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Regulatory And Functional Aspects Of Foxo3a Transcription Factor And Their Implications In Prostate Cancer

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REGULATORY AND FUNCTIONAL ASPECTS OF FOXO3A TRANSCRIPTION FACTOR AND THEIR IMPLICATIONS IN PROSTATE CANCER

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Advisor Date

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DEDICATION

I would like to dedicate this work to my husband, Nathan. You have always been there for me, encouraging me to persevere and believing in my abilities to fulfill my aspirations of becoming a scientist.

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First and foremost I would like to thank my two mentors, Dr. Guri Tzivion and Dr. Rafael Fridman. Guri, you have been an excellent mentor and I have the highest regard for your scientific knowledge and skill and consider myself privileged to have been able to work with you for so many years. Rafael, I want to thank you for taking me into your lab and guiding me through the completion of my graduate work.

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CHAPTER 1: INTRODUCTION

1.1 FoxO Transcription Factors: Background

The FoxO transcription factors are a family of Forkhead box proteins that include FoxO1a, FoxO3a, FoxO4 and FoxO6. The FoxO proteins are evolutionary conserved transcriptional regulators from *C.elegans* to mammals and function downstream of the insulin-PI3K/AKT pathway (Hannenhalli and Kaestner, 2009). They are similar in sequence and contain the highly conserved winged-helix domain, which mediates DNA binding. The FoxO transcription factors are involved in diverse cellular processes including cell proliferation, apoptosis, ROS (reactive oxygen species) response and longevity. FoxO target genes include pro-apoptotic proteins such as Bim (O'Connor et al., 1998), Bcl-6 (Tang et al., 2002) and Fas ligand (Brunet et al., 2001), the cell cycle regulators $p27^{KIP}$ (Medema et al., 2000),(Dijkers et al., 2002) and Gadd45 (Tran et al., 2002) as well as the oxidative response proteins MnSOD (Kops et al., 2002) and caveolin-1 (van den Heuvel et al., 2005). Given their role in proliferation and apoptosis and the frequent deregulation of their upstream effectors in cancer (Carnero et al., 2008), FoxO proteins and their target genes are considered attractive targets for cancer therapy (Myatt and Lam, 2007, Dansen and Burgering, 2008).

Cellular Role	Target Gene
Metabolism	PCK2 ↗
	Glucose-6-Phosphate ↗
	SCPx ↗
	HMG-Co-reductase ↗
	LPL ↗
	PPARGC1A ↗
Cell Cycle Arrest	$p27^{kip}$ ↗
	$p21^{wav}$ ↗
	p130 RB2 ↗
	Cyclin D ↘
	Cyclin G2 ↗
Apoptosis	Bim ↗
	FASLG ↗
	PUMA ↗
	TRAIL ↗
	BCL6 ↗
	Bcl-xL ↗
DNA Repair	Gadd45A ↗
Oxidative Stress Response	MnSOD ↗
	Catalase ↗
	Peroxiredoxin III ↗

Table 1. FoxO Target Genes: List of FoxO target genes, their cellular functions and effects of FoxO on their transcription (↗, up; ↘, down) [reviewed in (Weidinger et al., 2008)].

1.2 PI3-Kinase-Akt Pathway: Upstream Regulation of FoxO3a

The first receptor implicated in regulation of FoxO was the IGF-1 (Insulin-like Growth Factor-1) receptor. Upon cell stimulation with IGF-1, activation of phosphoinositide-3 kinase (PI3K) leads to activation of Akt, which translocates to the nucleus and phosphorylates FoxO, resulting in the exclusion of FoxO from the nucleus and inactivation of its transcriptional activity (Zheng et al., 2000b).

EGFR (Epidermal Growth Factor Receptor) is another receptor that can negatively regulate FoxO transcription factors through the PI3K-Akt pathway. Once ligand binding activates EGFR (i.e. EGF), it forms either a homodimer with another EGFR molecule or a heterodimer with one of the other three HER (Human Epidermal Receptor) receptors, resulting in activation of the intracellular receptor tyrosine kinase (RTK) through trans-tyrosine phosphorylation. Activation of the RTK can also be accomplished by other tyrosine kinases, such as Src and Abl (Henson and Gibson, 2006). Upon activation of the RTK, several pathways, including the MAP (Mitogen-Activated Protein) kinase pathway (Ras-Raf-MEK-ERK) and the PI3K-Akt pathway, are activated. Activation of these pathways promotes cell growth and proliferation, accompanied by inhibition of apoptosis (Henson and Gibson, 2006).

Class Ia PI3K molecules are important in activating PDK (3-phsophoinositide-dependent kinase) and Akt. This class of enzymes transmits signals directly from RTKs and G proteins (e.g. Ras) (Katso et al., 2001). PI3Ks are made of a heterodimer composed of a regulatory and a catalytic subunit. The regulatory subunits for class Ia PI3Ks include p85α, p85ß, p50α, p50ß, p55α and p55γ. All class Ia regulatory subunits contain two SH2 domains that bind to adaptor proteins (e.g. IRS-2) as well as Ras (Rodriguez-Viciana et al., 1994). The main function of these

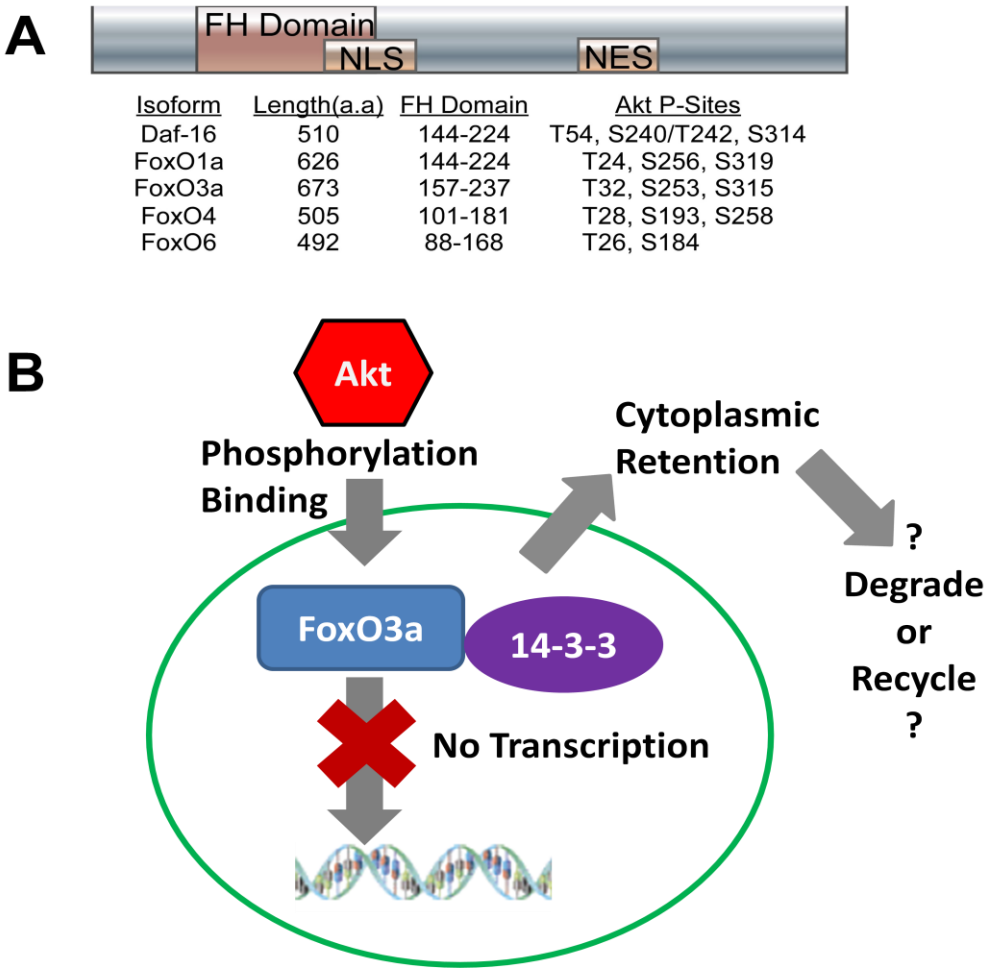
proteins is to recruit the catalytic p110 subunits to the plasma membrane to interact with tyrosine phosphorylated proteins and cause activation of various downstream pathways (Katso et al., 2001). The catalytic subunits of class Ia PI3Ks include p110α, p110ß and p110γ. When p85 is phosphorylated, p110 is activated. However, when p85 is unphosphorylated, it acts to inhibit p110 from converting the lipid $PI(4,5)P_2$ (Phosphatidylinositol bisphosphate) into $PI(3,4,5)P_3$ (Phosphatidylinositol trisphosphate). PIP_3 recruits PH (pleckstrin homology) domain-containing proteins to the plasma membrane (e.g. PDK1 and Akt). PTEN (Phosphate and tensin homologue deleted on chromosome 10) acts as an inhibitor of this process (Katso et al., 2001). PTEN dephosphorylates PIP_3, thus terminating the signal (Gericke et al., 2006). When PTEN is inactivated, there is an increase in PDK1 and Akt activation (Biondi, 2004), which affects many downstream molecules including the Forkhead transcription factors.

Several factors induce Akt activation including EGF, PDGF, insulin, IGF-1 as well as synthetic phosphatase inhibitors (i.e Calyculin A, Vanadate and Okadaic acid) (Galetic et al., 1999). Once PIP_3 has been generated, it attracts Akt and PDK1 to the plasma membrane through their PH domains and allows Akt to become phosphorylated and activated. Akt contains two amino acid residues that must be phosphorylated before it can be fully activated. The first is Threonine 308 located on the P-loop of the protein kinase domain. It has been shown that PDK1 catalyses phosphorylation at this site once both Akt and PDK1 are brought into close proximity by PIP_3 (Coffer et al., 1998). The second phosphorylation site is Serine 473, located in the regulatory domain located at the carboxy-terminus (Galetic et al., 1999). The kinase that phosphorylates and activates Akt on S473 was discovered to be mTORC2 (mTOR complex 2), which is composed of mTOR, GßL and rictor (Sarbassov et al., 2005). Once Akt is

phosphorylated and activated, it translocates to the nucleus where it phosphorylates many different substrates, including the FoxO transcription factors.

1.3 Direct Regulation of FoxO3a by Akt and 14-3-3

Experimental studies have largely focused on the regulation of FoxO by the PI3K pathway via Akt (Brunet et al., 1999, Tang et al., 1999, Obsil et al., 2003). These studies demonstrated that Akt phosphorylates FoxO3a on three residues: threonine 32, serine 253 and serine 315 (Brunet et al., 1999, Biggs et al., 1999, Tang et al., 1999). This phosphorylation leads to negative regulation by inducing the nuclear exclusion of FoxO, a step mediated by a family of adapter proteins designated 14-3-3 (Figure 1B). Additional details of this mechanism were discovered through studying the *C. elegans* FoxO, Daf-16. Phosphorylation of Daf-16 by Akt promoted its association with 14-3-3, which in turn blocked Daf-16 DNA binding and resulted in its cytoplasmic retention (Cahill et al., 2001). These details were confirmed with human FoxO in subsequent studies (Brunet et al., 2002, Obsil et al., 2003). The role of 14-3-3 in FoxO-DNA binding has been shown to be a complex phenomenon. Work performed with FoxO4 implicates the first two Akt phosphorylation sites on FoxO4 as 14-3-3 binding sites, both of which are required for strong FoxO-14-3-3 association. If either site is eliminated, there is a low affinity 14-3-3 interaction that allows for FoxO4-DNA binding (Obsil et al., 2003).



Isoform	Length(a.a)	FH Domain	Akt P-Sites
Daf-16	510	144-224	T54, S240/T242, S314
FoxO1a	626	144-224	T24, S256, S319
FoxO3a	673	157-237	T32, S253, S315
FoxO4	505	101-181	T28, S193, S258
FoxO6	492	88-168	T26, S184

Figure 1.1. FoxO Subfamily Protein Structure and Regulation by Akt and 14-3-3: The FoxO transcription factors all contain the conserved Forkhead (FH) or winged helix domain, which is composed of three α-helixes, three ß-strands and 2 wing-like loops (Boura et al., 2007). They also include a Nuclear Localization Sequence (NLS) and a C-terminus Nuclear Exclusion Sequence (NES) (**A**). Once Akt is activated, it will phosphorylate FoxO3a (T32, S253, S315), which open docking sites for the adaptor protein, 14-3-3. Once p-FoxO3a is bound by 14-3-3, it prevents association with DNA and causes nuclear exclusion. Once in the cytoplasm, p-FoxO3a can either be degraded by the proteosome or recycled back into the nucleus (**B**).

After phosphorylation of FoxO by Akt and cytoplasmic retention by 14-3-3, FoxO may be degraded via the proteosome (Plas and Thompson, 2003) (Figure 1B). This negative regulation of FoxO can be inhibited by the addition of PI3K inhibitors, such as LY294002 (Aoki et al., 2004). Later studies established Skp2 as a probable E3 ligase for FoxO1a (Huang et al., 2005) and that FoxO1a could be rescued from degradation by dephosphorylation by PP2a or a PP2a-like enzyme (Yan et al., 2008). However, despite these reports of FoxO3a degradation after cytoplasmic localization, there is also evidence that FoxO3a can be salvaged through dephosphorylation and back into the nucleus (Birkenkamp and Coffer, 2003). Finally, another study in *Arabidopsis* suggested that the actually binding of 14-3-3 to FoxO prevented its proteolytic cleavage (Cotelle et al., 2000). Therefore, it appears that the regulation of 14-3-3 of FoxO is far from complete and requires further study.

As mentioned above, Akt phosphorylates FoxO3a on three residues (Fig. 1.1A). These residues are located at an Akt phosphorylation recognition motif, RxRxxS/T, with the first site (T32) also containing a perfect 14-3-3 consensus binding motif RxxxpS/TxP (Yaffe et al., 1997). A triple mutation of the Akt phosphorylation sites has been shown to give rise to a reported constitutively active FoxO, which is primarily located in the nucleus, and can reverse and/or suppress cellular transformation (Kikuchi et al., 2007, Yang et al., 2005, Park et al., 2005). The individual Akt phosphorylation sites have been further analyzed by single mutations. It appears that the central phosphorylation site on FoxO1a, S256 (S253 in FoxO3a), is necessary for the phosphorylation of the T24 site (Nakae et al., 2000, Rena et al., 2001). The FoxO1a S256A mutant displays a dominant negative effect on Akt activity, raising the question of whether the triple mutant also functions as a dominant negative form for Akt rather than being a simply constitutively active variant. One drawback to using the constitutively active 3XA FoxO3a

variant is the inability to differentiate between negative regulation of Akt phosphorylation and 14-3-3 binding on FoxO3a's transcriptional activity. By identifying the key negative regulatory event, we could determine which event, Akt phosphorylation or 14-3-3 binding, would be the more effect therapeutic target.

Protein-protein interactions are another mode by which Akt can regulate and be regulated. Akt not only phosphorylates FoxO3a, but can form a stable interaction with this protein as well as with Daf-16 [(Zheng et al., 2000a) and unpublished data]. Akt binding proteins themselves can be divided into three main classes. First, there are proteins that can interact with and regulate Akt. The second class is comprised of proteins that are regulated by Akt via the protein-protein interaction. Finally, Akt can bind to a number of its phosphorylation substrates (reviewed in (Brazil et al., 2002)). The first member identified to be regulated solely by Akt binding was JIP (JNK interacting protein), which would prevent JIP from integrating into the JNK complex within neurons (Kim et al., 2002). This binding can either be advantageous (Ahn et al., 2006, Gervais et al., 2006, Lee et al., 2008) or detrimental to the activity of the binding substrate (Sun et al., 2004). A number of Akt phosphorylation substrates can form stable interactions with Akt (Goswami et al., 2005, Tang et al., 2007, Klein et al., 2005, Vandermoere et al., 2007), with the interaction having an inhibitory effect, such as with Par-4 and Merlin (Goswami et al., 2005, Tang et al., 2007), or a beneficial role in the activity of the substrate, as is the case with VCP and actin (Klein et al., 2005, Vandermoere et al., 2007). These studies demonstrate that Akt regulation can be exceedingly complex and occur by various mechanisms other than phosphorylation. Therefore, we wanted to determine the critical residues on FoxO3a that facilitate the Akt/FoxO3a interaction to gain insight on the purpose and role it plays in Akt regulation of FoxO3a.

1.4 The Role of FoxO3a in Prostate Cancer as a Tumor Suppressor

Many of the upstream regulators of FoxO are deregulated in cancer [reviewed in (Yang and Hung, 2009)], leading to a loss of FoxO transcriptional activity. In addition, FoxO itself has been determined to be a tumor suppressor (Paik et al., 2007). Loss of FoxO has been observed in cancers and its cytoplasmic localization and phosphorylation state are being used as a tumor prognostic factor in cancers such as gliomas, lymphomas, breast and prostate cancers (Chandramohan et al., 2008, Samuels et al., 2004).

FoxO3a is rendered nonfunctional in AML (acute myeloid leukemia) where it has been shown to form a fusion protein with MLL, which prevents its tumor suppressive function through the loss of the Forkhead, DNA binding domain (So and Cleary, 2003).

In prostate cancer, changes in several different pathways can lead to the inhibition of the tumor suppressive functions of FoxO3a. In early stages, most prostate cancers are shown to be androgen dependent. The androgen receptor was found to inhibit FoxO transcriptional function through the formation of a protein-protein interaction with FoxO1a and preventing its DNA-binding to target genes such as the Fas ligand (Li et al., 2003). After acquiring androgen independence, it is thought that the loss of FoxO activity is due predominantly to activation of the PI3K-Akt pathway. Also, chromosomal deletion at 13q14 is common in prostate cancer, resulting in the loss of FoxO1a (Dong et al., 2006). This same study also provided evidence that FoxO1a is transcriptionaly downregulated. Mutations in PTEN are common in late stage prostate cancer, leading to high levels of active Akt and continuous inhibition of FoxO, coupled with the loss of pro-apoptotic and cell cycle regulatory gene transcription (Modur et al., 2002).

1.5 Aims & Hypotheses

The FoxO family proteins are the main transcriptional regulators directly controlled by the PI3K-Akt pathway. Since PTEN loss is observed in approximately 70% of all human prostate cancers, leading to the inactivation of the FoxO3a transcription factor, FoxO3a emerges as a valid therapeutic target in these cancers. Thus, a better understanding of the mechanisms regulating FoxO3a activity downstream of PTEN through Akt and 14-3-3 could offer possible lines of therapy that increase FoxO3a activity.

Aim 1: Examine the regulation of FoxO3a by Akt and 14-3-3: A) Determine the mechanism of Akt-FoxO3a interaction and its effects on FoxO3a activity. B) Determine the role of Akt activity state on Akt-FoxO3a interaction. C) Examine the role of 14-3-3 in FoxO3a regulation.

Aim 2: Examine the regulation of FoxO3a transcriptional activity by Akt and 14-3-3: A) Determine FoxO3a transcriptional activity in cells with both regulated and deregulated PI3K-Akt pathway. B) Differentiate between the regulatory effects of Akt and 14-3-3 on FoxO3a transcriptional activity.

Aim 3: Determine the effects of reintroducing active FoxO3a in a prostate cancer cell model that has low endogenous FoxO3a activity due to PTEN loss.

The goal of these aims is to establish a more comprehensive view of the regulation of FoxO3a transcription factor by Akt and 14-3-3 and to demonstrate its potential use in prostate cancers carrying PTEN inactivation.

Hypotheses:

1) Akt-FoxO3a binding may involve the three Akt-phosphorylation recognition motifs present on FoxO3a.

