The Role of Transforming Growth Factor-β on the Regulation of FoxO Group Members and $p27^{kip1}$ in Fibroblast Cells

© Danielle Penny Ings, B.Sc.

A Thesis submitted to the School of Graduate Studies in the partial fulfillment of the requirements for the degree of

Master of Science, Medicine
Cancer and Development
Memorial University of Newfoundland

October 2017

Abstract

Transforming Growth Factor-β (TGF-β) regulates a variety of biological effects, including cellular proliferation, dependent on the cellular context. There is growing evidence that TGF-β regulates numerous pathways independent of Smad, including the PI3K/Akt pathway. Therefore, since FoxO is downstream of PI3K/Akt, we examined the role of TGF-β on the post-translational regulation of FoxO family members. It was found that TGF-β increased the phosphorylation of FoxO1, 3a, and 4, as well as the cytoplasmic localization of FoxO1 in fibroblasts via the PI3K/Akt pathway. In addition, since Akt and FoxO members can induce phosphorylation and transcription of $p27^{kip1}$ (a cyclin dependent kinase inhibitor), respectively, we examined both the post-translational and transcriptional regulation of $p27^{kip1}$ by TGF-β in fibroblasts. $p27^{kip1}$ phosphorylation increased following TGF-β addition, resulting in cytoplasmic localization in fibroblasts over time. $p27^{kip1}$ mRNA expression decreased following TGF-β treatment in fibroblasts, however, we were unable to show the decrease was dependent on PI3K or FoxO4. Therefore, the results demonstrated that TGF-β regulates the FoxO group post-translationally via the PI3K/Akt pathway, and FoxO group functionality through transcription and post-translational modifications of $p27^{kip1}$ in fibroblasts.

Acknowledgements

I would like to thank my supervisor Dr. Jules Doré for his support, guidance, patience and encouragement throughout the course of this project. I would also like to acknowledge and thank my committee members Dr. Karen Mearow and Dr. Daniel McPhee for their advice and help. A very big thank you to Elizabeth Kirby and the Kovacs lab for the qRT-PCR guidance and use of their lab space. A big thank you to my fellow lab mates during my time spent in the lab: Dr. Nicole White, Chris Hough and Nicole Pittman. Your support and knowledge is greatly appreciated. I am forever grateful for friends and family, especially my parents for their unwavering support, love and motivation during the course of my graduate studies.

Table of Contents

List of Figures

Abbreviations

ALK	Activin-like kinase
AMPK	AMP-activated protein kinase
ANOVA	Analaysis of Variance
Bad	Bcl-2-associated death promoter
BCA	Bicinchoninic Acid
BMPs	Bone Morphogenic Proteins
BSA	Bovine Serum Albumin
Cdc42	Cell division control protein 42
cdk	cyclin dependent kinase
CDKi	cyclin dependent kinase inhibitor
cDNA	complementary DNA
Cip1	CDK-interacting protein 1
CK1	Casein Kinase 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DYRK1A	Dual Specificity tyrosine-phosphorylation-regulated kinase 1A
EGF	Epidermal Growth Factor
EMT	epithelial-mesenchymal transition
ERK	Extracellular Signal Regulated kinase
FAK	Focal Adhesion kinase
FCS	Fetal calf serum
FRE	FoxO-recognition element
FYVE	FAB1, YOTB, Vac1, EEA1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDFs	Growth Differentiation Factors
GDP	Guanosine Diphosphate
Grb2	Growth factor receptor binding protein 2
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanosine Triphosphate
HRS	Hepatocyte Growth Factor-regulated tyrosine kinase
IgG	Immunoglobin G
Iκκ	Iκβ kinase
Imp	Importins
Ink4	Inhibitors of CDK4
i-Smads	Inhibitory Smads
JNK	c-Jun N-terminal kinase
Kip1	Kinase inhibiting protein 1
MAPK	Mitogen Activated Protein kinase
MMP-2	Matrix Metalloprotease 2
MMP-9	Matrix Metalloprotease 9
mRNA	messenger RNA

MST1	Mammalian Ste20-like kinase
NBCS	Newborn calf serum
NLS	Nuclear Localization Signal
Pak	p21-activated kinase
PBD	p21-binding domain
PBS	Phosphate-buffered Saline
PBST	Phosphate-buffered Saline with Tween
PDGF	Platelet Derived Growth Factor
PDK1	Phosphotidylinositol-dependent kinase 1
PDK2	Phosphotidylinositol-dependent kinase 2
PH	Pleckstrin Homology
PI3K	Phosphotidylinositol-3-OH kinase
PIP2	Phosphotidylinositol-4,5 biphosphate
PIP3	Phosphotidylinositol-3,4,5 triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein Phosphatase 2
PPM1G	3'-protein-phosphatase-1G
PTEN	Phosphatase and Tensin Homolog
PVDF	Polyvinylidene fluoride
Rac1	Ras-related C3 Botulinum Toxin Substrate 1
Raf	Rapidly Accelerated Fibrosarcoma
RIPA	Radioimmunopreciptation assay buffer
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
r-Smad	Regulatory Smads
RTK	receptor tyrosine kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SARA	Smad anchor for receptor activation
SGK	Serum and Glucocorticoid-regulated kinase
SH2	Src homology 2 domain
Shc	Src homology domain 2 containing
siRNA	small interfering RNA
Smad	*Sma* and *mad*-related protein
SNX9	Sorting Nexin 9
Sos	Son of Sevenless
TβRI	Transforming Growth Factor-β receptor I
TβRII	Transforming Growth Factor-β II
TBST	Tris Buffered Saline with Tween-20
TGF-β	Transforming Growth Factor-β

CHAPTER 1-Literature Review

1.1 TGF-β

1.1.1 TGF-β Family

Transforming Growth Factor-β (TGF-β) is a large superfamily of cytokines that include TGF-βs, Activins, Inhibins, Bone Morphogenic Proteins (BMPs), Growth Differentiation Factors (GDFs), and Müllerian Inhibiting Substance (Akhurst and Hata, 2012; Horowitz *et al.*, 2004; Katz *et al.*, 2013; Moustakas and Heldin, 2005). All family members have in common a conserved cysteine residue motif (Akhurst and Hata, 2012). TGF-βs direct a large array of cellular processes including embryonic development, cell migration, growth inhibition, epithelial-mesenchymal transition (EMT), invasion, extracellular matrix remodeling and immune system modulation (Akhurst and Hata, 2012; Bakin *et al.*, 2000; Horowitz *et al.*, 2004). TGF-βs act in a cell type specific manner; inhibiting cellular proliferation in non-transformed epithelial, endothelial and hematopoietic cells and induction of cellular proliferation in mesenchymal cells (Shin *et al.*, 2001; Massagué, 2008). These opposing functions of TGF-β, contributes to the complexity of this growth factor.

TGF-βs are found in all multicellular organisms (Akhurst and Hata, 2012) and there are three isoforms of TGF-βs in mammals that share a high degree of homology: TGF-β1, -β2 and -β3. TGF-β1 is the most abundant and most studied isoform (Katz *et al.*, 2013). A variety of cells including platelets (Katz *et al.*, 2013) and stromal cells (Colletta *et al.*, 1990) secrete inactive TGF-β as a latent homodimeric polypeptide bound to a latent complex protein. It only becomes an active ligand when the complex is

cleaved proteolytically by metalloproteases MMP-9 and MMP-2 (Yu and Stamenkovic, 2000), reactive oxygen species (ROS; Barcellos-Hoff & Dix, 1996), Thrombospondin-1 (Schultz-Cherry and Murphy-Ullrich, 1993), and/or acidic pH (Lyons *et al.*, 1988). Once activated the ligand can bind to receptors and initiate cell signalling.

1.1.2 TGF-β Receptors

Activated TGF-β ligand binds to a serine/threonine kinase transmembrane receptor and initiates cytoplasmic signalling pathways. The functional receptor is a heterotetramer of two type I receptor molecules and two type II receptor molecules (Katz *et al.*, 2013). In mammals, the entire TGF-β superfamily consists of 7 type I receptors (Activin-like kinases or ALKs) and 5 type II receptors and different combinations of receptors can occur depending on the ligand bound (Katz *et al.*, 2013). For TGF-β1, -β2 and -β3 ligands, it is the TGF-β type II receptor (TβRII) and the TGF-β type I receptor (TβRI; also referred to as ALK5) that is involved in eliciting the downstream signalling pathways (Miyazono *et al.*, 2010). Endoglin and β-Glycan (type III TGF-β receptor) are co-receptors that bind ligands through the type I and type II receptors (Katz *et al.*, 2013) to regulate ligand binding and signalling.

TGF-β ligand initially binds to the TβRII and results in the constitutively active type II receptor recruiting and phosphorylating/activating the type I receptor. The association of the two receptors triggers cross-phosphorylation of the Gly-Ser-rich (GS) domain of TβRI by TβRII, thereby activating the type I receptor kinase activity. This allows for the docking of substrates propagating signalling pathways, such as the Smad

pathway (Katz *et al.*, 2013; Moustakas and Heldin, 2005; Bakin *et al.*, 2000; Yi *et al.*, 2005; Miyazono *et al.*, 2012). Active receptor complexes are sequestered by the cell and initiate downstream signalling while receptor complexes absent of ligand are internalized and recycled back to the plasma membrane (Dore *et al.*, 2001). TGF-β receptor complexes are removed from the membrane via clathrin-mediated endocytosis (Figure 1) and membrane rafts. Clathrin-mediated endocytosis of TGF-β receptors targets localization to the early endosome. This location is necessary for TGF-β Smad signal transduction (Penheiter *et al.*, 2002). Membrane raft endocytosis of TGF-β receptors antagonistically promotes the association of inhibitory Smads with the receptor complex (DiGuglielmo *et al.*, 2003), preventing TGF-β signalling.

1.2 Smad Pathway

The canonical signalling pathway of TGF-β is the Smad pathway (Figure 1; Katz *et al.*, 2013; Miyazono *et al.*, 2012). There are three classes of Smads: Regulatory Smads (r-Smads), which include Smad-1, -2, -3, -5, -8; the common mediator Smad-4 (co-Smad); and inhibitory Smads (i-Smads) Smad-6, -7 (Katz *et al.*, 2013; Miyazono *et al.*, 2012). Smad-2 and Smad-3 are direct substrates of TGF-β receptors and activin receptors, and Smad-1, -5, -8 for bone morphogenic protein receptors (Moustakas *et al.*, 2001). Smad-2 and Smad-3 are recruited to the TGF-β receptor complex by SARA (Smad anchor for receptor activation; Tsukazaki *et al*., 2016). For Smad2/Smad3 phosphorylation to occur, the SARA/Smad/TGFβ receptor complex must be internalized

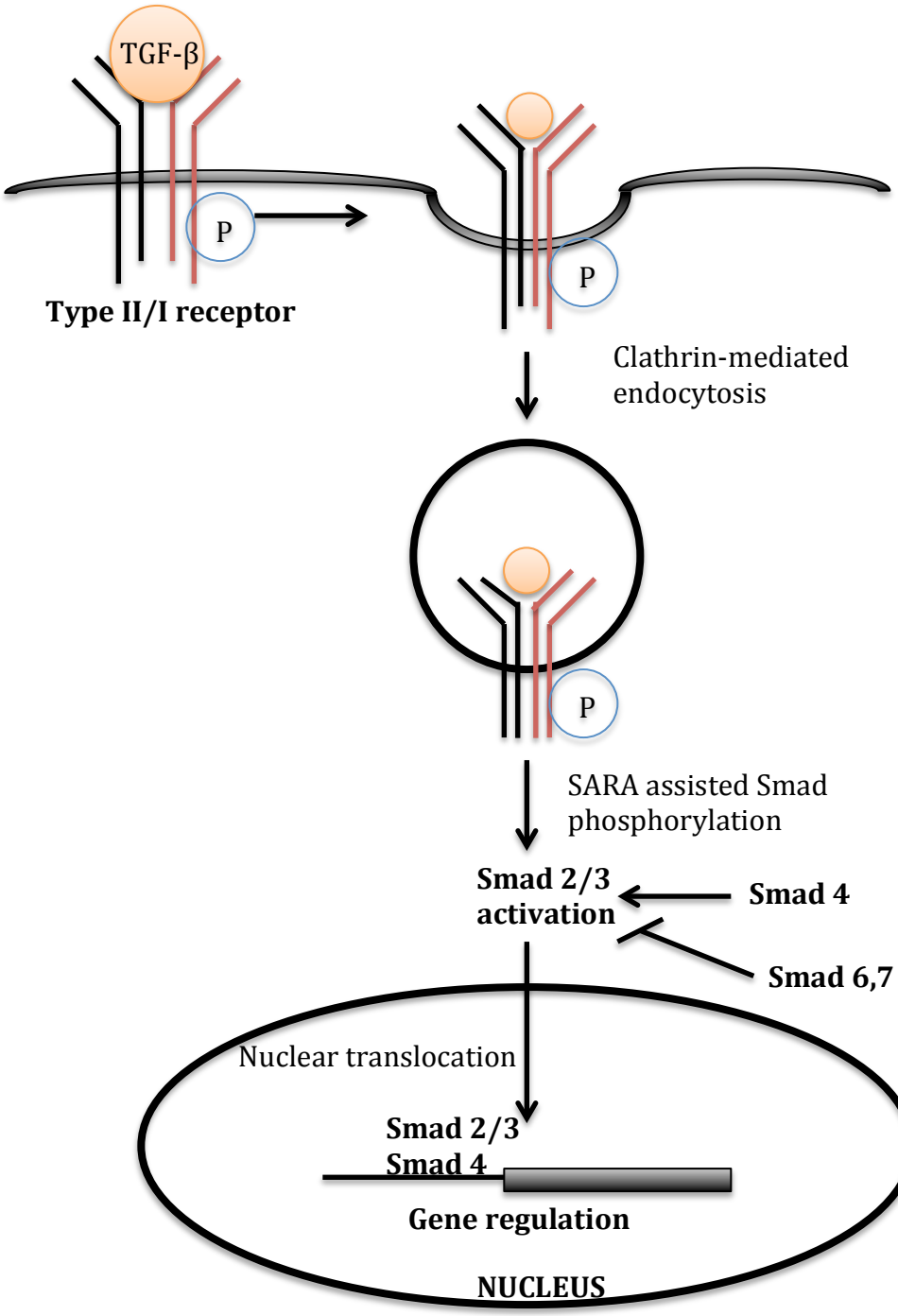

Fig. 1 Canonical TGF-β Smad signalling pathway. A depiction of clathrin-mediated endocytosis of TGF-β receptor complexes and the mechanism involved in Smad signalling.

to the early endosome (Penheiter *et al.*, 2002). Following phosphorylation by TβRI, pSmad-2 /3 associates with Smad-4 and dissociates from SARA, thereby mediating nuclear translocation (Massagué 2008; Miyazono et al. 2012; Moustakas & Heldin 2005; Shin et al. 2001; Yi et al. 2005; Tsukazaki et al. 2016). R-Smads and co-Smads shuttle between the nucleus and cytoplasm, although this mechanism is not well understood. Recently a study by Wilkes et al., (2015) suggests that the trafficking protein SNX9 (Sorting Nexins) promotes nuclear import of phosphorylated Smad-3 following TGF-β stimulation by binding pSmad3 to Imp8 and Impβ (importins) respectively, enabling it to move through the nuclear pore machinery. Nuclear Smad complexes activate specific genes with the help of DNA-binding cofactors and co-repressors (Katz *et al.*, 2013). Antagonistically, i-Smads block the association of r-Smads and co-Smad and thereby impede gene activation (Katz *et al.*, 2013; Miyazono *et al.*, 2012). In general, the Smad pathway is a well studied factor in the TGF-β signalling pathway.

1.3 Non-Smad TGF-β Signalling Pathways

The Smad pathway has been extensively studied with regards to TGF-β, however, it generated the question of how one simplified pathway could elicit all the diverse cellular functions of the large TGF-β family, let alone cell specific effects (Zhang, 2009; Moustakas and Heldin, 2005; Derynck and Zhang, 2003). Previously, it has been suggested that TGF-β promotes the activity of several other signalling pathways such as ERK, JNK, and p38 mitogen activated protein kinase (MAPK; Engel *et al*., 2000; Hong *et al*., 2011; Suzuki *et al*., 2007). As well, it has been reported that phosphatidylinositol-3-

OH kinase, RhoGTPases (cdc42/Rac1), Pak2, Akt and c-abl, to name a few, can be activated by TGF-β and in a cell type specific manner independent of Smads (Bakin *et al.*, 2000; Katz *et al.*, 2013; Miyazono *et al.*, 2012; Yi *et al.*, 2005; Hong *et al.*, 2011; Wilkes *et al.*, 2005; Suzuki *et al.*, 2007). There is some dispute in the literature whether or not these signalling pathways are truly Smad independent and it is still poorly understood whether or not these alternative pathways cross talk with or regulate the Smad pathway (Katz *et al.*, 2013; Hough *et al.*, 2012) and that they are truly independent of Smads (Moustakas and Heldin, 2005; Yi *et al.*, 2005). Accumulated findings in the literature suggest three mechanisms by which non-Smad signalling proteins respond to TGF-β: 1) Smad function is modified directly by non-Smad signalling pathways, 2) Smads directly interact and modulate the activity of other signalling pathways and 3) TGF-β receptors directly interact or phosphorylate non-Smad proteins. The first two mechanisms suggest a cross-talk between the Smad and non-Smad pathways, whereas the last mechanism is independent and therefore the non-Smad proteins would not affect the function or signal of Smads and vice versa (Moustakas and Heldin, 2005). A direct link between TGF-β receptors and the cytoplasmic effector molecules, other than Smads, is not completely understood, and requires further investigation.

1.3.1 TGF-β-induced Ras/Erk signalling pathway activation

One example of a Smad-independent TGF-β signalling pathway involves Ras/Erk. Ras is a large superfamily of G-proteins, which include the Rho and Rab families of proteins, having the common feature that they have GTPase activity (Robbins *et al.*,

1992). Ras is typically activated by receptor tyrosine kinases (RTKs), which most often leads to cellular proliferation or differentiation. Ras is highly implicated in cancer and mutated, constitutively active Ras can lead to uncontrolled cell growth (Bos, 1989; Campbell and Der, 2004). In the RTK pathway, binding of growth factors to the RTKs leads to dimerization and activation of the receptors, followed by auto- and trans-phosphorylation of tyrosine residues in the cytoplasmic domain of the RTK (Schlessinger, 2000; McKay and Morrison, 2007). The phosphotyrosine residues of RTK recruit and phosphorylate Shc (Src homology domain 2 containing), which then recruits the Grb2 (growth factor receptor binding protein 2)/Sos complex to the plasma membrane. Sos can then activate Ras by catalyzing the exchange of GDP for GTP. Active Ras in its GTP bound state can activate a Map kinase (MAPK) cascade by binding Raf (MAP3K), which then activates MEK1 (MAP2K) and finally Erk (MAPK; Ravichandran, 2001). It was first suggested that the ERK/MAPK pathway could be activated by TGF-β from observations of fast activation of $p21^{Ras}$ in rat intestine or mink lung epithelial cells (Mulder and Morris, 1992; Yan *et al.*, 1994). In the above epithelial cells the rapid GTP loading of Ras in response to TGF-β may cause the recruitment of Raf and lead to the activation of Erk through MEK1. The fast activation of Erk by TGF-β has also been observed in epithelial cells (Hartsough and Mulder, 1995), breast cancer cells (Frey and Mulder, 1997) and fibroblasts (Mucsi *et al.*, 1996). When the response of Erk activation to TGF-β is delayed, an indirect Smad related response has been suggested. In reports where activation has occurred within 5-10 minutes of TGF-β stimulation, it is comparable to the activation of Erk by EGF and therefore Smad-independent (Simeone *et al.*, 2001;

Olsson *et al.*, 2001). It is important to note that publications referring to the rapid responses (<30 minutes) initiate from a single lab and have not been replicated by others.

