in cells exposed to growth factors and nutrients. In response to mitogens, activation of PI 3-K leads to phosphorylation of the TSC2 and PRAS40 proteins by Akt, culminating in activation of

mTORC1 (Manning and Cantley, 2007). The activity of mTORC1 can also be stimulated by the Rag GTPase in response to nutrient stimulation (Sancak et al., 2008). Additionally, phosphorylation of Raptor by AMPK (5′ AMP-activated protein kinase) in response to a low energy state provides a negative regulatory mechanism to repress mTORC1 activity (Gwinn et al., 2008). Although mTORC2 is a key upstream kinase complex that functions to control Akt phosphorylation and downstream signaling, relatively little is known regarding the regulation of mTORC2. Recent studies indicate that the mTOR complexes might be multi-functional and contain activities other than protein kinases. For example, Raptor has been shown to form a complex with the Cullin-4 E3 ligase and this complex might be critical for mTOR kinase activity (Ghosh et al., 2008). Rictor has also been shown to associate with Cullin-4, although unlike Raptor it is not a WD40-repeat-containing protein (Ghosh et al., 2008). However, additional function(s) for Rictor and mTORC2 remain largely unknown.

The serum and glucocorticoid-inducible kinase (SGK) belongs to the AGC (protein kinase A, G and C) family of kinases, and its activity is stimulated by growth factors (Lang et al., 2006). There are three closely related family members designated SGK1, SGK2 and SGK3 (Loffing et al., 2006). One of the best-characterized SGK1 downstream targets is Foxo3a, which is involved in the regulation of apoptosis (Brunet et al., 2001). SGK1 has also been indicated in the regulation of Na+ retention through phosphorylation of NEDD4-2 to impair its ability to degrade the epithelial Na+ channel (ENaC) (Debonneville et al., 2001; Ichimura et al., 2005). SGK isoforms share

approximately 55% similarity in the kinase domains with other AGC family kinases including Akt and S6K. In vitro SGK recognizes the same phosphorylation consensus motif (RXRXXS/T, where X represents any amino acid) as Akt and S6K (McCormick et al., 2004). However, unlike Akt and S6K whose expression is relatively stable, SGK1 is a short-lived protein whose stability is controlled by the ubiquitin-proteasome pathway (Loffing et al., 2006). Both the NEDD4-2 (Brickley et al., 2002; Zhou and Snyder, 2005) and CHIP (C-terminal Hsc70-interacting protein) E3 ligases have been shown to ubiquitinate SGK1 (Belova et al., 2006). The first 60 amino acids of SGK1 are critical for NEDD4-2 mediated destruction of SGK1 (Bogusz et al., 2006; Brickley et al., 2002). In addition to growth factor stimulation, cellular stresses including osmotic stress, heat shock, oxidative stress and ultraviolet irradiation induce SGK1 expression by transcriptional mechanisms and thus influence cell survival, proliferation and differentiation (Lang and Cohen, 2001; Loffing et al., 2006). However, it remains unclear how SGK1 destruction by NEDD4-2 and CHIP are regulated by these cellular stresses and whether other unidentified E3 ligase(s) play a critical role in governing SGK1 destruction in response to these signaling events. Finally, both Akt and SGK are frequently amplified and/or overexpressed in cancers (Sahoo et al., 2005), although the underlying molecular mechanisms are unknown.

Here we evaluate the mechanism by which Rictor controls SGK1 stability. We found that by specific association with Cullin-1 and Rbx1, Rictor forms a functional E3 ubiquitin ligase complex that promotes the ubiquitination of SGK1, but not Akt1 or S6K1.

We also show that the AGC kinases phosphorylate Rictor at Thr1135 to disrupt the

Rictor/Cullin-1 complex and impair its E3 ligase activity and subsequent SGK1

ubiquitination. Our findings demonstrate a kinase-independent function for the Rictor

protein and provide a mechanistic explanation for the observed elevation of SGK1

expression in various human tumors.

(Gao et al., 2010)

3. MATERIALS AND METHODS

Cell culture and Cell Synchronization

Cell culture, including synchronization and transfection, has been described (Gao et al., 2009). Lentiviral shRNA virus packaging and subsequent infection of various cell lines were performed according to the protocol described previously (Boehm et al., 2005). Rictor –/– MEFs and control MEFs were kind gifts from Dr. Mark Magnuson (Shiota et al., 2006). Kinase inhibitors LY294002 (Sigma, L9908), Rapamycin (Calbiochem, 553210), Wortmannin (Sigma, 95455) and Akt 1/2 Inhibitor VIII (Calbiochem, 124018) were used as indicated.

In vitro Kinase Assay

mTORC2 *in vitro* kinase assay was performed as described previously (Sarbassov et al., 2005).

In vivo Ubiquitination Analysis

Cells were transfected with a plasmid encoding HA-Δ60-SGK1 along with Flag- or His-tagged ubiquitin. myc-Rictor or other expression vectors were co-transfected to assess their effects on SGK ubiquitination. Thirty-six hours after transfection, 10 μM MG132 was added to block proteasome degradation, and cells were harvested in EBC buffer or denaturing buffer (6 M Guanidine-HCL, 0.1 M NaH_2PO_4, 10 mM Tris-HCL, 10 mM immidazole, pH 8.0) containing protease inhibitor. 2 mg whole cell lysates were incubated with Flag beads or Ni-NTA resin for 4–10 hrs, followed by washing 4 times with NETN buffer or denaturing washing buffer (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-

HCL, 10 mM immidazole, pH 6.3). Then the washed pellet was boiled in SDS-containing lysis buffer and resolved on SDS-PAGE.

In vitro ubiquitination assay

The *in vitro* ubiquitination assays were performed as described previously (Jin et al., 2005). To purify the Cullin-1/Rictor E3 ligase complex, 293T cells were transfected with vectors encoding HA-Cullin-1, Myc-Rictor (WT or T1135E), and Flag-Rbx1. The Cullin-1/Rictor (E3) complexes were purified from the whole cell lysates using HA-agarose beads. Purified, recombinant SGK protein (purchased from Genway) were incubated with purified Cullin-1/Rictor (E3) complexes in the presence of purified, recombinant active E1, E2 (UbcH5a and UbcH3), ATP and ubiquitin. The reactions were stopped by the addition of 2X SDS-PAGE sample buffer and the reaction products were resolved by SDS-PAGE gel and probed with the indicated antibodies.

Gel Filtration Chromatography for Separation of Rictor Complexes

Two 10 cm plates of HeLa cells were washed with phosphate buffered saline, lysed in 0.5 ml of CHAPS lysis buffer (25 mM HEPES pH7.4, 150 mM NaCl, 1 mM EDTA, 0.3% CHAPS), and filtered through a 0.45 µm syringe filter. Total protein concentration was 6 mg/ml. 500 µl of lysate was loaded onto a Superdex 200 10/300 GL column (GE Lifesciences Cat. No. 17-5175-01). The experiment in was performed with this same column attached in series to a Superose 6 10/300 GL (GE Lifesciences Cat. No.17-5172-01). The sample was separated in the Superose 6 column first, and then the

Superdex 200 column. The gel filtration beads in each column have different size exclusion characteristics that complement each other and allow separation of very large and also smaller proteins. Chromatography was performed using an AKTAFPLC (GE Lifesciences Cat. No. 18-1900-26) and protein complexes were resolved by eluting with the same CHAPS buffer at 0.5 ml/min over 3 hours. Eluent was collected in either 250 μl or 500 μl fractions. 50 μl aliquots of these fractions were loaded onto SDS-PAGE minigels for SDS-PAGE and western blot analysis of the fractionated protein complexes. Prior to running cell lysates, the molecular weight resolution of the columns was first estimated by running native molecular weight markers (urease ~545 kDa, mouse monoclonal IgG ~180 kDa, human serum albumin ~68 kDa) and determining their retention times on Coomassie-stained SDS-PAGE protein gels.