2) 14-3-3 binding and not Akt phosphorylation of FoxO3a per se, serves as the main effector in the negative regulation of FoxO3a transcriptional activity.

3) Reactivation of FoxO3a transcriptional activity would be sufficient for inducing cell death or reversal of the transformed phenotype in a prostate cancer cell model that has deregulated PI3K-Akt pathway activity due to PTEN loss.

CHAPTER 2: MATERIALS & METHODS

2.1 Reagents

The p-FoxO1a/3a (T24/T32) (#9464), p-FoxO3a S256 (#9466), p-Akt S473 (#9271), FoxO3a (#9467), Akt (#9272), GST (#2624), PARP (#9542), Cleaved Caspase 3 (#9664) and AIF (#4642) antibodies were obtained from Cell Signaling Technology. Tubulin monoclonal antibody was purchased from Sigma Aldrich. GAPDH is from Trevigen. The HA mouse monoclonal antibody was produced in our laboratory from hybridomas. Cyclohexamide (239764) and LY294002 PI3 Kinase inhibitor (440202) were purchased form EMD/Calbiochem. Calyculin A (19-139) was purchased from Upstate/Chemicon. The MG132 proteosome inhibitor (C2211) was purchased from Sigma-Aldrich.

2.2 DNA Constructs and Mutagenesis

The pcDNA3-FoxO3a-HA tagged protein (and all FoxO3a variants and fragments) and pEBG-GST, pEBG-GST-Akt (and all variants) and pEBG-GST-14-3-3 were from the Tzivion lab. The pGL4-Renilla was a gift from Arun Rishi. The pGL3-FHE-Luciferase vector was purchased from addgene.com (deposited by Michael Greenburg). All mutations were performed in the Tzivion lab using Site Directed Mutagenesis (Stratagene) and primers designed by Melissa Dobson (with the exception of FoxO3a-3XA primers designed by Vitaly Balan). See Figure 2.1 for constructs.

2.3 Cell Lines and Culture

293T cells and HepG2 cells were maintained in high-glucose DMEM (Invitrogen) with either 10% newborn calf serum (293T) or 10% fetal bovine serum (HepG2) in a humidified incubator with 5% CO2. PC3 cells were maintained in RPMI media supplemented with 5% FBS.

All cells were maintained with penicillin and streptomycin (10u/ml or 1X). Cells for Luciferase reporter assays were performed in 60mm Nunc plates. All other experiments were performed in 100mm Corning plates.

2.4 Transfection

All cell lines were transfected with Fugene HD (Roche) according to the manufacturer's protocol for either 24 or 48 hours (specified in each figure/experiment).

2.5 Western Blot Analysis

Cells were lysed using 50mM Tris HCl pH 7.5, 100mM NaCl, 1% Triton, 1mM EDTA & EGTA, 50mM β-Glycerolphosphate (disodium) and protease inhibitors and protein concentration was determined using Bradford reagent. 50 ug of protein extract was resolved on SDS-PAGE, followed by transfer to PVDF membranes (0.2uM Immun-Blot PVDF Membrane 0.2uM BioRad #162-0177). The membrane was incubated with first antibody diluted in 5% milk in PBST for 45 min at RT followed by three washes with PBST, 5 min each, and incubation with a secondary antibody for 45 min under the same conditions, followed by 3 washes with PBST and two washes with 0.1M Tris-HCl, pH 8.8. The western was visualized using the ECL method, following the manufacturer's protocol and analyzed using BioRad ChemiDoc system and the Quantity One Software.

2.6 GST Pull Down

Cells transfected with the indicated GST-fusion proteins were collected, lysed [50mM Tris HCl pH 7.5, 100mM NaCl, 1% Triton, 1mM EDTA & EGTA, 50mM β-Glycerolphosphate (disodium) and protease inhibitors], and protein was measured using the Bradford method.

Equal amounts of protein (1-2mg) were incubated with 50ul GSH beads (Amersham/GE Healthcare #17-0756-01) for 90 minutes followed by 2x washes with incomplete lysis buffer (50mM Tris HCl pH 7.5, 100mM NaCl, 1% Triton, 1mM EDTA, 1mM EGTA, 50mM β-Glycerolphosphate), 1x wash with incomplete lysis buffer containing 0.5M LiCl and 2x washes with incomplete kinase buffer containing 40mM Tris HCl pH 7.5, 0.1mM EDTA and 5mM MgCl2. For the last wash, the GSH beads were transferred to a new tube followed by elution in 2X sample buffer and boiling at 90° C for 5 minutes. Samples were centrifuged and loaded onto SDS-PAGE. Total cell extracts were saved from each sample to verify protein expressions.

2.7 Protein Half-Life Studies

For the experiments elucidating the effect of 14-3-3 on steady-state levels of phosphorylated FoxO3a, 293T cells were transfected with Fugene HD for 24 hours. Fresh media was added for another 24 hours followed by treatment on the morning of the second day with either fresh media (control), pretreatment with the proteosome inhibitor MG132 (10uM concentration) for 2 hours followed by treatment with Cyclohexamide (25ug/ml) to stop translation or treatment with Cyclohexamide alone. For each time point (0= no drug, 1 hour, 3 hour, etc) the individual plate was collected, washed with cold 1X PBS and stored at -80 degrees Celsius. After all plates were collected, the cells were thawed on ice, lysed and analyzed by SDS-PAGE.

2.8 Luciferase Reporter Assays

HepG2 or PC3 cells (Nunc 60mm plates) were transiently transfected for 48 hours with the FHBE-Luciferase Reporter [obtained from Addgene, submitted by Michael Greenburg's lab (Brunet et al., 1999)] and the pGL4-Renilla expression vector (gift from Arun Rishi), collected

and lysed in 200ul lysis buffer [50mM Tris HCl pH 7.5, 100mM NaCl, 1% Triton, 1mM EDTA & EGTA, 50mM β-Glycerolphosphate (disodium) and protease inhibitors]. 50ul of the lysate was distributed to a white Nunclon flat bottom 96-well plate. An equal volume of the Dual-Glo Luciferase Assay System (Promega E2920) buffer A was added to each well and incubated for 15 minutes and luminescence was detected using a BioTek Synergy 2 system with Gen5 software. Stop and Glo reagent (Promega E2920) was added and incubated for 15 minutes before reading the Renilla activity. Activity is expressed as the ratio of Luciferase reading divided by the Renilla reading. Experiments were performed in duplicates or triplicates as indicated. Expression of the FoxO3a variants was monitored by western blotting.

2.9 Generation of PC3 Stable Cell Lines

PC3 cells were tested first for viability in the presence of G418. Death curves were performed and a concentration of 800ug/ml G418 was determined to be the optimal dosage. Next, PC3 cells were transfected with 10ug DNA/10cm plate for each of the following DNA expression vectors: pcDNA empty vector control or pcDNA containing wild-type FoxO3a, FoxO3a-3XA, FoxO3a-P34A or the DNA binding FoxO3a mutant, FoxO3a R211A/S215E. After 48 hours, plates were split 1:2 and left to recover for 24 hours. RPMI media containing 5% FBS and 800ug/ml of G418 were then added to the cells. PC3 stable cell lines were grown in 800ug/ml G418 until day 16, when media with 600ug/ml G418 was utilized.

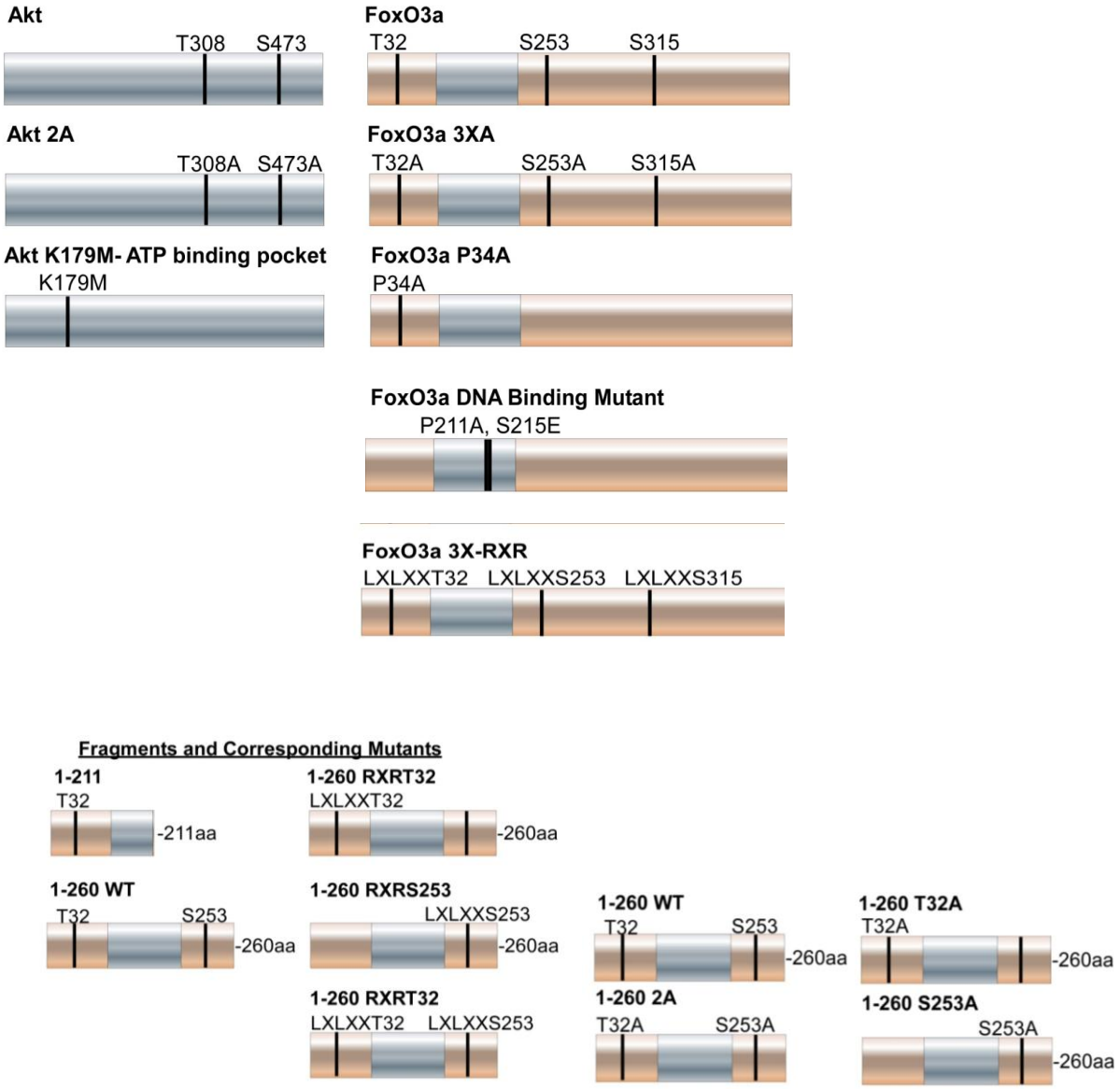


Figure 2.1 Akt and FoxO3a Constructs Depictions of Akt and FoxO3a constructs, mutations and fragments via Single or Multi-Site Directed Mutagenesis (Stratagene). All primers designed using Vector NTI (Invitrogen) software.

CHAPTER 3: RESULTS, REGULATION OF FOXO3A BY AKT

3.1 Akt-FoxO3a Binding and Steady-State Protein Levels

3.1.1 Effects of Akt activity state on FoxO3a Binding and Foxo3a Steady-State Protein Levels

It has been previously shown that FoxO3a can interact with both 14-3-3 and Akt (Lehtinen et al., 2006, Brunet et al., 2002, Zheng et al., 2000a). However, little is known regarding the effects or purpose of Akt binding to FoxO3a. To investigate the Akt/FoxO3a interaction, HEK-293T cells were transfected with expression vectors encoding HA-FoxO3a together with GST control, GST-Akt or GST-14-3-3 for 24 hours and GST pull-downs were analyzed for the presence of HA-FoxO3a using HA immunoblotting (Fig. 3.1). This experiment showed that both Akt and 14-3-3 are capable of binding FoxO3a, with 14-3-3 binding to phosphorylated FoxO3a (Fig. 3.1B). This experiment also revealed that expression of AKT increases the steady-state level of the co-transfected HA-FoxO3a when compared with the GST control (Fig. 3.1A). These results validate the initial discovery of Akt/FoxO3a binding.

To further understand the mechanism of Akt-FoxO3a binding, we wanted to determine if the activity state of Akt plays a role in Akt-FoxO3a interaction. To do this, we used two inactive Akt mutants: 1. Akt K179M, containing a mutation in the ATP binding pocket, rendering it an inactive kinase. 2. Akt T308A/S473A, containing alanine substitutions at the two activating phosphorylation sites, thus prohibiting its activation by the PI3K-PDK1 pathway (Fig. 3.2) (Andjelkovic et al., 1996, Alessi et al., 1996, Cichy et al., 1998).

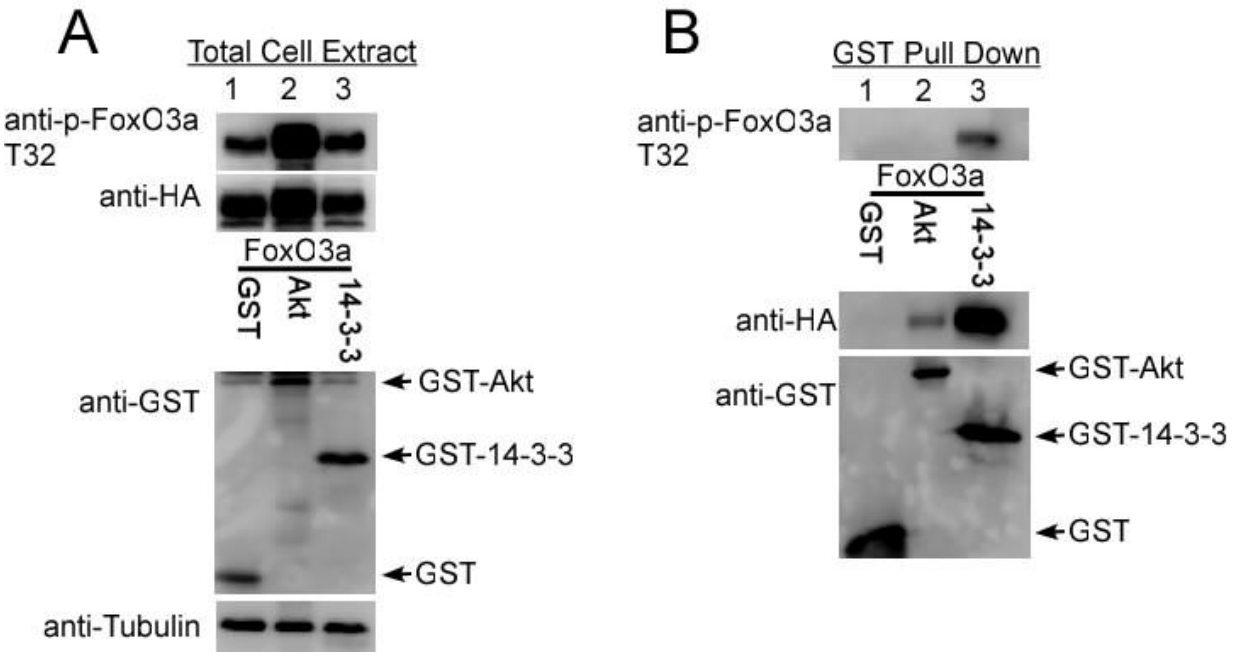


Figure 3.1. FoxO3a Binds to Akt and 14-3-3: 293T cells were transfected for 24 hours with HA-FoxO3a together with GST, GST-Akt or GST-14-3-3 using 18uL FuGENE HD. Cells were collected and lysed after 24 hours. A portion of the lysate was saved for total cell extract analysis (**A**) and a GST pull down was performed with the remainder (**B**); equal protein was incubated with 25ul of GSH bead slurry for 90min, washed and eluted with 2X sample buffer and boiling. Samples were analyzed by immunoblotting for p-FoxO3a, total FoxO3a (HA tag) and for the GST fusion proteins. Alpha-Tubulin blotting was used to confirm equal protein loading. The membrane was stripped after each immunoblotting using 8M Guanidine Hydrochloride, 100mM Glycine pH 2.4 stripping buffer. A representative experiment of at least five repeats is presented.

The ATP binding pocket mutant of Akt was unable to increase FoxO3a steady-state protein levels or its phosphorylation (Fig. 3.2A and C). In fact, the levels of phosphorylated FoxO3a were lower than when FoxO3a was co-expressed with the GST control vector, emulating the previous finding that the Akt K179M functions as a dominant negative form for endogenous Akt (Cichy et al., 1998).

Concerning Akt-FoxO3a interaction, neither the ATP binding mutant nor the phosphorylation site mutant was able to bind FoxO3a 3XA (Fig. 3.2B lanes 9 and 10) and both showed a dramatic decrease in binding to the wild type FoxO3a (Fig. 3.2B lanes 4 and 5, Fig 3.2C lanes 3 and 4 in pull down). These results suggest that either Akt needs to be active for binding FoxO3a or that these residues are important for binding or for maintaining a conformation of Akt that enables FoxO3a binding.

In conclusion, these experiments show that Akt needs its ATP binding pocket and the two activating phosphorylation residues (T308, S473) for its ability to phosphorylate, bind and increase steady-state levels of FoxO3a. However, it is unknown if this disruption in Akt function is due to the inhibition of kinase activity or if the mutations at the K179 and T308/S473 residues introduce a conformational change that is necessary for the above mentioned effects on FoxO3a.

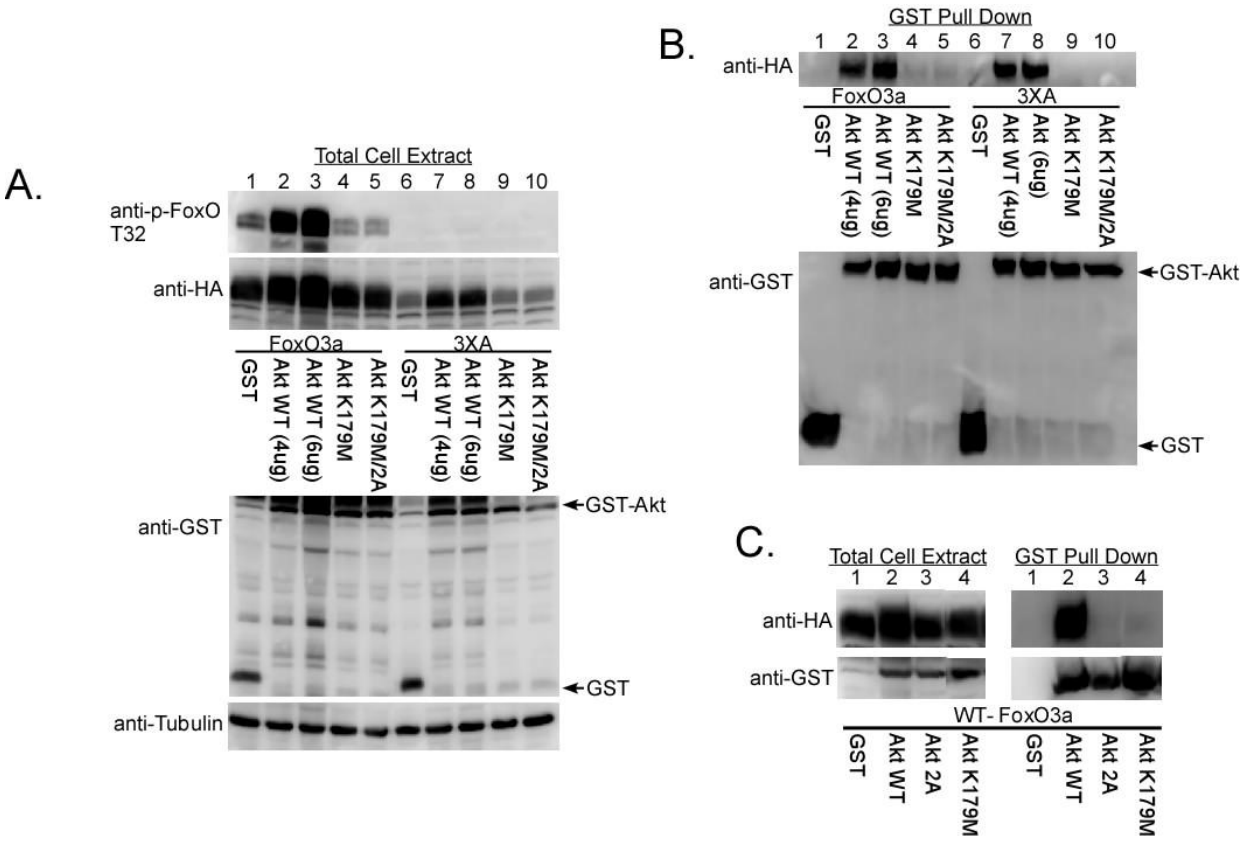


Figure 3.2. Akt Activity is Important for FoxO3a Binding: 293T cells were transfected for 24 hours with an HA-tagged wild type FoxO3a or the 3XA mutant together with the indicated GST fusion proteins or the GST control. Total cell lysates were analyzed by immunoblotting for anti-p-FoxO3a (T32) and anti-HA (**A & C**). A portion of the lysate was used for GST pull-down and immunoblotting as indicated (**B & C**).

