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GSK3 MEDIATES SIGNALING UPSTREAM OF AKT

by

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GSK3 MEDIATES SIGNALING UPSTREAM OF AKT

A

DISSERTATION

Presented to the Faculty of

The University of Texas

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Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Debra Lorita Smith, B.S.

Houston, Texas

May 2010

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DEDICATION

This dissertation is dedicated to my best friend, Shaun, for always encouraging me to reach my full potential. He has helped me countless times to look at the “big picture” and move past the bumps and potholes along what continues to be the long and winding road of my MD/PhD training.

I would also like to dedicate this dissertation to my parents. I am deeply grateful for their unconditional support and for their many sacrifices that have made it possible for me to succeed. My Mom frequently offers to “hop a plane” to come help me with anything I need.

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GSK3 MEDIATES SIGNALING UPSTREAM OF AKT

Publication No. _____

Debra Lorita Smith, B.S.

Supervisory Professor: Gordon B. Mills, M.D., Ph.D.

The phosphatidylinositol 3 kinase (PI3K)/Akt signaling network plays a pivotal role in multiple cellular functions. PI3K links the extracellular growth factor receptors to the serine-threonine kinase Akt which, through phosphorylation of numerous intracellular proteins, mediates increased cell growth, survival, motility and proliferation. The significance of the PI3K/Akt signaling network to human malignancy is demonstrated by cancer-associated genetic aberrations at multiple levels in the PI3K/Akt pathway in many tumor lineages. These aberrations include mutation or amplification of Akt, catalytic and regulatory subunits of PI3K, and growth factor receptors upstream of PI3K. Inactivating mutations and decreased expression of the PTEN, a major antagonist of PI3K signaling, also lead to constitutive hyperactivity of Akt.

Multiple small molecule inhibitors targeting components of the PI3K/Akt signaling pathway including Akt catalytic domain inhibitors, significantly slow tumor growth in

preclinical models. Strikingly, these Akt kinase inhibitors induce a paradoxical increase in phosphorylation of Akt activation sites. As Akt catalytic domain inhibitors are entering clinical trials, elucidation of the mechanism(s) underlying Akt inhibitor-induced Akt phosphorylation is needed to understand and anticipate drug effects.

In this study, we demonstrate an unexpected requirement of Glycogen Synthase Kinase 3 (GSK3), a serine-threonine kinase that participates in multiple cell signaling pathways downstream of Akt, for Akt inhibitor-induced Akt phosphorylation. siRNA-mediated knockdown of GSK3 in MDAMB231 breast cancer cells and in mouse embryonic fibroblasts (MEFs) abrogated induction of Akt phosphorylation by the Akt catalytic domain inhibitor A674563. Our discovery that GSK3 can function upstream of Akt led us to explore the role of GSK3 in mediating signaling from growth factors to Akt. Knockdown of GSK3 expression in MDAMB231 cells and in MEFs blocked stimulation of Akt phosphorylation by epidermal growth factor (EGF) and insulin-like growth factor (IGF1).

The results, taken together, demonstrate a novel signaling role for GSK3 upstream of Akt.

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LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
AFAP-110	Actin filament-associated protein of 110 kDa
AMPK	Adenosine monophosphate protein kinase
AP1G1	Adaptor-related protein complex 1, gamma 1 subunit
AP2B1	Adaptor-related protein complex 2, beta 1 subunit
APC	Adenomatosis polyposis coli
AR	Androgen receptor
ATM	Ataxia-telangiectasia mutated
BCAP	B-cell adaptor for PI3K
BH	Breakpoint cluster region homology
Ci	Cubitus interruptus
CNNM2	Cyclin M2
cIAP-1	Cellular inhibitor of apoptosis protein 1
CREB	Cyclic adenosine monophosphate response element-binding
CSFR	Colony-stimulating factor receptor
CTMB	Carboxy terminal modulator protein
CTNND1	Catenin, delta 1
DDR1	Discoidin domain receptor tyrosine kinase 1
DNA-PK	DNA-dependent protein kinase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EML4	Echinoderm microtubule associated protein like 4
ESYT2	Extended synaptotagmin-like protein 2
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR1	Fibroblast growth factor receptor 1
FRAT	Frequently rearranged in advanced T-cell lymphomas
Gab1	Grb2-associated binding protein 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFR	Growth factor receptor
GPCR	G-protein coupled receptors
Grb2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
Hh	Hedgehog
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor 1 receptor
IKK	Inhibitor of kappa B kinase
ILK	Integrin-linked kinase
IRS1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LEF	Lymphoid enhancer-binding factor

MAPK	Mitogen-activated protein kinases
MCL-1	Myeloid leukemia cell differentiation protein 1
Mdm2	Murine double minute protein 2
MEF	Mouse embryonic fibroblast
MEK1	Mitogen-activated protein kinase 1
MEKK4	Mitogen-activated protein kinase kinase 4
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MuSK	Muscle, skeletal receptor tyrosine protein kinase
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NEK6	(Never in mitosis gene A)-related kinase 6
NSCLC	Non-small cell lung cancer
NLS	Nuclear localization sequence
p70S6K	p70S6 kinase
p90RSK	p90 ribosomal S6 kinase
PDE2A	Phosphodiesterase 2A
PDGF	Platelet-derived growth factor
PDK1	Protein-dependent kinase 1
PDK2	Protein-dependent kinase 2
PH	Pleckstrin homology
PI3K	Phosphatidylinositide 3 kinase
PIK	Phosphatidyl inositol kinase

PI(3)P	Phosphatidylinositol 3-phosphate
PI(4)P	Phosphatidylinositol 4-phosphate
PIP2	Phosphatidylinositol bisphosphate
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol trisphosphate
PI(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PMS2	Post-meiotic segregation 2
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PRAS40	Proline-rich Akt substrate 40
PrINZ	7-n-propylindazole analog
Ptch	Patched
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
PTPN3	Protein tyrosine phosphatase, non-receptor type 3
RBM10	RNA binding motif protein 10
RPPA	Reverse phase protein array
RSK	Ribosomal s6 kinase
RTF	Regeneration and tolerance factor
RTK	Receptor tyrosine kinase

S6RP	S6 Ribosomal Protein
Ser	Serine
SH2	Src homology 2 domains
siRNA	Short interference RNA
SLC4A1	Solute carrier family 4, anion exchanger, member 1
SLITRK3	SLIT and NTRK-like family, member 3
TAO3	Thousand and one amino acid protein 3
TAP	T-cell-activating protein
TCF	T-cell factor
TCL1	T-cell leukemia 1
Thr	Threonine
TLR5	Toll-like receptor 5
TM	Turn motif
TRB3	Tribbles homolog 3
TSC2	Tuberous sclerosis protein 2
Tyr	Tyrosine
USP13	Ubiquitin specific peptidase 13
VEGFR2	Vascular endothelial growth factor receptor 2
YAP	Yes-associated protein

CHAPTER 1: INTRODUCTION

1.1 PI3K/Akt Pathway

Phosphoinositide 3-kinases (PI3Ks)

The phosphoinositide 3-kinase (PI3K) family of lipid kinases functions near the inner surface of the cell membrane to catalyze the phosphorylation of phosphatidylinositols (PtdIns) on the D3 position of the inositol ring. The PI3K enzymes are divided into three classes based on homology of the catalytic subunits and substrate specificity.

Class I PI3Ks can catalyze phosphorylation of the D3 site of PI(4)P or PI(4,5)P₂ PtdIns in vitro, but the preferred in vivo substrate is PI(4,5)P₂ (1). The phosphorylation of PI(4,5)P₂ (PIP₂) by Class I PI3Ks generates the important second messenger PI(3,4,5)P₃ (PIP₃) which activates a multitude of downstream signaling processes leading to important cellular process and outcomes. Class I PI3Ks are further divided into Class IA and Class IB based on different enzyme regulatory unit structure and activation downstream of tyrosine kinases (Class IA) versus G-protein coupled receptors (Class IB) (2).

Class II PI3Ks preferentially phosphorylate PtdIns and PtdIns-4-P (3). Class III PI3Ks regulate intracellular trafficking through phosphorylation of PI to generate PI(3)P (1). Detailed discussion of the important cellular processes mediated by Class II and Class III

PI3Ks is outside the scope of this dissertation. The interested reader is referred to the following sources for further detail (4, 5).

An extensive body of data implicates dysregulated signaling of Class IA PI3Ks in human cancers. This work is focused only on the cellular signaling network of Class IA PI3Ks. Throughout this work, use of the term PI3K will refer to the Class IA PI3K enzymes that are discussed in detail below.

Class IA PI3K Structure

Class IA PI3Ks exist as a heterodimer containing a catalytic subunit and a regulatory subunit. Multiple isoforms exist for both the catalytic and regulatory subunits. Three 110kD proteins, p110 α , p110 β , and p110 δ , encoded by distinct genes comprise the catalytic subunit isoforms. p110 α and p110 β are ubiquitously expressed, while p110 δ is expressed primarily in leukocytes (6). The p85 α and p85 β genes encode two 85kD regulatory subunit isoforms. Two truncated regulatory subunit isoforms, p55 α and p50 α , are produced as alternative splice variants of p85 α . A third gene, p55 γ , encodes p55PIK (p55 γ) and p50PIK (p50 γ), alternative translation initiation products (7). The regulatory subunit is commonly referred to as p85 as the two 85 kDa isoforms were the first isoforms discovered.

The three p110 catalytic isoforms possess an N-terminal regulatory subunit-binding domain and a C-terminal catalytic domain. The inner domains of the p110 isoforms are the Ras-binding domain (RBD) that interacts with Ras in its active GTP-bound state, the

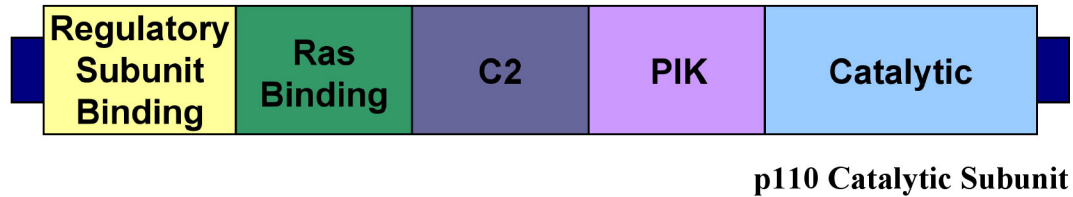
phospholipid-binding C2 domain, and the helical PIK domain that is conserved among the phosphatidylinositol kinase (PIK) superfamily. The domain structure of the p110 catalytic subunit is illustrated in Figure 1.1A.

Regulatory subunit isoforms all contain a proline-rich region, two Src homology 2 (SH2) domains and an inter-SH2 region that binds to p110. The larger (85kD) isoforms also contain an N-terminal Src homology 3 (SH3) domain, a breakpoint cluster region homology (BH) domain, and an additional proline-rich region. Domain structure of p85 is depicted in Figure 1.1B. p85 has multiple critical functions. It binds and stabilizes p110. Further it inhibits p110, creating a pool of p110 that can be rapidly activated through relief of the inhibitory effect of p85 by binding to phosphotyrosine residues at the cell membrane. Recently, p85 has also been demonstrated to bind and regulate PTEN, the key negative regulator in the PI3K pathway (8). Thus p85 provides an important point of integration of information transfer in the PI3K pathway.

Phosphoinositide 3-kinase Activation by Receptor Tyrosine Kinases

Class IA PI3Ks are activated primarily as a consequence of extracellular binding of growth factors to cell-surface receptors with intracellular tyrosine kinase domains. Binding of the growth factor alters the conformation of the receptor tyrosine kinase (RTK), activating transphosphorylation of intracellular tyrosine residues. The SH2 domains of the regulatory subunit of PI3K bind specific phosphotyrosine residues of the receptor tyrosine kinase. The binding of the PI3K regulatory subunit to the membrane-bound receptor brings

A



B

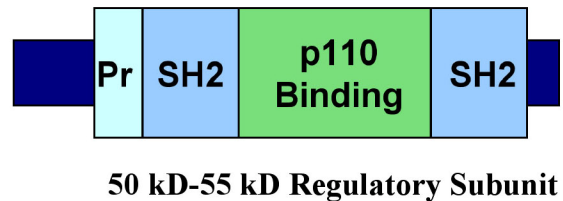
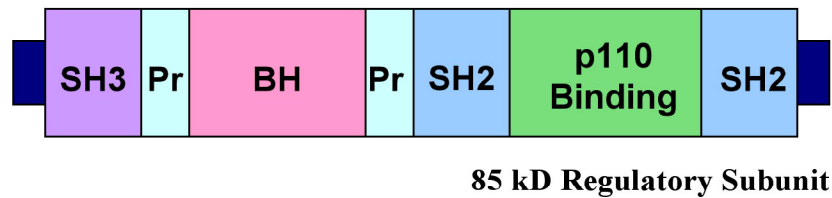


Figure 1.1 Domain structure of Class IA PI3K subunits. A. Domain structure of p110 catalytic subunit isoforms of Class IA PI3K contains a regulatory subunit-binding domain, a Ras-binding domain, a phospholipid-binding C2 domain, phosphatidyl inositol kinase (PIK) domain, and a catalytic domain. B. 85 kD regulatory subunits contain an N-terminal Src homology 3 (SH3) domain, proline-rich (PR) region, breakpoint cluster region homology (BH) domain, a second PR region, and two Src Homology 2 (SH2) domains separated by a p110-binding domain. The 50-55 kD regulatory subunits contain one N-terminal PR, and the two SH2 domains separated by an inter-SH2 p110-binding domain.

the PI3K catalytic subunit into proximity to its phosphoinositide substrate and also alters the conformation of the PI3K dimer releasing the inhibitory effect of p85 on the activity of the catalytic subunit. Phosphotyrosine residues on RTKs can also recruit PI3K to the cell membrane indirectly through adaptor molecules that bind the PI3K regulatory subunit. The catalytic subunit of PI3K can also be directly activated by Ras. Once activated, PI3K catalyzes the phosphorylation of PI(4,5)P₂ to generate PI(3,4,5)P₃ (PIP₃). PIP₃ recruits multiple proteins through specific phosphatidylinositol interaction domains such as the PH (pleckstrin homology) domains to form an activation nidus at the cell membrane.

Akt Structure and Activation of Akt Downstream of PI3K

Akt (aka PKB of the AGC protein kinase family) comprises three isoforms – Akt1, Akt2, and Akt3 - encoded by separate genes. The Akt structure consists of an N-terminal PH-domain, a hinge region, an activation loop, and a C-terminal tail region that includes a hydrophobic motif. Three Akt phosphorylation sites – the Thr308, Ser473, and Thr450 residues (Akt1 numbering) – regulate activation of Akt kinase activity. Figure 1.2 illustrates the Akt structural domains and phosphorylation sites.

Both Akt and the Akt-activating kinase PDK1 are recruited to the cell membrane by binding of their PH domains to PIP₃. In lower organisms, the effects of PI3K can be replaced by an activated Akt, suggesting that Akt is the major functional effector of PIP₃. However, in higher organisms, the multiple molecules recruited or activated by PIP₃ suggest a more complex relationship. Binding of the PH domain of Akt to PIP₃ removes an

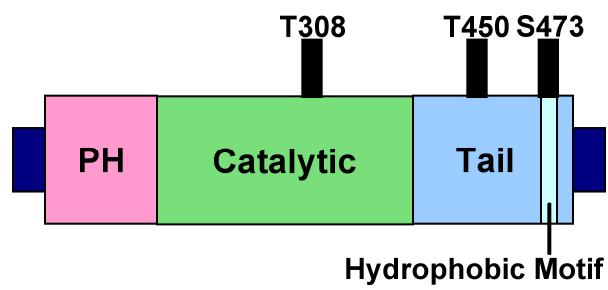


Figure 1.2 Domain structure and phosphorylation sites of Akt.

inhibitory effect of the PH domain on the activation loop, which allows PDK1 to phosphorylate Akt on its Thr308 residue within the activation loop.

Phosphorylation of the Ser473 residue within the hydrophobic motif of Akt is required for full activation of the kinase. Although the identity of the kinase responsible for phosphorylating Akt on this site remains controversial, mTORC2 has recently been established as a Ser473 kinase of Akt (9). However, PDK1, integrin-linked kinase (ILK), DNA-PK, PKC α , PKC β II, MK2, p38, NEK6, ATM and Akt itself have all been suggested to mediate this phosphorylation event under particular contexts (10-13).

A third less characterized phosphorylation site on the tail region of Akt between the kinase and hydrophobic motif domains is also important for Akt function. This phosphosite (Thr450) is thought to either stabilize the active form of Akt through cooperation with S473 phosphosite (14) or interact with the catalytic region of Akt to promote folding and stabilization (15). This tail/linker site, also called the turn motif (TM) phosphosite, requires mTORC2 for phosphorylation (15, 16).

Activation of Akt is also facilitated by binding interactions of Akt with Hsp90 and T-cell leukemia 1 (TCL1). The heat shock protein Hsp90 binds to Akt and increases its activity by preventing dephosphorylation by PP2A (17). TRB3 is a negative regulator of Akt that binds Akt and blocks its activation (18). T-cell leukemia 1 (TCL1) acts as an Akt coactivator that binds Akt to form oligomeric TCL1-Akt complexes, in which phosphorylation and activation of Akt is enhanced (19, 20).

Akt Inactivation

Akt is inactivated by dephosphorylation of its Thr308 and Ser473 residues by general and specific phosphatases. PP1 and PP2A are general phosphatases that dephosphorylate both the T308 and S473 sites (21-23). PHLPP1 and PHLPP2 are specific phosphatases whose only known substrates are Akt and PKC (24). The PHLPP phosphatases dephosphorylate only the hydrophobic motif site of its targets (Ser473 for Akt). Importantly, PHLPP phosphatases demonstrate differential activities against different Akt isoforms potentially contributing to the effects of the different isoforms (25).

Downregulation of PI3K/Akt Signaling

The major downregulator of PI3K/Akt signaling upstream of Akt is the PTEN phosphatase, which catalyzes the dephosphorylation of PIP3 (3,4,5) to PIP2 (4,5), thus reversing the reaction catalyzed by PI3K. Reduction of PIP3 (3,4,5,) levels in the cell membrane inhibits recruitment of Akt to the cell membrane required for its optimal activation.

Downregulation of Akt signaling also occurs through binding of carboxy terminal modulator protein (CTMB) to the hydrophobic motif of Akt, preventing phosphorylation of Akt on its Thr308 and Ser473 residues (26).

Akt Downstream Effects

Akt catalyzes the phosphorylation of numerous cellular proteins, with over 60 Akt substrates reported in the literature to date. Through regulation of a multitude of downstream signaling pathways that frequently interact with and amplify each other, Akt increases cell proliferation, cellular metabolism, motility, cell growth, and cell survival. Cell proliferation is increased through Akt's actions on cell cycle regulators including GSK3, p27, and p21 (27). Akt activation of the mTOR pathway increases protein synthesis and cell growth (28). Through regulation of the Bad, FoxO, Mdm2, IKK, and CREB pathways, Akt increases transcription of anti-apoptotic factors and decreases transcription of pro-apoptotic factors (29). These effects of Akt on cellular survival, growth, and proliferation in cancer cells enable tumor progression. Akt also contributes to cell motility, invasion, and angiogenesis (30, 31).

1.2 Aberrations of the PI3K/Akt Pathway in Cancer

Aberrations in the PI3K/Akt signaling network are prevalent in human cancers. Akt mutations have been reported in human tumors but are rare occurrences (32-34). Upregulated Akt signaling more often results from overexpression or amplification. Akt1 and Akt2 are overexpressed in colorectal cancer (35). Akt2 is amplified and overexpressed in a subset of ovarian cancers (36, 37). Amplification and/or overexpression of Akt2 also occur in a significant portion of head and neck squamous cell carcinomas (38), pancreatic

cancer (39-41), and hepatocellular carcinoma (42). Akt3 is overexpressed in estrogen receptor-negative breast cancers (43) and almost half of melanomas (44).

Whereas mutations of Akt are rarely observed, somatic missense mutations of the PIK3CA gene encoding the alpha isoform of the PI3K catalytic subunit occur in 20-35% of gastric (45), colorectal (45), glioblastoma (45), breast (46), liver (47), endometrial cancers (48) and ovarian cancers of endometrioid and clear cell origin (49). Amplification of the PIK3CA gene is a frequent event in breast (47), ovarian (50, 51), prostate (52), gastric (53), cervical (54), thyroid (47), lung (55), and head and neck squamous cell cancers (38).

The regulatory subunit of PI3K is also frequently aberrant, with p85 amplification or overexpression in most ovarian cancers (56) and mutation of p85 seen in glioblastoma, colorectal, pancreatic cancers (57).

Loss of function of the PTEN phosphatase frequently occurs in human cancers and results in constitutive activity of the PI3K/Akt pathway. Decreased PTEN function can result from mutation, deletion, or promoter methylation as well as through post-translational modification. PTEN alterations are seen in numerous tumor types including breast (58), colorectal (59), gastric (60), cervical (61), glioblastoma (62), acute lymphoblastic leukemia (ALL) (63), melanoma (64), prostate (52), endometrial (48), and ovarian cancers (65).

Activating mutations and amplifications of genes encoding receptor tyrosine kinase (RTK) growth factor receptors result in constitutively upregulated signaling through the

PI3K pathway. The HER2 RTK is amplified in several cancer types including breast (66), ovary (67), stomach (68), and bladder (69) cancers. The RTK EGFR is amplified or overexpressed in glioblastomas (70) and cancers of the breast (71), lung (72), and colorectum (73). An activating EGFR (HER1) mutation is seen in high-grade gliomas (74), glioblastomas (75) non-small cell lung cancer (NSCLC) (76), ovarian (77) , and breast carcinomas (78). While the EGFR and HER2 RTKs are inefficient activators of PI3K directly, they form heterodimers with HER3, which is a potent and efficient activator of PI3K due to multiple p85 binding sites in its intracellular tail.

Since Ras can directly activate PI3K, activating mutations of Ras upregulate PI3K/Akt pathway signaling. Ras mutations are present in almost all pancreatic adenocarcinomas (79) as well as subsets of myeloid leukemias (80) and lung (81), ovarian (82, 83), colorectal (84), and thyroid cancers (85).

1.3 Development of Akt Catalytic Domain Inhibitors as Anti-cancer Agents

The major importance of PI3K/Akt signaling has spurred development of numerous potential therapeutics targeting components of the PI3K/Akt pathway (86). Included among these drugs are Akt catalytic domain inhibitors as well as allosteric Akt inhibitors. Multiple structurally distinct series of Akt catalytic domain inhibitors are in development, including the indazole-pyridine based inhibitors from Abbott Laboratories (87) and the aminofurazan-derived compounds developed by GlaxoSmithKline (88).