Although TGF-β type I and type II receptors are classified as serine-threonine kinases, it has been reported that TβRII can autophosphorylate at three tyrosine residues: Y259, Y336, and Y424 (Lawler *et al.*, 1997). Alternatively, one study suggests that Src, a non-RTK, can phosphorylate TβRII on Y284, which can then serve as a docking site for Grb2 and Shc, allowing for the bridging of TGF-β activation of the MAPK pathway (Galliher and Schiemann, 2007). However, several earlier studies failed to show any phospho-tyrosine residues on the type I or type II TGF-β receptors (Luo and Lodish, 1997; Carcamo *et al.*, 1994), indicating more evidence is required.

Shc plays an essential role in TGF-β-induced Erk activation, as transfection of siRNA silencing Shc diminishes this response (Zhang, 2009). Moreover, Erk activation is important for EMT, an essential process necessary for embryonic development and implicated in tumor metastasis (Thiery, 2003; Lee *et al.*, 2006). In late tumorigenesis, TGF-β has been implicated in inducing EMT and tumor growth through both smad-dependent and smad-independent effects (Derynck and Akhurst, 2007; Massagué, 2008). TGF-β-stimulated Erk regulates genes required for disassembly of cell adherens junctions, remodeling of integrin-based cell-matrix adhesion and promote cell motility, all essential requirements of EMT and tumorigenisis (Zavadil *et al.*, 2001). It has been reported that the reduction of ShcA and Grb2 expression (by siRNA), leaves mammary epithelial or mammary tumor cells unresponsive to EMT induced by TGF-β, halting invasion and migration (Galliher-Beckley and Schiemann, 2008; Northey *et al.*, 2008). Moreover, Erk can phosphorylate and inhibit Smad activity, which may explain how

oncogenic Ras can override TGF-β-mediated growth arrest in cancer cells and elicit TGF-β as tumor promoting (Kretzschmar *et al.*, 1997; Funaba *et al.*, 2002). In stark contrast, a study by Suzuki et al. (2007), found that TGF-β stimulated Ras and Erk activity in mesenchymal cultures but not epithelial cells, and that Ras activation was not required for Smad2 and Smad3 phosphorylation or nuclear localization (Suzuki *et al.*, 2007). Other studies (Hough *et al.*, 2012; Blanchette *et al.*, 2001) have shown that Ras is not necessary for Erk activation and that Erk has no effect on Smad nuclear localization and actually increases Smad signalling. Conflicting reports make it difficult to determine the complete picture regarding TGF-β and the Ras/Erk pathway, however, evidence suggests that in response to TGF-β the Ras/Erk pathway may follow a Smad-independent function or it can cross talk with the Smad pathway, depending on the cellular context.

1.3.2 TGF-β-induced PI3K Activation

1.3.2.1 PI3K

The phosphatidylinositol-3-OH kinase (PI3K) pathway is involved in cell proliferation, growth, metabolism, glucose homeostasis, survival and motility (Shin *et al.*, 2002; Kato *et al.*, 2006). There are three classes of PI3Ks: class I PI3K, which is divided into IA and IB and primarily generate PIP3 as part of receptor mediated signalling cascades (Katso *et al.*, 2001), class II PI3Ks are generally involved in membrane trafficking and receptor internalization (Engelman *et al.*, 2006) and class III PI3Ks are involved in vesicle trafficking and crosstalk with class I (Backer, 2008). For my study, I will be focusing on the class I PI3Ks and will refer to them as PI3K.

PI3Ks are heterodimers consisting of catalytic and regulatory subunits (Carracedo and Pandolfi, 2008; van der Heide *et al.*, 2004). The regulatory subunit of PI3K IA, termed p85, typically binds to tyrosine-phosphorylated membrane receptors through its SH2 domains and then recruits p110 the catalytic subunit to produce active PI3K (Carracedo and Pandolfi, 2008; Katso *et al.*, 2001). Once activated, PI3K locates lipid substrates (Figure 2) and phosphorylates the 3'-hydroxy group of the inositol ring of phosphatidylinositol in PIP2 (phosphatidylinositol 4,5 biphosphate) to generate PIP3 (phosphatidylinositol 3,4,5 triphosphate; van der Heide et al., 2004). PIP3 recruits a variety of proteins containing either a FYVE finger (a double-zinc-binding domain named after four cysteine rich proteins from which it was found; FAB1, YOTB, Vac1, EEA1) or pleckstrin homology (PH) domains, to the plasma membrane, affecting their localization, conformation and activity (Carracedo and Pandolfi, 2008; Gaullier *et al.*, 1998). For example, hepatocyte growth factor-regulated tyrosine kinase (predominantly localized to early endosomes) and phosphatidylinositol-dependent kinase 1 (PDK1; activates Akt) contain FYVE and PH domains, respectively (Pullan *et al.*, 2006; Carracedo and Pandolfi, 2008) and are both recruited to the plasma membrane by PIP3.

The PI3K pathway has been reported to be associated with TGF-β mediated EMT and migration and can be activated by TGF-β in various cell types (Bakin *et al.*, 2000; Kato *et al.*, 2006; Shin *et al.*, 2001; Runyan *et al.*, 2004; Wilkes *et al.*, 2005; Hough *et al.*, 2012). PI3Ks are widely known to be activated by RTKs and growth factor stimulation such as insulin, platelet derived growth factor receptors (PDGF), and nerve growth factor (Carracedo and Pandolfi, 2008; Yi *et al.*, 2005). The most established pathway downstream of PI3K is the Akt pathway (Figure 2), but more recently it has been reported

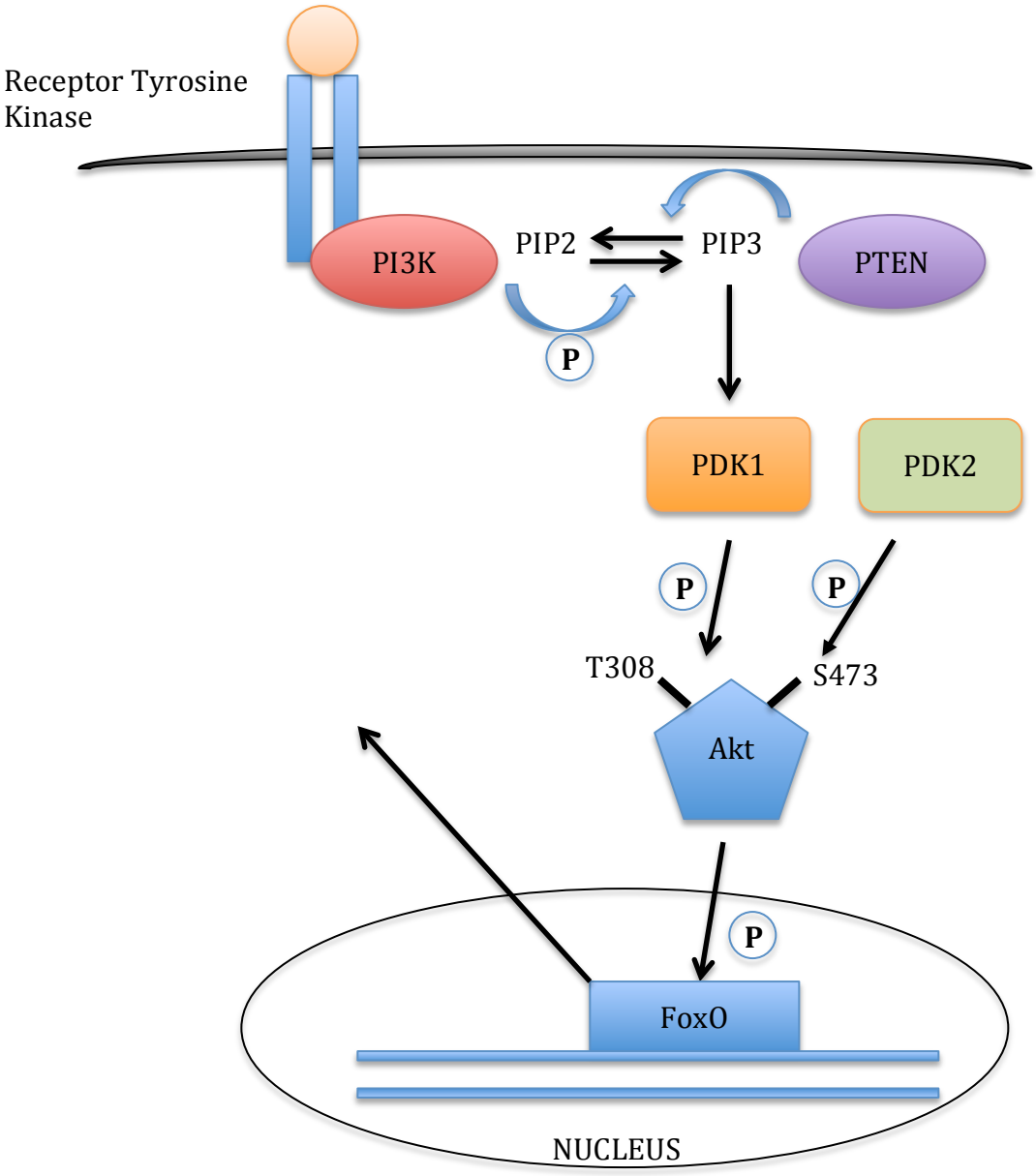

Fig. 2 Receptor Tyrosine Kinase mediated PI3K/Akt signalling pathway. A simplified depiction of FoxO regulation via the PI3K/Akt signalling pathway

that p21-activated kinase-2 (Pak2) can be activated by PI3K following TGF-β stimulation (Wilkes *et al.*, 2005). This occurred in a cell type specific manner independent of Smads (Wilkes *et al.*, 2005), supporting the reported non-Smad pathway hypothesis referred to previously.

1.3.3 TGF-β-induced PI3K/Pak2 pathway

The p21-activated kinases (Paks) are divided into two subfamilies and were first found to be effectors of p21-Rho GTPases. The two subfamilies consist of group I, Pak1, 2 and 3, and Group II, Pak4, 5, and 6. All family members bind GTP-bound Rho family members at the amino-terminal p21-binding domain (PBD). The Cdc42 and/or Rac1 family members have been shown to bind to and activate group I Paks, including Pak2, an important serine/threonine kinase upstream of ERK. (Radu *et al.*, 2014).

Pak2 activation occurs primarily in response to stresses such as radiation or serum starvation, and can regulate downstream Erk through Raf and Mek1 (Eblen *et al.*, 2002). Several studies by Wilkes et al. have elucidated TGF-β specific activation of Pak2 via TGF-βR/PI3K (Wilkes *et al.*, 2005; Wilkes *et al.*, 2003). They initially found that Pak2 was activated by TGF-β in a Smad-2 and Smad-3 independent manner only in fibroblasts, and not in epithelial cells. This was significant in that this highlighted TGF-β cell type specific regulation of a pathway in one cellular context only and independently of Smads. It was also shown that Pak2 activation by TGF-β was dependent on CDC42/rac1 and it created a proliferative response in fibroblasts (Wilkes *et al.*, 2003). Follow-up studies found that the Pak2 activation by TGF-β is PI3K-dependent and that PI3K independently

regulates both Pak2 and Akt and is spatially upstream of Pak2 (Wilkes *et al.*, 2005). They determined that PI3K activation was dependent on TGF-β kinase activity, but the p85 and p110 regulatory subunits of PI3K was not directly phosphorylated by TGF-β receptors I or II (Wilkes *et al.*, 2005). Wilkes and Leof, 2006, also determined that unlike the activation of Smad2 and Smad3, TGF-β activation of PI3K does not require endocytosis (Wilkes and Leof, 2006; Wilkes *et al.*, 2005), offering a differentiating mechanism for Smad-dependent and Smad-independent TGF-β pathways.

Very little is known about the mechanism for TGF-β receptor binding and transduction to the regulatory subunits of PI3K, however, it has been reported that TGF-β induced the non-receptor tyrosine kinase Fak binding to the p85 regulatory subunit of PI3K in a cell type specific manner. Fak acts as a signalling scaffold and it could help explain why fibroblasts can activate the non-smad pathways, Pak2 and Akt (Hong *et al.*, 2011). However, since epithelial cells also express Fak, there must be other players regulating this signalling pathway.

1.4 PI3K/Akt Pathway

The canonical PI3K pathway involves the activation of Akt, an important serine/threonine kinase implicated in several forms of cancer. Activated PI3K recruits serine/threonine kinases PDK1 and Akt (Protein Kinase B), both containing PH domains, to the plasma membrane (Carracedo and Pandolfi, 2008; Pullan *et al.*, 2006). PIP3 binding activates PDK1, which phosphorylates and partially activates Akt (Figure 2). A second enzyme(s), referred to as “PDK2”, also phosphorylates Akt to stimulate full

activity (Manning and Cantley, 2007; Guertin *et al.*, 2006; Jacinto *et al.*, 2006). Active Akt then leaves the plasma membrane and then transmits the signal initially elicited by PI3K (Carracedo and Pandolfi, 2008). PTEN (Phosphatase and Tensin Homolog) negatively regulates the PI3K pathway (Figure 2) by dephosphorylating PIP3 at the 3' position and abrogating Akt binding and activation, therefore also its signal (Carracedo and Pandolfi, 2008; van der Heide *et al.*, 2004), providing an important off switch.

Akt, or formerly Protein Kinase B (PKB), is a serine/threonine kinase belonging to the AGC family of protein kinases, structurally related to Protein Kinase A (PKA), Protein Kinase G (PKG) and Protein Kinase C (PKC; van der Heide et al., 2004). There are three isoforms of Akt (Akt1/PKBα, Akt2/PKBβ, Akt3/PKBγ) containing three functionally distinct domains (van der Heide *et al.*, 2004; Manning and Cantley, 2007): an N-terminal domain, a catalytic domain, and a C-terminal regulatory hydrophobic motif (van der Heide *et al.*, 2004). The pleckstrin homology domain responsible for the recruitment of Akt to the plasma membrane, has high affinity for PIP3, and is found within the N-terminus domain. The catalytic kinase domain and the C-terminal domain share an 85-90% homology between all three Akt isoforms and activation of Akt depends on the phosphorylation of threonine/serine residues located within these two domains (Liu *et al.*, 2014; Mundi *et al.*, 2016).

Akt is phosphorylated/activated by PDK1 and PDK2 at phosphorylation sites thr308 and ser473, respectively (positions are with respect to Akt1 isoform; Shin *et al.*, 2001). Once activated, Akt detaches from the plasma membrane and translocates to the cytosol and nucleus (Figure 2), where it phosphorylates serine or threonine residues on a variety of target proteins (van der Heide *et al.*, 2004). Akt has many functions, dependent

on its target proteins, and can be inhibitory or stimulatory. It promotes cell survival by inactivating proapoptotic factors GSK3β, Bad and Caspase 9 (van der Heide *et al.*, 2004; Shin *et al.*, 2002). It also promotes cell cycle progression and gene transcription by inhibiting GSK3β and modulating cellular localization of ForkheadO group of transcription factors and $p27^{kip1}$ (Shin *et al.*, 2002). TGF-β-induced PI3K/Akt regulation has been reported to be important for EMT (Bakin *et al.*, 2000), which suggests a two-armed model for the TGF-β signalling by PI3K, through the Pak2 pathway (Hough *et al.*, 2012), as well as Akt.

1.5 FoxO Family

As previously stated, Akt phosphorylates the group O Forkhead (FoxO) transcription factors. FoxOs belong to the Forkhead family of transcription factors with all members containing a 100-amino acid Forkhead domain, responsible for DNA binding (Shin *et al.*, 2001; Essaghir *et al.*, 2009). FoxOs were first discovered in *Caenorhabditis elegans* as Daf-16, a transcription factor regulated by Akt (Burgering and Kops, 2002; Shin *et al.*, 2002). Mutations in Daf-16 created a forked-head phenotype in *C. elegans* during larvae dauer formation thus the nomenclature (Ogg *et al.*, 1997; Shin *et al.*, 2001). FoxOs have since been identified in other organisms including zebrafish, Drosophila, mice, rats and humans (van der Heide *et al.*, 2004). The mammalian orthologs of Daf-16 are FoxO1 (previously called FKHR), FoxO3a (previously called FKHRL1), FoxO4 (previously called AFX), and FoxO6, all comprise the Forkhead Box O group (Shin *et al.*, 2001; Essaghir *et al.*, 2009). FoxO1, 3a and 4 are ubiquitously expressed at different

levels depending on the tissue, whereas FoxO6 is expressed only in the central nervous system (Anderson *et al.*, 1998; Jacobs *et al.*, 2003), and was not included in this study.

FoxO expression and activity are controlled by several posttranslational modifications such as phosphorylation, acetylation, methylation and ubiquitination (Huang and Tindall, 2007; Tzivion *et al*., 2011). Each modification uniquely regulates FoxO activity, whether by degradation or physical localization of the protein within the cell. A negative or positive effect on FoxO activity depends not only on the modification type, but also the specific regulator (Huang and Tindall, 2007). FoxOs are activated by JNK (c-Jun N-terminal kinase) and MST1 (Mammalian Ste20-like kinase) during oxidative stress and translocate FoxO to the nucleus to induce genes involved in stress defence (Huang and Tindall, 2007; Wang *et al.*, 2005). AMPK (AMP-activated protein kinase) phosphorylation of FoxO, during nutrient stress, targets genes involved in energy metabolism and stress resistance. In contrast, negative regulators of FoxO re-locate it from the nucleus to cytoplasm. Akt is an important negative regulator of FoxO, however, SGK (serum and glucocorticoid-regulated kinase), CK1 (Casein Kinase 1) DYRK1A, ERK, and Iκκ (Iκβ kinase) are other negative regulators of FoxO (Liu *et al.*, 2008). The target phosphorylation sites of FoxO by these regulators differs from one another, as well as the phosphorylation sites targeted by Akt (Huang and Tindall, 2007), allowing for differential FoxO modulated regulation.

FoxOs can induce cell growth arrest and apoptosis, but are blocked from these functions when phosphorylated by Akt (Essaghir *et al.*, 2009). All mammalian orthologs of FoxO contain three Akt phosphorylation sites (T32, S253, S315; numbered relative to FoxO3a) and a Nuclear Localization Signal (NLS) within the forkhead domain (Carter

and Brunet, 2008; Shin *et al.*, 2001; Essaghir *et al.*, 2009; van der Heide *et al.*, 2004; Greer and Brunet, 2005; Huang and Tindall, 2007). The preferred Akt phosphorylation site (S253) is located within the NLS, and T32 and S315 are located N- and C-terminally outside of the forkhead domain, respectively (Huang and Tindall, 2007; Obsilova *et al.*, 2005; Greer and Brunet, 2005). Unphosphorylated FoxOs are predominantly localized in the nucleus where they activate target genes containing a FoxO-recognition element (FRE). While active they can induce cell death by directing transcription of FasL, Bim and Trail or inhibit the cell cycle through expression of $p27^{kip1}$ and $p21^{waf1}$ (Carter and Brunet, 2008; Shin *et al.*, 2002; Essaghir *et al.*, 2009). Once phosphorylated by Akt, FoxOs are recognized by 14-3-3 shuttling proteins that bind the now phosphorylated Akt sites (S253 and T32) and translocate FoxOs from nucleus to cytoplasm, where they are sequestered and can be targeted for ubiquitination and degradation by proteasomes (Essaghir *et al.*, 2009; van der Heide *et al.*, 2004; Shin *et al.*, 2001). The 14-3-3 proteins act as a chaperone protein and in part masks the NLS allowing for the movement of FoxO from nucleus to cytoplasm (Obsilova *et al.*, 2005; Greer and Brunet, 2005). The "inactive state" of FoxO in the cytoplasm can also be rapidly reversed, for example with the phosphatase PP2A (Yan *et al.*, 2008), allowing FoxO to return to the nucleus where it can again activate transcription of genes involved in numerous functions, such as cell cycle regulation.

