Glycogen Synthase Kinase 3 is Required for Optimal AKT Activation

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GLYCOGEN SYNTHASE KINASE 3 IS REQUIRED FOR OPTIMAL AKT ACTIVATION

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GLYCOGEN SYNTHASE KINASE 3 IS REQUIRED FOR OPTIMAL AKT ACTIVATION

A THESIS
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for the Degree of

MASTER OF SCIENCE

by
Debora S. Bruno, M.D.
Houston, Texas
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DEDICATIONS

This dissertation is dedicated to my husband, Francisco Almeida, for being my greatest and steadiest supporter. He has been my most wonderful friend through all the years we have been in the United States, training and working as physicians. Francisco has always inspired me with his altruism, kindness, medical knowledge and most of all, capability to constantly adapt his views as new evidence is presented to him.

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GLYCOGEN SYNTHASE KINASE 3 IS REQUIRED FOR OPTIMAL AKT ACTIVATION

Debora S. Bruno, M.D.
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The phosphatidylinositol 3-kinase (PI3K) pathway, through its major effector node AKT, is critical for the promotion of cell growth, division, motility and apoptosis evasion. This signaling axis is therefore commonly targeted in the form of mutations and amplifications in a myriad of malignancies. Glycogen synthase kinase 3 (GSK3) was first discovered as the kinase responsible for phosphorylating and inhibiting the activity of glycogen synthase, ultimately antagonizing the storage of glucose as glycogen. Its activity counteracts the effects of insulin in glucose metabolism and AKT has long been recognized as one of the key molecules capable of phosphorylating GSK3 and inhibiting its activity. However, here we demonstrate that GSK3 is required for optimal phosphorylation and activation of AKT in different malignant cell lines, and that this effect is independent of the type of growth factor stimulation and can happen even in basal states. Both GSK3α and GSK3β isoforms are necessary for AKT to become fully active, displaying a redundant role in this setting. We also demonstrate that this effect of GSK3 on AKT phosphorylation and full activation is dependent on its kinase activity, since highly specific inhibitors targeting GSK3 catalytic activity also promote a reduction in phosphorylated AKT. Analysis of reverse phase protein array screening of MDA-MB-231 breast cancer cells treated with RNA interference targeting GSK3 unexpectedly revealed an increase in levels of phosphorylated MAPK14 (p38). Treatment with the selective p38 inhibitor SB 202190 rescued AKT activation in that cell line, corroborating the importance of unbiased proteomic analysis in exposing cross-talks between signaling networks and demonstrating a critical role for p38 in the regulation of AKT phosphorylation.
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ABBREVIATIONS

AP-1 – Activating protein - 1
APC - Adenomatous polyposis coli
APE – AKT-phosphorylation enhancer
ASK1- Apoptosis signal-regulating kinase 1
ATCC - American Type Culture Collection
ATF – Activating transcription factor
ATM – Ataxia telangiectasia mutated
β-TrCP1 - β-transducin repeats-containing protein-1
CDK1 – Cyclin-dependent kinase 1
CDK2- Cyclin-dependent kinase 2
Ci – Cubitus interruptus
cIAP – Cellular inhibitor of apoptosis protein - 1
CK1 – Casein kinase 1
DDX3 – DEAD-box polypeptide 3
DISC – Death-inducing signaling complex
DLK1 – Dual leucine zipper-bearing kinase 1
DVL - Disheveld
4EBP1 – Eukaryotic translation initiation factor 4E binding protein 1
EDTA – Ethylenediaminetetraacetic acid
EGF – Epidermal growth factor
EGFR– Epidermal growth factor receptor
eIF2 – Eukaryotic initiation factor 2
eIF2B – Eukaryotic initiation factor 2B
eNOS – endothelial nitric oxide synthase
ER – Endoplasmic reticulum
ERK – Extracellular signal-regulated kinase
FBS – Fetal bovine serum
FRAT – Frequently rearranged in advanced T cell lymphoma
FZD – Frizzled receptor
GAPDH – Glyceraldehyde-3-phosphate dehydrogenase
GBM – Glioblastoma multiforme
GLUT – Glucose transporter
GPCR – G-protein coupled receptor
GSK – Glycogen synthase kinase
GTPase – Guanosine Triphosphatase
HEPES – N-2-Hydroxyethylpiperazine-N‘-2-ethanesulfonic acid
HER – Human epidermal growth factor receptor
HM – Hydrophobic motif
IGF -1– Insulin-like growth factor 1
IGF- 1R - Insulin-like growth factor 1 receptor
IKK – Inhibitor of kappa B kinase
ILK – Integrin-linked kinase
IR – Insulin receptor
IRS – Insulin receptor substrate
JNK – c-Jun N-terminal kinase
LEF – Lymphoid enhancer factor
LRP6 – Low density lipoprotein receptor-related protein 6
LST8 – Lethal with SEC13 protein 8
MAPK – Mitogen activated protein kinase
MCL-1 – Myeloid cell leukemia 1
MMP – Matrix metalloproteinase
mSIN1 – Mammalian stress activated protein kinase-interacting protein 1
mTOR – mammalian target of rapamycin
mTORC1 – mTOR Complex 1
mTORC2 – mTOR Complex 2
MVBs – Multivesicular bodies
NF-κB – Nuclear factor kappa B
NSCLC – Non-small cell lung cancer
PBS - Phosphate Buffered Saline
PAGE – Polyacrylamide gel electrophoresis
PDK – Phosphoinositide-dependent kinase
PH – Pleckstrin homology
PHLLP – PH domain leucine-rich repeat phosphatase
PIK – Phosphatidylinositol kinase
PIKK – PI3K-like kinase
PI3K – Phosphatidylinositol 3-kinase
PIP3 – Phosphatidylinositol triphosphate
PKA – Protein kinase A
PKB – Protein kinase B
PKC – Protein kinase C
PP2A – Protein phosphatase 2A
PP1A – Protein phosphatase 1A
PRAS40 – Proline rich AKT substrate of 40 kDa
Proctor – Protein associated with Rictor
P90RSK – p90 ribosomal protein S6 kinase
P70S6K – p70 ribosomal protein S6 kinase
PtdIns(4,5)P2 – Phosphatidylinositol 4,5 biphosphate
PtdIns(3,4,5)P3 – Phosphatidylinositol 3,4,5 triphosphate
PTEN – Phosphatase and tensin homolog deleted on chromosome 10
Raptor – Regulatory associated protein of mTOR
RBD – Ras-binding domain
Rheb – Ras homolog enriched in brain
Rictor – Rapamycin-insensitive companion of mTOR
RISC – RNA-induced silencing complex
RPMI – Roswell Park Memorial Institute
RPPA - Reverse phase protein array
RTK – Receptor tyrosine kinase
SAPK – Stress activated protein kinase
SDS – Sodium dodecyl sulfate
S6K – Ribosomal protein S6 kinase
SH – Src homology
TAK1 – TGFβ- activated kinase 1
TCF – T cell factor
TGFβ - Transforming growth factor β
TK – Tyrosine kinase
TNF- Tumor necrosis factor
TRAF6 – TNF receptor associated factor 6
TSC – Tuberous sclerosis complex
VEGF – Vascular endothelial growth factor
VEGFR – Vascular endothelial growth factor receptor
Vps – Vacuolar protein-sorting
INTRODUCTION:

1. The Phosphatidylinositol 3-kinase (PI3K) pathway

The phosphatidylinositol 3-kinases are members of a family of intracellular lipid kinases that phosphorylate the 3’-hydroxyl group of the inositol ring of phosphatidylinositides (Engelman et al., 2006). The phosphorylated 3’-hydroxyl group acts as an intracellular second messenger, an anchor to proteins possessing pleckstrin homology (PH) domains (Isakoff et al., 1998; Lemmon and Ferguson, 2000), such as AKT and phosphoinositide-dependent kinase 1 (PDK1). PtdIns(3,4,5)P3 can activate AKT (also known as Protein Kinase B or PKB) by causing a translocation to the membrane of AKT itself and of its upstream Thr308-directed protein kinase, PDK1 (Alessi et al., 1997b; Stephens et al., 1998). Therefore, following activation of the insulin receptor and other receptor tyrosine kinases that respond to a variety of growth factors, PI3K ultimately orchestrates the recruitment and activation of one of the most important molecules in cell signaling. As such, AKT is responsible for controlling growth, metabolism, proliferation, and apoptosis evasion. Understandably, the PI3K pathway is affected by mutations and amplifications in a variety of cancers (Yuan and Cantley, 2008), constituting a desirable target for new therapeutic agents. However, altering the function of the effector nodes in this pathway for therapeutic reasons requires a vast understanding of the possible regulatory and intricate feedback loops that can be offset by such therapeutic approaches.

1.1. Classes of phosphatidylinositol 3-kinases

Three different classes (I-III) of phosphatidylinositol 3-kinases exist, which differ based on their substrate preference and sequence homology (Engelman et al., 2006).

Class I PI3Ks primarily generate PtdIns(3,4,5)P3 from PtdIns(4,5)P2. Class IA and IB PI3Ks differ primarily on their regulatory subunits. Class IA PI3Ks are heterodimers consisting of a regulatory subunit (p85α, p85β or p55γ) and a catalytic subunit (p110) (Fruman et al., 1998). Each of the regulatory subunits of the Class I of PI3Ks is encoded by
a distinct gene (PIK3R1, PIK3R2 and PIK3R3, respectively). The basic structure of the p85 regulatory units contains 2 Src-homology 2 (SH2) domains that flank one p110-binding domain. Through their SH2 domains, the p85 regulatory subunits bind to phosphorylated tyrosine residues on activated receptor tyrosine kinases (RTKs) or adaptor molecules such as insulin receptor substrate 1 (IRS1). This binding is fundamental for the relief of the inhibitory action that p85 exerts on the p110 catalytic subunit of the PI3K molecule (Yu et al., 1998). Three different genes encode the p110 catalytic subunit isoforms p110α, p110β and p110δ (PIK3CA, PIK3CB and PIK3CD, respectively) (Fruman et al., 1998). While p110α and p110β are ubiquitously expressed, the p110δ subunit is predominantly expressed in leukocytes (Engelman et al., 2006). The p110 catalytic subunit contains 5 distinct domains: an N-terminal p85-binding domain, a GTPase Ras-binding domain (RBD), a C2 domain, a PIK homology domain and the catalytic domain located in the C-terminal end of the subunit. Class IA PI3Ks are activated by the growth factor RTKs. While the insulin and insulin-like growth factor 1 (IGF-1) receptors utilize the IRS family of adaptor molecules to recruit Class IA PI3Ks to the plasma membrane and trigger their activity, other RTKs such as the epidermal growth factor receptor (EGFR) recruit Class IA PI3K directly. The small GTPase Ras can also directly activate Class IA PI3K by interaction with the RBD domain of the p110 catalytic subunit.

Class IB PI3Ks have a p101 regulatory subunit and a p110γ catalytic subunit. Since class IB PI3Ks do not possess a p85 regulatory subunit, they are typically not regulated by RTKs, and rather activated by the G-protein-coupled receptors.

Class II PI3Ks consist of a single p110-like catalytic subunit, in three different isoforms (PI3K C2α, β and γ). Although it is generally assumed that class II PI3Ks can phosphorylate both PtdIns and PtdIns(4)P in vitro, they seem to have a preferential activity for PtdIns, transforming it into PtdIns(3)P. In vivo studies also suggest that PtdIns(3)P might be the most abundant product generated by this class of PI3K (Falasca and Maffucci, 2007). Nevertheless, there is yet a considerable lack of information regarding the function and regulation of this class of PI3Ks.

Class III is represented solely by the mammalian homolog of the vacuolar protein-sorting
defective 34 (Vps34) molecule, first identified in *Saccharomyces cerevisiae*. Vps34 is indeed the only PI3K present in yeast. PtdIns is also the solo substrate of this class of PI3K identified, and accordingly, the only product it generates is PtdIns(3)P. In both yeast and mammalian cells, Vps34 is required for the induction of autophagy during nutrient deprivation (Backer, 2008). However, as Vps34 has also been implicated in the positive regulation of mTOR/raptor signaling through nutrient sensing (Byfield et al., 2005; Nobukuni et al., 2005), its role in autophagy might be context-dependent. More recently, Src has been demonstrated to phosphorylate and activate Vps34, in mammalian cells, leading to cellular transformation (Hirsch et al., 2010).