Effects on the cancer phenotype of Akt catalytic domain inhibitors have been evaluated in cancer cell lines, animal tumor models, and in clinical trials. The A443654 and A674563 inhibitors from Abbott Laboratories effectively slowed proliferation of MiaPaCa-2 pancreatic cancer cells in culture and in a xenograft tumor model (87). 3T3-Akt1 xenografts and a panel of glioblastoma multiforme cell lines showed increased apoptosis of tumor cells with A443654 treatment, indicating anti-apoptotic effects may be one mechanism of slowed tumor growth with this Akt inhibitor (87, 89). Local delivery of A443654 inhibited tumor growth and extended survival in a rat model of intracranial glioma (89), indicating potential therapeutic utility of A443654 and other Akt-targeting drugs in this disease. GSK690693, the most well characterized Akt catalytic domain inhibitor from GlaxoSmithKline, effectively inhibits proliferation of multiple cancer lines, including breast and prostate lines, and was also found to induce apoptosis in vitro (88). In vivo tumor growth inhibition with GSK690693 was demonstrated in xenograft models of ovarian, breast, and prostate cancer (88).

Akt catalytic domain inhibitors have recently entered clinical trials (<http://www.clinicaltrials.gov>). A phase I study of GSK690693 in solid tumors and lymphoma was initiated but discontinued because of a serious adverse event. A phase I study of GSK690693 in relapsed or refractory hematologic malignancies was subsequently withdrawn. Exelixis conducted a Phase I study of XL418, a catalytic domain inhibitor of both Akt and p70S6K, however this study was suspended due to low drug exposure.

In spite of these challenges in clinical development, Akt catalytic domain inhibitors demonstrate promise as potential anti-cancer therapeutics. Abbott and GlaxoSmithKline continue to introduce additional chemical modifications to their Akt catalytic domain inhibitors to produce compounds with more favorable pharmacological profiles (90-92). GlaxoSmithKline is currently recruiting patients for Phase I trials of two follow-up compounds - GSK2141795 and GSK21110183.

Phase I clinical trials of MK2206, an allosteric Akt inhibitor, have demonstrated acceptable toxicity with both single agent and combination therapy trials underway for multiple tumor types.

As these compounds are entering clinical trials, it is critical to understand their mechanisms of action and on- and off-target activities both to increase the likelihood that patients likely to benefit can be identified and to prevent unexpected patient toxicities. Further, an understanding of mechanisms of action will facilitate the development of rational combinatorial therapies.

1.4 Akt Inhibitor-induced Akt Phosphorylation

Strikingly, Akt catalytic domain inhibitors have been found to induce a paradoxical increase in Akt phosphorylation on its Thr308 and Ser473 activation sites. Inhibitor-induced Akt phosphorylation is characteristic of both the Abbott and GlaxoSmithKline Akt catalytic domain inhibitors. Induction of Akt phosphorylation with A443674, a highly selective Akt

inhibitor in the Abbott series, has been demonstrated in multiple cancer cell types (93) and in vivo in a xenograft model of MiaPaCa-2-derived (pancreatic cell line) tumors (87). The GSK690693 inhibitor from GlaxoSmithKline has also been shown to induce phosphorylation of Akt in cell lines derived from multiple tumor types (88). Importantly, although phosphorylation of Akt is normally associated with increased activity, in this case the increased phosphorylation of Akt is not associated with Akt activation due to blockade of the catalytic site by Akt inhibitor. Signaling mechanism(s) underlying induction of Akt phosphorylation in response to treatment with Akt catalytic domain inhibitors have not been well defined. This work reveals a role for Glycogen Synthase Kinase 3 (GSK3) in mediating Akt inhibitor-induced phosphorylation.

1.5 GSK3 Function and Regulation

GSK3, originally discovered as a regulator of glycogen metabolism, has since been found to interact broadly with multiple aspects of the signal transduction machinery of the cell. Through its involvement in multiple pathways, GSK3 plays a role in both development and disease processes, including Type II diabetes, inflammation, an array of neurological disorders, and cancer.

GSK3 Structure and Isoforms

The GSK3 structure consists of an N-terminal region, kinase domain, and C-terminal region. The two GSK3 isoforms GSK3 α and GSK3 β , encoded by different genes, are highly

homologous in their kinase domains but are divergent in the N-terminus and C-terminus regions. The N-terminus region of GSK3 α but not GSK3 β contains a glycine-rich domain (94).

The GSK3 α and GSK3 β isoforms are not equivalent, as illustrated by GSK3 knockout mice. GSK3 α knockout mice are viable (95), whereas GSK3 β knockout mice demonstrate massive liver apoptosis and die before birth (96). Mouse models have also provided evidence that GSK3 α regulates glycogen metabolism in the liver, and GSK3 β controls response of skeletal muscle to insulin (97). While GSK3 α and GSK3 β carry out distinct roles in tissue-specific glycogen metabolism, the two GSK3 isoforms appear to demonstrate redundancy in other signaling functions. For example, either GSK3 isoform can compensate for the absence of the other in GSK3 regulation of Wnt/ β -catenin signaling (98).

Modes of Regulation of GSK3

GSK3 mediates numerous functions through its involvement in multiple signaling pathways. GSK3 must be strictly regulated to allow specific downstream signaling effects in response to signals upstream of GSK3. Inactivating and activation phosphorylation, requirement for substrate priming, sequestration within protein complexes, and subcellular localization mechanisms regulate GSK3 function. Critically, the regulatory processes must ensure that GSK3 and the functions it regulates are not under homeostatic control, being activated in the appropriate contexts and returning to baseline after removal of the stimulus.

Phosphorylation

GSK3 is a constitutively active enzyme regulated by inactivation. Kinase activity is inhibited by phosphorylation on its Ser21/9 (GSK3 α /GSK3 β) residue within the N-terminal region. Inhibitory phosphorylation can be mediated by Akt (99), as well as PKA (100), atypical PKC (100), p90RSK (100), and ILK (101) as well as potentially by other kinases.

GSK3 also contains a tyrosine phosphorylation site at Tyr279/216 (GSK3 α /GSK3 β). This site is constitutively phosphorylated and contributes to maximal activation of the kinase. The mechanism regulating the phosphorylation of GSK3 on the Tyr279/216 site is controversial and still not well understood. The mechanism of tyrosine phosphorylation has been suggested to be an autophosphorylation event (102), but has also been suggested to occur through action of a separate kinase rather through an autoregulatory mechanism (103).

Substrate Priming

Most GSK3 substrates require prior phosphorylation by another kinase for efficient phosphorylation by GSK3. Priming phosphorylation generates the sequence Ser/Thr-X-X-X-Ser/Thr-PO₄, which GSK3 phosphorylates on the first Ser/Thr residue (104, 105).

Sequestration and Subcellular Localization

GSK3 is involved in multiple pathways. The formation of protein complexes such as the GSK3 protein complex downstream of Wnt signaling is believed to sequester GSK3, preventing regulation of GSK3 by other pathways. For example, inactivation of GSK3 by Akt does not increase β -catenin levels (106). However, crosstalk between the Wnt and Erk pathways has been described (107), and Erk interaction with GSK3 results in increased β -catenin (108), indicating the model of GSK3 sequestration may not be complete under different cellular contexts and stimuli.

GSK3, like many other kinases, functions in different contexts as a kinase and as a linker molecule. That is, some of the functions of GSK3 required its kinase activity, whereas others are dependent on its ability to bind to other cellular components independent of its kinase activity. In many cases, deconvoluting the two activities of GSK3 has been complex and may represent independent or integrative contributions from both functions.

GSK3 can be localized to the cytosol, nucleus, or mitochondria. Subcellular localization of GSK3 is an important regulatory mechanism that controls the availability of upstream and downstream mediators of GSK3 pathways. For example, stimulation of the intrinsic apoptotic signaling pathway with the DNA damaging agent camptothecin increases activity of the nuclear and mitochondrial pools of GSK3, but does not increase activity of GSK3 in the cytosol (109). The level of GSK3 in the nucleus varies during the cell cycle, and is regulated by nuclear export of GSK3 bound to other proteins and by a nuclear

localization sequence (NLS) motif of GSK3 (110). The role of GSK3 in these different locations in the cell remains to be fully elucidated.

1.6 GSK3 Signaling in Cancer

GSK3 in Wnt Signaling

Signaling by the Wnt family of ligands regulates stem cell function, cellular polarity, differentiation, and migration during development. GSK3 is a major component of the canonical Wnt signaling pathway that regulates β -catenin-mediated transcriptional events. Phosphorylation of β -catenin by GSK3 targets β -catenin for ubiquitinylation and degradation. In the absence of downregulation by GSK3, β -catenin accumulates and binds to TCF/LEF transcription factors to upregulate transcription of target genes, which promote tumorigenesis and invasiveness of cancer cells (111). GSK3 phosphorylation of β -catenin requires assembly of a protein complex known as the β -catenin destruction complex that consists of GSK3, β -catenin, Axin, and adenomatous polyposis coli (APC). Binding of Wnt ligand to its membrane-bound receptor disrupts the destruction complex and allows β -catenin to upregulate transcription of its targets.

Wnt also activates the mTOR pathway that plays important roles in regulating cell growth and bioenergetics. After priming by other kinases, GSK3 provides an activating phosphorylation for TSC2, a negative regulator of mTOR signaling. Wnt represses negative regulation of mTOR by GSK3 through a mechanism that involves axin and APC (112).

GSK3 in Hedgehog Signaling

Similar to the Wnt signaling pathway, binding of the secreted ligand Hedgehog (Hh) to its transmembrane receptor Patched (Ptch) results in disruption of a complex in which GSK3 directs proteolytic cleavage of Cubitus interruptus (Ci) protein. In the absence of cleavage to a shorter, repressor form, which is dependent on GSK3, Ci induces expression of Hh target genes. Germline mutations and sporadic mutations of the tumor suppressor Ptch leads to increased incidence of basal cell carcinoma and medulloblastoma (113, 114).

GSK3 in Growth Factor Signaling

Growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF) bind to their cell membrane receptors, leading to activation of Akt. Akt then phosphorylates GSK3 on its Ser21/Ser9 (GSK3 α /GSK3 β) residue, resulting in an inactive conformation of GSK3. Inhibition of GSK3 through Akt and other kinases as indicated above, promotes cell cycle progression since the active form of GSK3 phosphorylates and increases the proteosomal degradation rate of key cell cycle proteins including cyclin D1 (115), cyclin E (116), c-myc (117, 118), and p21 (119, 120).

GSK3 in JNK Signaling

GSK3 also interacts with the c-Jun N-terminal kinase (JNK) and p38 pathways, which activate downstream oncogenic transcription factors. JNK and p38 are preferentially

activated through stress stimuli, but can also be activated in response to growth factors (121) and Wnt (122, 123). GSK3 binds to and inhibits the MEKK4 kinase, preventing activation of downstream JNK and p38 (124). GSK3 also directly phosphorylates the JNK-activated c-Jun transcription factor on its Thr239 residue, targeting the oncogenic transcription factor for ubiquitination and proteosomal degradation (125).

GSK3 and Apoptosis

GSK3 promotes the intrinsic apoptosis pathway mediated by mitochondrial disruption and inhibits the extrinsic apoptosis pathway activated by death receptors (126). Intrinsic apoptosis signaling is promoted by direct interaction of GSK3 with pro-apoptotic and anti-apoptotic factors and regulation of transcription and translation factors that modulate expression of apoptotic signaling molecules. GSK3 phosphorylates and activates the pro-apoptotic Bcl-2 family member Bax (127) and phosphorylates and enhances the degradation of anti-apoptotic Bcl-2 family member MCL-1 (128). The p53 transcription factor is a key mediator of apoptosis. GSK3 has been shown to both physically modify p53 through phosphorylation (129) and promote its acetylation (130) through a binding interaction with the C-terminal basic domain of p53 (130-132), contributing to the apoptotic actions of p53 (131, 132). GSK3 inhibits protein translation through inhibitory phosphorylation of the translation initiation factor eIF2B (133), affecting mitochondrial cytochrome c release (134).

GSK3 inhibits the extrinsic apoptosis pathway mediated by death-domain containing receptors. GSK3 forms an anti-apoptotic complex with ddx3 and cIAP-1 near death receptors to prevent formation of the death-inducing signaling complex (135).

Regulation of NF-KB by GSK3 has been shown to either promote or inhibit apoptosis. These paradoxical observations result from NF-KB's ability to influence both intrinsic and extrinsic apoptotic signaling, and from variations of the role of NF-KB in different cell and signaling contexts (126).

1.6 Overview of Dissertation

We demonstrate that phosphorylation of Akt on its activation sites in response to cell treatment with Akt catalytic domain inhibitors or the EGF and IGF1 growth factors is regulated by GSK3 in the MDAMB231 breast cancer cell line and in mouse embryonic fibroblasts (MEFs). Additionally, we observed GSK3 dependence of basal Akt phosphorylation in the AU565 breast cancer cell line. This work establishes a novel role of GSK3 upstream of Akt in the PI3K/Akt pathway. The positioning of the effects of GSK3 both upstream and downstream of Akt raises important theoretical questions in terms of pathway homeostasis and offers important new information related to the activity of the PI3K pathway that are in or about to enter clinical trials.

CHAPTER 2: MATERIALS AND METTHODS

2.1 Antibodies

Rabbit polyclonal antibodies to phospho-S6RP (Ser235/236), phospho-S6RP (Ser240/244), phospho-Akt (Thr308), phospho-Akt (Ser473), Akt, rictor, raptor, p110 α , phospho-GSK3 (Ser21/9), phospho-p70S6K (Thr389), and phospho- β -catenin (Ser33/37/Thr41) were purchased from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibodies to phospho-PRAS40 (Thr246) and p85 and mouse monoclonal antibody to phosphotyrosine were purchased from Millipore (Billerica, MA). Mouse monoclonal antibody to PRAS40 (Clone 73P21) was purchased from MBL Intl Corp (Woburn, MA). Rabbit monoclonal antibodies to p110 β (Clone Y384) and p110 γ (Clone Y388) were purchased from Epitomics (Burlingame, CA). Mouse monoclonal antibody to GSK3 (Clone 1H8) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to GAPDH (Clone 6C5) was purchased from Ambion (Austin, TX). HRP-conjugated goat anti-rabbit secondary antibody and HRP-conjugated goat anti-mouse secondary antibody were purchased from Bio-Rad (Hercules, CA). The antibodies used in the RPPA study have been described previously (136).

2.2 Cell Lines

Tumor cell lines MDAMB231, A431, DU145, MDAMB468, SKOV3, AU565, T47D, MCF7, MDAMB415, BT549, SKBR3, and OVCAR3 were cultured in RPMI 1640 medium

supplemented with 5% fetal bovine serum (FBS). Mouse embryonic fibroblasts (MEFs) were cultured in DMEM medium supplemented with 5% FBS. All cells were maintained at 37 °C with 5% CO₂.

2.3 Small Molecule Inhibitors, Growth Factors, and Cell Treatments

The A674563 and A443654 Akt inhibitors were obtained through collaboration with Abbott Laboratories (Abbott Park, IL). QLT0454 was obtained through collaboration with QLT Incorporated. Rapamycin was purchased from Cell Signaling Technology. The GSK3 inhibitor SB216763 GSK3 inhibitor was purchased from Sigma (St. Louis, MO). LY294002 was purchased from Calbiochem (San Diego, CA). Epidermal growth factor (EGF) was purchased from R&D Systems (Minneapolis, MN). Insulin-like growth factor 1 (IGF-1) was purchased from Millipore (Billerica, MA). Cells were treated with the chemical inhibitors for the indicated times after serum-starvation overnight (16 to 24 hours) unless otherwise indicated.

2.4 siRNAs and Transient Transfection

siRNAs targeting rictor (pooled siRNA M-016984-00), raptor (pooled siRNA M-004107-00), p110 α (pooled siRNA M-003018-01), human GSK3 α (pooled siRNA L-003009-00), human GSK3 β (pooled siRNA L-003010-00), mouse GSK3 α (pooled siRNA L-059026-00), mouse GSK3 β (pooled siRNA L-041080-00), and nontargeted RISC-Free control siRNA were purchased from Dharmacon (Lafayette, CO). Pan-GSK3 siRNA #1

(siRNA 42839) was purchased from Ambion (Austin, TX). Pan-GSK3 siRNA #2 was purchased from Cell Signaling Technology (Danvers, MA). Cells were plated 24 hours prior to transfection at 40%-50% confluence. siRNAs were transfected using DharmaFECT™ (Dharmacon) according to the manufacturer's instructions. The culture medium was changed 24 hours after transfection, and cells were serum-starved 48 hours post-transfection. After overnight (16 to 24 hours) serum-starvation, cells were treated with inhibitors as indicated and then harvested.

2.5 Cell Lysis and Western Blotting

After cell treatment with various inhibitors, cell culture dishes were placed on ice; cells were washed twice with ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/mL aprotinin). Cell lysates were collected after centrifugation at 13,000 rpm for 10 minutes. The cellular protein concentration was determined by BCA reaction with reagents from Pierce (Rockford, IL). The proteins in the cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for one hour at room temperature and then incubated overnight with antibodies diluted in 5% BSA in TBS-T: anti-phospho-S6RP (Ser235/236) (1:1000 dilution), anti-phospho-S6RP (Ser240/244) (1:1000 dilution), anti-phospho-Akt (Thr308) (1:1000 dilution), anti-phospho-Akt (Ser473)

(1:1000 dilution), anti-Akt (1:1000 dilution), anti-ricor (1:1000 dilution), anti-raptor (1:1000 dilution), anti-p110 α (1:1000 dilution), anti-phospho-GSK3 (Ser21/9) (1:1000 dilution), anti-phospho-p70S6K (Thr389) (1:1000 dilution), anti-phospho- β -catenin (Ser33/37/Thr41) (1:1000 dilution), anti-phospho-PRAS40 (Thr246) (1:1000 dilution), anti-PRAS40 (Clone 73P21) (1:500 dilution), anti-p110 β (Clone Y384) (1:500 dilution), anti-p110 γ (Clone Y388) (1:1000 dilution), anti-GSK3 (Clone 1H8) (1:400 dilution), and anti-GAPDH (Clone 6C5) (1:3000 dilution). The membranes were washed in TBS-T and incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2500 dilution) or HRP-conjugated goat anti-mouse secondary antibody (1:2500 dilution) for one hour at room temperature. The membranes were washed with TBS-T and the proteins were visualized using ECL from Amersham Biosciences (Piscataway, NJ).

2.6 p85 Immunoprecipitation

For immunoprecipitation of p85, cell lysates were incubated with anti-p85 for 90 minutes. Immune complexes were captured by incubation with G-conjugated Sepharose beads for 90 minutes. Immunoprecipitates were washed three times with immunoprecipitation buffer (0.5% Triton X-100, 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride), washed once with Tris-buffered saline, and boiled in 2X SDS buffer before electrophoresis on SDS-PAGE gel.

2.7 Reverse Phase Protein Array (RPPA)

Serial dilutions of cell lysate are spotted onto nitrocellulose-coated slides using an Aushon Biosystems 2470 Arrayer. Each slide is stained for a different antibody using a BioGenex Autostainer and a Dako amplification kit. Slides are scanned to provide images for densitometry measurement by MicroVigene microarray image analysis software. For each sample, the relative signal intensities for each antibody are quantitated using a “SuperCurve” method, which fits all data onto a common curve and maps each sample data point onto the curve. Each slide contains positive and negative controls to ensure quality of slide printing and analysis. Each antibody has been validated for RPPA through correlation of western blot results with RPPA analysis and measurement of the RPPA signal dynamic range.

GSK3 REGULATES AKT INHIBITOR-INDUCED AKT PHOSPHORYLATION

3.1 Introduction

As a result of the importance of the PI3K/Akt pathway in contributing to the pathogenesis of human cancers, multiple potential inhibitors targeting molecules in this signaling network are under preclinical assessment with a number of potential therapeutics in early clinical trials. Both Akt catalytic domain and allosteric inhibitors are currently in development as anti-cancer therapeutics. Strikingly, inhibition of Akt kinase activity with Akt catalytic domain inhibitors results in a marked increase in phosphorylation on its Thr308 and Ser473 activation sites. This observation has been reported with at least three structurally distinct Akt catalytic domain inhibitors demonstrating hyperphosphorylation of Akt in multiple cancer cell lines. Thus understanding the underlying mechanisms and functional consequences of this process is of both theoretical and clinical relevance.

Akt inhibitor-induced phosphorylation of Akt has been demonstrated to be dependent on PI3K and mTORC2, known upstream regulators of physiologic Akt phosphorylation. It has also been demonstrated that Akt inhibitor-induced Akt phosphorylation signaling is not due to the well-known negative feedback signaling loop in which rapamycin analogs that inhibit mTORC1 induce IRS1-mediated phosphorylation of Akt. In a recent paper by Okuzumi et al., the authors proposed a model of intramolecular structural change of Akt upon inhibitor binding that renders Akt either more sensitive to phosphorylation or more resistant to dephosphorylation (137). Thus catalytic Akt inhibitors

can be used as probes of the mechanisms regulating Akt phosphorylation and dephosphorylation in a system where downstream targets of Akt are not activated and where the known mTORC1 mediated feedback loop is not activated. This isolation of upstream processes regulating Akt from the downstream signaling events may allow the identification of novel pathway regulators. Based on this opportunity as well as the need to understand the mechanism involved and possible consequences to facilitate implementation of Akt inhibitors into clinical trials, we decided to elucidate the underlying mechanisms responsible for Akt phosphorylation.

In the present study, we utilize siRNAs and small molecule inhibitors to target molecules in the PI3K/Akt signaling network to elucidate the molecular mediators of Akt catalytic inhibitor-induced Akt phosphorylation. Here we report the dependence of Akt inhibitor-induced Akt signaling on GSK3. GSK3 is a well-known downstream substrate of Akt. To our knowledge, this is the first report of a role of GSK3 acting as a positive upstream regulator of Akt.