3.1.2 Regions of FoxO3a Necessary for Akt-FoxO3a Binding and Increased FoxO3a Steady-State Protein Levels

After confirming that Akt binds FoxO3a, I set to determine the interaction point/s of Akt on FoxO3a in order to elucidate the effect of Akt binding on FoxO3a activity. We hypothesized that since Akt phosphorylates FoxO3a on the RxRxxS/T Akt phosphorylation recognition sequence, that these sites may be involved in the Akt-FoxO3a interaction. Therefore, the arginines in the three Akt phosphorylation recognition sites of FoxO3a were mutated to leucines (RxR → LxL), generating the mutant form described in figure 2.1 (FoxO3a 3X-RxR) as well as single RxR and double RxR mutants (Figure 3.3). Examining Akt binding to these mutants reveled that Akt binding to FoxO3a does not appear to be mediated by the phosphorylation recognition motif (Figure 3.3B and D). The RxR mutations, however, eliminated phosphorylation by Akt (Figure 3.3A and C) as well as the binding of FoxO3a to 14-3-3 (Figure 3.3B and D), confirming the previous observations that 14-3-3 binding to FoxO3a was dependent on FoxO3a phosphorylation on the Akt sites. It was notable that the 3X-RXR mutations decreased the protein expression to similar levels as observed with the 3XA mutant, however, Akt was still able to increase the steady state protein levels of both mutants (Figure 3.3A). This suggests that neither the RXR motifs nor the ability of Akt to phosphorylate FoxO3a are necessary for the increase in FoxO3a steady-state protein levels.

To investigate the relative roles of the three RXR motifs in the Akt-FoxO3a interaction, 1X-RxR mutant (RxRxxT32 → LxLxxT32) and a 2X-RxR mutant (RxRxxT32 → LxLxxT32 and RxRxxS235 → LxLxxS235) were generated. As can be seen in the total cell lysates (Figure 3.3C), Akt was able to increase the steady state protein levels of all the mutants to the same extent, indicating that these sites are not important for this Akt function. On the other hand, 14-3-3 was able to increase the steady state protein level of wild type FoxO3a, but not of

the RXR motif mutants, indicating that the RXR motifs are critical for this positive regulatory role of 14-3-3 on FoxO3a. Regarding the effects of the RXR mutations on Akt and 14-3-3 binding; Akt bound all forms with comparable efficiency (Figure 3.3D) with only a slight decrease in the ability to bind the 3X-RXR mutant, raising the possibility that the RXR motifs may be involved to some extent in Akt-FoxO3a binding. The binding to 14-3-3 was almost completely abolished by the RxR mutations, with even the 1X-RxR eliminating most of the binding, supporting previous observations that the T32 site serves as the main 14-3-3 binding point. In conclusion, the three RXR motifs are important for 14-3-3 binding and for the ability of 14-3-3 to increase FoxO3a steady-state protein levels. However, they do not seem critical for Akt-FoxO3a binding and for the ability of Akt to increase FoxO3a steady-state protein levels. Therefore, these experiments show that the RxR motifs are not involved in Akt-FoxO3a binding.

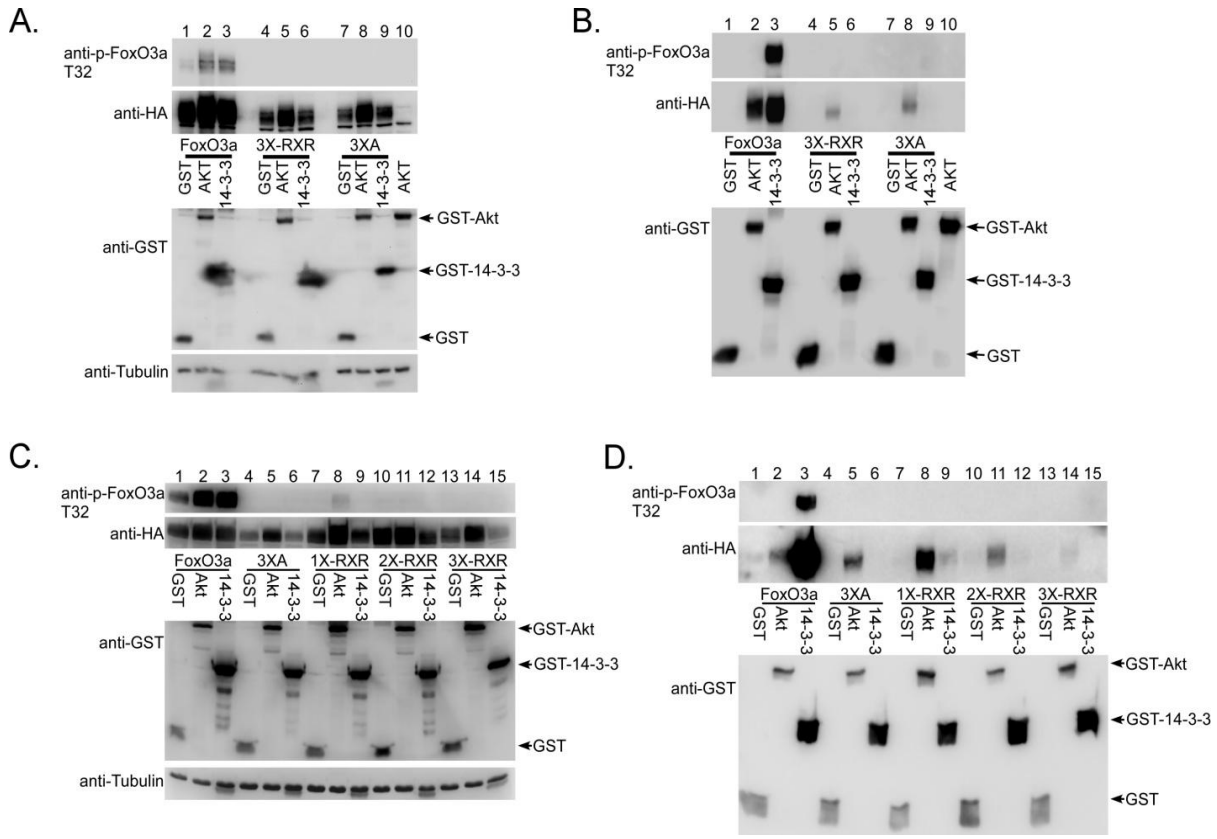


Figure 3.3. The Ability of Akt to Bind and Increase FoxO3a Steady-State Protein Levels is not Dependent on the Three RxRxxS/T FoxO3a Motifs: A & **B,** FoxO3a variants (Wild type, 3X-RXR or 3XA) were co-expressed in 293T cells for 24 hours with the indicated GST fusion proteins or the GST control. Total cell lysates were analyzed by immunoblotting using the indicated antibodies **(A)** or were used for GST pull-down before SDS-PAGE and Western blot analysis (**B). C** & **D,** The indicated FoxO3a mutants were co-transfected with GST control, GST-Akt or GST-14-3-3 for 24hrs. Total cell lysates were analyzed by immunoblotting using the indicated antibodies **(C)** or were used for GST pull-down before SDS-PAGE and Western blot analysis (**D).**

Since the RXR motif mutations were not capable of eliminating the Akt stabilizing effect on FoxO3a, several FoxO3a variants were generated to help elucidate this mechanism. These forms included N-terminal fragments and N-terminal fragments with RXR mutations (Fig. 2.1). As can be seen in Figure 3.4, it appears that the N-terminus fragment of FoxO3a that includes amino acids 1-260 is sufficient for Akt to increase the steady-state level of the protein (compare lanes 4 and 5, FoxO3a blot). In addition, the RxR motifs were not required for the stabilizing effect of Akt (compare lanes 5, 8, 11 and 14, FoxO3a blot). This experiment also showed that the ability of Akt to phosphorylate the T32 site was not dependent on the S253 site (Fig. 3.4, compares lane 5 and 11, pFoxO3a blot) as previously suggested (Nakae et al., 2000, Rena et al., 2001). The 1-211 fragment still served as a substrate to Akt, but the stabilizing effect of Akt was diminished (lanes 1-3). The ability of 14-3-3 to increase steady-state protein levels of FoxO3a was eliminated by all truncations, indicating that 14-3-3 needs the full-length protein for its stabilizing effect.

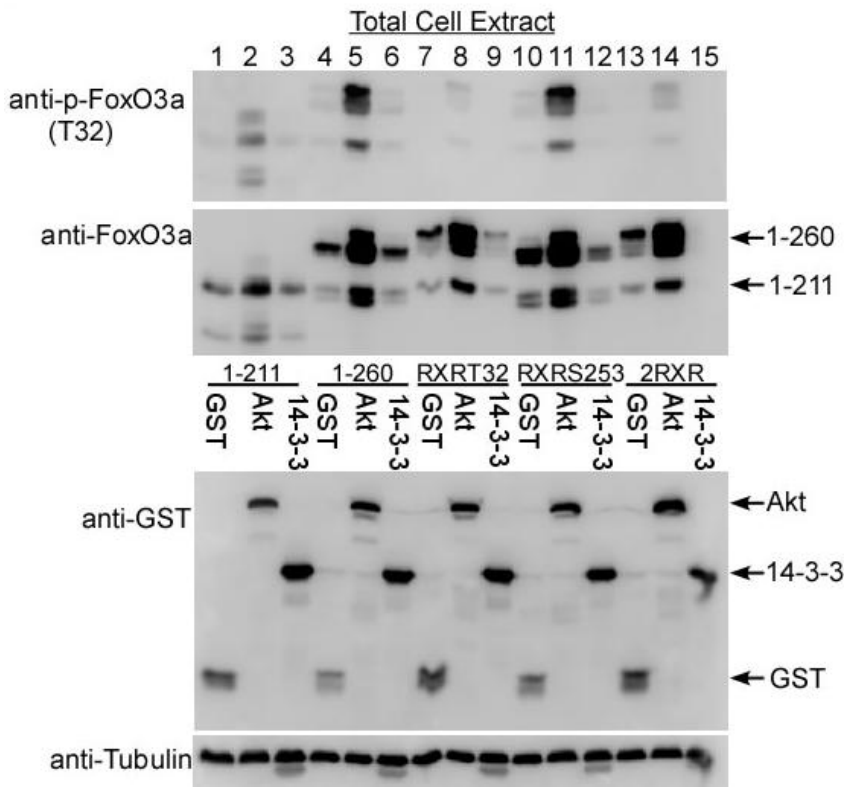


Figure 3.4. The Ability of Akt to Increase FoxO3a Steady-State Protein Levels Involves the N-terminal Protein Segment of FoxO3a: The indicated FoxO3a variants were cotransfected with GST fusion proteins (GST, GST-Akt and GST-14-3-3) for 24 hours. Cells were collected and lysed and equal amounts of lysate was run on SDS-PAGE and transferred to PVDF membrane.

3.2 Discussion, Regulation of FoxO3a by Akt

The presented results provide further insight into the role of Akt in FoxO3a regulation. The results confirm that Akt can form a stable interaction with FoxO3a (Fig 3.1), as has been previously observed (Lehtinen et al., 2006, Brunet et al., 2002, Zheng et al., 2000a). Though the actual domain/sequence of FoxO3a that mediates Akt binding has yet to be identified, the presented data indicates that the RxRxxS/T motif, which is essential for FoxO3a phosphorylation by Akt, is not required for Akt binding or for FoxO3a stabilization by Akt (Figure 3.3 B and D). In addition, the data shows that the N-terminal 1-260 fragment is sufficient for phosphorylation by Akt and for mediating the stabilizing effect of Akt (Figure 3.4). These findings suggest that Akt binds FoxO3a through a docking point that is different from the phosphorylation recognition motif, RxRxxS. This is an important novel finding that provides a new understanding regarding the binding of Akt to its substrate/s and the subsequent phosphorylation of the substrate. It also provides a new target for possible drug development, focusing on the Akt-substrate interaction. Interestingly, we were unable to detect phosphorylated FoxO3a binding to Akt, while detecting heavily phosphorylated FoxO3a associated with 14-3-3. This may suggest that Akt binds primarily to unphosphorylated FoxO3a and that following phosphorylation the complex dissociates, either as a consequence of phosphorylation-induced dissociation or because of competition with 14-3-3.

Another novel finding is the demonstration that mutations that prevent Akt activation impair the ability of Akt to bind FoxO3a: Mutation of either the ATP binding pocket (K179M) or the activating phosphorylation sites (T308/S473) eliminated the interaction of Akt with FoxO3a (Figure 3.2). These results suggest that the activation of Akt is important for stable association with FoxO3a or that these mutations affect the structure of Akt in a way that prevents association

with FoxO3a. Alternatively, it is possible that the inactive Akt variants have a different subcellular localization than active Akt and FoxO3a. Further elucidation of this question should focus on comparing the binding of active and inactive Akt to FoxO3a and determining the domain/s of Akt that mediate the binding. Whether this phenomenon is a common trait for other Akt substrates is also an important question as suggested by a work on the interaction between Akt and Par-4 (Goswami et al., 2005).

The finding that co-expression of Akt with FoxO3a increases the steady-state protein levels of FoxO3a independent of FoxO3a phosphorylation or the RxRxxS motif (Figure 3.3 and 3.4), raises the possibility that Akt can also serve as a positive regulator of FoxO3a. This positive effect was seen with wild type Akt but not with the K179M or T308/S437A mutants, suggesting that Akt activity was required. However, since these mutants also failed in binding FoxO3a, it is impossible at this stage to conclude which function of Akt is needed for the stabilizing effect; its ability to bind FoxO3a or its kinase activity. One indication that the kinase activity, at least toward FoxO3a, was not of importance is the fact that FoxO3a mutants lacking the Akt phosphorylation sites were stabilized to the same extent as wild type FoxO3a (Figure 3.2, 3.3 and 3.4).

Another possibility was that the over expression of Akt might have increased FoxO3a protein levels by affecting protein translation through the mTORC1 pathway (Ma and Blenis, 2009). This possibility was tested by using the mTORC1 inhibitor rapamycin, which showed no effect on the stabilizing effect of Akt on FoxO3a (data not shown). These data along with data from others describing similar events with PEA-15 and B23/NPM (Gervais et al., 2006, Lee et al., 2008) suggest that Akt may be directly increasing FoxO3a proteins levels and not through downstream signaling events or a feedback mechanism.

CHAPTER 4: RESULTS, REGULATION OF FOXO3A by 14-3-3

4.1 14-3-3 Decreases the Rate of FoxO3a Dephosphorylation

As shown above, both Akt and 14-3-3 are capable of binding FoxO3a, with 14-3-3 binding exclusively to phosphorylated-FoxO3a (Fig. 3.1 and 3.3). In addition, 14-3-3 was able to increase the steady-state protein levels of FoxO3a as well as phospho-FoxO3a. To investigate this effect, FoxO3a was co-expressed with Akt or 14-3-3 over a 24 or 48 hour period and the effects of 14-3-3 and Akt on total and phosphorylated FoxO3a steady-state levels was examined (Fig. 4.1). This experiment showed that 14-3-3 significantly increased the steady-state levels of both, total and phosphorylated FoxO3a, with the maximum effect seen at the 48h time point (Fig. 4.1A). Akt on the other hand displayed the maximum effect already at the 24h time point. In addition, 14-3-3 only protected wild type FoxO3a but not the Akt phosphorylation site FoxO3a 3XA mutant, suggesting that the phosphorylation-dependent 14-3-3 binding to FoxO3a was required for this stabilizing effect and implying that 14-3-3 stabilizes exclusively the phosphorylated pool of FoxO3a. Similar results were seen with the RxR FoxO3a mutants, to which 14-3-3 was unable to bind or exert a stabilizing effect (Fig. 3.3 and 3.4). These results indicate that 14-3-3 increases the steady-state protein levels of phosphorylated FoxO3a.

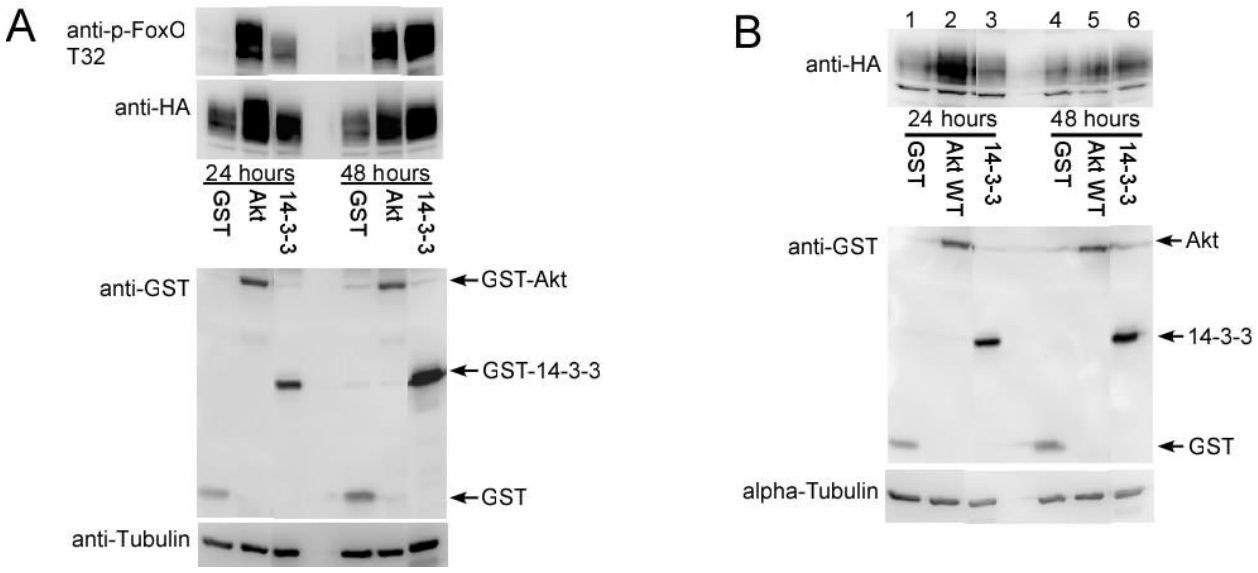


Figure 4.1. 14-3-3 Expression Increases the Steady-State Levels of Phosphorylated FoxO3a in a Time-Dependent Manner: HEK-293 cells were transfected with wild-type HA-FoxO3a (**A**) or the Akt phosphorylation mutant, HA-FoxO3a 3XA (**B**) together with GST control, GST-Akt or GST-14-3-3 and cell lysates were produced after 24 or 48 hours. Total cell lysates were analyzed by immunoblotting for p-FoxO3a, total FoxO3a (HA) and GST-fusion proteins as indicated. Tubulin immunoblotting served for verifying equal protein loading.