1.2. PI3K pathway and human disease

The PI3K pathway is at the center of growth and metabolism control, and alterations of its signaling are underlying causes of two of the most prominent diseases in developed countries: cancer and Type II Diabetes Mellitus.

1.2.1. PI3K pathway and Type II Diabetes Mellitus

The PI3K pathway has a central role in mediating the effects of insulin on cellular metabolism. Activation of this pathway by insulin and IGF leads to increased glucose uptake, as well as glycogen and protein synthesis. Therefore, Type II Diabetes Mellitus, which is characterized by insulin insensitivity, is associated with a decreased response of this pathway to insulin stimulation (Luo et al., 2006), and subsequent decrease in glucose utilization and storage. The class IA p110α catalytic subunit seems to be the key mediator of insulin metabolic actions in the liver. Mice with hepatic knockout of the p110α subunit exhibit impaired insulin sensitivity, glucose intolerance, and increased gluconeogenesis (Sopasakis et al., 2010). Interestingly, with the advance of clinical trials looking at the potential anti-cancer effects of drugs targeting this pathway, insulin resistance has become a potential toxicity as well as a pharmacodynamic marker of PI3K inhibition (Courtney et al., 2010). It seems though that with the development of inhibitors targeting specific p110 isoforms, it might be possible to selectively avoid the development of insulin resistance if only p110β or δ isoform inhibition is provided.
Also interesting is the finding that the p85 subunit’s dosage might interfere with the optimal activity of the p110 catalytic subunit. Studies utilizing transgenic mice lacking either p85α or p85β subunits have consistently demonstrated a paradoxical enhancement in insulin sensitivity due to increased PI3K signaling downstream of the IRS proteins (Mauvais-Jarvis et al., 2002; Terauchi et al., 1999). Existing in greater concentration than the p110 subunits, free p85 can actually interfere with the binding of the heterodimeric p85/p110 complexes to IRS1 by sequestering this adaptor protein into cytoplasmic foci (Luo et al., 2005), and therefore function as a hindrance to PI3K activation.

1.2.2. PI3K pathway and Cancer

Inappropriate signaling through the PI3K pathway has been undoubtedly one of the most frequently noted occurrences in human cancer. In 1985, it was demonstrated that the oncogenic polyoma middle T antigen, through its interaction with pp60c-src, was able to activate PI3K to generate the second messenger PtdIns(3,4,5)P3 (Whitman et al., 1985). Later on it was made clear that the single most important step in the oncogenic capabilities of the middle T antigen of polyomavirus was the generation of PtdIns(3,4,5)P3 (Ling et al., 1992). The PI3K pathway has every major node targeted by mutations or amplifications in a wide variety of solid tumors. Receptor tyrosine kinases upstream of PI3K, the p110α catalytic subunit of PI3K, its downstream major effector kinase AKT, and its negative regulator, the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) are all frequently altered in cancer (Yuan and Cantley, 2008).

Following the activation and therefore tyrosine phosphorylation of cytoplasmic tails of RTKs, only a small fraction of the available PI3K is recruited to the plasma membrane. Therefore, even slight increments in the engagement of these receptors can lead to many-fold increases in PI3K activity (Yuan and Cantley, 2008). The epidermal growth factor receptor tyrosine kinase family consists of four members: EGFR (HER1, erbB1), HER2 (erbB2, HER2/neu), HER3 (erbB3) and HER4 (erbB4). EGFR is amplified or mutated in a variety of malignant histologies and the use of tyrosine kinase inhibitors and monoclonal antibodies targeting EGFR has proved to be beneficial in several clinical settings. As an
example, in non-small cell lung cancer (NSCLC), deletions in exon 19 and L858R point mutations in exon 21 were reported to be associated with responses to treatment with tyrosine kinase inhibitors targeting EGFR (Lynch et al., 2004; Paez et al., 2004). On the other hand, HER2 has tumorigenic capabilities through overexpression alone (Moasser, 2007). Gene amplification and transcriptional deregulation lead to HER2 overexpression in approximately 20 to 25% of ovarian and breast cancers, conferring a more aggressive biological behavior (Slamon et al., 1989). HER2 overexpression is also seen in subsets of gastric, esophageal and endometrial cancer, also associated with worse prognostic features (Moasser, 2007). However, the discovery of this biological marker has also allowed the development of drugs that had profound impact in the survival in a subset of cancer patients whose tumors harbor HER2 overexpression. The use of the monoclonal antibody targeting HER2, trastuzumab, in addition to chemotherapy, has proved to increase the overall survival of breast cancer patients who are candidates for this therapy (Slamon et al., 2001). Also, dual inhibition of the EGFR and HER2 tyrosine kinases by the small molecule lapatinib has proved beneficial in women with metastatic breast cancer overexpressing HER2 that have developed resistance to trastuzumab (Geyer et al., 2006). Another most critical RTK involved in cancer cell signaling is the insulin-like growth factor receptor (IGF-1R). This receptor belongs to the insulin receptor (IR) family, and these two receptors, while highly homologous in their TK domains, differ considerably in their functions (Larsson et al., 2005). While IGF-1R regulates mostly cellular proliferation, differentiation, apoptosis evasion and motility, IR is mostly responsible for the control of glucose uptake and metabolism (Larsson et al., 2005). Following ligand binding, phosphorylation of the critical tyrosine residues Y1131, 1135 and 1136 release the auto-inhibitory conformation of the activation loop and the catalytic active TK goes on to phosphorylate adaptor proteins such as the insulin receptor substrates 1-4 (IRS 1-4) and Shc (Larsson et al., 2005). As with HER2, IGF1R does not appear to be mutated in cancers (Maki, 2010), while overexpression of this receptor has been demonstrated across a wide variety of human carcinomas of glandular and transitional cell origin, including breast, ovarian, endometrial, gastric, pancreatic, colon, lung and prostate carcinomas, as well as transitional cell carcinomas of the bladder (Ouban et al., 2003). In lung cancer, IGF1-R protein expression is higher in the squamous histology, and correlated with EGFR expression (Dziadziuszko et al., 2010). Interestingly though, a high IGF1R gene copy number harbors a positive prognostic value (Dziadziuszko et al.,
The most prevalent mutations in the PI3K pathway are the activating mutations in PIK3CA, the gene encoding the p110α catalytic subunit of PI3K, and inactivating mutations in the PTEN tumor-suppressor gene. PIK3CA has been found mutated in breast, endometrial, colorectal, urinary tract and ovarian cancers (Yuan and Cantley, 2008). The majority of these somatic mutations lie in two hotspot regions, encoding for the central helical domain and the carboxy-terminal kinase domain of the molecule, and conferring constitutive kinase activity (Yuan and Cantley, 2008). In mammary epithelial cells, expression of p100α mutants has been shown to promote a variety of changes associated with a malignant phenotype, including growth factor-independent proliferation, colony growth in soft-agar, and protection from anoikis (Isakoff et al., 2005). In contrast to p110α, no oncogenic mutations have been found in any of the other class I PI3K catalytic subunits.

PTEN, a dual lipid and protein phosphatase, targets primarily the main second messenger for the class I PI3K pathway, PtdIns(3,4,5)P3, creating PtdIns(4,5)P2 and relinquishing the activation of the main downstream effectors of PI3K, namely AKT, PDK1 and Rac1/cdc42 (Blanco-Aparicio et al., 2007). The loss of PTEN leads to constitutively high levels of PIP3, which are increased upon growth factor stimulation. Ultimately, PIP3 induction will lead to increased cell size, enhanced survival and cell cycle progression (Keniry and Parsons, 2008). PTEN is mutated or lost in both heritable and spontaneous cancers (Yuan and Cantley, 2008). The gene that encodes PTEN is located on chromosome 10q23, and germline nonsense and missense mutations that disrupt its phosphatase domain are responsible for Cowden disease, an autosomal dominant cancer predisposition syndrome associated with an elevated risk for tumors of the breast, thyroid and skin (Liaw et al., 1997). Somatic PTEN alterations are commonly seen in many sporadic tumor types, such as breast, prostate and endometrial cancers, melanomas and glioblastomas (more than 70% of GBMs harbor PTEN loss of heterozygosity) (Hollander et al., 2011). PTEN dosage has also been shown to correlate with an increased susceptibility for tumor development in mice, which suggests PTEN to be haploinsufficient. Indeed, in non-small cell lung cancer, decreased PTEN expression caused by epigenetic changes seems to be more prevalent than actual mutations or deletions of the gene (Hollander et al., 2011).
The three known AKT isoforms are derived from distinct genes (AKT1 from PKBα, AKT2 from PKBβ and AKT3 from PKBγ) and share more than 80% of sequence identity (Liao and Hung, 2010). Knockout mice studies have unveiled a prominent role for AKT1 in the regulation of apoptosis, AKT2 in the regulation of glucose homeostasis and AKT3 in brain development (Gonzalez and McGraw, 2009). Recent work exploring the differential activity of the PHLLPs (PH domain leucine-rich repeat phosphatases) has uncovered an intriguing differential regulation of downstream AKT targets by the different AKT isoforms (Brognard et al., 2007). For example, while GSK3α was specifically regulated by AKT2, GSK3β was regulated by all three AKT isoforms. As expected, overexpression of the AKT isoforms, dependent or not on gene amplification, has been reported in multiple cancers, including pancreatic, ovarian, gastric, colorectal, head and neck and breast cancers, as well as melanomas (Gonzalez and McGraw, 2009; Yuan and Cantley, 2008). Interestingly though, even as we see multiple members of this pathway being targeted by germline or somatic mutations, functional mutations of the three AKT isoforms are not commonly seen. This seems a counterintuitive observation, as AKT represents a key node in the PI3K pathway. In 2007, a unique mutation in the PH domain of AKT1 (E17K) was identified in breast, colorectal and ovarian clinical tumor specimens (8%, 6% and 2%, respectively) (Carpten et al., 2007). This mutation was mutually exclusive with respect to mutations in PIK3CA and complete loss of PTEN protein expression. In vitro experiments demonstrated that in the absence of serum, the E17K mutation led to an increase in AKT1 localization to the plasma membrane and subsequent phosphorylation. The E17K mutation alone was sufficient to transform cells in culture and to induce leukemia in mice (Carpten et al., 2007), suggesting that it may play a crucial role in cancer development. The molecular mechanism underlying E17K oncogenesis seems to reside in a broadened lipid selectivity that allows high-affinity binding of AKT1 to PtdIns(4,5)P2 (Landgraf et al., 2008). Subsequent studies have confirmed the presence of this mutation in a small subset of breast, colorectal, lung, endometrial and prostate cancers (Askham et al., 2010; Bleeker et al., 2008; Boormans et al., 2010; Kim et al., 2008; Malanga et al., 2008; Shoji et al., 2009). A second AKT1 point mutation (E49K) has been discovered in a small percentage of bladder tumors, resulting in elevated phosphorylation only of the Ser473 residue and much weaker transforming capabilities in vitro (Askham et al., 2010). An also rare, but functionally relevant E17K
mutation has been identified in the AKT3 isoform in melanoma (Davies et al., 2008).

2. AKT

2.1. Structure of AKT

The AKT molecule is composed by a central kinase domain flanked by an N-terminal pleckstrin homology (PH) domain and a carboxyl-terminal regulatory domain that contains the hydrophobic motif (HM) (Hanada et al., 2004). This hydrophobic motif is characteristic of the AGC (for protein kinases A, G and C) kinases, which include AKT, p70 ribosomal S6 kinase and the serum-glucocorticoid-inducible kinase (SGC). Thr308 and Ser473 residues, targets of phosphorylation for the full activation of AKT1 catalytic activity, are located on the molecule’s catalytic and HM domains, respectively (Liao and Hung, 2010).