3.2 Results

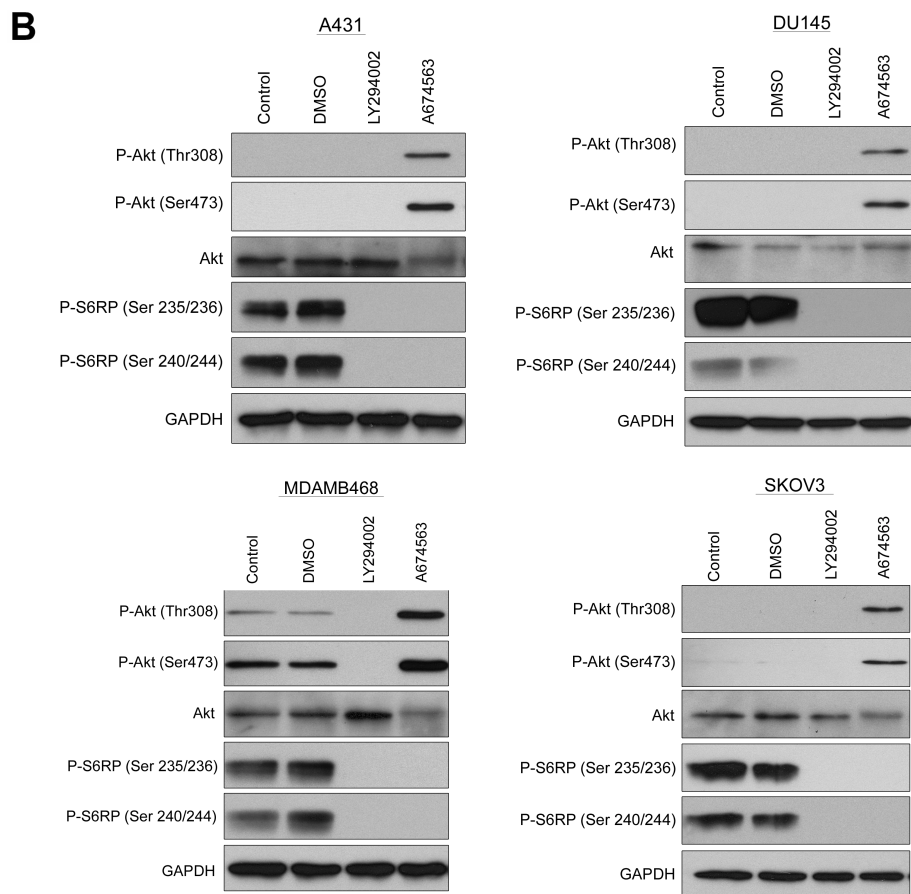
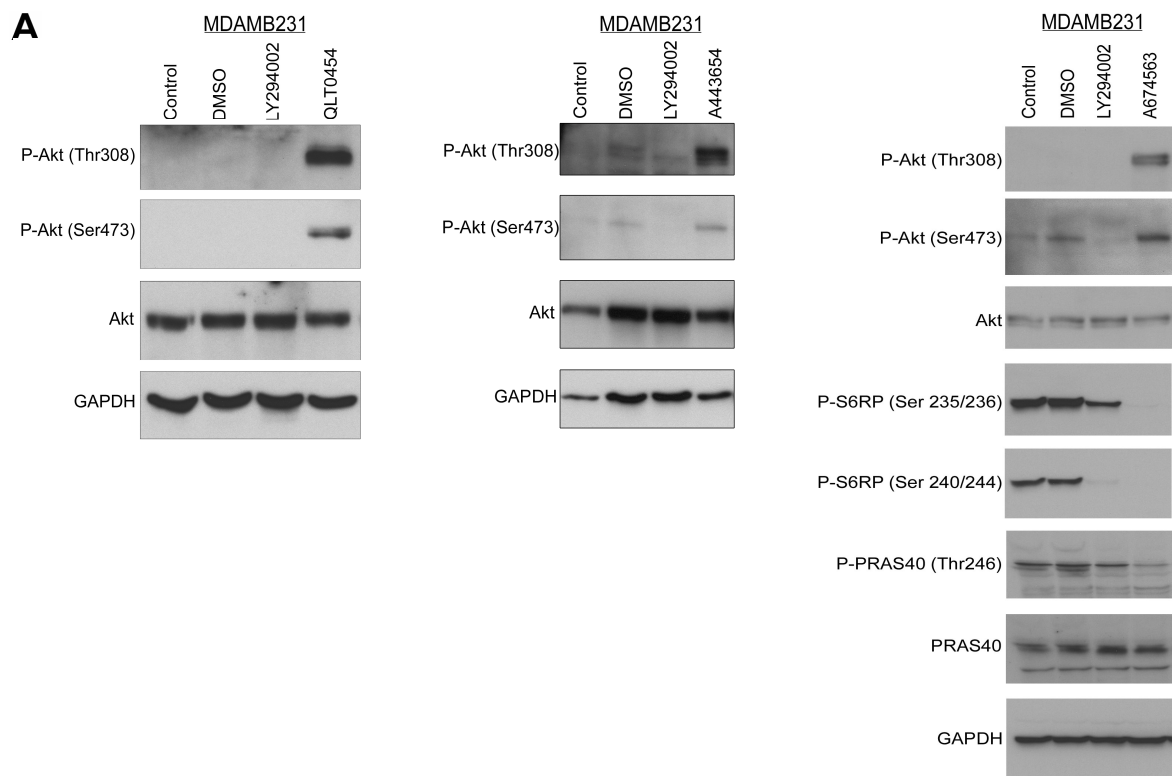
3.2.1 Treatment of multiple cancer cell lines with competitive reversible Akt inhibitors induces phosphorylation of Akt on its activation sites.

Three competitive reversible inhibitors of Akt catalytic activity that compete at the ATP binding site (87, 88, 93) have been demonstrated to increase phosphorylation. The Akt

inhibitors A674563 and A443654, generated by Abbott Laboratories, and GSK690693, produced by GlaxoSmithKline, result in inhibition of Akt activity as demonstrated by in vitro kinase assays with GSK3 β as substrate and decreased phosphorylation of Akt substrates in cultured human cancer cell lines. Inhibition of Akt kinase activity with these small molecule inhibitors is accompanied by increased phosphorylation of Akt on its activation sites (Thr308 and Ser473) – phosphorylation events that would result in increased activity of Akt were it not competitively inhibited by binding of the inhibitors to the ATP binding site of Akt.

To study the mechanism by which inhibition of Akt increases Akt phosphorylation, we determined the effect of three structurally distinct Akt inhibitors, the A674563 and A443654 inhibitors produced by Abbott Laboratories and a third inhibitor QLT0454 produced by QLT Inc., on phosphorylation of the Thr308 and Ser473 sites of Akt in the MDAMB231 breast cancer cell line (Figure 3.1A). All three inhibitors increase both pThr308 and pSer473 in serum-starved (Figure 3.1A) as well as serum-replete cells (not presented). The increase in phosphorylation of Akt was associated with inhibition of downstream phosphorylation of PRAS40 and S6 Ribosomal Protein (S6RP) indicating inhibition of Akt catalytic activity. The modest increase in total Akt levels likely reflects decreased reactivity of the total Akt antibody used with phosphorylated Akt, an effect observed in multiple studies. For subsequent experiments, the A674563 inhibitor was used. In addition to the MDAMB231 breast cancer cell line, phosphorylation of Akt was induced by A674563 in the A431 (epidermis), DU145 (prostate), MDAMB468 (breast), and SKOV3 (ovary) human cancer cell lines (Figure 3.1B). All cell lines tested with the catalytic domain

Figure 3.1 Competitive reversible Akt inhibitors induce phosphorylation of Akt in multiple cancer cell lines. A. MDAMB231 cells were serum-starved overnight and treated for 2 hours with DMSO (0.3%), LY294002 (20 uM), or one of three Akt inhibitors – A674563 (20 uM), A443654 (10 uM), or QLT0454 (20 uM). Cell lysates were analyzed for phosphorylation of Akt (Thr308 and Ser473) and S6RP (Ser235/236 and Ser240/244) by immunoblotting. Effect on PRAS40 phosphorylation (Thr246) of A674563 was also analyzed. GAPDH was used as a loading control. B. A431, DU145, MDAMB468, and SKOV3 cancer cell lines were serum-starved overnight and then treated with LY294002 (20 uM), A674563 (20 uM), or DMSO (0.3%) vehicle for 2 hours. Cell lysates were analyzed for phosphorylation of Akt (Thr308 and Ser473) and S6RP (Ser235/236 and Ser240/244). GAPDH was used as a loading control.



Akt inhibitors demonstrated an increase in phospho-Akt. Cell lines demonstrating Akt inhibitor-induced phosphorylation and not presented here include BT549 (breast), SKBR3 (breast), and OVCAR3 (ovary). These cell lines demonstrate multiple genomic aberrations including PTEN mutation, HER2 amplification, p53 mutation, and PIK3R1 mutations demonstrating that the process occurs in the presence of multiple genomic aberrations. No cell line was tested where the Akt catalytic domain inhibitors failed to induce increases in Akt phosphorylation. Thus Akt inhibitor-induced Akt phosphorylation is likely due to on-target effects and is generalizable across cellular lineages.

3.2.2 Akt catalytic domain inhibitor treatment is associated with increased signaling upstream of Akt.

Akt is activated downstream of multiple receptor tyrosine kinase growth factor receptors. The SH2 domains of p85, the regulatory subunit of PI3K, bind specific phosphotyrosine residues of the activated growth factor receptor, inducing an activating conformational change in the p110 catalytic subunit of PI3K and bringing it into proximity of its phosphoinositide substrate. PI3K is also recruited to the membrane by p85 binding of tyrosine-phosphorylated adaptor molecules complexed to an activated receptor tyrosine kinase. To determine if catalytic domain Akt inhibitors induce changes in the PI3K/Akt pathway upstream of Akt, we treated MDAMB231 cells with A674563, immunoprecipitated p85 α , and analyzed immunoprecipitates for tyrosine-phosphorylated proteins. A674563 induces increased tyrosine phosphorylation of a p85 α -associated protein in the 90-110 kDa size range (Figure 3.2), illustrating increased signaling in the PI3K/Akt pathway in response

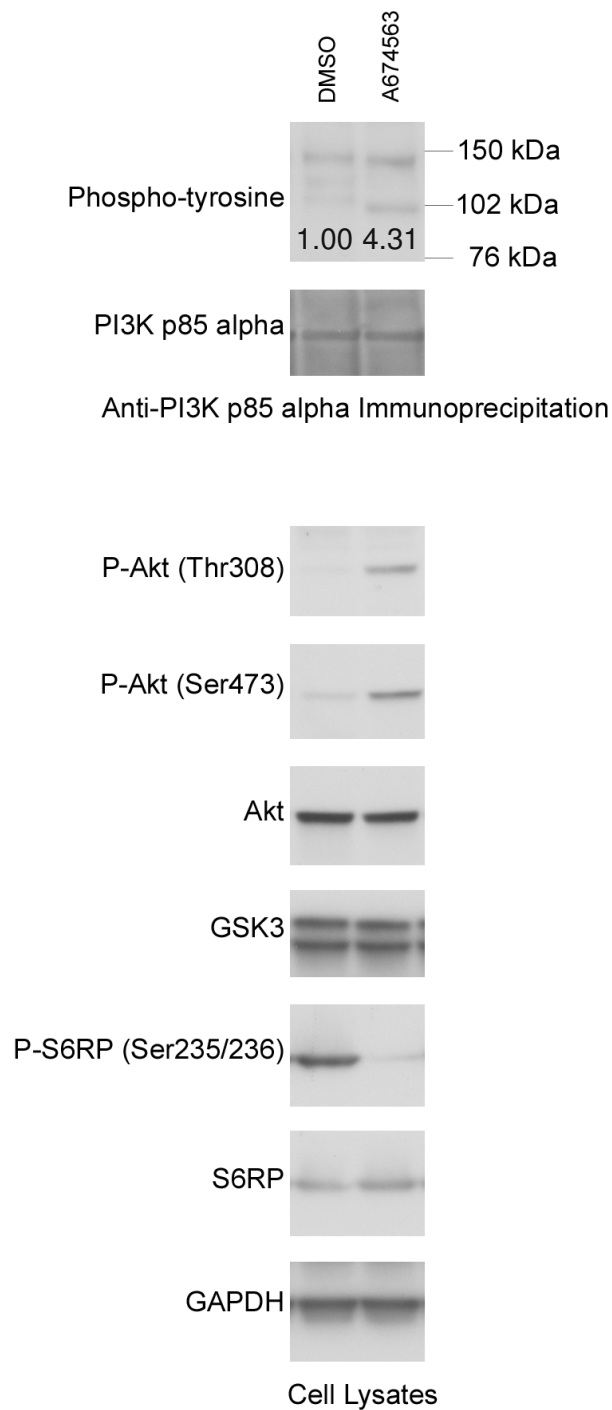


Figure 3.2 A674563 induces increased association of p85 with a tyrosine-phosphorylated protein. MDAMB231 cells were serum-starved overnight and treated for the indicated times with DMSO or A674563 (20 μ M). p85 was immunoprecipitated from cell lysates. Immunoprecipitates were probed for phosphotyrosine and p85 α by immunoblotting. Cell lysates were analyzed for pAkt (Ser473), Akt, pS6RP (Ser240/244), and S6RP. Immunoprecipitation procedure was performed by Dr. Yiling Lu.

to Akt catalytic domain inhibitors. In a recent paper, a model was introduced in which Akt hyperphosphorylation induced by Akt catalytic domain inhibitors results from a conformational change in Akt upon inhibitor binding that renders Akt more sensitive to activation by basal levels of signaling molecules upstream of Akt (137). However, our data suggests that hyperphosphorylation of Akt in response to treatment with Akt catalytic domain inhibitors is associated with an increase in upstream signaling, indicating that the inhibitors may induce a feedback loop that increases upstream signaling rather than simply rendering Akt more easily phosphorylated by upstream kinases.

3.2.3 A674563-induced Akt phosphorylation is inhibited by an allosteric PH domain Akt inhibitor.

Physiological Akt activation is preceded by recruitment of Akt to the cell membrane via binding of the PIP3 lipid to the PH domain of Akt. To determine if A674563-induced Akt phosphorylation also requires Akt recruitment to the cell membrane, we pretreated MDAMB231 cells with an allosteric Akt inhibitor, Akt Inhibitor VIII developed by Merck, that locks Akt into an inactive conformation in which the PH domain is inaccessible to bind PIP3 in the membrane (138). We pretreated cells with Akt Inhibitor VIII to prevent membrane translocation of Akt before treating cells with the A674563 Akt catalytic domain inhibitor. Results show abrogation of A674563-induced Akt phosphorylation with the allosteric inhibitor pretreatment (Figure 3.3), indicating requirement of Akt membrane translocation for this process. Importantly, phosphorylation of the Akt substrate PRAS40 was decreased, demonstrating effective Akt inhibition by Akt Inhibitor VIII and A674563.

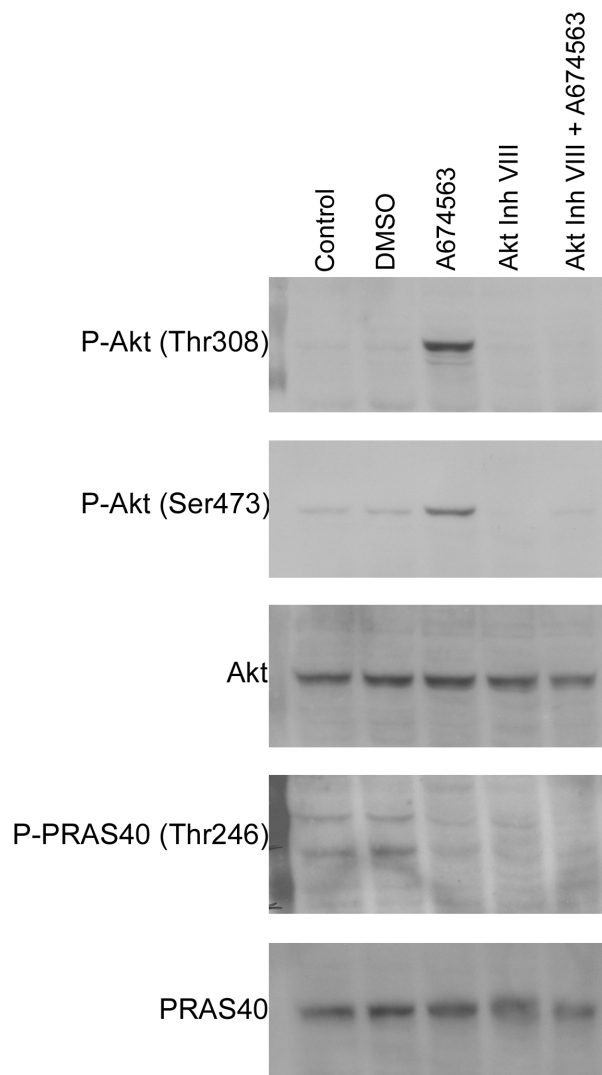


Figure 3.3 A674563-induced Akt phosphorylation is abrogated by treatment with an allosteric PH domain Akt inhibitor. MDAMB231 cells were serum-starved overnight and then treated with A674563 (20 uM for two hours) or preincubated with Akt Inhibitor VIII (5uM) for 1 hour before 2-hour treatment with A674563. Cell lysates were analyzed for phosphorylation of Akt (Thr308 and Ser473), Akt, and phosphorylation of PRAS40 (Thr246). GAPDH was used as a loading control.

3.2.4 Induction of Akt phosphorylation by A674563 is dependent on PI3K and mTORC2 but not mTORC1.

PI3K-mediated production of PIP3 recruits Akt to the cell membrane through binding of the Akt PH domain, facilitating phosphorylation and activation of Akt. To determine if PI3K is required for the Akt phosphorylation induced with A674563, we pretreated MDAMB231 cells with the PI3K inhibitor LY294002 prior to addition of A674563. As shown in Figure 3.4A, induction of Akt phosphorylation by A674563 was diminished in cells treated with LY294002. This result suggested that activity of PI3K is required for the A674563-induced phosphorylation of Akt. However LY294002 inhibits additional kinases including mTOR suggesting that other effects of LY294002 could explain, at least in part, inhibition of Akt phosphorylation. To further investigate the requirement of PI3K activity for A674563-induced Akt phosphorylation, expression of the p110 α isoform of the PI3K catalytic subunit was decreased using targeted siRNA (Figure 3.4B). The p110 α siRNA decreased p110 α to undetectable levels without altering p110 β or p110 γ levels. As shown in Figure 3.4B, knockdown of p110 α resulted in marked reduction in the level of Akt phosphorylation induced with A674563 treatment. Since MDAMB231 cells express all three class IA PI3K catalytic subunit isoforms (139), activity of the p110 β and p110 γ isoforms as well as low levels of p110 α , below detection by western blotting, remaining in the cells may account for the residual Akt phosphorylation induced with A674563 under conditions of p110 α knockdown. The reduction in Akt phosphorylation induced by A674563-mediated Akt inhibition in cells treated with pan-PI3K inhibitor LY294002 or p110 α -targeted siRNA demonstrates that PI3K activity is required to mediate the Akt phosphorylation induced by

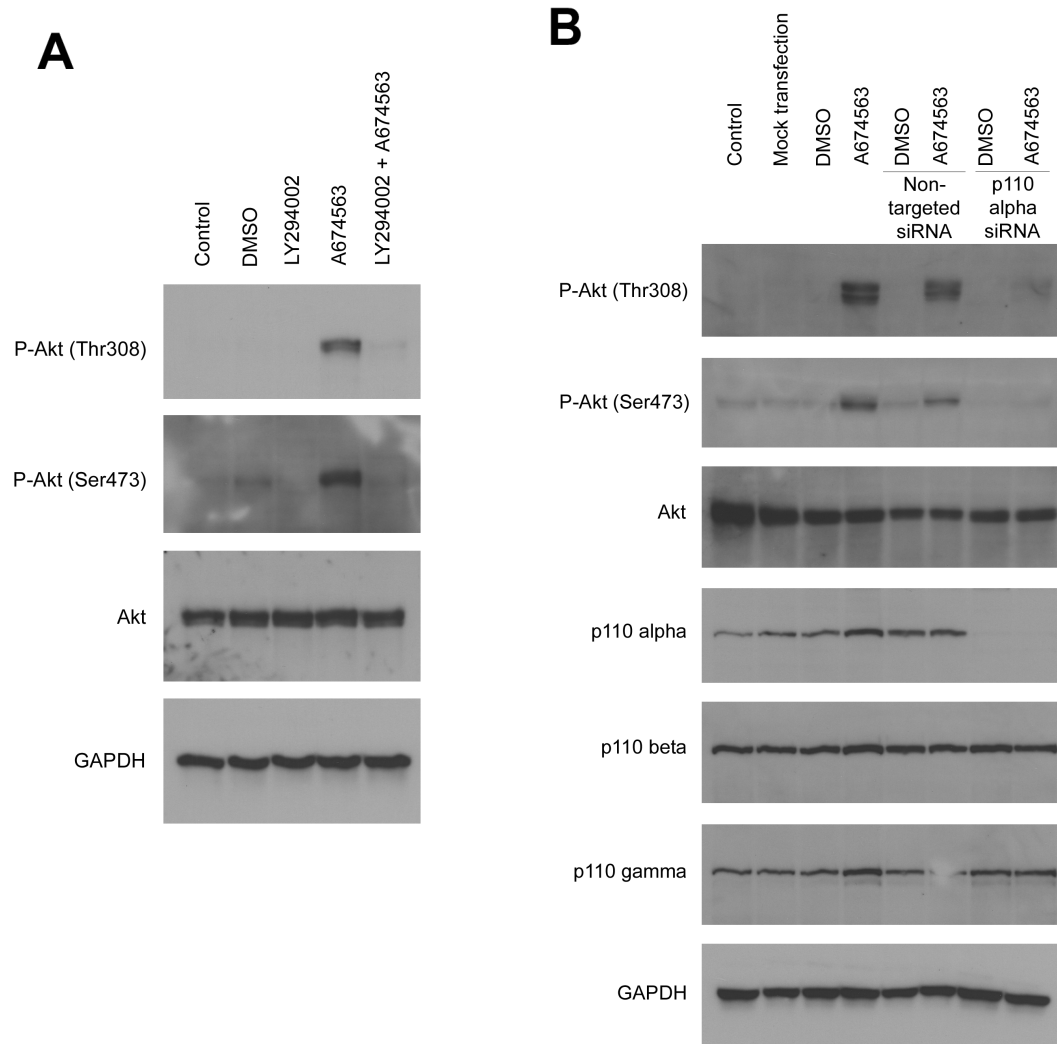


Figure 3.4 Induction of Akt phosphorylation by A674563 is dependent on PI3K. A. MDAMB231 cells were serum-starved overnight, pretreated with LY294002 for 1 hour, and then treated with A674563 for 2 hours. Cell lysates were analyzed for phosphorylation of Akt (Thr308 and Ser473) by immunoblotting. GAPDH was used as a loading control. B. MDAMB231 cells were transfected with either p110 α -targeting siRNA or control nontargeting siRNA. 48 hours post-transfection, cells were serum-starved overnight and then treated with A674563 or DMSO vehicle for 2 hours. Cell lysates were analyzed for Akt phosphorylation (Thr308 and Ser473), p110 α , p110 β , and p110 γ . GAPDH was used as a loading control.

inhibition of Akt with A674563. Further, these results demonstrate that p110 α is the PI3K isoform mediating this process. As p110 α is selectively activated as a consequence of activation of cell surface tyrosine kinase-linked receptors whereas p110 β is primarily activated as a consequence of G protein coupled receptors (140), the selective effect of targeting p110 α is compatible with the contention that upstream activation of PI3K as a consequence of p85 binding to tyrosine phosphorylated proteins plays a role.

The mTOR kinase forms two known protein complexes, mTORC1, the mTOR-raptor complex that functions downstream of Akt, and mTORC2, the mTOR-ricor complex that has been demonstrated to phosphorylate Akt on its Ser473 activation site (9). The requirement for mTOR-ricor in mediating Ser473 Akt phosphorylation that occurs in response to A674563 treatment was investigated using ricor-targeted siRNA to prevent formation of mTOR-ricor complex. As seen in Figure 3.5, ricor knockdown abrogates Akt Ser473 phosphorylation in response to A674563 treatment. This result demonstrates the dependence on mTOR-ricor for the increase in Ser473 Akt phosphorylation observed in response to treatment with A674563.