To investigate the mechanism of 14-3-3-dependent effects on phosphorylated FoxO3a, a time-course experiment comparing co-expression of FoxO3a with 14-3-3 or a GST control vector was preformed in the presence of the protein translation inhibitor cycloheximide and the proteosome inhibitor, MG132 (Fig. 4.2A). This experiment showed that, as previously observed, baseline phosphorylated FoxO3a steady-state protein levels are higher in the presence of 14-3-3 than with the GST control. Following cycloheximide treatment, p-FoxO3a levels decreased in both the control and 14-3-3 co-transfected cells. However, in the presence of MG132, 14-3-3 stabilized p-FoxO3a. These results show that 14-3-3 primarily inhibits FoxO3a dephosphorylation, rather than its degradation.

To further investigate this effect of 14-3-3 on p-FoxO3a and to support the idea that 14-3-3 inhibits FoxO3a dephosphorylation, I designed a FoxO3a mutation that would enable regulation by Akt (binding, phosphorylation and stabilization), but would interfere with 14-3-3 binding. The design of this mutant would also be able to distinguish between the negative regulatory events of Akt phosphorylation and 14-3-3 binding on FoxO3a. For this purpose, I took advantage of the 14-3-3-binding requirements, which necessitates a Proline at the +2 position of the phosphorylated residue; RxxpS/TxP (Muslin et al., 1996, Tzivion et al., 2001), P34 in FoxO3a, and generated a FoxO3a P34A mutant. Importantly, the +2 position does not play a role in Akt phosphorylation consensus as it only depends on the RxRxxS/T motif (Zhang et al., 2002). Testing the binding of the FoxO3a P34A mutant to Akt and 14-3-3 (Fig. 4.2D) revealed that the mutant was capable of binding Akt at least to the same extent as wild type FoxO3a (Fig. 4.2C compare lanes 2 and 5); but showed a dramatic decrease in its ability to bind 14-3-3 (Fig. 4.2D, compare lane 3 and 6). This result demonstrated a critical role of the proline at position 34 for 14-3-3 binding, while not affecting binding to Akt. In addition, comparing the

expression levels of the mutant when co-transfected with Akt or 14-3-3, it is notable that Akt increases the steady-state protein levels of the mutant and wild type FoxO3a, while 14-3-3 only affected wildtype FoxO3a but not the P34A mutant (Fig 4.2B compare lanes 1-3 with 4-6, HA blot). This result was similar to the results obtained with Akt phosphorylation site mutants that did not bind 14-3-3, i.e. the RxR and the 3XA FoxO3a mutants. Thus, the presented results establish the P34A mutant as a useful tool for studying the role of 14-3-3 binding in the stabilization of phospho-FoxO3a, without affecting its regulation by Akt. To this end, FoxO3a phosphorylation levels on the Akt phosphorylation sites were examined in control cells and cells treated with the PP2A phosphatase inhibitor calyculin A (Fig 4.2B). PP2A was shown in previous studies to be the main phosphatase regulating FoxO phosphorylation on these sites (Yan et al., 2008). As seen in this figure, the P34A mutation causes a profound decrease in steady-state FoxO3a phosphorylation at the S253 site (Fig. 4.2B compare lanes 1-3 with 4-6, p-FoxO blot). Similar results were obtained when examining FoxO3a phosphorylation on the T32 site (data not shown). The reduced phosphorylation could have been caused by the inability of Akt to phosphorylate the protein or by an increase in its dephosphorylation rate. The ability of calyculin A treatment to increase steady-state FoxO3a P34A phosphorylation levels to those of wildtype FoxO3a in the Akt-co-transfected samples (Fig 4.2B compare lanes 8 and 11), suggests that increased dephosphorylation rates rather than accessibility to Akt plays the main role in the low phosphorylation of the P34A mutant. This finding supports the view that 14-3-3 binding protects FoxO3a from dephosphorylation, resulting in increased steady-state FoxO3a levels. Supporting this notion, the addition of the phosphatase inhibitor increased the p-FoxO levels of wild type FoxO3a in the GST control sample to the same levels seen in the 14-3-3-co-expressing sample

(Fig. 4.2B compare lanes 1 & 3 with 7 & 9), indicating that 14-3-3 blocks FoxO3a dephosphorylation.

In conclusion, these experiments show that 14-3-3 protects the phosphorylated form of FoxO3a from dephosphorylation and that the development of a FoxO3a mutant that can be regulated by Akt, but not 14-3-3, can be used to determine the critical negative regulatory event for FoxO3a transcriptional inhibition; Akt phosphorylation versus 14-3-3 binding.

4.2 Discussion, Regulation of FoxO3a by 14-3-3

The regulation of FoxO3a by 14-3-3 has been studied extensively, establishing a negative regulatory role by inhibiting FoxO3a DNA binding and excluding the protein from the nucleus through a combined increased nuclear export and decreased nuclear import (Cahill et al., 2001, Brunet et al., 2002, Obsil et al., 2003, Plas and Thompson, 2003, Aoki et al., 2004). The presented results show a new positive role of 14-3-3 in FoxO3a regulation. This effect is reflected in the ability of 14-3-3 to increases the steady-state protein levels of total and phosphorylated FoxO3a (Fig 4.1). This effect is mediated by the ability of 14-3-3 to protect FoxO3a from dephosphorylation and potentially degradation (Figure 4.2A). Importantly, this effect of 14-3-3 was seen with wild type FoxO3a but not with FoxO3a mutants lacking the Akt phosphorylation sites (i.e., 3XA and RxR mutants, Fig. 3.3 and 4.1). To distinguish between Akt phosphorylation and 14-3-3 binding, a mutant was generated that abrogated 14-3-3 binding without affecting Akt binding or susceptibility to phosphorylation; FoxO3a P34A (Fig 4.2B and C). This mutant showed a marked decrease in phosphorylation on the Akt sites and was not stabilized by 14-3-3, supporting the novel view that 14-3-3 proteins can serve as positive regulators of FoxO3a by protecting it from dephosphorylation and degradation. The presented

findings correlate with the current literature, showing that significant levels of p-FoxO can be detected in the cytoplasm, suggesting that they are not immediately degraded. This implicates that there may be a time frame in which FoxO can be salvaged through dephosphorylation and shuttled back into the nucleus [reviewed in (Birkenkamp and Coffer, 2003)]. Therefore, the availability of unbound 14-3-3 in the cells, which could be modulated by environmental factors, could affect FoxO3a levels, leading to either stabilization and recycling of FoxO3a when 14-3-3 is available or to its degradation when unbound 14-3-3 levels are low. A positive 14-3-3 role in FoxO regulation was also demonstrated in *Arabidopsis*, suggesting that the binding of 14-3-3 to FoxO could prevent its proteolytic cleavage (Cotelle et al., 2000).

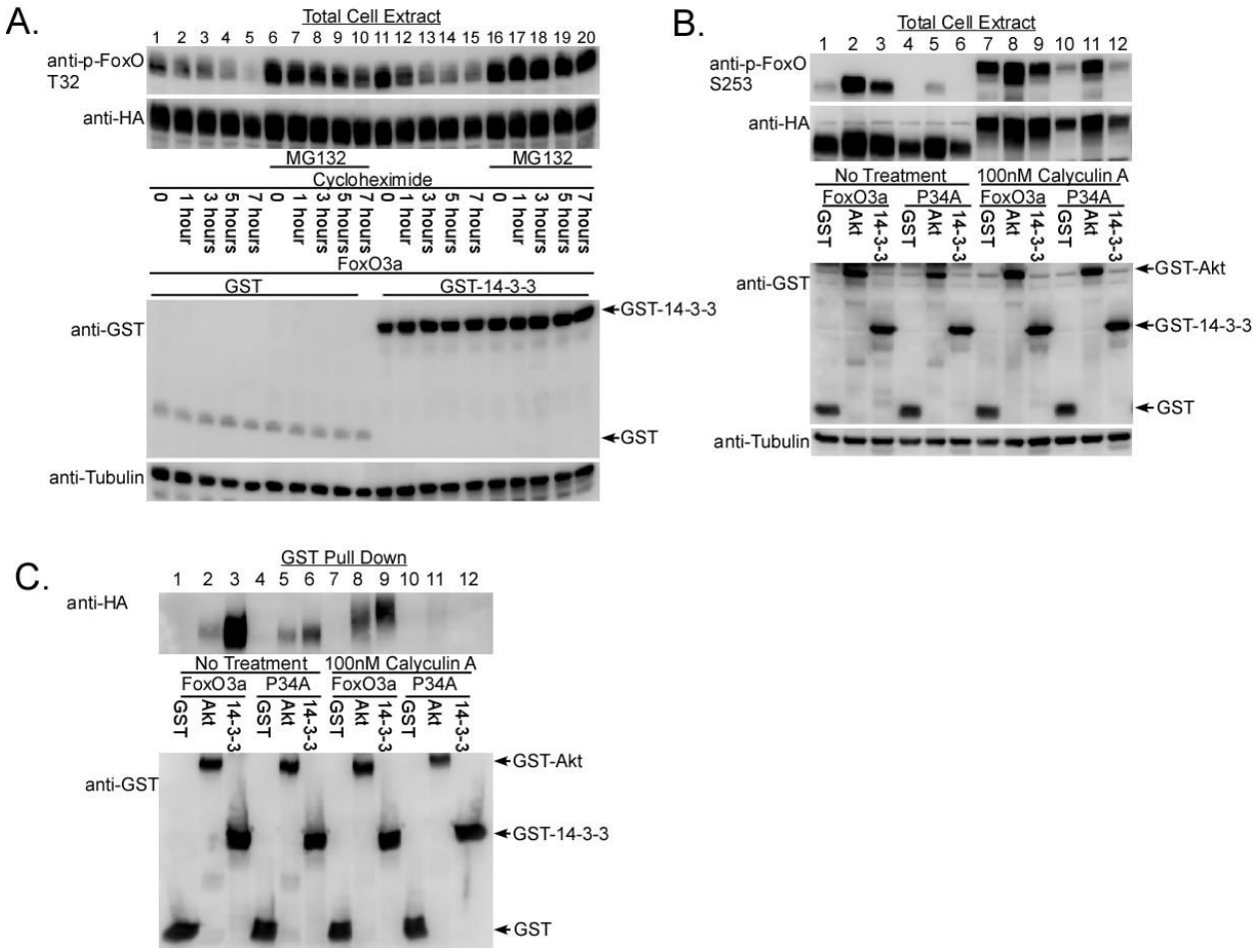


Figure 4.2. 14-3-3 Binding to p-FoxO3a Protects it from Dephosphorylation: A, HA-FoxO3a was transfected into 293T cells with either GST control or GST-14-3-3. Cells were treated with the protein translation inhibitor cyclohexamide alone or in combination with the protease inhibitor MG132 and collected at the indicated time points. Cells were lysed and equal protein amounts were analyzed for total and phospho-FoxO3a levels by immunoblotting. Presented are also GST and tubulin immunoblots showing equal protein loading. **B & C**, 293T cells were transfected with wildtype HA-FoxO3a or the P34A mutant together with GST control, GST-Akt or GST-14-3-3 as indicated for 48 hours. Transfected cells were treated with vehicle or with 100nM of the phosphatase inhibitor calyculin A for one hour. Cell lysates were analyzed directly **(B)** or following GST pull-down **(C)** by immunoblotting for total FoxO3a and p-FoxO3a (S253). Also shown are GST and tubulin immunoblots confirming equal GST expression and protein loading respectively.

CHAPTER 5: RESULTS, REGULATION OF FOXO3A TRANSCRIPTIONAL ACTIVITY BY AKT AND 14-3-3

Previous studies demonstrated negative effects of 14-3-3 and Akt on FoxO3a transcriptional activity and have defined the FoxO3a 3XA mutant that lacks the Akt phosphorylation sites as a constitutively active form (Brunet et al., 1999, Cahill et al., 2001). One caveat of using the 3XA mutant for studying biological functions of FoxO3a is its ability to serve as a dominant negative form for Akt (Zheng et al., 2000a). This effect is probably due to its ability to stably bind Akt, in contrast to the wild type protein that dissociates from Akt following its phosphorylation (see chapter 3 for details). The generation of the FoxO3a P34A mutant, which serves as an Akt substrate, but cannot be negatively regulated by 14-3-3, offers a new form that may serve as a constitutively active FoxO3a, but potentially lacking the dominant negative effect for Akt. Thus, this mutant can serve for studying the biological consequences of FoxO3a activation without the background noise of Akt inhibition. This chapter describes the characterization of the transcriptional activities of the P34A and other FoxO3a mutants and provides a new perspective on the regulation of FoxO3a by Akt and 14-3-3.

5.1 Choice of Cell Models for Studying FoxO3a Transcriptional Activities

In order to adequately study the transcriptional activity of the FoxO3a variants and their regulation by Akt and 14-3-3, the appropriate cell models had to be chosen. As noted above (Chapter 4), initial studies used HepG2 cells for studying FoxO3a transcriptional activities using luciferase reporter assays. These cells have relatively low basal levels of active Akt (Figure 5.1), and the PI3K-Akt pathway can be easily modulated by the addition of insulin or by serum deprivation. Therefore, these cells provided a good model for testing the activities of the different FoxO3a variants as well as the functions of the active and inactive Akt forms.

For studying the activity of the FoxO3a variants in a relevant tumor cell model, the PC3 human prostate cancer cell line was chosen. This cell line has high Akt activity due the loss of the PTEN tumor suppressor. The LNCaP prostate cancer cell line also has high p-Akt due to PTEN loss; however, these cells are difficult to transfect, making them unsuitable for monitoring the activity of transfected FoxO3a variants (data not shown). Various other cell lines were investigated including 293T and a mouse prostate cell model derived from wild type mice or from mice heterozygous or homozygous for PTEN inactivation (Fig 5.1). These cells, however, displayed high internal fluorescence, making them unsuitable for studying FoxO3a transcriptional activity using the luciferase reporter assay (data not shown). Embryonic fibroblasts derived from control or from $Akt1^{-/-}$ or $Akt1\&2^{-/-}$ mice (Fig. 5.1), which could have served a good model for studying FoxO3a regulation, also turned out to be difficult to transfect (data not shown). Therefore, the two cell models that were chosen were those that could be easily and efficiently transfected and either had a tightly regulated PI3K-Akt pathway (HepG2) or a constitutively active pathway (PC3).

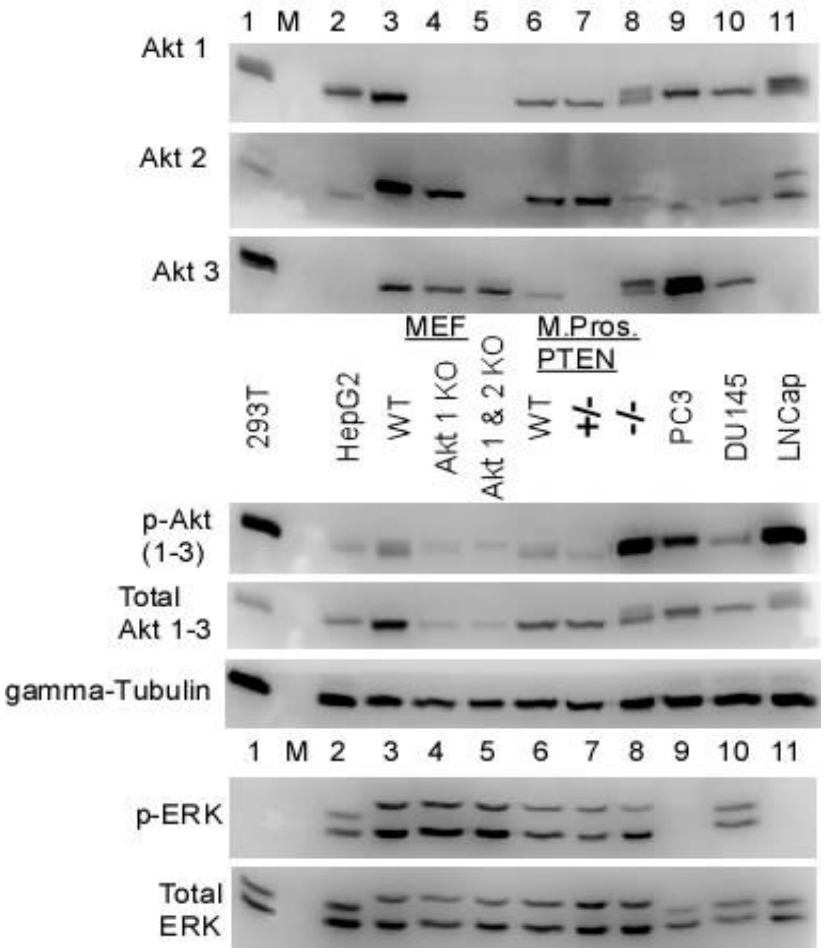


Figure 5.1. Akt Activity and Protein Levels in Cell Models Used in This Study: The indicated exponentially growing cells were lysed and equal protein amounts were analyzed by immunoblotting using the indicated antibodies. The membrane was stripped between blots using 8M guanidine, 100mM glycine pH 2.5 stripping buffer. MEF: mouse embryonic fibroblasts; KO: knockout; M. Pros. PTEN: prostate cells derived from wild type, heterozygous or homozygous PTEN knockout mice.

5.2 Regulation of FoxO3a Transcriptional Activity in HepG2 cells

As mentioned above, HepG2 cells have tightly regulated PI3K-Akt signaling pathway that can easily be activated or inactivated by insulin and serum deprivation. To investigate the activity of endogenous FoxO transcription factors in HepG2 cells and the effects of Akt variants on FoxO activity, Forkhead Binding Element (FHBE)-Luciferase and Renilla control reporter vectors were co-transfected with either, pcDNA empty vector, wild type GST-Akt or the dominant negative GST-Akt K179M vectors. The cells were serum-deprived in the absence or presence of insulin (20ug/ml) and luciferase activity was determined (Figure 5.2). This experiment demonstrated that insulin treatment decreases the transcriptional activity of endogenous FoxO by 50%. The expression of wild type Akt had little effect on FoxO activity in serum-deprived cells, illustrating the low activity of the PI3K-Akt pathway in serum-deprived HepG2 cells. Expression of the dominant negative Akt (K179M), however, increased FoxO activity by 50%, demonstrating that even in serum-deprived conditions there is a residual Akt activity. In the presence of insulin, wild type Akt increased FoxO inhibition by insulin, while the K179M mutant attenuated the inhibition by insulin. Together, these results demonstrate the activities of the wild type and the Akt K179M mutant and illustrate the PI3K-Akt pathway-mediated FoxO regulation in HepG2 cells.