2.2. Functional Roles of AKT

AKT orchestrates several signaling changes that ultimately can be described as hallmarks of malignant neoplastic transformation, such as evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion (Hanahan and Weinberg, 2000) (Figure 2.1).

AKT inhibits apoptosis by inactivating several pro-apoptotic proteins and activating anti-apoptotic ones. For instance, AKT is able to phosphorylate the Bcl-2 family member BAD at Ser136 and effectively block BAD-induced cell death (Datta et al., 1997), by permanently disrupting the BAD-Bcl-2 and BAD-Bcl-xL complex formation. AKT also is able to prevent translocation of another pro-apoptotic BCL-2 family member, BAX, from the cytoplasm to the mitochondria (Tsuruta et al., 2002). By phosphorylating FKHR1, a member of the Forkhead family of transcription factors, AKT induces its binding to 14-3-3 proteins in the cytosol, preventing its nuclear localization (Brunet et al., 1999). As a consequence, FKHR1-dependent transcription of the pro-apoptotic genes Bim and FasL is suppressed. In addition, AKT can phosphorylate and inactivate pro-caspase 9 at Ser196 (Cardone et al., 1998) and promote p53 degradation by phosphorylating Mdm2 and increasing its ability to ubiquitinate
p53 (Ogawara et al., 2002). AKT can also trigger activation of nuclear factor-kappa B (NF-κB), a master regulator of cellular survival functions, by activating IKK (Inhibitor of Kappa B Kinase) (Salminen and Kaarniranta, 2010).

AKT activation mediates cell cycle progression. By phosphorylating and inactivating GSK3, AKT allows for cyclin D1 and c-Myc, both downstream targets of GSK3, to remain free from ubiquitination and degradation and positively regulate G1/S cell cycle progression (Liang and Slingerland, 2003). AKT may also, through phosphorylation, inactivate the cyclin-dependent kinase inhibitors p21^{cip1} and p27^{kip1}.

AKT is required for cell migration/motility in different organisms. One of its substrates, Girdin/APE (AKT-phosphorylation enhancer) is an actin binding protein that is essential for the development of stress fibers and lamelipodia. By phosphorylating Girdin at Ser1416, AKT allows for its accumulation at the leading edge of migrating cells (Enomoto et al., 2005). In glioma cells, aberrant PI3K/AKT signaling leads to abnormalities in both cell proliferation and migration (Lefranc et al., 2005). AKT, through the activation of NF-κB (Kim et al., 2001), is also able to promote the production of matrix metalloproteinase-9 (MMP-9), resulting in the destruction of extra-cellular matrix compounds and cell invasiveness.

The PI3K/AKT pathway is an essential effector for the vascular endothelial growth factor receptor (VEGFR)-mediated angiogenesis. Besides VEGF, several other endothelial cell stimuli, including angiopoietin-1, hepatocyte growth factor and fluid shear stress also activate PI3K-AKT signaling, emphasizing the importance of this signaling pathway for endothelial cell viability (Shiojima and Walsh, 2002). Sustained AKT activation in endothelial cells has been demonstrated to induce the formation of enlarged, hyperpermeable blood vessels in non-tumor tissue (Phung et al., 2006). For instance, the production of nitric oxide, a pivotal regulator of vascular remodeling and angiogenesis, is known to be mediated by AKT, which phosphorylates the endothelial nitric oxide synthase (eNOS), enhancing its sensitivity to Ca²⁺ (Dimmeler et al., 1999).

As the major effector node of the PI3K signaling pathway, AKT’s role in cell metabolism is
quite remarkable: it regulates glucose transport, the synthesis of both protein and glycogen, and suppresses gluconeogenesis (Whiteman et al., 2002). Ultimately, it mediates the effects of insulin signaling in promoting cell growth. Constitutively active AKT induces glucose uptake into adipocytes in the absence of insulin by stimulating translocation of the glucose transporter 4 (GLUT4) to the plasma membrane (Cong et al., 1997; Kohn et al., 1996). It also promotes glycogen synthesis by phosphorylating and inhibiting the activity of glycogen synthase kinase 3 (GSK3). GSK3 is one of the kinases responsible for phosphorylating and hindering the activity of glycogen synthase, which adds activated glucosyl groups to growing polysaccharide chains – the final step in glycogen synthesis. GSK3 phosphorylates and inhibits eIF2B (eukaryotic initiation factor 2B), and since eIF2B is required for the recycling of eIF2, a factor necessary for all cytoplasmic translation initiation events, AKT promotes protein synthesis by phosphorylating GSK3 and inhibiting its activity (Proud, 2006). On the other hand, mTOR is a known regulator of both cell growth and cell proliferation, which are coordinated by at least two of its downstream targets: S6K1 and 4E-BP1. Ribosomal protein S6 kinases and proteins promote the initiation and elongation phases of translation, while 4E-BP1 binds to eIF4E and prevents the formation of eIF4E complexes needed for translation initiation (Averous and Proud, 2006). By phosphorylating S6K and 4E-BP1, mTORC1 activates and inhibits, respectively, these downstream targets, leading to an increase in protein synthesis. Insulin regulates mTORC1 signaling through the TSC1 (tuberous sclerosis complex 1)-TSC2 protein complex: this normally acts as a GTPase-activating complex toward the small G protein Rheb (Ras homolog enriched in brain), directly upstream of mTORC1 (Tee et al., 2003). By directly phosphorylating TSC2, AKT destabilizes TSC2 and disrupts its interaction with TSC1, allowing for the Rheb activity to remain intact (Inoki et al., 2002).
Figure 2.1. AKT and its targets. AKT commands several signaling events that lead to malignant neoplastic transformation by acting upon a variety of substrates. Here we illustrate a few of those substrates that have been highlighted in the text. The green arrows indicate activation/promotion and the black interrupted bars indicate inactivation/inhibition.

2.3. AKT phosphorylation and activation

The first indication that AKT plays a role in oncogenesis derived from the isolation of the transforming retrovirus AKT8, from an AKR mouse T-cell lymphoma (Staal et al., 1977). Subsequently it became clear that the oncogenic features of v-akt were related to its myristylation and differential subcellular localization: 40% of v-akt was found to be located at the plasma membrane, 30% at the nucleus with only 30% left at the cytoplasm of infected cells, whereas 90% of c-akt was located in the cytosol (Ahmed et al., 1993). Later on it was demonstrated that membrane-associated, but not cytosolic AKT, isolated from growth-factor stimulated hematopoietic cells, was catalytically active (Zhang and Vik, 1997), and that the PH domain of AKT was essential for its translocation to the plasma membrane (Bellacosa et al., 1998). The activity of AKT in mammalian cell lines was demonstrated to be controlled by the reversible phosphorylation of serine and threonine residues of that molecule, following either stimulation by serum or inhibition of protein phosphatase 2A (PP2A) activity (Andjelkovic et al., 1996). The identity of one of the kinases responsible for its
activation did not remain elusive for long. PDK1 was purified as one of the kinases that mediated the activation of AKT by insulin and growth factors (Alessi et al., 1997b), phosphorylating it at Thr308 (for AKT1) in the presence of lipid vesicles containing PtdIns(3,4,5)P3 or PtdIns(3,4)P2. The identity of the second kinase(s), PDK2, responsible for targeting Ser473 (in AKT1), however, has been a matter of much investigation and debate. It is however recognized that full activation of AKT is a multi-step process that results in the sequential phosphorylation of the Thr308 and Ser473 residues, in this order (Figure 2.2). Point mutants of those sites that prevent phosphorylation (T308A and S473A) show little activity, whereas phosphorylation-mimicking mutants (T308D and S473D) show constitutive kinase activity (Liao and Hung, 2010).

The catalytic site of PDK1 is located in its N-terminal domain, while its C-terminus contains the PH domain that interacts at a high affinity with PtdIns(3,4,5)P3 and one of its immediate breakdown products, PtdIns(3,4)P2. The binding to the plasma membrane is necessary for PDK1 to get into proximity to AKT and phosphorylate it, as experiments in which the PH domains of those molecules have been deleted demonstrate (Alessi et al., 1997a). However, PDK1 activity is not dependent on its interaction with the plasma membrane. This kinase is found constitutively phosphorylated and active, independent of growth factor stimulation (Alessi et al., 1997a). PDK1 possesses the intrinsic ability to phosphorylate its own T-loop residue Ser241 (Mora et al., 2004).

In certain contexts, several kinases, including PDK1, ataxia telangiectasia mutated (ATM), integrin-linked kinase (ILK), protein kinase C Beta II, and even AKT itself, have been implicated as PDK2, responsible for the phosphorylation of Ser473 (Balendran et al., 1999; Chan and Tsichlis, 2001; Kawakami et al., 2004; Persad et al., 2001; Toker and Newton, 2000; Viniegra et al., 2005). Later on, the identity of the hydrophobic motif kinase was attributed to the Rictor-mTOR complex (Sarbassov et al., 2005), also known as mTORC2 (mTOR Complex 2), in both *Drosophila* and human cells. The mammalian target of rapamycin (mTOR) serine/threonine kinase is a member of the PI3K-like kinase family (PIKK) and it is known to be part of two major complexes: mTORC1 and mTORC2.

The mTOR complex 1 (mTORC1) consists of mTOR, the regulatory associated protein of
mTOR (Raptor) and the mammalian ortholog of yeast LST8 (lethal with SEC13 protein 8). It can be activated through a pathway involving AKT-mediated phosphorylation of PRAS40 (proline-rich AKT substrate of 40 kD) and the tuberous sclerosis complex, events that ultimately lead to the activation of the Rheb GTPase (Alessi et al., 2009). mTORC1 activity is also regulated by the presence of amino acids and the energy balance within the cell. mTORC1 regulates protein translation through 4EBP1 (eukaryotic translation initiation factor 4E binding protein 1) and S6K1 (ribosomal S6 kinase 1). In addition to its role in promoting protein synthesis, S6K1 is also responsible for inhibiting IRS1 activity and IRS2 expression, which provides an important negative feedback for the PI3K pathway (Tremblay and Marette, 2001).

The second mTOR complex (mTORC2) results from the assembly of mTOR, rapamycin-insensitive companion of mTOR (Rictor), mLST8, mammalian stress activated protein kinase-interacting protein (mSIN1) and protein associated with Rictor (Protor). The activity of mTORC2 is controlled primarily by PI3K, being largely insensitive to nutrients or energy conditions (Alessi et al., 2009). In vitro, Rictor is required for mTORC2 to be able to phosphorylate AKT (Sabatini, 2006). Not much is known regarding the regulation of mTORC2. However, very recently it has been proposed that during endoplasmic reticulum (ER) stress, cells respond by downregulating activation of AKT through phosphorylation and inhibition of Rictor by GSK3β (Chen et al., 2011).
Figure 2.2. The process of AKT phosphorylation for its activation. Following activation of receptor tyrosine kinase, phosphorylated tyrosine residues in the RTK’s cytoplasmic tail become docking sites for the p85 regulatory subunit of PI3K. This relieves the inhibitory activity of p85 over the catalytic subunit p110 of PI3K, which in turn phosphorylates the 3’-OH group of PIP2, generating PIP3. PIP3 then becomes a docking site for molecules containing a PH domain, such as AKT and PDK1, which come into close proximity at the plasma membrane. PDK1 phosphorylates AKT at Thr308 and this induces a conformational change that allows for PDK2 (mTORC2 shown here) to phosphorylate AKT on Ser473. Fully active, now AKT can phosphorylate several of its targets. The phosphatases PP2A and PHLPPs target the phosphorylated residues on AKT and consequently decrease the activity of that molecule. PTEN is the phosphatase that targets PIP3, generating PIP2 and counteracting the effects of PI3K.