4. RESULTS

SGK1 Protein Expression is Regulated by Rictor

SGK1 is an unstable protein and previous studies have shown that in response to serum and growth factors, activation of PI 3-K leads to the induction of SGK1 (Park et al., 1999). Induced SGK1 expression occurs partially through the increased transcriptional levels of SGK1 mRNA, and partially through other layers of post-translational regulation (Loffing et al., 2006). However, the exact molecular mechanism(s) remain elusive. Recent studies have shown that the *Rictor*-containing mTORC2 complex phosphorylates SGK1 at Ser422 in the hydrophobic motif to fully activate SGK1 kinase activity (Garcia-Martinez and Alessi, 2008). We therefore first investigated if Rictor signaling can also influence SGK1 expression. We found that *Rictor*–/– MEFs have elevated SGK1 expression levels under serum-deprived conditions (**Figure 3-1A**). In agreement with previous studies (Webster et al., 1993), re-addition of serum led to a significant induction of SGK1 protein in both wild type and *Rictor* –/– MEFs. Elevated SGK1 expression in *Rictor* –/– MEFs has been reported (Huang et al., 2009), although the underlying molecular mechanism has not been characterized. Loss of Rictor is the primary mechanism for the observed elevation of SGK1 expression since re-introduction of wild-type *Rictor* into *Rictor*–/– MEFs dramatically reduced SGK1 expression (**Figure 3-1B**). Consistent with this, depletion of Rictor, but not Raptor, in HeLa cells also led to an accumulation of SGK1 protein, primarily in the early G1 phase of the cell cycle (**Figure 3-1C**). However, Akt1 and S6K1
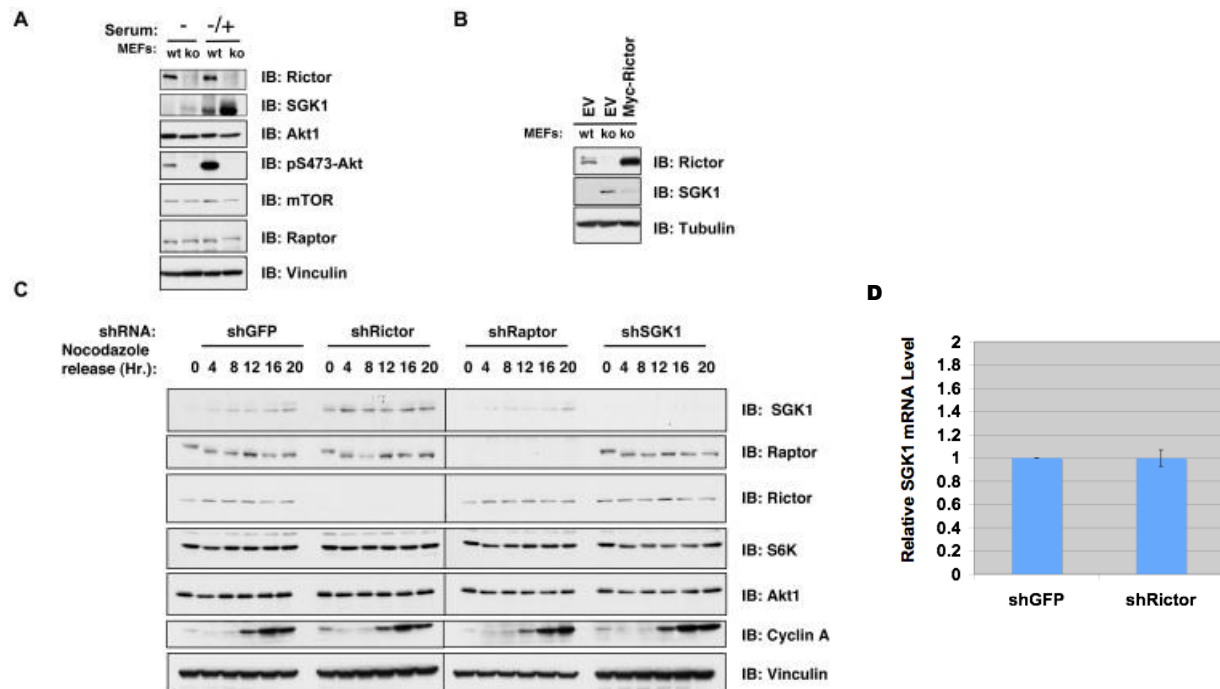
Figure 3-1: SGK1 expression is regulated by the Rictor pathway

(A) Whole cell lysates were isolated from wild type or Rictor –/– mouse embryonic fibroblasts (MEFs) in serum starvation conditions for 24 hours. In another experimental condition, 10% FBS was added to the serum-starved cells for 1.5 hours before harvesting. Equal amounts of whole cell lysates were immunoblotted with the indicated antibodies.

(B) Immunoblot analysis of wild type or Rictor–/– MEFs transfected with the Myc-Rictor plasmid (with empty vector as a negative control) together with pBabe-Puro retroviral empty vector constructs. Twenty-four hours post-transfection, the cells were treated with 1 µg/ml puromycin for 48–72 hours to kill the non-transfected cells prior to collecting the whole cell lysates for immunoblots.

(C) HeLa cells were infected with the indicated lentiviral shRNA constructs and selected with 1µg/ml puromycin to eliminate the non-infected cells. The resulting HeLa cell lines were arrested in the M phase by incubation with nocodazole for 18 hours and then released into the G1 phase. At the indicated time points, cell lysates were collected for immunoblot analysis.

(D) Quantitative RT-PCR analysis to determine the relative mRNA expression of *SGK1* in wild-type or *Rictor-/-* cell lines.

expression was not affected by depletion of endogenous Rictor in HeLa cells (**Figure 3-1C**). Depletion of Rictor does not significantly affect SGK1 mRNA levels (**Figure 3-1D**), indicating that post-translational modification(s) might contribute to the regulation of SGK1 by Rictor.

Rictor Promotes SGK1 Ubiquitination in a Cullin-1-dependent Manner

In support of the finding that Rictor regulates SGK1 stability, we detected an interaction between Rictor and SGK1 (**Figure 3-2A**). We used a cell-based ubiquitination assay to address whether Rictor and other mTOR components could promote SGK1 ubiquitination. To exclude the possible contribution of other known SGK1 E3 ligases, including NEDD4-2 and CHIP, we used a deletion mutant of SGK1 that lacks the amino-terminal 60 amino acids (Δ60-SGK1), which cannot be efficiently ubiquitinated by NEDD4-2 and CHIP (Brickley et al., 2002; Zhou and Snyder, 2005). Surprisingly, E3 ligase activity towards SGK1 is unique to Rictor as neither Raptor nor mTOR promotes SGK1 ubiquitination (**Figure 3-2B**). SGK1 was recently identified to be a specific downstream substrate of mTORC2 (Rictor), but not mTORC1 (Raptor) (Garcia-Martinez and Alessi, 2008). Therefore, we reasoned that SGK1 may specifically interact with Rictor, but not Raptor, which could explain why Raptor does not promote SGK1 ubiquitination, despite the fact that Raptor was recently shown to exist in a complex with Cullin 4 (Ghosh et al., 2008).
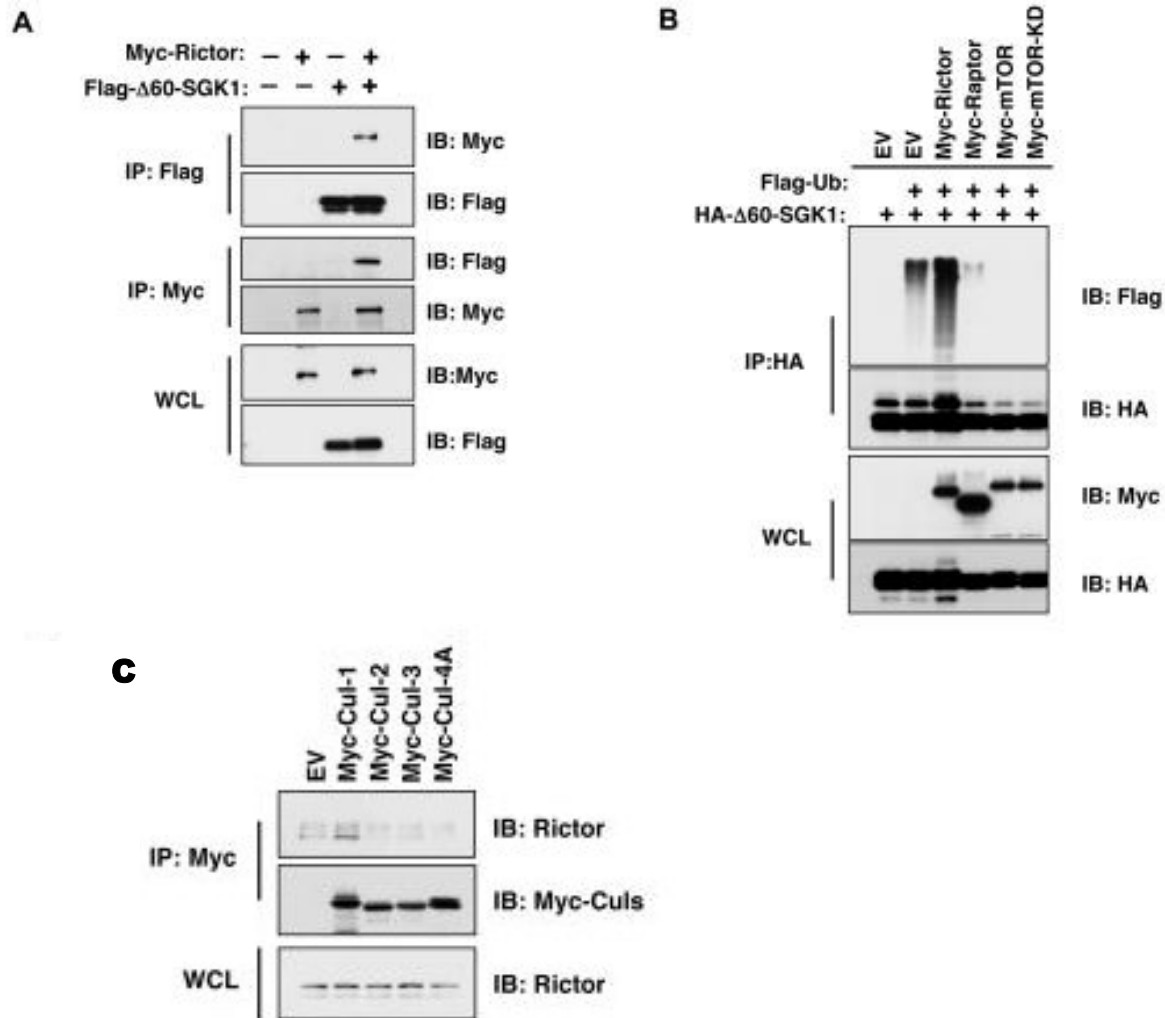
Figure 3-2: R Rictor promotes SGK1 ubiquitination in a Cullin-1 dependent manner

(A) Immunoblot analysis of whole cell lysates and immunoprecipitates derived from 293T cells transfected with Flag-Δ60-SGK1 and Myc-Rictor constructs. Thirty hours post-transfection, cells were treated with 10 µM MG132 for 10 hours to block the proteasome pathway before harvesting.

(B)Immunoblot analysis of whole cell lysates and anti-HA immunoprecipitates derived from 293T cells transfected with the indicated plasmids. Twenty hours post-transfection, cells were treated with the proteasome inhibitor MG132 overnight before harvesting.

(C)Immunoblot analysis of whole cell lysates and immunoprecipitates derived from 293T cells transfected with the indicated Myc-Cullin constructs. Thirty hours post-transfection, cells were treated with 10 µM MG132 for 10 hours to block the proteasome pathway before harvesting.

