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Dissecting the Roles of TGF-β Receptors in TGF-β-Induced PI3K-Akt Activation

by

Koy Yoon Saeteurn

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

DEDICATION

This dissertation is dedicated to my family, friends, and past and current mentors who have been huge advocates of my educational endeavors. They believed in me more than I believed in myself.

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Thanks to Jamie Smyth who helped at the initial stages of this work. Jamie created most of the TβRII tyrosine mutant constructs and performed the TIRF microscopy experiment.

I give many thanks to Bradley Webb for much of the resources I've used for protein overexpression and *in vitro* assays and for the helpful discussions and advice. Brad has been a great post doc mentor that I did not have in the lab. He provided me with various PI3K constructs and helped me with experimental protocols and, as a budding PI would, pushed me to do key experiments that would test possible hypotheses and answer them quickly.

I give thanks to all the members of the Derynck Lab whom I've worked with during my time in the lab. All of them have given me great advice and helped me experimentally at some time during my graduate work. Additionally, I would like to thank Katja Brückner and members of her lab for the helpful discussions.

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Last, but not least, I thank my parents for their initial skepticism which fed my determination to obtain higher education and then for their continued support as I pursued my degree. I thank all of my family for their respect and support. They have done more than they should to help me succeed in my graduate study and beyond.

ABSTRACT

TGF-β plays many roles in growth and development, and aberrant TGF-β signaling contributes to the pathogenesis and progression of various diseases, including fibrosis and the initiation and progression of cancer toward metastasis. TGF-β activates many downstream effectors, including Smad signaling, which regulates gene transcription, and PI3K-Akt signaling, which has roles in regulating cell proliferation and survival among other cellular processes. Both pathways are highly targeted in development of drug therapies.

It has been shown that the kinase activity of TβRI is needed for Akt activation through PI3K and that TGF-β receptors can associate with PI3K, but the mechanism of how TGF-β activates PI3K-Akt is yet to be determined. The PI3K-Akt pathway is known to be activated downstream of RTKs. Given that TGF-β receptors are dual-specificity kinases, we can reason that TGF-β receptors may activate PI3K-Akt similarly to RTKs, in that phosphorylated tyrosines on activated TGF-β receptors play key roles in recruitment and activation of PI3K.

In this study, we interrogate the mechanism of TGF-β-induced PI3K-Akt activation at the receptor level. We find that the PI3K p85α regulatory and the p110α catalytic subunits have major roles in TGF-β-induced Akt activation and EMT. We also find that the C-terminal SH2 domain of p85α, along with the iSH2 domain, is needed for interaction with TβRII. Efforts have been put in to exploring the role of TβRII tyrosine phosphorylation in the TβRII-p85α interaction, and it appears that loss of tyrosine phosphorylation sites attenuate TβRII-p85α

interaction. While the kinase activity of TβRI is needed for activation of Akt, it seems neither the kinase activity of TβRI nor TβRII is needed for PI3K interaction.

The TβRII-p85α interaction seems to be constitutive, with subtle fluctuations in interaction upon TGF-β stimulation or inhibition of the TβRI kinase. However, this fluctuation is too subtle to be conclusive. Some data suggest that the TβRII-p85α interaction could be one that is inhibitory. Data also suggest that the adaptor proteins ShcA and/or Grb2 may have a role in TGF-β-induced Akt activation. These theories have yet to be fully examined, but preliminary data and speculations are discussed in this dissertation.

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CHAPTER 1: Introduction

1.1 Background and Significance

The transforming growth factor-beta (TGF-β) family of cytokines is essential in regulating a multitude of cellular events in development and disease including germ-layer specification, heart valve development, cell growth and differentiation, and extracellular matrix (ECM) production (Gordon and Blobe 2008; Wu and Hill 2009). Mutations in TGF-β signaling pathways leading to aberrant TGF-β signaling is known to contribute to congenital defects, pathogenesis and progression of various diseases, including the initiation and progression of cancer toward metastasis, and fibrosis (Verrecchia and Mauviel 2007; Gordon and Blobe 2008; Goumans et al. 2009; Padua and Massagué 2009).

TGF-β signaling can induce activation of Smad and non-Smad signaling pathways which work in concert to regulate cellular processes, including epithelial-mesenchymal transition (EMT) wherein epithelial cells acquire invasive, mesenchymal characteristics. EMT is an integral process that takes place during various stages in development as well as in tumor metastasis and fibrosis. In TGF-β-driven EMT, different signaling pathways are activated, dependent on cell type and context. Major pathways activated in TGF-β-induced EMT include Smad signaling, which goes on to regulate gene transcription, and PI3K-Akt signaling, a pathway that is highly misregulated in cancer (Yuan and Cantley 2008; Lamouille et al. 2014; Mayer and Arteaga 2016; Moustakas and Heldin 2016). Signaling pathways known to be activated downstream of TGF-β are illustrated in Figure 1.

It has been shown that the kinase activity of TβRI is needed for activation of Akt (Lamouille and Derynck 2007; Wu and Derynck 2009) and that TGF-β receptors can associate with PI3K (Yi et al. 2005), but the mechanism of how TGF-β activates PI3K-Akt signaling at the receptor level is yet to be determined. The PI3K-Akt pathway is generally known to be activated downstream of receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs). TGF-β receptors have been classified as dual-specificity kinases, but are better understood for their roles as serine/threonine kinases, and less so for their tyrosine kinase activity. Nevertheless, that TGF-β receptors are dual-specificity kinases give way to reason that the TGF-β receptors may activate PI3K-Akt similarly to RTKs in that the PI3K binds to activated TGF-β receptors, either directly or indirectly, through phosphorylated tyrosines.

1.2 TGF-β Family and Smad Activation

The TGF-β family of polypeptides is encoded by 33 different genes in mammals. The family may be sub-divided into four major subfamilies that include TGF-β, bone morphogenetic protein (BMP), Activin/Nodal, and growth differentiation factor (GDF) (Morikawa et al. 2016). TGF-β ligands are secreted from the cell as latent peptides and are sequestered in the ECM. Removal of the latency-associated peptides is needed to activate the ligand (Horiguchi et al. 2012). Members of the TGF-β family share a common theme of activation wherein ligand binds to its cognate cell surface receptor, inducing activation of the type I receptor by the type II receptor through transphosphorylation (Hata and Chen 2016). This multimeric complex consists of two type I and two type II receptors held together by two ligands. The type I receptor subsequently phosphorylates receptor-regulated Smads (R-Smads) at their C-terminal SSXS motif, thereby activating them. R-Smads can then associate with the common mediator Smad (co-Smad),

Smad4, and translocate to the nucleus, and along with transcriptional co-factors, regulate the expression of many genes. In addition to R- and co-Smad, inhibitory Smads (I-Smads), Smad6 and Smad7, are induced to antagonize TGF-β signaling, a way of self-regulating the pathway. It is generally believed that Smad2 and Smad3 are activated downstream of TGF-β while Smad1, Smad5, and Smad8 are activated downstream of BMP.

Highly studied and relevant in disease is the TGF-β subclass of cytokines. The TGF-β subfamily consists of three members: TGF-β1, TGF-β2, and TGF-β3, encoded by three different genes. The ligand-bound type II TGF-β receptor (TβRII) recruits and activates the type I receptor (TβRI) through phosphorylation of TβRI GS domain. This leads to phosphorylation of Smad2 and Smad3 by TβRI and TGF-β-mediated transcriptional responses (Heldin and Moustakas 2016; Morikawa et al. 2016). While TGF-β1 and TGF-β3 have high affinity for TβRII, TGF-β2 has much lower affinity for TβRII. Instead, it is found to have preference for the type III TGF-β receptor, betaglycan (López-Casillas et al. 1993; Radaev et al. 2010)

1.3 Non-Smad Activation by TGF-β

TGF-β can activate pathways without direct involvement of Smads. These pathways include the extracellular-signal regulated kinase (ERK) mitogen-activated protein kinase (MAPK), p38 MAPK, c-Jun N-terminal kinase (JNK), Rho-like small GTPase pathways, and phosphatidylinositol-3-kinase (PI3K)-Akt pathways (Derynck and Zhang 2003; Moustakas and Heldin 2005; Zhang 2016). In turn, these pathways can regulate Smad signaling in various ways.

TGF-β induces p38 MAPK and JNK activation through TGF-β-activated kinase 1 (TAK1) and the E3 ligase TRAF6 (Sorrentino et al. 2008; Yamashita et al. 2008). TRAF6 associates with the TGF-β receptors and subsequently recruits TAK1. TAK1 is a MAPKKK that activates MKK3/6 and MKK4 upstream of p38 MAPK and JNK, respectively. Activation of p38 MAPK by TGF-β was found to be needed for TGF-β-induced EMT (Bakin et al. 2002). Phosphorylation of TβRII by Src was found to be necessary for p38 MAPK in cancer progression (Galliher and Schiemann 2007). Additionally, the transcriptional activity of the Smad pathway was shown to regulate p38 MAPK through Smad-dependent expression of GADD45 (Takekawa et al. 2002).

Studies show that TGF-β-mediated activation of Erk MAPK is needed for TGF-β-induced EMT (Xie et al. 2004). In another study, ShcA was found to be recruited and phosphorylated by activated TβRI, leading to recruitment of Grb2 and Sos, and subsequent activation of Ras leading to activation of Erk MAPK (Lee et al. 2007).

PI3K-Akt is another non-Smad pathway activated by TGF-β and is involved in EMT (Bakin et al. 2000; Wilkes et al. 2005). Akt phosphorylation can be induced by TGF-β, and the kinase activity of TβRI was shown to be required for Akt phosphorylation (Bakin et al. 2000; Lamouille and Derynck 2007). Additionally, Akt has been shown to regulate TGF-β signaling by sequestering Smad3 in the cytosol, inhibiting the induction of apoptosis and cell cycle arrest by TGF-β (Conery et al. 2004; Remy et al. 2004).

1.4 PI3K-Akt Signaling

PI3Ks are enzymes that phosphorylate phosphoinositide lipids at the 3'-hydroxyl group in the inositol ring (Whitman et al. 1988; Vanhaesebroeck et al. 1997; Engelman et al. 2006). The lipid generated by PI3Ks acts to recruit downstream effector proteins which regulate various cellular processes including cell survival, growth, proliferation, and migration (Katso et al. 2001; Vivanco and Sawyers 2002). There are three known classes of PI3K, Class I, II, and III, grouped by their structure and function (Engelman et al. 2006; Vanhaesebroeck et al. 2010). Of these, the Class I PI3Ks, and in particular, the Class IA PI3Ks, are most studied and have predominant roles in cancer (Yuan and Cantley 2008; Engelman 2009; Mayer and Arteaga 2016).

Class I PI3Ks, holoenzymes consisting of a regulatory subunit and a catalytic subunit, are further divided into Class IA and Class IB. The regulatory subunit can be activated by RTKs, GPCRs, or adaptor proteins while the catalytic subunit catalyzes the conversion of $PI(4,5)P_2$ (PIP2) to $PI(3,4,5)P_3$ (PIP3). The Class IB PI3K consists of a p110γ catalytic subunit that binds a p101 or p84/p87 regulatory subunit. Catalytic subunits of Class IA PI3K include p110α, p110β, and p110δ, encoded by their three respective genes. Regulatory subunits in the Class IA PI3K group include p85α, p85β, and p55γ, also encoded by three different genes, with p55α and p50α being splice variants of p85α [Figure 2A] (Engelman et al. 2006; Stephens and Hawkins 2011). p110γ has limited expression and is mainly found in leukocytes, heart, liver, skeletal muscle, and the pancreas (Vanhaesebroeck et al. 2010). p110α and p110β are ubiquitously expressed, but p110δ expression is largely limited to the immune system (Chantry et al. 1997).

The Class I PI3K catalytic subunits are structurally similar, with an N-terminal adaptor binding domain (ABD) that binds p85, a Ras binding domain (RBD), a C2 domain, a helical domain, and

a kinase domain. The regulatory subunits of Class IA have a p110 binding domain (termed the iSH2 domain) flanked by Src-homology 2 (SH2) domains which bind phosphorylated tyrosines in the pYXXM motif (Songyang et al. 1993; Songyang et al. 1994). The longer p85 regulatory protein also contains a SH3 domain which typically binds proline-rich sequences, and a BCR-homology (BH) that is also seen as a Ras-binding domain [Figure 2B]. The catalytic subunit is held inactive by the regulatory unit, and it is through binding of the regulatory subunit to phospho-tyrosine (pY) of activated receptors or adaptor proteins that a conformational change allows for activity of the catalytic unit (Yu et al. 1998a; Yu et al. 1998b).

PI3K phosphorylation of PIP2 to PIP3 provides a docking site for pleckstrin homology (PH)-Phox homology (PX)-, and FYVE-containing protein (Franke et al. 1995; Vanhaesebroeck et al. 2012). A direct downstream effector of PI3K is the serine/threonine protein kinase B (PKB), also known as Akt, as well as a kinase of Akt, PDK1 (3-phosphoinositide-dependent protein kinase 1), both of which harbor PH domains and are recruited to PIP3 (Franke et al. 1995). Localization of Akt at the cell membrane allows for its subsequent phosphorylation and activation by PDK1 and mTOR complex 2 at Threonine 308 and Serine 473, respectively (Alessi et al. 1997; Stokoe et al. 1997; Sarbassov et al. 2005). Regulation of this pathway include dephosphorylation of PIP3 by phosphatase and tensin homolog (PTEN) (Maehama and Dixon 1998). Figure 3 illustrates activation of this signaling pathway by cell surface RTKs.

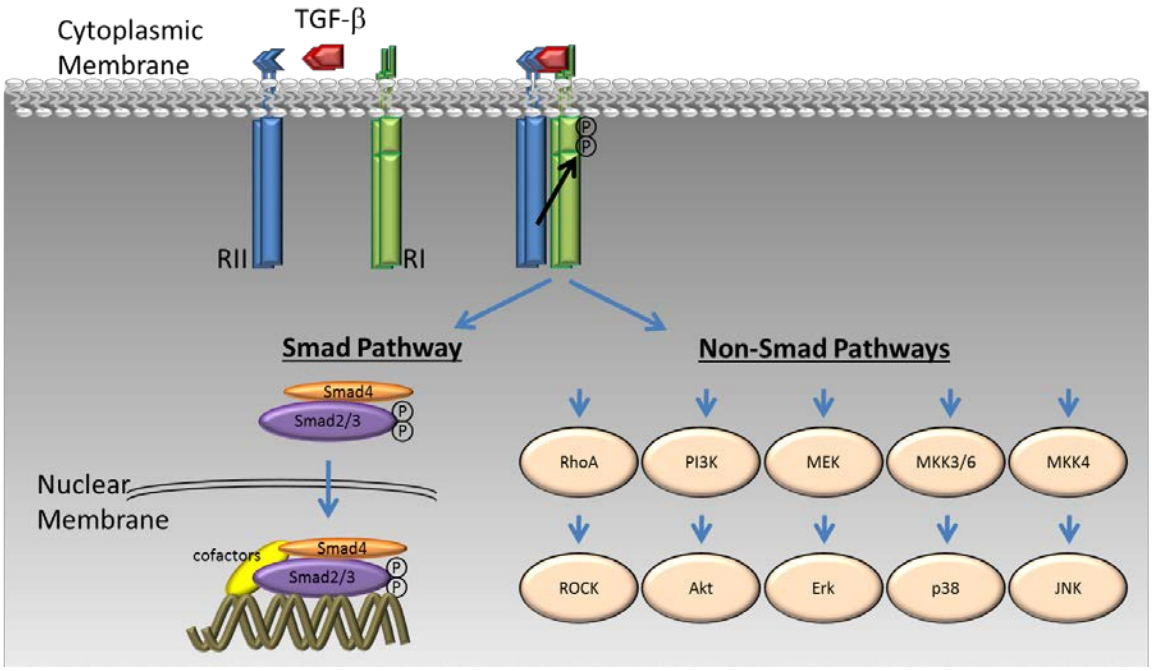

Figure 1. TGF-β Signaling TGF-β binds to cell surface receptors leading to downstream activation of the Smad signaling pathway, which regulates gene transcription, and non-Smad signaling pathways, which are usually activated downstream of RTKs and are implicated in EMT.

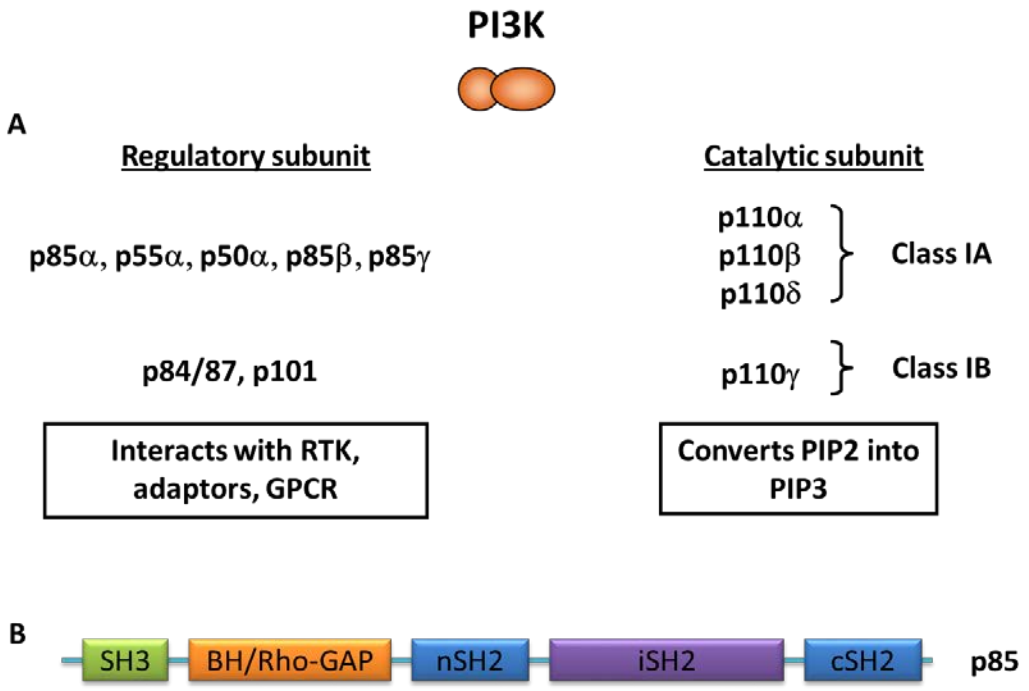

Figure 2. Class I PI3K Class I PI3 kinases are heterodimers consisting of a regulatory subunit that binds RTKs, adaptors, or GPCRs and a catalytic subunit that phosphorylates PIP2 (A). The general structure of p85 is also diagramed here, picture not to scale (B).

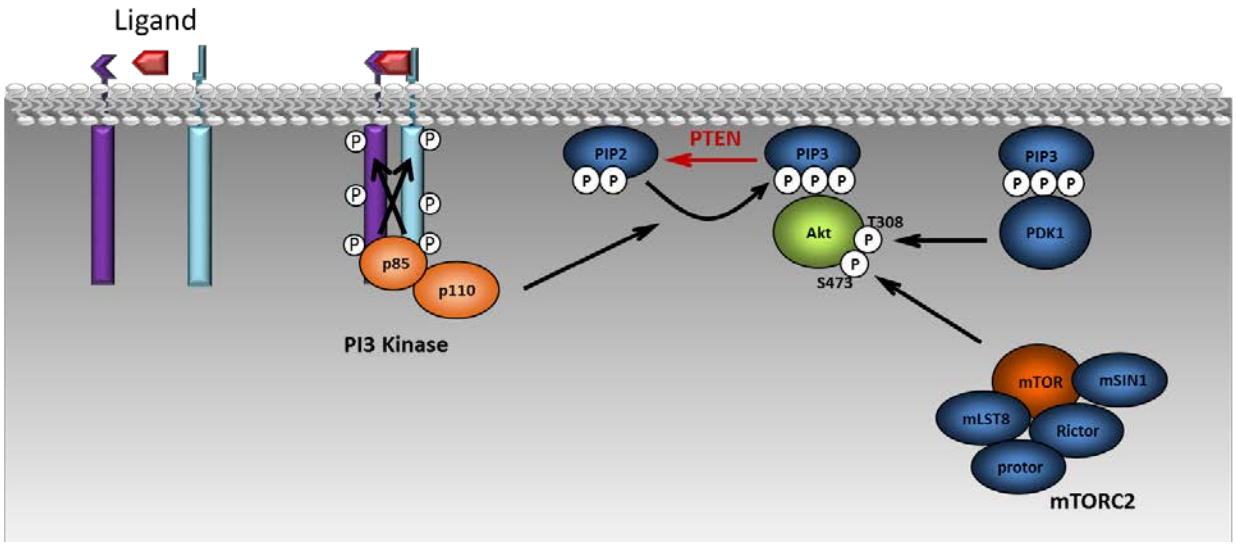

Figure 3. RTK Activation of PI3K-Akt, a Simplified Illustration Activated RTKs recruit PI3K, either directly or indirectly, to the plasma membrane. Binding to phosphorylated tyrosines in the pYXXM motif by the SH2 domains of the p85 regulatory subunit allows for released inhibition of the p110 catalytic subnit and conversion of PIP2 to PIP3, leading to subsequent recruitment and phosphrylation of Akt.