2.4. Phosphatases targeting AKT

The reversible phosphorylation of AKT is accomplished by the opposing activities of specific kinases (as previously discussed) and phosphatases (Figure 2.2). Protein phosphatase 2, also known as PP2A, comprises an actual family of phosphatases with a highly regulated, well-conserved catalytic subunit. Regulation is accomplished by post-translational modifications of its catalytic subunit (phosphorylation and methylation) as well as by interactions with a family of regulatory subunits capable of determining substrate specificity, subcellular localization and catalytic activity of the holoenzymes (Janssens and
The catalytic subunit of PP2A has been described to undergo phosphorylation at Tyr307 that is catalyzed by different kinases such as p60\textsuperscript{v-src}, p56\textsuperscript{lck}, as well as by the activation of epidermal growth factor and insulin receptors. This phosphorylation is enhanced in the presence of the PP2A phosphatase inhibitor okadaic acid, consistent with an autodephosphorylation mechanism (Chen et al., 1992). Indeed, more than 90% of the enzyme’s activity can be lost by this post-translational modification. AKT is one of PP2A’s substrates. Almost 10 years ago, it was discovered that PP2A not only had the ability to form a complex with AKT, but also to directly dephosphorylate the active kinase, reducing its activity by 36% +/- 23% (Ivaska et al., 2002). AKT activity has also been discovered to be directly repressed by the PH domain leucine-rich repeat phosphatases (PHLPPs) (Brognard et al., 2007; Gao et al., 2005). Differently from PP2A, these phosphatases target primarily AKT’s hydrophobic motif, (S473) while PP2A regulates mostly the PDK1 site Thr308 (Gao et al., 2005). The two PHLPP enzymes seem to have a differential preference for AKT isoforms: PHLPP1 attenuates the phosphorylation of AKT2 and AKT3 isoforms, while PHLPP2 dephosphorylates only AKT1 and AKT3 (Brognard et al., 2007). PP1 has also been assigned participation in the process of AKT inactivation by targeting the molecule’s Thr450 residue (Xiao et al., 2010). Phosphorylation of Thr450 by JNK has been described as a potential priming event for the molecule’s full activation (Shao et al., 2006).

Yet, the complete activation of AKT initiates with the recruitment of this molecule to the plasma membrane by means of interaction of its PH domain with the 3’OH group of PIP3 (Figure 2.2). Since the lipid phosphatase activity of PTEN dephosphorylates the 3-phosphoinositide products of PI3K, the activation of PDK1 and also AKT is substantially regulated by this tumor suppressor. It should be emphasized, however, that PTEN does not directly dephosphorylate AKT. The stability and consequently activity of PTEN seems to be controlled by the phosphorylation of multiple residues in its C-terminal domain (mainly Ser380, Thr382 and Thr383). As constitutively phosphorylated, the molecule remains mostly in the cytosol. Upon dephosphorylation, plasma membrane recruitment takes place as well as rapid degradation, which represents a negative feedback of its activity (Das et al., 2003).
3. Glycogen Synthase Kinase 3

Discovered as one of the kinases capable of phosphorylating and inactivating glycogen synthase (Embi et al., 1980), the final enzyme in glycogen biosynthesis, GSK3 was also, curiously, the first identified substrate of AKT (Cross et al., 1995). In mammalian tissues, GSK3 exists as 2 isoforms (GSK3α and GSK3β) that share 98% homology of their kinase domains, while differing substantially in their N-terminal and C-terminal sequences (Force and Woodgett, 2009). GSK3α has an extended glycine-rich N-terminal tail (Frame and Cohen, 2001) and therefore a higher molecular weight than the beta isoform (52 kDa versus 47 kDa). The two isoforms may or may not have redundant functions (Beurel and Jope, 2008; Doble et al., 2007; Hoeflich et al., 2000; Wilson and Baldwin, 2008) depending on the tissue in question and the targets studied. As an example, GSK3α and GSK3β appear to be redundant in regulating Wnt/β-catenin signaling at the same time that GSK3α primarily regulates glycogen storage in the liver and GSK3β does it so in the skeletal muscle (Force and Woodgett, 2009). Interestingly, total absence of GSK3β is embryonically lethal in mice, due to acute hepatocyte apoptosis (Hoeflich et al., 2000), while GSK3α knockout mice are viable, displaying enhanced glucose and insulin sensitivity accompanied by reduced fat mass (MacAulay et al., 2007). These differential roles are not a result of differences in expression of these isoforms. The curious and somewhat recent work from the Newton laboratory raises the possibility of the isoforms having different affinities when it comes to interacting with regulators such as the AKT isoforms (Brognard et al., 2007). Other possibilities include tissue-specific scaffolds that facilitate binding of one versus the other isoform to specific targets (Force and Woodgett, 2009). Also, two GSK3β species representing alternative splicing of exon 10 have been identified (Schaffer et al., 2003). The longer isoform of GSK3β containing exon 10 seems to be the most prevalent one in all tissues, with the exception of the brain. The functional consequence of exon 10 splicing is unknown.

The substrate recognition of this kinase is rather unusual. It preferentially targets primed phosphorylated molecules that contain the following consensus sequence: Ser/Thr-X-X-X-Ser/Thr-P, where the first Ser or Thr is the target residue, X is any aminoacid (often Pro), and the phospho Ser/Thr, 4 aminoacids C-terminal to the target residue, is the actual primed site. Different kinases will prime this site in different molecules. As an example, β-catenin, a
target of GSK3 in the Wnt signaling pathway, requires priming phosphorylation at Thr45, prior to being phosphorylated by GSK3 on Thr41, Ser37, and Ser33. In this case, casein kinase 1 (CK1) seems to be the kinase responsible for the priming (Hagen and Vidal-Puig, 2002). Although priming phosphorylation remarkably increases the efficiency of the GSK3 kinase activity, it is not strictly required.

As a constitutively active serine/threonine kinase, GSK3 for the most part either inactivates its substrates or flags them for destruction. The E3 ubiquitin ligase β-transducin repeats-containing protein-1 (β-TrCP1), for example, is able to recognize proteins with two phosphoserines located four residues apart. Therefore, some of GSK3 targets, such as β-catenin and c-myc, are targeted for ubiquitination and subsequent destruction by the proteasome (Cohen and Goedert, 2004).

3.1. Regulation of GSK3 activity

GSK3, like other protein kinases such as CDK2, p38γ and ERK2, requires phosphorylation of residues in its activation loop (T-loop) as a prerequisite for activity (Doble and Woodgett, 2003). The T-loop of GSK3α and GSK3β is phosphorylated at Y279 and Y216, respectively. However, although this event might substantially facilitate substrate phosphorylation (>200-fold) (Hughes et al., 1993), it is not absolutely required for kinase activity.

Since GSK3 is constitutively active in most cell types, negative regulation of its activity occurs basically through one of the following mechanisms: by inactivating its kinase domain, by altering GSK3 access to its substrates (protein-protein interactions) or by changing its ability to recognize its substrates (Doble and Woodgett, 2003). The activity of GSK3 kinase is negatively regulated by phosphorylation of the residues Ser21 and Ser9 in the alpha and beta isoforms, respectively. This can be accomplished, for example, upon insulin, IGF1 and EGF stimulation (Cross et al., 1994; Saito et al., 1994; Welsh and Proud, 1993). The phosphorylated serine residue transforms the molecule’s amino terminus into a “pseudosubstrate”, with the phosphoserine occupying the same binding site as the priming
phosphate of the substrate (Cohen and Frame, 2001). This blocks the substrate’s access to the active kinase site of GSK3. PI3K-induced activation of AKT results in AKT phosphorylation of both GSK3 isoforms (Cross et al., 1995) (Figure 3.1), but numerous other kinases can target those serine sites of GSK3, including p90RSK, p70S6K (Sutherland et al., 1993), PKA (Fang et al., 2000; Li et al., 2000) and PKC (Fang et al., 2002). This underscores the importance of GSK3 as a convergence node for multiple pathways and brings light into its potential as a cross-talk mediator.

Figure 3.1. GSK3 activity can be regulated by phosphorylation. In the PI3K pathway, after AKT becomes phosphorylated and fully active, it phosphorylates GSK3α/β on Ser21/9, respectively. This post-translational modification can inhibit the activity of GSK3 towards glycogen synthase. Glycogen synthase, no longer phosphorylated and inhibited, is free to finalize the multistep process of glycogen production, a very important end result of the insulin pathway for glucose utilization.

However, inhibition of GSK3 activity is not always achieved through phosphorylation. In the Wnt signaling pathway, GSK3 plays a central role as the kinase responsible for
controlling the amount of the transcriptional co-activator β-catenin present within the cytoplasm, and ultimately dictating the activity of key developmental gene expression programs. In this pathway, GSK3 regulation does not utilize the same phosphorylation events as in AKT signaling (Wu and Pan, 2010) (Figure 3.2). In the resting state, the multi-protein β-catenin destruction complex is anchored by AXIN1/2 and adenomatous polyposis coli (APC). Casein-kinase 1α (CK1α) and GSK3 sequentially phosphorylate β-catenin, targeting the residues Thr45 (CK1α) and Thr41, Ser37 and Ser33 (GSK3). After being hyperphosphorylated, β-catenin is recognized by β-Trcp, an E3 ubiquitin ligase subunit, and then ubiquitinated and proteasomically degraded (Clevers, 2006; MacDonald et al., 2009). Therefore, in a resting state, levels of free cytosolic β-catenin are kept at a minimum, which prevents it from reaching the nucleus. Without β-catenin, the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins represses the transcription of Wnt target genes. Upon Wnt stimulation, interactions between the frizzled (FZD) receptor and the cytoplasmic protein disheveld (DVL) and between DVL and Axin, are capable of localizing the Axin-GSK3 complex to the plasma membrane (Zeng et al., 2008). There, GSK3 and CK1α sequentially phosphorylate the Wnt co-receptor LRP6 (low density lipoprotein receptor-related protein 6), a post-translational modification that promotes the engagement of LRP6 with the scaffolding protein Axin, disrupting the stability of the β-catenin multi-protein destruction complex (Zeng et al., 2005). Hence, GSK3 interestingly plays both positive and negative regulatory roles in the Wnt signaling pathway. It has also been demonstrated that GSK3 is subject to another form of regulation in Wnt signaling, also by means of protein-protein interactions. After Wnt stimulation, a DVL-FRAT (frequently rearranged in advanced T cell lymphoma) complex may inhibit GSK3 activity towards β-catenin because FRAT can compete with axin for binding to GSK3 (Li et al., 1999).

GSK3 activity is dependent on its subcellular localization, since this protein has been isolated also in the nucleus and mitochondria. While most of the processes discussed previously take place in the cytoplasm, the presence of GSK3 in the nucleus grants access to several of its substrates, including cyclin D1 and c-myc. It has been proposed that GSK3 in the nucleus may have a role in alternative splicing. In the mitochondria, GSK3 regulates apoptosis (Ohori et al., 2008). In terms of regulation of the Wnt pathway, a recent study has implicated the formation of multivesicular bodies (MVBs) as an important regulatory event.
After Wnt stimulation, Wnt and GSK3 are both sequestered in these endosomal vesicles, which prevents the access of GSK3 to its target cytosolic protein β-catenin (Taelman et al., 2010).

**Figure 3.2. GSK3 activity is regulated by protein-protein interactions in the Wnt signaling pathway.** In the resting state, the multi-protein β-catenin destruction complex is anchored by Axin, APC and GSK3. Phosphorylation of APC and Axin by GSK3 induce conformational changes that keep that complex stable. Phosphorylation of β-catenin by GSK3 present in that complex, on the other hand, tags it for ubiquitination and destruction, preventing it from reaching the nucleus. Following the binding of one of the Wnt molecules to the FZD receptor, interactions between FZD and DVL are capable of pulling the Axin-GSK3 complex to the plasma membrane, where GSK3 can phosphorylate the Wnt co-receptor LRP6, a post-translational modification that promotes further engagement of LRP6 with Axin. Also, a DVL-FRAT complex formed after Wnt stimulation competes with Axin for binding to GSK3, again disrupting the structure of the APC-Axin-GSK3 multi-protein β-catenin destruction complex. As a result, β-catenin is no longer phosphorylated by GSK3 and, as its levels rise considerably, β-catenin translocates to the nucleus, where it can act as a transcriptional activator of Wnt target genes.