1.6 Cell Cycle Regulation

As previously stated, Akt and FoxO are both implicated in the regulation of cellular proliferation and growth, by influencing factors involved in the cell cycle

(Manning and Cantley, 2007; Huang and Tindall, 2007; Greer and Brunet, 2005; Burgering and Kops, 2002) The cell cycle is a complex and tightly controlled process, resulting in two identical daughter cells. It is the basis for the growth and proliferation of cells. Such an important process follows a highly regulated sequence of events with several checkpoints and regulatory proteins to ensure a proper functioning cell (Schafer, 1998). Failure to do so could lead to a multitude of diseases, including tumorigenesis. The cell cycle encompasses 4 phases: G1, S, G2, and M (mitosis). DNA replication and cell division into two daughter cells occur during S phase and M phase, respectively. In between these two phases are the Gap phases; G1, where the cell expands and prepares for DNA synthesis in S phase, and G2, where the cell prepares for M phase. Cells that are not actively dividing are in a senescent phase called G0. In the presence of growth factors, senescent cells leave G0 and enter G1 (Williams and Stoeber, 2012; Schafer, 1998). Each phase has specific stopping points called checkpoints where sensor mechanisms detect incomplete or aberrant cell cycle events and can trigger effectors necessary for cell cycle arrest until the problem is resolved or apoptosis is initiated (Williams and Stoeber, 2012) Cyclin dependent kinases (cdk's) and cyclin proteins catalyze the progression through the phases of the cell cycle (Schafer, 1998; Williams and Stoeber, 2012), and are important mediators of the cell cycle.

Cdk's are serine/threonine protein kinases that are activated at specific points in the cell cycle by phosphorylation on threonine and tyrosine residues (Malumbres, 2014; Schafer, 1998; Williams & Stoeber, 2012). The kinase activity of cdk is regulated by 4 mechanisms; binding to cyclins, activation/inactivation by phosphorylation on different threonine and tyrosine residues, and inactivation by binding of inhibitory molecules

(Malumbres, 2014; Schafer, 1998). Cdks are inactive as a monomer and require binding to cyclin to increase their enzymatic activity. It is the cdk-cyclin complexes that allow cell cycle progression to occur (Vervoorts and Lüscher, 2008). Cyclins are temporally expressed by the cell at specific points in the cell cycle to ensure that cdks can only be activated at certain times (Vervoorts and Lüscher, 2008). Different cdk-cyclin complexes occur during different phases of the cell cycle and some cdks can bind multiple cyclins allowing for substrate specificity (Malumbres, 2014) For example, cyclin D-CDK4, cyclin D-CDK6, and cyclin E-CDK2 complex promote progression through the G1/S restriction point, committing the cell to DNA synthesis. Cyclin A-CDK2 initiates S phase, and cyclin B-CDK1 regulates progression through G2 and initiating mitosis (Williams and Stoeber, 2012; Planas-Silva and Weinberg, 1997; Nigg, 2001). Inhibition of these cyclin-cdk complexes halts cell cycle progression at any point.

The Ink4 and Cip1/Kip1 families are effector proteins that inhibit Cyclin/cdk complexes. The Ink4 family, p16, p15, p18 and p19, interact primarily with cyclin D/cdk 4,6 complexes, during G1 phase. The Cip/Kip family, $p21^{cip1/waf1}$, $p27^{kip1}$ and $p57^{kip2}$, are less specific as they can bind all G1 and S cyclin/cdk complexes (Reynisdottir *et al.*, 1995). When active, the Ink4 and Cip/Kip inhibitors cause G1 or G2 arrest in response to antiproliferative agents and DNA damage (Schafer, 1998; Malumbres, 2014), to prevent downstream genetic errors from occurring.

1.7 Cyclin Dependent Kinase Inhibitor, $p27^{kip1}$

$p27^{kip1}$ is classified as a tumour suppressor and a cyclin-dependent kinase inhibitor (CDKi; Dijkers *et al*., 2000). It is a part of the Cip/Kip family of CDKi's, composed of $p21^{cip1/waf1}$, $p27^{kip1}$ and $p57^{kip2}$. All contain a homologous amino-terminal domain comprising cyclin and cdk binding regions, in order to bind cyclin-cdk complexes and interfere with their catalytic activity (Reynisdottir and Massague, 1997; Vervoorts and Lüscher, 2008). Target complexes for $p27^{kip1}$ include cyclin D-cdk4/6 and cyclin E-cdk2, both found in late G1 phase, and cyclin A-cdk2 found at the G1/S transition and throughout S phase (Reynisdottir and Massague, 1997). $p27^{kip1}$ inhibits these complexes and halts cell cycle progression (Roy and Banerjee, 2015; Reynisdottir *et al.*, 1995; Dijkers *et al.*, 2000). Upregulation of $p27^{kip1}$ is linked to cell cycle arrest in G0/G1 by interacting with these cyclin-cdk complexes (Dijkers *et al.*, 2000), contributing to its title as a tumour suppressor.

Having such an integral role in cell cycle regulation, $p27^{kip1}$ is highly regulated at both gene expression and post-translationally. $p27^{kip1}$ gene expression is regulated by the binding of transcription factors and adapter proteins to the promotor site of $p27^{kip1}$. There are several forkhead transcription factor-binding sites in the $p27^{kip1}$ promotor, suggesting that $p27^{kip1}$ is a target site for FoxO family members. Studies have found that $p27^{kip1}$ mRNA and protein is greatly elevated following FoxO3a and FoxO4 activation (Dijkers *et al.*, 2000; Medema *et al.*, 2000). Post-translational modification of $p27^{kip1}$ by its phosphorylation at several sites also plays a pivotal role in its regulation. Phosphorylation of $p27^{kip1}$ at T157 and T198, followed by binding to 14-3-3 proteins has been shown to shuttle $p27^{kip1}$ from the nucleus to the cytoplasm, where it is targeted for ubiquitin

degradation (Liang *et al.*, 2002; Shin *et al.*, 2002; Motti *et al.*, 2004). Relocalization to the cytoplasm renders $p27^{kip1}$ incapable of binding cyclin/cdk complexes and therefore allows cell cycle progression (Viglietto *et al.*, 2002). It has been found that the cellular localization of $p27^{kip1}$ in some tumor cells is associated with poor clinical prognosis (Vervoorts and Lüscher, 2008), and therefore cellular localization of $p27^{kip1}$ plays an important role on its function.

1.8 Hypothesis and Objectives

TGF-β regulates a variety of biological effects including cellular proliferation. It has been shown to inhibit proliferation in epithelial cells and promote proliferation in mesenchymal cells. Studies by Wilkes et al., 2005, have reported that TGF-β regulates the Pak2 pathway via PI3K in fibroblast cells and not epithelial cells, independently of Smads. The canonical Smad pathway is well understood, but growing evidence supports Smad-independent TGF-β pathways, including the PI3K/Akt pathway. Since TGF-β reportedly activates the PI3K/Akt pathway (Bakin *et al.*, 2000; Miyazono *et al.*, 2012; Yi *et al.*, 2005; Hong *et al.*, 2011; Wilkes *et al.*, 2005; Kato *et al.*, 2006; Hough *et al.*, 2012), and FoxO is downstream of Akt, and they can induce transcription and phosphorylation of $p27^{kip1}$, respectively, I examined the role of TGF-β regulation of FoxO group members and $p27^{kip1}$ in fibroblast cells.

<u>Hypothesis:</u>

I hypothesized that TGF-β regulates the FoxO group of transcription factors post-translationally via the PI3K/Akt pathway in fibroblast cells. It is further hypothesized that

TGF-β regulates $p27^{kip1}$ both at the transcriptional and post-translational levels in fibroblasts.

Objectives:

The following objectives were proposed to verify the hypothesis:

1) Determine phosphorylation of individual FoxO group members by PI3K/Akt in fibroblast cells following TGF-β addition using Western blotting.
2) Compare cytoplasmic localization of FoxO group transcription factors before and after TGF-β stimulation in fibroblast cells using immunohistochemistry and confocal microscopy.
3) Determine the regulation of phosphorylation and nuclear-cytoplasmic shuttling of $p27^{kip1}$ by Akt following TGF-β stimulation in fibroblast cells by using Western blotting and immunohistochemistry, respectively.
4) Evaluate the change of PI3K/Akt directed $p27^{kip1}$ gene expression, via FoxO4, before and after TGF-β addition in fibroblast cells by qRT-PCR.

CHAPTER 2-Materials and Methods

2.1 Cell Culture

AKR-2B cells were grown in 10% (v/v) Fetal Calf Serum (FCS; PAA Laboratories, Etobicoke, ON, Canada) in high glucose Dulbecco's Modified Eagle Medium (DMEM; LifeTechnologies, Carlsbad, USA). Cells were maintained at 37°C in an incubator containing 5% CO_2/air. Cells were passaged at approximately 80% confluence for 18-20 serial passages (approximately 2 months) before discarding and new stock was thawed from liquid nitrogen.

2.2 Western Blotting

Following trypsinization and removal of cells from T175 flasks, AKR-2B cells were plated in six well plates at a final density of $3x10^5$ cells/well in 10% FCS in DMEM. Cells were allowed to attach for 24 hours. Growth media was removed and cells were serum deprived in 0.1% Newborn Calf Serum (NBCS; LifeTechnologies, Carlsbad, USA) in DMEM for 18 to 20 hours prior to experimentation. Cells were treated with 2 ng/mL of TGF-β1 for indicated time points up to 6 hours. LY294002 (LifeTechnologies, Carlsbad, USA) was diluted to a concentration of 10 μM (stock of 10 mM in DMSO) to inhibit PI3K. AKTVIII (Calbiochem; EMD Biosciences, La Jolla, CA, USA) was diluted to a concentration of 1 μM (1 mM stock in DMSO) to selectively inhibit AKT1 and AKT2. TGF-β receptor inhibitor (LY364947; Tocris Bioscience, Ellisville, MO, USA)

was diluted to a concentration of 0.5 μM (1 mM stock in DMSO) to inhibit the TGF-β Type I receptor kinase. Inhibitors were added 30 minutes prior to TGF-β addition. Following treatments, cells were washed three times in ice cold PBS and lysed in 60 μl of RIPA [10X PBS, 1X complete protease inhibitor (Roche Diagnostics, Mannheim, Germany), 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 50 mM β-Glycerophosphate, 50 mM Sodium Fluoride, 0.1% SDS, 0.1 μM Sodium Orthovanadate, 5 mM EDTA, 75 ng/μl PMSF (Sigma-Aldrich, St. Louis, MO, USA)]. All buffers and salts were purchased from EM Science (Merck Chemical; Darmstadt, Germany) unless otherwise stated. Cell lysates were centrifuged at 21,000xg at 4°C for 10 minutes. Supernatants were separated and stored at -80°C. Lysate total protein concentrations were determined using a BCA assay (Pierce/ThermoFisher) with a standard curve generated with a BSA/RIPA standard and measured using a Polarstar Optima plate reader (BMG Labtechnologies, Durham, NC, USA). Equivalent amounts of protein from each sample of total cellular lysate were separated in an 8.5% or 12% polyacrylamide gel and transferred to PVDF (Immobilon-P; EMD Millipore, Billerica, MA, USA) or Nitrocellulose (BioTrace NT; Pall Corp., Pensacola, FL, USA) membrane prior to antibody detection of specified protein. PVDF membranes were blocked in Blotto Buffer 5% (w/v) Non-fat dry milk in TBST [10 mM Tris-HCL pH 7.4, 150 mM NaCL, 0.1% (v/v) Tween-20], and nitrocellulose membranes were blocked in 5% BSA/TBST, each for one hour, then incubated overnight at 4° in primary antibody, diluted in the appropriate blocking buffer. Primary antibodies used include Phospho-FoxO1/3a (Thr24/Thr32; Cell Signalling #9464), FoxO1 (C29H4; Cell Signalling #2880), Phospho-FoxO3a (Ser318/321; Cell Signalling #9465), Phospho-FoxO4 (Ser193; Cell Signalling #9471), β-actin(AC-15; Sigma A5441), α-tubulin

(11H10; Cell Signalling #2125), Phospho-$p27^{Kip1}$ (T198; R&D systems), $p27^{Kip1}$ (sx53G8.5; Cell Signalling #3698). Membranes were washed 5 times for 5 minutes each in TBST and incubated for one hour at room temperature in either Goat anti-Rabbit IgG (Cell Signalling Technology, Danvers, MA, USA) or Goat anti-Mouse IgG (Santa Cruz Biotechnology; Santa Cruz, CA, USA) secondary antibodies in either Blotto Buffer (PVDF membrane) or 5% BSA/TBST (nitrocellulose membrane) at a 1:15 000 dilution. Membranes were then washed 5 times for 5 minutes each in TBST before incubated for 5 minutes with Supersignal West Pico Chemiluminscent Substrate (Pierce/ThermoFisher Scientific; Rockford, IL, USA) and then exposed to film (Hyperfilm; GE Healthcare Biosciences, Piscataway, NJ, USA) and developed. Multiple exposures were obtained from each membrane to ensure data was collected from within the linear response range of the film.

2.3 Immunocytochemistry

AKR-2B cells were plated at a concentration of $2x10^4$ cells/well in each well of a 4 chamber-slide (Lab-Tek; ThermoFisher Scientific, Rochester, NY, USA) and allowed to attach for 24 hours. Cells were serum deprived for ~18 to 20 hours prior to addition of TGF-β (2 ng/ml) for indicated time points for up to 6 hours. For signalling inhibition experiments LY294002 (10 μM) was added 30 minutes prior to TGF-β addition. Following treatments, cells were washed 2 times with ice cold PBS and fixed with 4% (w/v) Paraformaldehyde/PBS at 4°C for 30 minutes, with one change in fixative at 15 minutes. Cells were washed 2 times with PBS for 5 minutes each and permeabilized using

0.1% Triton X-100 for 2 minutes at room temperature. Cells were then washed twice with PBS and blocked for 1 hour at room temperature in blocking solution [PBS, 10% (v/v) FCS, 10% Fish Gelatin (w/v), 5% (v/v) normal goat serum, 0.02% (w/v) Sodium Azide]. FoxO1 (C29H4; Cell Signalling #2880) and $p27^{Kip1}$ (sx53G8.5; Cell Signalling #3698) primary antibodies diluted in blocking solution were added to slides and incubated overnight at 4°C. The following day slides were washed 5 times for 5 minutes each in PBST. FoxO1 slides were incubated for 1 hour at room temperature in mouse conformation specific anti-rabbit IgG (Cell Signalling Technology, Danvers, MA, USA) diluted in antibody dilution solution (1:400), followed by 2 washes for 5 minutes each in PBST. In order to detect FoxO1, an extra amplification step was used, slides were incubated in the dark for 30 minutes at room temperature in a combination of anti-rabbit-Rhodamine X and anti-mouse-Rhodamine X (Jackson Immunoresearch Laboratories, West Grove, PA, USA) diluted in blocking solution (1:300), followed by 5 washes for 5 minutes each in PBST. $p27^{Kip1}$ slides were incubated for 30 minutes at room temperature in mouse-AlexaFluor488 (LifeTechnologies; Carlsbad, CA, USA) diluted in antibody dilution solution (1:300), followed by 5 washes for 5 minutes in PBST. FoxO1 and $p27^{Kip1}$ slides were coverslipped with Vectashield (Vectorlabs, Burlingame, CA, USA). Cellular localization was visualized by fluorescence microscopy (DMIRII; Leica Microsystems, Wetzlar, Germany) with Openlab image acquisition software or confocal microscopy (Olympus, Tokyo, Japan).

2.4 Quantitative RT-PCR

AKR-2B cells were grown as per Western blotting protocols and treated with TGF-β (2 ng/ml) up to 6 hours. LY294002 (10 μM) in DMSO was added 30 minutes prior to TGF-β addition. Following TGF-β addition, cell media was aspirated and washed with ice cold PBS. RNeasy Plus mini prep kits (Qiagen Inc., Toronto, ON, Canada) were used to extract cellular RNA from fibroblasts as per the manufacturer's instructions. Sample RNA was collected in 30 μl of RNase free water and stored at -80°C. Sample RNA concentrations and quality were determined with an Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). Sample RNA of >8.0 RIN values were used for analysis. The Taqman RNA to CT kit (Applied Biosystems, Foster City, CA, USA) was used and Taqman probe sets for $p27^{kip1}$ (Mm00438168_m1) and GAPDH (Mm99999915_g1) were purchased from Applied Biosystems. Initially qRT-PCR was first performed with serial dilutions of template to determine linearity and efficiency of cDNA synthesis reaction. Based on this information 0.1 μg of sample RNA was used in the cDNA synthesis step. To each well of a 96 well plate, 20 μl reactions were added composed of; 10 μl RT PCR Mix, 0.5 μl RT enzyme mix, 0.1 μg RNA, 1μl TaqMan probe set and water to 20 μl. Individual samples were added to triplicate wells, for each probe. Plates were centrifuged at 2,000xg for 3 minutes and qRT-PCR was performed (Applied Biosystems, Prism 7000). GAPDH was used as an internal normalization control. Relative fold changes were calculated corrected for GAPDH expression levels using the ΔΔCT method (Pfaffl, 2001).

2.5 Infection of Cells with Virus

FoxO4-EGFP was expressed as a retroviral particle via the GP2 packaging cell line and VSV-G coat protein (Clontech, Palo Alto, CA, USA), while FoxO3a-A3-EGFP was expressed as an adenovirus [the generous gift of Dr. Ken Walsh, Skurk et al., 2004] in order to express these proteins in fibroblasts. AKR-2B cells were plated in 6 well plates at a concentration of $3x10^5$ cells/well in growth medium. Cells were allowed to attach over night before virus was added the next day with an MOI of 50:1 (adenovirus) or 5:1 (retrovirus). Medium was replaced 18 to 20 hours later with DMEM containing 0.1% NBCS to serum deplete cells for 18 hours. Cells were then treated with TGF-β (2 ng/ml) for 6 hours and either total cellular lysates or RNA was collected, as previously described.

2.6 Densitometry and statistical analysis

Western blotting images were scanned and densitometry was performed using Image J software. Graphpad Prism software was used to perform statistical analysis on western blot densitometry and qRT-PCR relative expression results. To test for significant differences between samples an ANOVA was performed followed by Tukey's or Dunnett's multiple comparisons test. Significant differences were accepted at $\alpha \leq 0.05$.

CHAPTER 3-Results

3.1 FoxOs are phosphorylated in fibroblasts when treated with TGF-β

Previous studies in the Doré lab by graduate student Kielley, D. (2008) examined the regulation of PI3K/Akt signalling by TGF-β, and found that in fibroblast cells TGF-β stimulation increased Akt phosphorylation dependent on PI3K (results not shown). These results suggest that TGF-β plays a role in the regulation of the FoxO group of transcription factors since they are downstream of Akt. To determine if TGF-β alters FoxO group member function I decided to first look at their phosphorylation in fibroblast cells following the addition of TGF-β. AKR-2B fibroblasts were treated with TGF-β1 (2 ng/ml) over a 240-minute period, as shown in Figure 3, which demonstrates FoxO1 phosphorylation at threonines 24 & 32 apparent at 30 minutes and increases through 240 minutes. FoxO1 phosphorylation was significantly increased above untreated steady state levels (time 0) beginning at 120 minutes and continuing through 240 minutes (Figure 3B). Relative levels of phosphorylated protein, as measured by densitometry of immunoblots, were corrected for loading differences using total FoxO1.