Since Rictor itself does not contain the ring-finger, PHD or HECT domain that possesses intrinsic E3 ligase activity, it is possible that Rictor associates with other co-factor(s) to form an E3 ligase complex. The Cullin-Ring complex comprises the largest family of E3 ubiquitin ligases (Petroski and Deshaies, 2005). Thus, we first determined which Cullin family member might contribute to SGK1 ubiquitination. We showed that Rictor specifically interacts with Cullin-1, but not other Cullin family members Cullin-2, 3 and 4 (**Figure 3-2C**). These data are indicative of a role for Cullin-1 in SGK1 ubiquitination.

Rictor forms a complex with Cullin-1, Rbx1 to promote SGK1 destruction

In support of a possible physiological role for both Rictor and Cullin-1 in regulating SGK1 stability, we detected the endogenous interaction between Rictor and Cullin-1 (**Figure 3-3A-B**). Furthermore, the endogenous interaction between Rictor and Cullin-1 could be detected in NP-40 containing EBC buffer (**Figure 3-3B**), a condition that has been shown to disrupt the mTORC2 complex (Hara et al., 2002; Kim et al., 2002), indicating that the intact Rictor/mTOR complex might not be required for interaction with Cullin-1. In support of this idea, we showed that Rictor, but not Sin1 or mTOR, specifically interacts with endogenous Cullin-1 (**Figure 3-3C**). Furthermore, Cullin-1 only specifically interacts with Rictor, but not Raptor, or other known mTORC2 components including mTOR, GβL and Sin1, (**Figure 3-3D**). Although mTOR interacts with Rictor more strongly than Cullin-1 in CHAPS buffer, mTOR/Rictor interaction is not detected in

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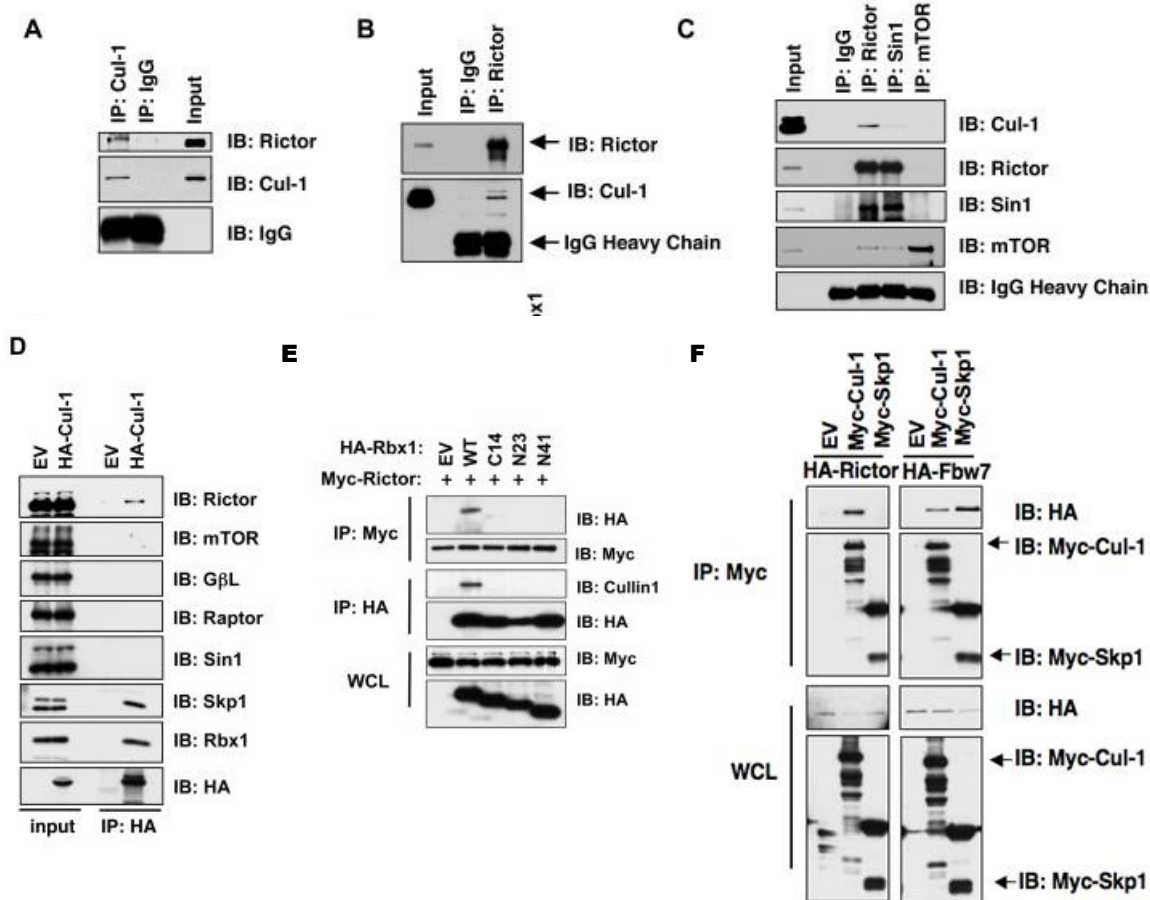
Figure 3-3: Rictor interacts with Cullin-1 and Rbx1

(A) Immunoblot (IB) analysis of 293T whole cell lysates (WCL) and anti-Cullin-1 immunoprecipitates (IP). Mouse IgG was used as a negative control for the IP. WCL were collected with CHAPS and IPs were washed with CHAPS buffer.

(B) IB analysis of 293T WCL and anti-Rictor IP. Rabbit IgG was used as a negative control for the IP procedure. WCL were collected with EBC buffer and IPs were washed with NETN buffer.

(C) IB analysis of 293T WCL and anti-Rictor, anti-Sin1, and anti mTOR IP. Rabbit IgG was used as a negative control for the IP. WCL were collected with EBC buffer and IPs were washed with NETN buffer.

(D) IB analysis of WCL and IP derived from HeLa cells transfected with the HA-Cullin-1 construct.

(E) IB analysis of WCL and IP derived from 293T cells transfected with Myc-Rictor together with the indicated HA-Rbx1 constructs. Thirty hours post-transfection, cells were pretreated with 10 µM MG132 for 10 hours to block the proteasome pathway before harvesting.

(F) IB analysis of WCL and IP derived from 293T cells transfected with HA-Rictor or HA-Fbw7 constructs together with the indicated Myc-Cullin-1 or Myc-Skp1 constructs. Thirty hours post-transfection, cells were pretreated with 10 μM MG132 for 10 hours to block the proteasome pathway before harvesting.

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EBC buffer **Figure 3-3E**) (Hara et al., 2002; Kim et al., 2002). These results suggest

that Cullin-1 interacts with Rictor *in vivo*.

Cullin-1 is an extensively studied member of the Cullin family (Cardozo and

Pagano, 2004; Harper et al., 2002; Nakayama and Nakayama, 2005). It complexes with

Rbx1, Skp1 and various F-box proteins to form a multi-protein SCF (Skp1, Cullin-1, F-

box protein) E3 ligase complex (Cardozo and Pagano, 2004; Schulman et al., 2000). To

further understand the physiological components of the Rictor/Cullin-1 complex, we

performed gel filtration chromatography. This unpublished data suggests there might be

two different pools of Rictor complex. One complex was estimated at a size over 600KD

(fractions 22–26), co-migrating with activated mTOR (as evidenced by S2481

phosphorylation) and Sin1, which might represent the mTORC2 complex. The other

Rictor-containing complex was detected at 300–400 KD (fractions 30–34), and co-

migrates with both Cullin-1 and Rbx1, but not Sin1. Since Sin1 is required for mTORC2

complex formation (Jacinto et al., 2006; Yang et al., 2006), the lack of Sin1 precludes a

possible existence of the mTORC2 complex in these fractions. In contrast, one Raptor

complex (mTORC1 complex) is detected, estimated to be over 600KD and co-migrating

with mTOR. Another detected Raptor peaks at around 180–250KD (fractions 36–40),

which might represent the free Raptor monomer. Consistent with the co-

immunoprecipitation data (**Figure 3-3D**), there is no detected co-migration between

Raptor and Cullin-1 at fractions 29–34 with anticipated size (around 250–400KD)

corresponding to a possible Raptor/Cullin-1 complex.

Using a series of Rbx1 mutants that are unable to interact with Cullin-1, we showed that the presence of Cullin-1 is required for Rbx1 and Rictor interaction (**Figure 3-3E**). However, we found that under ectopic overexpression conditions Rictor does not interact with Skp1 (**Figure 3-3F**). Altogether, these data support the hypothesis that a unique complex composed of Rictor, Cullin-1 and Rbx1 (and possibly other unknown partners) is involved in regulating SGK1 abundance. However, it requires further investigation to fully understand the role of Skp1 in this process.