Activation of mTOR-raptor, a downstream target of Akt, and its downstream target p70S6K leads to degradation of IRS1 and subsequent decrease in Akt activation contributing to homeostasis in the PI3K pathway. Inhibition of mTOR-raptor activity with rapamycin and its analogs blocks this negative feedback signaling resulting in increased phosphorylation of Akt. Increases in Akt phosphorylation levels generated by treatment with inhibitors of mTOR-raptor have been demonstrated in human cancers and cell lines (141-144). The

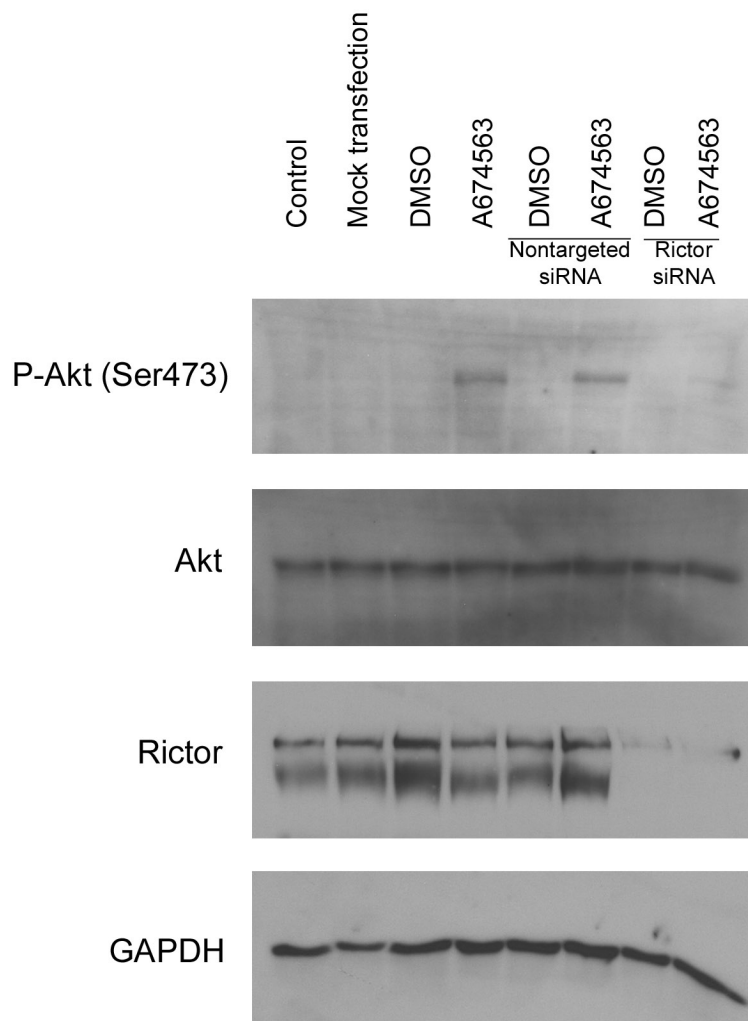


Figure 3.5 mTORC2 is required for A674563-induced phosphorylation of Akt on its Ser473 site. MDAMB231 cells were transfected with either siRNA targeting rictor or nontargeting control siRNA. 48 hours after transfection, cells were serum-starved overnight and then treated with A674563 or DMSO vehicle for 2 hours. Cell lysates were analyzed for Akt phosphorylation (Ser473) and rictor. GAPDH was used as a loading control.

increase in phosphorylated Akt levels resulting from treatment of cell lines with mTOR-raptor inhibitors is dependent on insulin-like growth factor signaling to IRS1 and requires supplementation of cell culture medium with serum or insulin-like growth factor (IGF) (141, 142, 144). Since Akt catalytic domain inhibitors prevent Akt-dependent activation of mTOR-raptor, we evaluated the possible role of mTOR-raptor inhibition in the A674563-induced increase in phosphorylated Akt levels. As seen in Figure 3.6A, treatment of MDAMB231 cells cultured in medium supplemented with fetal bovine serum (FBS) with the mTOR inhibitor rapamycin induces a modest increase in phosphorylation of Akt on its Thr308 and Ser473 activation sites. Effective inhibition of mTOR-raptor activity with rapamycin is indicated by decreased phosphorylation of p70S6K on its Thr389 residue. In comparison to the modest increases in Akt phosphorylation induced with rapamycin in serum-supplemented cells, treatment with A674563 induced marked increases in Akt phosphorylation on both activation sites. Treatment with rapamycin in MDAMB231 cells in culture medium that has not been supplemented with FBS did not generate detectable increase in Akt phosphorylation levels (Figure 3.6B). In contrast, the A674563-induced increase in Akt phosphorylation was readily observed in serum-starved cells, indicating a distinct mechanism of induction of Akt phosphorylation by the Akt catalytic domain inhibitor.

The ability of A674563 to increase Akt phosphorylation was neither increased nor decreased in cells pretreated with rapamycin (Figure 3.6A and 3.6B). Lack of effect of inhibition of mTOR-raptor on A674563-induced phosphorylation of Akt indicates that mTOR-raptor activity is not required for Akt inhibitor-induced Akt phosphorylation. To

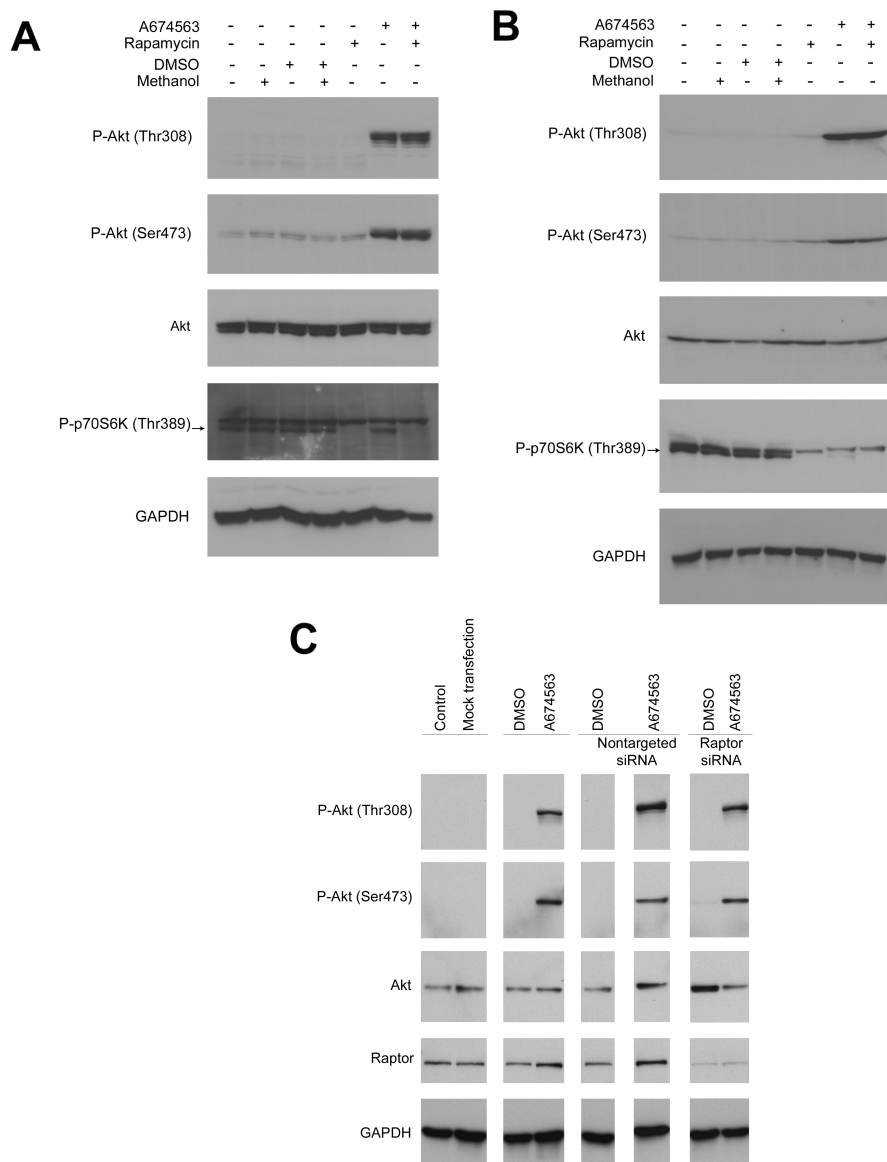


Figure 3.6 Akt phosphorylation induced by A674563 is not dependent on mTORC1. A. MDAMB231 cells maintained in medium supplemented with 5% FBS were treated with rapamycin for 3 hours, A674563 for 2 hours, or pretreated with rapamycin for 1 hour followed by 2-hour A674563 treatment. Cell lysates were analyzed for phosphorylation of Akt (Thr308 and Ser473) and p70S6K (Thr389). GAPDH was used as a loading control. B. MDAMB231 cells were serum-starved overnight followed by 3-hour rapamycin treatment, 2-hour A674563 treatment, or pretreated with rapamycin for 1 hour followed by 2-hour A674563 treatment. Cell lysates were analyzed for phosphorylation of Akt (Thr308 and Ser473) and p70S6K (Thr389). GAPDH was used as a loading control. C. MDAMB231 cells were transfected with either raptor-targeting siRNA or control nontargeting siRNA. 48 hours post-transfection, cells were serum-starved overnight and then treated with A674563 or DMSO vehicle for 2 hours. Cell lysates were analyzed for Akt phosphorylation (Thr308 and Ser473) and raptor. GAPDH was used as a loading control. The data presented is from a single experiment and extraneous data was removed solely for clarity.

further examine if there is any role for mTOR-raptor in induction of Akt phosphorylation by A674563, we knocked down the expression of raptor with targeted siRNA in MDAMB231 cells and treated cells with A674563. Figure 3.6C shows that knocking down expression of raptor does not interfere with A674563-induced Akt phosphorylation in serum-starved cells.

Taken together, these studies indicate that A674563-induced Akt phosphorylation occurs independently of the mTOR-raptor signaling loop and does not require the activity of mTOR-raptor.

3.2.5 Akt-inhibitor induced phosphorylation of Akt is dependent on GSK3.

In addition to mTOR-raptor, Akt phosphorylates numerous cellular signaling proteins upon activation. Akt phosphorylates Glycogen Synthase Kinase 3 (GSK3) on its Serine 21/9 (GSK3 α /GSK3 β) residue, inhibiting constitutive GSK3 downregulation cell cycle progression.

We evaluated dependence of A674563-induced Akt phosphorylation on GSK3 utilizing targeted siRNA to knock down expression of the GSK3 α and GSK3 β isoforms separately and in combination in MDAMB231 cells. Figure 3.7 illustrates that knockdown of GSK3 α or GSK3 β modestly decreased Akt phosphorylation induced by A674563. Knocking down expression of both the GSK3 α and GSK3 β isoforms resulted in marked decrease in A674563-induced Akt phosphorylation (Figure 3.7). Thus, GSK3 is required for Akt inhibitor-induced Akt phosphorylation and expression of both the GSK3 α and GSK3 β

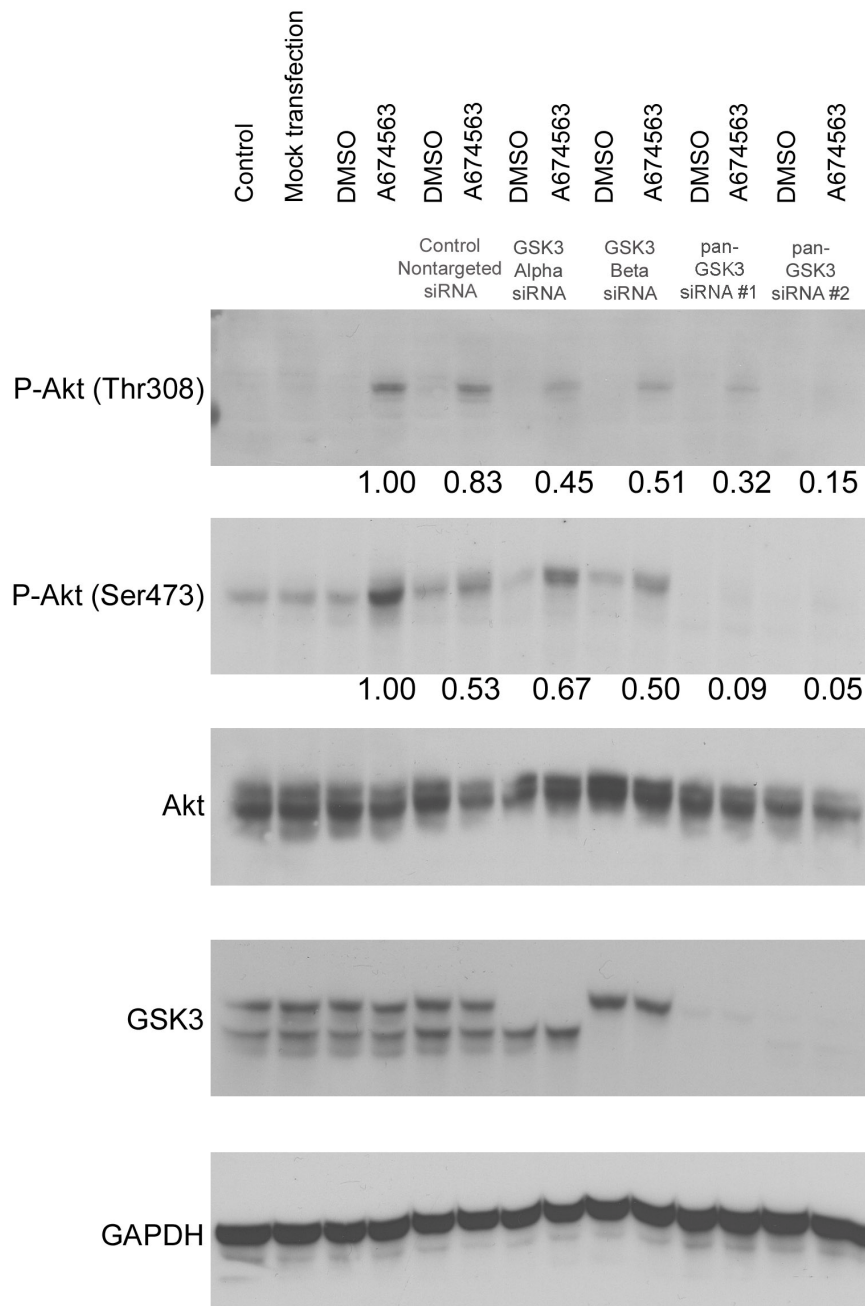


Figure 3.7 GSK3 is required for A674563-induced Akt phosphorylation in MDAMB231 cells. MDAMB231 cells were transfected with siRNA targeting GSK3 α (50 nM), GSK3 β (50 nM), or both isoforms of GSK3 (60 nM). Nontargeting siRNA was used as a control. 48 hours post-transfection, cells were serum-starved overnight and then treated with A674563 (20 μ M) or DMSO vehicle (0.2%) for 2 hours. Cell lysates were analyzed for Akt phosphorylation (Thr308 and Ser473) and GSK3 by immunoblotting. GAPDH was used as a loading control. Densitometry measurements were calculated using version 1.38x of ImageJ.

isoforms is required for maximum induction of Akt phosphorylation with A674563.

To determine if GSK3 α and GSK3 β are required for A674563 induction of Akt phosphorylation in another mammalian system, we used wild type mouse embryonic fibroblasts (MEFs) and GSK3 β knockout MEFs. Knockdown of the individual GSK3 isoforms in the wild type MEFs yielded no significant decrease in A674563-induced Akt phosphorylation, whereas knockdown of both isoforms markedly decreased phosphorylation of Akt in response to A674563 treatment (Figure 3.8A). Similarly, knockdown of GSK3 α in GSK3 β -/- MEFs yielded an almost complete loss of A674563-induced Akt phosphorylation (Figure 3.8B). These results demonstrate the requirement for GSK3 α and GSK3 β in MEFs to permit maximal induction of Akt phosphorylation in response to A674563 treatment.

3.2.6 A674563-induced Akt phosphorylation is independent of acute signaling effects of GSK3 kinase activity.

GSK3 has two mechanisms of signaling – one through its kinase activity and a second through its protein binding interactions. Abrogation of Akt inhibitor-induced Akt phosphorylation by decreased expression of GSK3 may result from loss of GSK3 signaling mediated by either its kinase activity or its protein binding interactions. Kinase activity of GSK3 affects signaling pathways both through mediation of rapid phosphorylation/dephosphorylation cascades and through phosphorylation events that alter protein stability or protein expression.

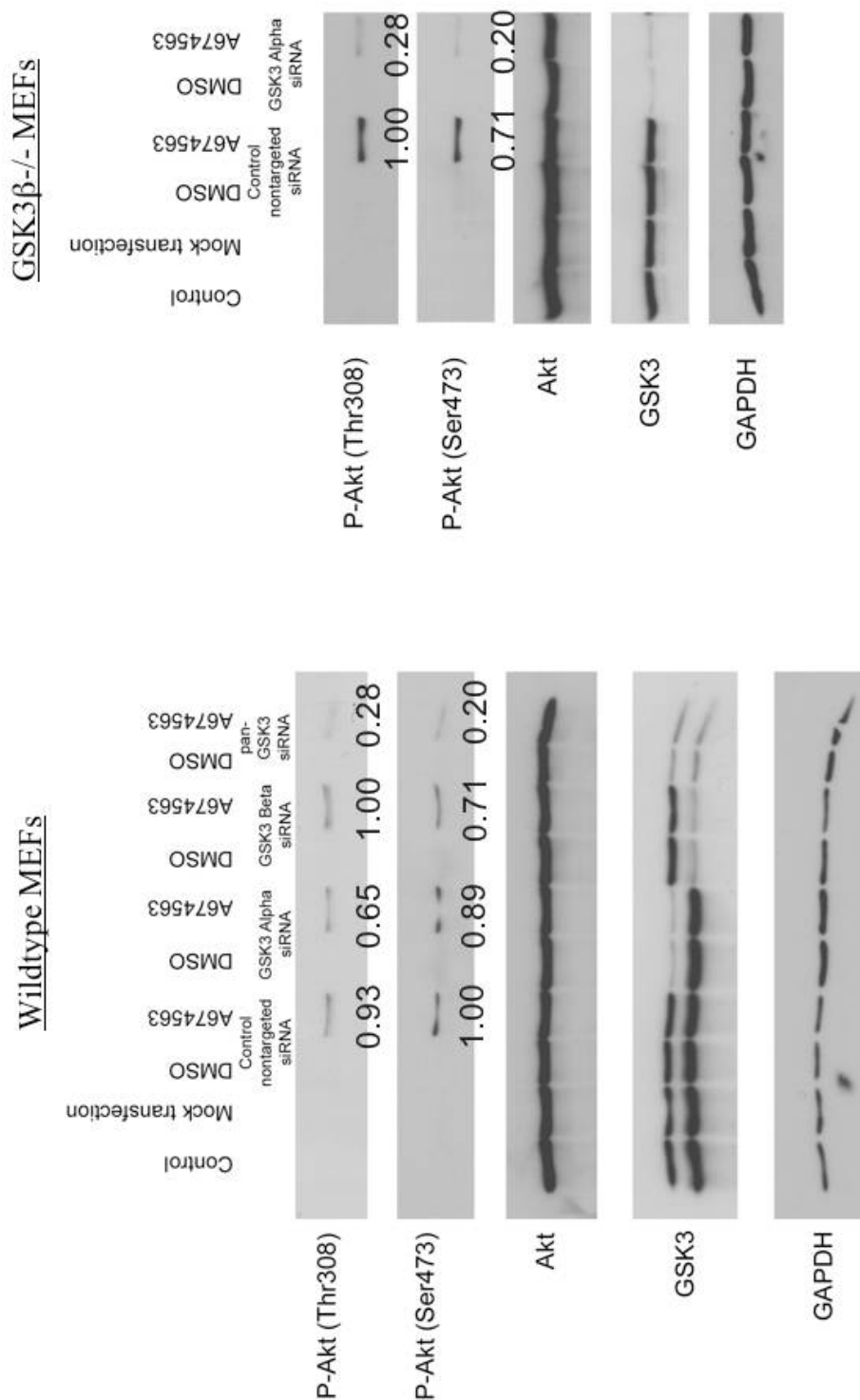


Figure 3.8 GSK3 is required for A674563-induced Akt phosphorylation. Wildtype MEFs were transfected with siRNA targeting GSK3 α (100 nM), GSK3 β (100 nM), or both isoforms of GSK3 (50 nM each GSK3 α and GSK3 β). GSK3 β -/- MEFs were transfected with GSK3 α -targeting siRNA (100 nM). Nontargeting siRNA (100 nM) was used as a control. 48 hours post-transfection, cells were serum-starved overnight and then treated with A674563 (20 μ M) or DMSO (0.2%) vehicle for 2 hours. Cell lysates were analyzed for Akt phosphorylation (Thr308 and Ser473) and GSK3 by immunoblotting. GAPDH was used as a loading control.

To investigate the role of acute signaling effects of GSK3 kinase activity in regulating A674563-induced Akt phosphorylation, we preincubated MDAMB231 cells for one hour with the pan-GSK3 kinase inhibitor SB216763 before exposing the cells to A674563. As seen in Figure 3.9, inhibition of GSK3 kinase activity did not prevent phosphorylation of Akt on its activation sites in response to treatment with A674563. Importantly SB216763 effectively inhibited GSK3 activity as indicated by blockade of β -catenin phosphorylation. This result indicates independence of Akt inhibitor-induced Akt phosphorylation on acute signaling effects mediated by the kinase activity of GSK3.

Effect of chronic inhibition of kinase activity of GSK3 on Akt inhibitor-induced Akt phosphorylation was not tested due to limitations of our reagents. In MDAMB231 cells, inhibition of GSK3 kinase activity by SB216763 (assayed by immunoblot of β -catenin phosphorylation on the Ser33/37/Thr41 sites) lasts for less than six hours even with addition of fresh inhibitor (data not presented).

Based on the independence of Akt inhibitor-induced phosphorylation on acute effects of GSK3 kinase activity, observed abrogation of A674563-induced Akt phosphorylation with knockdown of GSK3 expression for several days in the GSK3 knockdown studies may result from either the linker molecule function of GSK3 or from chronic loss of GSK3 kinase activity.