There are four members of the FoxO group (FoxO1, 3a, 4 and 6; Shin *et al.*, 2001; Essaghir *et al.*, 2009), however I was only able to analyze the phosphorylation of FoxO1, FoxO3a and FoxO4, following TGF-β addition, since antibodies were not available for phosphorylation sites on FoxO6. Similar to results for FoxO1, phosphorylation of FoxO3a at serines 318 & 321 appears at 30 minutes and increases to 180 minutes (Figure 4A). When corrected for loading differences using β-actin, phospho-FoxO3a levels were significantly above steady-state levels by 180 through 240 minutes. Although

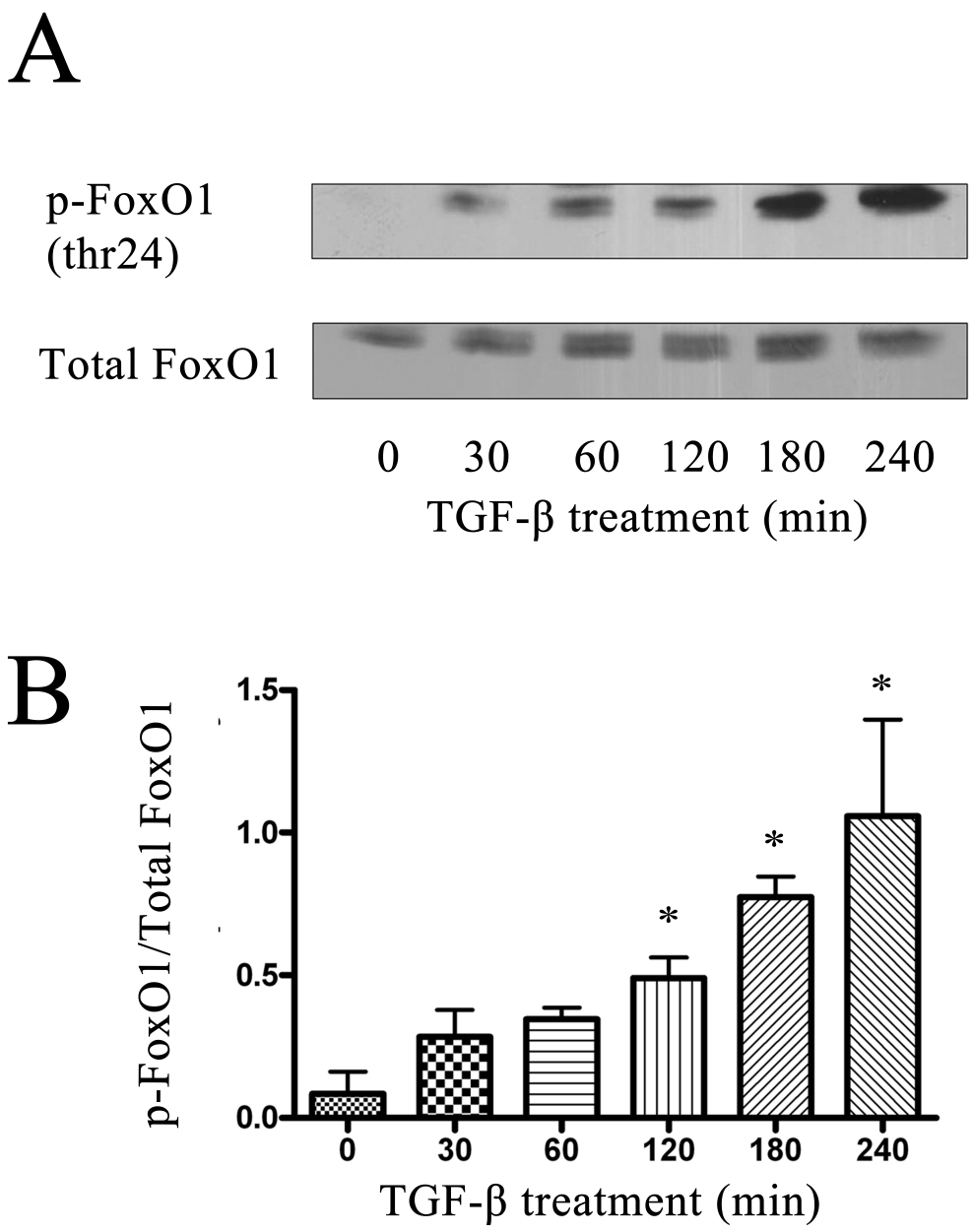

Fig. 3. TGF-β induced FoxO1 phosphorylation in fibroblasts. (A) AKR-2B cells were treated with TGF-β (2 ng/ml) for times ranging from 0 to 240 minutes. Cell lysates were probed with antibodies specific to phosphor-FoxO1. Blots were stripped and reprobed for total FoxO1 as a loading control. **(B)** The mean densitometric value of **(A)** was used to quantify and evaluate changes in phosphor-FoxO1 over time using total FoxO1 as the loading comparative control n=4 (*represents p>0.05).

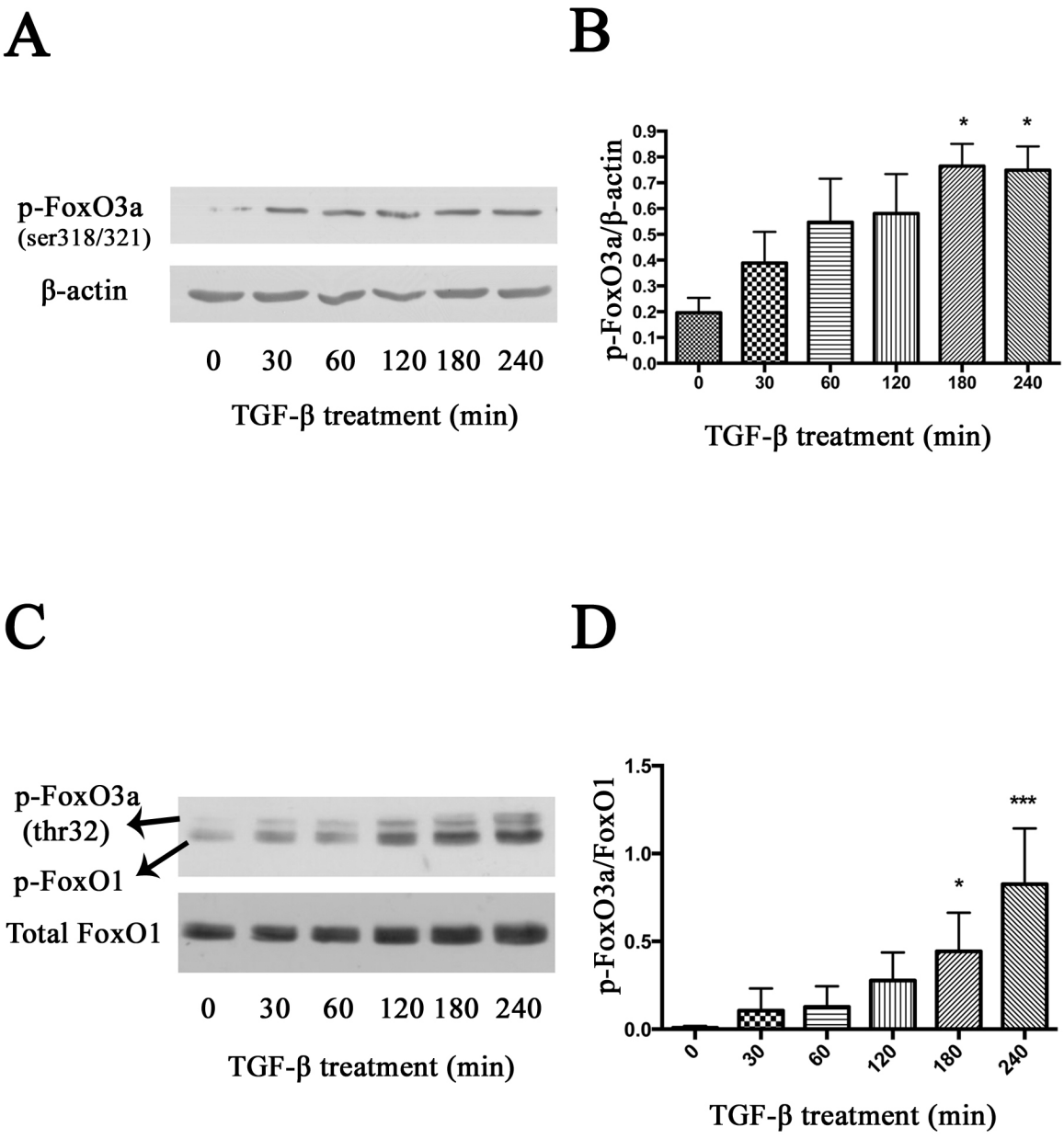

Fig. 4. TGF-β induced FoxO3a phosphorylation in fibroblasts. AKR-2B cells were treated with TGF-β (2 ng/ml) for a range of times from 0 to 240 minutes. Cell lysates were probed with an antibody specific to **(A)** phosphor-FoxO3a and **(C)** phosphor-FoxO1/3a. Blots were stripped and reprobed for **(A)** β-actin and **(C)** total FoxO1 as a loading control. The mean densitometric value of **(A)** and **(C)** was used to quantify and evaluate changes in phosphor-FoxO3a using **(A)** β-actin and **(C)** FoxO1 as the loading comparative control n=4 (*represents $p>0.05$ and *** represents $p>0.001$).

phosphorylation of serines 318/321 of FoxO3a is necessary for its nuclear exclusion. Figure 4B shows an increase of phosphorylated FoxO3a over 240 minutes at threonine 32, the Akt phosphorylation site. Utilizing the difference in molecular masses of FoxO3a and FoxO1 and the cross-reactivity of the antibody for the phospho-threonine motif, I was able to use total FoxO1 protein as the loading control for both FoxO1 and FoxO3a to determine the phosphorylation of FoxO3a was significantly increased above steady-state levels by 180 and 240 minutes. This data demonstrates that TGF-β regulates Akt directed phosphorylation of FoxO3a and that the kinase phosphorylates the S318/321 site.

In addition, I show that like the FoxO1 and FoxO3a members of the subfamily, FoxO4 is phosphorylated in response to TGF-β treatment (Figure 5A, B). Phosphorylation of FoxO4 was significantly increased above steady-state levels by 180 minutes when corrected for loading differences using β-actin. There was a slight decrease of p-FoxO4 at 240 minutes, however, insignificant compared to steady state levels.

3.2 PI3K and Akt regulation of FoxO1 phosphorylation

Having established that TGF-β stimulates the phosphorylation of three members of the FoxO family at Akt sites, my next step was to determine if the mechanism was via the known PI3K/Akt pathway. Using small molecular weight inhibitors and FoxO1-Thr24-phosphorylation as a marker of TGF-β/Akt activity I analyzed the effect inhibition of Akt or TGF-β on phosphorylation of FoxO1 would be. Figure 6 (A and B)

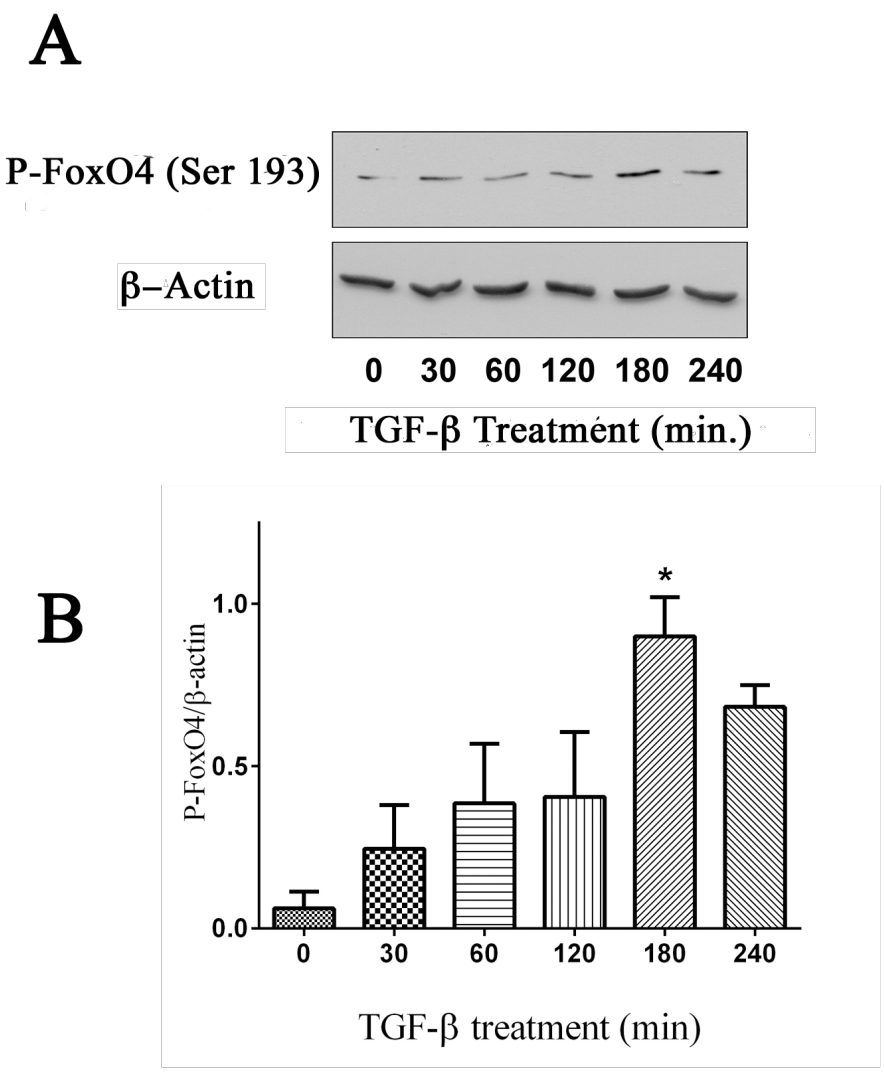

Fig. 5. TGF-β induced FoxO4 phosphorylation in fibroblasts. (A) AKR-2B fibroblasts were treated up to 240 minutes with TGF-β (2 ng/ml). Cell lysates were probed with an antibody specific to phosphor-S193-FoxO4. Blots were stripped and reprobed for β-actin as a loading control. **(B)** The mean densitometric value of **(A)** was used to quantify and evaluate change in phosphor-S193-FoxO4 overtime using β-actin as the loading comparative control n=3 (*represents $p<0.05$).

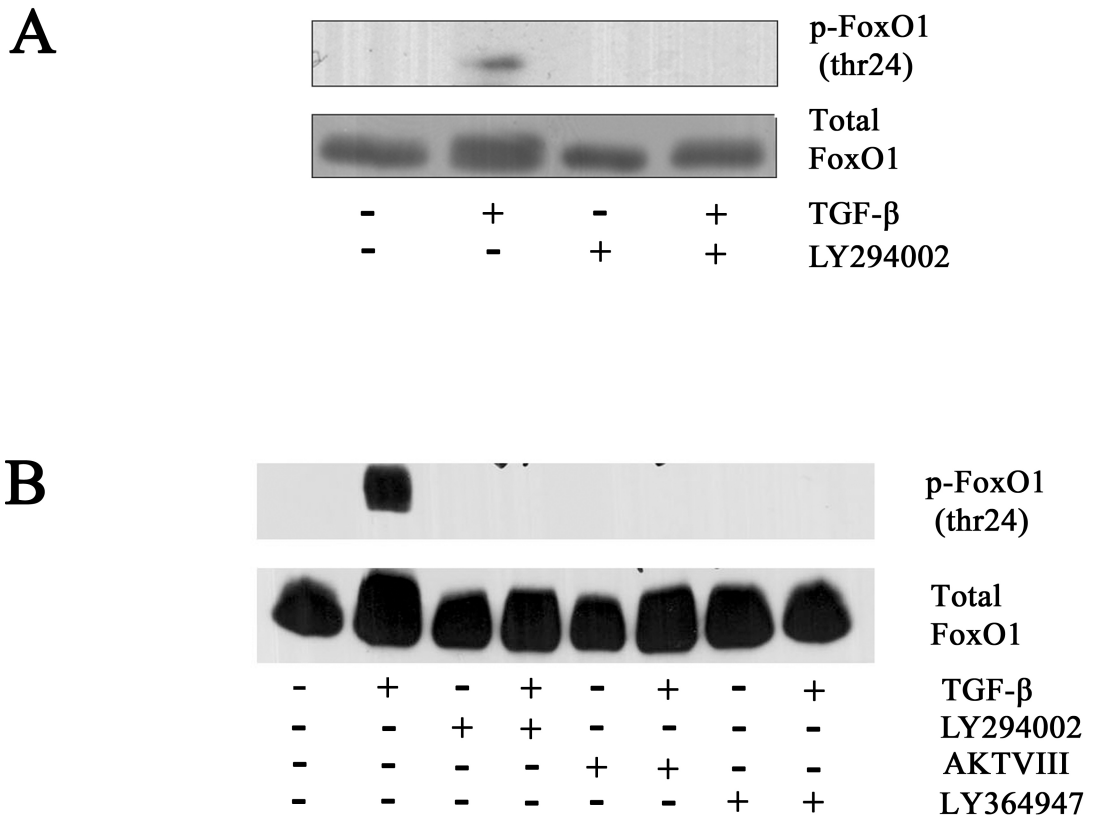

Fig. 6. TGF-β, PI3K and Akt aid in the regulation of FoxO1 phosphorylation in fibroblasts. (A) AKR-2B fibroblasts were untreated, treated with TGF-β (2 ng/ml) for 240 minutes, treated with the PI3K inhibitor LY294002 (10 μM) for 30 minutes, or treated with LY294002 (10 μM) 30 minutes prior to TGF-β (2 ng/ml) addition for 240 minutes. Cell lysates were probed with an antibody specific to phsopho-FoxO1. Blots were stripped and reprobed for total FoxO1as a loading control. **(B)** AKR-2B fibroblasts were treated as per **(A)** in the first 4 lanes, with additional treatments of an Akt inhibitor AKTVIII (1μM) or a TGF-β inhibitor LY364947 (0.5 μM) alone for 30 minutes or in addition to TGF-β (2 ng/ml) for 240 minutes. Cell lysates were probed for an antibody specific to phospho-FoxO1. Blots were stripped and reprobed for total FoxO1 as a loading control n=3.

shows an increase in FoxO1 phosphorylation following TGF-β addition, as previously shown. With the addition of LY294002 (PI3K inhibitor), Akt VIII (Akt1 and 2 isoform specific inhibitor) and LY364947 (Type I receptor kinase inhibitor) the phosphorylation of FoxO1 was negated. This demonstrates that FoxO1 T24 phosphorylation is regulated by TGF-β via the PI3K/Akt pathway in fibroblast cells.

3.3 Immunolocalization of FoxO1

As described in the literature, phosphorylation of FoxO members by Akt and other kinases correlates with the shuttling of FoxO members from nucleus to cytoplasm (van der Heide *et al.*, 2004) as part of their functional inactivation. Since I found that FoxO members are being phosphorylated following TGF-β addition via PI3K/Akt, I hypothesized that FoxO members are also being shuttled from nucleus to cytoplasm. To investigate whether this was occurring, we treated fibroblasts with TGF-β.