Rictor is Phosphorylated by AGC Kinases at T1135

Although Rictor is a key regulator of both Akt and SGK1, it remains largely unknown how its activity is regulated. In agreement with recent reports (Dibble et al., 2009; Julien et al., 2010; Treins et al., 2010) we found that S6K1 phosphorylates Rictor at Thr1135 (**Figure 3-4A-B**). Furthermore, ectopically expressed Rictor is phosphorylated in cells by Akt1 and SGK1 as well (**Figure 3-4A-B**). Moreover, although depletion of S6K leads to a reduction in Thr1135 phosphorylation, depletion of Akt1 or SGK1 delivers similar effects and depletion of any individual AGC kinase does not significantly affect endogenous Rictor Thr1135 phosphorylation as depletion of mTOR does (data not shown). This possibly indicates that all three AGC family kinases might function in a redundant manner towards Rictor phosphorylation at Thr1135. A similar mechanism has been reported for Foxo3a phosphorylation by AGC kinases (Tran et al., 2003; Vogt et al., 2005).
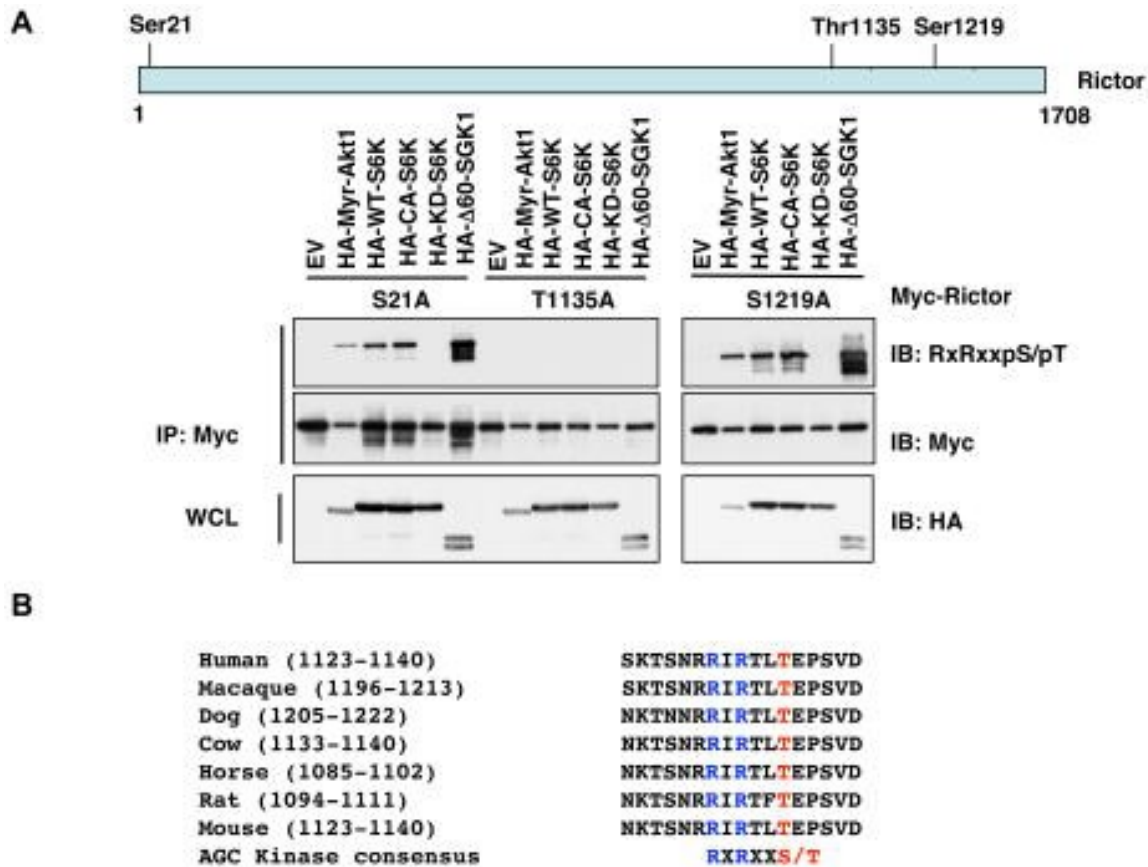
Figure 3-4: Rictor is phosphorylated at T1135.

(A) Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with the indicated Myc-Rictor constructs together with various HA-tagged AGC family kinases.
(B) Sequence alignment of the putative Rictor T1135 phosphorylation site across different species.

Phosphorylation of Rictor at T1135 disrupts the Cullin-1/Rictor interaction

Next we investigated the consequence of Rictor Thr1135 phosphorylation on mTORC2 complex activity. Using gel filtration chromatography, we detected co-migration of p-Thr1135-Rictor with an active form of mTOR (p-Ser2481) corresponding to the mTORC2 complex (fractions 14–20). However, p-Thr1135-Rictor did not co-migrate with Cullin-1 at 300–400KD (fractions 21–26). Similarly, wild-type-Rictor, but not phospho-mimetic Thr1135E-Rictor, co-migrated with Cullin-1 (**Figure 3-5A** and data not shown), indicating that phosphorylation of Rictor at Thr1135 might serve to disrupt the Rictor/Cullin-1 complex.

As shown in Figure 5B, insulin addition into serum starved HeLa cells leads to activation of the S6K and Akt kinase pathways (as shown by the increased Ser473-Akt and Thr389-S6K signals), which results in increased Rictor phosphorylation at Thr1135, coupled with a decrease in Cullin-1/Rictor interaction and enhanced 14-3-3/Rictor interaction, and consequently, increased SGK1 abundance. In a reciprocal set of experiments, we treated HA-Cullin-1 expressing cells cultured in 10% FBS-containing DMEM medium with LY290042 to inactivate PI 3-K and then monitored how this affects the Cullin-1/Rictor interaction. LY treatment induced a time-dependent decrease of Rictor Thr1135 phosphorylation, presumably due to the inactivation of S6K and Akt. Rictor phosphorylation at Thr135 is inversely correlated with its ability to interact with Cullin-1.
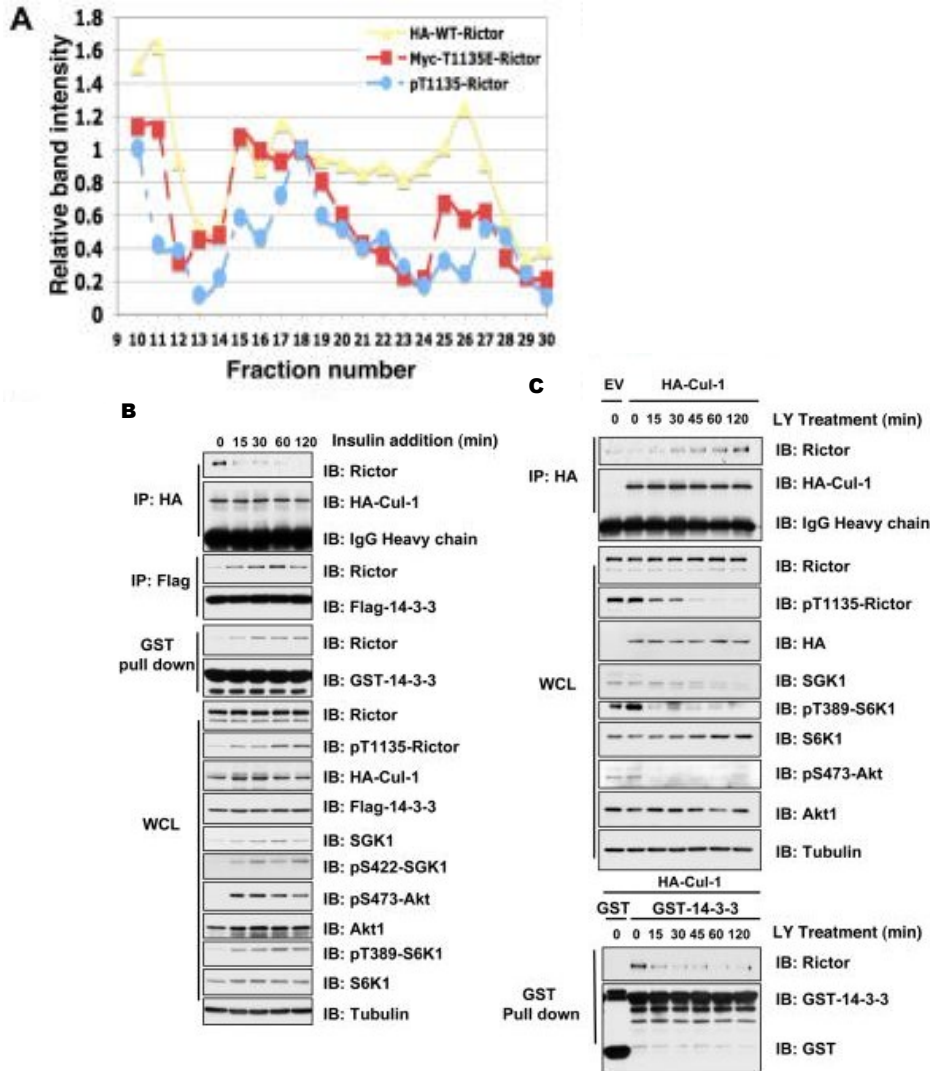
Figure 3-5: Phosphorylation of Rictor at T1135 disrupts the interaction between Rictor and Cullin-1

(A) HeLa cells were transiently transfected with HA-WT-Rictor and Myc-T1135-Rictor constructs. Thirty hours post-transfection, whole cell lysates were collected in CHAPS buffer and subjected to gel filtration chromatography. The relative band intensities for HA-WT-Rictor, Myc-T1135E-Rictor and p-T1135-Rictor at the indicated fractionations were quantitated.
(B) HA-Cullin-1 expressing HeLa cells transiently transfected with the Flag-14-3-3 construct. Eighteen hours post-transfection, cells were serum-starved for 24 hours. After the addition of 100nm Insulin, whole cell lysates (WCL) were collected at the indicated time points for immunoblot analysis with the indicated antibodies, for Flag-IP and GST pull-down assays to determine Rictor/14-3-3 interaction, and for HA-IP to determine Rictor/Cullin-1 interaction.
(C) HA-Cullin-1 expressing HeLa cells were treated with 20mM LY294002. At the indicated time points, whole cell lysates (WCL) were collected for immunoblot analysis with the indicated antibodies, for HA-IP to determine Rictor/Cullin-1 interaction, and GST-pulldown assays to determine Rictor/14-3-3 interaction.