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3.2. GSK3 substrates

GSK3 regulates over 100 putative proteins, including metabolic and signaling molecules, as well as structural proteins and transcription factors. Since a detailed discussion of how GSK3 regulates all these possible substrates is outside the scope of this brief introduction, we will highlight only a few of its targets.

3.2.1. GSK3 and metabolism

Perhaps one of the best known functions of GSK3 is that of being a negative regulator of glycogen synthesis in the PI3K/insulin signaling pathway. This role was identified back in 1979, when, after being purified from rabbit skeletal muscle, GSK3 was demonstrated to be able to phosphorylate three serine residues of glycogen synthase (or UDP-glucose-glycogen glucosyltransferase), reducing its activity (Embi et al., 1980; Rylatt et al., 1980). Also, inhibition of GSK3 activity results in an approximately twofold increase in glucose uptake due to a similar increase in protein expression of glucose transporter 1 (GLUT1). This is reportedly accomplished via phosphorylation and activation of the tumor suppressor and mTOR inhibitor TSC2 protein, by GSK3 (Buller et al., 2008). Such an extensive and central role in glucose metabolism has granted GSK3 the status of a highly targetable molecule for the treatment of Type II Diabetes Mellitus. GSK3 also negatively affects protein synthesis, by phosphorylating and inhibiting eIF2B (Proud, 2006), as mentioned above.

3.2.2. GSK3 and Wnt signaling

GSK3 participation in the Wnt signaling pathway is of fundamental importance, as previously described. Although regarded for so long to exert only a negative regulatory role in this pathway, by phosphorylating and targeting β-catenin for ubiquitination and degradation, it also participates in the phosphorylation and activation of the Wnt co-receptor LRP6. Axin and APC are also substrates of GSK3 in this pathway, as their phosphorylation enhances the interactions within the β-catenin destructing multi-protein complex, making it more stable. Through keeping β-catenin levels at check in the cytosol, GSK3 prevents its
nuclear translocation and the activation of target genes such as c-myc and cyclin D1, which would ultimately lead to an increase in cell proliferation. Therefore, the idea of therapeutic GSK3 inhibition is contemplated with great concern due to the possibility of malignancy development.

3.2.3. GSK3 and Alzheimer’s Disease

The typical histopathological findings in the brains of Alzheimer’s disease patients consist of multiple deposits of senile plaques composed of amyloid beta-protein, in combination with neurofibrillary tangles that result from hyperphosphorylated tau proteins (Takashima et al., 1993). GSK3 has been investigated as one of the most important links between these two pathological abnormalities. GSK3 not only hyperphosphorylates tau (Kremer et al., 2011), but also is positively regulated by amyloid beta-protein, which increases GSK3 catalytic activity and induces the appearance of tau proteins (Takashima et al., 1993). Studies using transgenic mice overexpressing human tau suggest that formation of tau neurofibrillary tangles might be prevented through inhibition of GSK3 kinase activity (Noble et al., 2005).

Among the known mechanisms that may contribute to the loss of neurons in Alzheimer’s brain disease, apoptosis has also received significant attention (Mines et al., 2011). The intrinsic apoptotic signaling pathway has predominated in studies of Alzheimer’s, and GSK3 mostly promotes this pathway (see discussion below). Therefore, the use of small molecules targeting GSK3 more and more is seen as a rational and promising strategy for the control of this neurodegenerative disorder.

3.2.4. GSK3 and apoptosis

GSK3 paradoxically can both promote and inhibit apoptosis. While GSK3 mostly (but not always) promotes cell death caused by the mitochondrial intrinsic apoptotic pathway, it inhibits the death receptor-mediated extrinsic apoptotic signaling pathway (Beurel and Jope, 2006). GSK3 promotes the intrinsic apoptotic pathway by several means, including the direct phosphorylation and resulting increased activity of Bax, a pro-apoptotic Bcl-2 family
member that elicits cytochrome c release from mitochondria (Linseman et al., 2004). GSK3 also mediates the expression of the pro-apoptotic Bim molecule (Hongisto et al., 2003) and phosphorylates anti-apoptotic MCL-1, targeting it for ubiquitination and degradation (Maurer et al., 2006). The induction of apoptosis is central to the tumor-suppressive activity of p53, which can promote the expression of a number of genes that are involved in apoptosis, including proapoptotic members of the Bcl-2 family. And GSK3β (but not GSK3α) has been demonstrated to phosphorylate and positively regulate the activity of phospho Ser33-p53 (Turenne and Price, 2001). However, during ER stress, GSK3β phosphorylates p53 on different residues (Ser315 ad Ser376), inducing its cytoplasmic localization and preventing its stabilization (Turenne and Price, 2001). The Mdm2 oncoprotein, which regulates abundance and activity of p53, can also be phosphorylated by GSK3. As Mdm2 becomes more stable with this post-translational modification, degradation of p53 is consequently enhanced (Kulikov et al., 2005).

The negative regulatory role of the extrinsic apoptotic pathway by GSK3 became clear when knockout GSK3β mice studies showed embryonic lethality due to massive hepatic degeneration (Hoeflich et al., 2000), a phenotype that was consistent with extensive TNF-induced hepatocyte cell death. Those investigators also were able to link the pro-apoptotic effects of GSK3β’s absence to an impaired transcriptional activity of NF-kappaB. Later studies have discovered the formation of a GSK3 complex with two other proteins called DEAD-box polypeptide 3 (DDX3) and cellular inhibitor of apoptosis protein-1 (cIAP-1) that caps all death receptors in different cell lines (Sun et al., 2008). This restrains the formation of the death-inducing signaling complex (DISC) and subsequent caspase-8 activation. In cancer cells that are resistant to death receptor stimulation, this anti-apoptotic complex is functional, and resistance can be overcome by the use of GSK3 inhibitors.

In conclusion, GSK3 is a complex regulator of apoptosis, displaying a myriad of effects that could both promote and protect from cell death. Further studies are needed to establish the major factors that would predict the final outcome if inhibition of GSK3 activity is undertaken in a variety of settings where apoptosis is a harmful event to be prevented or a desired outcome to be pursued.
3.2.5. GSK3 in Hedgehog signaling

The Hedgehog signaling pathway, first identified in *Drosophila* and one of the key regulators of animal embryo development, now is recognized as aberrantly activated in a variety of cancers, including basal cell carcinoma, medulloblastomas and pancreatic cancers (Katoh and Katoh, 2009). In this pathway, unstimulated cells have the cytosolic *Cubitus interruptus* (Ci) in *Drosophila* and Gli in mammals targeted for proteolysis. The result of such cleavage is the production of a truncated transcriptional repressor that undergoes nuclear translocation and prevents the transcription of Hh target genes. The binding of Hh ligand to patched receptors leads in turn to a rise in the levels of uncleaved Ci, which acts as a transcriptional activator. In *Drosophila*, GSK3 antagonizes Hedgehog signalling by phosphorylating Ci (after a primed phosphorylation by PKA) and facilitating its proteolytic cleavage (Jia et al., 2002).

3.2.6. GSK3 and the JNK pathway

The c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) pathway is one of the three recognized MAPK pathways (the other ones being the ERK and the p38 MAPK pathways). Though a variety of stimuli leads to JNK activation (UV light exposure, heat shock and other stress related conditions) growth factors, especially ligands for GPCRs (G-protein coupled receptors) usually induce only a modest increase in JNK activity, while ERK tends to be markedly active after GPCR stimulation. It has been demonstrated that GSK3 is responsible for inhibiting the activation of JNK following GPCR and EGFR stimulation, while mediating UV light-mediated JNK activation (Liu et al., 2004).

JNK activated proteins include some of the AP-1 transcription factors. The activating protein-1 (AP-1) homodimers and heterodimers consist of basic region-leucine zipper DNA binding proteins involved in the control of cell proliferation, survival and death (Shaulian and Karin, 2001). Those proteins belong mostly to the Jun (c-Jun, JunB and JunD), Fos and activating transcription factors (ATF) subfamilies. AP-1 proteins, after a variety of stimuli
(such as growth factors, inflammatory cytokines and UV radiation), are set to regulate the expression and function of cell cycle regulators. c-Jun is unique in its ability to stimulate cell proliferation by repressing tumor suppressor expression and inducing cyclin D1 transcription (Shaulian and Karin, 2001). The activity of c-Jun is controlled both at the transcription level and at the post-translational level. Different phosphorylation sites regulate its capability to bind to DNA (Morton et al., 2003) and stability. Phosphorylation of the c-Jun C terminal domain by GSK3 leads to the polyubiquitination and degradation of c-Jun (Wei et al., 2005).

3.3. GSK3 mediates signaling upstream of AKT

Work carried out in our lab (Lu et al., 2011) to identify regulators of intracellular signaling has utilized an siRNA screen targeting a total of 541 kinases and kinase-related molecules. Changes in signaling determined through a reverse phase protein array (RPPA) screen looking at 42 phospho and total proteins revealed that genetic ablation of GSK3 significantly blocks AKT phosphorylation, an unexpected observation (Lu et al., 2011).

Further work (Smith, 2010) exploring those findings led to confirmation that in three different cell lines GSK3 presence was necessary for AKT activation and phosphorylation. In GSK3 β/β− mouse embryonic fibroblasts, siRNA targeting the GSK3 α isoform abrogated AKT phosphorylation (Figure 3.3). Also, in the breast cancer cell lines MDA-MB-231 and AU565, targeting both GSK3 isoforms affected negatively AKT phosphorylation and activation (Smith, 2010). It remained unclear if that effect on AKT phosphorylation was dependent on the kinase activity of GSK3. This effect was probably a cell-context dependent signaling mechanism, since other cell lines also screened with siRNA (MDA-MB-415, T47D and MCF7) did not demonstrate dependency on GSK3 presence for AKT phosphorylation.
Figure 3.3. GSK3 mediates EGF signaling to AKT in Mouse Embryonic Fibroblasts (MEFs). GSK3β knockout MEFs were transfected with GSK3α siRNA or control nontargeting siRNA. 48 hours after transfection cells were serum-starved overnight and then stimulated with EGF (60 ng/ml). Cell lysates were analyzed for AKT, AKT phosphorylation (Thr308 and Ser473), and GSK3α. GAPDH was used as a loading control (Smith, 2010).
MATERIALS AND METHODS

4.1. Cells

Tumor cell lines MDA-MB-231, MDA-MB-468, SKBR3, HCC1569, HCC1964, BT474 and WM35 were obtained from the ATCC and cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS). All cells were maintained at 37°C with 5% CO₂.

4.2. Antibodies

Rabbit polyclonal antibodies against AKT, phospho-AKT (Thr308), phospho-AKT (Ser473), β-catenin, phospho-β-catenin (Ser33/37/Thr41), β-actin, glycogen synthase, phospho-glycogen synthase (Ser641), mTOR, Rictor, PDK1, phospho PDK1 (Ser241), phospho-Hsp27 (Ser82), PP2A catalytic subunit, phospho PP1A (Thr320), PTEN, phospho EGFR (Tyr1068), p38 MAPK, phospho p38 MAPK (Thr180/Tyr182), and phospho-Hsp 27 (Ser82) were purchased from Cell Signaling Technology (Beverly, MA). The rabbit monoclonal antibodies against phospho PTEN (Ser380/Thr382/383) (Clone 44A7), IGF-1 Receptor β (Clone 111A9) and phospho-IGF-1 Receptor β (Tyr1135/1136)/Insulin Receptor β (Tyr1150/1151) (Clone 19H7) were also obtained from Cell Signaling Technology (Beverly, MA). The monoclonal mouse antibody against GSK3 α/β (Clone 0011-A) and the rabbit polyclonal antibody against EGFR were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The mouse monoclonal antibody against PRAS40 (Clone 73P21) and the rabbit polyclonal antibody against phospho PRAS40 (Thr246) were obtained from Invitrogen (San Diego, CA). The rabbit monoclonal antibody against PP1A (EP1511Y) was obtained from Novus Biologicals (Littleton, CO). The rabbit monoclonal antibody against phospho PP2A (Tyr307) (Clone E155) was obtained from Epitomics (San Francisco, CA). The mouse monoclonal antibody against GAPDH (Clone 6C5) was purchased from Ambion (Austin, TX). Antibodies against PHLLP1 and PHLLP2 were generously provided from Dr. Alexandra C. Newton’s lab. The antibodies used in the reverse phase protein array experiment have been previously described (Hong et al., 2010).
4.3. Growth factors and small molecule inhibitors

Epidermal growth factor (EGF) was purchased from R&D Systems (Minneapolis, MN) and the insulin growth factor 1 (IGF-1) was purchased from Millipore (Billerica, MA). When cells were stimulated with growth factors, serum-starvation overnight was performed, followed by a 20-minute stimulation, and then lysis of the cells. The concentrations of the growth factors utilized were 60 ng/ml and 50 ng/ml for EGF and IGF-1, respectively. In the reverse phase protein array experiments, the concentrations were 60 ng/ml and 100 ng/ml, for EGF and IGF-1 respectively. The GSK3 inhibitors SB 216763, SB 415286 and CT 99021 were purchased from Sigma (St. Louis, MO). The p38 inhibitor SB 202190 was purchased from Tocris Bioscience (Bristol, UK). All small molecule inhibitors were dissolved in dimethyl sulfoxide.