AKR-2B cells were treated up to 360 minutes with TGF-β1 (2 ng/ml). Since I showed that the three common FoxO members were all phosphorylated by TGF-β addition (Figures 3,4,5), I used FoxO1 as an indicator for the FoxO family since antibodies were available for this member that worked in immunocytochemistry/immunofluorescence. Total FoxO1 was detected by immunofluorescence, and visualized by confocal microscopy. Figure 7A shows FoxO1 is localized to the nucleus in senescent, untreated fibroblasts. Over the course of 360 minutes of TGF-β treatment I show a progressive movement of FoxO1 from the nucleus

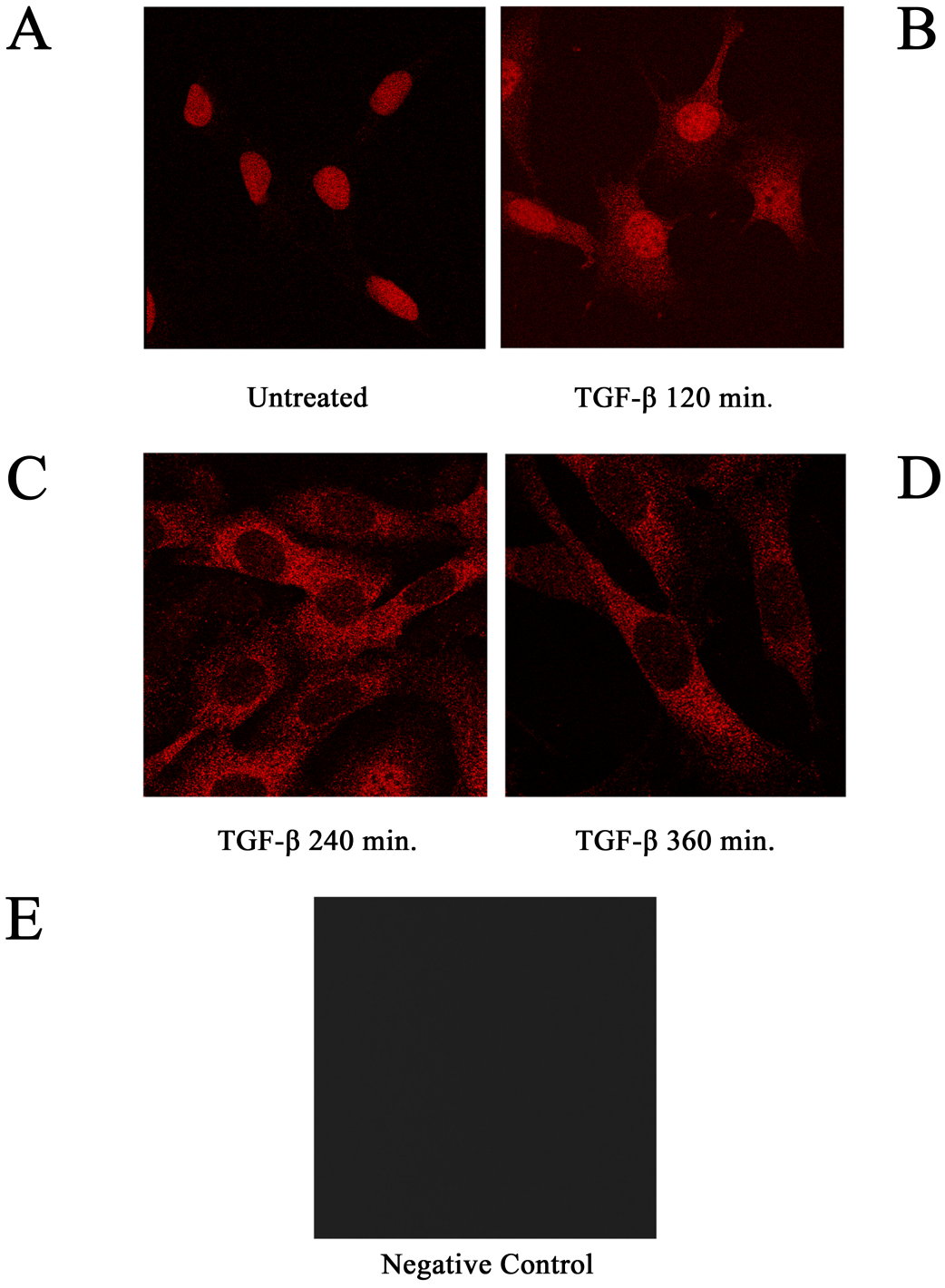

Fig. 7. FoxO1 *in vitro* cytoplasmic localization following TGF-β addition. AKR-2B fibrobasts were **(A)** untreated and treated with TGF-β (2 ng/ml) for **(B)** 120 minutes, **(C)** 240 minutes and **(D)** 360 minutes. Cells were incubated with FoxO1 antibody, detected with Rhodamine X conjugated secondary antibody, and visualised by confocal microscopy n=3.

into the cytoplasm. Colocalization of FoxO1 with a TOPRO nuclear stain was used to verify that FoxO1 was nuclear localized (results not shown). Taken together these results suggest that TGF-β regulates not only FoxO member's phosphorylation, but also subcellular localization in fibroblasts.

To determine whether the previously demonstrated PI3K-Akt pathway directs the relocalization of FoxO1, after the addition of TGF-β, I inhibited PI3K with the addition of LY294002. Figure 8 shows the redistribution of FoxO1 to the cytoplasm induced by TGF-β addition (Figure 8B) is inhibited by co-incubation with LY294002 and TGF-β (Figure 8D). Taken together, these results support my previous results that in fibroblast cells, TGF-β regulates FoxO member subcellular localization and does so via the PI3K pathway.

3.4 TGF-β regulation of $p27^{kip1}$

$p27^{kip1}$ is a tumor suppressor protein that inhibits the cell cycle (Dijkers *et al.*, 2000). Akt induces phosphorylation of $p27^{kip1}$, and is thought to be one of the primary mechanisms cells use to inhibit $p27^{kip\ 1}$ actions (Blagosklonny, 2002). As I previously show, Akt activation by TGF-β results in FoxO family members being inactivated by phosphorylation and their nuclear exclusion. Within the nucleus, active FoxO members induce the expression of $p27^{kip1}$ (Dijkers *et al.*, 2000; Shin *et al.*, 2002). Thus by activating Akt, I hypothesized that TGF-β is involved in the negative regulation of $p27^{kip1}$, both at the transcriptional and post-translational levels, in fibroblasts. To test this, TGF-β1 (2 ng/ml) was added to AKR-2B cells in a time dependent manner. Figure 9

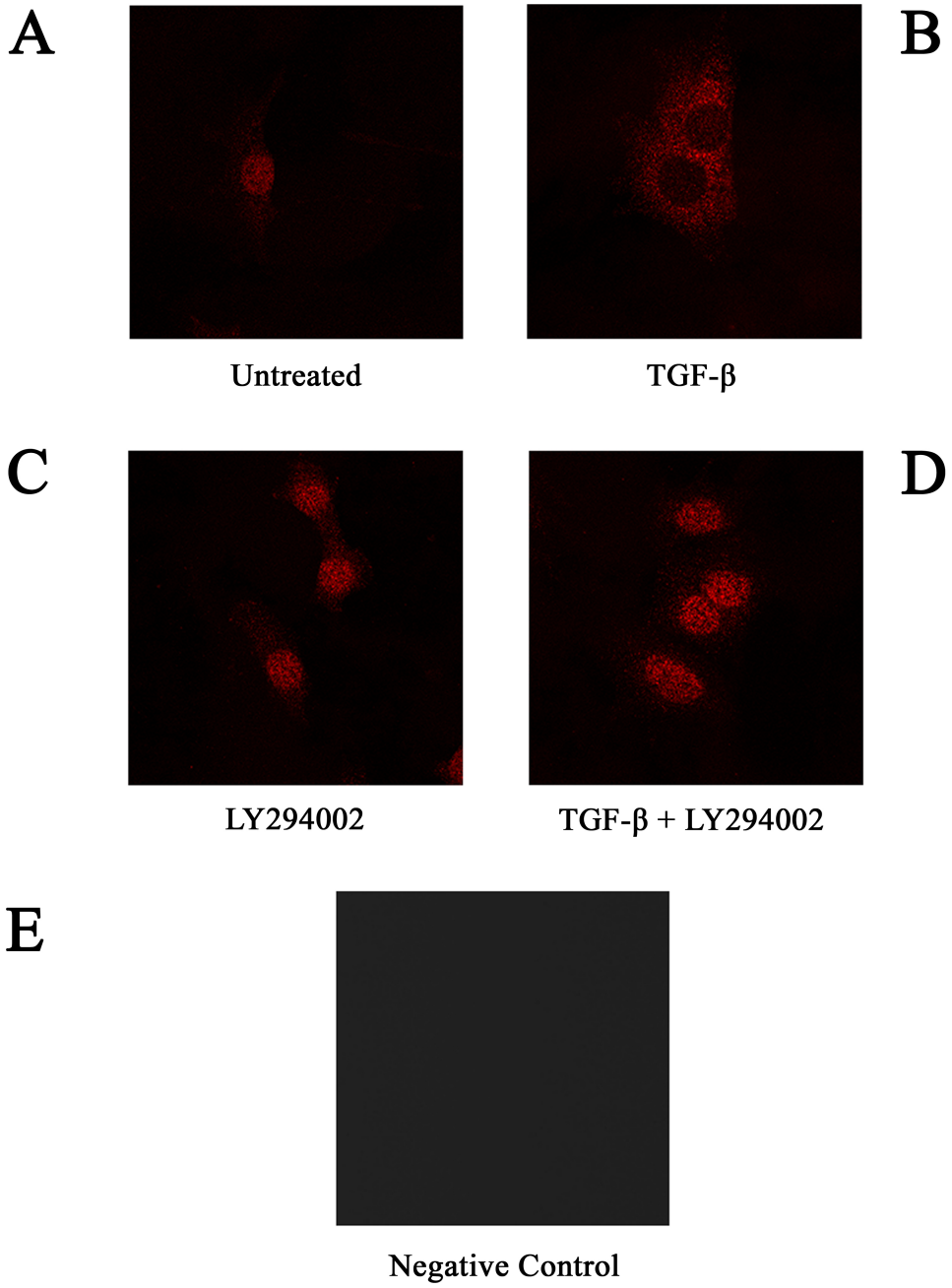

Fig. 8. PI3K aids in the localization of FoxO1 following TGF-β addition *in vitro.* AKR-2B fibroblasts were **(A)** untreated, **(B)** treated 240 minutes with TGF-β (2 ng/ml), **(C)** treated with PI3K inhibitor LY294002 (10 μM), and **(D)** LY294002 treatment 30 minutes prior to TGF-β (2 ng/ml) addition for 240 minutes. Cells were incubated with total FoxO1 antibody, detected using Rhodamine X conjugated secondary antibody and visualised by confocal microscopy n=3.

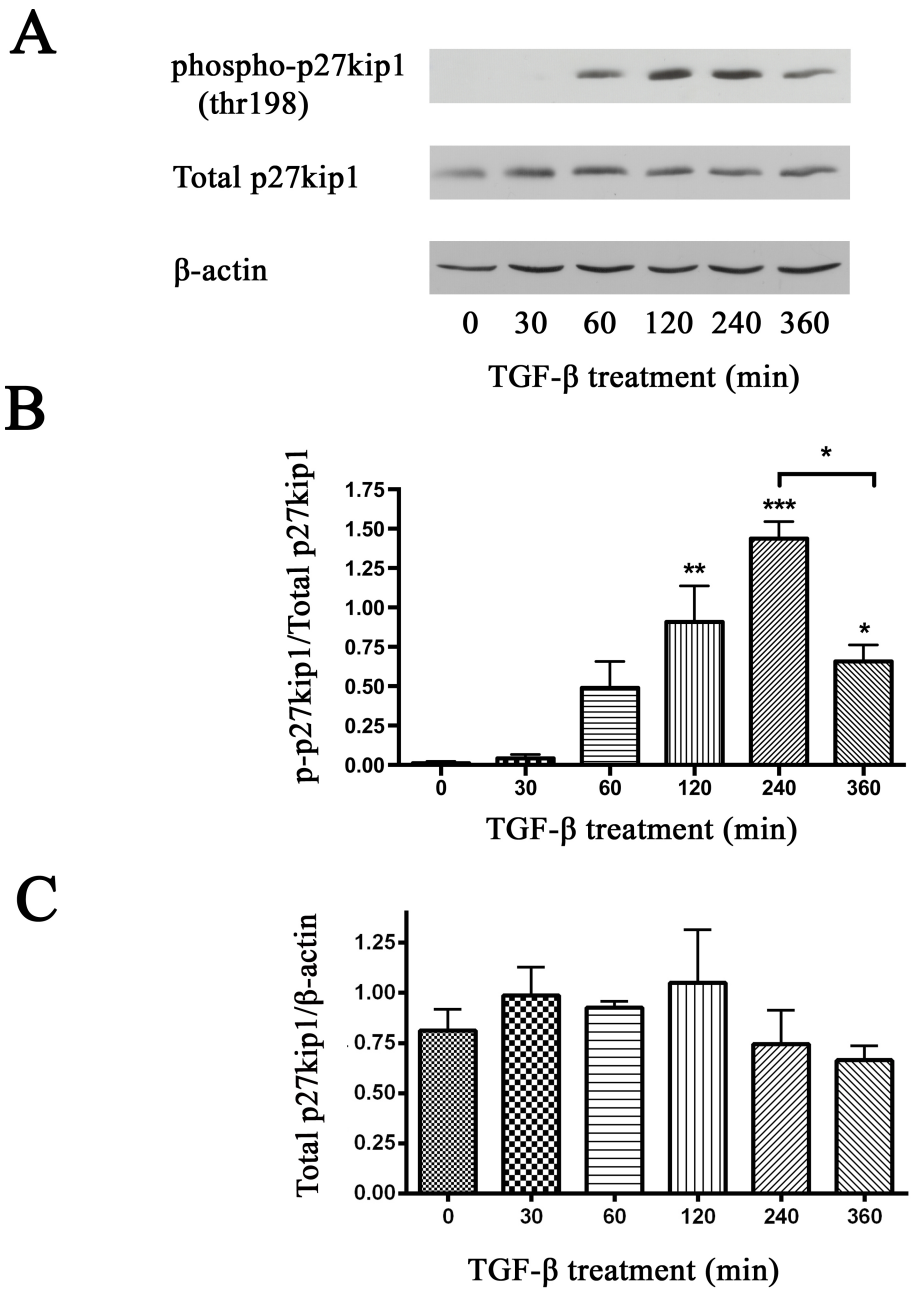

Fig. 9. $p27^{kip1}$ is phosphorylated in fibroblasts following TGF-β treatment. (A) AKR-2B fibroblasts were treated with TGF-β (2 ng/ml) for times ranging from 0 to 360 minutes. Cell lysates were probed with an antibody specific to phosphor-$p27^{kip1}$. Blots were stripped and reprobed for total $p27^{kip1}$ as a loading control, and stripped a second time and reprobed for β-actin as a secondary control. **(B)** The mean densitometric value of phosphor-$p27^{kip1}$ in **(A)** was used to evaluate changes in phosphor-$p27^{kip1}$ over time using total $p27^{kip1}$ as a comparative control **(C)** The mean densitometric value of total $p27^{kip1}$ in **(A)** was used to show no change in total $p27^{kip1}$ expression over time using β-actin as a comparative control n=3 (*=p>0.05, **=p>0.01, ***=p>0.001).

shows phospho-T198-p27^{kip1} being detectable at 60 minutes and increasing up to 240 minutes when compared to total p27^{kip1}, with significant increased levels above steady state by 120 and 240 minutes. Interestingly I noted a significant decrease in phospho-T198-p27^{kip1} from 240 minutes to 360 minutes of TGF-β treatment. Phosphorylation of p27^{kip1} at T198 has been associated with protein stability; therefore by using β -actin as a secondary control I assessed total p27^{kip1} protein levels relative to total cellular protein. Although not significantly different, the levels of total p27^{kip1} were reduced by approximately 0.25 fold from steady state levels by 240 and 360 minutes of TGF-β treatment.

The next step was to identify if the phosphorylation of p27^{kip1}, following TGF-β addition, was associated with shuttling p27^{kip1} from the nucleus to the cytoplasm. Total p27^{kip1} was visualized using immunofluorescence following TGF-β (2 ng/ml) addition. Figure 10A shows p27^{kip1} to be localized primarily in the nucleus in serum depleted, senescent, untreated AKR-2B fibroblasts. Similar results are seen after 120 minutes of TGF-β treatment (Figure 10B). By 240 and 360 minutes of TGF-β addition, Figure 10C, D shows the majority (approximately 90%) of p27^{kip1} to be found within the cytoplasm.

3.5 Target gene regulation by FoxO transcription factors

FoxO is a family of transcription factors that regulate expression of genes including p27^{kip1} (Dijkers *et al.*, 2000; Shin *et al.*, 2002). From previous data I showed that TGF-β induced the phosphorylation of the three common FoxO members and nuclear exit of FoxO1, suggesting that by removing FoxO members from the nucleus, TGF-β

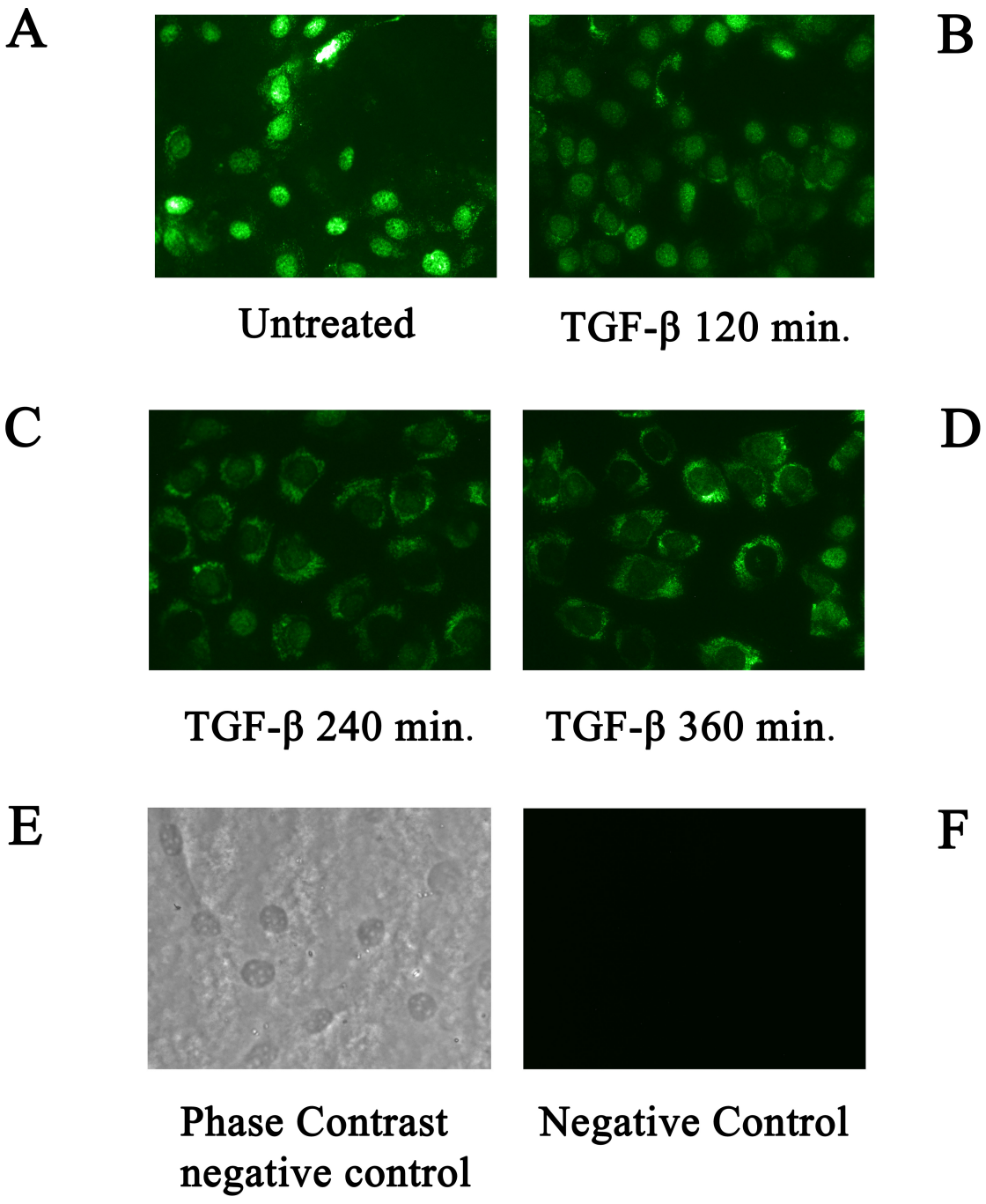

Fig. 10. p27^{kip1} becomes localized to the cytoplasm in fibroblasts following TGF-β addition *in vitro.* AKR-2B fibroblasts were **(A)** untreated and treated with TGF-β (2 ng/ml) for **(B)** 120 minutes, **(C)** 240 minutes and **(D)** 360 minutes. Cells were incubated with an antibody specific for total p27^{kip1}, detected using Alexafluor 488 and visualised by fluorescent microscopy. DMSO was used as a negative control **(E)** and **(F)** n=3.

would inhibit their nuclear functions as well. I tested this hypothesis by examining if TGF-β affected $p27^{kip1}$ transcription in fibroblasts. mRNA expression of $p27^{kip1}$ was measured following time-dependent addition of TGF-β to AKR-2B cells. qRT PCR, using probes specific for $p27^{kip1}$, demonstrated a TGF-β induced a decrease of $p27^{kip1}$ mRNA expression over time. A significant decrease in expression was seen by 240 minutes continuing through 360 minutes (Figure 11A).

To determine if the TGF-β induced reduction in $p27^{kip1}$ mRNA expression was actually regulated by the PI3K pathway, I used LY294002 to inhibit PI3K. Fibroblasts were untreated, treated with TGF-β only, LY294002 only and both TGF-β and LY294002 for 360 minutes. qRT-PCR measured $p27^{kip1}$ expression and its relative fold change was calculated using GAPDH mRNA as an internal standard. I found a significant decrease in $p27^{kip1}$ expression from cells treated with TGF-β for 360 minutes (Figure 11B), relative to untreated, similar to that seen previously in Figure 11A. Although reduced, fibroblasts treated with LY294002 showed no significant difference in $p27^{kip1}$ expression relative to either untreated fibroblasts or cells treated with TGF-β. Interestingly, fibroblasts treated with both TGF-β and LY294002 showed no significant change in $p27^{kip1}$ expression when compared fibroblasts treated with only LY294002.