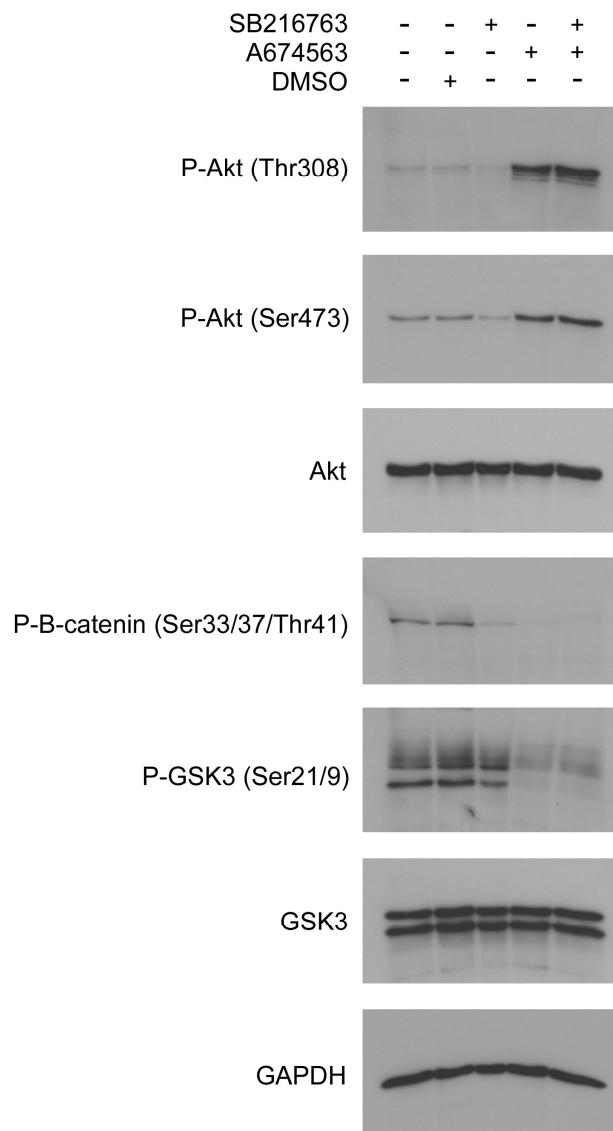


Figure 3.9 A674563-induced Akt phosphorylation is not dependent on acute effects of GSK3 kinase activity. MDAMB231 cells were serum-starved overnight, pretreated with SB216763 (10 uM) for 1 hour, and then treated with A674563 (20 uM) for 2 hours. Cell lysates were analyzed for phosphorylation of Akt (Thr308 and Ser473), β -catenin (Ser33/37/Thr41), and GSK3 (Ser21/9) by immunoblotting. GAPDH was used as a loading control.

3.2.7 GSK3 is not upstream of an A674563-induced p85-associated tyrosine phosphoprotein.

To determine the role of GSK3 in the A674563 induction of tyrosine phosphorylation of the p85-associated protein, expression of GSK3 was knocked down in MDAMB321 cells using targeted pan-GSK3 siRNA prior to A674563 treatment and p85 immunoprecipitation. As seen in Figure 3.10, A674563 induction of tyrosine phosphorylation of a protein in the 90-110 kDa size range is not decreased with GSK3 knockdown. This result suggests that if the A674563-induced tyrosine phosphoprotein is a driver of Akt-inhibitor induced Akt phosphorylation, then the effect of GSK3 is downstream of induction of tyrosine phosphorylation of this molecule and its association with p85 but upstream of the phosphorylation of Akt. Further studies are warranted to determine the identity and the role of the 90-110 kDa tyrosine phosphorylated protein in Akt inhibitor-induced Akt phosphorylation.

3.3 Discussion

The importance of the PI3K/Akt pathway in cancer is underscored by ongoing development of an array of small molecule inhibitors directed against molecules in the signaling network (86). Clinical trials of Akt inhibitors, including ATP competitive kinase inhibitors, allosteric inhibitors and phosphatidylinositol analogs, are currently underway. Evaluation of the global effects of Akt inhibition on PI3K signaling network have not been fully elucidated and could alter the outcomes of patient treatment.

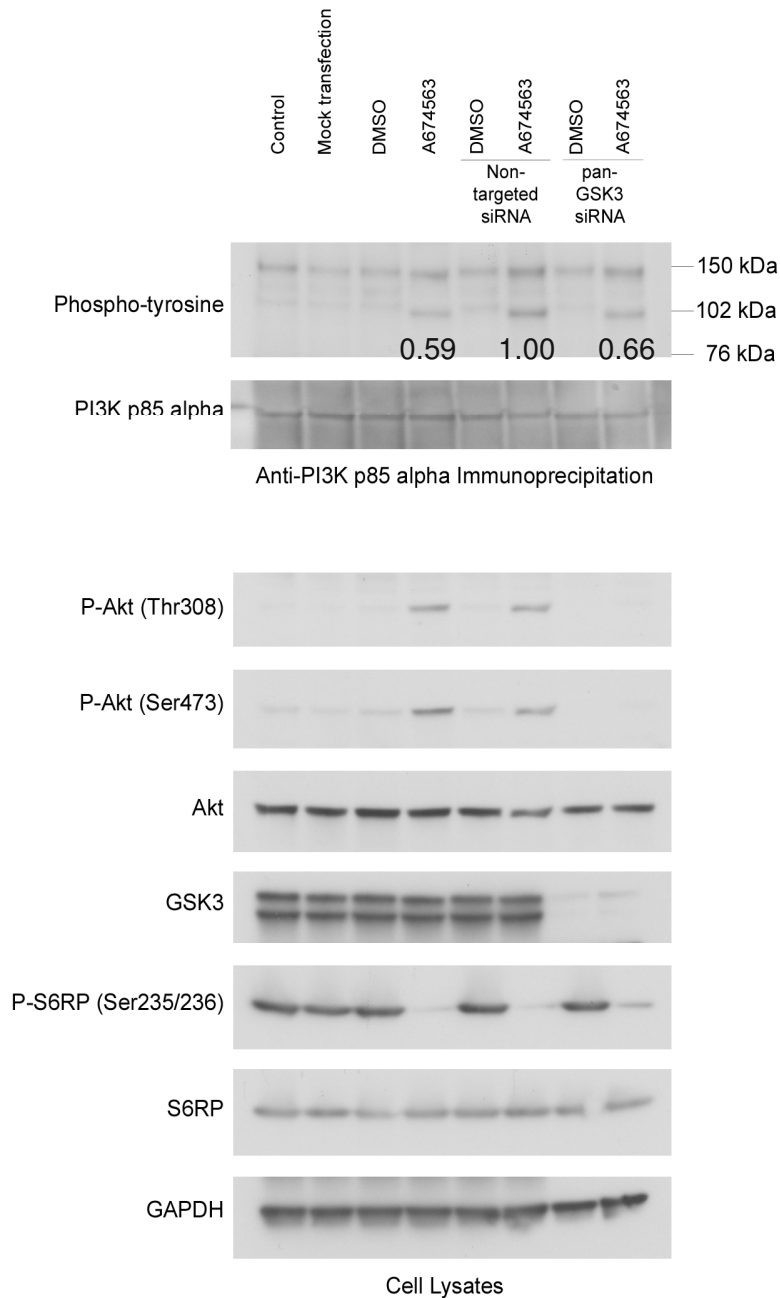


Figure 3.10 A674563-induced tyrosine phosphorylation of a 85-associated protein is independent of GSK3. MDAMB231 cells were transfected with either pan-GSK3 siRNA (60 nM) or control nontargeting siRNA. 48 hours post-transfection, cells were serum-starved overnight and then treated with A674563 or DMSO vehicle for 2 hours. p85 was immunoprecipitated from cell lysates. Immunoprecipitates were probed for phosphotyrosine and p85 α by immunoblotting. Cell lysates were analyzed for pAkt (Ser473), Akt, pS6RP (Ser240/244), and S6RP. Immunoprecipitation procedure was performed by Dr. Yiling Lu. Data from this experiment has been presented in part earlier in this work (Figure 3.2).

As demonstrated in the current work, the A674563 inhibitor induces a marked increase in phosphorylation of Akt on its activation sites. As expected based on previous studies with A443654, we show that induction of Akt phosphorylation with A674563 is dependent on PI3K and mTORC2. The hyperphosphorylation of Akt in response to A674563 treatment represents either an increase in the upstream signaling or a decrease in the rate of Akt dephosphorylation once it has been phosphorylated by PDK1 and PDK2. Our results show increased association of tyrosine-phosphorylated proteins with p85, the catalytic subunit of PI3K, indicating increased signaling upstream of Akt.

We demonstrate the dependence on GSK3 of Akt inhibitor-induced Akt phosphorylation. GSK3 has been known for more than a decade to be an important downstream mediator of Akt signaling, however, this places GSK3 both upstream and downstream of Akt. In both MDAMB231 breast cancer cells and MEFs, knocking down GSK3 isoforms individually has only a modest effect on the ability of A674563 to induce Akt phosphorylation, whereas knocking down both GSK3 isoforms yields a marked decrease in A674563-induced Akt phosphorylation. These results may indicate the existence of a pool of GSK3 α and GSK3 β mediating signaling to Akt. Depletion of either isoform results in only a small decrease in A674563-induced Akt phosphorylation likely due to incomplete compensation by the remaining isoform. In this model, knocking out both isoforms of GSK3 markedly decreases the entire GSK3 pool abolishing A674563-induced phosphorylation of Akt.

GSK3 mediates its effects through both its kinase activity and through protein-protein interactions. We did not observe dependence of Akt phosphorylation on GSK3 kinase activity. We did not test whether chronic inhibition of GSK3 diminishes Akt inhibitor-induced Akt phosphorylation. Since GSK3 mediates some of its signaling effects such as modification of gene transcription over longer periods than were tested in our studies, there is still a possibility that the kinase function of GSK3 is involved in signaling upstream of Akt phosphorylation. Alternatively, the linker molecule function of GSK3 may be solely responsible for its upstream role regulating Akt phosphorylation. We did not detect significant direct association of GSK3 with Akt itself (immunoprecipitation studies not presented). Thus GSK3 may bind and regulate a protein involved directly or indirectly in the processes of Akt phosphorylation or dephosphorylation.

The potential that GSK3 modulates a critical process or is physically positioned upstream Akt signaling represents a novel signaling process that warrants further investigation. Elucidation of the signaling mechanisms of Akt inhibitor-induced Akt phosphorylation is necessary to allow prediction of local and global cellular response to manipulation of specific pathway components and identification of valid therapeutic targets and rational combinations of pharmacological treatments. Indeed, both catalytic and allosteric Akt inhibitors are in clinical trials rendering this elucidation urgent.

CHAPTER 4: GSK3 REGULATES GROWTH FACTOR SIGNALING TO AKT

4.1 Introduction

Dysregulation of EGFR or IGF1R signaling leads to constitutively upregulated signaling through the PI3K/Akt and MAPK pathways and contributes to a variety of malignancies. EGFR is mutated, amplified, or overexpressed in a subset of cancers. No association of IGF1R mutations with cancer has been found, but IGF1R is overexpressed in some cancer cell types (145). Anti-EGFR therapies are in clinical use for multiple cancer types, and early-phase trials with anti-IGF1R therapies are underway. Development of resistance is a significant challenge with growth factor receptor (GFR) targeted cancer therapies. Frequently, activation of Akt signaling occurs through upregulation of alternate upstream pathways (146). Significant crosstalk occurs between the EGFR and IGF1R pathways, and subsequently tumors can acquire resistance to EGFR-targeted therapeutics through compensatory upregulation of signaling through the IGF1R pathway (147). Combination therapies targeting both EGFR and IGF1R or therapies targeting both a growth factor receptor pathway and a signal downstream of the GFR may effect improved treatment outcomes. Further elucidation of the signaling mechanisms regulating growth factor signaling to Akt is warranted to provide potential molecular targets for cancer treatment.

Glycogen Synthase Kinase 3 (GSK3) functions in a variety of signaling pathways, regulating important cellular functions such as cell polarity and glucose metabolism. GSK3 is constitutively active. However, Akt, as well as multiple other kinases, inhibits GSK3 by

phosphorylation of its Ser 21/9 (GSK3 β /GSK3 α) residue. GSK3 has been proposed to mediate many downstream effects of Akt by phosphorylation of cyclin D1 (115), cyclin E (116), c-myc (117, 118), and p21 (119, 120).

We have discovered that GSK3 is also involved in signaling upstream of Akt. This finding is novel and without precedence in the literature. Using targeted siRNAs to knock down GSK3 expression, we show here that GSK3 mediates EGF- and IGF1-stimulated activation of Akt in the MDAMB231 breast cancer cell line and in mouse embryonic fibroblasts (MEFs). We also found GSK3 controls Akt phosphorylation independent of growth factor stimulation in the HER2-positive AU565 breast cancer cell line. These findings define GSK3 as a regulator of upstream signaling to Akt. Thus GSK3, similar to multiple other components of the PI3K pathway, mediates critical up and downstream events in the PI3K pathway.

4.2 Results

4.2.1 EGF- or IGF1-stimulated activation of Akt is dependent on GSK3 in the MDAMB231 breast cancer cell line.

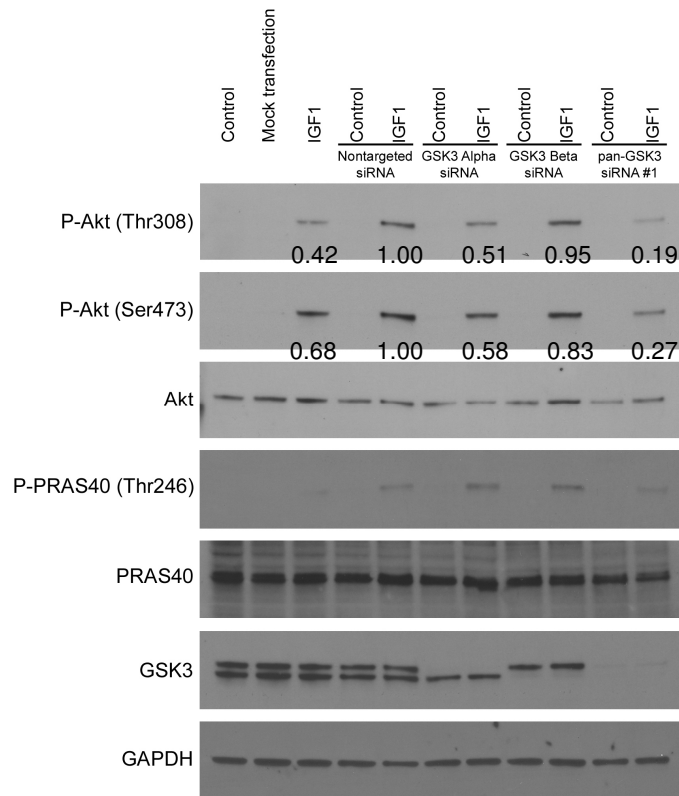
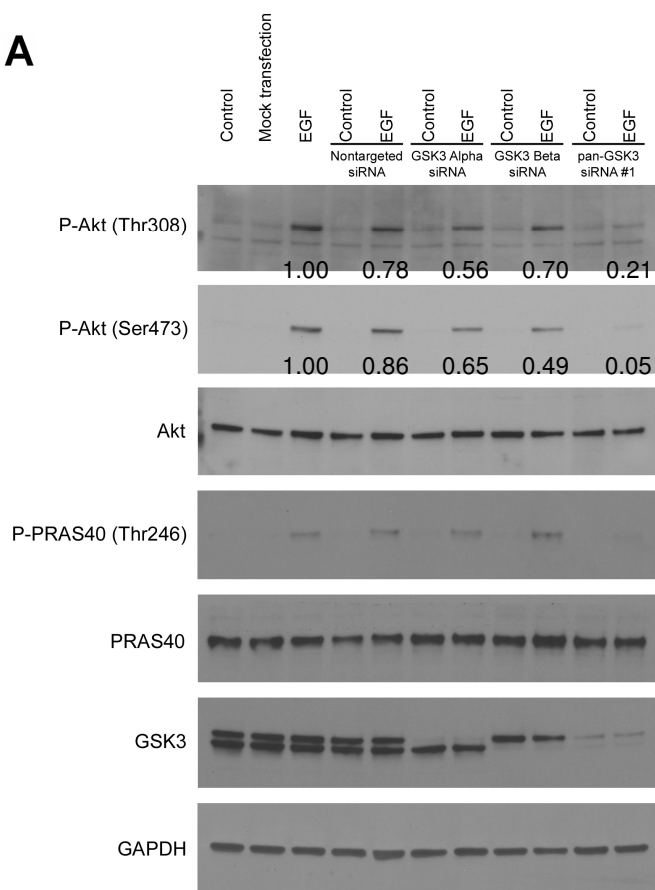
Activation of the PI3K/Akt pathway occurs in response to upstream activation of the EGF and IGF growth factor signaling systems. Tyrosine phosphorylation of the GFR recruits adaptor molecules to form a signaling complex at the cell membrane. Binding of the SH2 domain of the p85 regulatory subunit of PI3K to specific phosphotyrosine residues of

the GFR or linked adaptor molecules leads to activation of the p110 catalytic subunit of PI3K and subsequent activation of Akt. Downregulation of GFR signaling occurs through multiple mechanisms such as internalization and degradation of the growth factor receptor or dephosphorylation of GFR tyrosine residues by members of the protein tyrosine phosphatase (PTP) family.

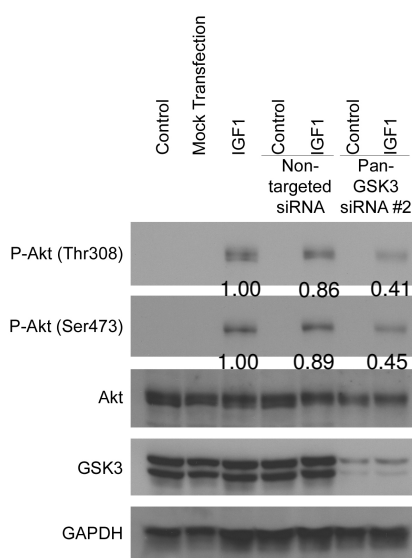
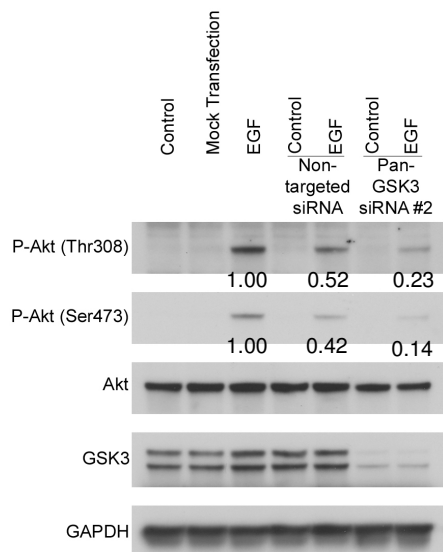
To investigate whether GSK3 plays a role in the signaling from growth factors to Akt, we determined the effects of GSK3 knockdown on the ability of EGF and IGF1 to stimulate Akt phosphorylation in the MDMB231 breast cancer cell line (Figure 4.1A). As seen in Figure 4.1A, knockdown of either the GSK3 α or GSK3 β isoforms with targeted siRNAs effected little or no change in the level of Akt phosphorylation induced by stimulation with either the EGF or IGF1 growth factor. Knockdown of expression of both isoforms of GSK3 resulted in significant reduction in EGF- and IGF1-induced Akt phosphorylation on both its Thr308 and Ser 473 activation sites (Figure 4.1A). Thus, GSK3 regulation of EGF and IGF1 signaling to Akt can be mediated by either isoform of GSK3. To verify that the decrease in growth factor stimulated Akt phosphorylation corresponds to a decrease in Akt activity, we determined the phosphorylation levels of the Akt substrate PRAS40. As seen in Figure 4.1A, we found decreased levels of phosphorylated PRAS40 (Thr246) in response to growth factor stimulation in cells in which the expression of GSK3 had been knocked down using siRNA. These results demonstrate that Akt phosphorylation and activation in response to the EGF and IGF1 growth factors is regulated by GSK3 in the MDAMB231 breast cancer cell line. GSK3 dependence of EGF- and IGF1-stimulated Akt phosphorylation was verified using an additional pan-GSK3 siRNA (Figure 4.1B).

Figure 4.1 GSK3 regulates EGF- and IGF1-stimulated Akt phosphorylation in the MDAMB231 breast cancer cell line. MDAMB231 cells were transfected with siRNA targeting GSK3 α , GSK3 β , or both isoforms of GSK3. Nontargeting siRNA was used as a control. Cells were serum-starved overnight and then treated with EGF (60 ng/mL) or IGF1 (50 ng/mL) for 20 minutes. Cell lysates were analyzed for Akt phosphorylation (Thr308 and Ser473), PRAS40 phosphorylation (Thr246), and GSK3 by immunoblotting. GAPDH was used as a loading control. B. An additional pan-GSK3 siRNA was tested for modulation of EGF- and IGF1-stimulated Akt phosphorylation. Cell lysates were analyzed for Akt phosphorylation (Thr308 and Ser473) and GSK3 by immunoblotting. GAPDH was used as a loading control.

A



B



4.2.2 Akt phosphorylation is dependent on GSK3 in the AU565 breast cancer cell line.

Since we discovered that EGF- and IGF1-stimulated activation of Akt is dependent on GSK3 in the MDAMB213 breast cancer cell line, we screened additional breast cancer cell lines to determine if GSK3 also regulates upstream signaling to Akt in these lines. Figure 4.2 shows that knockdown of GSK3 with targeted siRNA decreased Akt phosphorylation on both the Thr308 and Ser473 activation sites in the AU565 breast cancer cell line. Akt phosphorylation in AU565 cells is not responsive to IGF1 stimulation. AU565 cells express high levels of HER2 and have basally high levels of phosphorylation of Akt. As shown in Figure 4.2, GSK3 knockdown significantly decreased this basal level of Akt phosphorylation. The finding that GSK3 is required for basal Akt phosphorylation in the AU565 cell line demonstrates that GSK3 mediates signaling to Akt in multiple cell systems. As in the MDAMB231 cell line, the GSK3 α and GSK3 β isoforms function redundantly in controlling the phosphorylation of Akt, with significant decreases in Akt phosphorylation seen only when expression of both GSK3 isoforms is knocked down. GSK3 knockdown with targeted siRNA did not significantly decrease EGF- and IGF1-stimulated Akt phosphorylation in the MDAMB415, MCF7, or T47D breast cancer cell lines (data not presented). Taken together, these results indicate that the dependence on GSK3 of upstream signaling to Akt is cell context dependent or alternatively, that residual GSK3 present in these cells is sufficient to mediate signaling to Akt.