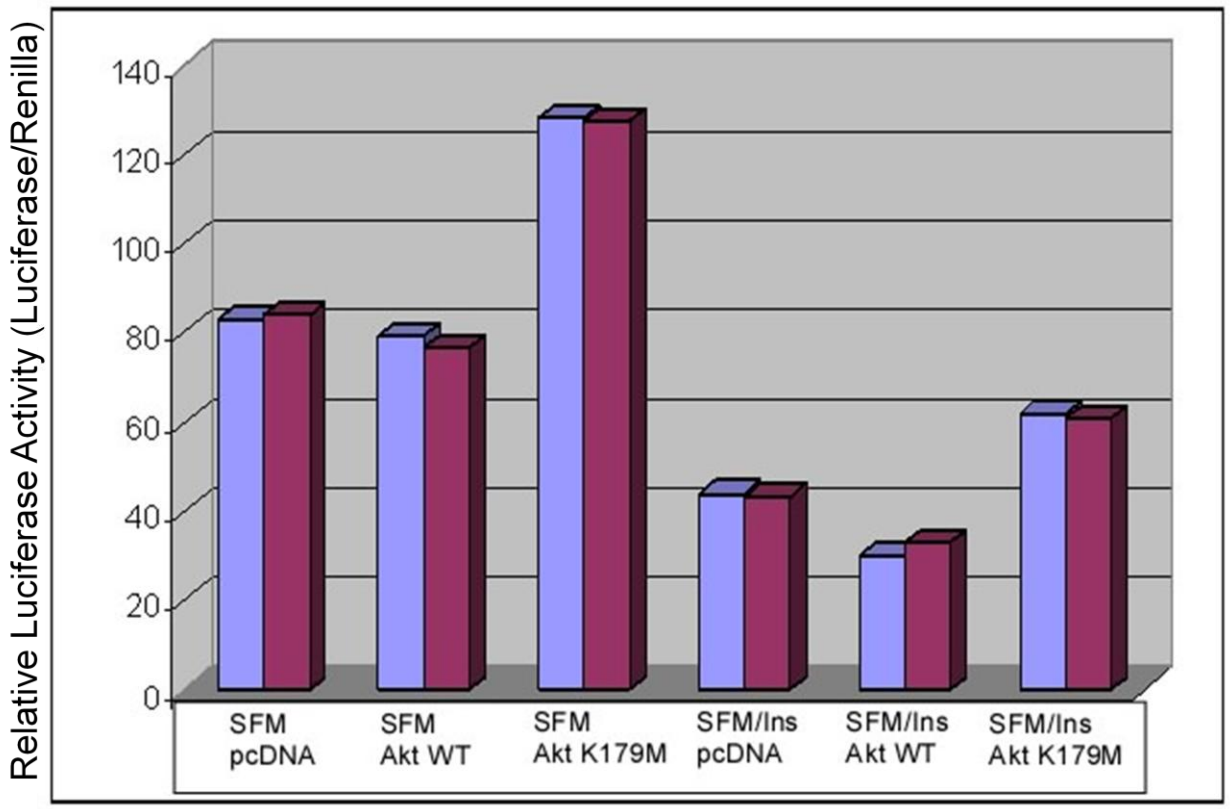


Figure 5.2. Regulation of Endogenous FoxO Transcriptional Activity by Insulin and Akt in HepG2 Cells: HepG2 cells were transfected in duplicates with FHBE-Luciferase and Renilla control vector together with pcDNA empty vector, GST-Akt or GST-Akt K179M for 24h. Cells were incubated for additional 24h with serum-free media (SFM) or with serum-free media supplemented with 20ug/ml insulin (Ins) and analyzed for Luciferase activity using the Promega Dual-Glo Luciferase Assay System. Presented are Luciferase/Renilla ratios with each bar representing an individual plate.

To investigate the transcriptional activities of the FoxO3a variants, HepG2 cells were transfected with the luciferase reporter constructs together with control empty vector or with wild type FoxO3a, the Akt phosphorylation mutant FoxO3a 3XA, the 14-3-3 binding mutant FoxO3a P34A or a DNA binding mutant FoxO3a R211A/S215E (Tsai et al., 2007). Luciferase activity was analyzed in cells growing in serum free media or in serum free media supplemented with 20ug/ml Insulin (Figure 5.3). The luciferase activity for the FoxO3a variants was calculated by subtracting the luciferase values obtained with the control vector, representing the activity of endogenous FoxO proteins. This experiment demonstrates that the 3XA and P34A FoxO3a mutants have increased basal transcriptional activity, compared to wild type FoxO in serum-deprived cells, while the DNA binding mutant functions as a dominant negative form for the endogenous FoxO proteins as reflected by a negative FoxO activity. Importantly, while the activity of the wild type FoxO3a is suppressed by insulin, by up to 4-fold, the activities of the 3XA and P34A mutants was only slightly reduced by insulin treatment. This result confirms previous findings that the 3XA mutant is an active form of FoxO3a and establish the P34A mutant as a novel constitutively active form of FoxO3a with activities up to 6-fold higher than wild type FoxO3a in the presence of insulin. The constitutive activity seen with the P34A mutant implies that 14-3-3 binding is the main effecter in FoxO3a regulation and that Akt phosphorylation by itself is insufficient for negative regulation of FoxO3a. Thus, there appears to be a two-layer regulation of FoxO3a, first, phosphorylation by Akt and second, through 14-3-3 binding, providing fine tuning of FoxO3a regulation that can be modulated both by controlling the activity of Akt and also by controlling 14-3-3 availability. Besides the new finding concerning the P34A mutant, the results seen with the DNA binding mutant provide a new concept. This appears to be the first description of a dominant negative effect on endogenous

FoxO activity by a FoxO DNA binding mutant and could prove an important tool for studying the biological consequences of FoxO inactivation. The observed dominant negative effect could be a result of competitive binding to FoxO transcriptional co-activators, thus preventing endogenous FoxOs from binding the necessary cofactors needed for transcribing target genes, or alternatively, through dimerization with endogenous FoxOs, generating inactive FoxO complexes.

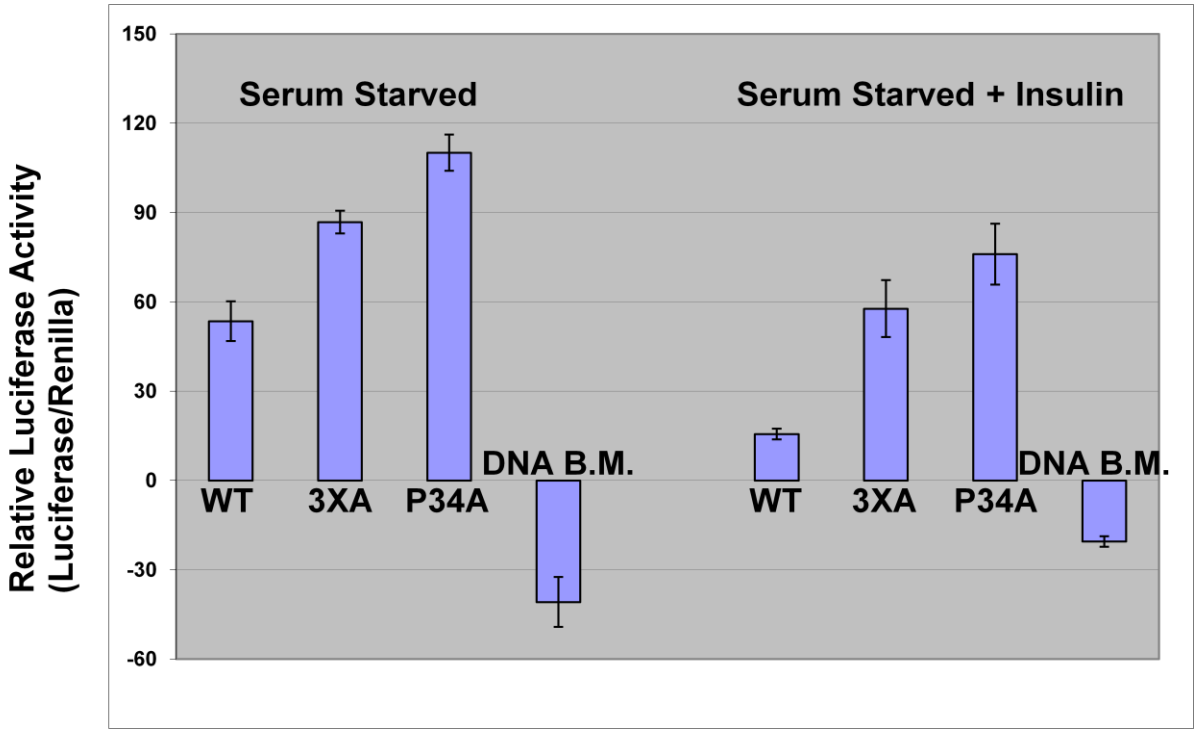


Figure 5.3. Transcriptional Activity of FoxO3a Variants in HepG2 Cells: HepG2 cells were transfected with FHBE-Luciferase and Renilla control vector together with control empty vector, FoxO3a (WT), FoxO3a 3XA, FoxO3a P34A or FoxO3a R211A/S215E (DNA B.M.) for 24 hours followed by changing the media to either serum-free media or serum-free media supplemented with 20ug/ml insulin and incubation for additional 24 hours. Cells were lysed and analyzed for Luciferase activity. For calculating the relative activity of the FoxO variants, the values obtained with the control pcDNA vector (representing the activities of endogenous FoxO) were subtracted form the values obtained with the FoxO variants. Data from two independent experiments, each with duplicate samples was combined and average readings and standard deviation of three samples is presented.

To further investigate the regulation of the transcriptional activity of the FoxO3a variants by Akt and 14-3-3, HepG2 cells were transfected with FHBE-Luciferase and Renilla reporters with the FoxO3a variants together with Akt or 14-3-3 (Fig. 5.4). Empty pcDNA3 and pEBG-GST vectors were used as controls. The results show that wild type FoxO3a has suppressed transcriptional activity when co-expressed with Akt or when exposed to insulin treatment (~3.6 fold decrease). The 3XA and P34A mutants displayed higher transcriptional activities than wild type FoxO3a and the addition of Akt or insulin did not significantly affect their function, confirming that both 3XA and P34A are constitutive active variants of FoxO3a. It is notable that the activity of the P34A mutant was suppressed by Akt (~1.6 fold decrease) under serum-deprived condition; however, its activity was still ~3 fold higher than wild type FoxO3a. This effect of Akt was not seen in the insulin-treated samples. The observed partial loss of activity could be explained by the fact that FoxO3a P34A displays some residual binding to 14-3-3 (Figure 4.2), probably through binding to the S253 or S315 sites. 14-3-3 co-expression resulted in a slight increase in the transcriptional activities of all FoxO3a variants, suggesting that it may have some effects on transcriptional co-activators of FoxO.

In conclusion, these results identify the P34A FoxO3a mutant as a novel constitutively active form, which is as active as the previously described 3XA FoxO3a form. This finding indicates that Akt phosphorylation is not sufficient to inhibit FoxO3a activity by itself and that 14-3-3 binding is essential for inhibiting FoxO3a transcriptional activity. Also, unexpectedly, the DNA binding mutant of FoxO3a that was used as a negative control in these experiments was found to function as a dominant negative form to endogenous FoxO transcriptional activity.

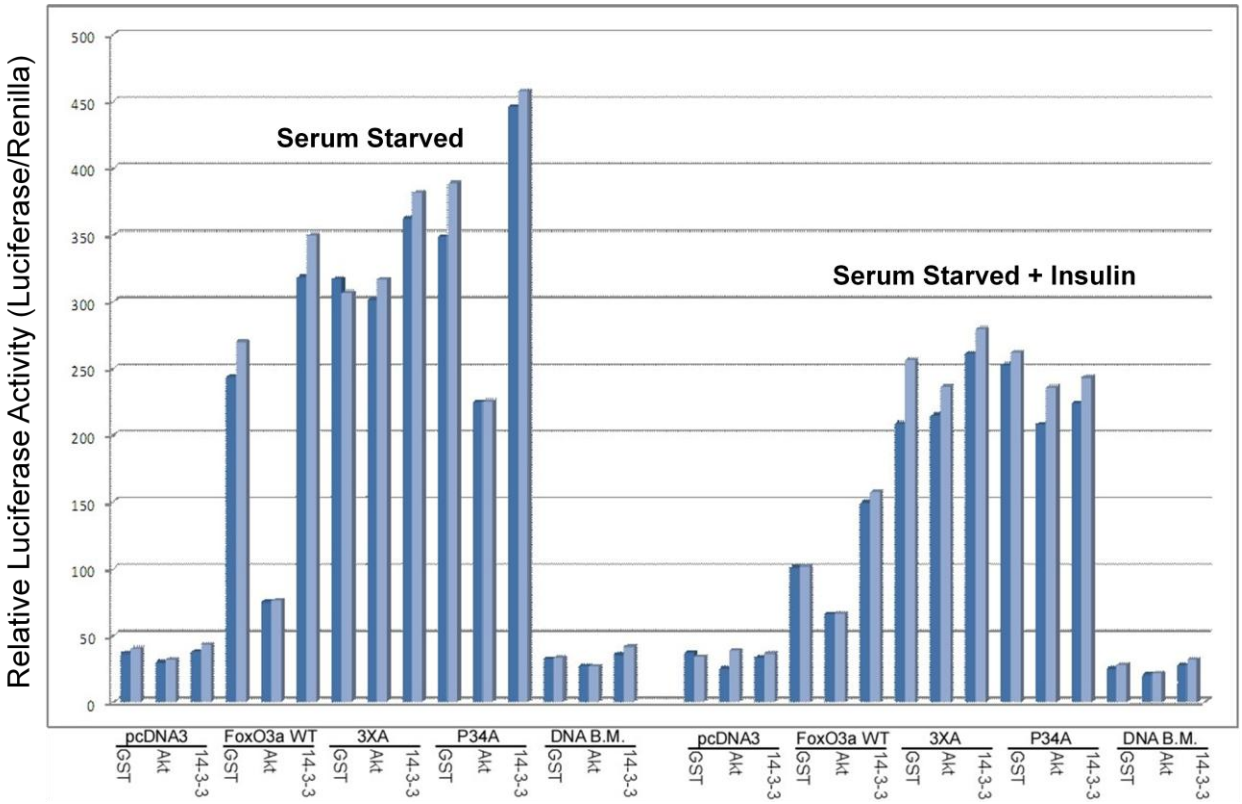
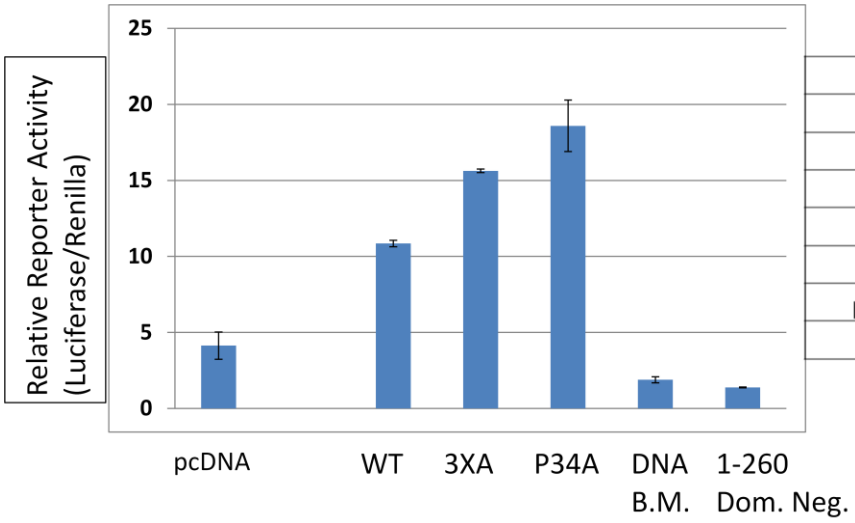


Figure 5.4. Effects of Exogenous Akt and 14-3-3 on the Transcriptional Activity of FoxO3a Variants: HepG2 cells were transfected in duplicates with FHBE-Luciferase and Renilla reporter vectors together with pcDNA empty vector or the indicated FoxO3a variants and with GST control vector, GST-Akt or GST-14-3-3 for 24 hours. Media was then changed to either serum-free media or to serum-free media supplemented with 20ug/ml insulin and cells were incubated for another 24 hours. Cells were lysed and analyzed for Luciferase activity. Readings of Luciferase/Renilla ratio are graphed with each bar representing an individual plate.

5.3 Regulation of FoxO3a Transcriptional Activity in PC3 cells

As noted in section 5.1, the PC3 cell line is a suitable cell system to test FoxO3a activity in a cancer cell model that caries PTEN inactivation and displays high Akt activity. To examine the activity of FoxO3a variants in PC3 cells, luciferase activity was determined in PC3 cells transfected with the FHBE-Luciferase and Renilla reporters together with control pcDNA vector or FoxO3a, FoxO3a 3XA, FoxO3a P34A, FoxO3a R211A/S215E or FoxO3a 1-260 fragment (Fig 5.5). This experiment showed that also in PC3 cells that exhibit high Akt activity, the 3XA and P34A FoxO3a forms display higher transcriptional activities than wild type FoxO3a, with the P34A mutant showing almost a two-fold increase. Interestingly, wild type FoxO3a showed up-to three-fold increase of FoxO transcriptional activity when compared to the pcDNA control, showing that the addition of wild type FoxO alone can increase overall FoxO activity, despite having high levels of active Akt. The DNA binding mutant displayed a dominant negative effect for endogenous FoxO transcriptional activity, confirming the results obtained in HepG2 cells. As a control for a dominant negative effect, the 1-260 FoxO3a N-terminal fragment, which contains the DNA binding domain but lacks the trans-activation domain was used (Cunningham et al., 2004). These results further confirmed the potential usefulness of the newly developed active and dominant negative FoxO3a forms for studying the effects of high FoxO3a transcriptional activity as well as the loss of FoxO transcriptional activity as exemplified in chapter 6.



Groups	P values
WT/3XA	0.0006
WT/P34A	0.0098
pcDNA /WT	0.0044
pcDNA/3XA	0.0008
pcDNA/P34A	0.0005
pcDNA/DNAB.M.	0.0210
pcDNA/1-260	0.0170

Figure 5.5. Transcriptional Activities of FoxO3a Variants in PC3 Prostate Cancer Cells: PC3 cells were transfected in triplicate with FHBE-Luciferase and Renilla reporter vectors together with control pcDNA vector or FoxO3a, FoxO3a 3XA, FoxO3a P34A, FoxO3a R211A/S215E (DNA B.M.) or FoxO3a 1-260 fragment (1-260 dominant negative) for 48 hours. Luciferase and Renilla activities were read and the student t-test was performed to generate P values. The results are a representative of three independent experiments.

5.4 Discussion, Regulation of Foxo3a Transcriptional Activity by Akt and 14-3-3

The functionality of FoxO3a variants were tested in two distinct cells lines; HepG2 and PC3. HepG2 cells have a normal and tightly controlled PI3K-Akt-FoxO pathway that can be regulated through the addition of insulin, whereas PC3 cells have uncontrolled, high Akt activity resulting in low levels of FoxO transcriptional activity.

Reporter assays for FoxO3a transcriptional activity in HepG2 cells established the P34A FoxO3a, which has a decreased capacity for 14-3-3 binding, but can be regulated by Akt in a similar fashion to wild type FoxO3a, as a new constitutive active FoxO3a form (Figure 5.3). This observation is significant since it demonstrates that though this FoxO3a form can be phosphorylated by Akt, unlike the 3XA form, which cannot be phosphorylated by Akt; it is constitutively active because of its lack of binding with 14-3-3. This leads to the hypothesis that the phosphorylation of FoxO3a by Akt in itself is not the main inhibitory event for preventing transcription, but rather the binding of 14-3-3 to FoxO3a is the critical event. This hypothesis is strengthened by findings reported by Obsil et al (Obsil et al., 2003) indicating that the FoxO4-14-3-3 binding motifs are necessary for the inhibition of FoxO-DNA binding.