Additionally, AKR-2B cells were infected with viruses containing an EGFP-FoxO4 fusion protein or an EGFP-FoxO4/AFX-A3 fusion protein (A3), where the three Akt phosphorylation sites are mutated and therefore cannot be inactivated. The expression of $p27^{kip1}$ was then measured using qRT-PCR. Figure 12A shows the relative fold change

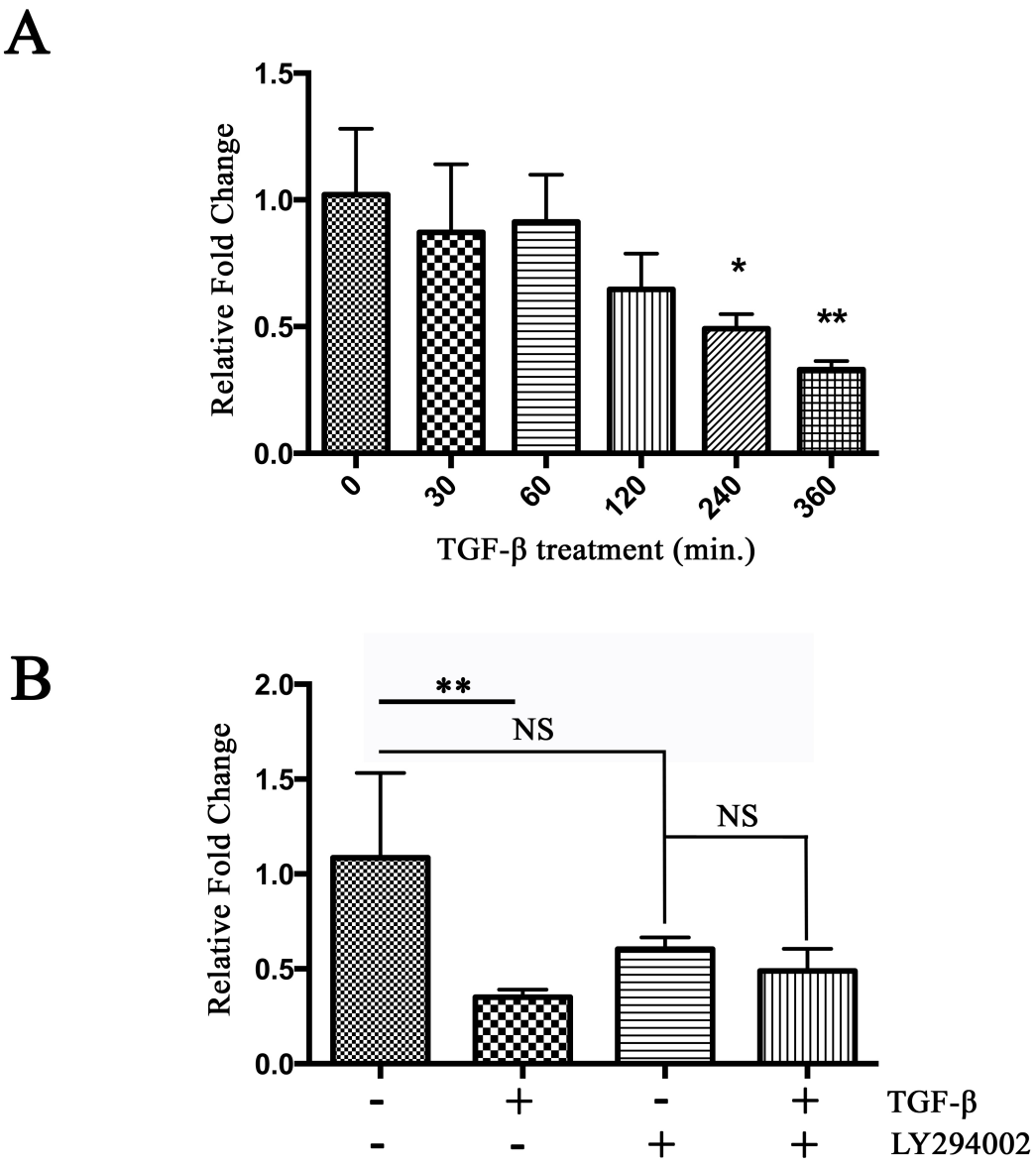

Fig. 11. TGF-β and PI3K regulation of $p27^{kip1}$ RNA expression in fibroblasts. **(A)** AKR-2B fibroblasts were treated with TGF-β (2 ng/ml) for 0 to 360 minutes. Cellular RNA was collected and qRT-PCR was performed using TaqMan probes specific for $p27^{kip1}$ and GAPDH as an internal control. **(B)** AKR-2B fibroblasts were untreated, treated with TGF-β (2 ng/ml) for 360 minutes, treated with the PI3K inhibitor LY294002 (10 μM) for 30 minutes or treated with LY294002 (10 μM) for 30 minutes prior to TGF-β addition for 360 minutes. Cellular RNA was collected and qRT-PCR was performed as per **(A)**. Both **(A)** and **(B)** were performed in triplicates on 3 or 4 independent experiments respectively. Relative fold changes were calculated and corrected for GAPDH expression levels using the ΔΔCT method (*=p>0.05, **=p>0.01).

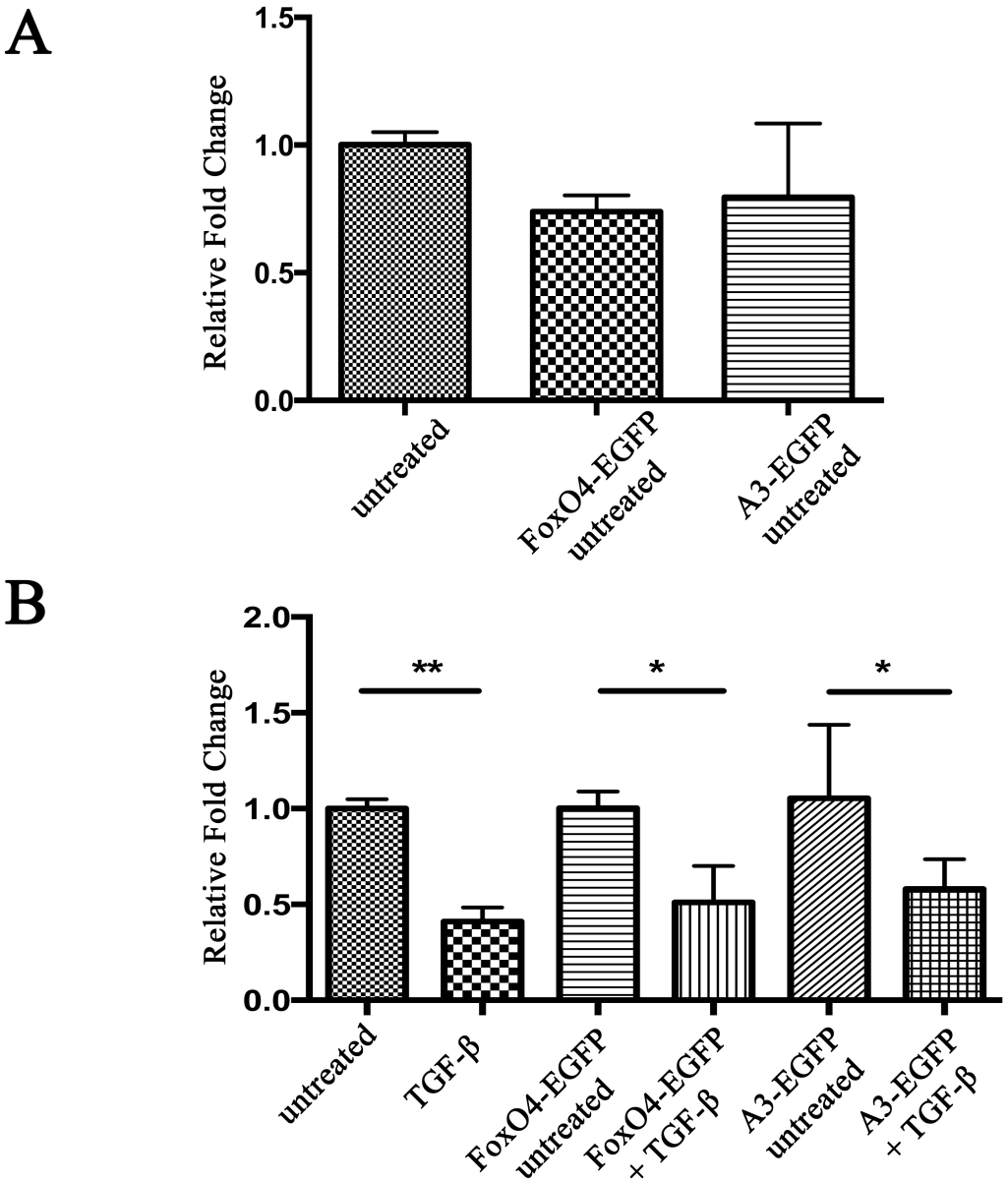

Fig. 12. $p27^{kip1}$ RNA expression following TGF-β addition in mutant infected fibroblasts. Normal AKR-2B fibroblasts and AKR-2B fibroblasts infected with an adenovirus containing a FoxO4-EGFP fusion protein or an A3-EGFP (3 mutated Akt phosphorylation sites) fusion protein was untreated or treated with TGF-β (2 ng/ml) for 360 minutes. Cellular RNA was collected and qRT-PCR was performed using TaqMan probes specific for $p27^{kip1}$ and GAPDH as an internal control. Relative fold changes were calculated and corrected for GAPDH expression levels using the ΔΔCT method. Experiments were performed in triplicates on 3 independent experiments. **(A)** shows that there was no significant relative fold change in $p27^{kip1}$ expression in infected untreated fibroblasts compared to normal untreated fibroblasts. **(B)** Relative fold change in RNA expression of $p27^{kip1}$ in TGF-β treated fibroblasts compared to untreated fibroblasts within the same infection type (*=p>0.05, **=p>0.01).

of $p27^{kip1}$ in wild type or FoxO4-A3 mutant infected, untreated fibroblasts compared to normal untreated AKR-2B cells to demonstrate there was no significant change in expression of $p27^{kip1}$ between untreated cells regardless of expression construct. Figure 12B shows the expression of $p27^{kip1}$ in AKR-2B cells treated with TGF-β compared to untreated fibroblasts expressing the same FoxO4 construct. The expression of $p27^{kip1}$ in normal AKR-2B cells treated with TGF-β decreased significantly compared to normal untreated cells. Similarly, $p27^{kip1}$ expression was significantly decreased in FoxO4-EGFP expressing TGF-β treated fibroblasts when compared to untreated infected cells. Interestingly, when AKR-2B cells expressed the A3 mutant of FoxO4 and treated with TGF-β they also significantly decreased expression of $p27^{kip1}$, compared to untreated A3-EGFP expressing fibroblasts.

CHAPTER 4-Discussion

4.1 FoxO regulation by TGF-β in fibroblasts

There is growing evidence that TGF-β mediates the regulation of several cellular target pathways outside of the canonical Smad pathway (Wilkes *et al.*, 2005; Hough *et al.*, 2012; Bakin *et al.*, 2000; Hong *et al.*, 2011; Katz *et al.*, 2013; Miyazono *et al.*, 2012; Suzuki *et al.*, 2007; Yi *et al.*, 2005), however the complete picture is still not well-defined. There is a definitive understanding that the effect of TGF-β is cell type specific. In epithelial, endothelial and hematopoietic cells, TGF-β inhibits cellular proliferation, while in fibroblasts, TGF-β induces cellular proliferation (Massagué, 2008; Shin *et al.*, 2001). Much is still unknown about the downstream targets of TGF-β regulation within different cell-types, or whether these target pathways are actually smad-independent. Wilkes *et al.,* 2005 found that TGF-β regulated the Pak2 pathway independently of Smads, but only in fibroblasts and not in epithelial cells, and this was dependent on PI3K. Previous studies from the Doré lab and others (Bakin *et al.*, 2000; Hong *et al.*, 2011; Hough *et al.*, 2012; Kato *et al.*, 2006; Miyazono *et al.*, 2012; Wilkes *et al.*, 2005; Yi *et al.*, 2005) reported that TGF-β activates the PI3K/Akt pathway. Since FoxOs are downstream of Akt, and FoxOs are transcription factors involved in the activation of target genes responsible for cell death or cellular proliferation/inhibition, I chose to look at TGF-β post-translational regulation of FoxOs by phosphorylation in fibroblasts.

Phenotypically normal fibroblasts were treated with TGF-β for a range of times up to 240 minutes. FoxO1, FoxO3a, and FoxO4 showed an increase in phosphorylation over

time at sites known to be phosphorylated by Akt (Figures 3, 4, and 5). These results are consistent with the well-known receptor tyrosine kinase activated PI3K/Akt signalling pathway, in that activated Akt phosphorylates FoxOs (Tzivion *et al*., 2011; Carracedo and Pandolfi, 2008; Yi *et al.*, 2005). This indicates that TGF-β regulation of FoxOs in fibroblasts follow a similar pathway as the RTK cellular signalling pathway.

As previously mentioned, the phosphorylation of FoxO's by Akt on specific sites (T32, S253, S315; relative to FoxO3a) results in its removal from the nucleus (Shin *et al*., 2001). In Figure 4a, I show that the phosphorylation of FoxO3a on serines 318/321 (responsible for the nuclear export of FoxO) when treated with TGF-β. A study by Rena *et al*. 2002, showed that following the phosphorylation of serine 318 by Akt, serine 321 can be phosphorylated by CK1, another known negative regulator of FoxO's, and is dependent on PI3K (Rena *et al*., 2002). Although the antibody used in this study is unable to differentiate between the phosphorylation sites, it is interesting to note that TGF-β treatment increased the phosphorylation of FoxO3a at these alternative phosphorylation sites and with further investigation might show that TGF-β can regulate FoxO3a by CK1.

To ensure that my findings are PI3K/Akt and TGF-β dependent, and that another mechanism is not regulating FoxO, I inhibited PI3K, TGF-β receptor and Akt using chemical inhibitors. I found that FoxO1 was not phosphorylated, even when TGF-β was added (Figure 6). Kato *et al*. 2006 previously found similar findings to my study using kidney mesangial cells. When cultured mesangial cells were treated with TGF-β they found significantly increased levels of p-Akt and p-FoxO3a, that were abrogated when treated with the same PI3K inhibitor (Kato *et al.*, 2006). Mesangial cells are found within

the kidney and there is evidence that they originated from mesenchyme, the same embryonic origin as fibroblasts (Faa *et al.*, 2012). Similarly, Kurebayeshi *et al*. 2016 found that in CD4+ T-cells, TGF-β induced Akt phosphorylation dependent on PI3K resulting in the phosphorylation and inhibition of FoxO1 and FoxO3a transcription factors. Although different types of cells were used in these studies (Kurebayeshi *et al*., 2016; Kato *et al*., 2006), they demonstrate TGF-β regulation of a FoxO family member dependent on the PI3K/Akt family and corroborates my findings that TGF-β regulates FoxO in a PI3K/Akt dependent manner.

4.2 FoxOs are localized to the cytoplasm following TGF-β stimulation

FoxO regulation is dependent on the specific regulator involved and type of modification (Huang and Tindall, 2007). The phosphorylation of FoxOs by Akt on (T32, S253, S315; numbered relative to FoxO3a), results in the inactivation and the removal of FoxO from the nucleus to the cytoplasm, by 14-3-3, where it is unable to bind to target transcription sites (Carter and Brunet, 2008; Essaghir *et al.*, 2009; Greer and Brunet, 2005; Huang and Tindall, 2007; Shin *et al.*, 2001; van der Heide *et al.*, 2004). Since I have shown that Akt is phosphorylating FoxO, I wanted to visualize the movement of FoxO from nucleus to cytoplasm following TGF-β addition over time. Figure 7 shows that FoxO1 moved from the nucleus to the cytoplasm following TGF-β addition over 360 minutes. I chose to look at FoxO1 specifically, because the antibodies for this member had the capability for immunofluorescence. Although, I did not show that FoxO3a and FoxO4 are undergoing nuclear-cytoplasmic shuttling in this study, I can infer similar

results, since there is evidence in the literature that FoxO3a and FoxO4 do in fact move from nucleus to cytoplasm when phosphorylated on known Akt phosphorylation sites (Kato *et al.*, 2006; Brownawell *et al.*, 2001) , as was shown in Figures 4 and 5. To ensure that the regulation of FoxO1 subcellular localization is PI3K/Akt dependent I used LY294002 to inhibit PI3K and I found that FoxO1 remained localized only to the nucleus (Figure 8).

As previously discussed, there are some contrasting views in the literature regarding Smad-independent TGF-β pathways. There are indications that in some cases there is a cross talk between the Smad pathway and the FoxO pathway. In 2004, Seoane *et al*., published a study where they showed that TGF-β induced binding of FoxO3a with Smads 3/4 to the $p21^{cip1/waf1}$ promoter site within the nucleus in HaCaT epithelial cell lines. Coimmunoprecipitation experiments showed that FoxO1, O3, O4 were bound to Smad 3 and Smad 4 in HaCaT cells following TGF-β addition (Seoane *et al.*, 2004). My study would suggest that in fibroblast cells, TGF-β regulates the PI3K/Akt pathway in a Smad-independent manner, since addition of TGF-β resulted in the phosphorylation and removal of FoxO from the nucleus, and therefore could not be bound to Smads at promotor sites. This could also provide insight for why TGF-β regulation is dependent on cell type.

4.3 $p27^{kip1}$ is phosphorylated and localized to the nucleus following TGF-β addition

$p27^{kip1}$ regulates the cell cycle by binding to cdk-cyclin complexes and hinders cell cycle progression (Dijkers *et al.*, 2000; Reynisdottir *et al.*, 1995). $p27^{kip1}$ can be regulated post-translationally and at the gene expression level. On a post-translational

level, when $p27^{kip1}$ is phosphorylated by Akt, like FoxO, it is targeted by 14-3-3 and moved to the cytoplasm (Liang *et al.*, 2002; Shin *et al.*, 2002; Motti *et al.*, 2004), where it is unable to bind cyclin-cdk complexes, and therefore would allow cell cycle to progress. Since Akt is activated in fibroblasts by TGF-β, as was found in previous studies in the Doré lab, we would expect to also see $p27^{kip1}$ being phosphorylated over time. Fibroblasts were treated with TGF-β, as per previous experiments and there was an increase in p-T198-$p27^{kip1}$ after TGF-β addition (Figure 9). It is important to note that there was a decrease in phosphorylation from 240-360 minutes. It can be ruled out that it is not due to turnover of $p27^{kip1}$, as there was no change in total $p27^{kip1}$ over time. Sun *et al*., (2016) has found that the phosphatase PPM1G dephosphorylates $p27^{kip1}$ at T198 (the same phosphorylation site as Akt), and could be the reason for the decrease in phosphorylation of $p27^{kip1}$ at 360 minutes. In contrast, the same report found that phosphorylated $p27^{kip1}$ had declined to undetectable amounts at just 2 hours. It is important to note that they used HeLa cells (an immortalized transformed cancer cell line) for this specific experiment and cells were not stimulated by specific growth factors. Instead, serum containing culture media was used to release cells from senescence. Given the differences in methodology, it would be difficult to assume that PPM1G is involved here without doing the required experiments.

Much like the FoxO family, Akt phosphorylated $p27^{kip1}$ is targeted by 14-3-3 and is removed from nucleus to cytoplasm. Since $p27^{kip1}$ was phosphorylated over time with TGF-β addition, I would expect to see $p27^{kip1}$ move from the nucleus to the cytoplasm over time. Consistent with the time line found in my western blots of p-$p27^{kip1}$, at 240 minutes, $p27^{kip1}$ was localized to the cytoplasm in comparison to untreated cells (Figure

11). At 360 minutes we still see the majority of cells having $p27^{kip1}$ located in the cytoplasm, with some cells showing its remaining localization in the nucleus. The movement of $p27^{kip1}$ into the cytoplasm would indicate cell cycle progression, as it is incapable of binding cyclin-cdk complexes outside of the nucleus.