CHAPTER 2: TGF-β-Induced Akt Phosphorylation and EMT

It has been seen many times that TGF-β can induce Akt phosphorylation in various epithelial cells, although to a lower degree than that induced by RTKs, for example the insulin receptor kinase. TGF-β-induced Akt activation, in turn, activates downstream effectors, including mTOR, resulting in increased protein translation (Lamouille and Derynck 2007).

We use the NMuMG murine mammary epithelial cell line to demonstrate the induction of Akt phosphorylation by TGF-β. TGF-β and PI3K-Akt signaling has been found to be misregulated in breast cancers, and NMuMG is a well-established line for TGF-β-induced Akt phosphorylation and EMT. TGF-β-induced Akt phosphorylation on Serine 473 and Threonine 308 is apparent by 30 minutes post TGF-β stimulation [Figure 4A]. Akt phosphorylation can be blocked by the pan PI3K inhibitor GDC-0941 [Figure 4B], indicating a role for PI3K upstream of Akt activation in TGF-β-induced PI3K-Akt signaling.

TGF-β is well-known to be an inducer of EMT, and TGF-β-induced EMT is used as a model for growth factor-induced EMT studies. EMT is characterized by key events including (1) dissolution of epithelial cell-cell junctions, loss of apical-basal polarity, and acquisition of front-rear polarity, (2) cytoskeletal reorganization with increased cell motility, and (3) gene reprogramming that results in activation of EMT transcription factors such as Snail1 or Snail2/Slug, ZEB1 or ZEB2, and Twist (De Craene and Berx 2013; Lamouille et al. 2014). EMT is also accompanied by a cadherin switch from epithelial (E) to neuronal (N) cadherin and an increase in metalloprotease expression, leading to ECM degradation and invasive cell behavior.

As illustrated in Figure 5, TGF-β-induced EMT results in rearrangement of actin, induction of fibronectin, and loss of E-cadherin at cell junctions. TGF-β-induced-EMT can be inhibited by blocking Akt phosphorylation (Lamouille and Derynck 2007; Lamouille et al. 2012; Serrano et al. 2013; Jo et al. 2015).

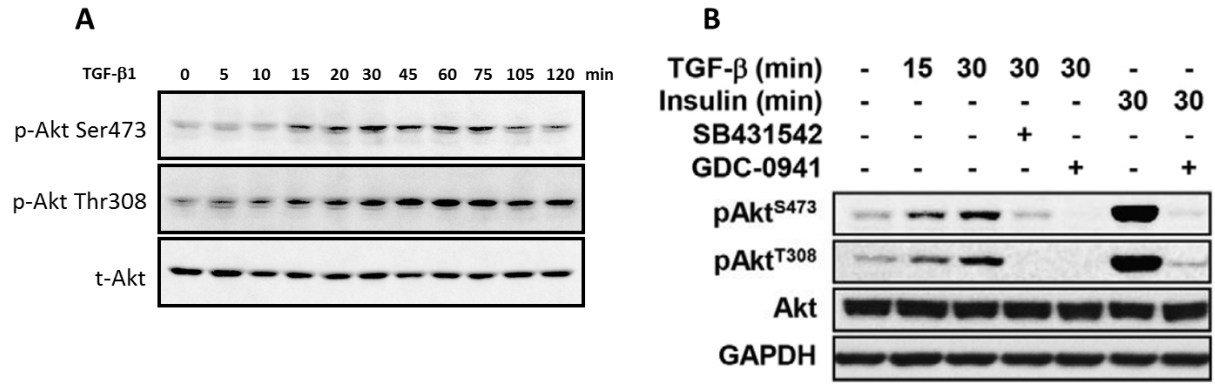

Figure 4. TGF-β-Induced Akt phosphorylation Stimulation of NMuMG cells with TGF-β1 induces Akt phosphorylation, apparent by 30 minutes (A), and this phosphorylation is blocked by the PI3K inhibitor GDC-0941 (B).

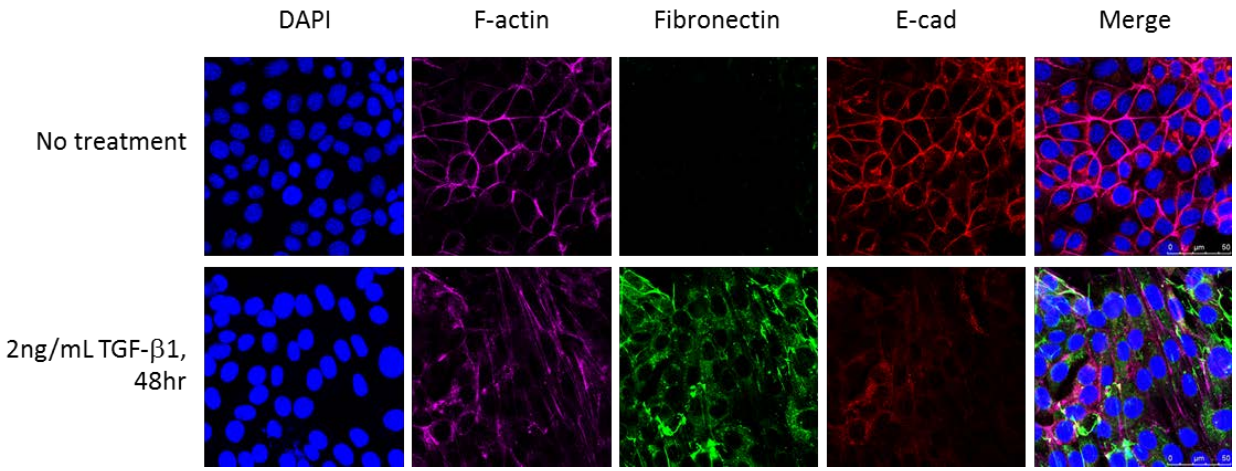

Figure 5. TGF-β-Induced EMT as Illustrated by Immunofluorescence Staining of EMT Markers TGF-β is a potent inducer of EMT. Immunofluorescence shows the rearrangement of actin, induction of fibronectin, and loss of E-cadherin as NMuMG cells undergo EMT.

CHAPTER 3: Specific PI3K Isoforms Needed for TGF-β-Induced Akt Activation and EMT

At this time, it is well established that the Class I PI3K activity upstream of Akt is responsible for TGF-β-induced Akt activation. There are various isoforms of Class I PI3K, and we wanted to determine which isoform(s) might be dominant in mediating TGF-β-induced Akt activation.

3.1 Chemical Inhibition of PI3K p110α Inhibits TGF-β-Induced Akt Activation and EMT

To clarify which PI3K catalytic subunit may be responsible for TGF-β-induced Akt phosphorylation, we recently obtained published p110 isoform-specific kinase inhibitors from the Shokat Lab at UCSF (Knight et al. 2006). NMuMG cells were serum starved and pre-treated with increasing concentrations of inhibitor, then Akt phosphorylation was induced with TGF-β. The Class 1B p110γ inhibitor did not seem to have an effect, whereas the Class 1A p110α inhibitor blocked TGF-β-induced Akt phosphorylation in a dose-dependent manner [Figure 6A]. Furthermore, inhibition of pl10β and p110δ largely did not block Akt activation, but inhibition of p110α obviously inhibited Akt phosphorylation in different epithelial cell lines including NMuMG, HaCaT, an aneuploid human keratinocyte cell line, and murine E4 squamous carcinoma cells derived from a lymph node metastasis of a skin carcinoma [Figure 6B].

3.2 Knockdown of PI3K p110α Expression Inhibits TGF-β-Induced Akt Activation and EMT

To further affirm that p110α is responsible for TGF-β-induced Akt phosphorylation, we used small interfering RNAs (siRNAs) to downregulate p110α expression. NMuMG cells were transiently transfected with a panel of siRNAs targeting p110α mRNA. We find that

phosphorylation of Akt at S473 and T308 were both attenuated with decreased p110α expression without significant loss of p85α [Figure 7].

We elected to use the fourth siRNA (si#4) from our panel to study the role of p110α in TGF-β-induced EMT. NMuMG cells were transfected with p110α siRNAs, then treated with TGF-β for 48 hours. We found that cells transfected with the p110α siRNA are attenuated in TGF-β-induced EMT as shown by phase-contrast microscopy [Figure 8A]. Immunofluorescence staining of EMT markers F-actin, E-cadherin, and fibronectin also illustrate the attenuation of EMT upon knockdown of p110α expression [Figure 8B]. Furthermore, inhibition of p110α by the p110α-specific kinase inhibitor resulted in a decrease in the mRNA level of an EMT transcription factor induced by TGF-β, *Snail*, although not to the extent of the TβRI kinase inhibitor SB431542, speaking to the roles of other TGF-β-activated signaling pathways involved in *Snail* regulation and EMT, namely Smads [Figure 8C]. Additionally, the pan Class I PI3K inhibitor only slightly further decreased *Snail* expression compared to inhibition of p110α alone, indicating that p110α, as opposed to other PI3 kinases, is a major contributor of TGF-β-induced *Snail* expression.

EMT in cancer is associated with increased cell migration and invasion, so we assessed the role of p110α in TGF-β-induced cell migration. Using a trans-well migration assay with fetal bovine serum (FBS) as an attractant. Cells pre-treated with TGF-β showed increased migration whereas cells treated with TGF-β in the presence of the p110α inhibitor are attenuated in their ability to migrate [Figure 9].

3.3 Knockdown of PI3K p85α Expression Inhibits TGF-β-Induced Akt Activation

The regulatory subunit largely known to be in complex with p110α is p85α. To determine if p85α is responsible for Akt activation, we used siRNAs targeting p85α. We found that knocking down p85α expression does indeed inhibit TGF-β-induced Akt phosphorylation [Figure 10]. However, TGF-β-induced EMT is not inhibited with decreased p85α expression [Figure 11], which can be expected since silencing of p85α expression by siRNA is incomplete and there are still active p110α subunits that can associate with residual p85α or other regulatory subunits.

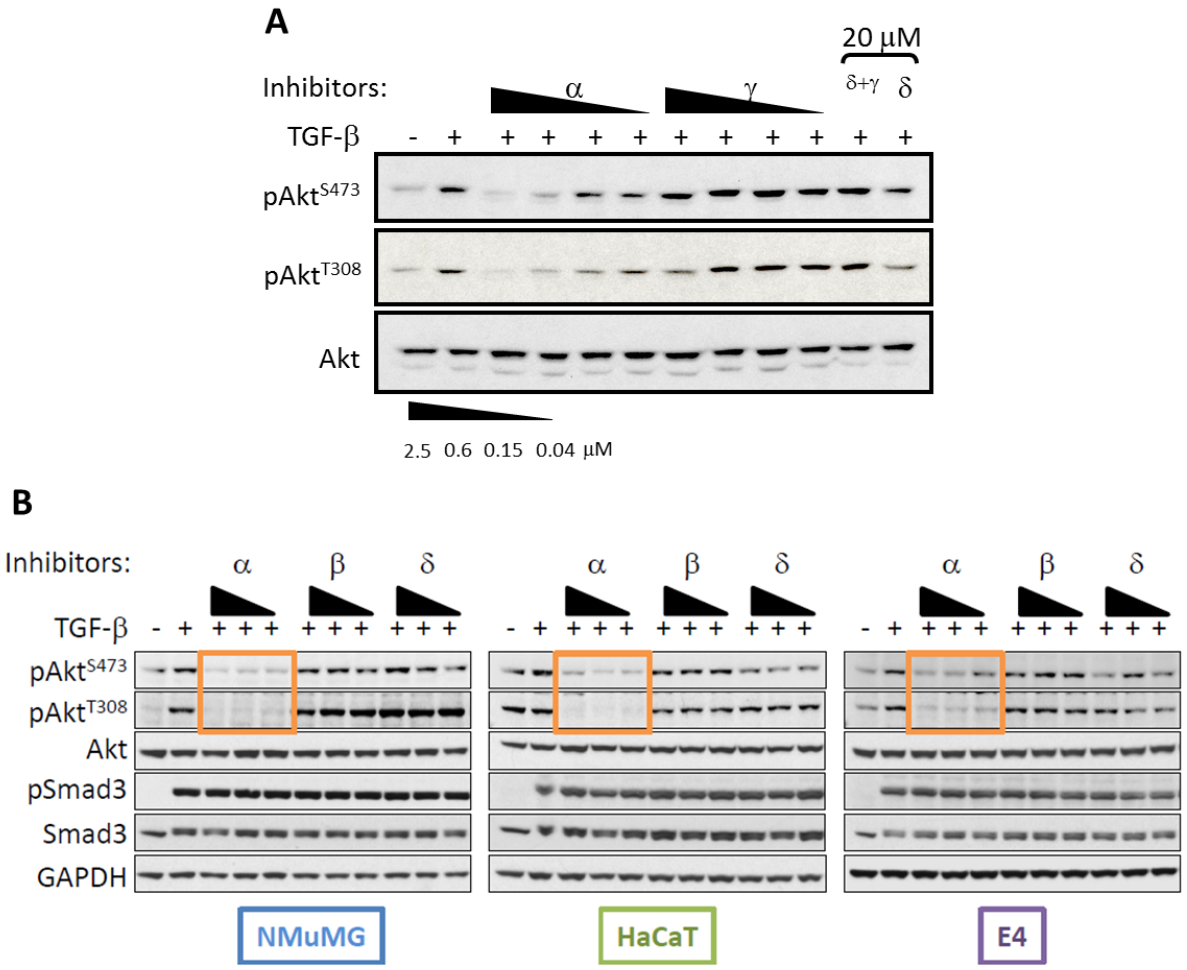

Figure 6. PI3K p110α is Responsible for TGF-β-Induced Akt Phosphorylation Use of Class I PI3K inhibitors show that chemical inhibition of Class 1B PI3K does not inhibit TGF-β-induced Akt phosphorylation (A) and inhibition of the specific Class 1A PI3K, p110α, attenuates TGF-β-induced Akt phosphorylation in various epithelial cell lines (B).

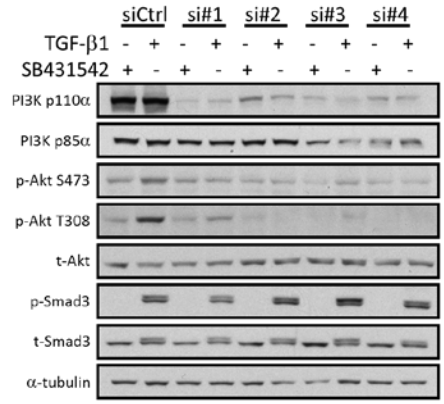

Figure 7. Knockdown of p110α Expression Attenuates TGF-β-Induced Akt Phosphorylation siRNA targeting p110α shows attenuation of TGF-β-induced Akt phosphorylation in NMuMG cells.

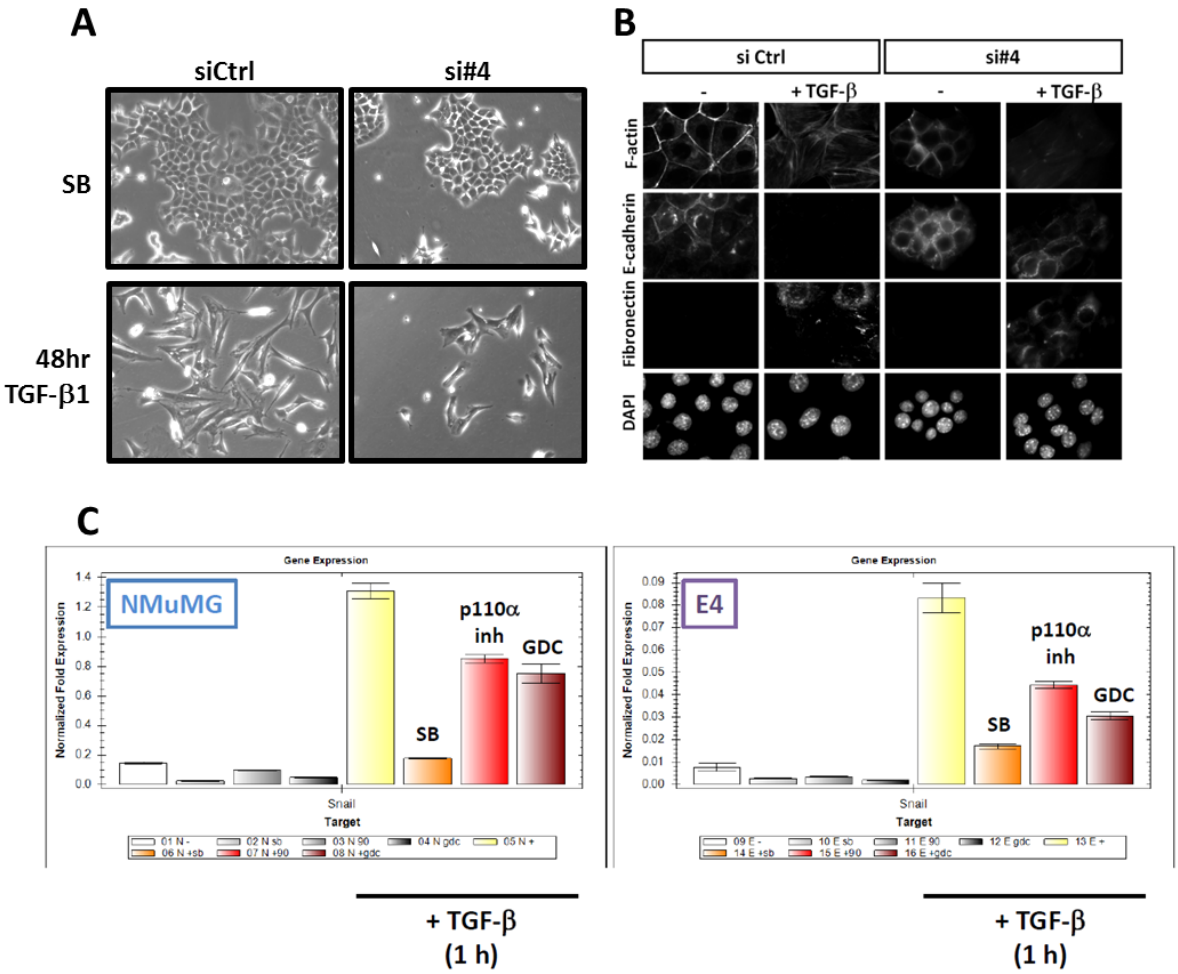

Figure 8. Knockdown of p110α Expression Attenuates TGF-β-Induced EMT siRNA knockdown of p110α expression shows attenuation of TGF-β-induced EMT as observed by phase-contrast (A) and fluorescence (B) microscopy. Chemical inhibition of p110α also reduces TGF-β-induced *Snail* mRNA, and the pan Class I PI3K inhibitor GDC-0941 only slightly further decreases this (C).

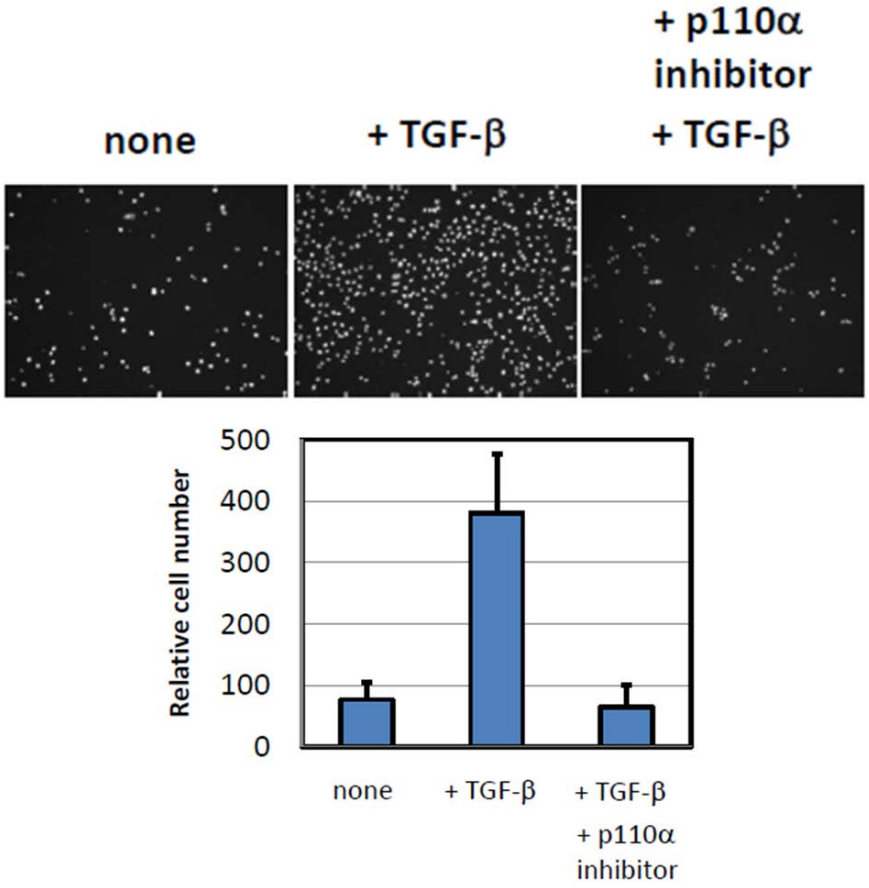

Figure 9. Inhibition of p110α Inhibits TGF-β-Induced Cell Migration Chemical inhibition of p110α shows decreased TGF-β-induced cell migration.