An interesting point observed in these studies was that over-expression of 14-3-3 was able to increase the activity all tested FoxO forms. Upon first glance, this would appear to be contrary to the hypothesis that 14-3-3 negatively regulates FoxO3a transcriptional activity. However, the fact that 3XA and P34A FoxO3a forms, which do not bind 14-3-3, displayed higher transcriptional activities suggests a different and possibly indirect mechanism; 14-3-3 proteins bind and regulate a very large number of proteins with diverse functions [reviewed in (Dougherty and Morrison, 2004, Tzivion et al., 2006)], thus it is possible that 14-3-3 proteins can

affect the transcriptional activity of FoxO proteins via regulation of FoxO cofactors or by affecting upstream regulators of FoxO function.

The presented data show that the DNA binding mutant not only is a transcriptionaly inactive FoxO3a variant, but that it behaves as a dominant negative form for endogenous FoxO transcriptional activity. This suggests that this variant, when expressed at high levels, can probably compete with endogenous FoxO proteins for transcription co-activators, thus resulting in the inhibition of endogenous FoxO proteins. Alternatively, the dominant negative effect could be mediated by dimerization with endogenous FoxOs, though FoxO dimerization has not been reported yet.

In PC3 cells, which have high, unregulated PI3K-Akt pathway activity due to the loss of PTEN, the FoxO3a variants showed similar transcriptional activity trends as in HepG2 cells; both 3XA and P34A had significantly higher activity compared to the wild type FoxO3a and the DNA binding mutant exerted a dominant negative effect on endogenous FoxO activity. These results showed that the PC3 cell line can serve as a good model for studying the ability of FoxO3a variants to reverse transformation characteristics in these cells and help in identifying downstream target genes that mediate these effects. The data also demonstrated that the transcriptional activities of the FoxO3a variants observed in HepG2 cells were not cell line specific, but rather represent a general trend.

CHAPTER 6: RESULTS, EFFECTS OF EXPRESSING FOXO3A VARIANTS IN PC3 PROSTATE CANCER CELLS

6.1 Generation of PC3 Stable Cell Lines Expressing FoxO3a Variants

In order to better understand the effects of varying FoxO3a activities on a cancer cell line with high, unregulated PI3K-Akt pathway, I set out to generate PC3 stable cell lines expressing different FoxO3a variants as summarized in Table 2. For this purpose, ~50% confluent PC3 cells in 10cm dishes were transfected for 48h with either a pcDNA vector control (this vector contains a neomycin resistance gene that allows selection with G418) or with pcDNA vector containing wild type FoxO3a, FoxO3a 3XA, FoxO3a P34A or FoxO3a R211A/S215E (DNA Binding Mutant). Following the 48h transfection, the cells were split in a 1:2 ratio and after a 24h recovery the media was replaced with media containing 800ug/ml G418 for selecting resistant cells. The G418 concentration used in the selection was determined in death-curve experiments of PC3 cells that showed 100% cell death at G418 concentrations of 500ug/ml and above (data not shown). Already before the addition of G418, there was a notable difference in cell confluence between the transfected samples, despite starting with an equal number of cells; The pcDNA empty vector and wild type FoxO3a transfected cells reached ~75% confluence while the samples containing the active mutants were ~40% confluent and had some dead floating cells. After two days of G418 treatment, the FoxO3a 3XA and P34A expressing cells did not look as healthy as the others (based on morphology, higher number of floating dead cells and a slower growth rate), suggesting that the presence of overactive FoxO3a is detrimental to the viability of PC3 cells.

At day 5 of G418 selection, the pcDNA expressing cells displayed no morphological changes and were growing at approximately the same rate as non-transfected, non-G418-treated PC3 cells, and thus were split at a 1:10 ratio. The wild type FoxO3a and DNA B.M.-transfected

cells showed some morphology changes, but did not show much growth inhibition, however, the 3XA and P34A-expressing cells exhibited slower growth rates, had more floating cells and did not look very healthy (Figure 6.1).

By day 16, the pcDNA control cells were 95% confluent and ready to be split again. Because the cells with the FoxO3a variants were growing at a much slower rate compared to the controls, the concentration of G418 was decreased to 600ug/ml. Cells with FoxO3a variants were split 1:2.5 on day 19 for passage 2. The next day, the DNA B.M. stable cells reached 85% confluence and had recovered from the passage, while the others were less confluent. The slow growth of the constitutive active (3XA and P34A) and wild type FoxO3a variants suggests that increased activity of FoxO3a is detrimental to PC3 cell growth.

While the PC3 stable cell lines were being maintained, one of the first things that needed to be determined was the expression level of the exogenous FoxO3a proteins. To investigate the exogenous FoxO3a protein levels in the PC3 stable cell lines, the first four passages of each stable cell line (except for the pcDNA control) were run on an SDS-PAGE and blotted for HA (the tag on all FoxO3a variants) (Figure 6.2A). It was clearly apparent that the overall level of the FoxO3a forms was much lower in the stable cell lines than what usually was seen in transiently transfected PC3 cells, as noticed by the length of time for blotting and increased amount of HA antibody used to detect the FoxO3a variants. This expression gradually decreased with each passage, reaching almost undetectable levels at passage 4. These results suggest that the expression of the FoxO3a variants negatively effected PC3 cell growth and/or viability.

To investigate why the transfected PC3 cell lines were growing so slowly and to determine if FoxO3a variants were inducing apoptosis in these cells, cell extracts from the first

two passages were blotted for various apoptosis markers (Figure 6.2B). This experiment demonstrated that cleaved PARP (active form) was increased slightly in the WT, 3XA and P34A FoxO3a stable cells, but not in the DNA binding mutant containing cell line. Cleaved AIF was present in all stable cell lines with the exception of the pcDNA controls. Finally, cleaved Caspase 3 was increased in the first passage of all FoxO3a variant cell lines. These results indicate that FoxO3a variants induce apoptosis in PC3 cells, providing an explanation for the observed slow growth rates and the gradual disappearance of the FoxO3a variants with each passage.

Cells from passage 4 were used for assaying FoxO transcriptional activity using the luciferase reporter assay (Fig. 6.3) to investigate the transcriptional activity of exogenous FoxO3a variants in the PC3 stable cell lines. Three separate experiments consistently showed increased FoxO transcriptional activity in P34A expressing PC3 cells. No significant changes were observed in cells expressing wild type FoxO3a, the 3XA mutants or the DNA binding mutant. The observation that the 3XA FoxO3a mutant was the least expressed of all the variants, may explains the lack of increased FoxO activity in cells transfected with this mutant. Apparently, the high level of Akt activity in the PC3 cells was able to keep the wild type FoxO3a activity at background levels in this passage. From these experiments, we conclude that the P34A FoxO3a mutant can induce increased FoxO activity in PC3 cells even when expressed at lower levels than wild-type FoxO3a, providing a good model for future studies examining biological consequences of FoxO3a activation in cancer cells with constitutively active Akt, such as the PC3 cells, as well as for determining transcriptional outputs.

Stable Cell Line	Day -3	Day -1	Day 1	Day 6	Day 16	Day 19
pcDNA3 Control	Transfection	Split 1:2	Selection (0.8mg/mL)	px 2	↘G418 (0.6mg/mL)	N/A
WT	Transfection	Split 1:2	Selection (0.8mg/mL)	N/A	↘G418 (0.6mg/mL)	px 2
3XA	Transfection	Split 1:2	Selection (0.8mg/mL)	N/A	↘G418 (0.6mg/mL)	px 2
P34A	Transfection	Split 1:2	Selection (0.8mg/mL)	N/A	↘G418 (0.6mg/mL)	px 2
DNA B.M.	Transfection	Split 1:2	Selection (0.8mg/mL)	N/A	↘G418 (0.6mg/mL)	px 2

Table 2: Generation of PC3 Stable Cell Lines Expressing FoxO3a Variants
PC3 cell were equally distributed into 100mm Corning plates and transiently transfected (Day -3) with 10ug of DNA for 48 hours. Cell were then split at a 1:2 ratio (Day -1) and allowed to recover for 24 hours. On day 1, selection was initiated by the addition of 0.8mg/ml G418 in RPMI media containing 5% fetal bovine serum. On day 2, the pcDNA control cell line was 95% confluent and was split 1:10 for passage 2 (px 2). The other cell lines were growing at a very slow rate, so lower levels of G418 media (0.6mg/mL) were used starting on Day 16. FoxO3a variant cell lines were split 1:2 for passage 2 (px 2) on day 19.

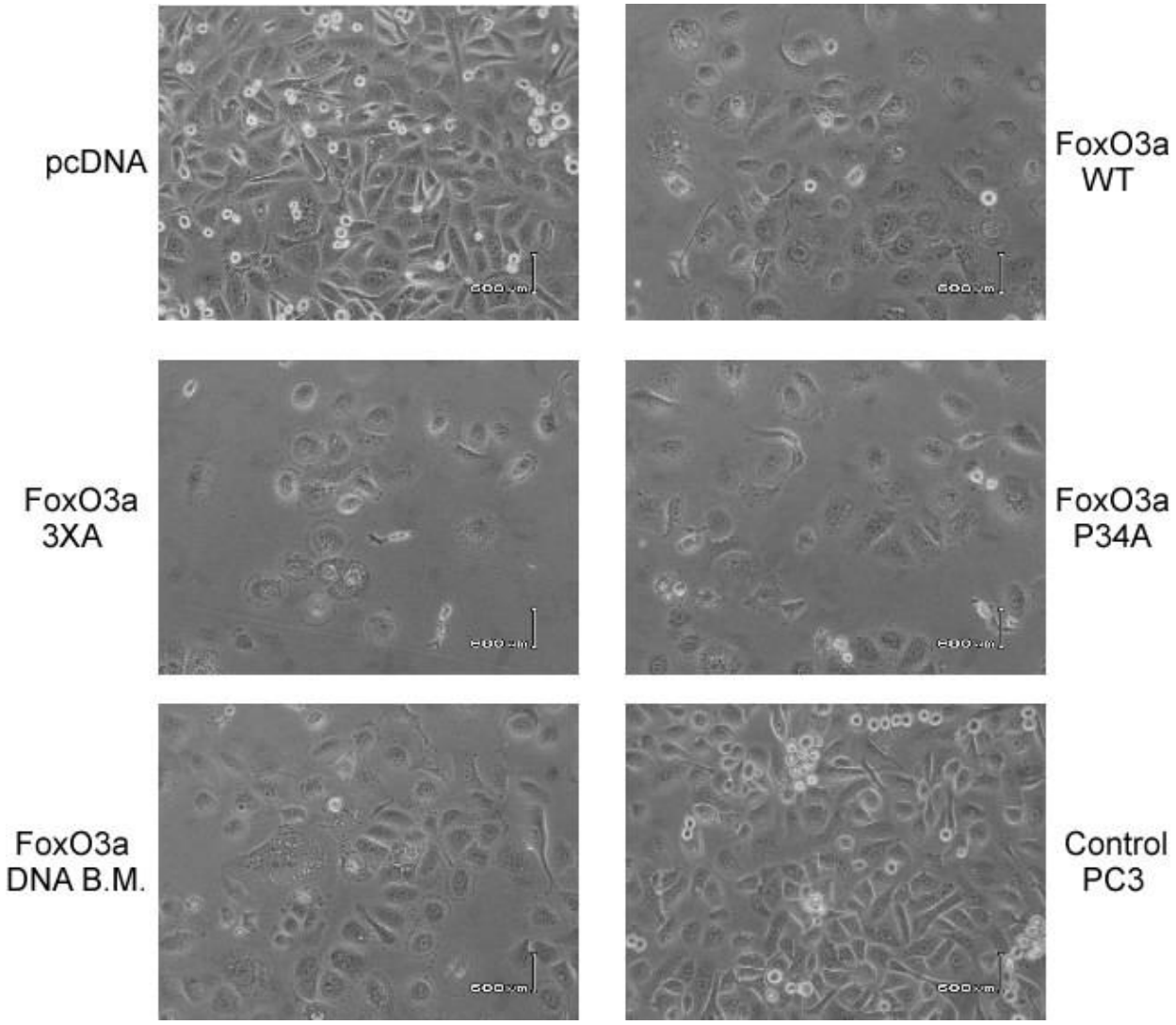


Figure 6.1. Morphology Changes in PC3 Cells Expressing FoxO3a Variants: Pictures were taken on Day 4 of passage 1 of all transfected cell lines. Control, non-transfected PC3 cells were maintained in parallel with the transfected cell lines.

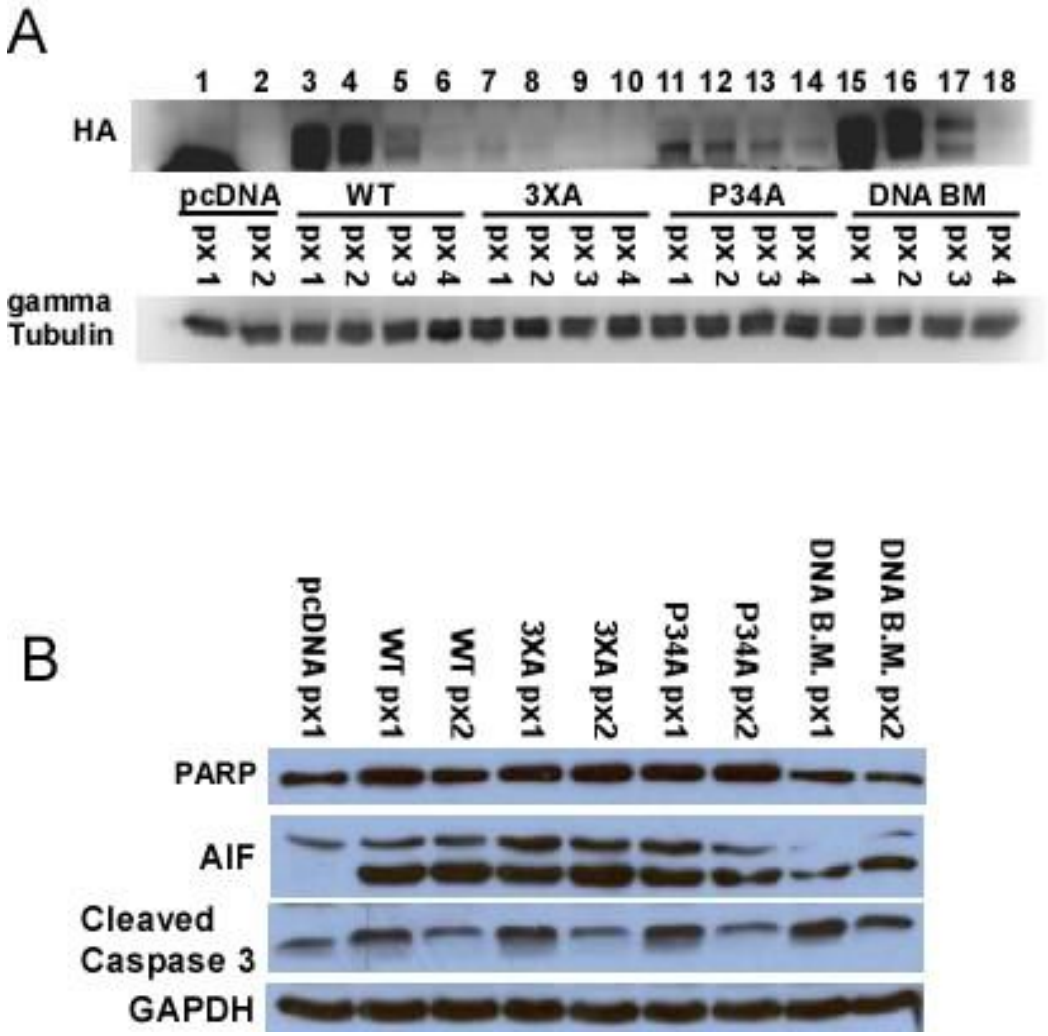


Figure 6.2. Expression of FoxO3a Variants Induces Apoptosis Markers in Stable PC3 Cell Lines: PC3 cells transfected with the indicated expression vectors were collected at the indicated passage and analyzed for FoxO3a expression **(A)** or for expression of the indicated apoptotic markers **(B)**. Tubulin **(A)** and GAPDH **(B)** immunoblots served as loading controls. The immunoblots presented in panel B were performed by A. Sosin. *The non specific band seen in the HA blot of lane 1 represents a cross-reacting band seen with all HA blots migrating just under the HA-FoxO3a band.

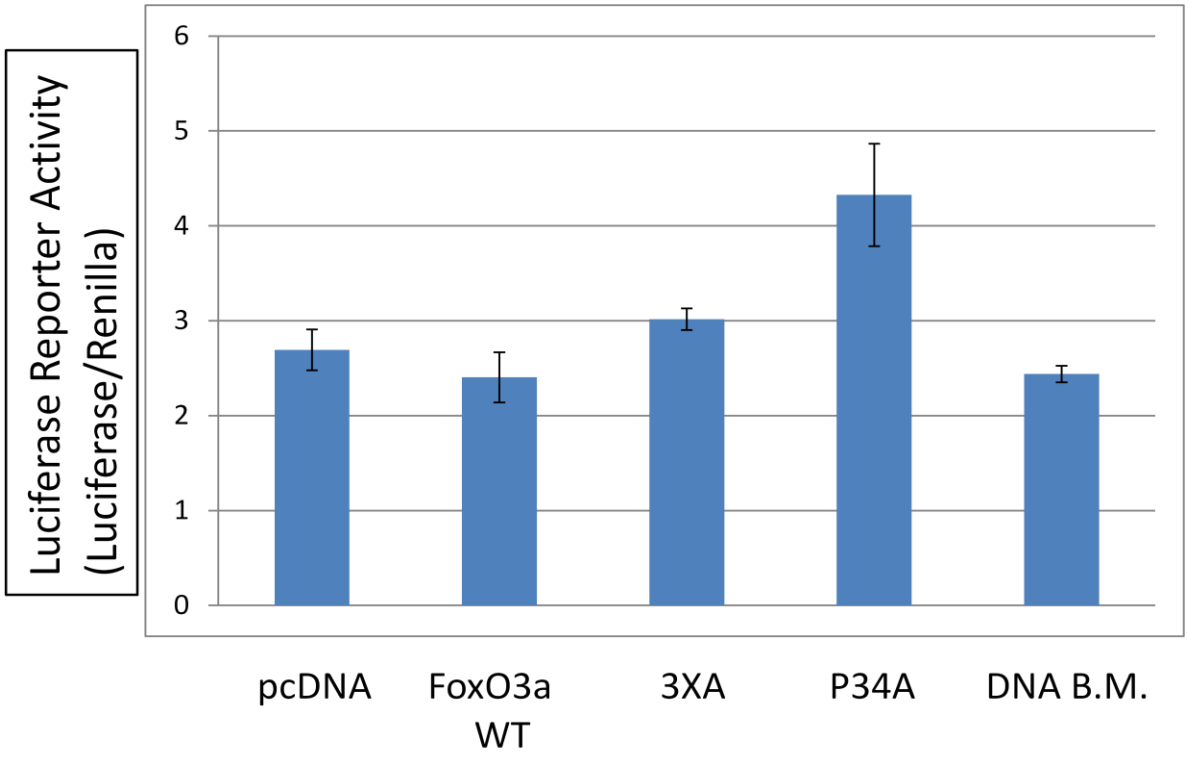


Figure 6.3. Increased FoxO Transcriptional Activity in FoxO3a P34A mutant-Expressing PC3 cells: PC3 cells from passage 4 expressing pcDNA control vector or the indicated FoxO3a mutants were transfected with FHBE-Luciferase and Renilla reporter vectors for 48 hours in RPMI media supplemented with 5% FBS and analyzed for luciferase activity.