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Phosphorylation of Rictor at T1135 reduces the ability of Rictor to ubiquitinate SGK1

As a result of reduced interaction with Cullin-1, T1135E Rictor is defective in promoting SGK1 ubiquitination (**Figure 3-6A**). Since T1135E has similar affinity as wild-type Rictor in binding to SGK1, the impaired E3 ligase activity towards ubiquitination of SGK1 might primarily be due to the disruption of the Cullin-1/Rictor association. Moreover, in keeping with impaired E3 ligase activity, compared with wild-type Rictor, T1135E Rictor is also compromised in promoting SGK1 degradation (**Figure 3-6B**). In summary, these data demonstrate that in addition to complexing with mTOR to form the mTORC2 complex as a means to phosphorylate Akt and SGK, by complexing with Cullin-1 and Rbx1, Rictor might have an additional function as an E3 ligase complex that controls the stability of SGK1 and likely additional targets (**Figure 3-7**). We also show that the E3 ligase activity of Rictor is subject to negative regulation by a variety of AGC kinases. Since PI 3-K positively regulates AGC kinase activity, frequent hyperactivation of this signaling axis might contribute to the elevated SGK1 expression levels detected in various cancers (**Figure 3-7**).
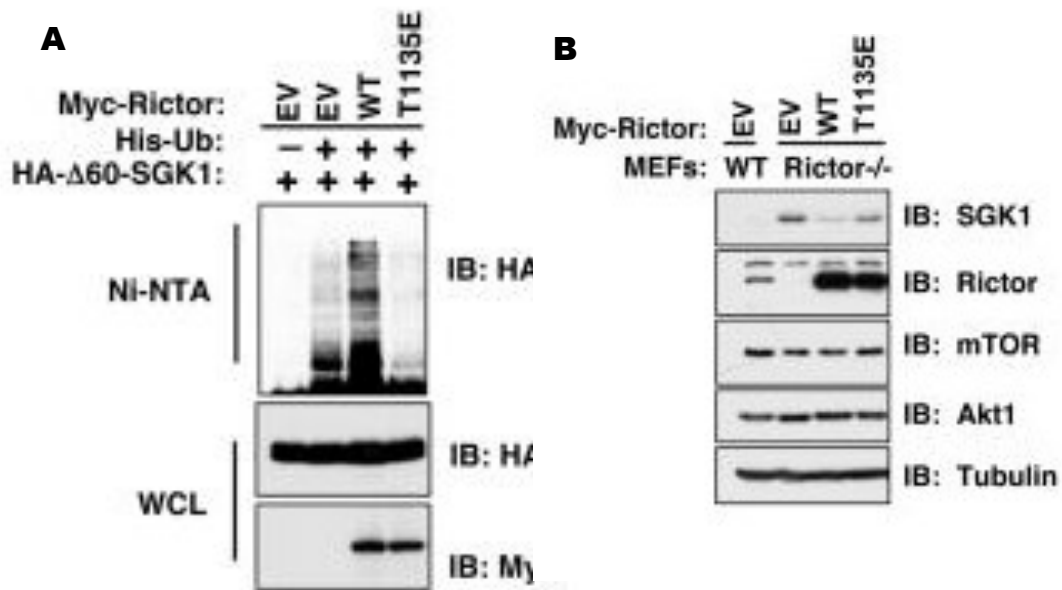
Figure 3-6: Phosphorylation of Rictor at T1135 reduces the ability of Rictor to ubiquitinate SGK1

(A) Immunoblot (IB) analysis of whole cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells Transfected with HA-D60-SGK1 together with His-Ub and various Myc-Rictor constructs. Twenty hours post-transfection, cells were treated with the proteasome inhibitor MG132 overnight before harvesting. The whole cell lysates were collected in EDTA-free lysis buffer and the His-pull down was carried out in the presence of 8M Urea to disrupt possible protein interactions.
(B) Wild type or Rictor–/– MEFs were transfected with the indicated Rictor plasmids (with empty vector as a negative control) together with the pBabe-Puro retroviral empty vector. Twenty-four hours post-transfection, the cells were treated with 1 µg/ml puromycin for 48–72 hours to kill the non-transfected cells prior to collecting the whole cell lysates for immunoblots

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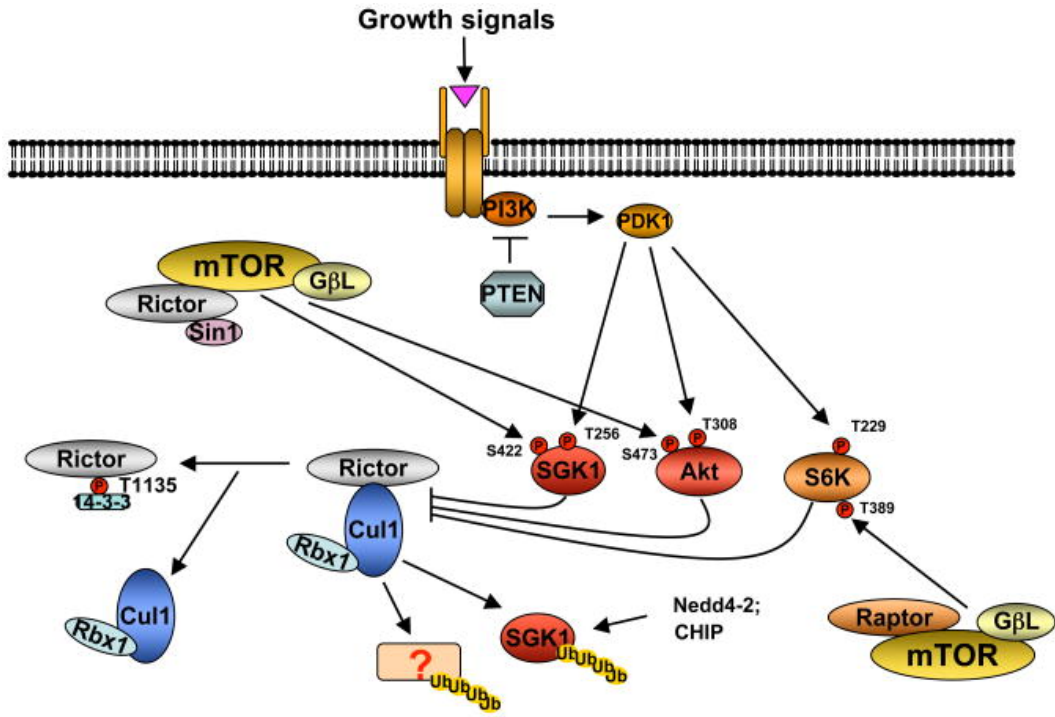
Figure 3-7: Proposed model for the Rictor/ Cullin-1 pathway to control SGK1 turnover

5. DISCUSSION

The data presented above provides experimental evidence for a possible function of Rictor in the ubiquitination of SGK1. To date, the only function attributed to Rictor in the mTORC2 complex is phosphorylation of the hydrophobic motifs of downstream targets such as Akt and SGK. Our data points to a specific association of Rictor with Cullin-1 and Rbx1 as part of a possible functional E3 ubiquitin ligase complex (**Figure 3-7).**

Although our results indicate that mTOR may not be required for Rictor E3 ligase activity (**Figure 3-2B**), it remains unclear whether these two complexes share common scaffolding proteins and what the crosstalk is between these two complexes. Recent studies demonstrate that many other kinase complexes also possess E3 ligase activity (Lu et al., 2002; Maddika and Chen, 2009). Since many ubiquitination processes require a prior phosphorylation event, the coupling of both the kinase and the E3 ligase in proximity provides for more efficient destruction (Carrano et al., 1999). It is possible that there are unknown Rictor ubiquitination substrate(s) that require prior phosphorylation by Rictor.

Using gel filtration assays, in addition to the well-known mTORC2 complex migrating at over 600KD, we detected a second possible Rictor-containing complex, co-migrating with Cullin-1 and Rbx1 at 300–400KD. This finding was supported by endogenous co-immunoprecipitation assays showing that only Rictor, but not other mTORC2 complex components including mTOR, GβL and Sin1 associates with Cullin-1 (**Figure 3-3A-D**). We also found that although Sin1 could efficiently immunoprecipitate endogenous Rictor, it could not immunoprecipitate endogenous Cullin-1 as Rictor IP

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does (**Figure 3-3D**). Since Sin1 is required for formation of the mTORC2 complex (Jacinto et al., 2006; Yang et al., 2006), these results suggest that Rictor might exist in two distinct complexes, the Sin1-containing mTORC2 complex, and the Cullin-1-containing complex. This was further supported by the observation that depletion of Rictor, Cullin-1 and Rbx1, but not mTOR, Raptor or Sin1 leads to upregulation of SGK1. However, additional studies are required to fully understand the possible role of mTOR and other TORC2 complex components in Rictor-mediated SGK1 turnover.

Our data also demonstrate that unlike Rictor, Raptor does not promote the ubiquitination of Akt1, SGK1 and S6K1 (**Figure 3-2B**). This suggests that the E3 ligase activity might be unique to the Rictor/Cullin-1 complex. However, under ectopic expression conditions, Skp1 is not detected in the Rictor/Cullin-1 complex. It is known that Skp1 serves as a bridging molecule to hold Cullin-1 and the F-box protein in a complex. The possible lack of Skp1 in the Cullin-1/Rictor complex suggests that Rictor might play a role equivalent to Skp1. However, further experimental investigation is required to fully understand the role of Skp1 for the Rictor/Cullin-1 complex to ubiquitinate SGK1, and more studies are also needed to determine whether a specific F-box protein is involved in this process.

6. CONCLUSIONS

Although SGK1 has been shown to be degraded by other E3 ligases including NEDD4-2 and CHIP, in both cases it is not known how the destruction is regulated and whether it is mediated by PI 3-K signaling. Our data suggest an alternative mechanism whereby multiple AGC kinases can negatively regulate Rictor/Cullin-1 E3 ligase activity without affecting its kinase activity, and this suggests a positive feedback loop to boost SGK1 activity post-stimulation. However, more studies are required to determine whether the ability of NEDD4-2 and CHIP to degrade SGK1 is also affected by the PI 3-K/Akt signaling. SGK1 overexpression has been reported in multiple cancers including breast cancer. Most interestingly, although SGK1 has been suggested to have redundant functions with Akt, simultaneous overexpression of both Akt and SGK1 has also been reported in breast cancers (Sahoo et al., 2005). Our work suggests that since SGK1, but not Akt, is subject to ubiquitination by the Rictor/Cullin-1 complex, elevated Akt activity might block the function of Rictor/Cullin-1, leading to accumulation of SGK1 (data not shown). Collectively, our results provide insight into how Rictor can influence SGK1 signaling by promoting its ubiquitination. Furthermore, we define a feedback mechanism that can negatively regulate the E3 ligase activity of the Rictor/Cullin-1 complex. This provides functional insight into Rictor regulation, as well as mechanistic information regarding the SGK1 stability controlled by the PI 3-K pathway, and how misregulation of this process contributes to SGK1 overexpression in human cancers.