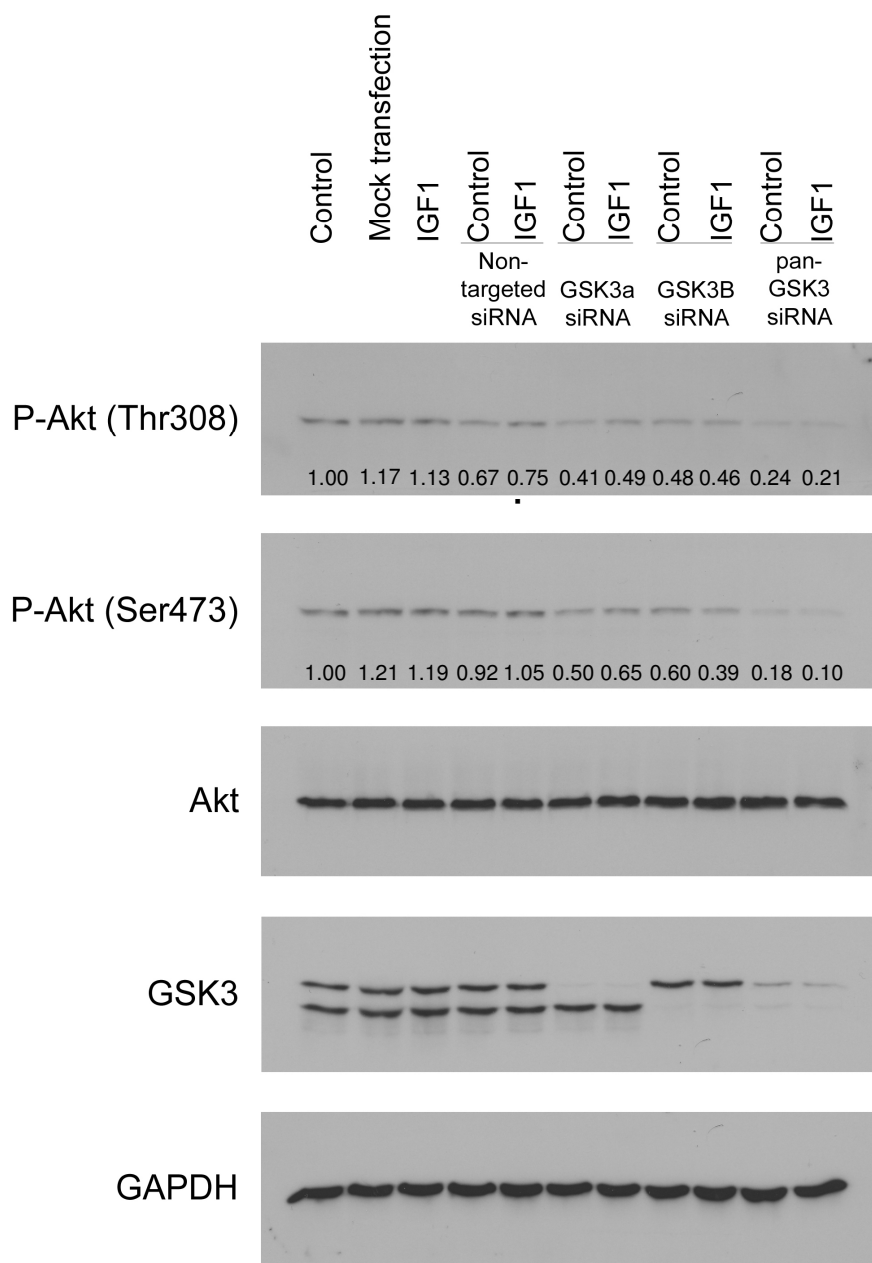


Figure 4.2 GSK3 regulates Akt phosphorylation in the AU565 breast cancer cell line. AU565 cells were transfected with siRNA targeting GSK3 α , GSK3 β , or both isoforms of GSK3. Nontargeting siRNA was used as a control. Cells were serum-starved overnight and then treated with IGF1 (50 ng/mL) for 20 minutes. Cell lysates were analyzed for Akt phosphorylation (Thr308 and Ser473), Akt, and GSK3 by immunoblotting. GAPDH was used as a loading control.

4.2.3 GSK3 regulates EGF and IGF1 signaling to Akt in Mouse Embryonic Fibroblasts (MEFs).

To further assess the role of GSK3 in optimal growth factor signaling to Akt in another mammalian system, we investigated EGF and IGF1 signaling in mouse embryonic fibroblasts (MEFs). We first stimulated wildtype and GSK3 knockout MEFs with IGF1 and determined effects on Akt phosphorylation by immunoblotting. Figure 4.3A illustrates no effects of loss of individual GSK3 isoforms on IGF1-stimulated phosphorylation of Akt.

Since in the MDAMB231 and AU565 cancer cell lines knockdown of the expression of both GSK3 isoforms was required to prevent Akt phosphorylation, we then investigated whether decreased expression of both GSK3 isoforms decreases growth factor induced Akt phosphorylation in MEFs. We utilized targeted siRNA to knock down expression of GSK3 α in GSK3 β knockout MEFs and evaluated Akt phosphorylation in response to stimulation of cells with EGF or IGF1. Figure 4.3B shows that decreased expression of both the GSK3 α and GSK3 β isoforms results in reduced ability of EGF to stimulate phosphorylation of Akt. A modest decrease in IGF1-stimulated Akt phosphorylation was also observed (Figure 4.3B). These results demonstrate that GSK3 regulates phosphorylation of Akt in MEFs induced by EGF and to a lesser degree, by IGF1.

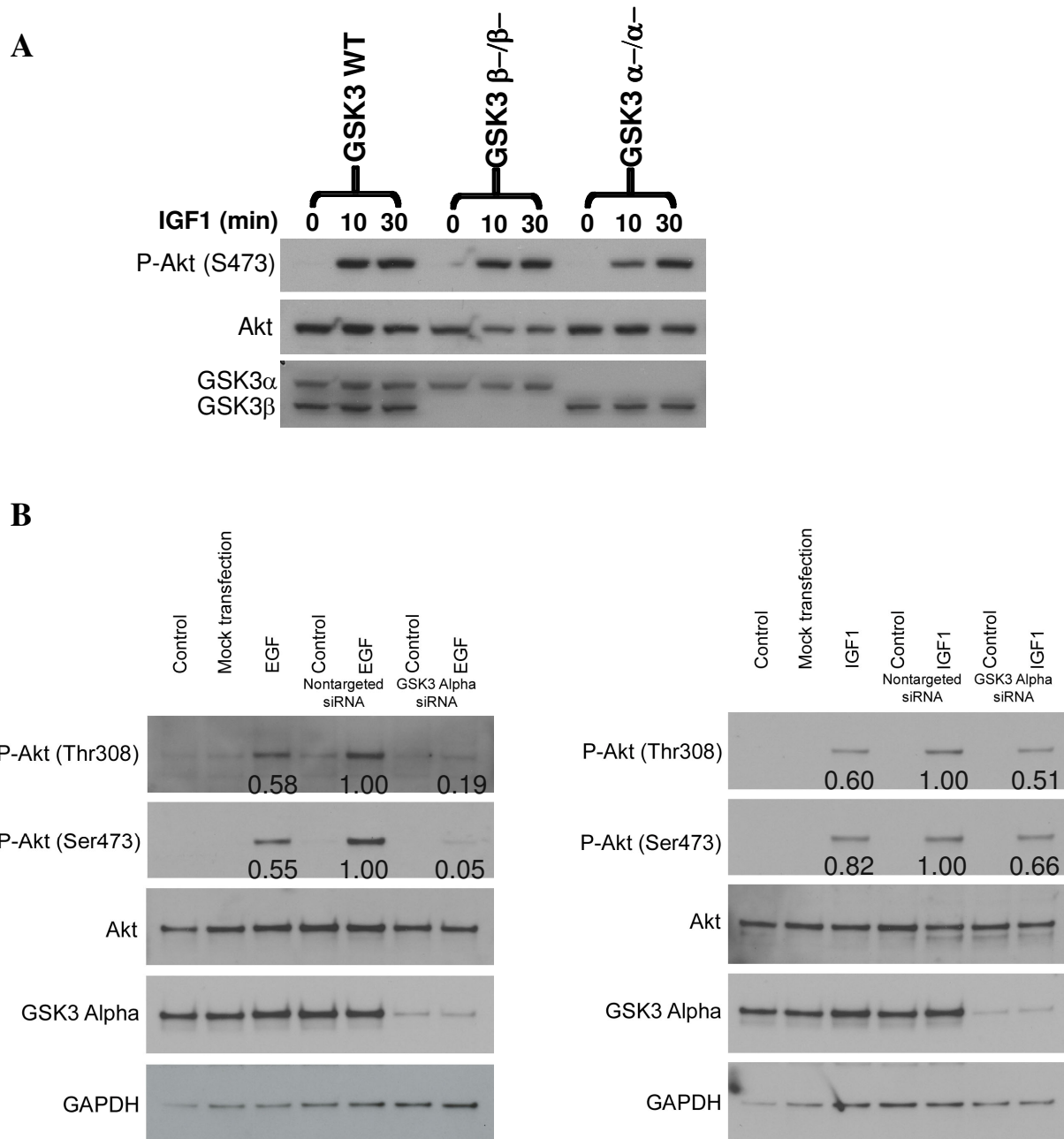


Figure 4.3 GSK3 mediates EGF and IGF1 signaling to Akt in Mouse Embryonic Fibroblasts (MEFs). A. Wildtype mouse embryonic fibroblasts (MEFs) and GSK3 β ^{-/-} and GSK3 α ^{-/-} MEFs knockouts were serum-starved overnight before stimulation with IGF1 for the indicated times. Cell lysates were analyzed for Akt phosphorylation (Ser473) and GSK3 by immunoblotting. B. GSK3 β knockout MEFs were transfected with GSK3 α siRNA or control nontargeting siRNA. 48 hours after transfection cells were serum-starved overnight and then stimulated with EGF (60 ng/mL) or IGF1 (50 ng/mL). Cell lysates were analyzed for Akt, Akt phosphorylation (Thr308 and Ser473), and GSK3 α . GAPDH was used as a loading control.

4.2.4 Akt activation by EGF and IGF1 is independent of acute signaling effects of GSK3 kinase activity.

GSK3 performs many of its signaling functions through substrate phosphorylation. To determine if GSK3 regulation of growth factor signaling to Akt is mediated by GSK3 kinase function, we chemically inhibited GSK3 kinase activity and measured Akt phosphorylation levels after stimulation of MDAMB231 cells with either EGF or IGF1. As seen in Figure 4.4, pretreatment of cells with GSK3 inhibitor SB216763 did not prevent EGF- or IGF1-induced Akt phosphorylation, indicating independence of Akt activation on acute effects of GSK3 kinase activity.

Since GSK3 regulation of Akt phosphorylation is not dependent on the acutely mediated effects of the kinase activity, remaining potential mechanisms of GSK3 regulation of upstream signaling to Akt include 1) non-kinase effects of GSK3 (protein binding-mediated interactions) and 2) signaling downstream of GSK3 kinase activity that are mediated over a longer timescale than we investigated in these studies.

4.2.5 GSK3 knockdown induces multiple changes in cell signaling.

To determine the effects of GSK3 on EGF and IGF1 signaling to a broad range of signaling proteins, we knocked down expression of GSK3 using targeted siRNA in MDAMB231 cells, stimulated cells with EGF or IGF1, and analyzed cell lysates by reverse

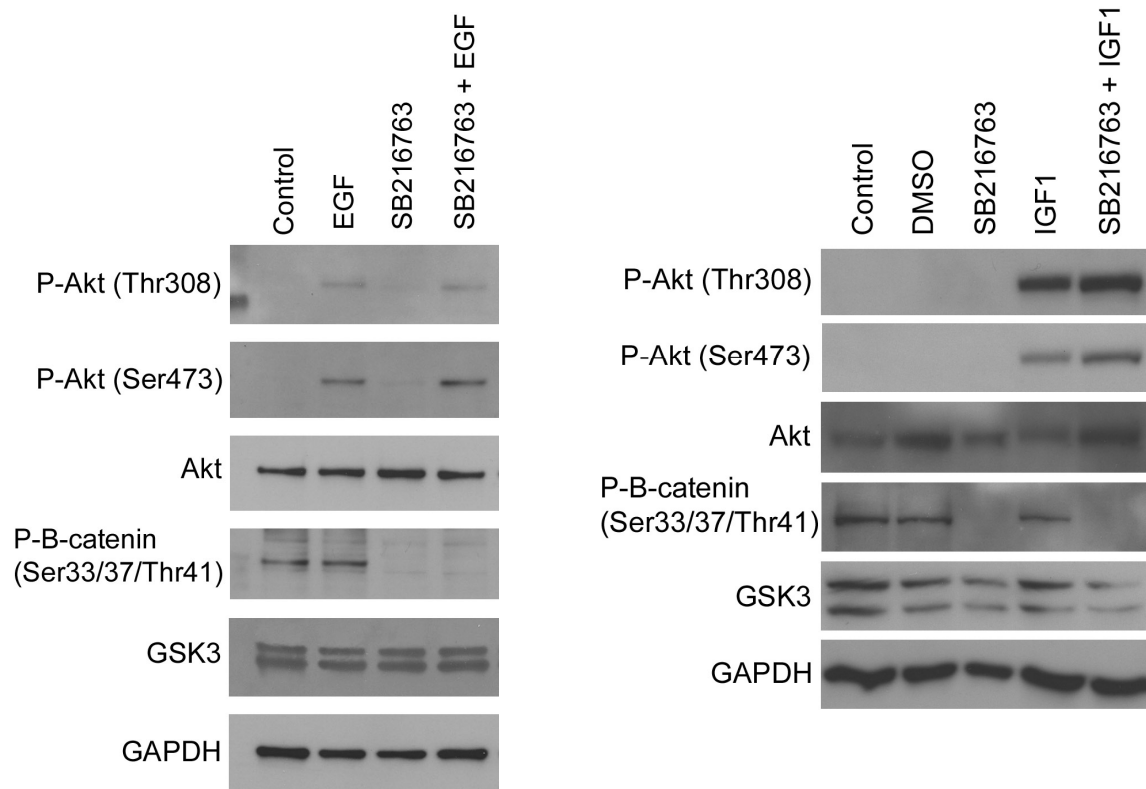


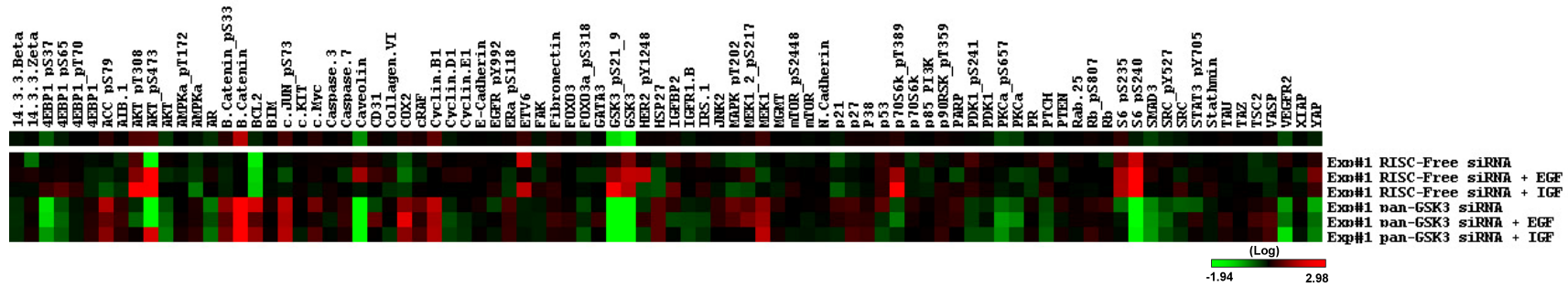
Figure 4.4 EGF and IGF1-stimulated Akt phosphorylation are not dependent on acute effects of GSK3 kinase activity. MDAM231 cells were serum-starved overnight before treatments with SB216763 (10 μ M) and either EGF (60 ng/mL) or IGF1 (50 ng/mL). Cells were treated for 1 hour with SB216763, 20 minutes with EGF or IGF1, or 1 hour with SB216763 followed by 20 minutes stimulation with EGF or IGF1. Cell lysates were analyzed for Akt, phospho-Akt (Thr308 and Ser473), phospho-B-catenin (Ser33/37/Thr41), and GSK3. GAPDH was used as a loading control.

phase protein array (RPPA) to measure the levels of 88 proteins and phospho-proteins in the PI3K pathway and other cell signaling pathways. Figure 4.5 illustrates the cell signaling profiles for the GSK3 knockdown experiments (results of two independent experiments are displayed). Among the 88 signals measured, there are marked differences between the two independent experiments for some proteins and phosphoproteins (i.e. Bcl-, ERK2, Fibronectin, PCNA, Stat3, YAP, and others), which may reflect differences in cell culture conditions or differences in handling of the lysate samples. Retesting of signaling changes of these proteins is warranted. However, both experiments demonstrated effective knockdown of GSK3 and expected concomitant decrease in EGF- and IGF1-induced Akt phosphorylation (illustrated in Figure 4.6 for Experiment #1).

GSK3 knockdown induced numerous additional cell signaling changes. Excluding GSK3, phospho-GSK3, and phospho-Akt, the changes of greatest magnitude (≥ 2 -fold change compared to appropriate control) are listed in Figure 4.7. Signals exhibiting at least a two-fold decrease with GSK3 knockdown for conditions of EGF stimulation, IGF1 stimulation, and no growth factor stimulation include total caveolin-1 levels and phosphorylation (activation) of p70S6K pathway proteins. β -catenin level was the only signal demonstrating at least a two-fold increase with GSK3 knockdown for all growth factor stimulation conditions. YAP protein levels were also changed more than two-fold with GSK3 knockdown for all growth factor stimulation conditions, but care must be taken in interpreting the significance of the YAP signal changes as the changes were in opposite directions for the two experiments (decreased YAP expression in Experiment #1 and increased YAP expression in Experiment #2). Additional 2-fold changes were seen with GSK3 knockdown in one or two of the three growth factor stimulation conditions. Bcl-2

levels were changed more than 2-fold for conditions of EGF stimulation and no growth factor stimulation. Changes in Bcl-2 levels with GSK3 knockdown were in opposite directions for the two independent experiments (increased Bcl-2 expression in Experiment #1 and decreased Bcl-2 expression in Experiment #2). GSK3 knockdown decreased expression of Smad3 and VEGFR2 more than 2-fold under the condition of no growth factor stimulation. Increased expression of Cox-2 with GSK3 knockdown was seen for the EGF-stimulated condition, and increased level of phospho-ACC (Ser79) was seen for the IGF1-stimulated condition. Changes for all signals induced by GSK3 knockdown under conditions of EGF-stimulation, IGF1-stimulation, and no growth factor stimulation (averages and values from each of the two independent experiments) are presented in Tables 1, 2, and 3 (found in the Appendix section), respectively.

Experiment #1



Experiment #2

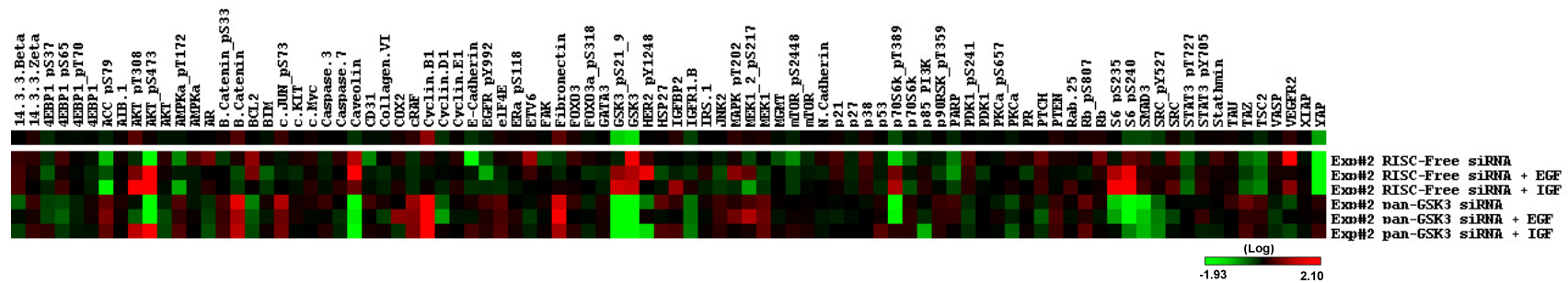
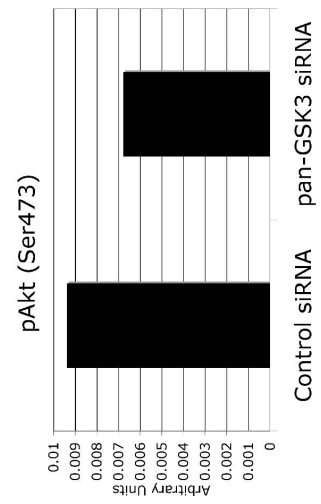
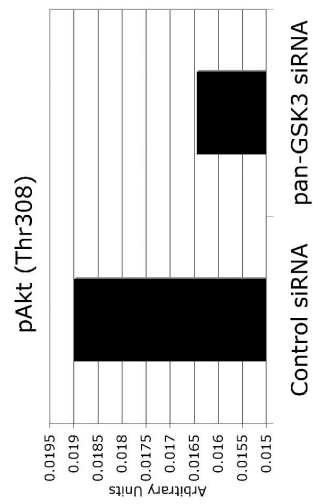
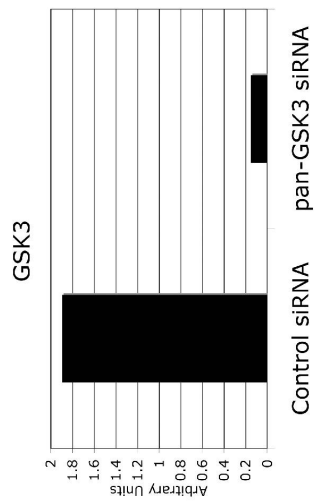


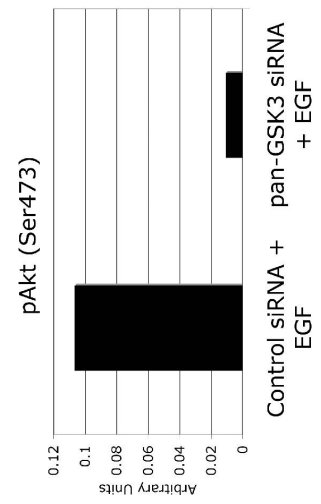
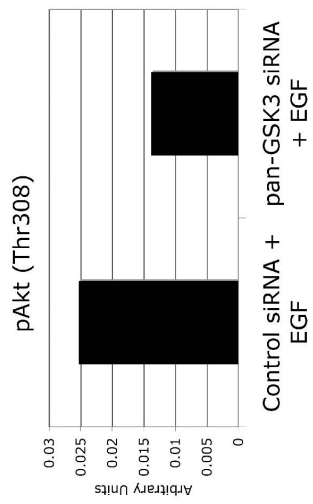
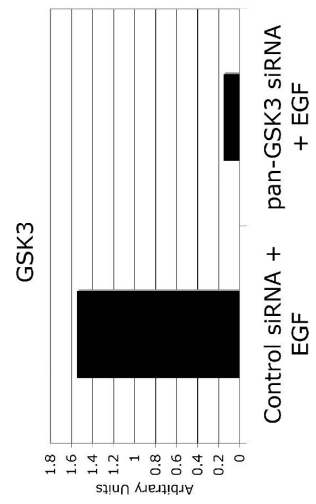
Figure 4.5 GSK3 knockdown induces multiple cell signaling changes. MDAMB231 cells were transfected with either pan-GSK3 siRNA or control nontargeting siRNA. Cells were stimulated for 20 minutes with either EGF (60 ng/mL), IGF1 (100 ng/mL), or no growth factor. Cell lysates were processed on a reverse phase protein array (RPPA) and stained for antibodies to each of the indicated proteins and phospho-proteins. RPPA procedure and initial data analysis performed by the Functional Proteomics Reverse Phase Protein Array Core Facility.