6.2 Discussion, Effects of Expressing Foxo3a Variants in PC3 Prostate Cancer Cells

The results presented in this chapter demonstrate that increased expression of FoxO3a, especially of active FoxO3a forms, is not well tolerated and even detrimental to PC3 prostate cancer cells, which carry an inactivation of the PTEN tumor suppressor gene, resulting in high Akt activation and low FoxO transcriptional activity. The suppressing effects of FoxO3a expression were detected at several levels: decreased cell growth, induction of apoptotic markers, changes in cell morphology and gradual loss of FoxO3a expression with each cell passage. In addition, there was a noticeable cell death in the initial passages in cells expressing the active FoxO3a forms P34A and 3XA. The decrease in FoxO3a expression with each passage negatively correlated with the expression of apoptotic markers indicating that the increased expression of FoxO3a induces apoptosis in PC3 cells. Importantly, the expression of the P34A mutant resulted in increased overall FoxO transcriptional activity, which was significantly higher than the one observed with wild type FoxO3a, indicating that this mutant can be highly active even in the presence of constitutively active Akt. Thus, this mutant can serve as a good model for studying the effects of FoxO activation in cancer models that have increased Akt activity such as the PC3 cells. The induction of apoptotic markers and decreased cell growth by FoxO3a expression also suggests that the decreased FoxO transcriptional activity due to the high PI3K-Akt activity in PC3 cells is an important factor in maintaining viability and continuous and rapid cell growth and that restoring FoxO activity is sufficient for inducing cell growth attenuation and even cell death in this prostate cancer cell model. Whether this is a common trait in prostate cancer cells that carry PTEN inactivation (almost 70% of all human metastatic prostate cancers) remains to be determined. In addition, this form could serve as a good tool for identifying FoxO target genes in cancer models that could reverse the transformed phenotype by defining the gene expression arrays in control and FoxO3a P34A expressing cells.

CHAPTER 7: SUMMARY & SIGNIFICANCE

The FoxO transcription factors are the key transcription factors downstream of the PI3K/Akt pathway. FoxO3a is important due to its ability to transcriptionaly regulate a multitude of proteins involved in cell cycle arrest, metabolism, apoptosis, DNA repair and oxidative stress response [reviewed in (Weidinger et al., 2008)]. The PI3K-Akt pathway is commonly deregulated in many cancers, including prostate cancer. Loss of the tumor suppressor PTEN causes an increase in PI3K-Akt signaling activity, which results in decreased FoxO transcriptional activity (Modur et al., 2002). Two key regulators of FoxO3a are Akt and 14-3-3. Following PI3K and Akt activation, Akt phosphorylates FoxO3a on three residues: T32, S253 and S315. These phosphorylations generate docking sites for the adapter protein 14-3-3, which blocks FoxO3a DNA binding and induces its nuclear exclusion (Cahill et al., 2001, Brunet et al., 1999, Tang et al., 1999, Obsil et al., 2003, Brunet et al., 2002). A detailed understanding of the complex regulation of FoxO3a including the interplay between FoxO3a, Akt and 14-3-3 can help in developing new approaches to fight cancers that display an increase in Akt activation. Some advances have been made in this area recently by observing that the chemotherapeutic drug Paclitaxol can increase FoxO activity by decreasing Akt activity and reducing the ability of 14-3-3 to bind FoxO3a [reviewed in (Yang and Hung, 2009)].

As noted above, Akt can negatively regulate FoxO3a by phosphorylating FoxO3a on three residues, prepping the molecule for 14-3-3 binding. It has also been shown that Akt can bind FoxO3a (Zheng et al., 2000b). The work described in Chapter 3 adds significant knowledge to our understanding of the Akt-FoxO interaction. It demonstrates that the Akt-phosphorylation site motif, RxRxxS/T, does not play an important role in Akt binding to FoxO3a. This is shown both by mutating the Akt phosphorylation sites, i.e., FoxO3 3XA, and demonstrating that this

mutation does not alter Akt binding and by mutating the two arginines in the motif, i.e., FoxO3a 3xRxR→ LxL, and showing that this mutant is also capable of Akt binding. This novel finding, which may represent a general trait for Akt substrates, implies that besides the established phosphorylation recognition motif, FoxO3a contains a docking point for a stable Akt association. This possibility opens the door for new ways to target Akt signaling by blocking Akt interaction with its targets (Fig 7.1B). Until now, the focus has been in developing drugs that target the kinase activity of Akt, thereby targeting all Akt substrates indiscriminately. The possibility of targeting Akt interaction with its substrate may allow the development of more target-specific inhibitors offering the potential to specifically inhibit targets associated with specific disease, such as cancer, while reducing non-specific toxic effects, which are a common characteristic of Akt kinase inhibitors. Importantly, both the 3XA and 3X-RxR mutants lost their ability to bind 14-3-3, consistent with previous studies demonstrating the importance of FoxO3a phosphorylation for 14-3-3 binding. Future work will have to narrow down the Akt interaction point/s on FoxO3a and to determine whether different Akt substrates share similarities in their docking points. An initial attempt in this study at this goal involved the generation of FoxO3a fragments, including 1-211, 1-260 and 1-334. These fragments were phosphorylated by Akt and their protein steady-state levels were increased by co-expression with Akt, similarly to full-length FoxO3a. Initial binding experiments also suggested that the 1-260 fragment was sufficient for Akt binding (data not shown), however, these experiments need to be repeated to offer conclusive evidence. It is also possible that there are several distinct binding points for Akt interaction, thus a comprehensive study is required to address this important question.

From the Akt side, the presented data established that mutations that interfere with Akt kinase activity or in Akt activation completely abolish Akt binding to FoxO3a. This

phenomenon may also represent a general characteristic for Akt binding to its substrates, a point that needs to be determined in future studies. It is yet unclear whether it is the activity state of Akt that determines the binding or whether the inactivating mutations used in this study affected the conformation of Akt in a way that it blocked the binding to FoxO3a. Another possibility is that the mutations affected the cellular localization of Akt: When inactive, Akt primarily is localized in the cytoplasm and is translocated to the nucleus after phosphorylation and activation at the plasma membrane. Therefore, it is possible that only wild type Akt is capable of being transported to the nucleus whereas the inactive Akt mutants (2A and K179M) remain in the cytoplasm. Initial experiments though showed Akt association with FoxO3a both in the nucleus and in the cytoplasm (data not shown), making this possibility less likely.

Another novel aspect described in this work is the observation that increased Akt expression results in increased expression of FoxO3a. This Akt-induced increase was seen with all FoxO3a forms tested including, 3XA, P34A, DNA B.M., the various Akt phosphorylation recognition site mutants (RxR→LxL) and the various N-terminal fragments (Figures 3.1, 3.3 and 3.4). This indicated that the increase was not dependent on the ability of Akt to phosphorylate FoxO3a, since it also increased expression of FoxO3a forms that cannot be phosphorylated by Akt, but rather on its ability to bind FoxO3a, since the inactive Akt mutants that failed to bind FoxO3a did not induce the increase in protein expression. This point requires, however, further elucidation since these Akt forms are also inactive and it is possible that the observed increase in FoxO3a expression is mediated by other Akt substrates that can affect protein translation or other general pathways that regulate protein stability and expression. To this end, experiments using rapamycin, an inhibitor of the mTORC1 complex downstream of Akt that has been shown to regulate protein translation showed that the Akt-induce increased expression of FoxO3a was not

dependent on this pathway (data not shown), leaving this question open. Together these results suggest that Akt may have a positive role in FoxO3a regulation besides its established negative role. This positive role requires an intact Akt capable of binding FoxO3a, but is not dependent on the ability of Akt to phosphorylate FoxO3a, suggesting that an inactive Akt can serve as a positive regulator of FoxO3a through direct interaction (Fig 7.1A). It is possible that Akt dissociates from FoxO3a after its phosphorylation, since no phosphorylated FoxO3a was detected in Akt pull downs, in contrast to 14-3-3 pull downs that showed plenty of phosphorylated FoxO3a.

The present work also uncovered a novel role of 14-3-3 in controlling FoxO3a protein levels by stabilizing phosphorylated FoxO3a (Chapter 4, reviewed in Fig 7.1A). The ability to increase p-FoxO3a protein levels was in a time dependent manner and required 14-3-3 binding to FoxO3a in as much as it was not observed with FoxO3a mutants that do not bind 14-3-3 (e.g., 3XA, P34A and 3X-RxR). Time course experiments using phosphatase and proteosome inhibitors demonstrated that 14-3-3 proteins primarily protect FoxO3a from dephosphorylation, suggesting a mechanism whereby 14-3-3 proteins maintain a recyclable pool of phosphorylated FoxO3a in the cytoplasm. This finding is supported by clinical data showing stable levels of p-FoxO3a in the cytoplasm of cancer patient samples (Kornblau et al., 2010), suggesting the protein is not automatically degraded following Akt phosphorylation. Thus, it appears that 14-3-3 not only serves as a negative regulator of FoxO3a by blocking DNA binding and excluding it from the nucleus, but it also plays a positive role by protecting FoxO3a from dephosphorylation (Fig. 7.1A). This novel function suggests that growth and stress condition that affect 14-3-3 availability (Tzivion et al., 2001) can modulate the levels of FoxO3a in the cell by limiting the levels of available 14-3-3.

In addition, the present work allowed the development of a novel active FoxO3a form by mutating a proline at the primary 14-3-3 binding site surrounding T32. This mutant remains a substrate for Akt, but because of lack of 14-3-3 binding, is constitutively active. Initial experiments showed that this mutant, unlike the 3XA form, which also is a constitutive form, does not serve as a dominant negative form for Akt (data not shown), thus making it a better tool than FoxO3a 3XA for studying the biological and transcriptional outputs of active FoxO3a without the "noise" of Akt inhibition.

In conclusion for this part, it appears that both Akt and 14-3-3 can function not only as negative FxoO3a regulators, but also as positive ones. Therefore, the availability of Akt and unbound 14-3-3 in the cell which can be modulated by different environmental conditions could dictate how FoxO3a will be processed; either through degradation or stabilization (summarized in Fig. 7.1A).

Luciferase transcriptional reporter assays in this study were conducted in two cell models: HepG2 cells, representing a cell with normal, tightly regulated PI3K-Akt pathway and PC3 prostate cancer cells, representing cancer cells with constitutively active Akt (Chapters 5 and 6). These experiments demonstrated the regulation of FoxO3a transcriptional activity by insulin and Akt in HepG2 cells and established the new FoxO3a P34A form as a constitutive form that cannot be regulated by insulin, even when Akt is over expressed, similarly to the previously describe 3XA form. Importantly, this FoxO3a form was highly active in PC3 cells that carry PTEN inactivation and highly active Akt, making it an important tool to examine the potential cancer suppressing activities of FoxO activation. An example for this potential is the observation in this study that expression of the FoxO3a P34A form in PC3 cells results in attenuated cell growth, increased expression of apoptotic markers and induction of

morphological changes and cell death in the expressing cells (Chapter 6, see Fig 7.1B). Further detailed analysis of the effects of this mutant on various transformation characteristics of cancer cells combined with transcriptional output analysis should provide important information on the tumor suppressive properties of FoxO proteins and open the door for potential development of novel anti-cancer agents.

Another interesting finding from the reporter assays is that the DNA binding mutant (R211A, S215E), behaves as a dominant negative form for endogenous FoxO transcriptional activity (chapter 5). The significance of this finding is that this mutant can help in studying the transcriptional outputs of FoxO proteins and in establishing potential oncogenic properties of inactivating FoxO mutations, suggesting that FoxO proteins can function both as tumor suppressors when normal and as oncogenes when inactivated by mutation through dominant negative effects on the endogenous FoxO.

In summary, through the generation of two new FoxO3a variants (P34A and the DNA binding mutant), the effects of high transcriptional activity and loss of FoxO transcriptional activity can be studied and their effects on cancer cell initiation and progression (both positive and negative) can be examined.

The attempt to generate PC3 stable cell lines expressing FoxO3a variants in order to study the long-term effects of FoxO3a activity on prostate cancer cells, exhibiting high Akt activity and low endogenous FoxO transcriptional activity, met with considerable difficulties. This was due primarily to the observed toxic effects of the FoxO3a proteins in PC3 cells, which caused a gradual loss of FoxO3a expression with each cell passage. This finding suggests that long-term expression of active FoxO3a is detrimental to PC3 cells, pointing out the importance

of the ability of Akt to inactivate endogenous FoxOs for maintaining the transformed state. Thus, it appears that enhancing FoxO3a activity is enough to inhibit or kill prostate cancer cells that are dependent on high Pi3K/Akt signaling (Fig 7.1B). This point can be further studied by developing viral vectors for high efficiency gene transfer of FoxO3a forms and examining their anti-cancer properties.

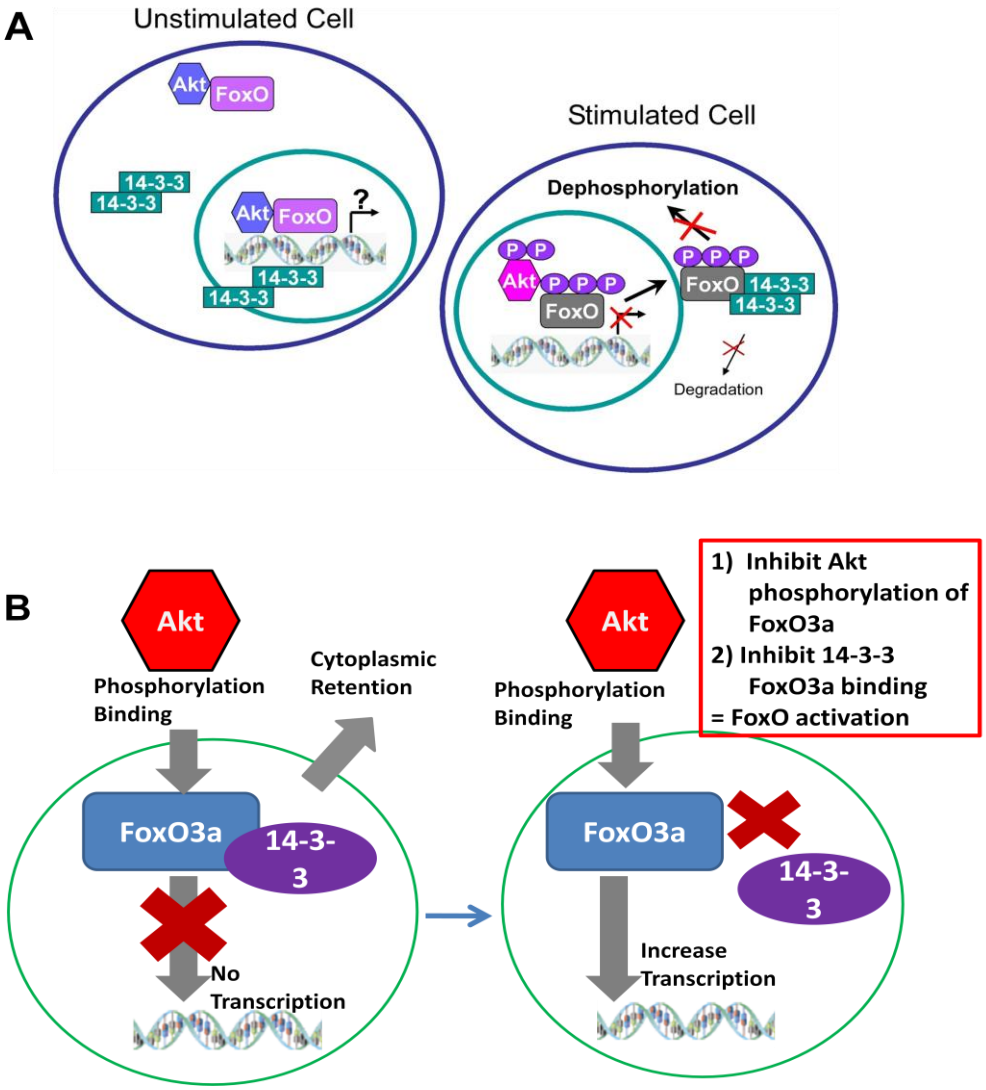


Figure 7.1. A model of FoxO3a Regulation by Akt and 14-3-3 and Clinical Implications in Prostate Cancer: In a resting cell, Akt binds and stabilizes un-phosphorylated FoxO3a. This effect represents a potential novel positive regulatory role of Akt in FoxO regulation. In a growth factor stimulated cell, Akt is active and phosphorylates FoxO. This phosphorylation disrupts the Akt/FoxO interaction (based on the results showing that Akt was not observed to bind to phosphorylated FoxO). 14-3-3 binding to phosphorylated-FoxO, blocks FoxO-DNA binding, induces nuclear exclusion and protects it from dephosphorylation. 14-3-3 may also protect phosphorylated-FoxO3a from degradation to some extent (**A**). PC3 cells and most human prostate cancers have a loss of PTEN, resulting in the loss of FoxO transcriptional activity. Targeting either the Akt-FoxO3a phosphorylation or FoxO3-14-3-3 binding could reactivate FoxO3a transcriptional activity, inducing apoptosis in prostate cancer cells (**B**).

REFERENCES

AHN, J. Y., LIU, X., LIU, Z., PEREIRA, L., CHENG, D., PENG, J., WADE, P. A., HAMBURGER, A. W. & YE, K. 2006. Nuclear Akt associates with PKC-phosphorylated Ebp1, preventing DNA fragmentation by inhibition of caspase-activated DNase. *EMBO J,* 25**,** 2083-95.

ALESSI, D. R., ANDJELKOVIC, M., CAUDWELL, B., CRON, P., MORRICE, N., COHEN, P. & HEMMINGS, B. A. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. *Embo Journal,* 15**,** 6541-6551.

ANDJELKOVIC, M., JAKUBOWICZ, T., CRON, P., MING, X. F., HAN, J. W. & HEMMINGS, B. A. 1996. Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. *Proc Natl Acad Sci U S A,* 93**,** 5699-704.

AOKI, M., JIANG, H. & VOGT, P. K. 2004. Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc Natl Acad Sci U S A,* 101**,** 13613-7.

BIGGS, W. H., 3RD, MEISENHELDER, J., HUNTER, T., CAVENEE, W. K. & ARDEN, K. C. 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A,* 96**,** 7421-6.

BIONDI, R. M. 2004. Phosphoinositide-dependent protein kinase 1, a sensor of protein conformation. *Trends Biochem Sci,* 29**,** 136-42.

BIRKENKAMP, K. U. & COFFER, P. J. 2003. Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors. *Biochem Soc Trans,* 31**,** 292-7.

BOURA, E., SILHAN, J., HERMAN, P., VECER, J., SULC, M., TEISINGER, J., OBSILOVA, V. & OBSIL, T. 2007. Both the N-terminal loop and wing W2 of the forkhead domain of transcription factor Foxo4 are important for DNA binding. *J Biol Chem,* 282**,** 8265-75.

BRAZIL, D. P., PARK, J. & HEMMINGS, B. A. 2002. PKB binding proteins. Getting in on the Akt. *Cell,* 111**,** 293-303.

BRUNET, A., BONNI, A., ZIGMOND, M. J., LIN, M. Z., JUO, P., HU, L. S., ANDERSON, M. J., ARDEN, K. C., BLENIS, J. & GREENBERG, M. E. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell,* 96**,** 857-68.