A study by Ciarallo *et al*., (2002), found that TGF-β modulated the phosphorylation of $p27^{kip1}$ from binding Cyclin D1 to binding and inhibiting the Cyclin E1-cdk2 complex in a human epithelial cell line that is resistant to TGF-β mediated G1 arrest. Although this was completed in a different cellular context, it does suggest that TGF-β may regulate $p27^{kip1}$, or supports the idea that an epithelial cell type that gains TGF-β resistance may be doing so by activating a fibroblast phenotype, ie EMT. To my knowledge this would be the first study to suggest that TGF-β may regulate the phosphorylation of $p27^{kip1}$ dependent on PI3K/Akt in any cell context.

4.4 TGF-β regulates $p27^{kip1}$ gene expression in fibroblasts

As previously mentioned, $p27^{kip1}$ is also regulated on the gene expression level by transcription factors binding to its promoter site, and studies have found that $p27^{kip1}$ mRNA and protein are elevated following FoxO activation (Dijkers *et al.*, 2000). Furthermore, there are FoxO binding consensus sequences within the $p27^{kip1}$ promoter (Dijkers *et al.*, 2000). Although there is much evidence that FoxOs are involved in the transcriptional regulation of $p27^{kip1}$, there is little evidence to suggest that TGF-β regulates $p27^{kip1}$ directly at the gene expression level. Microarray studies showed that $p21^{cip1}$, but not $p27^{kip1}$, was increased in HaCaT epithelial cells by TGF-β (Kang *et al.*,

2003; Seoane *et al.*, 2004), but the study did not provide any insight into mesenchymal cells.

Since I demonstrated that TGF-β regulates FoxOs by Akt phosphorylation and therefore its inactivation, I also wanted to see if there was an effect on $p27^{kip1}$ mRNA levels after being treated with TGF-β. I found that $p27^{kip1}$ mRNA expression decreased by 240 minutes of TGF-β addition, compared to our control, with continued reduction up to 360 minutes (Figure 11A). It is worth noting that the timing of 240 minutes is important since I found that FoxO phosphorylation (protein inactivation) was significantly increased at 120 and 180 minutes for FoxO1 and FoxO3a respectively, and that nuclear exclusion of FoxO1 occurred between 120 and 240 minutes of TGF-β addition. This would suggest that $p27^{kip1}$ mRNA expression decreased coincidentally with the removal of FoxO from nucleus to cytoplasm, since FoxO would be unable to promote its transcription.

Consistent with my methodology and to ensure that TGF-β is acting via the PI3K pathway, I inhibited PI3K by LY294002. I found that $p27^{kip1}$ expression decreased following LY294002 treatment but was not significant when compared to the expression in untreated cells. I found a decrease in $p27^{kip1}$ expression when cells are treated with LY294002 and TGF-β (Figure 11B), however, this decrease was not significant when compared to the expression in cells treated with LY294002 alone. Therefore, strictly speaking LY294002 blocked a TGF-β induced decrease in $p27^{kip1}$ expression. However, the difficulty in interpreting these results lies in the fact that there was inconsistency between our controls (untreated or LY294002). It is important to note that for this specific experiment, AKR-2B cultures were incubated with LY294002 for 30 minutes prior to TGF-β for 360 minutes, which was a longer incubation time than our other experiments

using LY294002. Perhaps the effect of LY294002 diminishes following such a long incubation time or it has a negative effect on the cells. LY294002 also stimulates apoptosis in cells depending on the concentration and length of incubation time (Semba *et al.*, 2002); however, the concentration I used would not normally induce apoptosis and cells were checked prior to cellular lysis to ensure no visual signs of apoptosis. Alternatively, since PI3K has a large number of biological effects in addition to regulating Akt activation, FoxO transcription factors utilize a number of co-factors in regulating gene expression (Katso *et al.,* 2006). It is also reasonable to assume that inhibition of one of these PI3K dependent co-factors decrease $p27^{kip1}$ expression independent of FoxO. In this case it is difficult to assume that the decrease in $p27^{kip1}$ mRNA expression is a result of a decrease in the ability of FoxO to promote $p27^{kip1}$ transcription following TGF-β stimulation.

Last, I looked at $p27^{kip1}$ mRNA expression following TGF-β addition in normal AKR-2B fibroblasts expressing FoxO4-EGFP fusion protein or an A3-EGFP fusion protein (constitutively active FoxO4) to gain an additional understanding of the role of TGF-β regulation of $p27^{kip1}$ via FoxO4 (Medema *et al.*, 2000). Figure 12A shows that the steady state expression of $p27^{kip1}$ mRNA was consistent among all three cell culture types, indicating no specific baseline effect was noted for any of the FoxO4 constructs. Consistent with my previous results, I found that $p27^{kip1}$ expression decreased following TGF-β addition in normal fibroblasts, as well as fibroblasts containing each FoxO4-EGFP fusion proteins. The fibroblasts containing the constitutively active FoxO4-EGFP protein also decreased expression of $p27^{kip1}$ mRNA expression following TGF-β addition. This is contradictory of what I expected to see since constitutively active FoxO4 should not be

phosphorylated, and should therefore remain in the nucleus and be capable of transcription. A study by Medema *et al*., (2000) used the same A3-FoxO4-EGFP found that FoxO4 did regulate the transcription of $p27^{kip1}$, suggesting that my results are not due to an issue regarding the ability of FoxO4 to regulate p27kip1 transcription, but would suggest that FoxO4 is not involved in TGF-β regulated $p27^{kip1}$ mRNA expression in fibroblasts. Keep in mind that Medema *et al*., used A14 cells (NIH3T3 overexpressing insulin receptor) and cells were stimulated with insulin. Furthermore, my results do not mean that FoxO1 or FoxO3a are not regulating the expression of $p27^{kip1}$ by TGF-β, as there are still endogenous levels of these FoxO members. Further studies looking at FoxO1 or FoxO3a TGF-β regulated $p27^{kip1}$ gene expression would be ideal to determine the contribution of each member of the FoxO family. This suggests that TGF-β does not regulate the expression of $p27^{kip1}$ by FoxO using some other PI3K/Akt dependent method, or that all FoxOs do not functionally overlap. Taken into account that when treated with a PI3K inhibitor I found a decrease in $p27^{kip1}$ gene expression as well (Figure 11), it is possibly the former explanation. However, since I show that TGF-β does cause Akt-dependent, inactivating phosphorylation of FoxO1, 3A and 4, as well as nuclear exclusion of FoxO1 and a large body of evidence supports FoxO regulation of $p27^{kip1}$ expression, more information using specific selective inhibition is needed before this explanation should be accepted.

4.5 Future directions

The data from this study provide an indication that TGF-β can regulate FoxO family members dependent on the PI3K/Akt pathway in fibroblast cells. However, there are still questions to be answered regarding this TGF-β non-canonical pathway.

I would like to highlight some experimental additions and fine-tuning I would make to this study. At the time of this study, antibodies for all FoxO family members did not exist, as per use of FoxO1 and 3a (T32/T34) antibodies in Figure 4 and β-actin as a control for FoxO4 in Figure 5. With reliable working antibodies, for all FoxO members, we could look at the cellular localization of FoxO3a and FoxO4 in addition to FoxO1, completing the full picture rather than using inference. Also, although previous studies from graduate students in the Doré lab show that TGF-β regulates Akt phosphorylation dependent on PI3K in fibroblasts, it would strengthen this study to have Akt phosphorylation blots alongside my FoxO phosphorylation blots.

With regards to the cellular localization of phospho-$p27^{kip1}$, it would be interesting to see if the relocalization to the cytoplasm was abrogated when pre-treated with LY294002, to determine if the localization was dependent on PI3K. Lastly, as my results could not determine if $p27^{kip1}$ gene expression was regulated by TGF-β via FoxO4 (Figure 12), it would be interesting to investigate FoxO1 and FoxO3a in the same way. Perhaps I would find similar results, indicating that TGF-β is regulating $p27^{kip1}$ by some other means, or that it is only FoxO4 that is not involved in TGF-β regulated $p27^{kip1}$ gene expression.

One of the curious aspects of TGF-β regulation is that it is dependent on cellular context. As previously mentioned, in non-transformed epithelial, endothelial, and

hematopoietic stem cells, TGF-β inhibits cellular proliferation, and in fibroblast cells it induces cell proliferation (Massagué, 2008; Shin *et al.*, 2001). In this study I only looked at the role of TGF-β regulation of FoxOs in fibroblast cells. If I were to look in epithelial cells I would expect to see the opposite effect on FoxOs, since TGF-β inhibits cellular proliferation in epithelial cells. Perhaps I would not see TGF-β have an effect at all on FoxO. This is an important point to highlight, since TGF-β is found to have such contrasting roles in epithelial cells and fibroblasts. There have been studies in the literature regarding TGF-β regulation in epithelial cells (Shin *et al.*, 2001; Kato *et al.*, 2006), however these are controversial because the cell lines that are being used in some studies are immortalized or cancer cells and not normal epithelial cells. In this case these cells are acting more similarly to fibroblasts. It would be important to ensure that these cells have not undergone EMT and therefore wouldn't give an appropriate representation of epithelial cells response to TGF-β.

Since the completion of the experiments in this thesis, new modern day techniques and technology have developed, and the use of some of these could provide novel findings to further this area of study. RNA-sequencing is a method used to measure the presence and quantity of RNA at a given point of time. This is an important technique used in discovery experiments to provide a global read-out of all RNA. If I used this technique to look at fibroblast cells prior to TGF-β treatment and those following treatment of TGF-β and focused on the differences of the two, it could provide us with a list of potential gene targets of TGF-β regulation. This list could include targets via the Smad pathway or other pathways, and further evaluation would be required, however, it would be an excellent starting point.

ChIp-sequencing is a method used to analyze protein interactions with DNA at specific moments of time. This would be an effective technology to utilize for verification of the binding of all or individual FoxOs to the $p27^{kip1}$ promotor site prior and following TGF-β. As this technique shows the global DNA binding interactions, the potential of utilizing this technique could also highlight novel targets of FoxOs regulated through TGF-β. If you looked at FoxO DNA interactions before the addition of TGF-β and compared it to the absence or addition of new interactions after TGF-β treatment, it could provide us with new gene targets. This list could then be cross-referenced with the RNA-sequencing list for a more concise list of targets for qRT-PCR to evaluate gene expression regulation by TGF-β/PI3K/FoxO.

4.6 Summary

The main purpose of this study was two-fold; to determine if TGF-β regulates the FoxO group of transcription factors post-translationally, via the PI3K/Akt pathway in fibroblasts, and to determine if TGF-β regulates FoxO group functionality through transcription and post-translational modifications of $p27^{kip1}$ in fibroblasts. Here I show that following TGF-β treatment; FoxO1, FoxO3a and FoxO4 are phosphorylated over time in AKR-2B fibroblast cells in response to TGF-β stimulation. Treatment with a PI3K, Akt and TGF-β inhibitors negated the phosphorylation of FoxO1, suggesting that TGF-β regulates FoxOs dependent of PI3K/Akt. The phosphorylation of FoxOs by Akt typically results in the localization from nucleus to cytoplasm, and I show here that FoxO1 does in fact relocate from nucleus to cytoplasm following TGF-β treatment and their effect was negated when treated with the PI3K inhibitor LY294002 further

supporting the hypothesis that FoxO group cellular localization, following TGF-β stimulation, is dependent on PI3K/Akt.

In fibroblast cells, TGF-β is known to induce cellular proliferation (Shin *et al.*, 2001). $p27^{kip1}$ is a cyclin-dependent kinase inhibitor which binds to cyclin/cdk complexes and halts cell cycle progression (Dijkers *et al.*, 2000; Reynisdottir *et al.*, 1995; Roy and Banerjee, 2015). FoxOs are transcription factors known to regulate $p27^{kip1}$ when active in the nucleus (Dijkers *et al.*, 2000; Shin *et al.*, 2002). Since, I found that FoxOs were phosphorylated, and therefore inactive following TGF-β addition in fibroblasts, I wanted to see if this would affect the gene expression of $p27^{kip1}$ downstream of FoxO. I was able to show that fibroblast cell cultures treated with TGF-β decreased $p27^{kip1}$ mRNA expression over time. However, when I treated cultures with LY294002, I was unable to show that the regulation of $p27^{kip1}$ mRNA expression was dependent on PI3K. I was also unable to show conclusively that TGF-β regulates $p27^{kip1}$ gene expression dependent on FoxO4. When AKR-2B cells expressed a FoxO4-EGFP fusion protein that is constitutively active and treated with TGF-β, $p27^{kip1}$ gene expression was reduced similar to normal AKR-2B cells. It is unclear whether this result is due to the fact that TGF-β does not regulate $p27^{kip1}$ gene expression through FoxOs in fibroblasts, or that this down-regulation is due to compensation by endogenous levels of FoxO1 and FoxO3a.

TGF-β regulates a substantial number of biological events, and it has contrasting effects in epithelial and fibroblast cells. The TGF-β canonical Smad pathway is well studied, however, growing evidence involving TGF-β regulation of multiple different pathways outside of the Smad pathway, could explain its diverse regulatory effects. This study supplements findings that in fibroblasts, TGF-β can regulate the PI3K/Akt pathway,

and its downstream target FoxO members, as well as the post-translational regulation of $p27^{kip1}$. Further verification is needed to conclude that the regulation of $p27^{kip1}$ gene expression by TGF-β is in fact through the PI3K/Akt/FoxO pathway. Figure 13 shows a graphic depiction summarizing my findings and my suggested TGF-β/PI3K pathway.

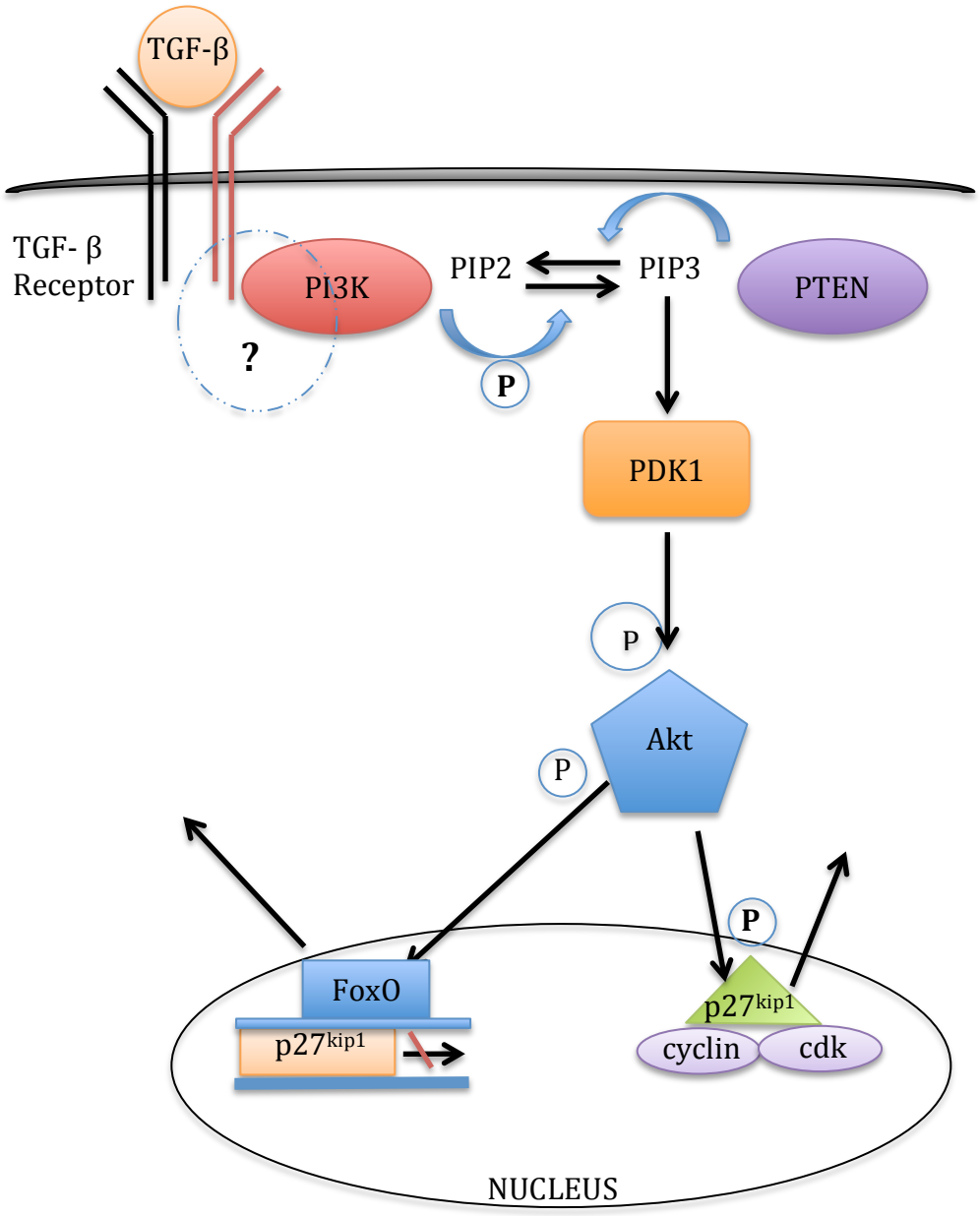

Fig. 13. TGF-β regulation of the PI3K pathway. A simplified depiction summarizing my findings of the role of TGF-β on FoxO and $p27^{kip1}$ in fibroblasts.

CHAPTER 5 – References

Akhurst, R., Hata, A. (2012) Targeting the TGFβ signalling pathway in disease. *Nature Reviews Drug Discovery*. **11**(10), 790–811.

Anderson, M.J., Viars, C.S., Czekay, S., Cavenee, W.K., Arden, K.C. (1998) Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. *Genomics*. **47**(2), 187–99.

Backer, J.M. (2008) The regulation and function of Class III PI3Ks: novel roles for Vps34. *The Biochemical Journal*. **410**(1), 1–17.

Bakin, A.V., Tomlinson, A.K., Bhowmick, N.A., Moses, H.L., Arteaga, C.L. (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *Journal of Biological Chemistry*. **275**(47), 36803–10.

Barcellos-Hoff, M.H., Dix, T.A. (1996) Redox-mediated activation of latent Transforming Growth Factor-beta 1. *Molecular Endocrinology*. **10**(9), 1077–1083.

Blagosklonny, M. V. (2002) Are p27 and p21 cytoplasmic oncoproteins? *Cell Cycle*. **1**(6), 391–3.

Blanchette, F., Rivard, N., Rudd, P., Grondin, F., Attisano, L., Dubois, C.M. (2001) Cross-talk between the p42/p44 MAP kinase and Smad pathways in transforming growth factor beta 1-induced furin gene transactivation. *Journal of Biological Chemistry*. **276**(36), 33986–33994.

Bos, J.L. (1989) ras Oncogenes in human cancer: a review. *Cancer Research*. **49**(17), 4682–4689.

Brownawell, A.M.Y.M., Kops, G.J.P.L., Macara, I.A.N.G. (2001) Inhibition of nuclear import by Protein Kinase B (Akt) regulates the subcellular distribution and activity of the Forkhead Transcription Factor AFX. *Molecular and Cellular Biology*. **21**(10), 3534–3546.

Burgering, B.M.., Kops, G.J.P. (2002) Cell cycle and death control: long live Forkheads. *Trends in Biochemical Sciences*. **27**(7), 352–360.

Campbell, P.M., Der, C.J. (2004) Oncogenic Ras and its role in tumor cell invasion and metastasis. *Seminars in Cancer Biology*. **14**(2), 105–114.

Carcamo, J., Weis, F.M.B., Ventura, F., Wieser, R., Wrana, J.L., Attisano, L., Massagué, J. (1994) Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor b and activin. *Molecular and Cellular Biology*. **14**(6), 3810–3821.

Carracedo, A., Pandolfi, P.P. (2008) The PTEN-PI3K pathway: of feedbacks and cross-talks. *Oncogene*. **27**(41), 5527–41.

Carter, M., Brunet, A. (2008) FOXO transcription factors. *Current Biology*. **17**(4), 113–114.