Figure 4.6 Effective knockdown of GSK3 and decrease of Akt phosphorylation with GSK3 siRNA transfection. Normalized linear values from RPPA analysis (Experiment #1) are depicted for Akt phosphorylation (Thr308 and Ser473) and GSK3.

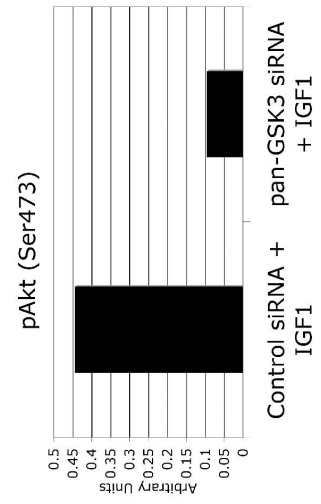
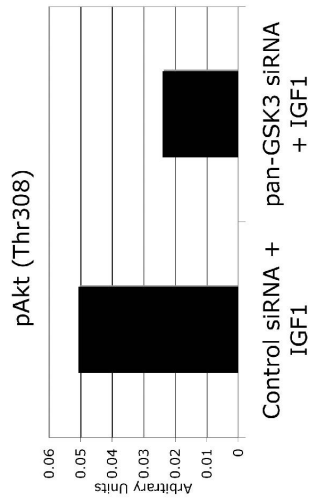
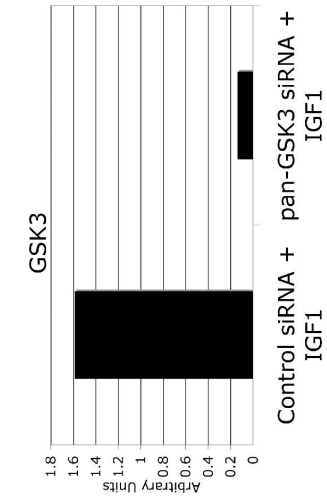
No Growth Factor



EGF Stimulation



IGF1 Stimulation



No Growth Factor Stimulation

Antibody	Average Fold Change (Exp #1 Fold Change, Exp #2 Fold Change)
B-catenin*	2.3 (2.9, 1.7)
Bcl-2*	±2.8 (3.4, -2.2)
Caveolin-1*	-2.9 (-2.3, -3.5)
P-S6RP (S240/S244)*	-5.3 (-4.7, -5.8)
Smad3*	-2.0 (-1.5, -2.4)
VEGFR2*	-2.2 (-1.7, -2.7)
YAP*	±2.4 (-1.7, 3.0)

EGF Stimulation

Antibody	Average Fold Change (Exp #1 Fold Change, Exp #2 Fold Change)
B-catenin*	2.4 (2.8, 2.0)
Bcl-2*	±2.0 (2.2, -1.8)
Caveolin-1*	-4.0 (-4.0, -4.0)
Cox-2**	2.0 (2.5, 1.5)
P-p70S6K (T389)*	-2.4 (-2.1, -2.8)
P-S6RP (S235/S236)*	-2.1 (-1.7, -2.6)
P-S6RP (S240/S244)*	-6.1 (-5.2, -7.0)
YAP*	±2.3 (-1.8, 2.8)

IGF1 Stimulation

Antibody	Average Fold Change (Exp #1 Fold Change, Exp #2 Fold Change)
P-ACC (S79)*	2.2 (1.8, 2.6)
B-catenin*	2.2 (2.4, 2.0)
Caveolin-1*	-3.2 (-2.8, -3.6)
P-S6RP (S240/S244)*	-4.0 (-4.2, -3.8)
YAP*	±2.6 (-1.6, 3.7)

Figure 4.7 RPPA antibody signals showing at least 2-fold change in response to GSK3 knockdown in MDAMB231 cells. Given fold change values are averaged from the two independent experiments, and fold change values from each individual experiment are included in parentheses. “Validated” antibodies, indicated by the “*” symbol, demonstrate performance for RPPA comparable to an ELISA assay. “Use with Caution” antibodies, indicated by the “**” symbol, provide high-quality useful information considered to need confirmation by additional technology.

4.3 Discussion

We have demonstrated here that GSK3 is required for optimal signaling upstream of Akt. While knockdown of GSK3 in human and MEF cells markedly decreased phosphorylation of Akt, this effect was not recapitulated by inhibition of GSK3 kinase activity. Thus either GSK3 functions as a linker molecule or long term inhibition of GSK3 kinase activity is required to reveal the function of GSK3 upstream of Akt.

GSK3 does not regulate signaling to Akt in all breast cancer cell lines. Differences in genetic background may play a role in whether or not Akt signaling will be sensitive to regulation by GSK3. The MDAMB231 breast cancer cell line that has CDKN2, p53, NF2, Ras and Raf mutation and the AU565 cell line that exhibits HER2 amplification and PTEN and p53 mutations demonstrated dependence on a GSK3 regulated event for optimal signaling to Akt. MDAMB415 that has a p53 mutation, MCF7 that has a CDKN2 and a helical domain PIK3CA mutation, and T47D that has catalytic domain PIK3CA and p53 mutations did not demonstrate dependence on GSK3 of Akt phosphorylation. The three cell tested that did not exhibit dependence on GSK3 of Akt phosphorylation (MDAMB415, MCF7, and T47D) all overexpress the estrogen receptor, which has been shown to activate Akt (148), and may be the major driver of signaling to Akt in these lines. Further investigation is required to characterize the cell context dependence of GSK3 regulation of signaling to Akt.

The RPPA approach allowed a screen of 88 proteins and phospho-proteins for signaling changes in response to GSK3 knockdown. We identified a series of proteins that showed marked changes in expression or phosphorylation status in response to GSK3 knockdown. Known mechanisms of modulation by GSK3 of these proteins and known mechanisms for each protein in the regulation of Akt phosphorylation are described below.

Caveolin-1:

Caveolin is the primary structural component of caveolae, which are plasma membrane microdomains containing large amounts of cholesterol and sphingolipids. Caveolins contain a scaffolding domain which binds and concentrates a number of signaling molecules including receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). No known mechanism of GSK3 modulation of caveolin-1 expression is reported in the literature. A significant body of literature does link expression of caveolin-1 to Akt activation. Caveolin-1 inhibits PP2A and PP1, preventing these phosphatases from dephosphorylation and inactivating Akt (149). In addition to inhibiting inactivation of Akt by phosphatases, caveolin-1 is colocalized with IGF1R (150) and augments the activation of Akt through IGF1 signaling. IGF1-stimulated phosphorylation of Akt has been shown to be reduced with knockdown of caveolin-1 in PC12 cells (150) and in an MCF-7-derived cell line (151). Similarly, Matthews et al. showed that IGF1-stimulated Akt phosphorylation was reduced in caveolin-1 knockout MEFs (152). Caveolin-1 also increased EGF-stimulated Akt phosphorylation in the MCF7 breast cancer cell line (153). Thus caveolin-1 is a strong candidate to explain the effects of GSK3 knockdown on Akt signaling.

p70S6K pathway:

GSK3 is known to inhibit the mTOR/p70S6K pathway through phosphorylation of TSC2 (112). Based on this connectivity, we might have expected knockdown of GSK3 to result in an increase in phosphorylation of p70S6K and subsequent increase in Akt phosphorylation through IRS1-mediated feedback. However, our experimental results show decreased phosphorylation of p70S6K and its substrate, S6 Ribosomal Protein (S6RP). No published studies have proposed signaling mechanisms that would decrease phosphorylation of p70S6K and S6RP in response to GSK3 knockdown, and decreased phosphorylation of these proteins is not known to reduce Akt phosphorylation. Thus it is likely that the effect of GSK3 knockdown on these proteins is through inhibition of Akt activity.

β -catenin and Smad3:

GSK3 phosphorylation of β -catenin and Smad3 targets these proteins for degradation (111, 154). Consequently, decreased expression of GSK3 results in accumulation of β -catenin and Smad3, as seen in our RPPA results. There are no known mechanisms for modulation of Akt phosphorylation by β -catenin and Smad3.

YAP:

Akt and other kinases have been reported to phosphorylate YAP on its Ser127 residue, promoting YAP binding to 14-3-3 and cytoplasmic retention of the YAP/14-3-3 complex (155, 156). However, Akt is not reported to regulate expression levels of YAP. YAP is not known to regulate signaling upstream of Akt, but YAP does interact with membrane proteins including NHERF family members and HER4 (156), which are known

to signal upstream of Akt. GSK3 knockdown was associated with markedly decreased expression of YAP in Experiment #1 and dramatically increased YAP expression in Experiment #2. Further experiments are needed to determine the significance of the changes in YAP expression in the two experiments. YAP protein levels are known to be cell density-dependent (157), and this may account for some of the differences seen between the two experiments.

Bcl-2:

GSK3 regulates function of NFkB and is known to affect transcription of NFkB target genes, including Bcl-2 (158, 159). Based on both this known regulation of Bcl-2 expression by GSK3 and the known regulation of Bcl-2 by Akt (160), we expect knockdown of GSK3 to result in the decreased Bcl-2 expression seen in Experiment #2. Further repeats of the experiment are needed to determine the significance of the unexpected and marked increase of Bcl-2 expression seen with GSK3 knockdown in Experiment #1. Bcl-2 has recently been implicated in upstream regulation of Akt in a pancreatic cancer cell line (161), and thus could potentially mediate GSK3 regulation of upstream Akt signaling.

VEGFR2:

VEGFR2 is a tyrosine kinase-containing member of the VEGFR family of receptors. Although studied mainly in the context of neovascularization, VEGF receptors are expressed on cancer cells. Caution needs to be used in interpretation of the VEGFR2 results as although the antibody is reported to be specific by the manufacturer, others in our laboratory have not been able to confirm this result and it likely interacts with multiple VEGFR

isoforms. Downregulation of VEGFR2 by GSK3 has not been noted previously. Further, none of the known effects of GSK3 would be predicted to decrease VEGFR2 levels. On the contrary, GSK3 β has been shown to decrease ability of kallikrein, a peptidase important in vasodilation and angiogenesis, to induce VEGFR2 mRNA expression levels in myocardial cells (162). VEGFR2 is a known upstream activator of Akt signaling (163), and thus, decreased VEGFR2 expression with GSK3 knockdown may result in concomitant decrease in Akt phosphorylation. Further evaluation of VEGFR2 as a potential intermediate in GSK3 signaling upstream of Akt is warranted.

Cox-2:

Cox-2 catalyzes the formation of pro-inflammatory prostaglandins. GSK3 is not known to modulate Cox-2 expression, and Cox-2 is not known to inhibit Akt phosphorylation. On the contrary, the inhibition of Cox-2 has been reported to inhibit phosphorylation of Akt (164, 165).

P-ACC (Ser79):

Phosphorylation of acetyl-CoA carboxylase (ACC), an important enzyme in fatty acid synthesis, on its Ser79 residue by AMPK inhibits ACC activity. GSK3 is not known to regulate ACC phosphorylation, and ACC activity is not known to regulate Akt phosphorylation. However, there is marked crosstalk between the Akt and AMPK pathway through the ability of Akt to upregulate glucose uptake and bioenergetics. Indeed, the increase in pACC levels is likely to reflect this effect in the MDAMB231 cells.

Decreased expression of caveolin-1 associated with GSK3 knockdown for all growth factor conditions tested provides a potential mechanism for GSK3 regulation of Akt phosphorylation. Alternatively, GSK3 regulation of Akt could be mediated by proteins exhibiting less than a two-fold change in signal intensity with GSK3 knockdown, proteins not currently known to affect Akt, or proteins not assayed in this experiment. Additional studies are required to elucidate in further detail the mechanism of GSK3 regulation of Akt phosphorylation.

Further studies elucidating the molecular events in GSK3 regulation of signaling upstream of Akt will define novel signaling interactions in the PI3K/Akt pathway and may identify potential molecular targets for anti-cancer therapy.

CHAPTER 5: DISCUSSION

In this work we have described a novel role for GSK3 in signaling upstream of Akt. Specifically, we have demonstrated that GSK3 functions in the pathways of signaling to Akt mediated by three separate ligands – Akt catalytic domain inhibitors, epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF1).

5.1 Models of Akt Catalytic Domain Inhibitor-induced Akt Phosphorylation

Currently two models have been described in the literature that have the potential to explain the increase in Akt phosphorylation that occurs with treatment of Akt catalytic domain inhibitors. In the first model, phosphorylation of Akt induced by Akt catalytic domain inhibitors is independent of decreased Akt substrate phosphorylation. In this model, inhibitor binding alters the interaction of the catalytic domain with other regions of Akt, resulting in stronger affinity for PIP3 of the Akt PH domain and increased susceptibility of the Thr308 and Ser473 sites to phosphorylation and/or decreased susceptibility to dephosphorylation by phosphatases (137). In the second model, upregulated phosphorylation of Akt results from disruption of a homeostatic negative feedback signaling loop that adjusts upstream signaling to Akt based on level of Akt activity towards its downstream substrates (93). The two models of increased Akt phosphorylation of Akt induced by Akt catalytic domain inhibitors are illustrated in Figure 5.1.

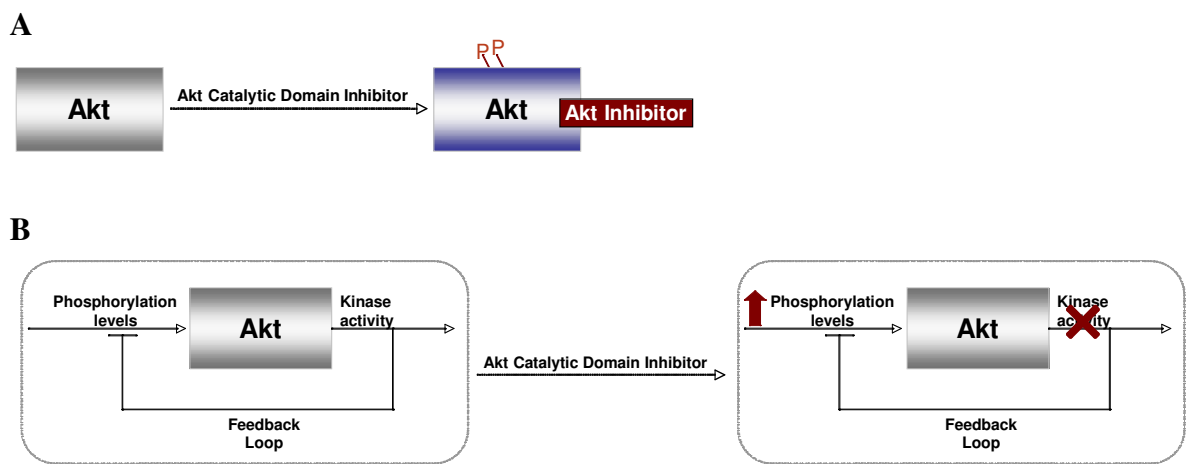


Figure 5.1. Two possible mechanisms of induction of Akt phosphorylation with catalytic domain Akt inhibitors. A. Pathway-independent model of intrinsic Akt kinase alteration by inhibitor binding to Akt catalytic domain. B. Feedback signaling model of Akt inhibitor-induced Akt phosphorylation.

The pathway-independent model of Akt inhibitor-induced Akt phosphorylation is based on studies of a mutant Akt, *asAkt* (analog sensitive Akt), with a modified ATP-binding pocket. To abolish any off-target signaling effects of the Akt inhibitor, an A443654-derived catalytic domain inhibitor, PrINZ, was developed that selectively binds to the *asAkt* mutant (137). Treatment of cells with PrINZ induces phosphorylation of Thr308 and Ser473 sites of *asAkt* mutant molecules but not wildtype Akt. This finding argues against an activation of signaling upstream of Akt, which would likely lead to phosphorylation of both endogenous Akt and *asAkt*.

However, we have provided evidence in this work of stimulation by A674563 of signaling upstream of Akt. Specifically, we demonstrated increased tyrosine phosphorylation of a p85-associated protein in response to cell treatment with A674563. The identity of this p85-associated tyrosine-phosphorylated protein has not yet been determined. Potential candidates, listed in Table 1, include proteins containing a p85-binding motif (p-YxxM) in the 90-110 kDa molecular weight range. The most likely candidates for identity of this protein will be determined by mass spectrometry analysis of the p-85-associated tyrosine phosphoprotein Western blot band that is induced by A674563 treatment. We have not yet investigated whether induction of p85-associated tyrosine phosphorylation is driving the increase in Akt phosphorylation, this but induction of tyrosine phosphorylation clearly shows that Akt catalytic domain inhibitors induce signaling changes other than alteration of Akt intramolecular structure. Dependence of Akt inhibitor-induced Akt phosphorylation on physiologic upstream regulators of Akt as reported in the literature and demonstrated in our work is consistent with both the kinase intrinsic and pathway-dependent models.

Table 4. List of 90-110 kDa proteins containing the p85-binding motif sequence. Candidate proteins were identified using the “Site Search” advanced search interface of PhosphoSitePlus (www.phosphosite.org), a manually curated phosphorylation site database. Protein abbreviations are included in the List of Abbreviations on pages xv-xix.

Protein	p85-Binding Motif Tyrosine Phosphosite	p85-Binding Motif Sequence
AFAP-110	Y54	SSSSDEE y YMNKVT
AP1G1	Y55	RNVAKLL y MHMLGYP
AP2B1	Y276	LLPKDSD y YNMLLKK
	Y737	THRQGH y MEMNFTN
AR	Y534	MDSYSGP y GDMRLET
Axl	Y779	ADCLDGL y ALMSRCW
	Y821	QEPDEIL y VNMDEGG
BCAP	Y263	CETVIS y YTDMEIEG
	Y419	GEEADAV y ESMAHLS
	Y444	PGCDED y ESMAAFV
	Y459	PAATEDL y VEMLQAS
Cbl	Y371	TQEQYEL y CEMGSTF
	Y731	QQIDSCT y EAMYNIQ
Cbl-b	Y363	TQEQYEL y CEMGSTF
CNNM2	Y713	EASAFS y YGVMALTA
CSFR	Y723	SSQGVDT y VEMRPVS
CTNND1	Y296	MGYDDL y GMMSDYG
	Y321	PRRRLRS y EDMIGEE
DDR1	Y881	PACPQGL y ELMLRCW
EML4	Y226	IINQEGE y IKMFMRG
EphA2	Y628	AGEFGEV y KGMLKTS
ESYT2	Y824	SEDGSDP y VRMYLLP
FGFR1	Y730	SNCTNEL y MMMRDCW
Kit	Y721	CSDSTNE y MDMKPGV
	Y900	EHAPAEM y DIMKTCW
Kv7.2	Y562	FKESLRP y DVMDVIE
Matrin 3	Y214	RSQESGY y DRMDYED
	Y250	YHKFDSE y ERMGRGP
	Y526	PYGKIKN y ILMRMKS
MuSK	Y554	RLHPNPM y QRMPLLL
PDE2A	Y754	DHFSRKD y QRMLDLM
PMS2	Y181	QRNIKKE y AKMVQVL
PTPN3	Y324	EKNVLSQ y WTMGSRN
RBM10	Y36	NRSRDHD y RDMDYRS
RTF	Y163	ESDSLDD y SCMQRLG
Sec5	Y113	VWVDEM y YDMRTDR
SLC4A1	Y8	MEELQDD y EDMMEEN
SLITRK3	Y213	NRLKVL y RGMLDHI
TAO3	Y305	RELDNLQ y RKMKKIL
TAP	Y655	HDNIVTH y KNMIREQ
TLR5	Y798	VVGSLSQ y QLMKHQS
TrkC	Y800	RVCPKEV y DVMLGCW
USP13	Y63	PNSEGGL y VCMNTFL

5.2 GSK3 Mediation of Akt Inhibitor-induced Akt Phosphorylation

This work demonstrates dependence on GSK3 of Akt inhibitor-induced Akt phosphorylation. Knockdown of GSK3 expression prevented A674563-induced phosphorylation of Akt in the MDAMB231 breast cancer cell line and in mouse embryonic fibroblasts. However, GSK3 dependence of Akt-inhibitor induced Akt phosphorylation was not tested in other cell lines so it is unclear whether GSK3 is required for the increased phosphorylation of Akt in the multiple cell lines where we and others have demonstrated that Akt inhibitors increase Akt phosphorylation. Knockdown of both the GSK3 α and GSK3 β isoforms is required for significant decrease of Akt inhibitor-induced Akt phosphorylation, indicating redundancy of the two isoforms in mediating this signaling function.

Requirement of GSK3 for induction of Akt phosphorylation indicates that GSK3 positively regulates an activator of Akt phosphorylation or negatively regulates a downregulator of Akt phosphorylation. Possible proteins GSK3 may modulate include regulatory binding partners of Akt or one of the numerous kinases or phosphatases in the PI3K/Akt signaling network. As demonstrated in Chapter 4, knockdown of GSK3 alters levels and phosphorylation of many different proteins. Thus, knockdown of GSK3 modulates functions of many proteins and deconvoluting those that are required for the effect of GSK3 knockdown is likely to prove challenging. The effect of complete knockdown of GSK3 on multiple signaling pathways is not surprising given that concurrent knockout of both GSK3 α and GSK3 β is embryonic lethal.

Since GSK3 functions as both a kinase and protein binding partner (linker molecule), effects of GSK3 on Akt phosphorylation may be mediated by either the kinase or linker molecule function of GSK3. Our studies showed no effect on A674563-induced Akt phosphorylation of preincubation of cells with GSK3 kinase inhibitor, indicating GSK3 kinase function is not required to mediate the phosphorylation of Akt. However, in addition to mediating rapid phosphorylation/dephosphorylation signaling cascades, GSK3 phosphorylates several proteins that modify activity of transcription factors, resulting in further changes in cell signaling after a period of several hours required to alter target gene expression. The observed abrogation of A674563-induced Akt phosphorylation with knockdown of GSK3 expression for several days may result from either the physical linker function of GSK3 or from chronic loss of GSK3 kinase activity.

GSK3 is a known substrate of Akt, with Akt inactivating the constitutive GSK3 kinase through serine phosphorylation. GSK3 mediation of Akt inhibitor-induced phosphorylation of Akt may result from two separate signaling functions of GSK3 upstream and downstream of Akt. Alternatively, GSK3 may serve as a downstream sensor of Akt kinase activity and mediate a feedback signaling loop to regulate upstream phosphorylation of Akt. The model of GSK3 as a sensor of Akt activity provides a potential mechanism of Akt inhibitor-induced Akt phosphorylation. In this model, inhibition of Akt decreases phosphorylation of GSK3, leading to accumulation of unphosphorylated GSK3, which upregulates upstream signaling to Akt. This potential mechanism is illustrated in Figure 5.2. Other potential downstream sensors of the proposed feedback loop include any of the many

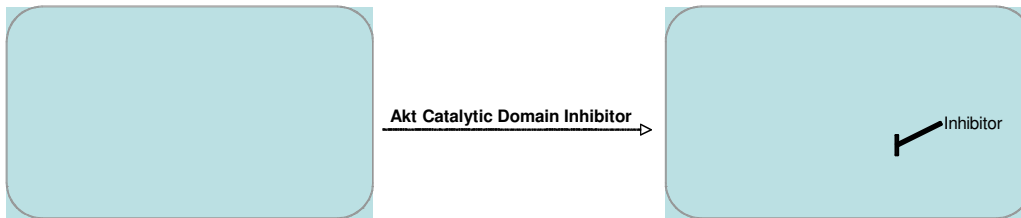


Figure 5.2. Potential mechanism of Akt feedback signaling through GSK3. Inhibition of Akt kinase activity leads to accumulation of unphosphorylated GSK3, which increases upstream signaling to increase levels of phosphorylated Akt. Increased font size indicates increased amount of molecule. Increased arrow thickness indicates greater signaling intensity.

proteins downstream of Akt. We have tested for mediation of Akt inhibitor-induced feedback signaling by the Akt downstream signaling kinases JNK1/2 (data not presented) and by the Akt downstream signaling protein complex mTORC1.