BRUNET, A., KANAI, F., STEHN, J., XU, J., SARBASSOVA, D., FRANGIONI, J. V., DALAL, S. N., DECAPRIO, J. A., GREENBERG, M. E. & YAFFE, M. B. 2002. 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *J Cell Biol,* 156**,** 817-28.

BRUNET, A., PARK, J., TRAN, H., HU, L. S., HEMMINGS, B. A. & GREENBERG, M. E. 2001. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol,* 21**,** 952-65.

CAHILL, C. M., TZIVION, G., NASRIN, N., OGG, S., DORE, J., RUVKUN, G. & ALEXANDER-BRIDGES, M. 2001. Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3-dependent and 14-3-3-independent pathways. *J Biol Chem,* 276**,** 13402-10.

CARNERO, A., BLANCO-APARICIO, C., RENNER, O., LINK, W. & LEAL, J. F. 2008. The PTEN/PI3K/AKT signalling pathway in cancer, therapeutic implications. *Curr Cancer Drug Targets,* 8**,** 187-98.

CHANDRAMOHAN, V., MINEVA, N. D., BURKE, B., JEAY, S., WU, M., SHEN, J., YANG, W., HANN, S. R. & SONENSHEIN, G. E. 2008. c-Myc represses FOXO3a-mediated transcription of the gene encoding the p27(Kip1) cyclin dependent kinase inhibitor. *J Cell Biochem,* 104**,** 2091-106.

CICHY, S. B., UDDIN, S., DANILKOVICH, A., GUO, S. D., KLIPPEL, A. & UNTERMAN, T. G. 1998. Protein kinase B/Akt mediates effects of insulin on hepatic insulin-like growth factor-binding protein-1 gene expression through a conserved insulin response sequence. *Journal of Biological Chemistry,* 273**,** 6482-6487.

COFFER, P. J., JIN, J. & WOODGETT, J. R. 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J,* 335 (Pt 1)**,** 1-13.

COTELLE, V., MEEK, S. E., PROVAN, F., MILNE, F. C., MORRICE, N. & MACKINTOSH, C. 2000. 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved Arabidopsis cells. *EMBO J,* 19**,** 2869-76.

CUNNINGHAM, M. A., ZHU, Q. & HAMMOND, J. M. 2004. FoxO1a can alter cell cycle progression by regulating the nuclear localization of p27kip in granulosa cells. *Mol Endocrinol,* 18**,** 1756-67.

DANSEN, T. B. & BURGERING, B. M. 2008. Unravelling the tumor-suppressive functions of FOXO proteins. *Trends Cell Biol,* 18**,** 421-9.

DIJKERS, P. F., BIRKENKAMP, K. U., LAM, E. W., THOMAS, N. S., LAMMERS, J. W., KOENDERMAN, L. & COFFER, P. J. 2002. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J Cell Biol,* 156**,** 531-42.

DONG, X. Y., CHEN, C., SUN, X., GUO, P., VESSELLA, R. L., WANG, R. X., CHUNG, L. W., ZHOU, W. & DONG, J. T. 2006. FOXO1A is a candidate for the 13q14 tumor suppressor gene inhibiting androgen receptor signaling in prostate cancer. *Cancer Res,* 66**,** 6998-7006.

DOUGHERTY, M. K. & MORRISON, D. K. 2004. Unlocking the code of 14-3-3. *J Cell Sci,* 117**,** 1875-84.

GALETIC, I., ANDJELKOVIC, M., MEIER, R., BRODBECK, D., PARK, J. & HEMMINGS, B. A. 1999. Mechanism of protein kinase B activation by insulin/insulin-like growth factor-1 revealed by specific inhibitors of phosphoinositide 3-kinase--significance for diabetes and cancer. *Pharmacol Ther,* 82**,** 409-25.

GERICKE, A., MUNSON, M. & ROSS, A. H. 2006. Regulation of the PTEN phosphatase. *Gene,* 374**,** 1-9.

GERVAIS, M., DUGOURD, C., MULLER, L., ARDIDIE, C., CANTON, B., LOVICONI, L., CORVOL, P., CHNEIWEISS, H. & MONNOT, C. 2006. Akt down-regulates ERK1/2 nuclear localization and angiotensin II-induced cell proliferation through PEA-15. *Mol Biol Cell,* 17**,** 3940-51.

GOSWAMI, A., BURIKHANOV, R., DE THONEL, A., FUJITA, N., GOSWAMI, M., ZHAO, Y., ERIKSSON, J. E., TSURUO, T. & RANGNEKAR, V. M. 2005. Binding and phosphorylation of par-4 by akt is essential for cancer cell survival. *Mol Cell,* 20**,** 33-44.

HANNENHALLI, S. & KAESTNER, K. H. 2009. The evolution of Fox genes and their role in development and disease. *Nature Reviews Genetics,* 10**,** 233-240.

HENSON, E. S. & GIBSON, S. B. 2006. Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: implications for cancer therapy. *Cell Signal,* 18**,** 2089-97.

HUANG, H., REGAN, K. M., WANG, F., WANG, D., SMITH, D. I., VAN DEURSEN, J. M. & TINDALL, D. J. 2005. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci U S A,* 102**,** 1649-54.

KATSO, R., OKKENHAUG, K., AHMADI, K., WHITE, S., TIMMS, J. & WATERFIELD, M. D. 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol,* 17**,** 615-75.

KIKUCHI, S., NAGAI, T., KUNITAMA, M., KIRITO, K., OZAWA, K. & KOMATSU, N. 2007. Active FKHRL1 overcomes imatinib resistance in chronic myelogenous leukemia-derived cell lines via the production of tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Sci,* 98**,** 1949-58.

KIM, A. H., YANO, H., CHO, H., MEYER, D., MONKS, B., MARGOLIS, B., BIRNBAUM, M. J. & CHAO, M. V. 2002. Akt1 regulates a JNK scaffold during excitotoxic apoptosis. *Neuron,* 35**,** 697-709.

KLEIN, J. B., BARATI, M. T., WU, R., GOZAL, D., SACHLEBEN, L. R., JR., KAUSAR, H., TRENT, J. O., GOZAL, E. & RANE, M. J. 2005. Akt-mediated valosin-containing protein 97 phosphorylation regulates its association with ubiquitinated proteins. *J Biol Chem,* 280**,** 31870-81.

KOPS, G. J., DANSEN, T. B., POLDERMAN, P. E., SAARLOOS, I., WIRTZ, K. W., COFFER, P. J., HUANG, T. T., BOS, J. L., MEDEMA, R. H. & BURGERING, B. M.

2002. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature,* 419**,** 316-21.

KORNBLAU, S. M., SINGH, N., QIU, Y., CHEN, W., ZHANG, N. & COOMBES, K. R. 2010. Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia. *Clin Cancer Res,* 16**,** 1865-74.

LEE, S. B., XUAN NGUYEN, T. L., CHOI, J. W., LEE, K. H., CHO, S. W., LIU, Z., YE, K., BAE, S. S. & AHN, J. Y. 2008. Nuclear Akt interacts with B23/NPM and protects it from proteolytic cleavage, enhancing cell survival. *Proc Natl Acad Sci U S A,* 105**,** 16584-9.

LEHTINEN, M. K., YUAN, Z., BOAG, P. R., YANG, Y., VILLEN, J., BECKER, E. B., DIBACCO, S., DE LA IGLESIA, N., GYGI, S., BLACKWELL, T. K. & BONNI, A. 2006. A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. *Cell,* 125**,** 987-1001.

LI, P., LEE, H., GUO, S., UNTERMAN, T. G., JENSTER, G. & BAI, W. 2003. AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. *Mol Cell Biol,* 23**,** 104-18.

MA, X. M. & BLENIS, J. 2009. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol,* 10**,** 307-18.

MEDEMA, R. H., KOPS, G. J., BOS, J. L. & BURGERING, B. M. 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature,* 404**,** 782-7.

MODUR, V., NAGARAJAN, R., EVERS, B. M. & MILBRANDT, J. 2002. FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. *J Biol Chem,* 277**,** 47928-37.

MUSLIN, A. J., TANNER, J. W., ALLEN, P. M. & SHAW, A. S. 1996. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell,* 84**,** 889-97.

MYATT, S. S. & LAM, E. W. 2007. The emerging roles of forkhead box (Fox) proteins in cancer. *Nat Rev Cancer,* 7**,** 847-59.

NAKAE, J., BARR, V. & ACCILI, D. 2000. Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single amino acid residue in the forkhead transcription factor FKHR. *EMBO J,* 19**,** 989-96.

O'CONNOR, L., STRASSER, A., O'REILLY, L. A., HAUSMANN, G., ADAMS, J. M., CORY, S. & HUANG, D. C. 1998. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J,* 17**,** 384-95.

OBSIL, T., GHIRLANDO, R., ANDERSON, D. E., HICKMAN, A. B. & DYDA, F. 2003. Two 14-3-3 binding motifs are required for stable association of Forkhead transcription factor FOXO4 with 14-3-3 proteins and inhibition of DNA binding. *Biochemistry,* 42**,** 15264-72.

PAIK, J. H., KOLLIPARA, R., CHU, G., JI, H., XIAO, Y., DING, Z., MIAO, L., TOTHOVA, Z., HORNER, J. W., CARRASCO, D. R., JIANG, S., GILLILAND, D. G., CHIN, L., WONG, W. H., CASTRILLON, D. H. & DEPINHO, R. A. 2007. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell,* 128**,** 309-23.

PARK, K. W., KIM, D. H., YOU, H. J., SIR, J. J., JEON, S. I., YOUN, S. W., YANG, H. M., SKURK, C., PARK, Y. B., WALSH, K. & KIM, H. S. 2005. Activated forkhead transcription factor inhibits neointimal hyperplasia after angioplasty through induction of p27. *Arteriosclerosis Thrombosis and Vascular Biology,* 25**,** 742-747.

PLAS, D. R. & THOMPSON, C. B. 2003. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem,* 278**,** 12361-6.

RENA, G., PRESCOTT, A. R., GUO, S., COHEN, P. & UNTERMAN, T. G. 2001. Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targetting. *Biochem J,* 354**,** 605-12.

RODRIGUEZ-VICIANA, P., WARNE, P. H., DHAND, R., VANHAESEBROECK, B., GOUT, I., FRY, M. J., WATERFIELD, M. D. & DOWNWARD, J. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature,* 370**,** 527-32.

SAMUELS, Y., WANG, Z., BARDELLI, A., SILLIMAN, N., PTAK, J., SZABO, S., YAN, H., GAZDAR, A., POWELL, S. M., RIGGINS, G. J., WILLSON, J. K., MARKOWITZ, S., KINZLER, K. W., VOGELSTEIN, B. & VELCULESCU, V. E. 2004. High frequency of mutations of the PIK3CA gene in human cancers. *Science,* 304**,** 554.

SARBASSOV, D. D., GUERTIN, D. A., ALI, S. M. & SABATINI, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science,* 307**,** 1098-101.

SO, C. W. & CLEARY, M. L. 2003. Common mechanism for oncogenic activation of MLL by forkhead family proteins. *Blood,* 101**,** 633-9.

SUN, L., LIU, L., YANG, X. J. & WU, Z. 2004. Akt binds prohibitin 2 and relieves its repression of MyoD and muscle differentiation. *J Cell Sci,* 117**,** 3021-9.

TANG, E. D., NUNEZ, G., BARR, F. G. & GUAN, K. L. 1999. Negative regulation of the forkhead transcription factor FKHR by Akt. *J Biol Chem,* 274**,** 16741-6.

TANG, T. T., DOWBENKO, D., JACKSON, A., TONEY, L., LEWIN, D. A., DENT, A. L. & LASKY, L. A. 2002. The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. *J Biol Chem,* 277**,** 14255-65.

TANG, X., JANG, S. W., WANG, X., LIU, Z., BAHR, S. M., SUN, S. Y., BRAT, D., GUTMANN, D. H. & YE, K. 2007. Akt phosphorylation regulates the tumour-suppressor merlin through ubiquitination and degradation. *Nat Cell Biol,* 9**,** 1199-207.

TRAN, H., BRUNET, A., GRENIER, J. M., DATTA, S. R., FORNACE, A. J., JR., DISTEFANO, P. S., CHIANG, L. W. & GREENBERG, M. E. 2002. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science,* 296**,** 530-4.

TSAI, K. L., SUN, Y. J., HUANG, C. Y., YANG, J. Y., HUNG, M. C. & HSIAO, C. D. 2007. Crystal structure of the human FOXO3a-DBD/DNA complex suggests the effects of post-translational modification. *Nucleic Acids Res,* 35**,** 6984-94.

TZIVION, G., GUPTA, V. S., KAPLUN, L. & BALAN, V. 2006. 14-3-3 proteins as potential oncogenes. *Semin Cancer Biol,* 16**,** 203-13.

TZIVION, G., SHEN, Y. H. & ZHU, J. 2001. 14-3-3 proteins; bringing new definitions to scaffolding. *Oncogene,* 20**,** 6331-8.

VAN DEN HEUVEL, A. P., SCHULZE, A. & BURGERING, B. M. 2005. Direct control of caveolin-1 expression by FOXO transcription factors. *Biochem J,* 385**,** 795-802.

VANDERMOERE, F., EL YAZIDI-BELKOURA, I., DEMONT, Y., SLOMIANNY, C., ANTOL, J., LEMOINE, J. & HONDERMARCK, H. 2007. Proteomics exploration reveals that actin is a signaling target of the kinase Akt. *Mol Cell Proteomics,* 6**,** 114-24.

WEIDINGER, C., KRAUSE, K., KLAGGE, A., KARGER, S. & FUHRER, D. 2008. Forkhead box-O transcription factor: critical conductors of cancer's fate. *Endocr Relat Cancer,* 15**,** 917-29.

YAFFE, M. B., RITTINGER, K., VOLINIA, S., CARON, P. R., AITKEN, A., LEFFERS, H., GAMBLIN, S. J., SMERDON, S. J. & CANTLEY, L. C. 1997. The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell,* 91**,** 961-71.

YAN, L., LAVIN, V. A., MOSER, L. R., CUI, Q., KANIES, C. & YANG, E. 2008. PP2A regulates the pro-apoptotic activity of FOXO1. *J Biol Chem,* 283**,** 7411-20.

YANG, H., ZHAO, R., YANG, H. Y. & LEE, M. H. 2005. Constitutively active FOXO4 inhibits Akt activity, regulates p27 Kip1 stability, and suppresses HER2-mediated tumorigenicity. *Oncogene,* 24**,** 1924-35.

YANG, J. Y. & HUNG, M. C. 2009. A new fork for clinical application: targeting forkhead transcription factors in cancer. *Clin Cancer Res,* 15**,** 752-7.

ZHANG, H., ZHA, X., TAN, Y., HORNBECK, P. V., MASTRANGELO, A. J., ALESSI, D. R., POLAKIEWICZ, R. D. & COMB, M. J. 2002. Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. *J Biol Chem,* 277**,** 39379-87.

ZHENG, D. Q., WOODARD, A. S., TALLINI, G. & LANGUINO, L. R. 2000a. Substrate specificity of alpha(v)beta(3) integrin-mediated cell migration and phosphatidylinositol 3-kinase/AKT pathway activation. *J Biol Chem,* 275**,** 24565-74.

ZHENG, W. H., KAR, S. & QUIRION, R. 2000b. Insulin-like growth factor-1-induced phosphorylation of the forkhead family transcription factor FKHRL1 is mediated by Akt kinase in PC12 cells. *J Biol Chem,* 275**,** 39152-8.

ABSTRACT

REGULATORY AND FUNCTIONAL ASPECTS OF FOXO3A TRANSCRIPTION FACTOR AND THEIR IMPLICATIONS IN PROSTATE CANCER

by

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The PI3K-Akt pathway is a critical mediator of growth factor signaling affecting many cellular functions. The deregulation of this pathway has been shown to be involved in the development of various cancers. One of the main targets of this pathway is FoxO3a, a transcription factor whose target genes are involved in important cellular processes such as apoptosis, cell cycle control, and glucose metabolism. FoxO3a is regulated by various post-translational modifications including acetylation, ubiquitination and phosphorylation. FoxO3a is directly phosphorylated by Akt on 3 residues: Threonine 32, Serine 253 and Serine 315. Phosphorylation by Akt generates binding sites for 14-3-3, a protein which regulates FoxO3a DNA binding, transcriptional activity, and nuclear localization. Once phosphorylated, FoxO3a accumulates in the cytoplasm where it is subsequently degraded. My goal was to further elucidate the functional significance of the Akt-FoxO3a interaction and the mechanisms associated with FoxO3a regulation by Akt and 14-3-3. Novel findings presented in this dissertation offer a more in-depth and complete understanding of FoxO3a regulation, where both

Akt and 14-3-3 can serve as positive regulators of FoxO3a besides their established negative roles. 14-3-3 binding is shown to stabilize FoxO3a protein by protecting it from dephosphorylation, providing a pool of FoxO3a that can be reactivated by dephosphorylation, while Akt seems to stabilize FoxO3a proteins and increase their steady-state protein levels by direct binding. In addition, the work provides a better understanding of the characteristics of Akt-FoxO3a interaction by demonstrating that the Akt phosphorylation site recognition motif, RxRxxS/T on FoxO3a does not mediate Akt binding, suggesting the presence of a distinct docking point for this interaction. An intact ATP binding pocket and intact activating phosphorylation sites of Akt were obligatory for the interaction. By mutating a residue located within an important region for FoxO3a-14-3-3 binding (P34A), it was demonstrated that 14-3-3 binding to FoxO3a plays a key role in blocking FoxO3a transcriptional activity and that Akt phosphorylation of FoxO3 is insufficient by-itself for negative FoxO3a regulation. The demonstration that the P34A FoxO3a mutant was active under conditions that were suppressive of wild type FoxO3a, established this mutant as a constitutive active FoxO3a variant, offering an important tool for future studies. Importantly, expression of the P34A FoxO3a mutant in PC3 prostate cancer cells, which have high levels of active Akt due to loss of the PTEN, resulted in decreased growth, increased expression of apoptotic markers and cell death, demonstrating the detrimental effects of active FoxO3a on PC3 cell viability. Overall, this data suggest that active FoxO3a may be capable of inducing apoptosis in cancer cells that rely on high PI3K-Akt pathway activity for their survival.

AUTOBIOGRAPHICAL STATEMENT

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2000 Faculty Honors Scholarship, Calvin College
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2000-2004 College of Science Dean's List- fall 2000, Winter 2001, Fall 2001, Winter 2002, Fall 2002, Fall 2003, Winter 2004
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2004 Recipient of the Honor's Undergraduate Research Grant
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2006-2007 Thomas C. Rumble University Fellowship
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Publications

Freeman DC, Brown ML, **Dobson M**, Jordon Y, Kizy A, Micallef C, Hancock LC, Graham JH, Emlen JM. "Developmental instability: measures of resistance and resilience using pumpkin (*Cucurbita pep* L.)." Biological Journal of the Linnean Society. 2003. 78, 27-41.

Leicht DT, Balan V, Kaplan A, Singh-Gubta V, Kaplan L, **Dobson M** and Tzivion G. (2007)" Raf Kinases; function, regulation and role in human cancer." BBA-MCR, Vol 1773, Iss 8, August 2007, pp1196-1212.

Dobson M, Ma S, Kaplan L and Tzivion G. (2010). "A Complex Bimodal Regulation of FoxO3a by Akt and 14-3-3." **In preparation.**

Singh-Gupta V, **Dobson M** and Tzivion G. (2010) "Differential Requirements for Raf and MEK activities during cell cycle progression revealed using specific kinase inhibitors." **In preparation.**

Dobson M and Tzivion G. (2010) FoxO3a: Signaling Gateway, Molecule Pages. UCSD and Nature.