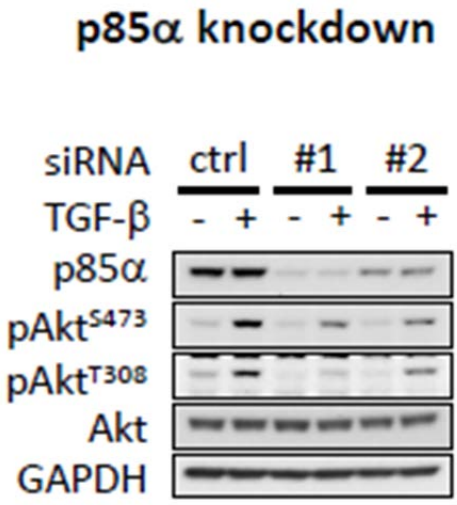

Figure 10. Knockdown of p85α Expression Attenuates TGF-β-Induced Akt Phosphorylation siRNA targeting p85α attenuates TGF-β-induced Akt phosphorylation in NMuMG cells.

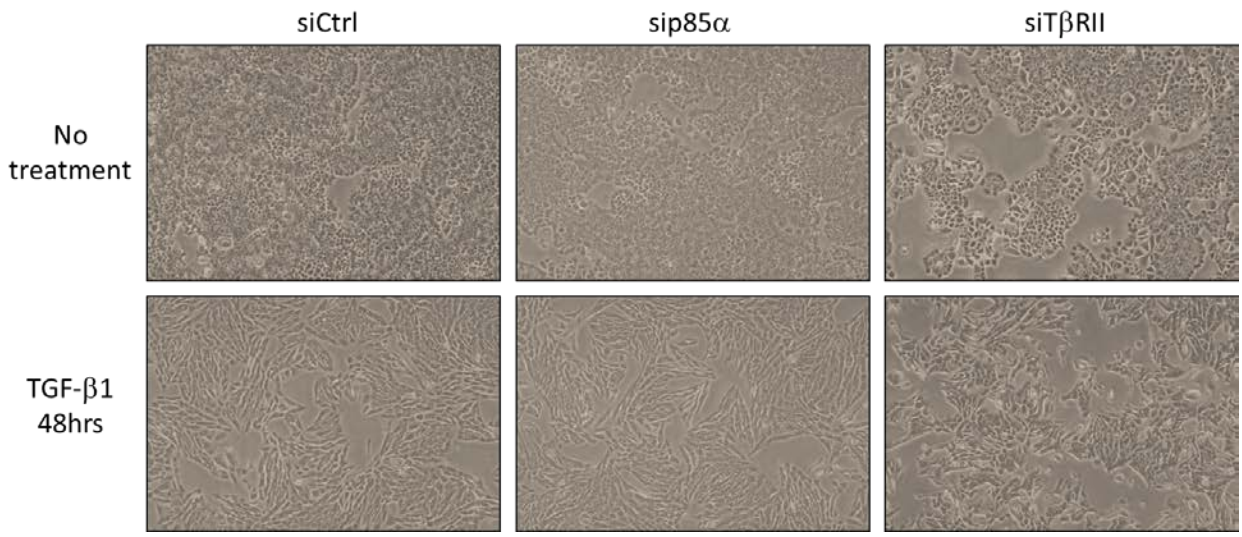

Figure 11. TGF-β-Induced EMT in p85α or TβRII Knockdown Cells NMuMG cells with knockdown of p85α expression are still able to undergo TGF-β-induced EMT whereas cells with decreased TβRII expression are attenuated.

CHAPTER 4: PI3K p85α Associates with TβRII

4.1 PI3K p85α Constitutively Interacts with TβRII when Overexpressed in 293T Cells

Others have shown that TGF-β receptors are able to interact with PI3K, with the interaction between TβRI and p85α being ligand-inducible (Yi et al. 2005). Similarly, we find constitutive association of p85α with TβRII when co-expressed in 293T cells [Figure 12A]. This interaction does not seem to significantly change upon TGF-β stimulation or with inhibition of the TβRI kinase. However, we do not find a ligand-induced TβRI-p85α interaction [Figure 12A] as seen by Yi et al. This could be a limitation of efficiency of the cells to make p85α protein when co-expressed with TβRI, as there seems to be lower levels of p85α protein expression when TβRI is co-expressed as opposed to when TβRII is co-expressed [Figure 12B].

4.2 Endogenous Interaction of PI3K p85α and TβRII in NMuMG Cells

While the TβRII-p85α interaction can be easily detected when overexpressed in 293T cells, the endogenous interaction proved more difficult to determine. It was clear that a single confluent 15-centimeter dish of NMuMG cells did not provide sufficient amounts of protein for detection of receptor-p85α interaction, particularly if it is a low-level interaction. Multiple attempts have been made in scaling up the amount of protein for endogenous co-immunoprecipitation (co-IP) of PI3K and TGF-β receptors. Preliminary results suggest that the endogenous TβRII-p85α interaction may also be constitutive since stimulation with TGF-β for a short period (5 minutes) or longer period (45 minutes) or inhibition of TβRI activity did not alter apparent TβRII-p85α interaction compared to untreated cells [Figure 13]. However, this observation remains largely inconclusive for various reasons as discussed Chapter 7.

4.3 The cSH2 and iSH2 Subdomains of PI3K p85α are Needed for Interaction with TβRII

What is clear thus far is that p85α associates with TβRII when both components are overexpressed. We moved on to ask how exactly p85α interacts with TβRII. p85α is a multi-domain protein that has two SH2 pY binding domains (nSH2 and cSH2) separated by an iSH2 domain that is need for interaction with the catalytic subunit, a SH3 domain, and a BH/Ras-binding domain. We proceeded to determine what region(s) of p85α is/are necessary for interaction with the receptor. We obtained a N-terminal-truncated mouse p85α construct (termed PI3K p85α ΔN) that contains the six amino acids upstream of the nSH2 and the remaining C-terminus of p85α from the Barber Lab (UCSF). This is essentially the p50α isoform of p85α. Initial results revealed that this C-terminus of p85α is sufficient for interaction with TβRII.

It has been observed that a conserved arginine in the SH2 domain coordinates pY binding (Mayer et al. 1992; Bibbins et al. 1993). This arginine corresponds to R358 and R649 in the nSH2 and cSH2 domains, respectively, of p85α. Using the PI3K p85α ΔN construct, as well as a cSH2-mutated construct (PI3K p85α ΔN R649A, also provided by the Barber Lab), we created several other p85α mutants to interrogate the region of p85α necessary for TβRII interaction [Figure 14]. Co-expression of these mutants with TβRII shows efficient and comparable expression of the p85α mutants [Figure 15A]. Co-IP data suggest that the cSH2 and the iSH2 are needed for association with the receptor, since complete loss of the cSH2 abrogates receptor binding, but the domain alone is insufficient for binding [Figure 15B]. *In vitro* interaction assays with the PDGFR-β pY751 peptide confirmed the loss of pY binding ability of the R358,649A

double mutant while single SH2 mutants and single SH2 domain constructs can still bind phosphorylated PDGFR (data not shown).

4.4 TβRII Tyrosine Residues are Needed for Efficient Interaction with PI3K p85α

TGF-β receptors have been grouped in the class of dual-specificity kinases, with the ability to phosphorylate serine and threonine as well as tyrosine (Hanks and Hunter 1995). While the Ser/Thr kinase ability has been explored, less is known about its role as a tyrosine kinase. A feature of RTKs is its ability to autophosphorylate (Hubbard and Miller 2007). It has been found that TβRII can autophosphorylate at tyrosines (Lawler et al. 1997). And while TβRI is found to be tyrosine-phosphorylated, its ability to autophosphorylate is less clear (Lee et al. 2007). Given the role of TGF-β receptors as dual-specificity kinases and the necessity of the p85α cSH2 in binding to TβRII, we wanted to determine if tyrosine phosphorylation of TβRII is needed for binding to p85α.

We first attempted to establish the ability of TGF-β receptors to autophosphorylate. GST fusion proteins containing the cytoplasmic domains of TβRI and TβRII were purified from bacteria and individually subjected to a protein kinase reaction. We are able to detect pY of TβRI and TβRII post kinase reaction which can be abrogated by treatment with Lambda Protein Phosphatase [Figure 16A]. Additionally, Flag-tagged wild-type TβRI purified from 293T cells subjected to a kinase reaction also seem to exhibit autophosphorylation capabilities, with detectable levels of pY that can be blocked by treatment with the TβRI kinase inhibitor [Figure 16B]. Granted, this tyrosine phosphorylation may have resulted from the kinase activity of other molecules that co-

purified with TβRI-Flag, i.e. TβRII. Nevertheless, this shows that the kinase activity of TβRI is needed for its tyrosine phosphorylation.

80% of the cytoplasmic domain of TβRII is its kinase domain. There are 9 tyrosine residues in the cytoplasmic domain, 8 of which are in the kinase domain. Not one of the 9 tyrosine residues on TβRII contain the pYXXM binding motif that is specific for p85α SH2 binding. There have been reports of tyrosine phosphorylation of TβRII on Y259, Y284, Y336, Y424, and Y470 (Lawler et al. 1997; Galliher and Schiemann 2007), and the phosphorylation of some of these tyrosines have roles in TGF-β-mediated signaling events (Galliher and Schiemann 2007; Chen et al. 2014). Recently, the first crystal structure of TβRII was published (Tebben et al. 2016). Using this crystal structure and the PyMOL molecular modeling software tool, we can see that of the 5 tyrosines in question, three (Y259, Y284, and Y336) look to be accessible to binding by other molecules [Figure 17]. However, one should note that this crystal structure was obtained by necessarily mutating six amino acids near and around Y424 and Y470, so it is possible that these tyrosine residues are exposed as well.

Using site-directed mutagenesis, we mutated the tyrosine residues individually and collectively to phenylalanine. Single tyrosine mutants did not seem to have detrimental effects on TβRII-p85α interaction (data not shown), so we proceeded to work with only multiple tyrosine mutants. We established that TβRII mutated at all five tyrosines (5YF) does not have a dominant negative effect on TGF-β signaling [Figure 18A] and is able to reach the cell surface [Figure 18B]. Co-expression of TβRII tyrosine mutants with wild-type p85α resulted in significantly attenuated

interaction of the TβRII 5YF mutant [Figure 19]. The TβRII Y336,424F also shows a significant loss of interaction.

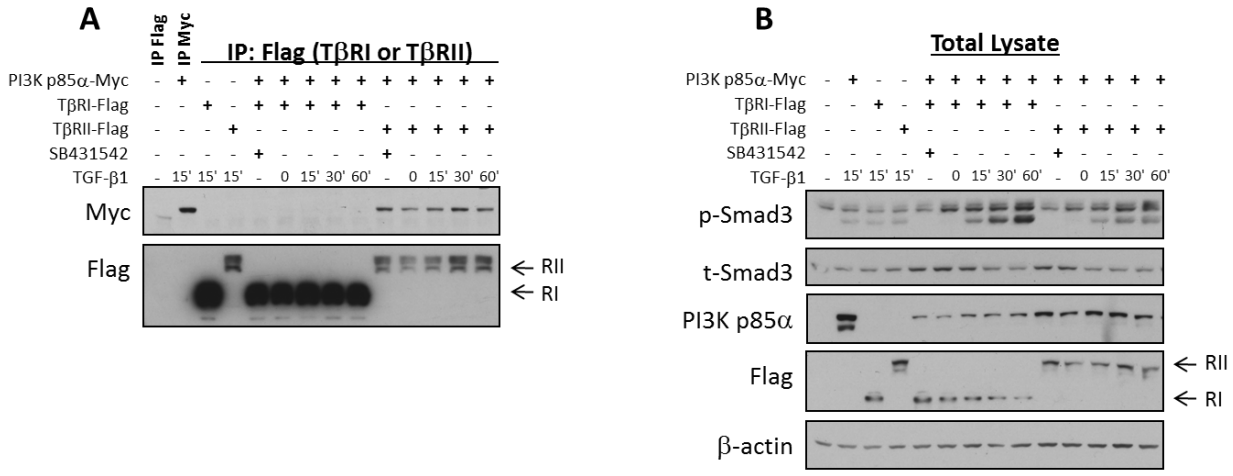

Figure 12. PI3K Constitutively Interacts with TβRII Myc-tagged p85α was co-expressed with wild-type TβRI- or TβRII-Flag in 293T cells. Immunoprecipitation of the receptors show that p85α-Myc constitutively interacts with TβRII (A). Total lysate blot shows all proteins were indeed overexpressed and the TGF-β ligand was active.

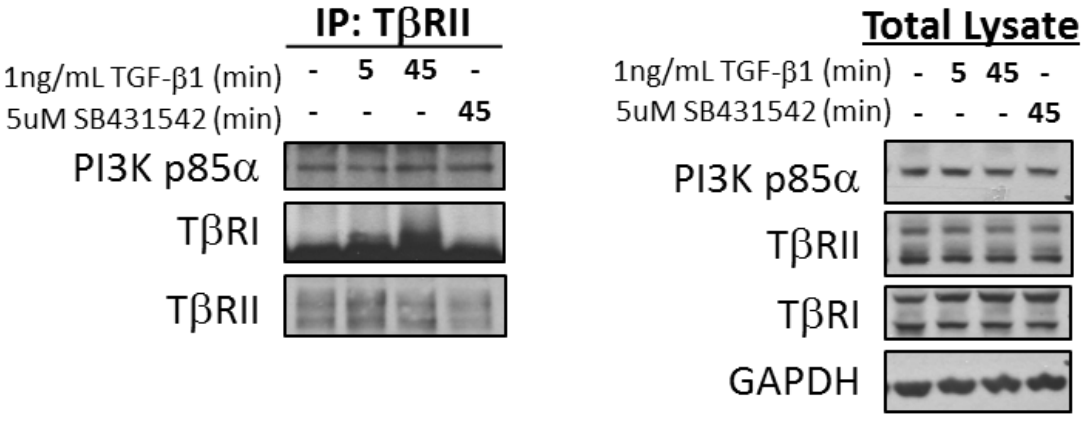

Figure 13. Endogenous TβRII-p85α Interaction Preliminary results suggest that endogenous TβRII-PI3K interaction may to be constitutive and remain unchanged with TGF-β1 or SB431542 treatment.

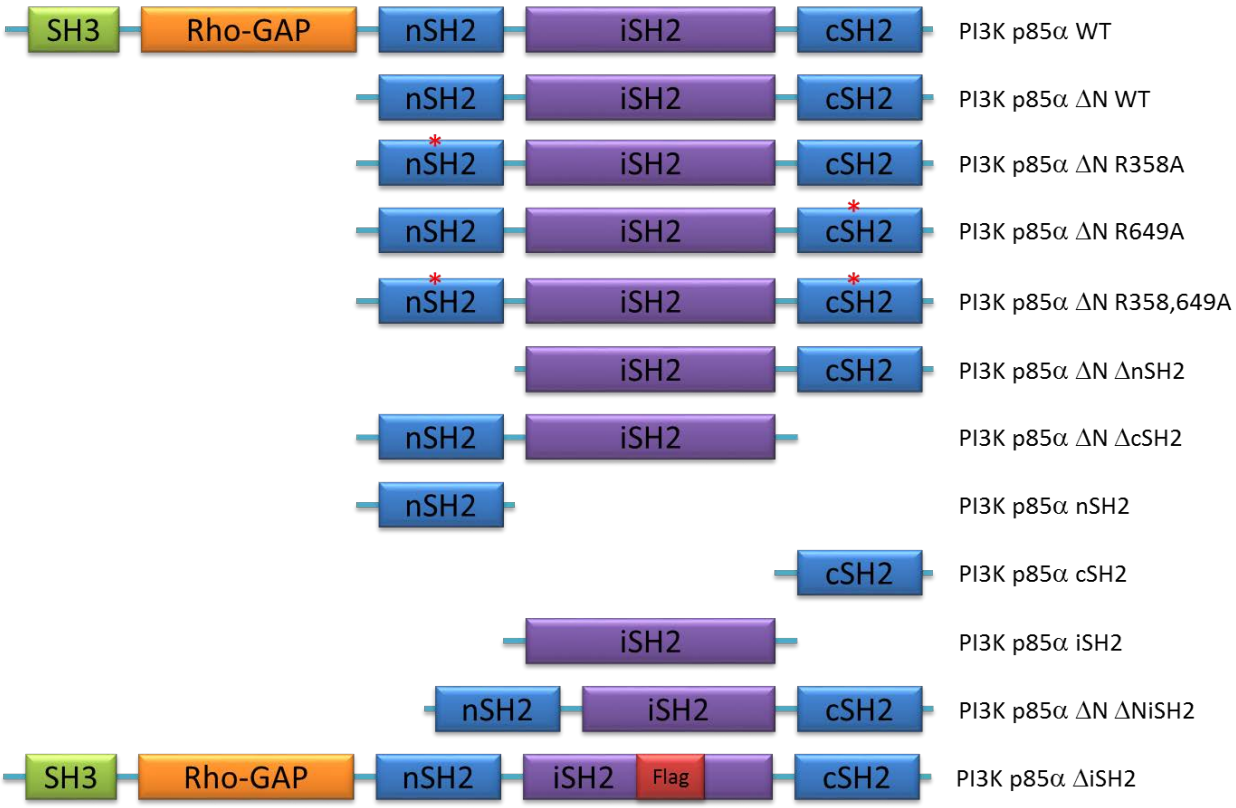

Figure 14. Mutant p85α Constructs Full-length and N-terminal-truncated p85α mutants (PI3K p85α ΔN) were made, some containing mutations in the pY binding pocket (*). Single-domain constructs were also created. Diagram not to scale.

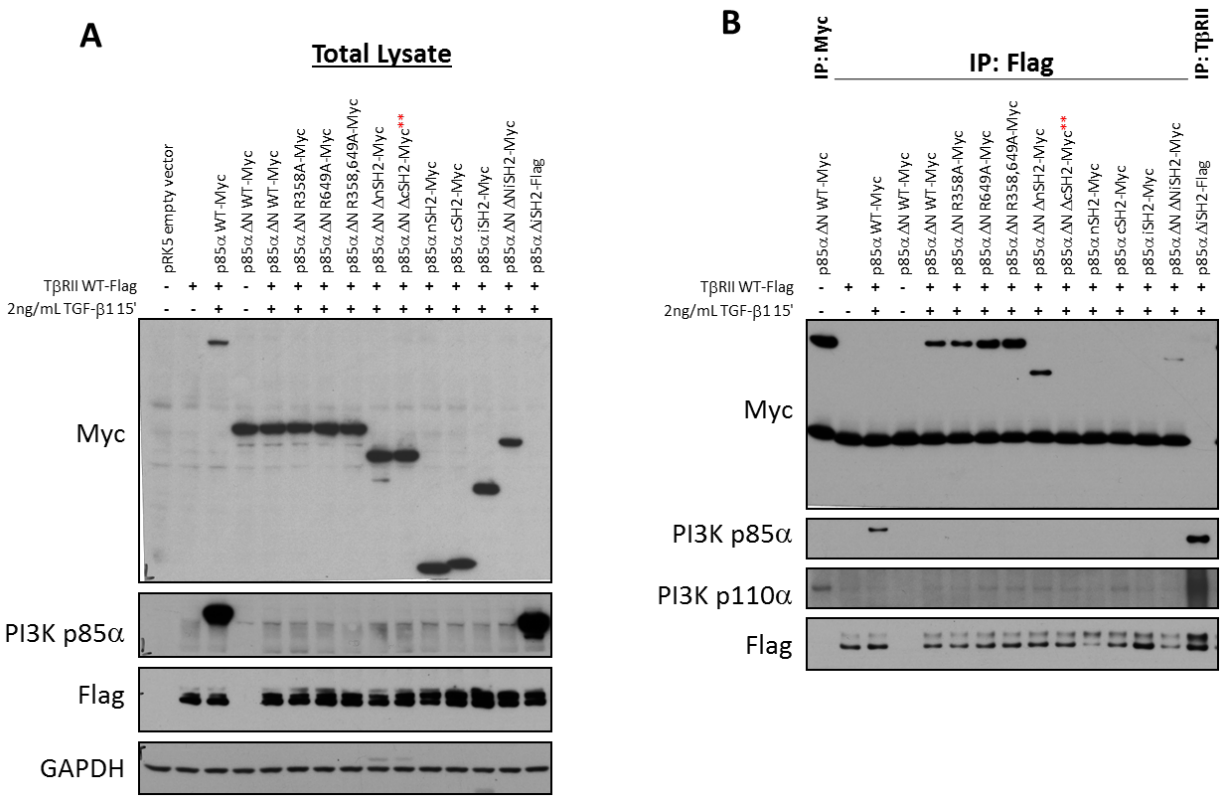

Figure 15. Interaction of Various p85α Constructs with TβRII Myc- or Flag-tagged p85α wild-type and mutant constructs were co-expressed with wild-type TβRII. Total lysate show all mutants were satisfactorily expressed (A). Protein samples were immunoprecipitated for TβRII (either with the Flag antibody or with a TβRII-specific antibody in the case of the Flag-tagged p85α construct) and blotted for p85α. Co-IP results show that the cSH2 (**) of p85α is needed for interaction, but this domain alone is not sufficient for interaction with TβRII (B).