5.3 GSK3 Regulation of Signaling from EGF and IGF1 to Akt

In addition to GSK3 playing a role in Akt-inhibitor induced Akt phosphorylation, we have discovered a role of GSK3 in regulating stimulation of Akt in response to the endogenous growth factors EGF and IGF1. The general pathway of signaling from EGF and IGF1 to Akt is known. EGF or IGF1 activate their respective tyrosine kinase receptors, leading to binding of signaling proteins to receptor phosphotyrosine residues. Participation of the regulatory subunit of PI3K in protein complexes (EGFR-Grb2-Gab1-PI3K and IGF1R-IRS-PI3K) formed by the growth factor receptor and adaptor molecules results in activation of PI3K and downstream Akt (166, 167). EGFR also heterodimerizes with other members of the EGFR family and in particular HER3 that contains multiple p85 binding motifs (168). Decreased EGF- and IGF1-stimulated Akt phosphorylation observed in our studies with knockdown of GSK3 expression in MDAMB231 breast cancer cells and mouse embryonic fibroblasts reveals a previously uncharacterized signaling event in these pathways. The observed decrease of the basally high levels of phosphorylated Akt with GSK3 knockdown in the AU565 breast cancer cell line likely indicates dependence on GSK3 of signaling to Akt from a constitutively active overexpressed HER2 receptor in this cell line. Since the HER2 receptor is known to dimerize with EGFR and other EGFR family

receptors (169), this result may represent GSK3 regulation of signaling to Akt downstream of HER2 homodimers or heterodimers of HER2 with other receptors in the EGFR family.

Regulation of EGF- and IGF-stimulated Akt phosphorylation by GSK3 was observed in only a subset of the cell lines tested, indicating cell context dependence of this signaling mechanism. GSK3 knockdown with targeted siRNA did not significantly decrease EGF- and IGF1-stimulated Akt phosphorylation in the MDAMB415, MCF7, or T47D breast cancer cell lines (data not presented). Screening a larger panel of cell lines will be necessary to reveal which cell characteristics determine sensitivity of EGF and IGF- stimulated Akt phosphorylation on GSK3 modulation.

As with GSK3 playing a role in Akt inhibitor-induced Akt phosphorylation, EGF- and IGF1-stimulated phosphorylation of Akt were not significantly affected by knockdown of individual GSK3 isoforms while knockdown of both the GSK3 α and GSK3 β isoforms markedly decreased induction of Akt phosphorylation by EGF and IGF1. Regulation of EGF- and IGF1-stimulated Akt phosphorylation by GSK3 was also independent of short-term GSK3 kinase signaling events. It is possible that regulation by GSK3 of both EGF/IGF1-stimulated and Akt inhibitor-induced Akt phosphorylation are mediated by the same mechanism.

In an effort to detect proteins or phosphoproteins that may be responsible for the effect of GSK3 on Akt phosphorylation, we screened multiple cell signaling pathways in unstimulated and EGF- or IGF1-stimulated MDAMB231 cells for changes associated with

decreased GSK3 expression. Knockdown of GSK3 yielded changes in expression or phosphorylation level of numerous proteins, including decreased expression of Caveolin-1, a known upstream activator of Akt. Regulation of expression of Caveolin-1 provides a potential mechanism by which GSK3 could alter ligand induced Akt phosphorylation.

5.4 Future Directions

Further studies are required to determine the signaling events by which Akt catalytic domain inhibitors increase Akt phosphorylation and the roles of GSK3 inhibitor-induced and EGF/IGF1-stimulated Akt phosphorylation.

Measurement of PIP3 lipid from lysates of cells treated with Akt catalytic domain inhibitors and comparison with basal levels will inform us whether or not the Akt inhibitors increase PI3K activity and induce accumulation of phosphatidylinositols. An increase in PIP3 levels with Akt inhibitor treatment would support the feedback signaling model of Akt inhibitor-induced Akt phosphorylation and would in turn direct our studies to evaluate potential molecules involved in Akt feedback signaling. Identification through mass spectrometry of the tyrosine phosphoprotein induced by A674563 treatment would enable siRNA-targeted knock down of its expression and assessment of any role of this protein in Akt inhibitor-induced Akt phosphorylation. The existence of a feedback loop would require a molecular sensor of changes in Akt kinase activity that communicates with upstream Akt signaling molecules. We would need to determine if GSK3 mediates this sensor function. Overexpression of GSK3 in MDAMB231 cells will aid our studies of GSK3 regulation of

Akt phosphorylation. An increase in phosphorylation of Akt in response to overexpression of GSK3 would indicate that GSK3 is not merely serving a permissive role in signaling upstream of Akt but is sufficient to drive phosphorylation of Akt.

We need to evaluate long-term signaling effects of the GSK3 kinase function in GSK3 regulation of Akt inhibitor-induced and EGF/IGF1-stimulated Akt phosphorylation. In MDAMB231 cells, inhibition of GSK3 kinase activity by SB216763 (assayed by immunoblot of β -catenin phosphorylation on the Ser33/37/Thr41 sites) lasts for less than six hours even with addition of fresh inhibitor (data not presented). Therefore, we could not study long-term GSK3 kinase inhibition with this reagent. Additional chemical inhibitors of GSK3 kinase activity need to be evaluated for use in long-term inhibition studies. Alternatively, we may employ GSK3 β -Y216F MEFs that produce a mutant GSK3 β with markedly decreased kinase activity for these studies.

Multiple growth factors such as FGF (fibroblast growth factor) and PDGF (platelet-derived growth factor) are known to activate Akt signaling. GSK3 dependence of signaling to Akt from growth factors other than EGF and IGF1 needs to be tested to determine the scope of the role of GSK3 in upstream Akt signaling. Importantly, we would need to determine why GSK3 knockdown in some lines but not others blocks signaling to Akt. Indeed, a detailed RPPA study comparing the effects of GSK3 knockdown in lines where GSK3 is required to induce optimal Akt phosphorylation to those where GSK3 is not required could identify potential candidates. This screen could be further improved by comparing the effects of GSK3 α and GSK3 β siRNA alone, which are insufficient to block

Akt phosphorylation with the pan-GSK3 siRNA that efficiently blocks Akt activation. This could be extended further by comparison to the effect of inhibition of GSK3 kinase activity with a chemical inhibitor that does not decrease ligand-induced increase of Akt phosphorylation.

While we have identified GSK3 as an upstream regulator of Akt activation, we have not determined the precise placement of GSK3 in the signaling pathway. Determining if knock down of GSK3 prevents the massive increases in levels of PIP3 known to occur with EGF and IGF1 stimulation will inform us if GSK3 signals upstream or downstream of this event. However, the demonstration that tyrosine phosphorylation of a protein that associates with p85 induced by Akt catalytic domain inhibition occurs despite GSK3 knockdown argues that the effect of GSK3 knockdown is distal to receptor activation and proximal to Akt phosphorylation. The recruitment of p85 to phosphotyrosine-containing molecules also argues that the effect of GSK3 knockdown is distal to p85 and PI3K activation.

Decreased expression of Caveolin-1, a known upstream activator of Akt, was correlated with GSK3 knockdown. Knockdown of Caveolin-1 expression using targeted siRNAs and measurement of Akt phosphorylation in response to EGF and IGF1 stimulation will reveal if Caveolin-1 is activating signaling to Akt downstream of EGF and IGF1. Positive results would then be followed up by overexpression studies to determine if Caveolin-1 is downstream of GSK3 in the Akt signaling pathway.

5.5 Concluding Remarks

Inhibition of signaling pathways upstream of Akt represents a major strategy being evaluated for control of cancers dependent on the PI3K/Akt signaling pathway. Therapies targeting EGF and IGF1 signaling are in the clinic, and Akt catalytic domain inhibitors are in clinical trials. Discovery of a novel function of GSK3 in mediating signal from these ligands to Akt may improve understanding and anticipation of drug effects. Further elucidation of the molecular events mediating GSK3 control of signaling to Akt may identify additional druggable targets in the pathway and facilitate development of more effective cancer therapeutic strategies.

CHAPTER 6: REFERENCES

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APPENDIX

Table 1. Changes in expression levels and phosphorylation status in response to GSK3 knockdown in MDAMB231 cells.

Table 2. Changes in expression levels and phosphorylation status in EGF-treated MDAMB231 cells in response to GSK3 knockdown.

Table 3. Changes in expression levels and phosphorylation status in IGF1-treated MDAMB231 cells in response to GSK3 knockdown.

Table 1. Changes in expression levels and phosphorylation status in response to GSK3 knockdown in MDAMB231 cells. Given fold change values are averaged from the two independent experiments, and fold change values from each individual experiment are included in parentheses. “Validated” antibodies, indicated by the “*” symbol, demonstrate performance for RPPA comparable to an ELISA assay. “Use with Caution” antibodies, indicated by the “**” symbol, provide high-quality useful information considered to need confirmation by additional technology.

Antibody	Average Fold Change (Exp #1 Fold Change, Exp #2 Fold Change)
14-3-3 Beta*	-1.3 (-1.1, -1.5)
14-3-3 Zeta*	1.2 (1.4, 1.0)
P-4EBP1 (S37)*	-1.4 (-1.8, -1.1)
P-4EBP1 (S65)*	-1.4 (-1.3, -1.5)
P-4EBP1 (T70)**	-1.1 (-1.1, -1.1)
4EBP1*	1.1 (1.1, 1.1)
P-ACC (S79)*	1.9 (1.6, 2.2)
AIB-1*	1.2 (1.3, 1.0)
P-Akt (T308)*	±1.2 (-1.1, 1.2)
P-Akt (S473)*	-1.4 (-1.5, -1.2)
Akt*	-1.3 (-1.3, -1.2)
P-AMPK (T172)*	1.4 (1.0, 1.8)
AMPK**	±1.3 (1.2, -1.3)
Androgen Receptor*	-1.5 (-1.3, -1.6)
P-B-catenin (S33/S37/T41)**	1.5 (1.5, 1.4)
B-catenin*	2.3 (2.9, 1.7)
BCL2*	±2.8 (3.4, -2.2)
BIM*	1.0 (1.1, 1.0)
P-c-Jun (S73)**	1.7 (1.7, 1.7)
c-Kit*	1.0 (1.0, 1.0)
c-Myc**	1.2 (1.3, 1.0)
Caspase 3**	1.1 (1.0, 1.3)
Caspase 7**	±1.1 (1.1, -1.1)
Caveolin-1*	-2.9 (-2.3, -3.5)
CD31*	±1.5 (-1.3, 1.7)
Collagen VI*	1.1 (1.1, 1.1)
Cox-2**	±1.5 (1.8, -1.1)
c-Raf*	1.6 (1.3, 1.8)
Cyclin B1*	1.7 (1.5, 1.9)
Cyclin D1*	-1.2 (-1.1, -1.2)
Cyclin E1*	-1.1 (-1.0, -1.1)
E-cadherin*	1.8 (1.0, 2.6)
P-EGFR (Y992)*	±1.3 (-1.2, 1.5)
P-ER alpha (S118)*	±1.2 (1.2, -1.1)
ETV6**	-1.8 (-1.9, -1.6)
FAK**	1.2 (1.0, 1.3)
Fibronectin**	1.6 (1.0, 2.1)
FoxO3**	1.0 (1.0, 1.0)
P-FoxO3 (S318)**	-1.2 (-1.2, -1.2)
GATA3*	1.0 (1.0, 1.1)
P-GSK3 (S21/9)*	-4.4 (-4.7, -4.1)

GSK3*	-11.1 (-9.7, -12.5)
P-HER2 (Y1248)*	-1.4 (-1.2, -1.7)
Hsp27**	1.3 (1.4, 1.3)
IGFBP2*	±1.1 (-1.1, 1.1)
IGFR1 beta**	-1.2 (-1.1, -1.3)
IRS1*	±1.1 (-1.2, 1.1)
JNK2**	1.3 (1.2, 1.3)
P-MAPK (T202)*	1.3 (1.6, 1.0)
P-MEK1/2 (S217)*	1.5 (1.5, 1.5)
MEK1*	1.4 (1.5, 1.3)
MGMT*	1.2 (1.1, 1.3)
P-mTOR (S2448)*	1.3 (1.1, 1.5)
mTOR*	1.1 (1.1, 1.2)
N-Cadherin*	-1.1 (-1.0, -1.1)
p21**	±1.4 (1.5, -1.2)
p27*	1.2 (1.3, 1.1)
p38**	±1.2 (1.1, -1.3)
p53*	1.1 (1.1, 1.2)
P-p70S6K (T389)*	-1.3 (-1.3, -1.4)
p70S6K*	1.4 (1.3, 1.5)
PI3K p85*	-1.0 (-1.1, -1.0)
P-p90RSK (T359)**	1.2 (1.1, 1.3)
PARP**	1.4 (1.1, 1.7)
P-PDK1 (S241)*	-1.4 (-1.4, -1.4)
PDK1*	-1.1 (-1.2, -1.0)
P-PKC alpha (S657)*	-1.2 (-1.4, -1.0)
PKC alpha*	-1.3 (-1.3, -1.4)
Progesterone Receptor*	1.0 (1.0, 1.0)
Patched**	-1.4 (-1.4, -1.5)
PTEN*	1.1 (1.1, 1.1)
Rab25**	1.0 (1.0, 1.1)
P-Rb (S807)*	1.2 (1.1, 1.3)
Rb*	±1.3 (1.3, -1.3)
P-S6RP (S235/S236)*	-1.7 (-1.7, -1.7)
P-S6RP (S240/S244)*	-5.3 (-4.7, -5.8)
Smad3*	-2.0 (-1.5, -2.4)
P-Src (Y527)*	-1.3 (-1.3, -1.2)
Src*	-1.3 (-1.3, -1.4)
P-STAT3 (Y705)*	-1.1 (-1.2, -1.0)
Stathmin*	-1.1 (-1.1, -1.2)
Tau**	1.0 (1.0, 1.1)
TAZ*	1.4 (1.0, 1.8)
TSC2**	1.5 (1.2, 1.8)
VASP**	±1.2 (1.2, -1.2)
VEGFR2*	-2.2 (-1.7, -2.7)
XIAP**	1.1 (1.0, 1.1)
YAP*	±2.4 (-1.7, 3.0)

Table 2. Changes in expression levels and phosphorylation status in EGF-treated MDAMB231 cells in response to GSK3 knockdown. Given fold change values are averaged from the two independent experiments, and fold change values from each individual experiment are included in parentheses. “Validated” antibodies, indicated by the “*” symbol, demonstrate performance for RPPA comparable to an ELISA assay. “Use with Caution” antibodies, indicated by the “**” symbol, provide high-quality useful information considered to need confirmation by additional technology.

Antibody	Average Fold Change (Exp #1 Fold Change, Exp #2 Fold Change)
14-3-3 Beta*	-1.2 (-1.2, -1.3)
14-3-3 Zeta*	-1.0 (-1.1, -1.0)
P-4EBP1 (S37)*	±1.4 (-1.8, 1.1)
P-4EBP1 (S65)*	-1.4 (-1.4, -1.4)
P-4EBP1 (T70)**	-1.1 (-1.1, -1.10)
4EBP1*	1.2 (1.2, 1.2)
P-ACC (S79)*	1.6 (1.4, 1.8)
AIB-1*	1.1 (1.2, 1.1)
P-Akt (T308)*	-1.8 (-1.9, -1.8)
P-Akt (S473)*	-10.1 (-11.5, -8.7)
Akt*	-1.2 (-1.2, -1.3)
P-AMPK (T172)*	1.2 (1.1, 1.3)
AMPK**	1.2 (1.2, 1.1)
Androgen Receptor*	-1.3 (-1.3, -1.2)
P-B-catenin (S33/S37/T41)**	1.3 (1.4, 1.2)
B-catenin*	2.4 (2.8, 2.0)
BCL2*	±2.0 (2.2, -1.8)
BIM*	1.1 (1.0, 1.2)
P-c-Jun (S73)**	1.7 (1.9, 1.5)
c-Kit*	1.1 (1.0, 1.2)
c-Myc**	±1.0 (1.0, -1.1)
Caspase 3**	±1.1 (-1.1, 1.1)
Caspase 7**	±1.2 (1.3, -1.1)
Caveolin-1*	-4.0 (-4.0, -4.0)
CD31*	1.1 (1.1, 1.1)
Collagen VI*	-1.1 (-1.1, -1.0)
Cox-2**	2.0 (2.5, 1.5)
c-Raf*	1.4 (1.2, 1.6)
Cyclin B1*	1.9 (1.6, 2.2)
Cyclin D1*	-1.4 (-1.3, -1.5)
Cyclin E1*	-1.2 (-1.2, -1.1)
E-cadherin*	1.0 (1.0, 1.0)
P-EGFR (Y992)*	1.4 (1.0, 1.8)
P-ER alpha (S118)*	1.2 (1.3, 1.1)
ETV6**	-1.2 (-1.1, -1.4)
FAK**	1.0 (1.0, 1.0)
Fibronectin**	1.7 (1.1, 2.3)
FoxO3**	1.1 (1.1, 1.0)
P-FoxO3 (S318)**	1.1 (1.0, 1.1)
GATA3*	1.3 (1.3, 1.2)
P-GSK3 (S21/9)*	-6.8 (-6.8, -6.8)

GSK3*	-10.6 (-11.0, -10.2)
P-HER2 (Y1248)*	-1.7 (-1.5, -1.9)
Hsp27**	1.4 (1.6, 1.2)
IGFBP2*	±1.1 (-1.1, 1.1)
IGFR1 beta**	-1.2 (-1.2, -1.2)
IRS1*	-1.2 (-1.2, -1.1)
JNK2**	1.3 (1.1, 1.4)
P-MAPK (T202)*	±1.1 (1.2, -1.1)
P-MEK1/2 (S217)*	1.2 (1.2, 1.3)
MEK1*	1.7 (1.8, 1.6)
MGMT*	-1.0 (-1.0, -1.1)
P-mTOR (S2448)*	1.1 (1.0, 1.2)
mTOR*	1.1 (1.0, 1.1)
N-Cadherin*	1.0 (1.0, 1.0)
p21**	±1.2 (1.2, -1.3)
p27*	1.1 (1.2, 1.1)
p38**	±1.2 (1.1, -1.2)
p53*	±1.2 (-1.3, 1.1)
P-p70S6K (T389)*	-2.4 (-2.1, -2.8)
p70S6K*	1.2 (1.1, 1.2)
PI3K p85*	±1.2 (1.1, -1.2)
P-p90RSK (T359)**	1.2 (1.1, 1.3)
PARP**	1.6 (1.4, 1.7)
P-PDK1 (S241)*	-1.3 (-1.2, -1.4)
PDK1*	±1.1 (-1.1, 1.1)
P-PKC alpha (S657)*	±1.3 (-1.5, 1.1)
PKC alpha*	-1.2 (-1.2, -1.1)
Progesterone Receptor*	1.2 (1.2, 1.2)
Patched**	-1.4 (-1.3, -1.5)
PTEN*	1.2 (1.0, 1.3)
Rab25**	1.0 (1.0, 1.0)
P-Rb (S807)*	1.1 (1.0, 1.2)
Rb*	±1.1 (1.0, -1.2)
P-S6RP (S235/S236)*	-2.1 (-1.7, -2.6)
P-S6RP (S240/S244)*	-6.1 (-5.2, -7.0)
Smad3*	-1.8 (-1.6, -2.0)
P-Src (Y527)*	-1.4 (-1.3, -1.6)
Src*	±1.2 (1.0, -1.3)
P-STAT3 (Y705)*	1.1 (1.1, 1.1)
Stathmin*	-1.1 (-1.1, -1.1)
Tau**	1.1 (1.1, 1.0)
TAZ*	1.2 (1.0, 1.4)
TSC2**	1.4 (1.3, 1.5)
VASP**	±1.3 (1.4, -1.2)
VEGFR2*	-1.2 (-1.3, -1.1)
XIAP**	1.1 (1.1, 1.0)
YAP*	±2.3 (-1.8, 2.8)

Table 3. Changes in expression levels and phosphorylation status in IGF1-treated MDAMB231 cells in response to GSK3 knockdown. Given fold change values are averaged from the two independent experiments, and fold change values from each individual experiment are included in parentheses. “Validated” antibodies, indicated by the “*” symbol, demonstrate performance for RPPA comparable to an ELISA assay. “Use with Caution” antibodies, indicated by the “**” symbol, provide high-quality useful information considered to need confirmation by additional technology.

Antibody	Average Fold Change (Exp #1 Fold Change, Exp #2 Fold Change)
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P-4EBP1 (S37)*	±1.5 (-1.7, 1.3)
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c-Kit*	-1.1 (-1.1, -1.0)
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GATA3*	1.1 (1.0, 1.2)
P-GSK3 (S21/9)*	-9.0 (-10.3, -7.7)

GSK3*	-11.6 (-11.2, -12.0)
P-HER2 (Y1248)*	-1.5 (-1.0, -1.9)
Hsp27**	1.4 (1.6, 1.3)
IGFBP2*	-1.3 (-1.3, -1.2)
IGFR1 beta**	-1.4 (-1.2, -1.6)
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P-STAT3 (Y705)*	±1.2 (-1.1, -1.3)
Stathmin*	-1.1 (-1.2, -1.1)
Tau**	±1.1 (1.1, -1.1)
TAZ*	1.3 (1.0, 1.6)
TSC2**	1.3 (1.1, 1.6)
VASP**	±1.2 (1.3, -1.2)
VEGFR2*	-1.8 (-1.9, -1.8)
XIAP**	-1.1 (-1.0, -1.1)
YAP*	±2.6 (-1.6, 3.7)

VITA

Debra Lorita Smith was born in Pampa, Texas on September 20, 1977, the daughter of Lorita Kay Smith and Melvin Lee Smith. After completing her high school education at the Texas Academy of Mathematics and Science, Denton, Texas in 1996, she entered the University of Texas at Austin. She received the degree of Bachelor of Science in Chemical Engineering in May, 2000. For the next year, she worked as a research assistant in the Department of Human Genetics at Baylor College of Medicine, Houston, Texas. In May of 2001, she entered the dual degree M.D./Ph.D. program at the University of Texas Health Science Center at Houston.

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