4.4. siRNAs and Transient Transfections

siRNAs targeting human GSK3α (pooled siRNA L-003009-00), human GSK3β (pooled siRNA L-003010-00), and nontargeting RISC-Free control siRNA were purchased from Dharmacon (Lafayette, CO). Pan-GSK3 siRNA was purchased from Ambion (Austin, TX). Cells were subjected to transient transfections using Lipofectamine™ RNAiMAX Transfection Reagent purchased from Invitrogen (San Diego, CA) according to the manufacturer’s instructions. Most cell lines were able to be efficiently transfected by the forward method, where cells were plated 24 hours prior to transfection at approximately 50% confluence. For the forward transfections, serum-free medium was substituted for regular medium 20 minutes prior to transfection, when siRNAs or RISC-Free control was added to the cells, together with the transfection reagent. Regular medium was then substituted for serum-free medium 6 to 8 hours after transfection. The cell lines T47D and SKBR3 were subjected to reverse transfection, where cells and transfection reagent in combination with siRNAs or RISC-Free control were mixed at the same time cells in suspension were re-platted at approximately 60% confluence. Cells in the reverse transfection were plated in regular, serum-containing medium. The culture medium was
changed after the cells attached to the surface, approximately 24 hours following transfection. In the experiments using transient transfection, cells were typically harvested 72 hours after transfection was performed.

4.5. Western Blotting

Cells were washed by ice-cold phosphate buffered saline (PBS), scraped from the cell culture dish, collected, pelleted and resuspended in lysis buffer pH 7.5 (1% Triton X 100, 10% glycerol, 10 mM EDTA, 10 mM NaCl, 50 mM HEPES), with the addition of the protease inhibitor mixture from Roche Applied Science (Branford, CT), 10µg/mL aprotinin, 1 mM sodium vanadate and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at maximum speed for 15 minutes and the clarified supernatant containing the protein was collected. Protein concentrations were measured by protein assay from Bio-Rad (Hercules, CA) according to the manufacturer’s instructions. Samples containing 25-30 µg of total protein were prepared with lamelli buffer and resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with antibodies following the protocols provided by the manufacturer’s.

4.6. Reverse Phase Protein Array (RPPA)

Cellular proteins were denatured by 1% SDS (with β-mercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab, Bend, OR) using an Aushon 2470 Arrayer (Aushon BioSystems, Inc, Billerica, MA). Each slide was probed with a different validated primary antibody plus a biotin-conjugated secondary antibody using a BioGenex Autostainer and a Dako amplification kit. Slides were then scanned to provide images for densitometry measurement by MicroVigene microarray image analysis software. Relative signal intensities for each antibody were then quantitated using a “SuperCurve” method (http://bioinformatics.mdanderson.org/OOMPA) which fits all data onto a common curve and maps each sample data point onto the curve. Positive and negative controls were present in each slide to ensure quality of slide printing and its analysis. Antibodies used for
RPPA have been validated through correlation of western blot results with RPPA analysis and measurement of the RPPA signal dynamic range.
RESULTS

5.1. GSK3 alpha and beta isoforms play a redundant role in AKT phosphorylation

In the breast cancer cell line MDA-MB-231, the use of siRNA targeting both isoforms of GSK3 caused a remarkable decrease in the phosphorylation of AKT on both sites required for its full activity (Thr308 and Ser473), while pooled siRNA targeting each isoform of GSK3 separately did not (Figure 5.1). AKT phosphorylates proline-rich AKT substrate of 40 kDa (PRAS40) on Thr246 (Kovacina et al., 2003). Levels of phosphoThr246 PRAS40 as measured by western blot correlated with the decrease in AKT phosphorylation indicating that the decreased phosphorylation lead to decrease in AKT activity. Both EGF and IGF were utilized as inducers of PI3K pathway activation in this model and the effect on AKT activation caused by the decrease in GSK3 dosage is seen irrespective of the receptor tyrosine kinase activated. This result underscores the generalizability of the observed effect and discards a feedback loop involving IRS-1 as major cause of attenuation of AKT activation as IRS-1 is not required for EGFR signaling.
Figure 5.1. GSK3 isoforms alpha and beta have a redundant role in AKT phosphorylation in the MDA-MB-231 cell line. MDA-MB-231 cells were treated with transfection reagent alone (mock) or transiently transfected with non-targeting siRNA (scramble), siRNA targeting GSK3α, GSK3β or both GSK3 isoforms. Cells were lysed 72 hours after transfection. Cells were serum-starved the evening before lysis and then stimulated with IGF 50 ng/ml (+) or EGF (+) 60 ng/ml for 20 minutes followed by lysis and immunoblotting. The symbol (-) denotes no growth factor stimulation.

Targeting both isoforms of GSK3 in the Her-2 amplified breast cancer cell line SKBR3, where AKT is activated due to HER2 amplification independent of exogenous ligand, also led to decreased AKT phosphorylation and activity (Figure 5.2).
Figure 5.2. Knock-down of GSK3 isoforms alpha and beta in the HER-2 overexpressing breast cancer cell line SKBR3 impairs AKT activation. SKBR3 cells were transiently transfected with non-targeting siRNA (scramble) or siRNA targeting both isoforms of GSK3. Cells were lysed 72 hours after the transfection. The cells that were serum-starved the evening before lysis were stimulated with IGF 50 ng/ml (+) for 20 minutes followed by lysis and immunoblotting. The symbol (-) denotes no growth factor stimulation.

A much weaker dependency on GSK3 for the phosphorylation of AKT was observed in the melanoma cell line WM35 (Figure 5.3A) and complete independence was seen in the breast cancer cell line T47D (Figure 5.3B). Thus GSK function is required for optimal AKT phosphorylation in many but not all lines and across multiple mechanisms of cellular activation.
5.3 The effect of GSK3 inhibition on optimal AKT phosphorylation is cell-specific. (A) WM 35 melanoma cells were transiently transfected with non-targeting siRNA (scramble) or siRNA targeting GSK3α/β. Cells were lysed 72 hours after the transfection. Cells were serum-starved the evening before lysis and were stimulated with IGF 50 ng/ml (+) for 20 minutes followed by lysis and immunoblotting. The symbol (-) denotes no growth factor stimulation. (B) T47D cells were transiently transfected with non-targeting siRNA (scramble) or siRNA targeting GSK3α/β. Cells were lysed 72 hours after the transfection. Cells were serum-starved the evening before and were stimulated with IGF 50 ng/ml (+) for 20 minutes followed by lysis and immunoblotting. The symbol (-) denotes no growth factor stimulation.

5.2 The kinase activity of GSK3α/β is required for the optimal phosphorylation of AKT

GSK3 is known to be embedded in multi-protein complexes and protein-protein interactions as well as subcellular localization are essential to some of its regulatory functions. Therefore, we asked if the observed effects on AKT phosphorylation could also be demonstrated by the use of small molecules targeting only the kinase activity of GSK3. CT
99021 is an ATP-mimetic that is both a highly potent and specific inhibitor of both GSK3 isoforms (Bain et al., 2007). In MDA-MB-231 cells, treatment with CT 99021 led to a remarkable decrease in AKT phosphorylation, compared to vehicle alone (Figure 5.4). Ser33/37 and Thr41 of β-catenin that are targets for phosphorylation by GSK3 were measured as a marker of GSK3 kinase activity. As expected, total β-catenin levels increased modestly in the presence of GSK3 inhibition. Similar results were observed in two other breast cancer cell lines, HCC 1954 and HCC 1569 (Figure 5.5).

![Figure 5.4](image.png)

**Figure 5.4.** The kinase activity of GSK3α/β is required for the optimal phosphorylation of AKT in the MDA-MB-231 cell line. MDA-MB-231 cells were treated with the GSK3 kinase inhibitor CT 99021 (5µM) for 24 hours or DMSO at 0.025% as a control. Cells were stimulated (+) or not (-) with IGF (50 ng/ml) for 20 minutes following serum-starvation overnight and lysed for immunoblotting.
Figure 5.5. The kinase activity of GSK3α/β is required for the optimal phosphorylation of AKT in two other cell lines. (A) HCC1954 cells were treated with the GSK3 kinase inhibitor CT 99021 (2.5µM and 5µM) for 24 hours or DMSO at 0.025% as a control. Cells were stimulated (+) or not (-) with IGF (50 ng/ml) for 20 minutes following serum-starvation overnight and lysed for immunoblotting. (B) HCC1569 cells were treated with the GSK3 kinase inhibitor CT 99021 (2.5µM and 5µM) for 12 and 24 hours or DMSO at 0.025% as a control. Cells were stimulated (+) or not (-) with IGF (50 ng/ml) for 20 minutes following serum-starvation overnight and lysed.
The effects observed above were not restricted to the drug CT 99021. Two other GSK3 inhibitors, SB 216763 and SB 415286, were tested to see if they could also affect AKT phosphorylation. Compared to vehicle, the use of two different concentrations of those inhibitors led to a decrease in AKT phosphorylation (Figure 5.6).

**Figure 5.6. Inhibition of GSK3 kinase activity with different ATP mimetics leads to a decrease in AKT phosphorylation.** MDA-MB-231 cells were treated with the GSK3 kinase inhibitors SB 216763 at two different concentrations (5µM and 10µM), SB 415286 at two different concentrations (10µM and 20µM) or DMSO as a control, for 24 hours. All cells were stimulated with IGF (50 ng/ml) for 20 minutes following serum-starvation overnight and lysed for immunoblotting.

In BT 464 breast cancer cells, however, despite a remarkable inhibition of GSK3 activity (as measured by reduced levels of phosphorylated Ser641 in Glycogen Synthase), almost no effect on AKT phosphorylation was seen (Figure 5.7), underscoring the cell-specificity of the dependency of GSK3 kinase activity for full AKT phosphorylation and activation.
Figure 5.7. The effect of GSK3 kinase inhibitors on AKT phosphorylation is cell-specific. BT474 cells were treated with the GSK3 kinase inhibitor CT 99021 (2.5µM and 5µM) for 12 and 24 hours or DMSO at 0.025% as a control. Cells were stimulated (+) or not (-) with IGF (50 ng/ml) for 20 minutes following serum-starvation overnight and lysed for immunoblotting.

5.3. Inhibition of GSK3 does not lead to changes in levels of putative kinases involved in AKT phosphorylation

In the canonical PI3K pathway, AKT activation occurs following the phosphorylation of Thr308 and Ser473 residues by PDK1 (Alessi et al., 1997b) and PDK2 respectively. In most conditions and cell types, PDK2 is mTORC2 (Sarbassov et al., 2005). We hypothesized that inhibition of GSK3 could have led to a decrease in the levels of these kinases, which would in turn impact the phosphorylation of AKT. In MDA-MB-231 cells that had been treated with siRNA targeting GSK3α/β and exhibiting a remarkable decrease in AKT phosphorylation and activity, we measured by immunoblotting the total levels of PDK1, as well as phosphorylated Ser241 on its activation loop (necessary for its activity) (Casamayor
et al., 1999), mTOR and RICTOR (Figure 5.8). No differences were detected between control and treatment groups.