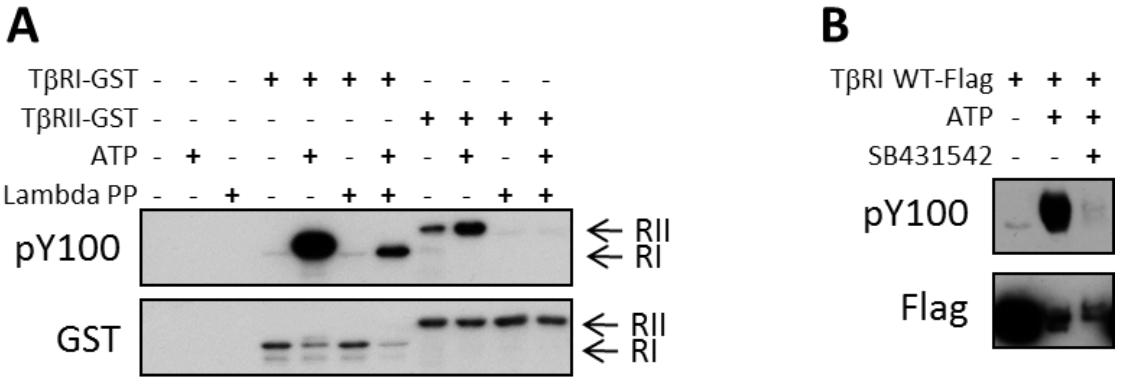

Figure 16. Autophosphorylation of TGF-β Receptors GST- or Flag-tagged receptors were purified from bacteria or transfected 293T cells, respectively, and subjected to a kinase reaction. Data suggest the receptors can autophosphorylate on tyrosines, and in the case of TβRI, this can be blocked by the TβRI kinase inhibitor SB431542.

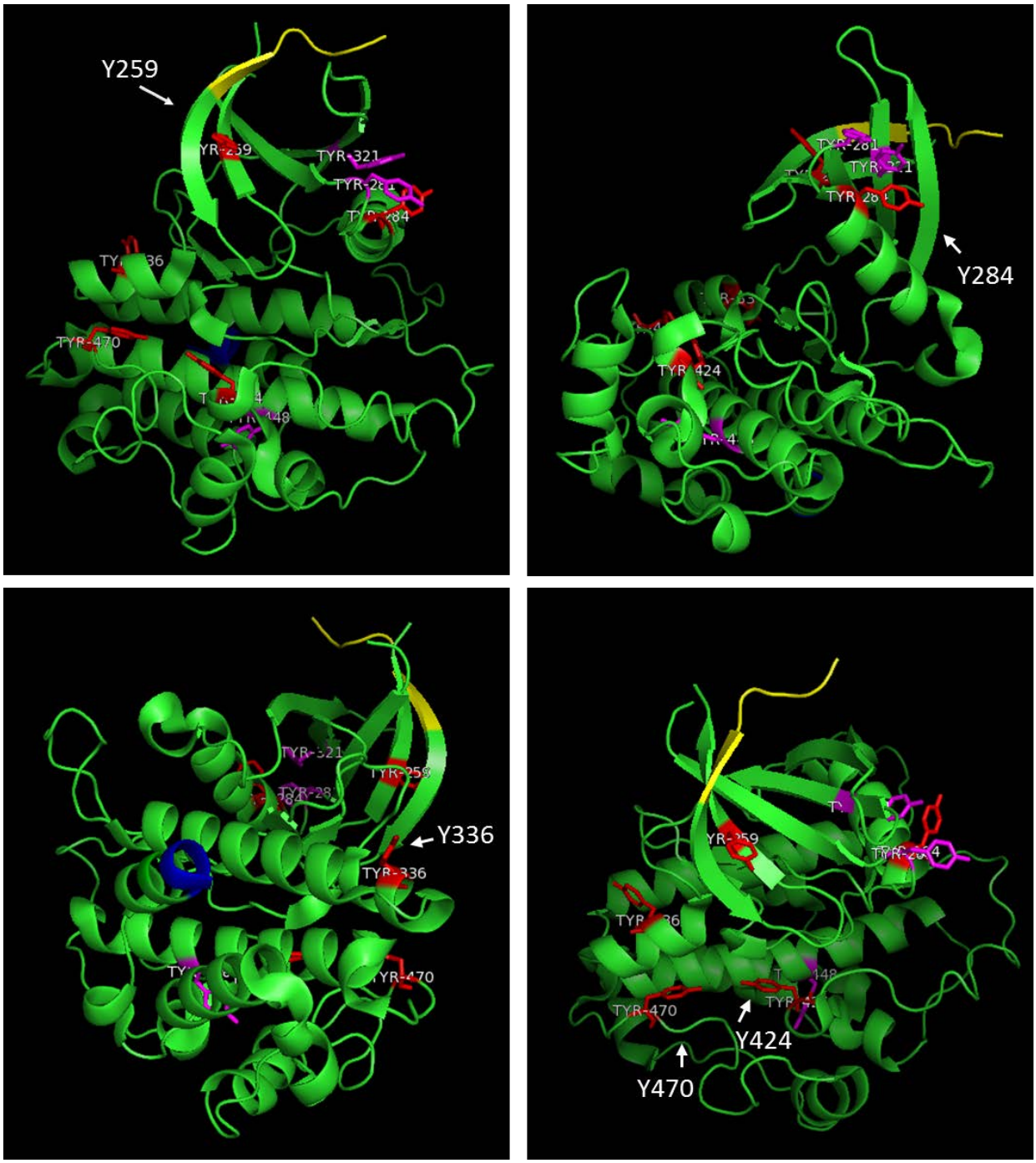

Figure 17. Crystal Structure of the TβRII Kinase Domain Views of the TβRII kinase structure in four different rotations. Tyrosine residues for this study are shown in red and labeled with arrows, remaining tyrosines are shown in magenta. Yellow labels the N-terminal and blue labels the C-terminal ends of the kinase.

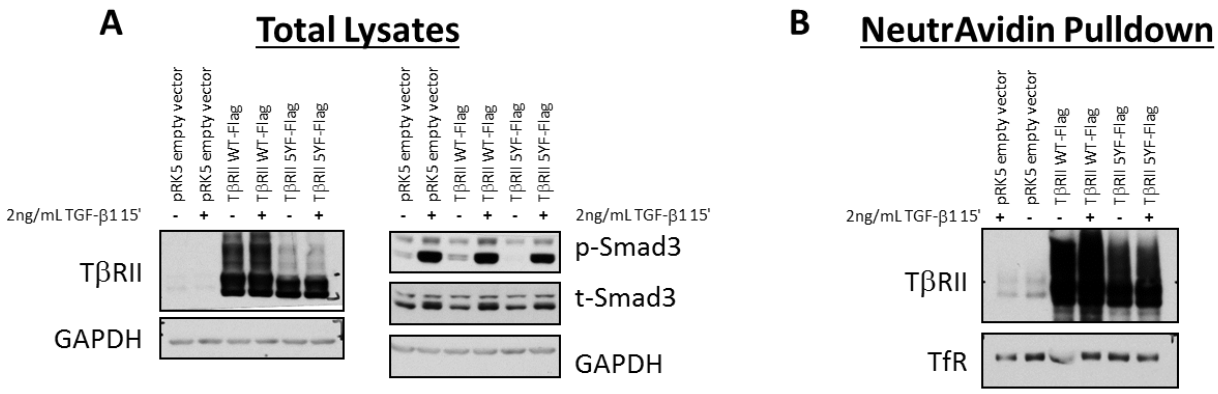

Figure 18. Assessment of the TβRII 5YF Mutant TβRII wild-type and 5YF mutant were transiently transfected in NMuMG cells. The 5YF mutant has noticeably reduced expression as compared to wild-type, but TGF-β-induced Smad3 activation still persists (A), and the mutant is able to express at the cell surface (B).

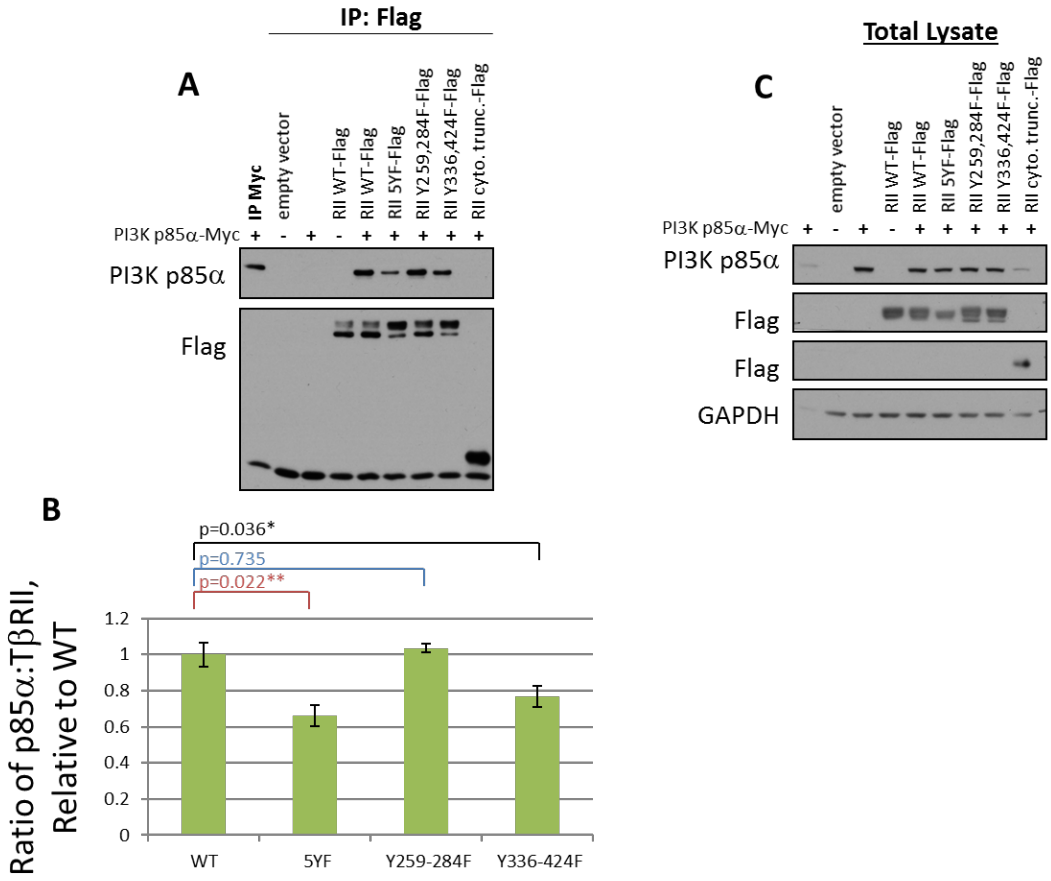

Figure 19. Pulldown of p85α by Mutant TβRII Wild-type, tyrosine-mutated, or cytoplasmically truncated TβRII were co-transfected with wild-type p85α. Samples were immunoprecipitated for TβRII and blotted for p85α (A). The ratio of p85α signal to TβRII signal was quantified using the densitometry and the ImageJ software; TβRII with all 5 tyrosine residues mutated is significantly attenuated in its ability to co-IP p85α (B). Total lysates show expression of over-expressed constructs (C).

CHAPTER 5: Roles for TGF-β Receptor Kinase Activity in PI3K Recruitment and Akt Activation

5.1 Inhibition of TβRI Kinase Inhibits TGF-β-Induced Akt Phosphorylation

Studies in TGF-β signaling regularly involve inhibition of the TβRI kinase activity since it is seen as the receptor by which downstream effectors are predominately activated. Therefore, in TGF-β-induced Akt activation studies, it has been shown many times, and we show here, that inhibition of the TβRI kinase activity results in loss of TGF-β-induced Akt activation. Figure 20 illustrates that inhibition of the TβRI kinase activity by SB431542 results in attenuated TGF-β-induced Akt phosphorylation. However, it does not inhibit activation of Akt downstream of other growth factor signaling, e.g. insulin. It has also been established that SB431542 inhibits TGF-β-induced EMT. In all, we can conclude that the kinase activity of TβRI is needed for TGF-b-induced activation of Akt and EMT.

5.2 Loss of TβRII Kinase Activity in TGF-β-Induced Signaling and TβRII-PI3K Interaction

An inhibitor of the TβRII kinase has not been described, but a kinase-dead TβRII (K277R) mutant has been used in studies (Wrana et al. 1992; Perlman et al. 2001). However, the efficiency of expression of this mutant is low compared to wild-type, and concentrations of 2-3 times that of wild-type is needed for transfection to achieve measurable protein expression. We have to balance this with the fact that transfecting cells with exogenous DNA at high doses is also detrimental to cell health, so we attempted to create a different TβRII kinase-dead mutant that has better expression potential. There are two conserved aspartic acid residues in the

activation loop of kinases that have been described to have important roles in catalysis (Kannan and Neuwald 2005; Kornev et al. 2006). These are known as the “DFG” and “HRD” motifs. However, whether mutations in these motifs have an inhibitory or activating affect is yet to be clearly defined and seems to be kinase-specific (Till et al. 2001; Meyer et al. 2006). TβRII contains both these motifs at D379 (HRD) and at D397 (DFG). We first attempted to mutate D379 in the HRD motif to asparagine.

Analysis of the TβRII D379N mutant shows that the mutant is expressed at comparable levels to wild-type but is slightly attenuated in TGF-β-induced Smad3 activation [Figure 21A]. While the D397N mutant does not have observable pY signal compared to wild-type [Figure 21B], it is still able to associate with p85α [Figure 21C]. The readout in kinase activity in this case is Smad3 activation since it is known that TβRII kinase activity is needed for activation of TβRI leading to Smad phosphorylation.

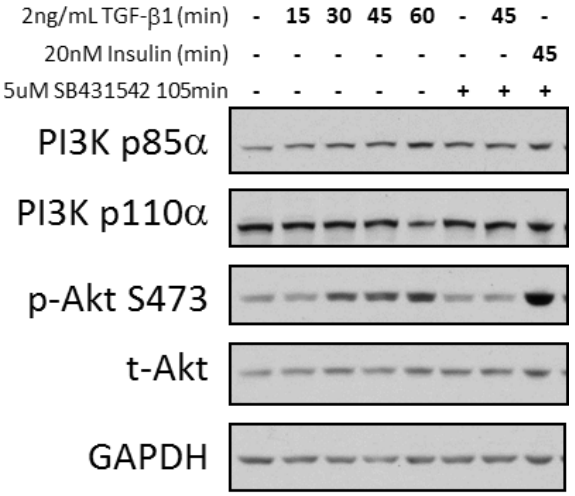

Figure 20. Inhibition the TβRI Kinase Activity on Akt Phosphorylation SB431542 inhibition of the TβRI kinase activity results in attenuation of TGF-β-induced Akt phosphorylation, but not Akt phosphorylation as a result of insulin signaling.

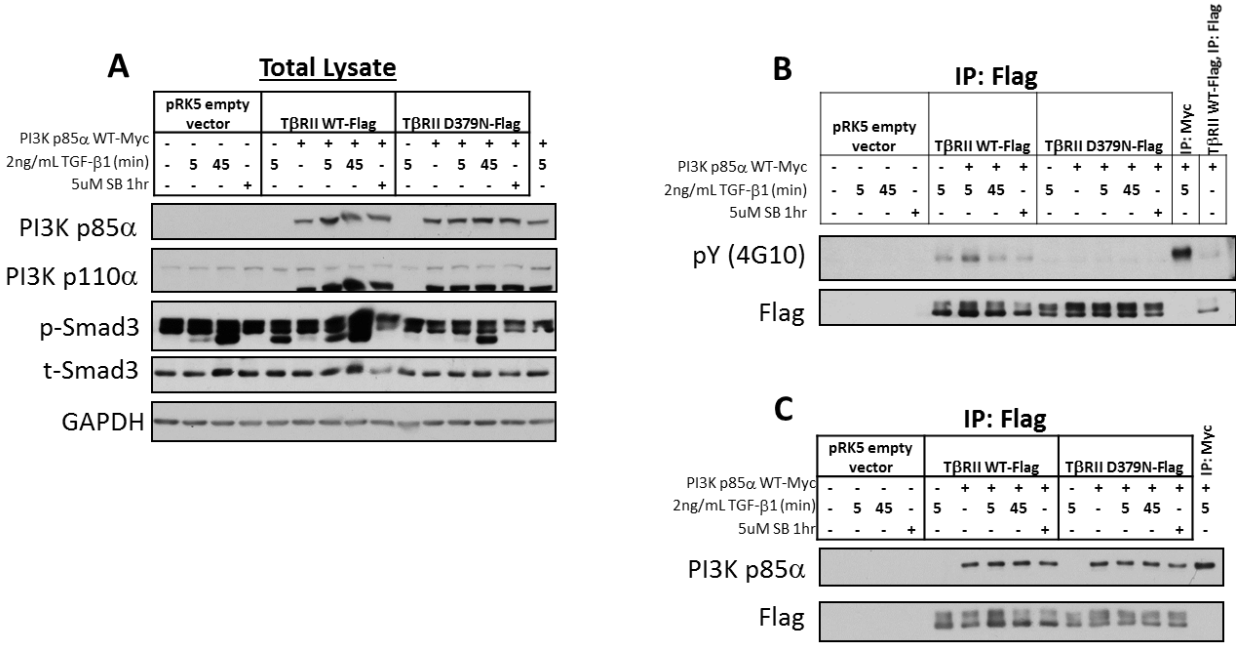

Figure 21. Effect of the TβRII D379N Kinase Mutant The TβRII D379N mutant expresses at comparable levels to wild-type but is slightly attenuated in TGF-β-induced Smad3 activation (A). The D397N mutant does not have observable pY signal compared to wild-type (B), but is still able to associate with p85α (C).

CHAPTER 6: Localization of TGF-β-Regulated PI3K-Akt Signaling

Protein trafficking can dictate the specificity, kinetics, and amplitude of an activated growth factor. Ligand-induced endocytosis of cell surface receptors usually involves post-translation modifications, either phosphorylation or ubiquitination, of the receptor (Bonifacino and Traub 2003; Hicke and Dunn 2003), and increasing evidence suggest that the nature of the signaling responses, e.g. which pathways are activated by a particular ligand/receptor complex, depend on the endocytosis and routing of the activated receptors. TGF-β receptor association with clathrin-coated pits had been seen as a mode of activation of Smad signaling and receptor turnover, and it was thought that receptor association with caveolin lipid rafts led to receptor degradation as this was associated with the expression of inhibitory Smad7 and the Smurf E3 ubiquitin ligase that targets TGF-β receptors for degradation (Razani et al. 2001; Penheiter et al. 2002; Di Guglielmo et al. 2003).

Studies suggest that rather than leading to receptor degradation, receptor association with caveolin may lead to activation of some non-Smad signaling pathways. It has been shown that TGF-β-mediated Erk MAPK signaling involves association with lipid rafts during epithelial plasticity (Zuo and Chen 2009), and recently, it was shown that ShcA regulates TGF-β-mediated EMT by sequestering TGF-β receptors in caveolin-associated compartments, inhibiting autocrine TGF-β signaling to Smads (Muthusamy et al. 2015). The activation of Akt by TGF-β has also been found to depend on the presence of caveolin (Meyer et al. 2011; Meyer et al. 2013), and the activity of PI3K has been seen to regulate different types of vesicle trafficking, including clathrin-coated pits (Basquin et al. 2013; Bhattacharya et al. 2016; Naguib 2016).

We suspect that the compartmentalized interaction of TGF-β receptors and PI3K in Akt activation may be significant in our study. To begin to show this, we first attempted to show that TGF-β stimulation recruits PI3K-Akt to the plasma membrane. Using total internal reflection fluorescence (TIRF) microscopy, we find that TGF-β stimulation causes recruitment of Akt to the plasma membrane [Figure 22]. However, cells stimulated with TGF-β in the presence of the p110α inhibitor (#90) attenuates this recruitment. This suggests that p110α activity at the plasma membrane, converting PIP2 to PIP3, is required for TGF-β-induced Akt activation at the cell surface.