CHAPTER IV

DISCUSSION

1. SUMMARY OF THESIS

The objective of my thesis work was to investigate Akt-independent mechanisms of PI 3-K signaling in breast cancer. Gaining a greater understanding of PI 3-K pathways that mediate oncogenic *PIK3CA* signals will help to identify new therapeutic targets and better understand the utility of current targets. In chapter 2, I identify a mechanism of PI 3-K-dependent SGK3 activation. I show that the phosphoinositide phosphatase INPP4B functions as the regulator between SGK3 and Akt signaling downstream of PI 3-K. Moreover, I establish that SGK3 in turn promotes oncogenic phenotypes under conditions of low Akt activity and *PIK3CA* mutation. I determine that the metastasis suppressor, NDRG1, is a critical SGK3 substrate that supports enhanced invasive migration in breast cancer cells. I further demonstrate that NDRG1 phosphorylation by SGK3 leads to FBW7 E3 ligase recruitment and subsequent proteolytic degradation. The carboxyl-terminal phosphorylation of NDRG1 is the first identified post-translational modification that targets its destruction. PI 3-K pathway control of NDRG1 degradation is a previously unknown mechanism of *PIK3CA*-mediated cancer signaling through its downregulation of a metastasis suppressor.

In chapter 3, I explore a novel mTORC2-independent function for Rictor. I identify the Rictor-Cullin1 complex as an SGK1 E3 ligase. Furthermore, I show that Rictor is phosphorylated by AGC family kinases and this inhibits E3 ligase formation, effectively serving as a positive signaling feedback loop to SGK1. The active Rictor-Cullin1 complex targets SGK1 directing degradation by the proteasome. Rictor-mediated SGK1 turnover is the first-identified PI 3-K-dependent post-translational regulation that

modulates SGK degradation. This mechanism highlights a mechanism by SGK1 is

overexpressed in human cancers as a result of hyperactive PI 3-K signaling. The UPS

plays a prominent coordinating role in both aspects of my thesis work, namely SGK1

regulation by Rictor-Cullin1 and SGK3 regulation by PI 3-K AND INPP4B. The

implication of these findings in the broader context of PI 3-K signaling in breast cancer

is discussed below.

2. DISCUSSION

The prevalence of mutations in genes in the PI 3-K pathway across human cancers has enabled the development of multiple small molecule inhibitors currently in pre-clinical and clinical evaluation (Zardavas et al., 2013). These inhibitors include drugs that target PI 3-K itself, or those target its upstream regulators such as RTKs, or its downstream effectors such as Akt (Baselga, 2011). Data emerging from the use of these inhibitors in vitro and in genetically engineered mouse models have revealed that although we know much concerning the major mechanisms by which PI 3-K relays signals to phenotypes associated with malignancy, we do not yet have a complete picture (Zardavas et al., 2013). Since the PI 3-K pathway harbors multiple genes that encode both oncogenes and tumors suppressors, and multiple feedback loops exist within the signal relay network, it is unlikely that a single linear signaling mechanism accounts for the majority of aberrations in cancer. Moreover, many of proteins that transduce PI 3-K signaling exist in multiple isoforms, arguing that one must consider redundancy and specificity as a major component of signal relay, that must also be taken into account when considering the development of small molecule inhibitors. In this context, the best-understood effector of PI 3-K is the Akt kinase and a multitude of studies have linked PI 3-K and Akt signaling in the regulation of proliferation, survival, metabolic, and motility processes with implications for oncogenic signaling (Luo et al., 2003). It is not surprising therefore that there are currently a number of small molecule Akt inhibitors under clinical evaluation, along with pan PI 3-K and isoform-specific PI 3-K inhibitors. Given that some compounds of these have demonstrated limited efficacy

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either in the laboratory or in the clinic, there is an obvious and unmet need for the development of additional inhibitors that target effectors distinct from Akt (Zardavas et al., 2013). This is especially important since studies have revealed that *PIK3CA* and *PTEN*-mutant tumors do not always display elevated phospho-Akt levels, implying that additional effectors may be transducing the PI 3-K and PI(3,4,)P2 and PI(3,4,5)P3 signals (Vasudevan et al., 2009). A number of such effectors have received limited attention, including novel GEFs for Rho family GTPases, Tec family kinases and SGK isoforms (Cherfils and Zeghouf, 2013; Sommer et al., 2013; Vargas et al., 2013; Vasudevan et al., 2009). A recent study by the Garraway laboratory demonstrated that in certain *PIK3CA*-mutant cell lines and tumors that do not display high phospho-Akt levels and in which Akt is dispensable for growth and proliferation, the SGK3 isoform is required for *PIK3CA*-dependent signaling (Vasudevan et al., 2009).

Although the Garraway study clearly implicated SGK3 as an effector of *PIK3CA* in cancer, the mechanism by which SGK3 responds to either PI(3,4,)P2 and PI(3,4,5)P3 has remained elusive. This is especially difficult to rationalize since unlike Akt, SGK3 does not posses a PH domain capable of binding these two second messengers. Instead, SGK3 has a PX domain that binds PI(3)P exclusively, and PI(3)P is generally considered to be a phosphoinositide that is constitutively present in endomembranes and not function as a classical second messenger (Tessier and Woodgett, 2006b). Nonetheless, PI 3-K inhibitor experiments have clearly demonstrated that SGK3 is indeed a PI 3-K effector and must therefore respond to elevated PI(3,4,)P2 and PI(3,4,5)P3. The mechanism by which SGK3 is activated downstream of PI 3-K has

remained elusive, in part due to the lack of reagents and tools to study SGK3 phosphorylation and activation (Tessier and Woodgett, 2006a). My thesis work has focused on identifying such the mechanism by which SGK3 can be activated downstream of growth factor-stimulated PI 3-K. Specifically, I show that a critical component of this pathway is the INPP4B 4' lipid phosphatase, that interconverts PI(3,4)P2 to PI(3)P. Expression of INPP4B enhances IGF-stimulated SGK3 phosphorylation, whereas catalytically-inactive INPP4B cannot. Conversely, INPP4B silencing with shRNA attenuates SGK3 phosphorylation, protein kinase activity and phosphorylation of the downstream substrate NDRG1 as determined in cell-based assays. Since the product of INPP4B activity, PI(3)P, can directly bind to SGK3, I propose that INPP4B is rate-limiting in the activation of SGK3 downstream of *PIK3CA*, and thus INPP4B levels ultimately determine whether Akt, or SGK3 is the primary effector of this pathway in human tumors. One important aspect is the source of PI(3)P in membranes. It is largely accepted that the class III PI 3-K VPS34 that is localized at endosomes is responsible for the synthesis of the main pool of PI(3)P in endomembranes (Backer, 2008). However, we show that expression of exogenous VPS34 does not enhance SGK3 activity. Moreover, VPS34 deletion in fl/fl MEFs also has no effect on the IGF-stimulated SGK3 phosphorylation. Therefore, the pool of PI(3)P generated by VPS34 is unlikely to be directly involved in SGK3 activation. Instead, I propose that it is the PI(3)P that is interconverted from PI(3,4)P2 by INPP4B in cells that have elevated levels of this phosphatase combined with PI 3-K pathway mutations that lead to increased PI(3,4,)P2 and PI(3,4,5)P3. As such, this model

dictates that SGK3 is a positive transducer of INPP4B activity. The model that elevated

INPP4B can positively regulate signaling, rather than simply being viewed as a tumor

suppressor whose loss promotes Akt activation, also implies that other PX domain-

containing proteins that sense PI(3)P could be regulated by this alternate pathway.

Furthermore, the concept that INPP4B can promote PI 3-K signaling by its presence,

and not just by its loss, characterizes new potential combination therapies in the

PIK3CA pathway. Combination therapies targeting PI 3-K and SGK3 may hold promise

for those tumors that harbor *PIK3CA* mutation, SGK3 amplification and constitutive or

elevated INPP4B. Luminal ER positive tumors are likely to represent one such

molecular subtype, since these tumor have the highest frequency of *PIK3CA* mutations,

do not frequently lose INPP4B by LOH, and SGK3 is often over-expressed and/or

amplified.

In Chapter 2, I show that SGK3 is focally amplified in breast cancer with an overall

amplification rate of 54%. I then show that SGK3 and INPP4B regulate oncogenic

phenotypes such as growth in 3D and invasive migration in cells that harbor oncogenic

PIK3CA but display low-level Akt phosphorylation/activation. The relative number of

tumors and tumor cell lines that exhibit minimal Akt activity and in which Akt is

dispensable for proliferation emphasizes the importance of understanding alternate

mechanisms of PI 3-K-driven cancer progression. The ability of SGK3 to mediate

tumorigenic phenotypes is likely due in part to its overlapping substrates with Akt. The

number of substrates shared between Akt and SGK3 emphasizes the ability of SGK3 to

regulate oncogenic phenotypes via shared signaling pathways. However, identifying

specific mechanisms by which activated SGK3 might regulate cancer phenotypes that

are not shared with Akt holds promise for new drug targets and biomarkers for clinical

benefit. In my studies I identify an exclusive SGK substrate, NDRG1, as a mediator of PI

3-K signaling in the SGK3 pathway. Moreover, my data reveal the mechanism by which

a post-translational modification leads to NDRG1 destruction by the proteasome. These

data also highlight a novel mechanism by which PI 3-K/SGK3 regulates degradation of a

breast cancer metastasis suppressor (**Figure 4-1**). In this model oncogenic *PIK3CA*

promotes invasive migration in breast cancer through the INPP4B/SGK3 signaling axis.

The ability of SGK3 to promote progression adds to our knowledge of the mechanisms

of PI 3-K signal relay, and further indicate that SGK3 is an effector that can transduce

signals in the absence of productive Akt activation.