Ciarallo, S., Subramaniam, V., Hung, W., Lee, J., Kotchetkov, R., Sandhu, C., Milic, A., Slingerland, J.M. (2002) Altered p27(Kip1) phosphorylation, localization, and function in human epithelial cells resistant to transforming growth factor beta-mediated G(1) arrest. *Molecular and Cellular Biology*. **22**(9), 2993–3002.

Colletta, A.A., Wakefield, L.M., Howell, F. V, van Roozendaal, K.E., Danielpour, D., Ebbs, S.R., Sporn, M.B., Baum, M. (1990) Anti-oestrogens induce the secretion of active transforming growth factor beta from human fetal fibroblasts. *British Journal of Cancer*. **62**(3), 405–409.

Derynck, R., Akhurst, R.J. (2007) Differentiation plasticity regulated by TGF-beta family proteins in development and disease. *Nature Cell Biology*. **9**(9), 1000–1004.

Derynck, R., Zhang, Y.E. (2003) Smad-dependent and Smad-independent pathways in TGF-beta family signaling. *Nature*. **425**(6958), 577–584.

DiGuglielmo, G.M., Le Roy, C., Goodfellow, A.F., Wrana, J.L. (2003) Distinct endocytic pathways regulate TGF-[beta] receptor signaling and turnover. *Nature Cell Bioliogy*. **5**(5), 410–421.

Dijkers, P., Medema, R., Pals, C. (2000) Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27KIP1. *Molecular and Cellular Biology*. **20**(24), 9138–9148.

Dore, J.J.J., Yao, D., Edens, M., Garamszegi, N., Sholl, E.L., Leof, E.B. (2001) Mechanisms of Transforming Growth Factor-beta Receptor endocytosis and intracellular sorting differ between fibroblasts and epithelial cells. *Molecular Biology of the Cell*. **12**(3), 675–684.

Eblen, S.T., Slack, J.K., Weber, M.J., Catling, A.D. (2002) Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating rormation of MEK1-ERK complexes. *Molecular and Cellular Biology*. **22**(17), 6023–6033.

Engel, M.E., McDonnell, M.A., Law, B.K., Moses, H.L. (2000) Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *The Journal Of Biological Chemistry*. **274**(52), 37413–37420.

Engelman, J.A., Luo, J., Cantley, L.C. (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nature Reviews Genetics*. **7**(8), 606–619.

Essaghir, A., Dif, N., Marbehant, C.Y., Coffer, P.J., Demoulin, J.-B. (2009) The transcription of FOXO genes is stimulated by FOXO3 and repressed by growth factors. *Journal of Biological Chemistry*. **284**(16), 10334–42.

Faa, G., Gerosa, C., Fanni, D., Monga, G., Zaffanello, M., Eyken, P. Van, Fanos, V. (2012) Morphogenesis and molecular mechanisms involved in human kidney development. *Journal of Cellular Physiology*. **227**(3), 1257–1268.

Frey, R.S., Mulder, K.M. (1997) TGFbeta regulation of mitogen-activated protein kinases in human breast cancer cells. *Cancer Letters*. **117**(1), 41–50.

Funaba, M., Zimmerman, C.M., Mathews, L.S. (2002) Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. *Journal of Biological Chemistry*. **277**(44), 41361–41368.

Galliher-Beckley, A.J., Schiemann, W.P. (2008) Grb2 binding to Tyr284 in TbetaR-II is essential for mammary tumor growth and metastasis stimulated by TGF-beta. *Carcinogenesis*. **29**(2), 244–251.

Galliher, A.J., Schiemann, W.P. (2007) Src phosphorylates Tyr284 in TGF-beta type II receptor and regulates TGF-beta stimulation of p38 MAPK during breast cancer cell proliferation and invasion. *Cancer Research*. **67**(8), 3752–3758.

Gaullier, J.-M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H., Aasland, R. (1998) FYVE fingers bind PtdIns(3)P. *Nature*. **394**(6692), 432–433.

Greer, E.L., Brunet, A. (2005) FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene*. **24**(50), 7410–25.

Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J., Sabatini, D.M. (2006) Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Developmental Cell*. **11**(6), 859–871.

Hartsough, M.T., Mulder, K.M. (1995) Transforming growth factor beta activation of p44mapk in proliferating cultures of epithelial cells. *Journal of Biological Chemistry*. **270**(13), 7117–7124.

Hong, M., Wilkes, M.C., Penheiter, S.G., Gupta, S.K., Edens, M., Leof, E.B. (2011) Non-Smad Transforming Growth Factor-β signaling regulated by Focal Adhesion Kinase Binding the p85 subunit of phosphatidylinositol 3-Kinase. *Journal of Biological Chemistry*. **286**(20), 17841–17850.

Horowitz, J.C., Lee, D.Y., Waghray, M., Keshamouni, V.G., Thomas, P.E., Zhang, H., Cui, Z., Thannickal, V.J. (2004) Activation of the pro-survival phosphatidylinositol 3-kinase/AKT pathway by transforming growth factor-beta1 in mesenchymal cells is mediated by p38 MAPK-dependent induction of an autocrine growth factor. *Journal of Biological Chemistry*. **279**(2), 1359–67.

Hough, C., Radu, M., Doré, J.J.E. (2012) TGF-Beta induced Erk phosphorylation of Smad linker region regulates Smad signaling. *PLoS ONE*. **7**(8).

Huang, H., Tindall, D.J. (2007) Dynamic FoxO transcription factors. *Journal of Cell Science*. **120**(15), 2479–87.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., Su, B. (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell*. **127**(1), 125–137.

Jacobs, F.M.J., van der Heide, L.P., Wijchers, P.J.E.C., Burbach, J.P.H., Hoekman, M.F.M., Smidt, M.P. (2003) FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. *Journal of Biological Chemistry*. **278**(38), 35959–67.

Kang, Y., Chen, C.-R., Massagué, J. (2003) A self-enabling TGFβ response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in Epithelial Cells. *Molecular Cell*. **11**(4), 915–926.

Kato, M., Yuan, H., Xu, Z.-G., Lanting, L., Li, S.-L., Wang, M., Hu, M.C.-T., Reddy, M. a, Natarajan, R. (2006) Role of the Akt/FoxO3a pathway in TGF-beta1-mediated mesangial cell dysfunction: a novel mechanism related to diabetic kidney disease. *Journal of the American Society of Nephrology*. **17**(12), 3325–35.

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. *Annual Review of Cell and Developmental Biology*. **17**, 615–675.

Katz, L., Li, Y., Chen, J., Al., E. (2013) Targeting TGF-β signaling in cancer. *Expert Opinion on Therapeutic Targets*. **17**(7), 743–760.

Kielley, D. (2008) Transforming growth factor beta regulates cell cycle and growth via Akt. Masters thesis, Memorial University of Newfoundland.

Kretzschmar, M., Doody, J., Massague, J. (1997) Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. *Nature*. **389**(6651), 618–622.

Kurebayashi, Y., Baba, Y., Minowa, A., Nadya, NA., Azuma, M., Yoshimura, A., Kayasu, S., Nagai, S. (2016) TGF-β-induced phosphorylation of Akt and FoxO transcription factors negatively regulates induced regulatory T cell differentiation. *Biochemical and Biophysical Research Communication.* **480**(1), 114-119.

Lawler, S., Feng, X.H., Chen, R.H., Maruoka, E.M., Turck, C.W., Griswold-Prenner, I., Derynck, R. (1997) The type II transforming growth factor-beta receptor autophosphorylates not only on serine and threonine but also on tyrosine residues. *Journal of Biological Chemistry*. **272**(23), 14850–14859.

Lee, J.M., Dedhar, S., Kalluri, R., Thompson, E.W. (2006) The epithelial–mesenchymal transition: new insights in signaling, development, and disease. *Journal of Cell Biology*. **172**(7), 973–981.

Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M.K., Han, K., Lee, J.-H., Ciarallo, S., Catzavelos, C., Beniston, R., Franssen, E., Slingerland, J.M. (2002) PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nature Medicine*. **8**(10), 1153–60.

Liu, P., Kao, T.P., Huang, H. (2008) CDK1 promotes cell proliferation and survival via phosphorylation and inhibition of FOXO1 transcription factor. *Oncogene*. **27**(34), 4733–44.

Liu, P., Wang, Z., Wei, W. (2014) Phosphorylation of Akt at the C-terminal tail triggers Akt activation. *Cell Cycle*. **13**(14), 2162–2164.

Luo, K., Lodish, H.F. (1997) Positive and negative regulation of type II TGF-beta receptor signal transduction by autophosphorylation on multiple serine residues. *EMBO Journal*. **16**(8), 1970–1981.

Lyons, R.M., Keski-Oja, J., Moses, H.L. (1988) Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *Journal of Cell Biology*. **106**(5), 1659–1665.

Malumbres, M. (2014) Cyclin-dependent kinases. *Genome Biology*. **15**(6), 122.
Manning, B.D., Cantley, L.C. (2007) AKT/PKB Signaling: Navigating Downstream. *Cell*. **129**(7), 1261–1274.

Massagué, J. (2008) TGFbeta in cancer. *Cell*. **134**(2), 215–30.

McKay, M.M., Morrison, D.K. (2007) Integrating signals from RTKs to ERK/MAPK. *Oncogene*. **26**(22), 3113–3121.

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**(6779), 782–787.

Miyazono, K., Ehata, S., Koinuma, D. (2012) Tumor-promoting functions of transforming growth factor-β in progression of cancer. *Upsala Journal of Medical Sciences*. **117**(2), 143–52.

Miyazono, K., Kamiya, Y., Morikawa, M. (2010) Bone morphogenetic protein receptors and signal transduction. *Journal of Biochemistry* . **147**(1), 35–51.

Motti, M.L., De Marco, C., Califano, D., Fusco, A., Viglietto, G. (2004) Akt-dependent T198 phosphorylation of cyclin-dependent kinase inhibitor p27kip1 in breast cancer. *Cell Cycle*. **3**(8), 1074–1080.

Moustakas, A., Heldin, C.-H. (2005) Non-Smad TGF-beta signals. *Journal of Cell Science*. **118**(Pt 16), 3573–84.

Moustakas, A., Souchelnytskyi, S., Heldin, C.-H. (2001) Smad regulation in TGF-β signal transduction. *Journal of Cell Science*. **114**(24), 4359–4369.

Mucsi, I., Skorecki, K.L., Goldberg, H.J. (1996) Extracellular signal-regulated kinase and the small GTP-binding protein, Rac, contribute to the effects of transforming growth factor-beta1 on gene expression. *Journal of Biological Chemistry*. **271**(28), 16567–16572.

Mulder, K.M., Morris, S.L. (1992) Activation of p21ras by transforming growth factor beta in epithelial cells. *Journal of Biological Chemistry*. **267**(8), 5029–5031.

Mundi, P.S., Sachdev, J., McCourt, C., Kalinsky, K. (2016) AKT in cancer: new molecular insights and advances in drug development. *British Journal of Clinical Pharmacology*. **82**(4), 943–956.

Nigg, E.A. (2001) Mitotic kinases as regulators of cell division and its checkpoints. *Nature Reviews Molecular Cell Biology*. **2**(1), 21–32.

Northey, J.J., Chmielecki, J., Ngan, E., Russo, C., Annis, M.G., Muller, W.J., Siegel, P.M. (2008) Signaling through ShcA is required for transforming growth factor beta- and Neu/ErbB-2-induced breast cancer cell motility and invasion. *Molecular and Cellular Biology*. **28**(10), 3162–3176.

Obsilova, V., Vecer, J., Herman, P., Pabianova, A., Sulc, M., Teisinger, J., Boura, E., Obsil, T. (2005) 14-3-3 Protein interacts with nuclear localization sequence of forkhead transcription factor FoxO4. *Biochemistry*. **44**(34), 11608–17.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature*. **389**(6654), 994–999.

Olsson, N., Piek, E., Sundstrom, M., ten Dijke, P., Nilsson, G. (2001) Transforming growth factor-beta-mediated mast cell migration depends on mitogen-activated protein kinase activity. *Cellular Signalling*. **13**(7), 483–490.

Penheiter, S.G., Mitchell, H., Garamszegi, N., Edens, M., Dore, J.J.E., Leof, E.B. (2002) Internalization-dependent and -independent requirements for Transforming Growth Factor Receptor signaling via the Smad pathway. *Molecular and Cellular Biology*. **22**(13), 4750–4759.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids.* **29**(9).

Planas-Silva, M.D., Weinberg, R.A. (1997) The restriction point and control of cell proliferation. *Current Opinion in Cell Biology*. **9**(6), 768–772.

Pullan, L., Mullapudi, S., Huang, Z., Baldwin, P.R., Chin, C., Sun, W., Tsujimoto, S., Kolodziej, S.J., Stoops, J.K., Lee, J.C., Waxham, M.N., Bean, A.J., Penczek, P.A. (2006) The endosome-associated protein Hrs is hexameric and controls cargo sorting as a "Master Molecule." *Structure*. **14**(4), 661–671.

Radu, M., Semenova, G., Kosoff, R., Chernoff, J. (2014) PAK signaling during the development and progression of cancer. *Nature Reviews Cancer*. **14**(1), 13–25.

Ravichandran, K.S. (2001) Signaling via Shc family adapter proteins. *Oncogene*. **20**(44), 6322–6330.

Rena, R., Woods, Y.L., Prescott, A.R., Peggie, M., Unterman, T.G., Williams, M.R., Cohen, P. (2002) Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion. *EMBO Journal.* **21**(9), 2263-2271.

Reynisdottir, I., Massague, J. (1997) The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. *Genes & Development*. **11**(4), 492–503.

Reynisdottir, I., Polyak, K., Iavarone, a, Massague, J. (1995) Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes & Development*. **9**(15), 1831–1845.

Robbins, D.J., Cheng, M., Zhen, E., Vanderbilt, C.A., Feig, L.A., Cobb, M.H. (1992) Evidence for a Ras-dependent extracellular signal-regulated protein kinase (ERK) cascade. *Proceedings of the National Academy of Sciences*. **89**(15), 6924–6928.

Roy, A., Banerjee, S. (2015) p27 and leukemia: cell cycle and beyond. *Journal of Cellular Physiology*. **230**(3), 504–509.

Runyan, C.C.E., Schnaper, H.W., Poncelet, A.A.-C. (2004) The phosphatidylinositol 3-Kinase/Akt pathway enhances Smad3-stimulated mesangial cell collagen I expression in response to Transforming Growth Factor-β1. *Journal of Biological Chemistry* . **279**(4), 2632–2639.

Schafer, K.A. (1998) The cell cycle: a review. *Vet Pathology*. **35**(6), 461–478.

Schlessinger, J. (2000) Cell signalling by receptor tyrosine kinases. *Cell*. **103**(2), 211–225.

Schultz-Cherry, S., Murphy-Ullrich, J.E. (1993) Thrombospondin causes activation of latent transforming growth factor-beta secreted by endothelial cells by a novel mechanism. *Journal of Cell Biology*. **122**(4), 923–932.

Semba, S., Itoh, N., Ito, M., Harada, M., Yamakawa, M. (2002) The *in vitro* and *in vivo* effects of 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), a specific inhibitor of phosphatidylinositol 3'-kinase, in human colon cancer cells. *Clinical Cancer Research*. **8**(6), 1957–1963.

Seoane, J., Le, H., Shen, L., Anderson, S., Massagué, J. (2004) Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell*. **117**(2), 211–223.

Shin, I., Bakin, a V, Rodeck, U., Brunet, A., Arteaga, C.L. (2001) Transforming growth factor beta enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. *Molecular Biology of the Cell*. **12**(11), 3328–39.

Shin, I., Yakes, F., Rojo, F., Shin, N. (2002) PKB / Akt mediates cell-cycle progression by phosphorylation of p27 Kip1 at threonine 157 and modulation of its cellular localization. *Nature Medicine*. **8**(9), 1145–1152.

Simeone, D.M., Zhang, L., Graziano, K., Nicke, B., Pham, T., Schaefer, C., Logsdon, C.D. (2001) Smad4 mediates activation of mitogen-activated protein kinases by TGF-beta in pancreatic acinar cells. *American Journal of Physiology Cell Physiology*. **281**(1), 311–9.

Sun, C., Wang, G., Wrighton, K.H., Songyang, Z., Feng, X.H., Lin, X. (2016) Regulation of p27Kip1 phosphorylation and G1 cell cycle progression by protein phosphatase PPM1G. *America Journal of Cancer Research.* **6**(10), 2207-2209.

Suzuki, K., Wilkes, M.C., Garamszegi, N., Edens, M., Leof, E.B. (2007) Transforming growth factor beta signalling via Ras in mesenchymal cells requires p21-activated kinase 2 for extracellular signal-regulated kinase-dependent transcriptional responses. *Cancer Research*. **67**(8), 3673–3682.

Thiery, J.P. (2003) Epithelial-mesenchymal transitions in development and pathologies. *Current Opinion in Cell Biology*. **15**(6), 740–746.

Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L., Wrana, J.L. (2016) SARA, a FYVE Domain Protein that Recruits Smad2 to the TGFbeta Receptor. *Cell*. **95**(6), 779–791.

Tzivion, G., Dobson, M., Ramakrishnan, G. (2011) FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. *Biochimica et Biophysica Acta-Molecular Cell Research.* **1813**(11), 1938-1945.

van der Heide, L.P., Hoekman, M.F.M., Smidt, M.P. (2004) The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochemical Journal*. **380**(2), 297–309.

Vervoorts, J., Lüscher, B. (2008) Post-translational regulation of the tumor suppressor p27KIP1. *Cellular and Molecular Life Sciences*. **65**(20), 3255–3264.

Viglietto, G., Motti, M., Bruni, P., Melillo, R. (2002) Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27Kip1 by PKB/Akt-mediated phosphorylation in breast cancer. *Nature Medicine*. **8**(10), 32–35.

Wang, M.C., Bohmann, D., Jasper, H. (2005) JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. *Cell*. **121**(1), 115–125.

Wilkes, M.C., Repellin, C.E., Kang, J.H., Andrianifahanana, M., Yin, X., Leof, E.B. (2015) Sorting nexin 9 differentiates ligand-activated Smad3 from Smad2 for nuclear import and transforming growth factor β signaling. *Molecular Biology of the Cell.* **26**(21), 3879-3891.

Wilkes, M.C., Leof, E.B. (2006) Transforming growth factor beta activation of c-Abl is independent of receptor internalization and regulated by phosphatidylinositol 3-kinase and PAK2 in mesenchymal cultures. *Journal of Biological Chemistry*. **281**(38), 27846–27854.

Wilkes, M.C., Mitchell, H., Penheiter, S.G., Doré, J.J., Suzuki, K., Edens, M., Sharma, D.K., Pagano, R.E., Leof, E.B. (2005) Transforming growth factor-beta activation of phosphatidylinositol 3-kinase is independent of Smad2 and Smad3 and regulates fibroblast responses via p21-activated kinase-2. *Cancer Research*. **65**(22), 10431–10440.

Wilkes, M.C., Murphy, S.J., Garamszegi, N., Leof, E.B. (2003) Cell-type-specific activation of PAK2 by transforming growth factor beta independent of Smad2 and Smad3. *Molecular and Cellular biology*. **23**(23), 8878–8889.

Williams, G.H., Stoeber, K. (2012) The cell cycle and cancer. *Journal of Pathology*. **226**(2), 352–364.

Yan, L., Lavin, V.A., Moser, L.R., Cui, Q., Kanies, C., Yang, E. (2008) PP2A regulates the pro-apoptotic activity of FOXO1. *Journal of Biological Chemistry*. **283**(12), 7411–7420.

Yan, Z., Winawer, S., Friedman, E. (1994) Two different signal transduction pathways can be activated by transforming growth factor beta 1 in epithelial cells. *Journal of Biological Chemistry*. **269**(18), 13231–13237.

Yi, J.Y., Shin, I., Arteaga, C.L. (2005) Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase. *Journal of Biological Chemistry*. **280**(11), 10870–6.

Yu, Q., Stamenkovic, I. (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes & Development*. **14**(2), 163–176.

Zavadil, J., Bitzer, M., Liang, D., Yang, Y.C., Massimi, A., Kneitz, S., Piek, E., Bottinger, E.P. (2001) Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proceedings of the National Academy of Sciences*. **98**(12), 6686–6691.

Zhang, Y.E. (2009) Non-Smad pathways in TGF-beta signaling. *Cell Research*. **19**(1), 128–39.