We therefore attempted to establish if TGF-β recruits PI3K to the plasma membrane. Cell surface biotinylation of NMuMG cells shows that while TGF-β induced recruitment of its own receptors to the cell surface, the recruitment of PI3K is not obvious [Figure 23]. This is not surprising since PI3K is ubiquitous and is associated with a number of cell plasma membrane-associated proteins. Minor induction of PI3K recruitment by TGF-β may be too sensitive for our system to detect. To fully understand the role of TGF-β receptor in regulation of PI3K-Akt activation at the plasma membrane, it is necessary to isolate the plasma membrane fraction since roles for PI3K activity have been described in other intercellular membrane fractions (Davis et al. 2015; Naguib 2016). Unfortunately, the attempt to describe compartmentalization the TGF-β receptor-PI3K interaction has not been completed in this study.

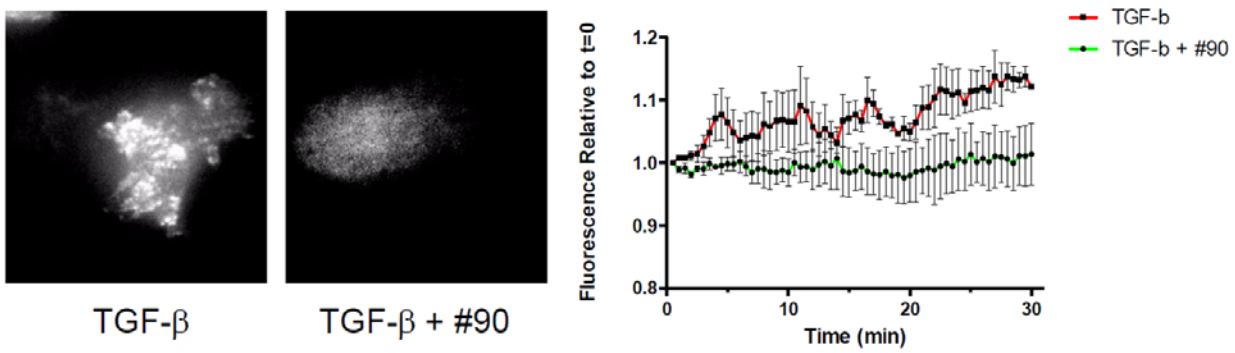

Figure 22. TGF-β-Induced Akt Recruitment to the Cell Surface TIRF microscopy shows that upon TGF-β stimulation, Akt is recruited to the plasma membrane as indicated by the increase in fluorescence. Inhibition of the PI3K p110α catalytic subunit suggests that PI3K activity is needed for TGF-β-induced recruitment of Akt to the plasma membrane.

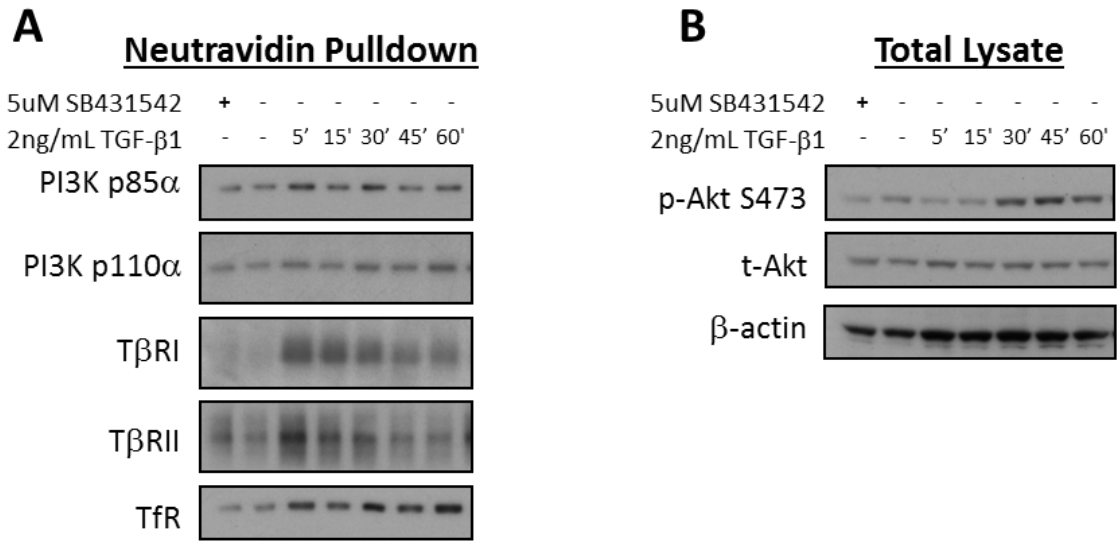

Figure 23. Cell Surface Association of PI3K TGF-β receptors are clearly recruited to the cell surface upon TGF-β stimulation, but recruitment of PI3K is not apparent (A). Total lysates show TGF-β-activated Akt is apparent after 30 minutes of stimulation (B).

CHAPTER 7: Discussion and Future Directions

Both TGF-β signaling and PI3K-Akt signaling pathways are major determinants of cell survival and behavior, and mutations in either pathway can lead to adverse outcomes including cancer progression. Being a messenger of extracellular activity, TGF-β can elicit a plethora of events, including activation of the PI3K-Akt pathway. TGF-β-induced PI3K-Akt activation has been shown to induce EMT, and inhibition of PI3K activity attenuates this transition. It has also been established that the kinase activity of TβRI is needed for TGF-β-induced Akt phosphorylation and EMT. Previous studies have shown that the p85α regulatory subunit of PI3K can bind to TGF-β receptors. In this study, we aimed to understand the mechanism of TGF-β-induced PI3K activity, leading to Akt phosphorylation and EMT, at the receptor level with the understanding that TGF-β receptors are dual-specificity kinases and may activate PI3K-Akt similarly to RTKs.

7.1 Discussion of Results

In our studies, we used NMuMG cells as is a well-established cell line for TGF-β-induced Akt phosphorylation and EMT. Additionally, these cells express relatively high levels of TGF-β receptors and PI3Kα relevant for our studies. We used 293T cells for overexpression studies since they are easily transfectable.

We began by identifying the specific PI3K isoforms which have major contributions in TGF-β-induced Akt activation and EMT. Using chemical inhibitors that target different PI3K catalytic subunits, we determined that the ubiquitously expressed p110α catalytic subunit is a major mediator of TGF-β-induced Akt phosphorylation and EMT. Inhibition of p110α strongly blocked

TGF-β-induced Akt phosphorylation in various cell lines of epithelial origin including NMuMG, HaCaT, and E4 cells. Additionally, p110α inhibition attenuated the TGF-β-induced EMT morphology and induction of the EMT transcription factor Snail. Decreased expression of the p85α regulatory subunit by siRNA also attenuated TGF-β-induced Akt phosphorylation. However, decreased p85α expression continued to exhibit full TGF-β-induced EMT. This is not surprising as the catalytic subunit remains functional.

We can find constitutive interaction of TβRII with wild-type p85α. A truncated version of p85α (PI3K p85α ΔN), closely resembling the p50α isoform, is able to interact with TβRII. We further reduced the region of interaction to the cSH2 and iSH2 domains of p85α. Interestingly, while a complete loss of the cSH2 domain of p85α abrogated interaction with TβRII, the R649A point mutation in the phospho-tyrosine binding pocket of the cSH2 domain resulted in increased TβRII association. The mutation of R649 had been shown to result in SHORT (short stature, hyperextensibility, hernia, ocular depression, Rieger anomaly, and teething delay) syndrome due to impaired interaction with insulin receptor substrate (Chudasama et al. 2013). Since cells are exposed to many growth factors, the increase in TβRII-p85α R649A interaction might be simply explained by the fact that a mutation resulting in loss of interaction with IRS-1 allows for more p85α interaction with the TGF-β receptor.

While it has been reported, and we have confirmed many times, that the TβRI kinase activity is needed for Akt activation and EMT, there does not seem to be detectable TβRI-p85α interaction, nor did we find a TGF-β-inducible interaction. Additionally, neither inhibition of TβRI kinase activity by SB431542 nor activation of its kinase activity by ligand stimulation had obvious

effects on TβRII-p85α interaction. Some results hint that inhibition of TβRI kinase activity might promote this interaction, but this remains to be validated. To determine the role of TβRII kinase activity in PI3K interaction, we created a point mutation in the activation loop of TβRII since the previously reported kinase-dead TβRII K277R mutant does not express at comparable levels to wild-type TβRII. We found that while this mutant, D379N, was attenuated in TGF-β-induced Smad3 activation and tyrosine phosphorylation of the receptor seems to be abrogated, p85α could still be found to interact with it. We conclude, then, that while the kinase activity of TβRI is needed for Akt phosphorylation, the kinase activities of neither TβRI nor TβRII significantly alter receptor-PI3K interaction. However, we have to tread this carefully since SB431542 not only inhibits TβRI kinase activity, but also Alk4 and Alk7 activity, though neither are thought to be bound and activated by TGF-β (Heldin and Moustakas 2016). Additionally, the kinase activity of the TβRII D379N mutant remains to be fully evaluated. It would be ideal to mutate the D397 residue in the DFG motif and evaluate its kinase activity to determine which mutation is a better representation of a kinase-dead TβRII.

With the knowledge that TGF-β receptors are dual-specificity kinases, we hypothesized that the receptor tyrosine kinase activity, and phosphorylated tyrosine(s) of the receptors, might have a role in recruitment of PI3K, similar to RTKs. To this end, we analyzed possible tyrosine phosphorylation sites of the receptors, mutating tyrosines to phenylalanines, and assessing its ability to associate with PI3K. We found that simultaneous mutation of five potential tyrosine phosphorylation sites in TβRII resulted in a significant loss of p85α interaction. Nevertheless, there was still interaction. TβRII Y336 may have a role in this interaction as a Y336,424F double mutant also seemed to have attenuated interaction with p85α. In other studies, a Y336N mutation

in TβRII had been found to occur in patients with Loeys-Dietz syndrome (Loeys et al. 2005), a disorder affecting connective tissues, speaking to a functional role of Y336 in TGF-β signaling. This mutation seemingly leads to increased phospho-Smad2 in the nucleus. If we suggest that the Smad and non-Smad signaling pathways are in contention for activation by TGF-β receptors, this may suggest that the Y336 mutation, leading to phospho-Smad2 nuclear accumulation, is a result of loss of PI3K association due to the mutation in the tyrosine residue. For this reason, it is important to understand the partition of signaling by receptor compartmentalization, which we have not presented in this study.

We did begin to explore the interaction of PI3K with TGF-β receptors at the plasma membrane by first asking if TGF-β stimulation resulted in recruitment of PI3K to the membrane. This did not seem to be the case, but the result is not conclusive. There may be a slight increase in PI3K at the cell surface at the earlier time points after TGF-β stimulation, just prior to Akt phosphorylation, which may be a genuine TGF-β-induced recruitment, but the loading control, transferrin receptor, also has this pattern of fluctuation. This is not surprising as TGF-β has been found to stimulate the cell surface expression of other receptors (Ungerleider et al. 2016). A better cell surface loading control, one that is not affected by TGF-β stimulation, is needed to truly assess TGF-β-induced upregulation of PI3K at the plasma membrane. However, TGF-β stimulation did indeed recruit Akt to the plasma membrane, and the p110α inhibitor abrogated this, indicating that TGF-β-induces PI3K activity at the plasma membrane, even if not its recruitment.

Lastly, the receptor-PI3K interaction has been largely studied in an overexpression system. The endogenous interaction needs to be shown to bear relevance. Attempts in establishing receptor-PI3K interaction has been hampered by inefficient receptor antibodies and low levels of endogenous interaction. We found that a single confluent 15-centimeter dish of NMuMG cells is not enough to detect co-IP of PI3K with TGF-β receptors. However, we have to be careful with how much more protein we collect for detection of endogenous interaction (e.g. four 15-centimeter dishes) as this requires much more reagents and can overload the system. Additionally, an increase in protein overall may result in protein aggregation and unspecific interactions, particularly since there have been issues with obtaining good antibodies to TGF-β receptors.

7.2 A Role for ShcA in TGF-β-Induced PI3K-Akt Activation

Reports have suggested the involvement of adaptor proteins in PI3K-Akt activation. PI3K can be recruited by binding to adaptor proteins, for example Shc and Grb2, in interlukin-3 (IL-3)/IL-2 or insulin-like growth factor-I signaling (Gu et al. 2000; Radhakrishnan et al. 2008). These adaptor proteins are also known to be recruited to the TGF-β receptors (Galliher and Schiemann 2007; Lee et al. 2007). Some studies suggest a role for focal adhesion kinase (FAK) in TGF-β-induced PI3K-Akt signaling (Hong et al. 2011; Xue et al. 2014). Hong et al. found that FAK, without its kinase activity or tyrosine phosphorylation, was needed to bridge the TGF-β-PI3K-Akt activation in a specific cell type, not in epithelial cells. Xue et al. also suggested a role for FAK and ShcA in recruitment and activation of PI3K-Akt in the non-small cell lung cancer cell line A549, an epithelial cell line.

Shc proteins are intracellular adaptors that relay signals from membrane-associated proteins and contain a phospho-tyrosine binding (PTB) domain and a SH2 domain, which also binds phosphorylated tyrosine (Ravichandran 2001; Wills and Jones 2012). While four unique members of the Shc family, A-D, have been identified, ShcA is more ubiquitously expressed and well characterized. In turn, ShcA has three isoforms: $p46^{ShcA}$, $p52^{ShcA}$, and $p66^{ShcA}$. Shc activity has been primarily studied as an adaptor involved in Erk MAPK activation downstream of Ras, through recruitment of Grb2 and Sos (van der Geer et al. 1996; Lee et al. 2007). It is also known to be involved in the PI3K-Akt signaling pathway (Besset et al. 2000; Pelicci et al. 2002; De Falco et al. 2005; Ursini-Siegel et al. 2012). Additionally, ShcA has been shown to be recruited to the TGF-β receptors to mediate Erk and Smad signaling and EMT through receptor compartmentalization (Lee et al. 2007; Muthusamy et al. 2015).

It was recently reported that knockdown of ShcA expression abrogates TGF-β-induced Akt phosphorylation and increases basal Smad phosphorylation (Muthusamy et al. 2015). We show that knockdown of ShcA expression does indeed inhibit TGF-β-induced Akt phosphorylation [Figure 24A]. We've seen that knocking down ShcA expression or inhibiting TβRI kinase individually leads to inhibition of Akt activation. Here, we find that simultaneous decreased expression of ShcA and inhibition of the TβRI kinase might enhance detection of endogenous TβRII-p85α interaction [Figure 24B]. This suggests that the absence of ShcA, freeing p85α to bind TβRII, in addition to the inability of TβRI to release inhibition of TβRII on p85α through its kinase activity, results in increased TβRII-p85α interaction, and this interaction can be more easily detected endogenously.

While knockdown of p85α expression attenuated TGF-β-induced Akt phosphorylation, it did not seem to have an effect on TGF-β-induced EMT [Figure 11]. However, we find that Smad3 phosphorylation looks to be slightly enhanced with decreased p85α expression [Figure 25]. This, with the observation by Muthusamy et al. that knockdown of ShcA expression enhances autocrine TGF-β signaling leading to Smad activation and EMT, suggests that when there is inhibition of TGF-β-induced non-Smad pathways, i.e. PI3K-Akt and Erk MAPK, TGF-β can more effectively signal through the Smad pathway to induce EMT.

$p66^{ShcA}$ has been described as an antagonist of Erk MAPK activation (Migliaccio et al. 1997). With the recent finding that ShcA can regulate TGF-β signaling, and that knockdown of ShcA expression inhibits TGF-β-induced Akt phosphorylation while enhancing Smad phosphorylation, there may be value in teasing out which ShcA isoform might be a major mediator of TGF-β signaling and Akt activation. It will, however, be difficult to knockdown expression the $p46^{ShcA}$ and $p52^{ShcA}$ isoforms without affecting the larger splice variants.

7.3 Other Preliminary Findings and Future Directions

Given the above observations that: (1) SH2-mutated p85α can interact with TβRII, (2) a point mutation in the phospho-tyrosine binding pocket of the p85α cSH2 domain shows an increase in interaction with TβRII, but a complete loss of the cSH2 domain abrogates TβRII interaction, (3) the cSH2 domain alone cannot interact with TβRII but needs the iSH2 domain for interaction, and (4) the kinase- and tyrosine-mutated TβRII can still interact with p85α, it seems likely that the activation of PI3K-Akt by TGF-β differs from traditional RTK activation of PI3K, and more

than just tyrosine phosphorylation of TβRII is needed for receptor interaction with PI3K. The biological factors that govern TβRII-p85α interaction still remains to be elucidated.

A recent study suggests that some SH2 domain-containing proteins may have higher affinity for lipids than for phosphorylated tyrosine, and can be recruited to the membrane via a lipid-binding module in the SH2 domain (Park et al. 2016). Various PI3K regulatory subunits had been identified as having alternative cationic patches with affinity for lipids. This can potentially suggest that rather than p85α binding to TβRII directly (or indirectly via adaptor proteins), it is only spatially associated with the receptor through membrane-associated proximity.

Simply testing empirical variables of buffer stringency by increasing salt concentration in the IP lysis buffer initially seemed to increase TβRII-p85α binding [Figure 26A], and relatively high concentration of sodium dodecyl sulfate (SDS) in the IP incubation buffer was needed to abrogate TβRII-p85α interaction [Figure 26B], suggesting the interaction maybe through ionic bonds, and possibly something more. However, more experiments could be done to exclude the fact that simple membrane co-localization is why we find constant association of the two molecules. Better plasma membrane solubilization or using chimeric receptors or receptors that cannot be membrane-associated might begin to address this, but these experiments have to be carefully considered since the role of PI3K in regulating trafficking of different intercellular membrane-bound vesicles adds another layer of complexity in truly dissociating the role of the plasma membrane in TβRII-p85α interaction. Being able to isolate the plasma membrane from other cellular membranes will help to elucidate the role of the plasma membrane and receptor-

PI3K compartmentalization in TGF-β-induced PI3K-Akt activation, but these experiments have not been completed for this study.

Showing that the TGF-β receptors interact with PI3K is a good start, but the interaction is not relevant if it is without a biological consequence. To test the relevance of the TβRII-PI3K interaction, we performed a PI3K activity assay. A PI3K fusion protein consisting of the p85α and pll0α subunit was purified from bacteria and incubated with its PIP2 substrate in the presence or absence of a di-phosphorylated PDGFR-β peptide (pY740 + pY751) with increasing amounts of purified TβRII. Increasing TβRII seemed to have an inhibitory effect on PI3K activity [Table 1]. This does not abrogate the fact that TGF-β induces PI3K-Akt activation through activity of the receptors. It is possible that in a quiescent state, TβRII holds p85α in an inactive confirmation, and ligand-induced activation of the TβRI kinase leads to tyrosine phosphorylation of TβRII or recruitment other proteins to the receptor complex, allowing for release of the inhibitory effect of TβRII on PI3K, without dissociation of the TβRII-PI3K complex. In line with this, knockdown of TβRII expression seems to result in increased basal levels of phospho-Akt [Figure 25].

We did begin to explore the possibility of TβRII as negative regulator in PI3K activity by transfecting increasing amounts of wild-type TβRII in NMuMG cells. It cannot be expected that increasing TβRII will inhibit TGF-β-induced Akt activation since ligand binding to the TGF-β receptors is needed for activation of signaling pathways. However, it may be possible that TβRII might inhibit PI3K activity by displacing p110α association with p85α. Increasing TβRII expression did not displace p110α interaction with p85α, so it does not seem that TβRII is

competing with p110α for interaction with p85α (data not shown). This experiment may be repeated and confirmed with lower levels of transfected TβRII in a TβRII-null background.

A protein alignment of TβRII and p110α shows a string of six amino acids (ND/GMIVT) found in both proteins. This sequence is in the adaptor binding domain (ABD) of p110α, whereas this corresponding sequence in TβRII is in the extracellular domain, unlikely to bind p85α, but this has not been ruled out experimentally. Figure 19 suggests that a cytoplasmically truncated TβRII is not able to associate with p85α, but the caveat is that co-expression of the cytoplasmically truncated TβRII with p85α regularly results in decreased p85α protein expression, possibly due to the cells' preference to translate shorter exogenous protein.

Superimposition of the TβRII kinase domain and p110α does not show very good alignment, but it is interesting to note that the best alignment between TβRII and p110α, as aligned by PyMOL, is around the p110α ABD [Figure 27]. This figure also shows (in yellow) the ND/GMIVT sequence found in TβRII and p110α, respectively. Whether TβRII is indeed a negative regulator of PI3K activity, and whether it could displace p110α binding to p85α is intriguing. The TGF-β receptors do not have the canonical pYXXM motif recognized by the p85 SH2 phospho-tyrosine binding domain, so there is certainly a novel mode of interaction between TβRII and PI3K. We should also keep in mind that the iSH2 domain, a p110-interacting domain, is needed for TβRII association with p85α.

With the observations in this study, we suggest that while TGF-β receptors are considered dual-specificity kinases, they do not act similarly to RTKs in activation of PI3K-Akt. We suggest a

model wherein the constitutive TβRII-p85α interaction is one that is inhibitory, and this interaction, in part, involves the cSH2 domain of p85α and phosphorylated tyrosines of TβRII. Upon ligand binding and TβRI kinase activation, PI3K activation is achieved through induced association of PI3K with TβRI as suggested by Yi et al. or with TβRI-recruited adaptor proteins, e.g. ShcA, presumably through binding of the p85α nSH2 domain to a second phospho-tyrosine, without loss of association with TβRII [Figure 28].