Initial studies showed that INPP4B is primarily expressed in luminal A and luminal B

molecular subtypes in breast cancer. The expression of INPP4B in ER positive

cancers, and its loss by LOH in ER negative breast cancers implies that lipid

phosphatase activity may not exclusively function in a signal termination capacity in the

context of PI 3-K signaling, at least in ER positive cancers. Instead, I propose that in

ER positive breast cancers, INPP4B catalytic activity diverts the signal towards PI(3)P

and recruitment and activation of SGK3. This event in turn promotes downstream

SGK3 signaling, while inhibiting *PIK3CA*-stimulated Akt activation, since PI(3,4,)P2 is

eliminated.   In this model, those rate-limiting factors that determine activation of Akt

versus SGK3, are INPP4B protein levels and SGK3 amplification. Although other

factors likely determine the ability of *PIK3CA* to drive SGK3 activation, I propose that the
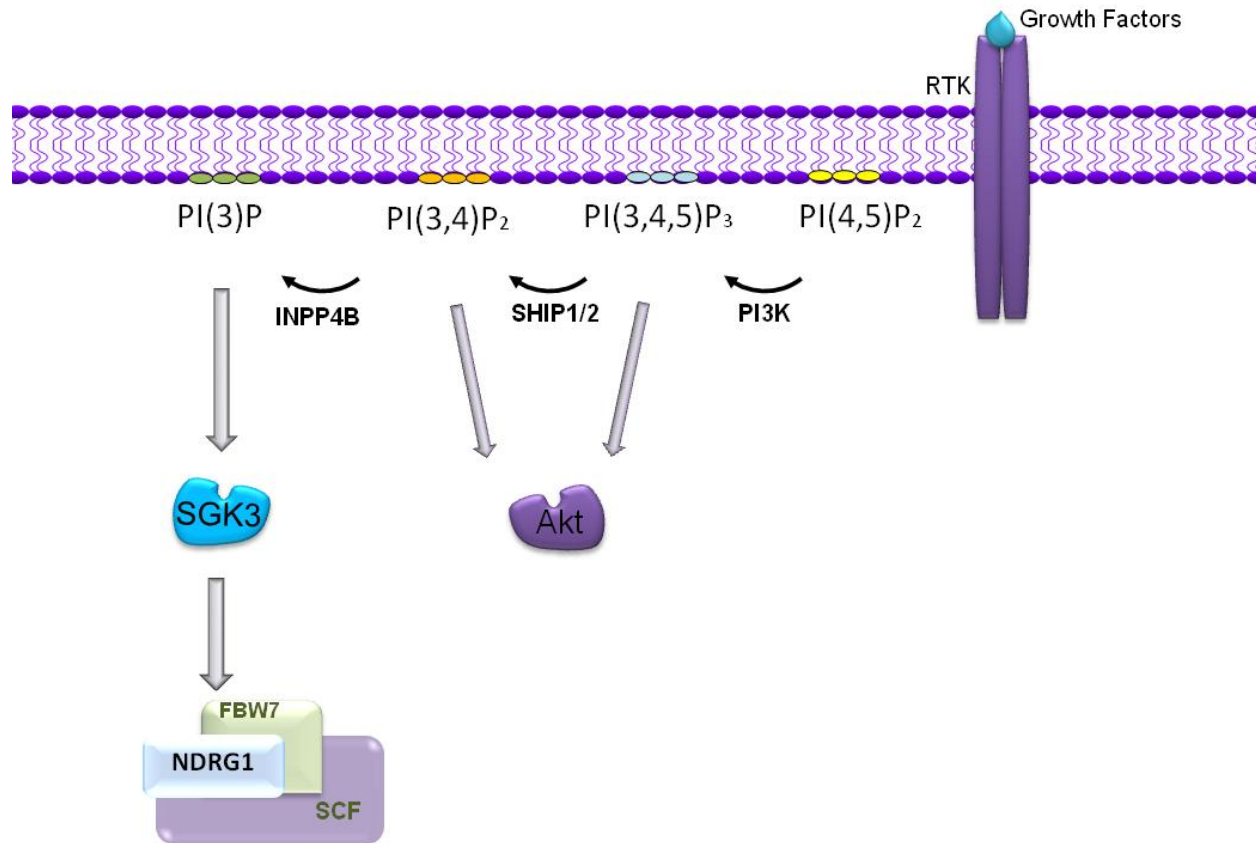
Figure 4-1: Model of PI 3-K regulation of Metastasis Suppressor NDRG1 through SGK3

SGK3 activation via the PI 3-K is mediated by INPP4B catalytic activity likely through its conversion of PI(3,4)P2 to produce PI(3)P which recruits and binds the SGK3 PX domain.

primary determinant is INPP4B expression. It is also worth noting that this is not

necessarily an all-or-nothing mechanism. It is likely that in certain breast cancers both

Akt and SGK3 are activated simultaneously, possibly under conditions where the levels

of INPP4B allow for some *de novo* PI(3)P synthesis but the pool of PI(3,4,)P2 is also

retained. The mutual induction of SGK3 and INPP4B in ER+ cells differentiates ER+

from ER- PI 3-K signaling.

The fact that INPP4B functions as a positive mediator of PI 3-K signaling in ER

positive tumors, and not solely as a signal terminator changes the way we view INPP4B

as a therapeutic target. In triple negative breast cancers, INPP4B has been shown to

function as a tumor suppressor, such that *INPP4B* LOH is commonly observed

(Gewinner et al., 2009). A tumor with this profile will likely respond very differently to

Akt inhibitors compared to an ER positive tumor with high INPP4B and high SGK3. My

studies highlight the emerging concept of personalized medicine and the evaluation of

the specific genetic background of a tumor to be able to administer the appropriate drug

treatment. As such, I propose that INPP4B can function both as an oncogene and

tumor suppressive, depending on the genetic background, although this concept has not

yet been tested *in vivo*. However, the notion that genes can function as both oncogenes

and tumor suppressors depending on the context is not new. For example, studies in

Notch signaling and Mouse Double Mutant 2 (MDM2) have helped to establish the

concept of dual modalities of a single gene (Manfredi, 2010; South et al., 2012). Thus,

the notion that a gene and its protein product have a unique function in all tumor

settings is an over-simplification, and this must be taken into account when devising

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treatment strategies. Although the model that *INPP4B* can function as an oncogene or tumor suppressor, and that SGK3 can mediate the oncogenic function, it is likely that there exist additional INPP4B effectors that are yet to be described, and that likely contribute to oncogenic function.

In Chapter 3 we identify a new E3 ubiquitin ligase complex composed of Rictor and Cullin1, which directs the degradation of SGK1 at the proteasome. The Rictor-Cullin1 E3 complex is the first example of an mTORC2-independent function for Rictor. Interestingly, the mTORC1 subunit, Raptor, was previously shown to have mTOR-independent E3 ligase functions in complex with Cullin4 (Ghosh et al., 2008). Duality in the abilities of kinase complexes to intersect and perform E3 ligase functions leading to degradation increases the probability of coordination in cellular events. This raises the probability that the two Rictor- containing complexes crosstalk or signal feedback to one another. The turnover rate for each complex is also likely to have diverse effects on the processing rate of the competing complex.

Rictor is phosphorylated at Thr1135 by members of the AGC family of kinases including Akt and S6K, and this event functions to negatively regulate the Rictor-Cullin complex. PI 3-K-mediated Thr1135 regulation modulates the levels of SGK1 and this mechanism increases our understanding of SGK regulation at the post-translational level. The participation of Akt in promoting SGK1 expression adds to its known role in the phosphorylation of WNK1, where by WNK1 functions as a scaffolding protein for PDK-1-mediated phosphorylation of SGK1 at Thr256. The dual mechanisms of Akt in the regulation of SGK1 activity and expression underscore the importance of signal

integration necessary for efficient pathway output. The Rictor-Cullin1 complex is the

first SGK E3 ligase identified that functions in a PI 3-K-dependent manner. A logical

extension of this finding is that oncogenic *PIK3CA* and *PTEN* mutations perturb SGK1

degradation. Under conditions of deregulated PI 3-K activity, SGK1 protein levels

increase leading to hyperactivation of SGK1 downstream targets.

3. FUTURE DIRECTIONS

My work has identified a novel mechanism by which PI 3-K transduces lipid signals to SGK3, specifically through the activity of the lipid phosphatase INPP4B. In addition, I have identified a mechanism of regulation of the SGK substrate NDRG1. A number of open questions remain regarding the details by which SGK3 responds to PI 3-K signaling. One obvious question is the mechanism of localization that determines activation SGK3, given that previous studies have localized SGK3 primarily at the endosome, consistent with the notion that endosomal membranes contain the bulk of the intracellular PI(3)P pool . However, my model dictates that the activity of INPP4B on PI(3,4)P2 thereby generating PI(3)P drives SGK3 activation. Since PI(3,4)P2 and PI(3,4,5)P3 are largely generated at the plasma membrane, this implies that the resulting PI(3)P must also accumulate at the plasma membrane, thus allowing for SGK3 recruitment through the PX domain leading to activation. By inference, INPP4B is unlikely to be responsible for PI(3)P production at the endosomal membrane, consistent with published findings demonstrating that endosomal localization of SGK3 is *PIK3CA*-independent (Tessier and Woodgett, 2006a). In this model, the two distinct pools of PI(3)P exist, one endosomal generated by VPS34 that primarily functions in vesicle trafficking, and a second INPP4B-generated pool at the plasma membrane that is responsible for mediating PIK3CA signals. In both cases, SGK3 senses PI(3)P, but may ultimately dictates specific functions. At the same time, we cannot exclude the possibility that the two pools are interchangeable, or that the plasma membrane pool of PI(3)P and SGK3 integrates with the endosomal pools. Regardless, obtaining formal proof for a

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plasma membrane localization of PI(3)P and SGK3 has proven challenging. We attempted to make use of Total Internal Reflection Fluorescence (TIRF) microscopy to specifically detect SGK3 plasma membrane recruitment. Unfortunately, the overexpression of Green Fluorescent Protein (GFP)-tagged SGK3 generated considerable background fluorescence that precluded a consistent and robust evaluation of SGK3 membrane recruitment. An alternate method to investigate distinct SGK3 pools would be the use of self-quenching SNAP-tags to label SGK3 and examine SGK3 localization with fluorescent confocal and TIRF microscopy. SNAP-tags should theoretically have the advantage of reduced background allowing for increased resolution. Alternatively, the development of more specific and high affinity antibodies for phospho-SGK3 at pThr320 and pSer486 for use in IHC applications would be an important development. Such antibodies would allow for the examination of localization of endogenous SGK3 especially in cells and tissues with known PI 3-K genetic lesions. Further insight would be gained by examining the phosphoprotein profile IGF-1 stimulation or *PIK3CA* mutation in cells in which INPP4B has been silenced. Membrane interactions could be isolated after such treatments followed by mass spectrometry analysis to determine candidates of positively and negatively regulated INPP4B targets. This information would expand our understanding and add perspective to SGK3 signaling and may aid in the targeting of ER-positive breast cancers with combination therapy.