![Image](image.png)

**Figure 5.8.** GSK3 inhibition does not affect levels of known kinases involved in AKT signaling. MDA-MB-231 cells were transiently transfected with non-targeting siRNA (scramble) or siRNA targeting both isoforms of GSK3. Cells were lysed 72 hours after the transfection. Cells were serum-starved the evening before the treatment and were stimulated with IGF 50 ng/ml (+) for 20 minutes followed by lysis and immunoblotting.

5.4. Inhibition of GSK3 does not lead to changes in levels of putative phosphatases involved in AKT dephosphorylation

The phosphorylated state of AKT requires an equilibrium between the activities of kinases and phosphatases that target phosphosites. As presented above, Thr308 in AKT is a target of protein phosphatase 2A or PP2A (Ivaska et al., 2002) while the PH domain leucine-rich
repeat phosphatases (PHLPP) 1 and 2 have selectivity for the hydrophobic phosphorylation motif of AKT, Ser473 (Brognard et al., 2007; Gao et al., 2005). Also, PP1A has been demonstrated to regulate AKT1 signal transduction by dephosphorylating the AKT residue Thr450, which seems to be an important phosphorylated residue required to prime AKT for activation (Xiao et al., 2010). Phosphorylation of Tyr307 of PP2A and of Thr320 of PP1A results in inactivation of these enzymes (Janssens and Goris, 2001; Kwon et al., 1997).

We hypothesized that an increase in the total levels or modifications that increase the activity of these important phosphatases could be causes for a decrease in AKT phosphorylation. There was, however, no difference in those levels as measured by immunoblotting following treatment of MDA-MB-231 cells with siRNA targeting GSK3α/β (Figure 5.9).

The phosphatase and tensin homologue PTEN is a tumor suppressor that reverses the action of PI3K by catalyzing the removal of the 3’ phosphate of phosphoinositides, which results in a diminished amount of AKT being mobilized to the plasma membrane for phosphorylation and activation. Several studies have indicated that PTEN is constitutively phosphorylated on Ser370, Ser380, Thr382, Thr383 and Ser385 in the C-terminal tail and dephosphorylation of these residues leads to increased membrane localization and decreased stability (Das et al., 2003). No increase in total levels of PTEN or decrease in its most stable, phosphorylated form was observed in MDA-MB-231 cells treated with siRNA targeting GSK3α/β (Figure 5.9).
Figure 5.9. **GSK3 inhibition does not affect levels of known phosphatases involved in AKT signaling.** MDA-MB-231 cells were transiently transfected with non-targeting siRNA (scramble) or siRNA targeting both isoforms of GSK3. Cells were lysed 72 hours after the transfection. Cells were serum-starved the evening before the treatment and were stimulated with IGF 50 ng/ml (+) for 20 minutes followed by lysis and immunoblotting.

5.5. Inhibition of GSK3 does not lead to changes in levels of receptor tyrosine kinases

Ultimately, PI3K activation initiates with the activation and phosphorylation of the receptor tyrosine kinases (RTKs) implicated in each signaling event. A decrease in total or activated/phosphorylated levels of IGF1R and EGFR upon IGF and EGF stimulation,
respectively, could potentially lead to a decrease in PI3K activation and AKT phosphorylation. However, no differences in receptor levels were detected between treatment groups and controls (Figure 5.10). Thus changes in the various kinases, phosphatases and RTKs known to regulate AKT activity are not sufficient to explain the mechanism by which GSK3 regulates AKT phosphorylation.

Figure 5.10. GSK3 inhibition does not affect levels of growth factor receptors. MDA-MB-231 cells were transiently transfected with non-targeting siRNA (scramble) or siRNA targeting both isoforms of GSK3. Cells were lysed 72 hours after the transfection. The cells that were serum-starved the evening before the treatment were stimulated with IGF 50 ng/ml or EGF 60 ng/ml (+) for 20 minutes followed by lysis and immunoblotting.
5.6. Reverse Phase Protein Array identifies an increase of MAPK phospho-p38 (Thr180) as a result of GSK3 inhibition

After performing a candidate molecule based interrogation on levels of the proteins expected to be involved in the process of AKT phosphorylation, we decided to perform a broader and more comprehensive analysis of the proteomic changes associated with the inhibition of GSK3. Reverse-phase protein arrays (RPPA) use micro-scale, cell lysate dot blots that are printed to a substrate, followed by quantitative immunochemical protein detection (Spurrier et al., 2008). Because it can generate 1,000 times more data points using 10,000 times less sample volume than an ordinary western blot, RPPA provides a high-throughput and objective analysis of multiple proteins in very small amounts of sample. Following 72 hours of transfection of MDA-MB-231 cells with either GSK3α, GSK3β, pan-GSK3 siRNA or control non-targeting siRNA, cells were stimulated with IGF or no growth factor and lysed. Cell lysates were then processed on a reverse phase protein array and stained for 88 antibodies targeting different proteins and phosphoproteins. Two different siRNA experiments were conducted for this analysis. The average expression of the measured proteins was plotted in bar graphs for further visualization and comparison. An expected fall in the levels of GSK3 and phosphorylated AKT at Ser473 and Thr308 residues provided an important validation for the experiments (Figures 5.11A, 5.11B and 5.11C). We then looked for proteomic changes of at least 50% in the treatment groups compared to controls and detected an unexpected increase in the phosphorylated form of the MAPK p38 (Figure 5.11D), indicating the potential activation of this pathway following GSK3 inhibition in this cell line. Western blot analysis of samples from a different experiment in MDA-MB-231 cells confirmed the unexpected increase in phosphorylated p38 levels (pThr180/Tyr182) (Figure 5.11E).
Figure 5.11. Reverse Phase Protein Array identifies an increase of MAPK phospho-p38 (T180) as a result of GSK3 inhibition. MDA-MB-231 cells were transfected with siRNA targeting either GSK3 alpha (siα), beta (siβ), or both isoforms of GSK3 (siα/β) or control non-targeting siRNA (Scr). Seventy-two hours later, cells were stimulated (+) for 20 minutes with IGF (100 ng/ml) or no growth factor (-), following overnight serum-starvation. Cell lysates were processed on a reverse phase protein array and stained for antibodies targeting 88 different proteins and phosphoproteins. The bars represent the average of expression levels obtained in two experiments. (A) Average of GSK3 total levels by RPPA analysis in two different experiments. (B) Average of phospho-AKT S473 levels by RPPA analysis in two different experiments. (C) Average of phospho-AKT T308 levels by RPPA analysis in two different experiments. (D) Average of phospho-p38 T180 levels by RPPA analysis in two different experiments. (E) MDA-MB-231 cells were transfected with non-targeting siRNA (scramble) or siRNA targeting both isoforms of GSK3. Cells were lysed 72 hours after the transfection. Cells were serum-starved the evening before and stimulated with IGF 50 ng/ml (+) for 20 minutes followed by lysis and immunoblotting. The symbol (-) denotes no growth factor stimulation.
5.7. Inhibition of p38 activity restores optimal AKT phosphorylation despite GSK3 inhibition

The p38 mitogen-activated protein kinase (MAPK) pathway is strongly activated by stress, but also plays important roles in the immune response and regulation of cell survival and differentiation (Cuadrado and Nebreda, 2010). MAPK kinase 3 (MKK3), MKK6 and sometimes MKK4 activate p38 MAPK by phosphorylation at Thr180 and Tyr182 (Raingeaud et al., 1995). The two major groups of proteins that are regulated by p38 MAPK-mediated phosphorylation are transcription factors and protein kinases, such as MAPK-activated protein kinase-2 (MAPKAPK-2) (Rouse et al., 1994). MAPKAPK-2 phosphorylates a myriad of targets, including the heat-shock protein of 27 kDa (Hsp27) (Ahlers et al., 1994). The finding of an unexpected increase in phosphorylated p38 levels in MDA-MB-231 cells treated with siRNA targeting GSK3 led us to hypothesize that p38 could be critical for the effect of GSK3 inhibition on AKT phosphorylation and activation in that cell line. To test that hypothesis we used a p38 kinase inhibitor, SB202190, in MDA-MB-231 cells that had been previously treated with siRNA targeting GSK3α/β. We observed that treatment of those cells with SB202190 completely restored AKT phosphorylation in response to IGF in the presence of GSK3 knockdown (Figure 5.12), as measured by immunoblotting. Thus, GSK3, through modulating p38 activity, is required for optimal AKT phosphorylation in MDA-MB-231 cells.
Figure 5.12. Inhibition of p38 activity restores optimal AKT phosphorylation despite GSK3 inhibition. MDA-MB-231 cells were transfected with non-targeting siRNA (scramble) or siRNA targeting both isoforms of GSK3. Forty-eight hours later, cells were treated with 10 μM of the p38 inhibitor SB202190 for 3 hours or vehicle (DMSO). Cells were serum-starved the evening before and were stimulated with IGF 50 ng/ml (+) for 20 minutes followed by lysis and immunoblotting. The symbol (-) denotes no growth factor stimulation.
DISCUSSION

As previously discussed, GSK3 plays a remarkable role in a number of metabolic and cell fate determining events, therefore constituting a desirable target for the treatment of a variety of disorders, some of them increasingly prevalent in the past years, such as Type II Diabetes Mellitus and Alzheimer’s dementia. It is thought, for example, that a significant proportion of the therapeutic effects of lithium as a mood stabilizer stems from the capability of this drug to inhibit GSK3, even though this inhibition is far from selective. Accordingly, a number of novel, potent and fairly selective small-molecule GSK3 inhibitors have been developed (Medina and Castro, 2008). The majority of them constitute ATP-competitive inhibitors, and some new compounds exhibit substrate-competitive inhibition activity. None of these inhibitors has demonstrated isoform selectivity so far. SB 216763 and SB 415286 are two potent and selective maleimide compounds that, by ATP-competitive action, inhibit GSK3 with K(i)s of 9 nM and 31 nM, respectively (Coghlan et al., 2000). In the presence of 0.1 mM ATP, 10 µM SB 216763 and SB 415286 inhibit GSK3 kinase activity by 96% and 83%, respectively (Coghlan et al., 2000). CT 99021 is even a more potent and specific inhibitor of GSK3, capable of not inhibiting any other kinase at 1 µM, except for CDK2-cyclin A (IC50 – 1.4 µM) (Bain et al., 2007). Therefore, it has been recommended using CT 99021 to inhibit GSK3 in cells, as it is the most potent and specific inhibitor available (Bain et al., 2007). Currently, tideglusib (NP 031112), a non-ATP competitive GSK3 inhibitor of the thiadiazolidindione family, has been granted fast track status by the Food and Drug Administration for the treatment of the neurodegenerative disorder progressive supranuclear palsy. This compound is also undergoing Phase II trials in Alzheimer’s disease patients.

However, one of the major concerns regarding the use of GSK3 inhibitors stems from the knowledge that this molecule exerts pivot antagonistic effects to major pathways involved in cell proliferation, such as Wnt, PI3K and JNK/c-Jun. What if prolonged GSK3 inhibition leads to the activation of mediators of cell proliferation and tumor promotion? In this study, we document the unexpected dependency of AKT, the major effector node in the PI3K pathway, on GSK3. It is only when GSK3 is present that AKT becomes phosphorylated at Thr308 and Ser473 residues, with an observed increased phosphorylation of typical targets.
This is an important shift from the current paradigm of GSK3 as a tumor suppressor. Through the use of both siRNA technique and small, very potent and specific GSK3 inhibitors, we have been able to demonstrate that GSK3 activity is needed for AKT to become fully active in a variety of cell lines, including human breast cancer and human melanoma. Previous work in our lab has confirmed the need of GSK3 for the full activation of AKT in murine embryonic fibroblasts, a non-malignant cell line.