Studying TGF-β signaling has many challenges due to the extensive crosstalk and regulation of and by other major signaling pathways. Recent studies infer that TGF-β signaling can upregulate various cell surface receptors, including its own receptors, enhancing other signaling pathways in parallel with TGF-β signaling, making the study of molecular mechanisms of the TGF-β signaling pathways more difficult. Additionally, promiscuity amongst TGF-β family of ligands is not unheard of. Recently, it was discovered that GDF-15 can signal through the TβRI/TβRII complex (Artz et al. 2016) when it had been thought to signal through Activin and BMP receptors. In our own studies, TGF-β1 seems to induce Smad1 and Smad5 activation in NMuMG cells, albeit at lower levels than Smad2 and Smad3 activation. Care will have to be taken to resolve whether signaling events are direct effects of ligand activation, a result of downstream signaling events, or off-target effects due to excessive stimulation of the system.

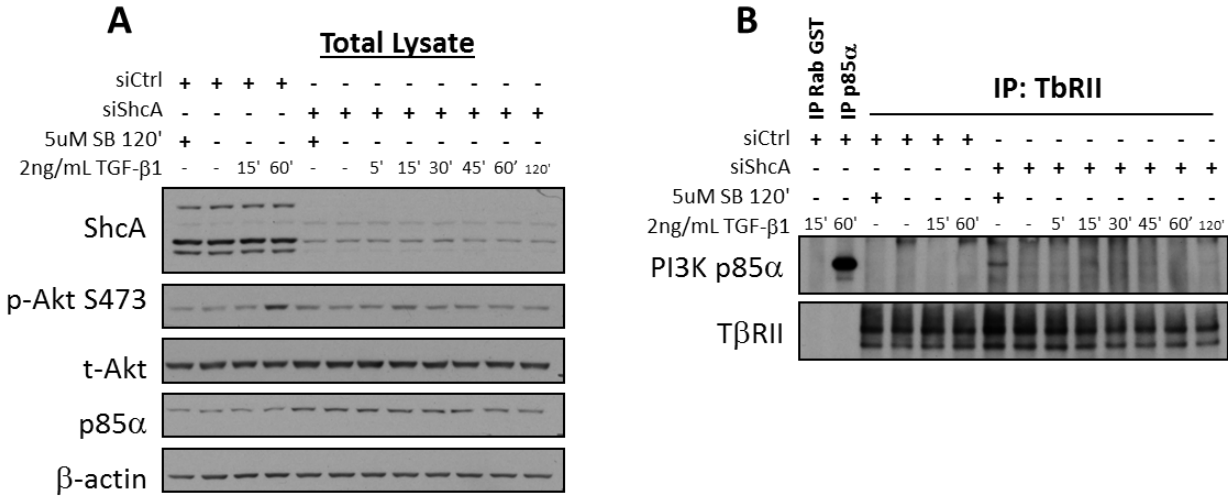

Figure 24. Knockdown of ShcA Expression on Akt Activation and TβRII-p85α Interaction Loss of ShcA expression seems to inhibit TGF-β-induced Akt phosphorylation (A) and loss of ShcA in addition to inhibition of the TβRI kinase might enhance TβRII-p85α interaction (B).

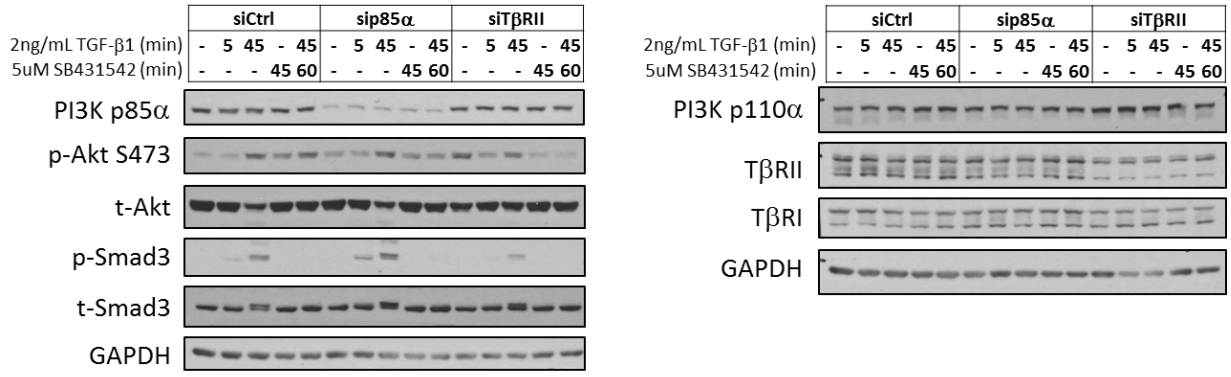

Figure 25. Effects of Knockdown of p85α or TβRII Expression on Signaling Decreased p85α expression seems to have enhanced Smad3 activation while decreased TβRII expression possibly increases basal p-Akt levels and p110α levels.

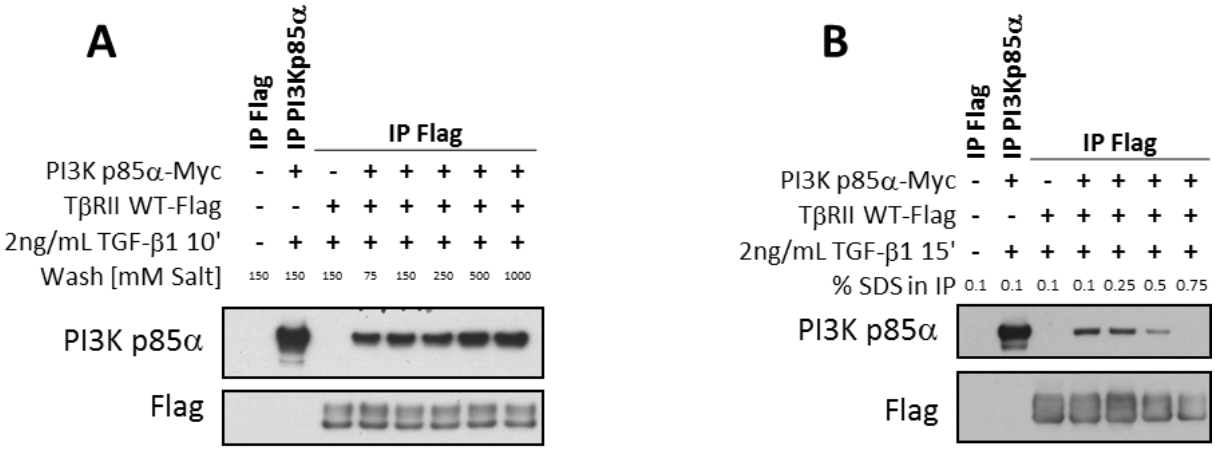

Figure 26. TβRII-p85α Interaction in Varying Salt and Detergent Concentrations A test of increasing salt (NaCl) concentration seem to enhance TβRII-p85α interaction (A), and incubation in relatively high concentrations of SDS buffer (with constant 150mM NaCl) was needed for TβRII-p85α dissociation.

Activator + Kinase	**PIP3 (pmole)**	**Background subtracted**
10pmole di-phospho PDGFR peptide + PI3K	12.03	3.493
10pmole di-phospho PDGFR peptide + PI3K + 50ng RII-GST	11.06	2.523
10pmole di-phospho PDGFR peptide + PI3K + 250ng RII-GST	7.173	-1.364
10pmole di-phospho PDGFR peptide + PI3K + 500ng RII-GST	6.594	-1.943
PI3K only	8.537	0

Table 1. PIP3 ELISA Assay to Assess PI3K Activity Purified PI3K enzyme was incubated with PI(4,5)P2 substrate in the presence or absence of pY PDGFR peptide with increasing amounts of TβRII-GST. The amount of PIP3 produced was detected using a colorimetric assay.

Figure 27. Alignment of TβRII Kinase and p110α The kinase domains of TβRII (orange) and p110α (red) do not have good sequence alignment. Blue/cyan: p85α iSH2 domain, magenta: p85α nSH2 domain, yellow: p110α (NGMIVT), green: remaining p110α.

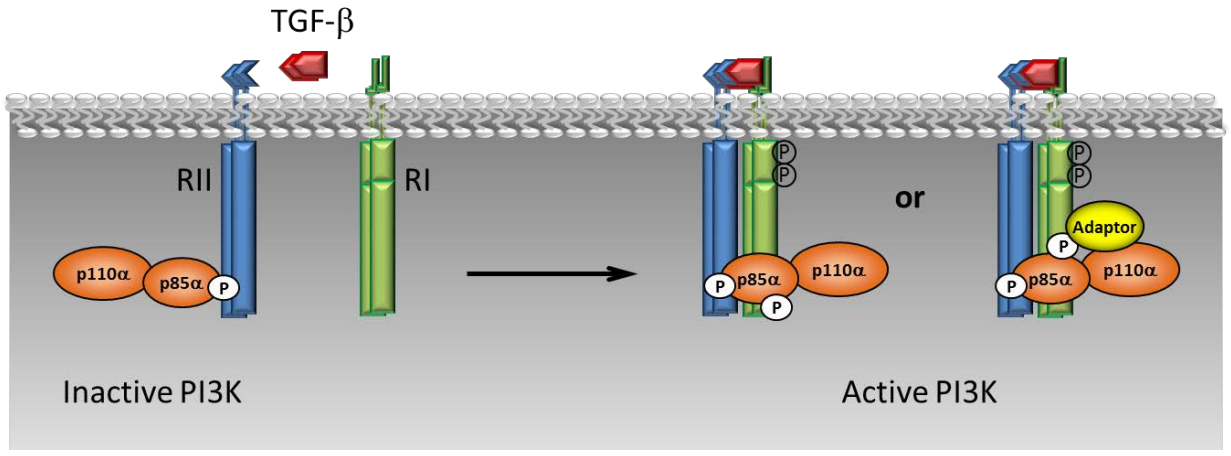

Figure 28. Model of TGF-β-Induced PI3K Activation PI3K is constitutively bound to TβRII, presumably through the cSH2 (and iSH2) domain of p85α and phosphorylated tyrosine(s) of TβRII. Activation of the receptors by ligand binding provides a second pY binding site, either on TβRI, TβRII, or an adaptor protein, for the p85α nSH2 domain, releasing inhibition of the p110α kinase and allowing for PI3K activity.

CHAPTER 8: Cell Lines Created

8.1 Knockdown of TβRI Expression in NMuMG Cells by Lentiviral shRNA Infection

We began this study by exploring the activity of TβRI in TGF-β-induced non-Smad signaling. Recent studies suggested a role for TβRI tyrosine phosphorylation in TGF-β-induced Erk MAPK signaling (Lee et al. 2007), and we were interested in studying this further. However, detection of TβRI proved difficult due to lack of reliable antibodies to the receptor. Our goal, then, was to stably knock down endogenous TβRI expression and re-introduce wild-type or tyrosine mutant TβRI to determine the role of TβRI tyrosine phosphorylation in TGF-β signaling.

We used commercially available short hairpin RNA (shRNA) targeting mouse *Tgfbr1* (the gene encoding TβRI) in a pLKO.1 expression vector. We first determined the efficacy the shRNA to knock down endogenous TβRI expression in NMuMG cells by transient transfection. At this time, the TβRI antibody available was not efficient in detection of TβRI, but we can see a clear inhibition of TGF-β-induced Smad3 activation with the "sh107" shRNA [Figure 29A]. Using RT-PCR, we also find that this shRNA is able to down-regulate *Tgfbr1* expression by 80% compared to wild-type [Figure 29B]. We subsequently used the shRNA vector to produce lentiviral particles in 293T cells and infected wild-type NMuMG cells. Cells were selected for puromycin resistance and positive clones were assessed for TβRI expression and TGF-β-induced Smad3 activation (data not shown). We show that TGF-β-induced EMT is attenuated in the cell line where TβRI expression is knocked down [Figure 30]. We attempted to recover this by re-introducing wild-type TβRI by transient transfection. Cells seem to reactivate TGF-β-induced EMT, but are very sickly [Figure 30]. Further propagation of this cell line seems to invoke

recovery of TβRI expression, so it is advisable to continually check for TβRI down regulation and supplement the knockdown with transient transfection of siRNA targeting TβRI.

8.2 Creating a Flag-tagged TβRII NMuMG Cell Line by CRISPR-Cas9 Genome Editing

While the TβRII-p85α interaction can be easily detected when overexpressed in 293T cells, endogenous interaction proved more difficult to detect. Another limitation is the availability of reliable TGF-β receptor antibodies for IP or Western. To be able to better immunoprecipitate endogenous TβRII, we attempted to generate a C-terminal 3xFlag-tagged TβRII NMuMG cell line. The 3xFlag tag was designed to be just upstream of the TβRII stop codon and in-frame with the coding sequence. Using the CRISPR-Cas9 system with integrated puromycin selection, we were able to generate clones that were positively selected. Amplification of the tagged region confirmed insertion or modification at this site [Figure 31]. Genomic DNA of clones were sequenced, and only Clone 7 was confirmed to contain an in-frame 3xFlag tag.

We tested the functional ability of this 3xFlag-tagged TβRII protein by IP and determined its availability at the cell surface by cell surface biotinylation [Figure 32A]. Curiously, the Flag-tagged protein immunoprecipitated with the Flag antibody was harder detect by immunoblotting with its own antibody than with a TβRII antibody. We also tested the ability of this cell line to transduce TGF-β signaling, and we confirmed that the cell line can continue to respond to TGF-β, activating Smad3 and Akt similarly to wild-type NMuMG cells [Figure 32B].

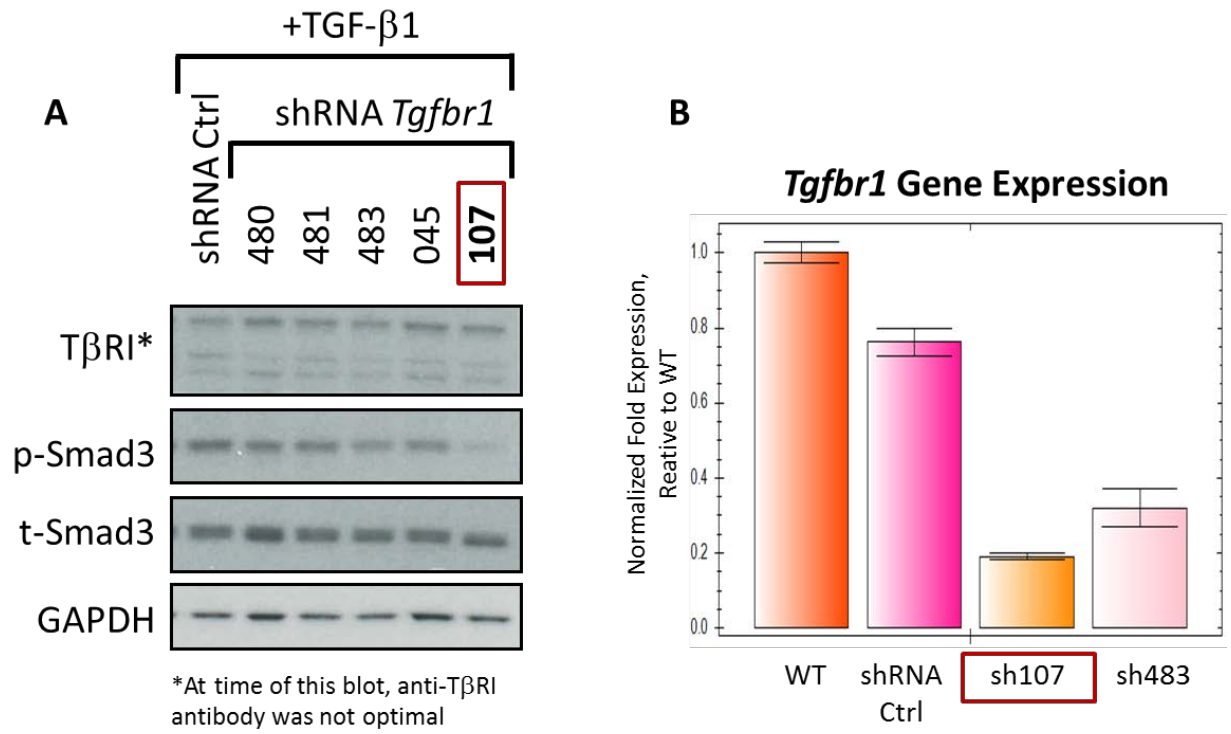

Figure 29. Knockdown of *Tgfbr1* Transient transfection of shRNAs targeting *Tgfbr1*, the gene encoding TβRI, in NMuMG cells inhibit TGF-β-induced Smad3 phosphorylation (A) and RT-PCR shows downregulation of *Tgfbr1* by 80% (B).

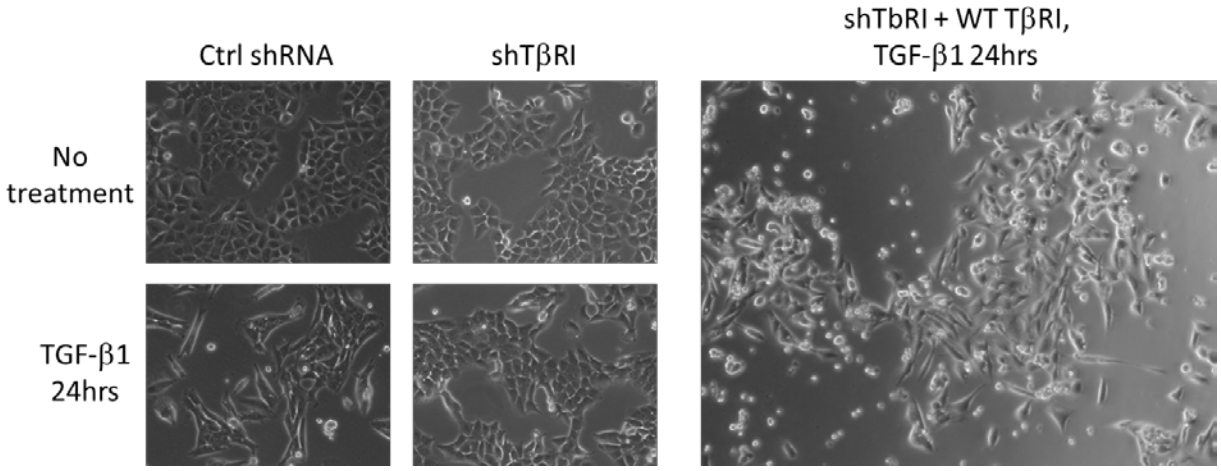

Figure 30. TGF-β-Induced EMT After Stable Expression of shRNA Targeting *Tgfbr1* TGF-β-induced EMT is attenuated in cells with stable knockdown of TβRI expression. Re-introducing wild-type TβRI seems to recover this, but cells are very sickly.

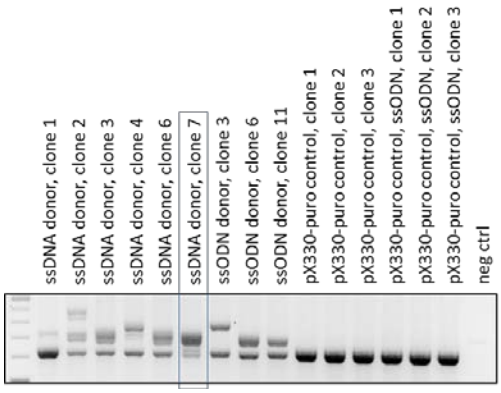

Figure 31. PCR Amplification of 3xFlag Insertion Region at the TβRII Locus Clonal selection yielded several clones that carried an insertion in the 3' end of the *Tgfbr2*, the gene encoding TβRII. Sequencing of the amplified bands indicated that only 1 of the clones (Clone 7) had a 3xFlag insertion in frame with the TβRII coding sequence.