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Another important step forward will be to generate genetically engineered mouse models that recapitulate finding that SGK3 can function as a *PIK3CA* and INPP4B effector. This would be especially interesting in the setting of ER-positive tumors, and examination of subsequent tumor growth and metastasis. One would predict that INPP4B silencing would mimic the phenotypes seen by SGK3 silencing. Although SGK3 isoform knockout mice have been made, they have only informed the contribution of SGK in embryonic development. Studies in adult mice in the context of tumor phenotypes have not been performed. Moreover, there is no combined SGK1/SGK2/SGK3 knockout mouse to address possible SGK familial signaling roles. Another gap in understanding is the nature of SGK3-specific substrates, or at least SGK-specific substrates that are not shared with Akt, other than NDRG1. Identifying such substrates will likely provide an important advance in our understanding of the mechanisms by which SGKs mediate cancer phenotypes. Finally, if specific total and phospho-specific SGK3 antibodies that are of sufficient quality for IF can be developed, one obvious application will also be to use confocal microscopy in the context of growth of tumor cells in 3D culture to determine changes in SGK1 and SGK3 localization, phosphorylation and activity to provide insight into spatial-temporal regulation of SGK.

The data pointing to NDRG1 as a metastasis suppressor through its modulation of Wnt signaling and E-cadherin recycling highlights a potential for a therapeutic strategy. However, a more detailed analysis of the NDRG1 phospho-degron remains an important issue, since NDRG1 does not possess a canonical FBW7 degron sequence. However, it does share similarities in the SGK3 and GSK3β decapeptide repeat to

typical degrons. The priming of the phospho-degron by GSK3β and the tandem

phosphorylated serine and threonine sites are common characteristics of FBW7 sites.

A logical extension of the studies presented in this thesis would be to mutate the

NDRG1 carboxyl-terminal sites to alanine, and evaluate binding to FBW7 (**Figure 4-2**).

Additionally, mass spectrometry analysis of the phosphorylation sites should be carried

out under conditions of stimulation and in combination with a panel of protein kinase

inhibitors (SGK, GSK3β, PI 3-K).
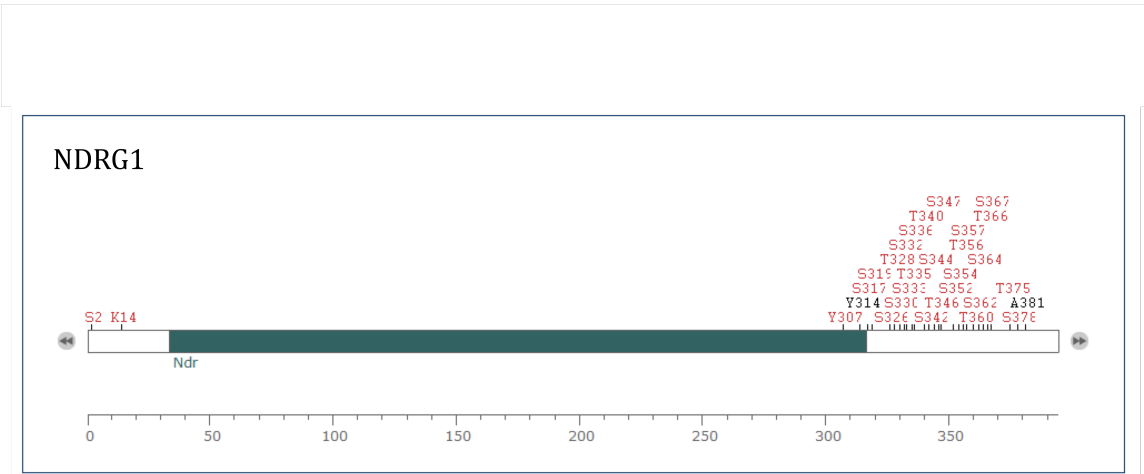
Figure 4-2: Phospho-site analysis for NDRG1

Mapping of known phosphorylation sites for NDRG1 produced from mass spectrometry evaluation. Adapted from phosphosite.org.

The studies outlined in Chapter 3 provide evidence for an mTORC2- independent function for Rictor. One logical extension of these studies is to determine if scaffolding proteins common to Rictor in both the E3 ligase complex and mTORC2 exist. The absence of Skp1 in the Rictor-Cullin1 complex suggests that there may be alternate scaffolding proteins bridging Cullin1 to its F-box protein. The possibility remains that Rictor alone could mediate a bridging function or there could be additional components of the Rictor-Cullin1 complex that are essential to assemble a functional complex. Understanding which precise subunits within the E3 ligase complex directly interact will increase our understanding of complex formation. This knowledge will also guide the development of drugs that interrupt SGK1 degradation as a viable therapeutic option. Another important consideration is the consequence of the Rictor Thr1135 phosphorylation. There are two main models: Thr1135 phosphorylation could simply prevent the *de novo* assembly of Rictor-Cullin1, or alternatively Thr1135 phosphorylation could lead to spatial inhibition of the binding of complex components, and ultimate disassembly. The phosphorylation of Thr1135 generates a positive feedback loop that promotes the accumulation of SGK1. The deregulation of this feedback in cells with oncogenic PI 3-K pathway mutations may be causal with respect to SGK1 overexpression in human cancer. In this context, published studies have established a significant correlation between SGK1 overexpression and high levels of pAkt1 S473 in breast tumor samples (Sahoo et al., 2005). Additional examination of tumor samples with high SGK1 levels for enhanced Thr1135 Rictor phosphorylation will

help to establish the amplitude of the feedback loop. This same feedback loop

negatively regulates Rictor, adding to our understanding of Rictor regulation and

downstream signaling consequences. The existence of two Rictor complexes begs

questions of stoichiometry between the pools. Although technically challenging, it will

be interesting to examine the ability of one mTORC2 complex to sequester Rictor at the

expense of the activity of the other. Our studies also establish the need to determine the

extent of Rictor-Cullin1 as an E3 ligase and if its role in the UPS extends to other

proteins or is exclusive to SGK1. It would be interesting to investigate the ability of

Rictor to function as both a priming kinase within the phospho-degron and then to

sequentially function as an E3 ligase for poly-ubiquitination. The synchronization of

priming and poly-ubiquitination would challenge the participation of DUBs and provide

insight into the mechanistic organization of cellular processes.

The synergy of Akt isoforms and SGK isoforms as effectors of *PIK3CA* and other

PI 3-K pathway lesions likely leads to alterations in expression and activation of SGK1

and SGK3 under distinct phases of tumor development. Examining expression patterns

of SGK1 in 3D spheroid growth and changes in expression resulting from altered Akt or

SGK3 activity would help to explore the dynamic relationship between SGK isoforms

and other AGC family kinase members. Moreover, since no specific SGK isoform-

specific inhibitors have been developed, I propose our work provides the impetus for the

development of such drugs, as they may provide an unmet need for tumors with specific

pathway alterations.

4. FINAL NOTE

In summary, the data described in this thesis identifies two Akt-independent PI 3-K mediators, SGK1 and SGK3, that are hyperactivated in human cancers. The contribution of SGK3 amplification to *PIK3CA* signaling in breast cancers provides insight for a potentially novel therapeutic target. This thesis identifies INPP4B as a switch that determines pathway output to Akt or SGK3 in the PI 3-K pathway. I propose that INPP4B functions as both as an oncogene in luminal type breast cancer in the context of *PIK3CA* mutations, in addition to its well-documented role as a tumor suppressor in basal like/triple negative breast cancers. This concept reinforces the notion that complete understanding of the genetic context of a given tumor must be considered so that ultimately, the appropriate treatment regimen is prescribed. Furthermore, the work described in this thesis reveals a new pathway by which PI 3-K regulates the degradation of a metastasis suppressor, NDRG1, through SGK3. This finding also argues that there exists specificity between SGK and Akt signaling, rather that redundancy between the two kinase pathways. The identification of an mTORC2-independent function for Rictor proposes a more coordinated view of protein degradation. AGC kinase negative feedback regulation of Rictor ligase activity synergizing with Thr1135 phosphorylation highlights a mechanism by which PI 3-K activity promotes the overexpression of SGK1 and likely enhancement of *PI3KCA* signaling. The chapters of this thesis are related in that they focus on poly-ubiquitination and proteasomal degradation as a central feature to control signaling events. The deregulation of ubiquitination during oncogene-driven cancer progression

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is also a central theme in this thesis. Most obvious is the concept that SGK isoforms

contribute to breast cancer cell phenotypes in the context of PI 3-K signaling. These

findings provide a more comprehensive understanding of PI 3-K biology and the

emerging concept of importance of Akt-independent signaling. Finally, from a

therapeutic perspective, the data outlined in this thesis advocate the development and

implementation of combination therapies using small molecule inhibitors targeting SGK

and Akt isoforms for maximal efficacy.

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