Although GSK3 isoforms do not always demonstrate overlapping functional roles, in our studies either GSK3α or GSK3β presence is sufficient to promote AKT activation/phosphorylation. It is only when siRNA targeting both GSK3 isoforms is effectively utilized that AKT phosphorylation and activation is impaired. Since these isoforms share 98% homology within their kinase domains, but differ substantially in their N- and C-terminal sequences (Force and Woodgett, 2009), we raised the question whether the effect on AKT phosphorylation was dependent primarily on the kinase activity of GSK3. GSK3 is well known for regulating other molecules by means of protein-protein interactions, and this is mostly exemplified in the Wnt signaling pathway. Using three different ATP-competitive inhibitors of GSK3, we have recapitulated the effect we had seen initially with RNA interference, establishing that the kinase activity of GSK3 is needed for full AKT activation and phosphorylation. Ideally, we would like to confirm this finding in GSK3 kinase inactive mutants, and this idea should be tested in future experiments.

The ability of GSK3 to phosphorylate insulin receptor substrate-1 (IRS-1) has been described as a potential inhibitory mechanism for insulin resistance in Type 2 Diabetes (Eldar-Finkelman and Krebs, 1997; Liberman and Eldar-Finkelman, 2005). As IRS-1 is an important docking molecule for IGF-1R (Baserga, 1999), we raised the question whether the observed effect of GSK3 inhibition on AKT activation was not another representation of a feedback loop involving IRS-1. However, we were able to detect similar effects when EGF was used as a stimulus for the PI3K pathway activation and in cells where the PI3K pathway is activated by overexpression of HER2 indicating that the effect of GSK3 on AKT phosphorylation cannot be explained solely by feedback inhibition of IRS-1. Dependency on GSK3 is also demonstrated in the non-stimulated states of cells that already bear a high
baseline level of AKT activity and phosphorylation (HER2 overexpressing cell lines SKBR3 in Figure 5.2 and HCC 1954/HCC 1569, in Figure 5.5).

Cell lines with different genetic mutations have been interrogated for this effect of GSK3 on AKT phosphorylation (Table 1). At this point, we have not been able to establish a commonality in the signaling processes that drive proliferation and survival in these cell lines and that could explain the mechanism of this observed effect. Having said that, one possible pattern can be described here. The one that mostly stands out is the fact that all HER2 overexpressing cell lines tested displayed either dramatic or some effect on AKT phosphorylation after GSK3 inhibition. HER2, one of the 4 members of the EGFR/ErbB RTK family, upon homodimerization or heterodimerization (Wang et al., 2011) with other members of the EGFR family, commands the activation of PI3K and MAPK pathways (Karunagaran et al., 1996; Waterman et al., 1999), leading to increased proliferative and migration capabilities. PI3K pathway activation in breast cancers, defined as PTEN loss and/or PIK3CA mutations, has been associated with resistance to trastuzumab and lapatinib and also shorter survival times (Wang et al., 2011). Trastuzumab resistance has also been associated with signaling ignited by IGF1R (Nahta and Esteva, 2006). Therefore, it is at least very encouraging to find that GSK3 inhibition in the setting of HER2 overexpression negatively impacts the activation of AKT, the major effector node of the PI3K pathway. A greater number of HER2 overexpressing cell lines needs to be screened and the interaction of GSK3 inhibitors with HER2 targeting therapy should be investigated.
Table 1. Cell lines screened for the effect of GSK3 inhibition on AKT phosphorylation. Depicted in green are the cell lines where GSK3 presence/activity is clearly necessary for full AKT phosphorylation and activation. In yellow, the cell lines where GSK3 inhibition decreases some of AKT activation/phosphorylation, but not dramatically. In red, cell lines where no effect was observed in AKT phosphorylation following GSK3 inhibition.

In an attempt to determine if inhibition of GSK3 activity was ultimately leading to a change in the levels of the putative kinases and phosphatases involved in the canonical AKT activation pathway, we measured such levels by immunoblotting following RNA interference targeting GSK3. We could not detect changes that could potentially explain such a change in levels of AKT phosphorylation and activation. Moreover, the levels of IGF1-R and EGFR, as well as their respective active and phosphorylated forms, remained unchanged following GSK3 inhibition. We are aware though of the multiplicity of steps involved in AKT activation, including other post-translational modifications and changes in subcellular localization that can prime that molecule for migration to the plasma membrane vicinities and its further interaction with PIP3, PDK1 and PDK2. For example, it has been suggested that phosphorylation of Tyr315 and Tyr326 by Src is a step necessary for the full
activation of AKT (Chen et al., 2001). Also, in a recent study, it has been demonstrated that, in addition to phosphorylation, AKT undergoes lysine-63 chain ubiquitination, a process mediated by the E3 ubiquitin ligase TRAF6 (TNF receptor associated Factor 6) (Yang et al., 2009). K63 ubiquitination regulates protein trafficking and in this case it leads to increased mobilization of AKT to the plasma membrane and increased phosphorylation on Thr308 and Ser473. We have not investigated if other post-translational modifications of AKT such as tyrosine phosphorylation or ubiquitination were affected by GSK3 inhibition. We have also not yet determined if GSK3 inhibition ultimately leads to changes in AKT subcellular localization. These all constitute future directions for the current study. A myriad of molecules has been described in one cell system or another to interact with AKT and interfere with its activation. The list of proteins that act as scaffold molecules allowing for proper interaction of AKT with its kinases and also phosphatases is extensive and beyond the scope of this discussion.

Reverse phase protein array technology has recently emerged as a tool that allows measuring and profiling signaling pathways (Hennessy et al., 2011; Kornblau et al., 2009). By assessing the expression and post-translational phosphorylation levels of proteins, RPPA provides information that cannot be obtained by gene microarray analysis. Since the previous investigation of the levels of kinases and phosphatases involved in the canonical activation pathway of AKT provided no evidence of remarkable changes in that cell line, we utilized this tool to uncover unexpected changes in signaling that could stem from GSK3 knockdown. In two different experiments in the MDA-MB-231 cell line, phosphorylated and therefore active MAPK14 or p38 levels were elevated by at least 50%. This was independent of growth factor stimulation.

While the p38 MAPK pathway has very important roles in the control of cellular responses to different stresses, such as heat and osmotic shock as well as UV radiation, it can also control the proliferation, differentiation, survival and migration of specific cell types. The functions of p38 MAPK in cancer development are certainly complex, and while some cells use this signaling pathway to antagonize cell proliferation, others subvert it to facilitate proliferation, survival and invasion (Wagner and Nebreda, 2009). There are 4 genes that encode p38 MAPKs: MAPK14, MAPK11, MAPK12 and MAPK13 (which encode p38α,
p38β, p38γ and p38δ, respectively). Most of the published literature on p38 MAPKs refers to p38α, as it is the most abundant and ubiquitous of the p38 isoforms (Wagner and Nebreda, 2009). The serine/threonine kinase p38α is activated by dual phosphorylation of its Thr180 and Tyr182 (Raingeaud et al., 1995), typically after stimulation with pro-inflammatory cytokines and environmental stress. The classic activating kinases are the MAP2Ks MKK3 and MKK6 (MKK4, a established activator of JNK, has also been described to phosphorylate p38). Several different molecules can possibly function as MAP3Ks (or kinases of MAP2Ks) for MKK3 and MKK6, including ASK1 (apoptosis signal-regulating kinase 1), DLK1 (dual leucine zipper-bearing kinase 1) and TAK1 (TGFβ-activated kinase 1) (Cuadrado and Nebreda, 2010). Once active, p38 MAPK targets innumerous substrates, mostly kinases (such as MAPK-activated kinase 2 or MAPK2) and transcription factors. Hsp27 is one of the targets of MAPK2 and its phosphorylation status correlates with p38 activity. The stimulation and activation of p38 MAPK by growth factors is typically poor.

The relationship between AKT and p38 has been described as antagonistic or as cooperative at times, depending on the cell-context. For instance, cells expressing constitutively active PI3K and AKT can have inhibited p38 activation (Berra et al., 1998), and the mechanism for that might be the MEKK3 phosphorylation and inhibition of its activity, leading to down-regulation of MKK3/6 and p38 activation (Gratton et al., 2001). In concordance with those observations, another study investigated the interplay between AKT and p38, and established a negative regulatory role for AKT in p38 activation through the phosphorylation and consequent inhibition of ASK1, a known MAP3K (Liao and Hung, 2003). Other studies, relying mostly on work with chemical inhibitors, have ascribed a positive regulatory role for p38 of AKT. In human neutrophils, for example, p38-dependent active MAPK2 can function as PDK2 for AKT (Rane et al., 2001).

Interestingly though, there is some evidence that GSK3 and p38 participate directly in a feedback loop in different cell systems. For instance, p38 has been implicated in the phosphorylation and inhibition of GSK3β, by targeting its residue Thr390 (Al-Mulla et al., 2011; Thornton et al., 2008). On the other hand, GSK3β can prevent p38 activation by binding to MEKK4 (a possible MAP3K for p38) and preventing its dimerization. (Abell et
In our study, the use of a p38 inhibitor in MDA-MB-231 cells was able to revert completely the negative effects on AKT phosphorylation caused by GSK3 inhibition. This validated the unexpected finding that stemmed from a broad proteomic analysis in that cell line. However, it would be interesting not only to test this finding through inhibition of p38 using a different technique, but also to investigate its generalizability in other cell lines.

GSK3, like many other molecules that participate in the convergence of multiple signaling axes, could, depending on the specificities of each tissue, the presence or lack of different stresses or stimuli, and possibly even the duration of such events, promote different outcomes in terms of cell growth, differentiation and proliferation. Interestingly, a recent study has implicated GSK3β, but not GSK3α, as a kinase able to phosphorylate and inhibit Rictor (essential for the kinase function of mTORC2) by targeting it at Ser1235, under ER stress (Chen et al., 2011). This could explain the fall in AKT phosphorylation and activity that is seen in situations that trigger ER stress, such as hyperosmolar states, and provides emerging corroboratory evidence for a regulatory role that GSK3 possesses in AKT activation.

Since GSK3 is rapidly becoming an attractive target for the treatment of multiple disorders, considerable concern arises with interfering with the activity of a protein long regarded as a tumor suppressor. In this study, we demonstrate that, in a variety of cell lines, GSK3 kinase activity is necessary for the optimal phosphorylation and activation of AKT. These findings add to an emerging number of studies that implicate GSK3 as a driver of tumorigenesis, rather than a tumor suppressor. For example, ablation of GSK3β in colorectal cancer cells activates p53-dependent apoptosis and antagonizes tumor growth (Ghosh and Altieri, 2005). Specifically, inhibition of GSK3 activity can decrease the proliferation of human colon cancer cells in rodents (Shakoori et al., 2007). GSK3 activity has also been linked to proliferation of ovarian cancer cell lines, with the use of pharmacological inhibitors suppressing cancer cell growth in vitro and in vivo (Cao et al., 2006). Similar findings have been described in multiple myeloma (Cao et al., 2006) and chronic lymphocytic leukemia cells (Ougolkov et al., 2007).
CONCLUSIONS

GSK3 is required for optimal phosphorylation and activation of AKT in different malignant cell lines as well as murine fibroblasts. The isoforms GSK3α and GSK3β have a redundant role in regulating AKT activation. This effect is independent of the type of growth factor utilized. It can also be observed in basal states, in HER2 overexpressing cell lines. The kinase activity of GSK3 is required for this effect on AKT activation, since very specific ATP-competitive inhibitors of GSK3 reproduce the effect seen with RNA interference targeting GSK3. The levels of the putative kinases and phosphatases typically involved in AKT activation and de-phosphorylation were found to be unchanged after GSK3 inhibition. And so were the levels of the growth receptors. Analysis of a reverse phase protein array screen in MDA-MB-231 cells revealed a greater than 50% increase in the levels of phospho-p38, independent of growth factor stimulation. A pharmacological inhibitor of p38 rescued completely AKT phosphorylation and activation in the presence of GSK3 inhibition, suggesting this molecule plays an important role in this signaling event. GSK3 might well constitute a desirable target for cancer treatment in the future.


angiogenesis is induced by sustained Akt signaling and inhibited by rapamycin. Cancer Cell 10, 159-170.


VITA

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