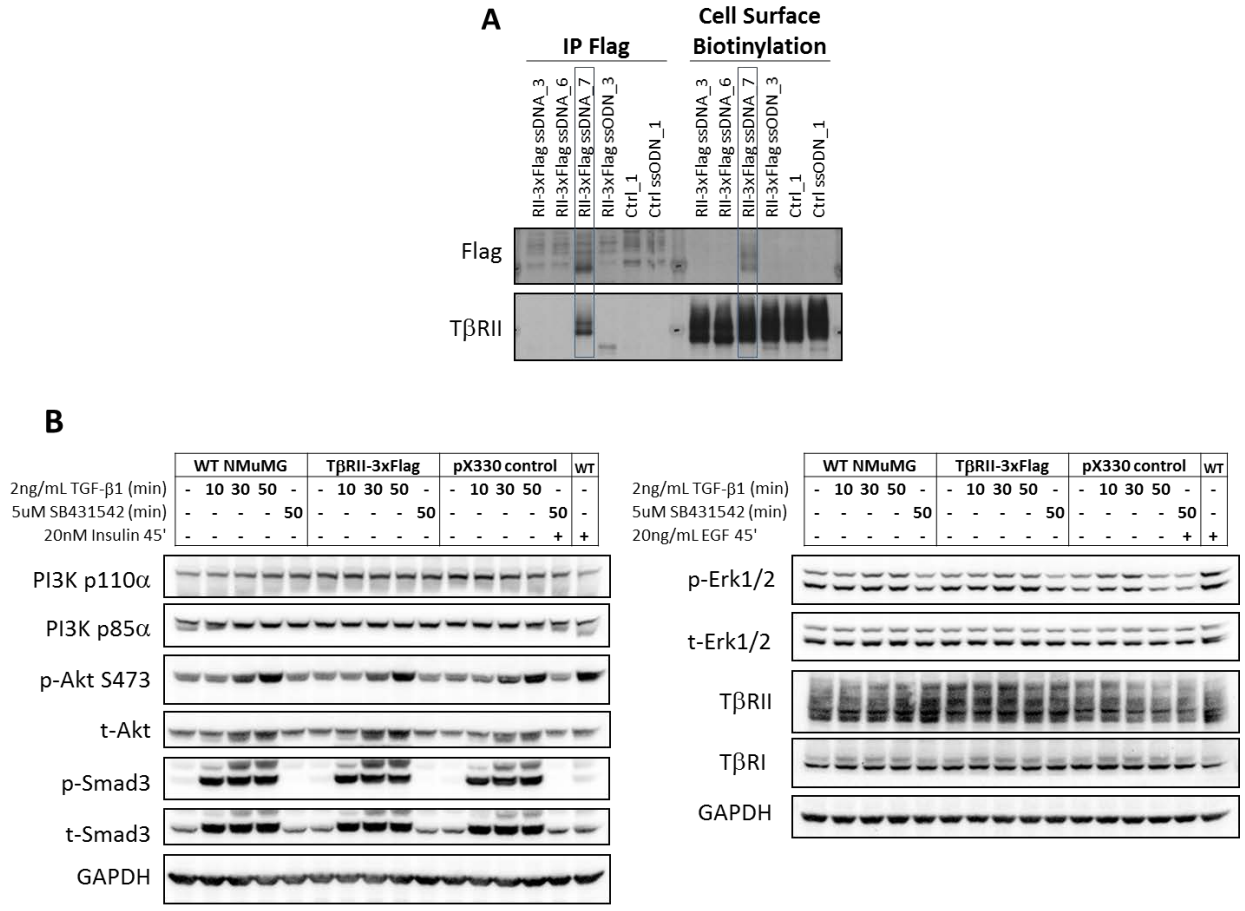

Figure 32. Functional Analysis of TβRII-3xFlag Cell surface biotinylation shows that the Flag-tagged TβRII is detected at the cell surface (A). The TβRII-3xFlag cell line is able to induce signaling similar to control and wild-type NMuMG cells (B)

MATERIALS AND METHODS

Cell Culture

NMuMG, HaCaT, E4, and 293T cells were cultured in DMEM with 10% Fetal Bovine Serum (FBS). NMuMG culture medium was supplemented with 10µg/mL insulin (Sigma-Aldrich). Cells were maintained at 37°C and 5% CO2 in a humidified incubator.

Expression Plasmids, Peptides, RNAi, and Transfections

pRK5 expression plasmids encoding Flag-tagged rat TβRI and human TβRII have been described (Feng et al. 1995). TβRII tyrosine mutant constructs were derived from the pRK5-TβRII-Flag plasmid described, in the most part by Jamie Smyth. The TβRII Y259,284,336,424F mutant provided by Jamie was used for generation of the TβRII 5YF (Y259,284,336,424,470F) mutant. Myc-PI3K p85α-6xHis was a gift from Frank McCormick's lab (UCSF). PI3K p85α-p110α-His protein, GFP- or GST-tagged PI3K p85α ΔN plasmid and protein, respectively, GFP-PI3K p85α ΔN R649A plasmid, and PDGFR-β pY751 and pY740 + pY751 peptides (Biomatik) were gifts from Bradley Webb (Diane Barber's lab, UCSF, unpublished). Flag-tagged PI3K p85α ΔiSH2 in a pBSSK backbone was purchased from Addgene and sub-cloned into a pRK5 plasmid. siRNAs targeting mouse PI3K p85α, PI3K p110α, or ShcA were purchased from Qiagen. siRNA targeting the 3'UTR of mouse TβRII was custom-designed and purchased from Thermo Scientific 5'GGATAGCGTTAGCACTTGACA (Budi et al. 2015). Cells were transfected using Lipofectamine 2000 (Life Technologies) or RNAiMAX (Life Technologies) for RNAi, according to manufacturer's protocol.

The vector constructs for TβRI, TβRII, and p85α contain coding sequence from different species (rat, mouse, human, or bovine). However, we have duly noted that the kinase domains of TβRI and TβRII are highly conserved amongst these species. We also note that PI3K p110α and the C-terminal domain of p85α that include the nSH2, iSH2, and cSH2 domains are highly conserved across species.

Cloning and Point Mutation Generation

PI3K constructs were sub-cloned into the pRK5-Myc mammalian expression vector to control for expression levels. PI3K SH2 and iSH2 domains were amplified from the PI3K p85α ΔN plasmid and cloned into pRK5-Myc. Cloning primers are listed on Table 1. All cloning primers contained an EcoRI restriction site on the forward primer and a BamHI restriction site on the reverse primer. DNA fragments were amplified using a high-fidelity DNA polymerase (Q5 High-Fidelity DNA Polymerase, New England BioLabs or Pfu Turbo DNA polymerase, Agilent Technologies) according to manufacturer protocol. Primers for point mutations were generated using the online primer design program provided by Agilent Technologies. Mutagenesis primers are listed on Table 2. Mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer protocols.

Growth Factors and Inhibitors

PI3K catalytic subunit inhibitors were provided by Kevan Shokat's lab (UCSF) and described in (Knight et al. 2006). The TβRI kinase inhibitor SB4315242 was purchased from Sigma-Aldrich and used at 5μM. The pan PI3K inhibitor GDC-0941 was purchased from Selleck Chemicals and

used at 10μM. TGF-β1 ligand was purchased from Humanzyme. Insulin was purchased from Sigma-Aldrich.

Cell Treatment, Lysis, and Immunoprecipitation

For growth factor-stimulated assays, cells were washed once with pre-warmed phosphate-buffered saline (PBS) then serum-starved with DMEM only (or DMEM + 0.5% FBS) for 5-6 hours before stimulation with 1-2ng/mL TGF-β1. Cells were pre-treated with inhibitors for 1 hour prior to TGF-β treatment where appropriate. Cells were harvested with RIPA [20mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% SDS, 0.25% Na Deoxycholate, 2mM Na3VO4, 5mM NaF, and cOmplete Protease Inhibitor cocktail (Roche)] or co-IP buffer [50mM Tris, pH 7.5, 150mM NaCl, 2mM EDTA, 1% NP-40, 5% glycerol, 2mM Na_3VO_4, 5mM NaF, and cOmplete Protease Inhibitor cocktail]. Protein concentration was quantified using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) with bovine serum albumin (BSA) as standard, and a SpectraMax M5 plate reader. Protein was normalized for immunoprecipitation.

For immunoprecipitation of endogenous proteins in NMuMG cells, cells were serum-starved then treated with TGF-β1 (or SB431542) for indicated times. For immunoprecipitation of overexpressed proteins in 293T cells, cells were transfected with appropriate constructs and subsequently treated with TGF-β1 (or SB431542) where indicated. Cells were harvested with co-IP buffer and subjected to immunoprecipitation with anti-TβRII (endogenous IP) or with anti-Flag M2 (overexpression IP) antibody overnight with Protein A Sepharose or Protein G Sepharose beads (GE Healthcare), respectively. Absorbed proteins were eluted from the beads with lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) and analyzed by SDS–

polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. ImageJ was used to quantify the density ratio of the p85α and TβRII bands in Figure 19. Both upper and lower bands of TβRII was used in this quantification.

Cell Surface Biotinylation

NMuMG cells were grown to 70-90% confluency before treatment. Cells were washed with ice-cold PBS, and incubated for 30 minutes with EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) on ice. Non-reacted biotin was quenched with 0.1mM glycine for 10 minutes on ice. Cells were lysed in co-IP lysis buffer. Protein was quantified and normalized for absorption with NeutrAvidin beads (Thermo Scientific) overnight. Samples were eluted with LDS sample buffer subjected to SDS-PAGE, followed by immunoblotting.

Antibodies

TβRII antibody was from Santa Cruz Biotechnology. TβRI and t-Smad3 antibodies were from Abcam. PI3K p85α, PI3K p110α, p-Smad3, p-Akt S473, p-Akt T308, t-Akt, p-Erk1/2, t-Erk1/2, p-Tyr-100 (pY100) antibodies were from Cell Signaling. ShcA and E-cadherin antibodies were from BD Biosciences. Flag (M2) and Fibronectin antibodies were from Sigma-Aldrich. GAPDH antibody was from Proteintech. Transferrin Receptor antibody was from Life Technologies.

RNA Extraction and RT-PCR

RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad) and real-time PCR performed with iQ SYBR Green Supermix (Bio-Rad). *Gapdh* and *Rpl19* were used for normalization of *Tgfbr1* and *Snail* expression,

respectively. The CFX96 Real-Time System and CFX Manager software (Bio-Rad) were used for RNA expression analysis. RT-PCR primers for mouse *Tgfbr1* are: 5'GCTGCAATCAGGACCACTG and 5'CTTCATTTGGCACACGGTGG. Primers for *Gapdh* are: 5'GTGAAGGTCGGTGTGAACG and 5'AAGATGGTGATGGGCTTCCC. Primers for *Snail* and *Rpl19* been described (Lamouille et al. 2012).

Immunofluorescence Microscopy

Cells were fixed with 4% PFA for 30 minutes, permeabilized in 2% PFA and 0.25% Triton X-100 for 10 minutes, and blocked with PBS and 3% BSA. The slides were incubated for 2 hours with anti–E-cadherin and anti-fibronectin antibodies in PBS and 3% BSA, washed, then incubated with secondary Alexa Fluor–conjugated antibodies and Alexa Fluor Phalloidin (Life Technologies) in PBS and 3% BSA for 1 hour in the dark. Slides were mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies) and imaged with a Leica DMI 4000B microscope.

Live-Cell Imaging by TIRF Microscopy

Cells were maintained in Hank's balanced salts solution (HBSS, Life Technologies) with 10% FBS at 37°C and stimulated with TGF-β1 at time T=0 in the presence or absence of the p110α inhibitor (PIK-90). Through-the-objective TIRF illumination was achieved using a 486 nm DPSS laser source (Spectral Applied Research), and a Cascade II 512 camera (Photometrics). GFP-tagged Akt dynamics images were acquired every 30 seconds for 30 minutes.

Invasion Assay

After stimulating cells with TGF-β or not for 48 hours, cells were trypsinized and 50,000 cells were added to Matrigel-coated inserts (BioCoat Matrigel Invasion Chamber; Becton Dickinson) in DMEM with 0.2% FBS. These were then placed in companion plates with DMEM 10% FBS for 24 hours. Cells in upper chambers were removed, filters were fixed in methanol for 5 minutes at -20°C, and mounted using ProLong Gold Antifade Reagent with DAPI.

GST Protein Expression and Purification

The cytoplasmic domains of human TβRII or rat TβRI were amplified as described (Feng et al. 1995) and was cloned into a pGEX vector as described (Lee et al. 2007). The pGEX vector containing receptor-GST fusion proteins were used to transform B21 DE3 competent cells. One clone was used to grow a mini culture which was then used to inoculate a larger culture (~$1/10^{th}$ volume). Bacteria culture was incubated at 37°C, shaking at 225rpm. At OD_{600} of ~0.5, GST protein expression was induced with 0.25mM IPTG and culture flasks were incubated at 18°C, shaking at 225rpm. GST-protein was extracted as described (Lawler et al. 1997), absorbed with Glutathione Sepharose beads (GE Healthcare), washed 4 times, and eluted with reduced L-glutathione (Sigma). Eluted protein samples were flash-frozen and stored in aliquots at -80°C. Protein concentrations were approximated with coomassie blue staining and BSA standards.

***In Vitro* Kinase Assay**

GST fusion proteins containing the cytoplasmic tail of TβRI or TβRII were purified from bacteria (described above). Full-length TβRI-Flag in a pRK5 plasmid was transfected into 293T cells and immunoprecipitated with anti-Flag antibody and Protein G Sepharose beads (described in immunoprecipitation method above). Purified proteins were incubated in total volume of 30μL

kinase buffer (25mM HEPES, pH 7.4, 2mM $MnCl_2$, 10mM $MgCl_2$, 0.01% Triton X-100, 20μM DTT). 100μM ATP or 5μM SB431542 was added where appropriate. Reactions were incubated at 37°C for 30 minutes. Select samples were subsequently treated with 120U of Lambda Protein Phosphatase (New England Biolabs) in the proper volume of Protein MetalloPhosphatase buffer (New England Biolabs) at 30°C for 1 hour. Reactions were stopped by adding LDS sample buffer when appropriate. Protein G-bound Flag-tagged receptors were washed 3 times with kinase buffer (without ATP) after the kinase reaction and prior to addition of sample buffer. Note that NaF and Na_3VO_4 were absent in this particular kinase reaction to avoid inhibition of Lambda Protein Phosphatase activity.

PI3K Activity Assay

PI3K activity was assayed (in duplicates) using the Pico 96-well ELISA Assay (Echelon) according to manufacturer's protocol. Briefly, 5μM of $PI(4,5)P_2$ substrate was incubated with 0.25μg/mL of a purified PI3K fusion protein (GST-p85α-p110α-His, provided by Bradley Webb, unpublished) only, with 10pmole of a PDGFR di-pY peptide (provided by Bradley Webb), or with PDGFR di-pY peptide and increasing amounts of RII-GST. Kinase reactions were allowed to proceed for 3 hours at 37°C with gentle shaking, then stopped with 2.4mM EDTA. Reactions were then incubated with a PI(3,4,5)P3 detector protein and transferred onto a PIP3 detection plate. PI3K activity was measured using colorimetry to and a SpectraMax M5 plate reader.

Knockdown of Endogenous TβRI Expression by shRNA

293T cells were seeded in 10-centimeter dishes the day prior to transfection. Cells were co-transfected with 13.3μg of pLKO.1-puro plasmids containing non-targeting shRNA or shRNA

targeting mouse *Tgfbr1* (Sigma-Aldrich), 10µg of Δ8.9 plasmid containing gag, pol and rev genes, and 6.7µg of VSV-G expressing envelope plasmid using Lipofectamine 2000. Media of cells were changed the next day and viral production was allowed to proceed for another day. Viral supernatant was collected and filtered through a 0.45µm filter, supplemented with 4µg/mL polybrene, and 2mL of supernatant was used for infection of NMuMG cells that were seeded in 6-well plates the day prior. The infected plated was sealed with parafilm and centrifuged at 1800rpm in a Sorvall Legend XTR centrifuge for 45 minutes at 32°C. Viral supernatant was removed and infection with centrifugation was repeated. After the second infection, the viral supernatant was left on the cells for 1 hour in the cell culture incubator before removal and replaced with regular media. Infected NMuMG cells were allowed to recover for a day before trypsinization and re-plating onto 6cm dishes and subsequent selection with 3µg/mL puromycin (InvivoGen).

CRISPR-Cas9 Genome Editing of Endogenous TβRII

pX330 plasmid was provided by Stanley Qi's Lab (Stanford University) and modified with a puromycin selection cassette. The online CRISPR guide RNA design tool created by Feng Zhang's lab (Massachusetts Institute of Technology, http://crispr.mit.edu/) was used to define guide RNAs with proximity the stop codon of *Tgfbr2* in the mouse genomic sequence. Guide RNAs were designed for cloning into the pX330 vector at the BbsI restriction sites. Efficiency of guide RNAs were assessed using the Surveyor Mutation Detection Kit (IDT) and the following "surveyor" primers:

Surveyor Primer F2: 5'AGCTCCAGGCTCAATGAGAA,

Surveyor Primer R2 5'CAAGGCATAGGACGCTCTCT.

The most efficient guide RNA was selected for use in genome editing (vector sequences in bold):

Forward 5'**CACCG**GTCCTCTAGCCAAAGACCAG

Reverse 5'**AAAC**CTGGTCTTTGGCTAGAGGAC**C**

Donor sequence was designed as a gBlock Gene Fragment (IDT) that included mouse genomic sequence around exon 8 of *Tgfbr2*, a 3xFlag sequence (bold, underlined) adjacent and upstream of the stop codon, and the Cas9 targeted PAM sequence mutated (bold).

gBlock Gene Fragment sequence (guide RNA sequence italicized):

gttgtgttggggagggccacctttgaaatatcttacagtcaagcatatttgacatacaaagacagtcacgtcagagggcctagcttaatgtcaa
tggagatgacgggggtgtctgagtcaccctaggcaattctggaagcaacttataagtcattctgtgaaagacaggctttaccaaatgagctca
ggttaatgcctgccccactctgggcttgagggtccagctccattcaaacagaagggtacctatgggctagcacggttacagtttgaaagaag
aaaatctccagaggccatgaaagagtcaagggaaagagagagcccagcttaaagtgaggaacggaagtccttcagaccctgtaatggat
atatctgttacattctatacaatgtatgccaccatgagtagaccctaggcactggagagatggcactattgcaaattagggtgaggacctgag
ctcgaatccccagtacccatgtaaaaagctggtggccatgatgtaatcctgtaagacaagcacagaaggaagaggaggcagaaggatccc
aaatcagtgagctccaggctcaatgagaaaccctgtctcaaagagtaaggtgtctttgaggaagacagctgacagtgacctttgacccacac
ctacctacacatgaacacatagaaagagtgagccttattctgaactgtggaccttgttaatgatgtattgatactaactccatggagaccaccca
ctgacggtgcccttgggacccccctcctttcctctgcagggcatccagatcgtgtgtgagactttgaccgagtgctgggaccatgaccccga
agcccgtctcacagcacagtgtgtggcagagcgcttcagtgagctggagcatccggagagactctctggcaggagctgctcccaggaga
agattccagaagatggctcgctgaacactaccaaa**GACTACAAAGACCACGATGGTGACTACAAAGAC**
CACGATATCGACTACAAAGACGATGACGATAAAtaatagctttttctgggcaggctgggccaagcctcg
agaagcc*GTCCTCTAGCCAAAGACCAG*ag**A**cagcaggattctctcctgactgatgcttctggaaaaccaaggacttgctcc
cttcttccccaggagctgccccgtgtttagaagcggcagcagcagcagcaacaaccatagcggcggtggcagcggcgggggatgagtg
acagagagcgtcctatgccttggagactgtcatggcataagctgtgctagcacctcctcaggaaatgagattgatttttacaacagccaataa

cgtttgcactttattaatgcctgtgtgtaaatacgaatagctatgttttatatatatctatatatctatatgtctatatctctctatatatagccatactctg
caaggagacaaagaaaatgatcaaatgtgttccccggggaattagtttttattggagagctctagaatggagcagaagggactcgggatag
cgttagcacttgacaatcagtcacacaagcaacgatcccctgacagcagggttgggggcacaattgtatgagaaggatccatgccttgcag
cctgctttggccacaaaacactttgttttgcaataatgaccctctacagtagggtgctttatggaccagggagctgagctccagtccagcactg
agtcccaggatctcccatgtgtctttgcttctcttggttgttatctttgacattcaagccccactctgacttgtgaaccttctgacttagccttgaaac
ttggccccatttttctgcttttacgggctaccaaaaatcaaagaagaccgttccccacccatgaaattggcctaccatctactaataagattgagt
tctttgatcctttcctgtgcataagtaactgttatttgttccctgccattatcctttgttgatttttttaaaacaaggcacaccctacactcagccc.

This sequence was first amplified, then single-stranded DNA was made using only one of the primers used for amplification, and gel-purified. Primers:

F1 5’CCTAGGCACTGGAGAGATGGC

R1 5’CTGCTGTCAGGGGATCGTTGC

NMuMG cells in 6-well plates were co-transfected with 0.5µg of pX330 plasmid containing the specific guide RNA and 500ng of gel-purified single-stranded DNA. 48 hours after infection, puromycin-positive clones were selected for with 3µg/mL puromycin. Cells were then trypsinized and seeded for clonal expansion at 0.5cell/well in 96-well plates. Genomic DNA was extracted from viable clones using QuickExtract (Epicentre), and edited genomes were assessed using the Surveyor Mutation Detection Kit (described above). Positive clones were expanded and the genomic region around the Flag insertion was sequenced. The 3xFlag tag was confirmed to be present and in-frame for the clone used in experiments presented.

Construct	Forward Primer	Reverse Primer
mouse PI3K p85α ΔN ΔnSH2	5' GAG AAT TCT AAA TAC CAG CAG GAT CAA G	5' GAG GAT CCT CAT CGC CTC TGT TGT GC
mouse PI3K p85α ΔN ΔcSH2	5' GAG AAT TCT TTG CAG GAT GCT GAA TGG TAC	5' GAG GAT CCT CAT CGC CTC TGT TGT GCA TAC GTC TTC TCG TCA TGG TG
mouse PI3K p85α ΔN ΔNiSH2 (5')*	5' GAG AAT TCT TTG CAG GAT GCT GAA TGG TAC	5' CCT TTG AAT TTC TTT CTC GTT GCC TTC GCG GAT TTC CTG GGA AGT ACG GGT GTA CTC CTC
mouse PI3K p85α ΔN DNiSH2 (3')*	5' GAG GAG TAC ACC CGT ACT TCC CAG GAA ATC CGC GAA GGC AAC GAG AAA GAA ATT CAA AGG	5' GAG GAT CCT CAT CGC CTC TGT TGT GC
mouse PI3K p85α nSH2	5' GAG AAT TCT TTG CAG GAT GCT GAA TGG TAC	5' GAG GAT CCT CAA TCC TGC TGG TAT TTG G
mouse PI3K p85α cSH2	5' GAG AAT TCA GAT GAT GAG GAT TTG CCC C	5' GAG GAT CCT CAT CGC CTC TGT TGT GC
mouse PI3K p85α iSH2	5' GAG AAT TCT AAA TAC CAG CAG GAT CAA G	5' GAG GAT CCT CAT CGC CTC TGT TGT GCA TAC GTC TTC TCG TCA TGG TG

Table 2. List of Cloning Primers *mouse PI3K p85α ΔN ΔNiSH2 was constructed by 2-part PCR – first by amplification of the 5' and 3' fragments, then a fusion of these fragments was amplified using products from the first amplification and the EcoRI-containing 5' primer and the BamHI-containing 3' primer.

Mutation	Forward Primer	Reverse Primer
human TβRII D379N	5' CCA TCG TGC ACA GGA ACC TCA AGA GCT CC	5' GGA GCT CTT GAG GTT CCT GTG CAC GAT GG
human TβRII Y470F	5' GTA ATG CAG TGG GAG AAG TAA AAG ATT TTG AGC CTC CAT TTG	5' CAA ATG GAG GCT CAA AAT CTT TTA CTT CTC CCA CTG CAT TAC
mouse PI3K p85α R358A	5' ACT GCT GAT GGG ACC TTT TTG GTA GCA GAC GCA TCT ACT	5' AGT AGA TGC GTC TGC TAC CAA AAA GGT CCC ATC AGC AGT

Table 3. List of Site-Directed Mutagenesis Primers Complimentary forward and reverse primers were designed using the online mutagenesis primer design program provided by Agilent Technologies.

CONTRIBUTIONS

Initial experiments for this study was performed by Samy Lamouille. Samy did much of the work to determine the specific PI3K subunits that regulate TGF-β-induced Akt phosphorylation and EMT in epithelial cells and produced many figures [Figures: 4A, 4B, 6A, 6B, 8B, 8C, 9, 10].

Jamie Smyth made the all the single and most of the multiple TβRII tyrosine mutant constructs in the pRK5-Flag vector. Jamie also obtained the TIRF microscopy image in TGF-β-induced Akt cell surface recruitment [Figure 22].

REFERENCES

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* **7**: 261-269.

Artz A, Butz S, Vestweber D. 2016. GDF-15 inhibits integrin activation and mouse neutrophil recruitment through the ALK-5/TGF-βRII heterodimer. *Blood* **128**: 529-541.

Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. 2002. p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. *J Cell Sci* **115**: 3193-3206.

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. 2000. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* **275**: 36803-36810.

Basquin C, Malardé V, Mellor P, Anderson DH, Meas-Yedid V, Olivo-Marin JC, Dautry-Varsat A, Sauvonnet N. 2013. The signalling factor PI3K is a specific regulator of the clathrin-independent dynamin-dependent endocytosis of IL-2 receptors. *J Cell Sci* **126**: 1099-1108.

Besset V, Scott RP, Ibáñez CF. 2000. Signaling complexes and protein-protein interactions involved in the activation of the Ras and phosphatidylinositol 3-kinase pathways by the c-Ret receptor tyrosine kinase. *J Biol Chem* **275**: 39159-39166.

Bhattacharya S, McElhanon KE, Gushchina LV, Weisleder N. 2016. Role of phosphatidylinositol-4,5-bisphosphate 3-kinase signaling in vesicular trafficking. *Life Sci* **167**: 39-45.

Bibbins KB, Boeuf H, Varmus HE. 1993. Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides. *Mol Cell Biol* **13**: 7278-7287.

Bonifacino JS, Traub LM. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* **72**: 395-447.

Budi EH, Muthusamy BP, Derynck R. 2015. The insulin response integrates increased TGF-β signaling through Akt-induced enhancement of cell surface delivery of TGF-β receptors. *Sci Signal* **8**: ra96.

Chantry D, Vojtek A, Kashishian A, Holtzman DA, Wood C, Gray PW, Cooper JA, Hoekstra MF. 1997. p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. *J Biol Chem* **272**: 19236-19241.

Chen X, Wang H, Liao HJ, Hu W, Gewin L, Mernaugh G, Zhang S, Zhang ZY, Vega-Montoto L, Vanacore RM et al. 2014. Integrin-mediated type II TGF-β receptor tyrosine dephosphorylation controls SMAD-dependent profibrotic signaling. *J Clin Invest* **124**: 3295-3310.

Chudasama KK, Winnay J, Johansson S, Claudi T, König R, Haldorsen I, Johansson B, Woo JR, Aarskog D, Sagen JV et al. 2013. SHORT syndrome with partial lipodystrophy due to impaired phosphatidylinositol 3 kinase signaling. *Am J Hum Genet* **93**: 150-157.

Conery AR, Cao Y, Thompson EA, Townsend CM, Ko TC, Luo K. 2004. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nat Cell Biol* **6**: 366-372.

Davis WJ, Lehmann PZ, Li W. 2015. Nuclear PI3K signaling in cell growth and tumorigenesis. *Front Cell Dev Biol* **3**: 24.

De Craene B, Berx G. 2013. Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer* **13**: 97-110.

De Falco V, Guarino V, Malorni L, Cirafici AM, Troglio F, Erreni M, Pelicci G, Santoro M, Melillo RM. 2005. RAI(ShcC/N-Shc)-dependent recruitment of GAB 1 to RET oncoproteins potentiates PI 3-K signalling in thyroid tumors. *Oncogene* **24**: 6303-6313.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**: 577-584.

Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL. 2003. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nat Cell Biol* **5**: 410-421.

Engelman JA. 2009. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* **9**: 550-562.

Engelman JA, Luo J, Cantley LC. 2006. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* **7**: 606-619.

Feng XH, Filvaroff EH, Derynck R. 1995. Transforming growth factor-beta (TGF-beta)-induced down-regulation of cyclin A expression requires a functional TGF-beta receptor complex. Characterization of chimeric and truncated type I and type II receptors. *J Biol Chem* **270**: 24237-24245.

Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**: 727-736.

Galliher AJ, Schiemann WP. 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 Res* **67**: 3752-3758.

Gordon KJ, Blobe GC. 2008. Role of transforming growth factor-beta superfamily signaling pathways in human disease. *Biochim Biophys Acta* **1782**: 197-228.

Goumans MJ, Liu Z, ten Dijke P. 2009. TGF-beta signaling in vascular biology and dysfunction. *Cell Res* **19**: 116-127.

Gu H, Maeda H, Moon JJ, Lord JD, Yoakim M, Nelson BH, Neel BG. 2000. New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. *Mol Cell Biol* **20**: 7109-7120.

Hanks SK, Hunter T. 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* **9**: 576-596.

Hata A, Chen YG. 2016. TGF-β Signaling from Receptors to Smads. *Cold Spring Harb Perspect Biol* **8**.

Heldin CH, Moustakas A. 2016. Signaling Receptors for TGF-β Family Members. *Cold Spring Harb Perspect Biol* **8**.

Hicke L, Dunn R. 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* **19**: 141-172.

Hong M, Wilkes MC, Penheiter SG, Gupta SK, Edens M, Leof EB. 2011. Non-Smad transforming growth factor-β signaling regulated by focal adhesion kinase binding the p85 subunit of phosphatidylinositol 3-kinase. *J Biol Chem* **286**: 17841-17850.

Horiguchi M, Ota M, Rifkin DB. 2012. Matrix control of transforming growth factor-β function. *J Biochem* **152**: 321-329.

Hubbard SR, Miller WT. 2007. Receptor tyrosine kinases: mechanisms of activation and signaling. *Curr Opin Cell Biol* **19**: 117-123.

Jo E, Park SJ, Choi YS, Jeon WK, Kim BC. 2015. Kaempferol Suppresses Transforming Growth Factor-β1-Induced Epithelial-to-Mesenchymal Transition and Migration of A549 Lung Cancer Cells by Inhibiting Akt1-Mediated Phosphorylation of Smad3 at Threonine-179. *Neoplasia* **17**: 525-537.

Kannan N, Neuwald AF. 2005. Did protein kinase regulatory mechanisms evolve through elaboration of a simple structural component? *J Mol Biol* **351**: 956-972.

Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol* **17**: 615-675.

Knight ZA, Gonzalez B, Feldman ME, Zunder ER, Goldenberg DD, Williams O, Loewith R, Stokoe D, Balla A, Toth B et al. 2006. A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* **125**: 733-747.

Kornev AP, Haste NM, Taylor SS, Eyck LF. 2006. Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *Proc Natl Acad Sci U S A* **103**: 17783-17788.

Lamouille S, Connolly E, Smyth JW, Akhurst RJ, Derynck R. 2012. TGF-β-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion. *J Cell Sci* **125**: 1259-1273.

Lamouille S, Derynck R. 2007. Cell size and invasion in TGF-beta-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. *J Cell Biol* **178**: 437-451.

Lamouille S, Xu J, Derynck R. 2014. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* **15**: 178-196.

Lawler S, Feng XH, Chen RH, Maruoka EM, Turck CW, 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. *J Biol Chem* **272**: 14850-14859.

Lee MK, Pardoux C, Hall MC, Lee PS, Warburton D, Qing J, Smith SM, Derynck R. 2007. TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *EMBO J* **26**: 3957-3967.

Loeys BL, Chen J, Neptune ER, Judge DP, Podowski M, Holm T, Meyers J, Leitch CC, Katsanis N, Sharifi N et al. 2005. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat Genet* **37**: 275-281.

López-Casillas F, Wrana JL, Massagué J. 1993. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* **73**: 1435-1444.

Maehama T, Dixon JE. 1998. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**: 13375-13378.

Mayer BJ, Jackson PK, Van Etten RA, Baltimore D. 1992. Point mutations in the abl SH2 domain coordinately impair phosphotyrosine binding in vitro and transforming activity in vivo. *Mol Cell Biol* **12**: 609-618.

Mayer IA, Arteaga CL. 2016. The PI3K/AKT Pathway as a Target for Cancer Treatment. *Annu Rev Med* **67**: 11-28.

Meyer C, Godoy P, Bachmann A, Liu Y, Barzan D, Ilkavets I, Maier P, Herskind C, Hengstler JG, Dooley S. 2011. Distinct role of endocytosis for Smad and non-Smad TGF-β signaling regulation in hepatocytes. *J Hepatol* **55**: 369-378.

Meyer C, Liu Y, Kaul A, Peipe I, Dooley S. 2013. Caveolin-1 abrogates TGF-β mediated hepatocyte apoptosis. *Cell Death Dis* **4**: e466.

Meyer RD, Mohammadi M, Rahimi N. 2006. A single amino acid substitution in the activation loop defines the decoy characteristic of VEGFR-1/FLT-1. *J Biol Chem* **281**: 867-875.

Migliaccio E, Mele S, Salcini AE, Pelicci G, Lai KM, Superti-Furga G, Pawson T, Di Fiore PP, Lanfrancone L, Pelicci PG. 1997. Opposite effects of the p52shc/p46shc and p66shc

splicing isoforms on the EGF receptor-MAP kinase-fos signalling pathway. *EMBO J* **16**: 706-716.

Morikawa M, Derynck R, Miyazono K. 2016. TGF-β and the TGF-β Family: Context-Dependent Roles in Cell and Tissue Physiology. *Cold Spring Harb Perspect Biol* **8**.

Moustakas A, Heldin CH. 2005. Non-Smad TGF-beta signals. *J Cell Sci* **118**: 3573-3584.

-. 2016. Mechanisms of TGFβ-Induced Epithelial-Mesenchymal Transition. *J Clin Med* **5**.

Muthusamy BP, Budi EH, Katsuno Y, Lee MK, Smith SM, Mirza AM, Akhurst RJ, Derynck R. 2015. ShcA Protects against Epithelial-Mesenchymal Transition through Compartmentalized Inhibition of TGF-β-Induced Smad Activation. *PLoS Biol* **13**: e1002325.

Naguib A. 2016. Following the trail of lipids: Signals initiated by PI3K function at multiple cellular membranes. *Sci Signal* **9**: re4.

Padua D, Massagué J. 2009. Roles of TGFbeta in metastasis. *Cell Res* **19**: 89-102.

Park MJ, Sheng R, Silkov A, Jung DJ, Wang ZG, Xin Y, Kim H, Thiagarajan-Rosenkranz P, Song S, Yoon Y et al. 2016. SH2 Domains Serve as Lipid-Binding Modules for pTyr-Signaling Proteins. *Mol Cell* **62**: 7-20.

Pelicci G, Troglio F, Bodini A, Melillo RM, Pettirossi V, Coda L, De Giuseppe A, Santoro M, Pelicci PG. 2002. The neuron-specific Rai (ShcC) adaptor protein inhibits apoptosis by coupling Ret to the phosphatidylinositol 3-kinase/Akt signaling pathway. *Mol Cell Biol* **22**: 7351-7363.

Penheiter SG, Mitchell H, Garamszegi N, Edens M, Doré JJ, Leof EB. 2002. Internalization-dependent and -independent requirements for transforming growth factor beta receptor signaling via the Smad pathway. *Mol Cell Biol* **22**: 4750-4759.

Perlman R, Schiemann WP, Brooks MW, Lodish HF, Weinberg RA. 2001. TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol* **3**: 708-714.

Radaev S, Zou Z, Huang T, Lafer EM, Hinck AP, Sun PD. 2010. Ternary complex of transforming growth factor-beta1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily. *J Biol Chem* **285**: 14806-14814.

Radhakrishnan Y, Maile LA, Ling Y, Graves LM, Clemmons DR. 2008. Insulin-like growth factor-I stimulates Shc-dependent phosphatidylinositol 3-kinase activation via Grb2-associated p85 in vascular smooth muscle cells. *J Biol Chem* **283**: 16320-16331.

Ravichandran KS. 2001. Signaling via Shc family adapter proteins. *Oncogene* **20**: 6322-6330.

Razani B, Zhang XL, Bitzer M, von Gersdorff G, Böttinger EP, Lisanti MP. 2001. Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. *J Biol Chem* **276**: 6727-6738.

Remy I, Montmarquette A, Michnick SW. 2004. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. *Nat Cell Biol* **6**: 358-365.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**: 1098-1101.

Serrano I, McDonald PC, Lock FE, Dedhar S. 2013. Role of the integrin-linked kinase (ILK)/Rictor complex in TGFβ-1-induced epithelial-mesenchymal transition (EMT). *Oncogene* **32**: 50-60.

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**: 767-778.

Songyang Z, Shoelson SE, McGlade J, Olivier P, Pawson T, Bustelo XR, Barbacid M, Sabe H, Hanafusa H, Yi T. 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol Cell Biol* **14**: 2777-2785.

Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, Zhang S, Heldin CH, Landström M. 2008. The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. *Nat Cell Biol* **10**: 1199-1207.

Stephens L, Hawkins P. 2011. Signalling via class IA PI3Ks. *Adv Enzyme Regul* **51**: 27-36.

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT. 1997. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277**: 567-570.

Takekawa M, Tatebayashi K, Itoh F, Adachi M, Imai K, Saito H. 2002. Smad-dependent GADD45beta expression mediates delayed activation of p38 MAP kinase by TGF-beta. *EMBO J* **21**: 6473-6482.

Tebben AJ, Ruzanov M, Gao M, Xie D, Kiefer SE, Yan C, Newitt JA, Zhang L, Kim K, Lu H et al. 2016. Crystal structures of apo and inhibitor-bound TGFβR2 kinase domain: insights into TGFβR isoform selectivity. *Acta Crystallogr D Struct Biol* **72**: 658-674.

Till JH, Ablooglu AJ, Frankel M, Bishop SM, Kohanski RA, Hubbard SR. 2001. Crystallographic and solution studies of an activation loop mutant of the insulin receptor tyrosine kinase: insights into kinase mechanism. *J Biol Chem* **276**: 10049-10055.

Ungerleider N, Han C, Zhang J, Yao L, Wu T. 2016. TGFβ signaling confers sorafenib resistance via induction of multiple RTKs in hepatocellular carcinoma cells. *Mol Carcinog*.

Ursini-Siegel J, Hardy WR, Zheng Y, Ling C, Zuo D, Zhang C, Podmore L, Pawson T, Muller WJ. 2012. The ShcA SH2 domain engages a 14-3-3/PI3'K signaling complex and promotes breast cancer cell survival. *Oncogene* **31**: 5038-5044.

van der Geer P, Wiley S, Gish GD, Pawson T. 1996. The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediate protein-protein interactions. *Curr Biol* **6**: 1435-1444.

Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. 2010. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* **11**: 329-341.

Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD. 1997. Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* **22**: 267-272.

Vanhaesebroeck B, Stephens L, Hawkins P. 2012. PI3K signalling: the path to discovery and understanding. *Nat Rev Mol Cell Biol* **13**: 195-203.

Verrecchia F, Mauviel A. 2007. Transforming growth factor-beta and fibrosis. *World J Gastroenterol* **13**: 3056-3062.

Vivanco I, Sawyers CL. 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* **2**: 489-501.

Whitman M, Downes CP, Keeler M, Keller T, Cantley L. 1988. Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* **332**: 644-646.

Wilkes MC, Mitchell H, Penheiter SG, Doré JJ, Suzuki K, Edens M, Sharma DK, Pagano RE, Leof EB. 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 Res* **65**: 10431-10440.

Wills MK, Jones N. 2012. Teaching an old dogma new tricks: twenty years of Shc adaptor signalling. *Biochem J* **447**: 1-16.

Wrana JL, Attisano L, Cárcamo J, Zentella A, Doody J, Laiho M, Wang XF, Massagué J. 1992. TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* **71**: 1003-1014.

Wu L, Derynck R. 2009. Essential role of TGF-beta signaling in glucose-induced cell hypertrophy. *Dev Cell* **17**: 35-48.

Wu MY, Hill CS. 2009. Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell* **16**: 329-343.

Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. 2004. Activation of the Erk pathway is required for TGF-beta1-induced EMT in vitro. *Neoplasia* **6**: 603-610.

Xue X, Wang X, Liu Y, Teng G, Wang Y, Zang X, Wang K, Zhang J, Xu Y, Wang J et al. 2014. SchA-p85-FAK complex dictates isoform-specific activation of Akt2 and subsequent PCBP1-mediated post-transcriptional regulation of TGFβ-mediated epithelial to mesenchymal transition in human lung cancer cell line A549. *Tumour Biol*.

Yamashita M, Fatyol K, Jin C, Wang X, Liu Z, Zhang YE. 2008. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. *Mol Cell* **31**: 918-924.

Yi JY, Shin I, Arteaga CL. 2005. Type I transforming growth factor beta receptor binds to and activates phosphatidylinositol 3-kinase. *J Biol Chem* **280**: 10870-10876.

Yu J, Wjasow C, Backer JM. 1998a. Regulation of the p85/p110alpha phosphatidylinositol 3'-kinase. Distinct roles for the n-terminal and c-terminal SH2 domains. *J Biol Chem* **273**: 30199-30203.

Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM. 1998b. Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol* **18**: 1379-1387.

Yuan TL, Cantley LC. 2008. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27**: 5497-5510.

Zhang YE. 2016. Non-Smad Signaling Pathways of the TGF-β Family. *Cold Spring Harb Perspect Biol*.

Zuo W, Chen YG. 2009. Specific activation of mitogen-activated protein kinase by transforming growth factor-beta receptors in lipid rafts is required for epithelial cell plasticity. *Mol Biol Cell* **20**: 1020-1029.

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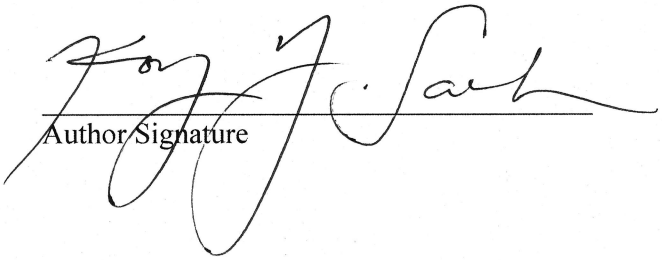

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